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Title: Immunostaining and Dye Penetration Experiments to Define Core Pleated Septate Junction Proteins in *Drosophila* Embryonic Epithelia

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**, done

If **Yes**, can you record movies/images using your own microscope camera?

Yes

SCOPE shot: **4.1.2**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? Down the hall in the same building. A 2-3 minute walk.

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 18

Number of Shots: 51 (11 SC, 1 Scope)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

Answers are slightly edited in accordance with the journal guidelines

INTRODUCTION:

REQUIRED: ~~Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Jae Lee:** Our research investigates the developmental, non-occluding functions of septate junction proteins, focusing on identifying and characterizing new components involved in their structure and biological roles.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the research contexts or systems in which this protocol can be applied.**~~

- 1.2. **Jae Lee:** This protocol is meant to characterize the organization and occluding function of pleated septate junctions in the ectodermal epithelia of *Drosophila* embryos.

- 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~**What does this protocol allow researchers to study or measure?**~~

- 1.4. **Jae Lee:** This protocol allows researchers to confidently determine whether a gene encodes a core or accessory component of the pleated septate junction in *Drosophila* embryonic tissues.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the most important consideration or challenge when performing this protocol.**~~

- 1.5. **Jae Lee:** Embryo staging is absolutely critical while performing this technique as this requires stage 16 embryos. The septate junction matures over time until it is fully established in stage 16.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.6. **Jae Lee:** We believe this protocol can be effectively implemented in open-ended genetic screens using RNA interference lines to identify new pleated septate junction genes.

- 1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Collecting *Drosophila* Embryos and Imaging Immunostained Embryos

Demonstrator: Jae Lee

2.1. To begin, cross healthy male and female *Drosophila* flies of the appropriate genotypes and place them in embryo collection cages with fresh apple juice plates smeared with yeast paste [1].

2.1.1. WIDE: Talent placing the prepared cages with flies in the designated incubator.

2.2. Allow the adults to lay fertilized eggs on the apple juice plates [1]. Remove the plate from the egg collection cage [2] and age the embryos for 13 to 16 hours after egg laying at 25 degrees Celsius to reach stage 16 [3]. Then, fix the stage 16 embryos in an appropriate fixative solution and perform immunostaining with the desired antibodies [4].

2.2.1. Shot of embryos visible on the surface of the apple juice plate during the laying period.

2.2.2. Talent lifting the apple juice plate out of the egg collection cage.

2.2.3. Talent placing the plate aside for aging.

2.2.4. Talent picking up stage 16 embryos and placing them in a fixation container.

2.3. For best results, image the embryos on a confocal microscope with a 40x oil immersion objective [1]. Use control embryos to set the laser intensity and gain to achieve a strong fluorescent signal without oversaturating any pixels [2]. Identify stage 16 embryos that do not express balancer-encoded green fluorescent protein using midgut morphology [3]. Focus the scan on the salivary gland or hindgut [4] and position the z scan to include the largest possible diameter of the lumen to visualize the full lateral membrane of the epithelial cell [5].

2.3.1. Talent positioning a slide with embryos on the confocal microscope and selecting the 40x oil immersion objective.

2.3.2. SCREEN: 2.3.2-final.mp4.

2.3.3. SCREEN: 2.3.3-final.mp4. 00:04-00:16

2.3.4. SCREEN: 2.3.4-final.mp4. 00:04-00:16.

2.3.5. SCREEN: 2.3.5-final.mp4.

2.4. Set a suitable line average and image a polarized tissue in at least 20 control and 20 *cora4* mutant stage 16 embryos [1]. Record data only from embryos in which the adherens junction marker is tightly localized to the apical lateral region of the membrane [2]. In wildtype stage 16 embryos, assess enrichment of pleated septate junction proteins along apical region of the lateral membrane, near the adherens junction [3]. Record the persistent distribution of the pleated septate junction marker all along the lateral membrane in the mutant [4].

