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Title: A Single Cell Dissociation Approach for Molecular Analysis of Urinary Bladder in the Mouse Following Spinal Cord Injury

Authors and Affiliations: Hussein Atta[1,2*], Ali Hashemi Gheinani[1,2*], Amanda Wacker[1], Yaser Heshmati[3,4,5], Alex Bigger-Allen[1,6], George Lambrinos[1,2], Yao Gao[2,7], Diane R. Bielenberg[2,7], and Rosalyn M. Adam[1,2]

[1]Department of Urology, Boston Children's Hospital
[2]Department of Surgery, Harvard Medical School
[3]Division of Hematology/Oncology, Harvard Medical School Boston
[4]Dana-Farber Cancer Institute, Boston
[5]Broad Institute
[6]Biological Biomedical Sciences Program, Division of Medical Sciences, Harvard Medical School
[7]Vascular Biology Program, Boston Children's Hospital

Corresponding Authors:
Ali Hashemi Gheinani
Ali.HashemiGheinani@childrens.harvard.edu

Rosalyn Adam
rosalyn.adam@childrens.harvard.edu

Co-authors:
Hussein.atta@childrens.harvard.edu
Ali.HashemiGheinani@childrens.harvard.edu
alw16e@my.fsu.edu
yaser.heshmati@childrens.harvard.edu
aab589@g.harvard.edu
George.Lambrinos@childrens.harvard.edu
yao.gao@childrens.harvard.edu
diane.bielenberg@childrens.harvard.edu

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **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 by the script return deadline

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

Protocol length
31 steps
60 shots

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Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Rosalyn Adam**: This protocol is significant, because it uses biological information to facilitate the generation of a single cell suspension with a high cell viability **[1]**.

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

REQUIRED:

1.2. **Rosalyn Adam**: The main advantage of this technique is the ability to generate single cell suspensions that retain a high cell viability and an appropriate representation of the isolated cell types **[1]**.

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

Introduction of Demonstrator on Camera

1.3. **Rosalyn Adam**: Demonstrating the surgical procedure will be Hussein Atta and Ali Hashemi Gheinani, Research Fellows from my laboratory, and Yaser Heshmati, **[1][2]]**.

1.3.1. INTERVIEW: Author saying the above
1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital.

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Protocol

2. Mouse Preparation

2.1. After confirming a lack of response to pedal reflex in an adult, anesthetized mouse **[1-TXT]**, palpate the most prominent spinous process in the thoracic spine **[2-TXT]**.

2.1.1. WIDE: Talent pinching toe *Videographer: More Talent than mouse in shot*
TEXT: Anesthesia: 3% isoflurane

2.1.2. Spine being palpated **TEXT: *i.e.*, typically T13 spinous process**

2.2. Shave a longitudinal rectangle area on the back of the mouse from the lower neck to just below the most prominent spinous process **[1]** and 1 centimeter on each side of the midline **[2]**.

2.2.1. Fur being shaved from neck to T13

2.2.2. Side of mouse being shaved

2.3. Disinfect the exposed skin three times with sequential 10% povidone iodine **[1]** and 70% ethanol wipes in a circular fashion, starting from the site of incision and working outward **[2]**.

2.3.1. Skin being wiped, with povidone iodine container visible in frame
Videographer: More Talent than mouse in shot

2.3.2. Skin being wiped, with ethanol container visible in frame

2.4. After the last wipe, cover the mouse with a sterile 4- x 4-inch piece of gauze sponge with a window over the surgical field **[1]**.

2.4.1. Talent placing sponge onto mouse

3. Spinal Cord Injury (SCI)

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3.1. To induce the spinal cord injury, first make a 1.5-centimeter incision in the middle of the back **[1]** and use sharp, blunt dissection to separate the muscles from the spinous processes and the laminae of the T9, T10, and T11 vertebrae **[2]**.

3.1.1. WIDE: Talent making incision *Videographer: More Talent than mouse in shot*

3.1.2. Muscles and tissues being dissected

3.2. Use fine scissors to sharply divide the interspinous ligaments between T9 and T10 and T10 and T11 **[1]** before performing a bilateral laminectomy of the T10 spinous process **[2]**.

