Submission ID #: 61609
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18790793

Title: A Neonatal Imaging Model of Gram-Negative Bacterial Sepsis

Authors and Affiliations: Brittany G. Seman[1*], Jessica M. Povroznik[1,2*], Jordan K. Vance[1], Travis W. Rawson[1], and Cory M. Robinson[1,2]
*These authors contributed equally

[1]Department of Microbiology, Immunology, & Cell Biology, West Virginia University School of Medicine
[2]Vaccine Development Center at West Virginia University Health Sciences Center

Corresponding Author:
Cory M. Robinson
cory.robinson1@hsc.wvu.edu

Co-Authors:
brittany.seman@hsc.wvu.edu
jessica.povroznik@hsc.wvu.edu
jkv0003@mix.wvu.edu
twr0001@mix.wvu.edu

Author Questionnaire

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

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as reasonably possible.

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **49**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Cory Robinson**: Our protocol allows bacterial dissemination and burden tracking in an experimental neonatal sepsis model in real time and the ability to correlate other markers of disease with the pathogen burden **[1]**.

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

REQUIRED:

1.2. **Brittany Seman**: This protocol allows us to longitudinally analyze sepsis in individual neonatal mice, providing the opportunity to observe and compare infection dynamics and bacterial dissemination in real time **[1]**.

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

OPTIONAL:

1.3. **Jessica Povroznik**: Preclinical testing of interventions for neonatal sepsis can be performed with this method, allowing monitoring of the effects on host control of the bacteria **[1]**.

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

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at West Virginia University.

Protocol

2. Subscapular Inoculation

2.1. Begin by placing age-matched pups into either high- or low-dose litter groups within a biosafety level 2 cabinet **[1-TXT]**.

2.1.1. WIDE: Talent placing pup(s) into cage **TEXT: Randomly assign pups as control or infected**

2.2. On postnatal day 3 or 4, record the weights of all the pups prior to inoculation **[1-TXT]**.

2.2.1. Talent placing pup onto balance **TEXT: Separate dam from pups during inoculation procedure to ensure pups not moved during infection**

2.3. Load insulin syringes with PBS or the high- or low-dose *E. coli-lux* inoculum in the biosafety cabinet **[1-TXT]** and place the loaded syringes on ice **[2]**.

2.3.1. Talent loading syringe, with PBS and inocula containers visible in frame **TEXT: *e.g.*, low dose: 2 x 10^6 CFUs; high dose: 7 x 10^6 CFUs**
2.3.2. Talent placing syringe(s) on ice

2.4. Place the neonate to be injected onto a clean surface within the biosafety cabinet **[1]** and raise the skin at the nape of the neck as if to scruff the pup **[2]**.

2.4.1. Talent placing neonate onto clean surface *Videographer: Important step*
2.4.2. Skin being lifted *Videographer: Important step*

2.5. Insert the needle, bevel up, just beneath the skin in the space created between the skin and the muscle **[1]**. When the needle can be felt under the skin, inject 50 microliters of the inoculant while simultaneously releasing the pinched portion of the skin to prevent backflow **[2]**.

2.5.1. Needle being inserted *Videographer: Important/difficult step*
2.5.2. Inoculant being injected/skin being released *Videographer: Important/difficult step*

2.6. Then remove the needle slowly and with care **[1]**. When all of the pups have been injected in the same manner, return the pups to their dams **[2]**.

2.6.1. Needle being removed *Videographer: Important step*

2.6.2. Talent placing pup into cage *Videographer: Important step*

3. In Vivo Bacterial Burden Imaging

3.1. Immediately after the injection and at the appropriate experimental timepoints thereafter, place the cage with *E. coli-lux*-infected neonatal mice and dam into a biosafety level-2 laminar flow hood **[1]** and open the software in the microCT (micro-C-T) computer **[2-TXT]**.

3.1.1. WIDE: Talent placing cage into hood
3.1.2. Talent opening software, with monitor visible in frame **TEXT: CT: computed tomography**

3.2. After initializing the system and waiting for the CCD (C-C-D) temperature to lock at 37 degrees Celsius **[1]**, place up to four anesthetized pups onto the imaging box in the imaging chamber in the prone position **[2-TXT]**.

3.2.1. Talent initializing system
3.2.2. Talent placing pup(s) into box *Videographer: More Talent than pup; Important/difficult step* **TEXT: Anesthesia: 5% -> 2-4% isoflurane**

3.3. Shut the imaging chamber door **[1]** and select the **Luminescent** imaging option **[2]**.

3.3.1. Talent shutting door
3.3.2. SCREEN: screehshot_1: 00:02-00:07

3.4. Select **Open Filter** and **Next [1]** and set the excitation filter to **Block** and the emission filter to **Open [2]**.