2.4.1. SCREEN: 2.4.1-final.mp4.

2.4.2. SCREEN: 2.4.2-final.mp4 (Authors: Hover the cursor over the specified area for the shots 2.4.2, 2.4.3 and 2.4.4). *Video editor: Please focus on the area where the cursor is hovering for the shots 2.4.2, 2.4.3 and 2.4.4. Please focus the orange region being highlighted by the cursor for this shot*

2.4.3. SCREEN: 2.4.4-final.mp4. *Please highlight/focus the green region being highlighted by the cursor for this shot*

2.4.4. SCREEN: 2.4.3-final.mp4. *Please highlight/focus the green region being highlighted by the cursor for this shot*

3. Preparation for Rhodamine-Labeled Dextran Dye Injection

3.1. Make a fresh dye solution by dissolving rhodamine-labeled 10 kilodalton Dextran to 1 milligram per milliliter in 0.5 molar sodium phosphate at pH 7.5 with 5 millimolar potassium chloride [1].

3.1.1. Talent preparing the dye solution by dissolving rhodamine-labeled 10 kilodalton Dextran in sodium phosphate buffer with potassium chloride.

3.2. Using a needle puller, pull a 1 millimeter outer diameter by 0.5 millimeter inner diameter capillary tube with fiber into a glass needle [1].

3.2.1. Talent placing the capillary tube into the needle puller and generating a glass needle. **Videographer's NOTE: Do NOT use takes 1 or 2 for shot 3.2.1**

3.3. Now, load the glass needle into the needle holder on a micromanipulator [1] and arrange the micromanipulator next to an inverted microscope [2]. Orient the needle as

close to parallel to the microscope slide as possible and lower it to the same z (zee) plane as the side of the slide [3].

3.3.1. Talent securing the glass needle into the needle holder on the micromanipulator.

3.3.2. Talent positioning the micromanipulator adjacent to the inverted microscope.
Videographer's NOTE: Shots 3.3.2 and 3.3.3 were combined into just 3.3.2

3.3.3. Talent adjusting the needle orientation to be parallel to the microscope slide and lowering it to the same z plane as the slide edge.

3.4. Break the tip of the needle by striking it against the side of the microscope slide [1]. Raise the needle above the level of the slide [2] and remove it for loading [3].

3.4.1. Talent gently striking the needle tip against the side of the microscope slide to break the tip.

3.4.2. Talent lifting the needle above the slide surface. **Videographer's NOTE: Shots 3.4.2 and 3.4.3 were combined into just 3.4.2**

3.4.3. Talent removing the needle from the holder for loading.

3.5. Next, fill the capillary tube with dye using the smallest available pipette tip [1].

3.5.1. Talent loading dye into the capillary tube using a small pipette tip.

3.6. Reload the filled needle into the needle holder [1]. Test the flow of the 10 kilodalton Dextran by injecting a small amount into a drop of halocarbon oil on the surface of a microscope slide [2].

3.6.1. Talent securing the filled needle back into the needle holder.

3.6.2. Talent injecting a small amount of dye into a drop of halocarbon oil on a microscope slide to confirm flow.

4. Injecting Rhodamine-Labeled Dextran and Imaging Embryos

4.1. Using a 22 by 22 millimeter number 2 thickness coverslip, create an indented line on an apple juice plate [1]. Line up approximately 25 embryos each of control and cora4 mutant at stage 16 or 17 on the apple juice plate. Ensure that their posterior ends align along a line and that the ventral surfaces face upward [2].

