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Title: Establishment of Zone-Enriched Primary Cultures from the Mouse Adrenal Cortex

Authors and Affiliations:

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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? **No**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

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

Filming will take place in two rooms within the same facility: the laboratory and an animal room located on a lower floor.

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. **Yes**

If **Yes**, please provide the **full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author willing to participate.

Jean Lucas Kremer, first and correspondent author.

Current Protocol Length

Number of Steps: 19

Number of Shots: 35

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**

INTRODUCTION:

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

- 1.1. **Jean Lucas Kremer:** My research investigates adrenal microenvironment regulation, focusing on ECM and Wnt signaling in zG and zF differentiation and tumor development.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Describe the research gap or limitation in existing methods and how this protocol addresses them.

- 1.2. **Jean Lucas Kremer:** Existing methods cannot clearly distinguish adrenal zones; this protocol enriches zG and zF, enabling the study of different cell types.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 2.17.1.*

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

- 1.3. **Jean Lucas Kremer:** This protocol can be applied to study adrenal physiology in zG and zF, including zonation and cell differentiation.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 2*

CONCLUSION:

What does this protocol allow researchers to study or measure?

- 1.4. **Jean Lucas Kremer:** This protocol enables the study of zone-specific physiology and differentiation, including hormone production, signaling pathways, and extracellular matrix composition.

1.4.1. 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.

- 1.5. **Jean Lucas Kremer:** Future studies can explore adrenal zonation and differentiation mechanisms and use these cells to build 3D models and study ECM interactions.

1.5.1. 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.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What value do you expect this video article to have for other researchers working in your field?

- 1.6. **Jean Lucas Kremer**, **Enter author title**: (authors will present their testimonial statements live)
 - 1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

How does the video format of your article help you to share your work with the broader scientific community?

- 1.7. **Jean Lucas Kremer**, **Enter author title**: (authors will present their testimonial statements live)
 - 1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Identification and Collection of the Adrenal Glands of the Mouse

Demonstrator: Jean Lucas Kremer

- 2.1. To isolate the adrenal gland for zone-enriched primary culture, use 10–15 young adult male mice, approximately 6–7 weeks of age [1].
 - 2.1.1. Establishing shot of the talent with the mouse in the cage. **TXT: Mice Used: Cyp11b2tm1.1(cre)BrIt/+ and Gt(ROSA)26Sortm4(ACTB-tdTomato-EGFP) Luo/J**
- 2.2. After euthanizing the mouse, place it in a dorsal decubitus position on a sterile flat surface [1-TXT] and disinfect the animal fur with 70% alcohol [2].
 - 2.2.1. WIDE: Talent placing the mouse in a dorsal decubitus position on a sterile flat surface. **TXT: Euthanasia: CO₂ inhalation**
 - 2.2.2. Talent disinfecting the animal fur with 70% alcohol.
- 2.3. Using scissors, make an initial incision in the inguinal region of the abdomen [1]. Extend the incision laterally toward the thorax on both sides to expose the peritoneum [2].
 - 2.3.1. Talent making an initial incision in the inguinal region of the abdomen.
 - 2.3.2. Talent extending the incision laterally toward the thorax on both sides to expose the peritoneum.
- 2.4. Retract the skin carefully [1]. Then, gently retract the abdominal muscles to expose the cavity, avoiding blood vessel damage to maintain clear visualization of the adrenal glands [2]. Change surgical instruments to minimize microbial contamination [3].
 - 2.4.1. Talent retracting the skin.
 - 2.4.2. Talent retracting the abdominal musculature to expose the abdominal cavity.
 - 2.4.3. Talent places a fresh set of surgical instruments in the working area.
- 2.5. Now, identify the left adrenal gland located medially and superior to the left kidney in the retroperitoneum [1] with an oval shape and pink coloration surrounded by white adipose tissue [2].
 - 2.5.1. Talent indicating the region above the left kidney.