3.4.1. SCREEN: screenshot_1: 00:08-00:19
3.4.2. SCREEN: screenshot_1: 00:20-00:23

3.5. Select 500, 520, 560, 580, 600, and 620 nanometers **[1]**.

3.5.1. SCREEN: screehshot_1: 00:24-01:06 *Video Editor: please speed up*

3.6. Then image the pups **[1]** before returning them to the cage with the dam with monitoring until anesthesia recovery **[2]**.

3.6.1. SCREEN: SCREEN: screenshot_2: 00:54-03:07 *Video Editor: please speed up*
Author NOTE: I don't know if it really needs to go all the way to 03:07, you get the idea by 01:04, but it adds a bunch of imaging panels, so the additional imaging panels can be included (or not) at the discretion the video editor.

3.6.2. Talent placing pup into cage *Videographer: More Talent than pup in shot*

4. Tissue Harvest

4.1. At the appropriate experimental end point, in a biosafety cabinet, douse the euthanized neonate with 70% ethanol to prevent contamination **[1-TXT]** and place the animal on its right side **[2]**.

4.1.1. WIDE: Talent spraying ethanol onto pup *Videographer: More Talent than pup in shot* **TEXT: Euthanasia: anesthesia + decapitation**
4.1.2. Talent placing mouse onto side *Videographer: More Talent than pup in shot*

4.2. Use forceps to grasp the skin between the abdomen and rear left leg **[1]** and use fine-tipped surgical scissors to make a skin incision **[2]**.

4.2.1. Skin being grasped *Videographer: Important step*
4.2.2. Incision being made *Videographer: Important step*

4.3. Continue the incision toward the back of the animal until the entire spleen is exposed **[1]**.

4.3.1. Incision being made

4.4. Use the forceps and scissors to remove the spleen from the abdomen **[1]** and place it in the solution appropriate for its downstream application **[2]**.

4.4.1. Spleen being removed *Videographer: Important step*
4.4.2. Talent placing spleen into solution *Videographer: Important step*

4.5. To obtain the lungs, peel back the skin of the chest completely **[1]** and, entering at the base of the sternum with the scissors held vertically, cut upward until the rib cage is split **[2]**.

4.5.1. Skin being peeled
4.5.2. Ribs being split

4.6. Use forceps to grasp the right and left lungs individually **[1]** and remove the lungs from the thoracic cavity **[2]**. Remove the heart from the lung tissue with scissors **[3]**.

4.6.1. Lung being grasped *Videographer: Can combine 4.6.1. and 4.6.2. as appropriate*
4.6.2. Lungs being removed *Videographer: Can combine 4.6.1. and 4.6.2. as appropriate*

4.6.3. Heart being removed

4.7. Then place the lung in the solution appropriate for its downstream application **[1]**.

4.7.1. Talent placing lung into solution

5. In Vitro Bacterial Killing Assay

5.1. To perform an in vitro bacterial killing assay, place the uninfected spleen into a 40-micrometer nylon strainer within a sterile 60-millimeter Petri dish containing 5 milliliters of PBS supplemented with 10% fetal bovine serum **[1]** and use a sterile, 3-milliliter syringe plunger to disaggregate the tissue into a single cell suspension **[2]**.

5.1.1. WIDE: Talent placing spleen into strainer
5.1.2. Spleen(s) being mashed

5.2. Transfer the cells to a 15-milliliter centrifuge tube **[1]** and collect them by centrifugation **[2-TXT]**.

5.2.1. Talent adding cells to tube
5.2.2. Talent placing tube(s) into centrifuge **TEXT: 5 min, 350 x g, RT**

5.3. Resuspend the pellet in 1 milliliter of red blood cell lysis buffer per 3-4 spleens **[1]**.

5.3.1. Shot of red pellet, then buffer being added to tube, with buffer container visible in frame

5.4. After 5 minutes at room temperature, wash the spleens in PBS **[1]** and resuspend the pellet in 250 microliters of PBS supplemented with bovine serum albumin and 2-millimolar EDTA for counting **[2]**.

5.4.1. Talent adding PBS to tube, with PBS container visible in frame
5.4.2. Shot of white pellet, then PBS+BSA+EDTA being added to tube, with PBS+BSA+EDTA container visible in frame

5.5. Next, isolate the Ly6B.2 (lie-six-B-two)-positive cells with the appropriate immunomagnetic beads according to manufacturer's protocol **[1]** and seed the enriched Ly6B.2-positive cells at a 1 x 10^5 cells/100 microliters of complete medium per well density in a black or white 96-well plate **[2-TXT]**.

5.5.1. Talent adding cells to magnet, with bead kit visible in frame

5.5.2. Talent adding medium to cells, with medium container visible in frame **TEXT: See text for all medium and solution preparation details**

5.6. Prepare the bacterial inoculum at the desired multiplicity of infection in a final volume of 100 microliters per well **[1]** and add 100 microliters of bacterial inoculum or medium alone to each well for a 1-hour incubation in the cell culture incubator **[2]**.