- 4.1.1. Talent pressing the edge of a 22 by 22 millimeter number 2 thickness coverslip into the apple juice plate to create an indented line.
- 4.1.2. SCOPE: Scope-4.1.2.mov 00:12-00:23.
- 4.2. Then, apply a piece of double-sided tape to a 22 by 22 millimeter number 2 coverslip [1]. Touch the tape to the embryo line and lightly rub the coverslip to transfer the embryos from the plate onto the double-sided tape [2].
 - 4.2.1. Talent placing double-sided tape onto a 22 by 22 number 2 coverslip.
 - 4.2.2. Talent pressing the taped coverslip onto the aligned embryos and gently rubbing to transfer them onto the tape.
- 4.3. Now, attach the coverslip to a microscope slide by adding a drop of halocarbon oil to the slide and allowing capillary action to create a bond [1]. Orient the coverslip closer to the side of the slide opposite any frosted portion [2]. Desiccate the embryos for 3 to 5 minutes in a container of desiccant [3] and cover the embryos completely with halocarbon oil [4].
 - 4.3.1. Talent placing a drop of halocarbon oil on a microscope slide and lowering the coverslip to form a bond by capillary action. **Videographer's NOTE: All the shots in 4.3 were combined into just 4.3.1 - AND do NOT use take 1 for this shot**
 - 4.3.2. Talent adjusting the position of the coverslip toward the side of the slide opposite the frosted end.
 - 4.3.3. Talent placing the slide in a desiccant container.
 - 4.3.4. Talent adding halocarbon oil to fully cover the embryos.
- 4.4. Mount the slide with embryos onto the inverted microscope with the embryos facing up and away from the objective [1]. Align the micromanipulator so that the needle faces the posterior end of the embryos at an angle as close to parallel to the embryos as possible [2] and adjust the z axis of the micromanipulator so that the needle tip aligns with the midpoint of the embryo along the dorsal ventral axis [3].
 - 4.4.1. Talent placing the slide onto the inverted microscope stage with embryos facing up. **Videographer's NOTE: All the shots in 4.4 were combined into just 4.4.1**
 - 4.4.2. Talent positioning the micromanipulator so the needle is oriented toward the posterior end of the embryos at a near-parallel angle.
 - 4.4.3. Talent adjusting the z axis control to align the needle tip with the midpoint of an embryo.

- 4.5. Next, inject the embryos by quickly moving the stage into the needle so that the needle tip enters the hemocoel [1]. Inject approximately 0.2 to 0.5 nanoliters of dye using a 10, 25, or 50-milliliter syringe [2]. Quickly withdraw the needle [3], move the stage to the next embryo for dye injection [4].
- 4.5.1. Talent advancing the stage so the needle tip penetrates the posterior end of an embryo. **Videographer's NOTE: All the shots in 4.5 were combined into just 4.5.1**
- 4.5.2. Shot of syringe delivering dye into the embryo.
- 4.5.3. Talent withdrawing the needle from the embryo.
- 4.5.4. Talent repositioning the stage to align the next embryo with the needle.
- 4.5.5. ~~Shot of the moving to the next injection.~~ **NOTE: Delete**
- 4.6. Then, add additional halocarbon oil over the embryos [1]. Cover the embryos with a 22 by 22 millimeter number 2 coverslip to create a uniform oil layer between the coverslips and prevent crushing [2].
- 4.6.1. Talent dispensing additional halocarbon oil onto the embryos. **Videographer's NOTE: All the shots in 4.6 were combined into just 4.6.1**
- 4.6.2. Talent gently placing a 22 by 22 number 2 coverslip over the embryos to form a uniform oil layer.

5. ~~Imaging Embryos Post-Dye Injection~~

NOTE: Treat this as a part of section 4, show 5.1.1 right after 4.6.2

- 5.1. Perform imaging on a fluorescent compound microscope or a confocal microscope 10 minutes after dextran dye injection [1]. On the confocal microscope, acquire a z stack through approximately one quarter of the embryo depth along the dorsal ventral axis to assess the lumen of the trachea and hindgut [2]. ~~Acquire deeper sections or reorient the embryos with the ventral surface at the top to visualize the salivary glands [3].~~
- 5.1.1. Talent positioning the slide on a confocal microscope.
- 5.1.2. SCREEN: 5.1.2-final.mp4.
- 5.1.3. ~~Shot of adjusting the embryo orientation on the slide to position the ventral surface at the top for salivary gland imaging.~~ **Videographer's NOTE: We did not do shot 5.1.3 because the provider said it was not needed**
- 5.2. Examine the lumen of the trachea, salivary gland, or hindgut for accumulation of labeled dextran [1]. Record the genotype and developmental stage of each embryo and

whether labeled dextran accumulates in any tubular organ [2]. Note embryos in which the perivitelline space is strongly stained with dye as these may represent inadvertent injection into that space [3].

5.2.1. SCREEN: 5.2.1-final.mp4 .

5.2.2. Talent documenting genotype, developmental stage, and dextran accumulation in a notebook.

5.2.3. SCREEN: 5.2.3-final.mp4 00:20-00:27.

Videographer's NOTE: And then last thing - apologies but the headshots I took are unusable.