- 2.5.2. Close-up of the left adrenal gland showing its oval shape and pink coloration surrounded by white adipose tissue.
- 2.6. Using blunt forceps, carefully displace the adjacent organs to improve visualization of the adrenal gland [1]. Using scissors, remove most of the surrounding adipose tissue with short, controlled cuts to isolate the adrenal gland [2-TXT]. Avoid directly grasping the adrenal gland to prevent damage to its capsule [3].
 - 2.6.1. Talent gently displacing adjacent organs with blunt forceps to expose the adrenal gland.
 - 2.6.2. Talent removing surrounding adipose tissue with short, controlled cuts using scissors.
 - 2.6.3. Close up of the talent removing the adrenal gland.
- 2.7. Immediately place the isolated adrenal gland into a P60 culture dish containing 10 milliliters of pre-warmed DMEM with F-12 medium, until all samples have been collected [1].
 - 2.7.1. Talent placing the isolated adrenal gland into a P60 culture dish containing pre-warmed medium.
- 2.8. Next, identify the right adrenal gland, located lateral and superior to the right kidney in the retroperitoneum [1], noting that its proximity to the liver, ascending colon, and nearby blood vessels may make visualization more challenging [2]. Carefully handle the renal veins and renal arteries to avoid accidental cuts and bleeding [3].
 - 2.8.1. Talent exposing the abdominal cavity and indicating the region above the right kidney.
 - 2.8.2. Shot showing the anatomical proximity of the adrenal gland to the liver and ascending colon.
 - 2.8.3. Shot highlighting major blood vessels near the adrenal gland.
- 2.9. After isolating the right adrenal gland using the same procedure as for the left adrenal gland, place both glands together in the same dish [1].
 - 2.9.1. Talent placing both adrenal glands together in the same culture dish.
- 2.10. Wipe the culture dish clean with 70% alcohol before transferring the glands to the laminar flow hood [1].
 - 2.10.1. Talent wiping the culture dish with 70% alcohol.
- 2.11. Then, wash the adrenal glands with PBS three times at 25 degrees Celsius using sequential dishes [1]. Collect the glands in the fourth dish after washing [2].
 - 2.11.1. Talent transferring the adrenal glands between dishes containing phosphate buffered saline for washing.
 - 2.11.2. Talent collecting the washed adrenal glands in the fourth dish.

- 2.12. Maintain separated tissues in pre-warmed DMEM to preserve viability during preparation of multiple glands [1].
- 2.12.1. Talent placing separated tissues into a dish containing pre-warmed medium.
- 2.13. Using fine forceps, grasp the adrenal gland by the adherent adipose tissue [1]. Carefully dissect the fat on the filter paper with controlled movements to avoid damaging the capsule [2].
- 2.13.1. Talent grasping the adrenal gland by the adherent adipose tissue with fine forceps.
- 2.13.2. Talent dissecting fat from the gland on filter paper using controlled movements.
Videographers's NOTE: 2.13.1 and 2.13.2 are combined in one shot.
- 2.14. Ensure the gland is smooth and intact and free of excess adipose and connective tissue [1]. Transfer the adrenal glands carefully onto fresh filter paper to facilitate precise microdissection [2].
- 2.14.1. Close-up of a clean adrenal gland with a smooth and intact surface.
- 2.14.2. Talent transferring the adrenal glands onto fresh filter paper.
- 2.15. Using fine forceps, gently pinch the capsule of the adrenal gland [1]. Using a scalpel blade, make a radial incision in the gland without completely transecting the tissue [2].
- 2.15.1. Talent pinching the capsule of the adrenal gland with fine forceps.
- 2.15.2. Talent making a radial incision in the gland with a scalpel blade without fully cutting through. **Videographers's NOTE: 2.15.1 and 2.15.2 are combined in one shot.**
- 2.16. Using fine forceps, apply slight mechanical pressure to expel the inner contents of the cortex outward through the incision [1]. Expose the inner glandular material while keeping the capsule and zona glomerulosa connected to the tip of the forceps [2].
- 2.16.1. Talent applying gentle pressure with forceps to expel inner contents of the gland.
- 2.16.2. Close-up showing the capsule and outer layer held by forceps while inner material is exposed.
- 2.17. Identify the expelled inner glandular material as the zona fasciculata and medulla [1]. Maintain separation of the outer and inner zones during handling [2].
- 2.17.1. Talent highlighting zona fasciculata and medulla
- 2.17.2. Shot showing clear separation between outer and inner zones of the gland.
- 2.18. Initiate enzymatic digestion only after all glands have been processed to ensure proper mechanical separation of the outer and inner zones [1]. After centrifugation, resuspend the collected cells in culture medium [2]. **NOTE: VO is added for additional shot.**

