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Title: A Neonatal Mouse Model of Necrotizing Enterocolitis and Lamina Propria Isolation for Immune Cell Profiling

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? **No**
- 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: 21

Number of Shots: 46

Introduction

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

~~REQUIRED: What is the scope of your research? What questions are you trying to answer?~~

- 1.1. **Misty Good:** Our research focuses on the disease affecting medically fragile infants called necrotizing enterocolitis. We aim to uncover the mechanisms involved in the immune response in NEC and develop strategies for early diagnosis and prevention of the disease.

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

~~What are the most recent developments in your field of research?~~

- 1.2. **Lauren Bolzan:** Single-cell and multi-omics approaches are uncovering immune cell signatures in NEC, offering new insights into mechanisms and potential biomarkers.

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

~~What are the current experimental challenges?~~

- 1.3. **Danielle Sklar:** Modeling NEC is challenging because the disease is multifactorial. Replicating the human intestinal environment, immune responses, and microbial influences in neonatal mice requires careful optimization.

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

~~What research gap are you addressing with your protocol?~~

- 1.4. **Gergely Mozes:** It is very challenging to study intestinal immune cell responses in neonatal NEC. Our protocol fills this gap by demonstrating how to isolate lamina propria immune cells for analysis flow cytometry.

- 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:3.7*

~~What advantage does your protocol offer compared to other techniques?~~

- 1.5. **Gergely Mozes:** Our model integrates human-derived microbiome, hypoxia, and formula feeding, making it physiologically relevant, allowing for detailed immune cell profiling in neonatal mice.

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.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill School of Medicine

Protocol

2. Preparation and Administration of NEC Formula in a Murine Model of Necrotizing Enterocolitis

Demonstrators: Lauren Bolzan, Journie Roper, Gergely Mozes

- 2.1. To begin, acquire a culture of enteric bacteria cultured from an infant with *necrotizing enterocolitis totalis* or NEC (*N-E-C*) [1]. Pipette a 20-microliter aliquot of the culture into a 15-milliliter culture tube containing 2 milliliters of Luria-Bertani broth [2-TXT].
 - 2.1.1. WIDE: Talent holding a vial of *NEC totalis* culture.
 - 2.1.2. Talent pipetting 20 microliters of bacterial culture into the 15-milliliter culture tube. **TXT: Day 0, 3 PM**
- 2.2. Incubate the bacterial culture at 37 degrees Celsius for 16 hours in an orbital shaker set to 1 g, ensuring the tube lid is kept in a vented position [1]. Concurrently, incubate a second culture tube containing only 2 milliliters of Luria-Bertani broth as a control to check for any contamination [2].
 - 2.2.1. Talent placing the tube into an orbital shaker with the lid in the vented position and starting overnight incubation at 37 degrees Celsius.
 - 2.2.2. Talent labeling and placing the control tube with Luria-Bertani broth into the same shaker.
- 2.3. The next day, at 7 AM, pipette 125 microliters of the overnight bacterial culture into a T75 (*T-Seventy-five*) cell culture flask with a vented lid containing 25 milliliters of Luria-Bertani broth [1]. Prepare a total of four T75 flasks [2] and incubate in an upright position at 37 degrees Celsius for 2 hours in an orbital shaker set at 1 g [3].
 - 2.3.1. Talent pipetting the overnight culture into the T75 flask with 25 milliliters of broth.
 - 2.3.2. Shot of 4 prepared T75 flasks.
 - 2.3.3. Talent placing the upright flasks into the orbital shaker and starting the 2-hour incubation.
- 2.4. At 9 AM on experimental days 1, 2, and 3, combine 16 milliliters of infant formula with 8 milliliters of puppy milk in a sterile 50-milliliter centrifuge tube to prepare the formula mixture [1]. Now, remove the supernatant from two 50-milliliter centrifuge tubes containing the pelleted bacterial culture [2].
 - 2.4.1. Talent pouring 16 milliliters of infant formula and 8 milliliters of puppy milk into a sterile centrifuge tube.
 - 2.4.2. Talent carefully decanting the supernatant from both centrifuge tubes, leaving the bacterial pellet undisturbed.