Results

6. Results

- 6.1. In wild-type embryos, Macroglobulin complement-related or Mcr (*M-C-R*) was uniformly localized along the lateral membrane at stage 13 [1], became enriched in the apical region of the lateral membrane at stage 14 [2], and was strongly localized to the apical lateral region just basal to the adherens junction by stage 16 [3].
 - 6.1.1. LAB MEDIA: Figure 1ABC. *Video editor: Highlight panel A' and mark the region shown by red arrow.*
 - 6.1.2. LAB MEDIA: Figure 1ABC. *Video editor: Highlight panel B' and mark the region shown by red arrow.*
 - 6.1.3. LAB MEDIA: Figure 1ABC. *Video editor: Highlight panel C'.*
- 6.2. Coracle antibody staining defined the mature pleated Septate Junctions in stage 16 wild-type embryos, similarly to Mcr [1].
 - 6.2.1. LAB MEDIA: Figure 1D. *Video editor: Highlight panel D' showing Coracle staining at the apical lateral region just below the E-cadherin signal.*
- 6.3. In *cora4* mutant embryos, Mcr failed to achieve strong apical localization by stage 16 and remained distributed along the lateral membrane [1].
 - 6.3.1. LAB MEDIA: Figure 1EFG. *Video editor: Highlight panel G' and mark the region shown by red arrow.*
- 6.4. E-cadherin localization was similar in wild-type and *cora4* mutant stage 16 embryos [1].
 - 6.4.1. LAB MEDIA: Figure 1C'' and Figure 1G''.
- 6.5. In wild-type stage 17 embryos injected with rhodamine-labeled 10-kilodalton dextran, the dye surrounded the trachea but did not penetrate the lumen [1]. In *cora4* mutant stage 17 embryos, the dye accumulated in the lumen of the trachea within 10 minutes of injection [2].
 - 6.5.1. LAB MEDIA: Figure 3 top panels. *Video editor: Highlight the tracheal outline indicated by red arrows.*
 - 6.5.2. LAB MEDIA: Figure 3, bottom panels. *Video editor: Highlight the tracheal lumen indicated by red arrows*