2.18.1. Talent placing processed glands together in preparation for enzymatic digestion.

Added shot: 2.18.2: Talent resuspending the collected cells in culture medium.

2.19. After establishing the adrenal cell culture, perform molecular characterization of cultured cells using gene expression analysis and immunostaining [1].

2.19.1. Talent at the computer screen looking at the captured images of the cells.

Results

3. Results

- 3.1. Primary adrenal cell cultures were successfully established from the outer fraction enriched for zona glomerulosa cells [1] and the inner fraction enriched for zona fasciculata cells [2].
 - 3.1.1. LAB MEDIA: Figure 2A. *Video editor: Show the full image labeled OF-zG Enriched*
 - 3.1.2. LAB MEDIA: Figure 2B. *Video editor: Show the full image labeled IF-zF Enriched*
- 3.2. The lineage tracing mouse model enabled visualization of zona glomerulosa-specific expression through mGFP driven by the Cyp11b2 (*Sip-Eleven-b-Two*) promoter [1].
 - 3.2.1. LAB MEDIA: Figure 2C.
- 3.3. mGFP expression was predominantly observed in zona glomerulosa-enriched cultures [1], while Zona fasciculata-enriched cultures primarily expressed mTomato [2].
 - 3.3.1. LAB MEDIA: Figure 2D–F. *Video editor: Highlight the green fluorescent cells in the panels labeled OF zG-Enriched*
 - 3.3.2. LAB MEDIA: Figure 2G–I. *Video editor: Highlight the red fluorescent cells in the IF zF-Enriched panels*
- 3.4. Higher relative expression levels of Cyp11b2, Dab2 (*Dab-Two*), and Sonic hedgehog were observed in outer fraction cultures compared to inner fraction cultures [1].
 - 3.4.1. LAB MEDIA: Figure 2J–L. *Video editor: Highlight the higher point or bar for OF compared to IF*
- 3.5. Inner fraction cultures showed higher relative expression of Cyp11b1 and Akr1b7 (*A-K-R-One-B-Seven*) compared to outer fraction cultures [1].
 - 3.5.1. LAB MEDIA: Figure 2M and N. *Video editor: Highlight the higher point or bar for IF compared to OF*
- 3.6. Aldosterone secretion was higher in zona glomerulosa-enriched cultures than in zona fasciculata-enriched cultures [1]. In outer fraction cultures, aldosterone secretion further increased upon stimulation with angiotensin II, potassium, and adrenocorticotrophic hormone [2].
 - 3.6.1. LAB MEDIA: Figure 2O. *Video editor: Highlight the taller bar for OF compared to IF*
 - 3.6.2. LAB MEDIA: Figure 2P. *Video editor: Highlight the bar labeled AngII, K+ and ACTH*

3.7. Corticosterone secretion was higher in zona fasciculata-enriched cultures compared to zona glomerulosa-enriched cultures [1]. Zona fasciculata-enriched cultures showed increased corticosterone secretion in response to adrenocorticotrophic hormone and forskolin stimulation [2].