- 2.5. Thoroughly resuspend each of the two pellets in 2 milliliters of the prepared formula mix [1]. Then transfer both resuspended mixtures into the remaining formula mix to create a total of 24 milliliters of NEC formula [2].
 - 2.5.1. Talent pipetting 2 milliliters of formula mix into each tube and resuspending the pellets.
 - 2.5.2. Talent transferring both resuspended mixtures into one tube containing the remaining formula to reach 24 milliliters total.
- 2.6. Now, label six 50 milliliter centrifuge tubes with the six feeding time points [1-TXT]. Pipette 4 milliliters of the NEC formula into each of the six labeled tubes [2].
 - 2.6.1. Talent writing time points on labels and attaching them to six centrifuge tubes.
TXT: Feeding points: 10:00, 13:00, 16:00, 19:00, 22:00, 07:00
 - 2.6.2. Talent pipetting 4 milliliters of NEC formula into each labeled centrifuge tube.
- 2.7. Next, prepare six single-use aliquots of lipopolysaccharide stock at 5 milligrams per milliliter [1-TXT]. ~~Add lipopolysaccharide to the NEC formula immediately before each feeding time point [2].~~ **NOTE: The VO is edited for the deleted shot**
 - 2.7.1. Talent aliquoting the lipopolysaccharide stock into low-binding microtubes. **TXT: Store in low-binding microtubes at - 20 °C**
 - ~~2.7.2. Talent pipetting the lipopolysaccharide aliquot into NEC formula.~~ **NOTE: Not filmed, redundant information**
- 2.8. Just before feeding, thaw one lipopolysaccharide aliquot and vortex for 15 seconds [1]. Add a calculated volume of the lipopolysaccharide to a 4 milliliter NEC formula tube and mix well by pipetting [2].
 - 2.8.1. Talent vortexing the thawed lipopolysaccharide aliquot.
 - 2.8.2. Talent pipetting the calculated volume of lipopolysaccharide into NEC formula and mixing by pipetting.
- 2.9. Now, fill a 1 milliliter syringe with the prepared NEC formula [1] and attach it to a neonatal PICC (*Pick*) Line [2-TXT].
 - 2.9.1. Talent drawing NEC formula into a syringe. ‘
 - 2.9.2. Talent securing a PICC line to the syringe. **TXT: PICC: Peripherally Inserted Central Catheter Line**
- 2.10. To administer the NEC formula to each pup via oral gavage, gently restrain the pup by the loose skin at the base of the neck and hold it upright [1]. Using forceps, guide the PICC line into the oropharynx and down into the stomach [2-TXT].
 - 2.10.1. Talent gripping the pup’s loose skin and holding it upright.
 - 2.10.2. Talent using forceps to insert the PICC line into the oropharynx and stomach.
TXT: If resistance is encountered, remove PICC line, reposition and attempt re-

insertion

- 2.11. Dispense 80 microliters of NEC formula slowly into the stomach [1]. Monitor the pup for signs of aspiration during feeding [2].
 - 2.11.1. Talent dispensing the formula slowly.
 - 2.11.2. Shot of the pup during administration.
- 2.12. After feeding, remove the PICC line [1] and observe the pup for any laboured breathing or vomiting [2]. If regurgitation occurs, blot the pup's mouth and nose using a low-lint laboratory wipe [3-TXT].
 - 2.12.1. Talent carefully withdrawing the PICC line.
 - 2.12.2. Talent checking the pup's breathing and signs of distress.
 - 2.12.3. Talent blotting the mouth and nose of the pup with a wipe. **TXT: Repeat feeding at all time points**

3. Isolation of Immune Cells from Lamina Propria of Neonatal Mice

Demonstrators: Gergely Mozes, Lauren Bolzan, Journie Roper

- 3.1. Obtain the intestinal tissue from the euthanized neonatal mice [1]. With a pair of scissors, cut the intestine longitudinally to open it [2], then section it transversely into 1-to-1.5-centimeter segments [3].
 - 3.1.1. Shot of intestines from neonatal mice.
 - 3.1.2. Talent cutting open the intestine along its length on a dissection board.
 - 3.1.3. Talent sectioning the opened intestine into 1 to 1.5 centimeter pieces.
- 3.2. Transfer the intestinal segments into a 15-milliliter centrifuge tube containing 10 milliliters of pre-digestion solution that has been cooled on ice [1]. Then incubate the tube in a rotating incubator at 37 degrees Celsius for 20 minutes to begin tissue dissociation [2].
 - 3.2.1. Talent using forceps to place intestinal pieces into a centrifuge tube with pre-chilled pre-digestion solution.
 - 3.2.2. Talent placing the tube in the rotating incubator and starting a 20-minute timed incubation at 37 degrees Celsius.
- 3.3. Gently swirl the tube to dislodge loosened tissue [1], then pour the contents through a 100-micrometer cell strainer placed on top of a 50-milliliter centrifuge tube [2].
 - 3.3.1. Talent gently swirling the tube.
 - 3.3.2. Talent pouring the tube contents through a 100 micrometer cell strainer into a

50 milliliter centrifuge tube.