1. Drosophila

Pronunciation link: https://www.merriam-webster.com/dictionary/Drosophila

IPA: /drə'säfələ/

Phonetic Spelling: druh·saa·fuh·luh

2. genotypes

Pronunciation link: https://www.merriam-webster.com/dictionary/genotype

IPA: /'jen-ə-, tīp/

Phonetic Spelling: jeh·nuh·type

3. embryos

Pronunciation link: https://www.merriam-webster.com/dictionary/embryo

IPA: /'em-brē-, ō/

Phonetic Spelling: em·bree·oh

4. incubator

Pronunciation link: https://www.merriam-webster.com/dictionary/incubator

IPA: /'in-kyə-, bā-tər/

Phonetic Spelling: in·kyuh·bay·ter

5. fertilized

Pronunciation link: https://www.merriam-webster.com/dictionary/fertilize

IPA: /'fər-tə-, līz/

Phonetic Spelling: fur·tuh·lize

6. fixative

Pronunciation link: https://www.merriam-webster.com/dictionary/fixative

IPA: /'fik-sə-tiv/

Phonetic Spelling: fik·suh·tiv

7. immunostaining

Pronunciation link: No confirmed link found

IPA: /ɪ,mjuːnoʊ'steɪnɪŋ/

Phonetic Spelling: ih·myoo·noh·stay·ning

8. antibodies

Pronunciation link: https://www.merriam-webster.com/dictionary/antibody

IPA: /'an-tē-,bä-dē/

Phonetic Spelling: an·tee·baa·dee

9. confocal

Pronunciation link: https://www.merriam-webster.com/dictionary/confocal

IPA: /kän'fō-kəl/

Phonetic Spelling: kahn·foh·kuhl

10. fluorescent

Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescent

IPA: /flō're-sənt/, /flə're-sənt/

Phonetic Spelling: flor·ess·uhnt, fluh·ress·uhnt

11. morphology

Pronunciation link: https://www.merriam-webster.com/dictionary/morphology

IPA: /môr'fāl-ə-jē/

Phonetic Spelling: mor·faa·luh·jee

12. salivary

Pronunciation link: https://www.merriam-webster.com/dictionary/salivary

IPA: /'sal-ə-ver-ē/

Phonetic Spelling: सल·uh·ver·ee

13. lumen

Pronunciation link: https://www.merriam-webster.com/dictionary/lumen

IPA: /'lü-mən/

Phonetic Spelling: loo·muhn

14. epithelial

Pronunciation link: https://www.merriam-webster.com/dictionary/epithelial

IPA: /,e-pə-'thē-lē-əl/

Phonetic Spelling: eh·puh·thee·lee·uhl

15. polarized

Pronunciation link: https://www.merriam-webster.com/dictionary/polarize

webster.com/dictionary/polarize)

IPA: /'pō-lə-rīz/

Phonetic Spelling: poh-luh-rize

16. adherens

Pronunciation link: No confirmed link found

IPA: /əd'hɪrənz/

Phonetic Spelling: uhd-heer-uhnz

17. pleated

Pronunciation link: https://www.merriam-webster.com/dictionary/pleated

IPA: /'plē-təd/

Phonetic Spelling: plee-tuhd

18. septate

Pronunciation link: https://www.merriam-webster.com/dictionary/septate

IPA: /'sep-tāt/

Phonetic Spelling: sep-tayt

19. mutant

Pronunciation link: https://www.merriam-webster.com/dictionary/mutant

IPA: /'myü-tənt/

Phonetic Spelling: myoo-tuhnt

20. Rhodamine

Pronunciation link: [https://www.merriam-

webster.com/dictionary/rhodamine](https://www.merriam-webster.com/dictionary/rhodamine)

IPA: /'rō-də-,mēn/

Phonetic Spelling: roh·duh·meen

21. Dextran

Pronunciation link: https://www.merriam-webster.com/dictionary/dextran

IPA: /'dek-,stran/

Phonetic Spelling: dek·stran

22. kilodalton

Pronunciation link: No confirmed link found

IPA: /'kɪləˌdɒltən/

Phonetic Spelling: kil·uh·dawl·tuhn

23. molar

Pronunciation link: https://www.merriam-webster.com/dictionary/molar

IPA: /'mō-lər/

Phonetic Spelling: moh·ler

24. capillary

Pronunciation link: https://www.merriam-webster.com/dictionary/capillary

IPA: /'kəp-ə-,ler-ē/

Phonetic Spelling: kap·uh·lair·ee

25. micromanipulator

Pronunciation link: No confirmed link found

IPA: /ˌmaɪkroʊməˈnɪpjəˌleɪtər/

Phonetic Spelling: my·kroh·muh·nip·yuh·lay·ter

26. inverted

Pronunciation link: https://www.merriam-webster.com/dictionary/inverted

IPA: /ɪn-ˈvər-təd/

Phonetic Spelling: in·vur·tuhd

27. desiccant

Pronunciation link: https://www.merriam-webster.com/dictionary/desiccant

IPA: /ˈde-si-kənt/

Phonetic Spelling: dess·ih·kuhnt

28. hemocoel

Pronunciation link: No confirmed link found

IPA: /ˈhi:məˌsi:l/

Phonetic Spelling: hee·muh·seel

29. perivitelline

Pronunciation link: No confirmed link found

IPA: /ˌperiˈvɪtəlɪn/

Phonetic Spelling: peh·ree·vit·uh·line

30. Macroglobulin

Pronunciation link: https://www.merriam-webster.com/dictionary/macroglobulin

IPA: /ˌma-krō-'glä-byə-lən/

Phonetic Spelling: ma·kroh·glob·yuh·lin

31. Coracle

Pronunciation link: https://www.merriam-webster.com/dictionary/coracle

IPA: /'kôr-ə-kəl/

Phonetic Spelling: kor·uh·kuhl

32. trachea

Pronunciation link: https://www.merriam-webster.com/dictionary/trachea

IPA: /trā-kē-ə/

Phonetic Spelling: tray·kee·uh