5.6.1. Talent adding medium to bacteria, with medium container visible in frame
5.6.2. Talent adding bacteria to well(s)

5.7. At the end of the incubation, replace the medium with 200 microliters of fresh complete medium supplemented with 100 micrograms/milliliter of gentamicin to remove any remaining extracellular bacteria **[1]** and return the cells to the cell culture incubator for an additional 2 hours **[2]**.

5.7.1. Talent adding medium to well(s), with medium and gentamicin containers visible in frame
5.7.2. Talent placing plate into incubator

5.8. At the end of the incubation and at each experimental time point thereafter, use a plate reader to measure the luminescence in each well of the lidded culture plate **[1]** before returning the plate to the cell culture incubator until the next reading **[2-TXT]**.

5.8.1. Talent placing plate onto plate reader
5.8.2. Talent placing plate into incubator **TEXT: Discard in BSL2 waste after last measurement**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.4.-2.6., 3.2., 4.2., 4.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
2.5. The person doing the injection must have steady hands, be able to feel the needle go just under the skin, inject slowly, and remove the needle without pulling the inoculum out in the process.
3.2. The person anesthetizing the pups must be able to anesthetize them with enough isoflurane (typically more than an adult needs), but not over anesthetize to promote mortality.

Results

6. Results: Representative Gram-Negative Bacterial Sepsis Imaging in Murine Neonates

6.1. While most animals exhibit high levels of bacteria in the blood at 24 hours post infection **[1]**, some pups have low or undetectable bacteria in the blood **[2]**, suggesting clearance of the infection by this time point **[3]**.

6.1.1. LAB MEDIA: Figure 1C *Video Editor: please emphasize black and grey data points between 6-10 CFU*
6.1.2. LAB MEDIA: Figure 1C *Video Editor: please emphasize black and grey data points between 0-4 CFU*
6.1.3. LAB MEDIA: Figure 1C

6.2. In addition, neonatal Ly6B.2-positive splenic cells infected with bioluminescent *E. coli* for 1 hour before the removal of extracellular bacteria and subsequent gentamicin treatment **[1]** express a high level of luminescence at 3 hours **[2]** that decreases over time, indicative of bacterial clearance **[3]**.

6.2.1. LAB MEDIA: Figure 5
6.2.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize data bars at 3 h*
6.2.3. LAB MEDIA: Figure 5 *Video Editor: please sequentially add/emphasize data bars at 6, 20, and 48 hours*

6.3. Live animal imaging of the luminescent bacteria **[1]** further confirms the increase in bacteria dissemination and growth in neonatal pups over time at 10- and 24-hours post infection **[2]**.

6.3.1. LAB MEDIA: Figure 2A
6.3.2. LAB MEDIA: Figure 2A *Video Editor: please sequentially emphasize 0, 10, and 24 hpi images*

6.4. Intravital imaging in conjunction with microCT **[1]** facilitates the identification of infection foci within the brain **[2]**, lungs **[3]**, and other peripheral tissues **[4]**.

6.4.1. LAB MEDIA: Figure 2B
6.4.2. LAB MEDIA: Figure 2B *Video Editor: please emphasize right white arrow in 10 hpi image*
6.4.3. LAB MEDIA: Figure 2B *Video Editor: please emphasize bottom white arrow in 24 hpi image*

6.4.4. LAB MEDIA: Figure 2B *Video Editor: please emphasize left white arrow in 10 hpi image and top right arrow in 24 hpi image*

6.5. The lungs of some highly infected mice **[1]** demonstrate opaque regions **[2]** consistent with inflammatory consolidation that co-localize to luminescent bacterial signals **[3]**.

6.5.1. LAB MEDIA: Figure 3A *Video Editor: please emphasize white arrow in Inoculum images*
6.5.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize opaque region indicated by white arrow in top Inoculum image*
6.5.3. LAB MEDIA: Figure 3A *Video Editor: please emphasize blue signal indincated by white arrow in bottom Inoculum image*

6.6. These regions of presumed inflammatory exudate are not observed in uninfected control lungs **[1]**.

6.6.1. LAB MEDIA: Figure 3A *Video Editor: please emphasize white arrow in Control image*

6.7. A significant increase in inflammatory cytokine expression **[1]** relative to controls is also observed in both low and high inoculum groups **[2]**, correlating with a notable thickening of the alveolar wall, increased alveolar hemorrhaging, and inflammatory infiltration in these animals **[3]**.

6.7.1. LAB MEDIA: Figure 4A
6.7.2. LAB MEDIA: Figure 4A *Video Editor: please add/emphasize asterisks*
6.7.3. LAB MEDIA: Figures 4B, 4C, and 4D *Video Editor: please emphasize yellow arrows in both images*

Conclusion

7. Conclusion Interview Statements

7.1. **Brittany Seman**: The most important things to remember are to execute proper technique when performing the injections and to pay strict attention to the neonates when administering the anesthesia **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.5., 3.2.)

7.2. **Cory Robinson**: Using this technique, we can visualize neonatal sepsis in real time to study interventions and host-directed therapies aimed at improving the immune response **[1]**.

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