- 3.4. Transfer the remaining tissue fragments into a new 15-milliliter centrifuge tube containing 10 milliliters of pre-heated digestion solution [1-TXT]. Incubate the sample with continuous rotation for 40 minutes at 37 degrees Celsius [2].
 - 3.4.1. Talent transferring undigested fragments into a new centrifuge tube with warm digestion buffer. **TXT: Pre-heat digestion solution to 37 °C**
 - 3.4.2. Talent placing the tube into the rotating incubator for 40-minute incubation.
- 3.5. Then transfer the contents into an automated tissue dissociation tube [1]. Run the program with four 15-second alternating clockwise and counterclockwise rotation cycles [2].
 - 3.5.1. Talent loading sample into the automated dissociation system.
 - 3.5.2. Talent activating the rotation program.
Videographer: Please capture the instrument screen for this shot
- 3.6. When the dissociation is complete, gently vortex the cell suspension in the dissociation tube [1] and then centrifuge it at 300 g for 5 minutes at 4 degrees Celsius [2].
 - 3.6.1. Talent briefly vortexing the dissociation tube.
 - 3.6.2. Talent placing the tube in a centrifuge and starting the spin at 300 g for 5 minutes.
- 3.7. Carefully aspirate the supernatant without disturbing the pellet [1]. Resuspend the cells in 1 milliliter of ice-cold fluorescence-activated cell sorting buffer [2].
 - 3.7.1. Talent aspirating the supernatant with a pipette, avoiding the pellet.
 - 3.7.2. Talent resuspending the pellet in 1 milliliter of cold FACS buffer.
- 3.8. Now, place a 100-micrometre cell strainer over a 50-milliliter centrifuge tube [1], transfer the resuspended cells through the strainer [2], and wash it with 2 milliliters of cold fluorescence-activated cell sorting buffer [3].
 - 3.8.1. Talent setting the strainer over the tube.
 - 3.8.2. Talent pouring the suspension through the strainer.
 - 3.8.3. Talent rinsing the strainer with additional buffer.
- 3.9. Count the isolated cells and assess viability using trypan blue or a viability dye [1]. Centrifuge the desired volume for flow cytometry at 300 g for 5 minutes at 4 degrees Celsius [2].
 - 3.9.1. Talent pipetting cell sample onto a counting slide and checking viability under the microscope.
 - 3.9.2. Talent centrifuging the required volume in preparation for flow cytometry.

Results

4. Results

- 4.1. A significant reduction in survival in the NEC treatment group was observed compared to the dam-fed controls [1]. Gross intestinal examination of NEC mice demonstrated hallmark features of NEC, including visible signs of distension, air accumulation, and pneumatosis intestinalis compared to healthy controls [2].
 - 4.1.1. LAB MEDIA: Figure 1A. *Video editor: Highlight dotted curved for NEC*
 - 4.1.2. LAB MEDIA: Figure 1B (Macroscopy). *Video editor: Highlight the NEC panel*
- 4.2. Hematoxylin and eosin staining revealed patchy villus destruction and disrupted intestinal architecture in NEC mice, compared to intact villi in controls [1].
 - 4.2.1. LAB MEDIA: Figure 1B (H&E). *Video editor: Highlight the NEC image*
- 4.3. Expression of *Il1b* (*Eye-L-one-B*), *Cxcl2* (*C-X-C-L-Two*) and *Lcn2* (*L-C-N-Two*) messenger RNA was significantly higher in the NEC group than in controls [1].
 - 4.3.1. LAB MEDIA: Figure 1C *Video editor: Highlight the NEC bar in all 3 graphs*
- 4.4. Total CD45-positive immune cells in the intestinal lamina propria were not significantly different between NEC and control groups [1]. Macrophage levels were significantly elevated in NEC intestines compared to controls [2].
 - 4.4.1. LAB MEDIA: Figure 2B (Left).
 - 4.4.2. LAB MEDIA: Figure 2B (Right). *Video editor: Highlight the taller NEC bar*
- 4.5. NEC mice showed an increased proportion of myeloid cells, particularly monocytes and macrophages, relative to other immune subtypes [1].
 - 4.5.1. LAB MEDIA: Figure 2C (Myeloid pie charts). *Video editor: Highlight the pink and red wedges in the NEC myeloid pie chart of NEC*