3.7.1. LAB MEDIA: Figure 2Q. *Video editor: Highlight the taller bar for IF compared to OF*

3.7.2. LAB MEDIA: Figure 2R. *Video editor: Highlight the bar labeled ACTH and Forskolin*

Pronunciation guides:

1. Adrenal

Pronunciation link: <https://www.merriam-webster.com/dictionary/adrenal>

IPA: /ə'driː.nəl/

Phonetic Spelling: uh·dree·nuhl

2. Decubitus

Pronunciation link: <https://www.merriam-webster.com/dictionary/decubitus>

IPA: /dɪ'kjuː.bə.təs/

Phonetic Spelling: dih·kyoo·buh·tuhs

3. Inguinal

Pronunciation link: <https://www.merriam-webster.com/dictionary/inguinal>

IPA: /'ɪŋ.gwə.nəl/

Phonetic Spelling: ing·gwuh·nuhl

4. Peritoneum

Pronunciation link: <https://www.merriam-webster.com/dictionary/peritoneum>

IPA: /,per.ə.tə'niː.əm/

Phonetic Spelling: peh·ruh·tuh·nee·uhm

5. Retroperitoneum

Pronunciation link: <https://www.merriam-webster.com/dictionary/retroperitoneum>

IPA: /,re.trəʊ.per.ə.tə'niː.əm/

Phonetic Spelling: reh·troh·peh·ruh·tuh·nee·uhm

6. Adipose

Pronunciation link: <https://www.merriam-webster.com/dictionary/adipose>

IPA: /'æd.ə.pɒs/

Phonetic Spelling: ad·uh·pohs

7. Thorax

Pronunciation link: <https://www.merriam-webster.com/dictionary/thorax>

IPA: /'θɔːr.æks/

Phonetic Spelling: thor·aks

8. Laminar

Pronunciation link: <https://www.merriam-webster.com/dictionary/laminar>

IPA: /'læm.ə.nər/

Phonetic Spelling: lam·uh·ner

9. Phosphate

Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>

IPA: /'fɑːs.feɪt/

Phonetic Spelling: faas·fayt

10. Microdissection

Pronunciation link: <https://www.merriam-webster.com/dictionary/microdissection>

IPA: /,maɪ.kroʊ.dɪ'sek.ʃən/

Phonetic Spelling: my·kroh·dih·sek·shuhn

11. Enzymatic

Pronunciation link: <https://www.merriam-webster.com/dictionary/enzymatic>

IPA: /,en.zaɪ'mætɪk/

Phonetic Spelling: en·zy·mat·ik

12. Cortex

Pronunciation link: <https://www.merriam-webster.com/dictionary/cortex>

IPA: /'kɔːr.teks/

Phonetic Spelling: kor·teks

13. Capsule

Pronunciation link: <https://www.merriam-webster.com/dictionary/capsule>

IPA: /'kæp.sjuːl/

Phonetic Spelling: kap·syool

14. Medulla

Pronunciation link: <https://www.merriam-webster.com/dictionary/medulla>

IPA: /mə'dʌl.ə/

Phonetic Spelling: muh·duh·luh

15. Aldosterone

Pronunciation link: <https://www.merriam-webster.com/dictionary/aldosterone>

IPA: /æl'dɑː.stə.roʊn/

Phonetic Spelling: al·daa·stuh·rohn

16. Corticosterone

Pronunciation link: <https://www.merriam-webster.com/dictionary/corticosterone>

IPA: /ˌkɔːr.tɪ'kɑː.stə.roʊn/

Phonetic Spelling: kor·tuh·kaa·stuh·rohn

17. Angiotensin

Pronunciation link: <https://www.merriam-webster.com/dictionary/angiotensin>

IPA: /ˌæŋ.dʒi.oʊ'ten.sɪn/

Phonetic Spelling: an·jee·oh·ten·sin

18. Adrenocorticotropic

Pronunciation link: <https://www.merriam-webster.com/dictionary/adrenocorticotropic>

IPA: /əˌdriː.noʊˌkɔːr.tɪˌkoʊ.trə'pɪk/

Phonetic Spelling: uh·dree·noh·kor·tuh·koh·troh·pik

19. Forskolin

Pronunciation link: <https://www.merriam-webster.com/dictionary/forskolin>

IPA: /fɔːr'skoʊ.lɪn/

Phonetic Spelling: for·skoh·lin

20. Immunostaining

Pronunciation link: <https://www.merriam-webster.com/dictionary/immunostaining>

IPA: /ɪˌmjuː.noʊ'steɪ.nɪŋ/

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