1. Enteric
Pronunciation link: <https://www.merriam-webster.com/dictionary/enteric>
IPA: /ɛnˈtɛrɪk/
Phonetic Spelling: en·teh·rik
2. Necrotizing
Pronunciation link: <https://www.merriam-webster.com/dictionary/necrotizing>
IPA: /ˈnɛkrəˌtaɪzɪŋ/
Phonetic Spelling: neh·kruh·ty·zing
3. Enterocolitis
Pronunciation link: <https://www.merriam-webster.com/dictionary/enterocolitis>
IPA: /ˌɛn.tə.roʊ.kəˈlaɪ.tɪs/
Phonetic Spelling: en·tuh·roh·kuh·ly·tuhs
4. Aliquot
Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>
IPA: /ˈæliˌkwɑːt/
Phonetic Spelling: al·uh·kwaat
5. Luria-Bertani
Pronunciation link: No confirmed link found
IPA: /ˈlʊriə bərˈtɑːni/
Phonetic Spelling: lu·ree·uh ber·taa·nee
6. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /ˈɪŋkjəˌbeɪt/
Phonetic Spelling: ing·kyuh·bayt
7. Orbital
Pronunciation link: <https://www.merriam-webster.com/dictionary/orbital>
IPA: /ˈɔːrbɪtəl/
Phonetic Spelling: or·buh·tl
8. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈnɛntənt/
Phonetic Spelling: soo·pruh·nay·tuhnt
9. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsɛntrɪˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
10. Resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /ˌriːsəˈspɛnd/
Phonetic Spelling: ree·suh·spend
11. Lipopolysaccharide
Pronunciation link: <https://www.merriam-webster.com/dictionary/lipopolysaccharide>
IPA: /ˌlaɪpəʊˌpɑːliˈsækəraɪd/
Phonetic Spelling: ly·poh·pol·ee·sak·uh·ryd

12. Vortex
Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>
IPA: /'vɔːr,tɛks/
Phonetic Spelling: vor·teks
13. Neonatal
Pronunciation link: <https://www.merriam-webster.com/dictionary/neonatal>
IPA: /,niːoʊ'nertəl/
Phonetic Spelling: nee·oh·nay·tl
14. Oropharynx
Pronunciation link: <https://www.merriam-webster.com/dictionary/oropharynx>
IPA: /,ɔːroʊ'færɪŋks/
Phonetic Spelling: or·oh·fa·ringks
15. Gavage
Pronunciation link: <https://www.merriam-webster.com/dictionary/gavage>
IPA: /gə'vɑːʒ/
Phonetic Spelling: guh·vaazh
16. Aspiration
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspiration>
IPA: /,æspə'reɪʃən/
Phonetic Spelling: as·puh·ray·shuhn
17. Regurgitation
Pronunciation link: <https://www.merriam-webster.com/dictionary/regurgitation>
IPA: /rɪ,gəːdʒə'teɪʃən/
Phonetic Spelling: ree·gur·juh·tay·shuhn
18. Lamina Propria
Pronunciation link: <https://www.merriam-webster.com/dictionary/lamina%20propria>
IPA: /'læmɪnə 'prɒpɹiə/
Phonetic Spelling: la·muh·nuh proh·pree·uh
19. Euthanized
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>
IPA: /'juːθəˌnaɪzd/
Phonetic Spelling: yoo·thuh·nyzd
20. Longitudinally
Pronunciation link: <https://www.merriam-webster.com/dictionary/longitudinally>
IPA: /,lɔːndʒə'tuːdənəli/
Phonetic Spelling: lon·juh·too·duh·nuh·lee
21. Transversely
Pronunciation link: <https://www.merriam-webster.com/dictionary/transversely>
IPA: /trænz'vɜːsli/
Phonetic Spelling: tranz·vurss·lee
22. Dissociation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>
IPA: /dɪˌsoʊsi'eɪʃən/
Phonetic Spelling: dih·soh·see·ay·shuhn

23. Micrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪˈkraːmətər/
Phonetic Spelling: my·krah·muh·ter
24. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊˈresəns/
Phonetic Spelling: floo·res·uhns
25. Cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /saɪˈtɑːmətri/
Phonetic Spelling: sy·tom·uh·tree
26. Pneumatosis intestinalis
Pronunciation link: No confirmed link found
IPA: /ˌnuːməˈtoʊsɪs ɪnˌtɛstəˈnælɪs/
Phonetic Spelling: noo·muh·toh·suhs ɪnˌtes·tuh·nal·is
27. Hematoxylin
Pronunciation link: <https://www.merriam-webster.com/dictionary/hematoxylin>
IPA: /ˌhiːmətɑːkˈsɪlɪn/
Phonetic Spelling: hee·muh·tok·suh·lin
28. Eosin
Pronunciation link: <https://www.merriam-webster.com/dictionary/eosin>
IPA: /ˈiːoʊsɪn/
Phonetic Spelling: ee·oh·sin
29. Villus
Pronunciation link: <https://www.merriam-webster.com/dictionary/villus>
IPA: /ˈvɪləs/
Phonetic Spelling: vil·uhs
30. Macrophage
Pronunciation link: <https://www.merriam-webster.com/dictionary/macrophage>
IPA: /ˈmækroʊˌfeɪdʒ/
Phonetic Spelling: mak·roh·fayj
31. Myeloid
Pronunciation link: <https://www.merriam-webster.com/dictionary/myeloid>
IPA: /ˈmaɪəˌlɔɪd/
Phonetic Spelling: my·uh·loyd
32. Monocytes
Pronunciation link: <https://www.merriam-webster.com/dictionary/monocyte>
IPA: /ˈmɑːnəˌsaɪts/
Phonetic Spelling: mon·uh·syts