3.2.1. Ligaments being divided *Videographer: Important/difficult step*

3.2.2. Spinous process being excised *Videographer: Important/difficult step*

3.3. When the laminae have been completely excised, use the scissors to transect the spinal cord **[3.4.1]**. To completely cut the lateral columns, delicately sweep the tip of the fine scissors on both sides **[1]**.

3.3.1. Scissors being swept on at least one side *Videographer: Important step*

3.4. Use a sterile cotton tip application to compress any bleeding **[2-TXT]**.

3.4.1. Spinal cord being transected NOTE: Shot should be moved before 3.3.1, may be slated differently.

3.4.2. Cotton tip being pressed onto bleeding site *Videographer: Important step*
TEXT: Bleeding should be minimal

3.5. After achieving hemostasis, close the skin with 7-0 polyglactin 910 continuous sutures **[1]** and subcutaneously deliver 1 milliliter of saline solution to prevent postoperative dehydration **[2]**.

3.5.1. Suture being placed Video

3.5.2. Saline being injected

3.6. Then return the animal to a fresh cage with monitoring until full recumbency **[1-TXT]**.

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3.6.1. Talent placing mouse into cage *Videographer: More Talent than mouse in shot*
TEXT: See text for full post-operative care details

3.7. Every 12 hours, holding the animal with one hand **[1]** and massaging the lower abdomen with the other **[2]**, use the index finger and thumb to locate and gently compress the distended urinary bladder to stimulate manual bladder expression until the animal is able to urinate on its own **[3-TXT]**.

3.7.1. Talent grasping mouse

3.7.2. Hand being placed around abdomen

3.7.3. Bladder being located/urination being initiated **TEXT: Perform Credé maneuver 2x/10-14 d**

4. Tissue Perfusion and Procurement

4.1. For downstream analysis of the bladder cell types of interest, at the appropriate experimental time point, perform a midline laparotomy from the pelvis to the diaphragm **[1]** and cut the diaphragm away from the ribs **[2]**.

4.1.1. WIDE: Talent making incision *Videographer: More Talent than mouse in shot*
TEXT: Anesthesia: 3% isoflurane

4.1.2. Diaphragm being cut

4.2. Following the bone-cartilage border parallel to the sternum, open the thorax along the ribs on both sides of the animal, starting at the diaphragm and proceeding to the first rib **[1]**.

4.2.1. Thorax being opened

4.3. After fixing the anterior thoracic wall over the animal's head, use fine scissors to cut away the pericardium **[1]** and connect a 23-gauge needle to the perfusion apparatus **[2]**.

4.3.1. Shot of wall fixed over head, then pericardium being removed

4.3.2. Talent fixing needle to apparatus

4.4. Insert the needle into the left ventricle **[1]** and slowly advance the needle into the aorta without puncturing the tissue **[2]**.

4.4.1. Needle being inserted

4.4.2. Needle being advanced

4.5. When the needle is in place, begin infusing the tissue with PBS at a 15 milliliter/minute flow rate **[1]** and use the tips of a pair of fine scissors to quickly make a small cut in the right atrium **[2]**.

4.5.1. PBS being infused

4.5.2. Incision being made

4.6. When the drainage is clear and a lightened liver color can be observed **[1-TXT]**, stop the perfusion **[2]** and free the bladder from the vascular pedicles and urethra **[3]**.

4.6.1. Shot of clear drainage and/or liver color **TEXT: *i.e.*, 3.5-4 min perfusion**

4.6.2. Talent stopping perfusion

4.6.3. Bladder being dissected

4.7. Then place the bladder in a microcentrifuge of ice-cold Tyrode's solution **[1]**.

4.7.1. Talent placing bladder into tube, with solution container visible in frame

5. Cell Dissociation and Single Cell Suspension Preparation

5.1. To prepare a single cell suspension, when all of the bladders have been collected, tare a 1.5-milliliter centrifuge containing 100 microliters of Tyrode's solution **[1]**.

5.1.1. WIDE: Talent placing tube onto balance, with solution container visible in frame *Videographer: Important step*

5.2. After weighing, mince the bladders in a 10-centimeter Petri dish containing 100 microliters of Tyrode's solution **[1]** and use a wide-bore pipet tip to transfer the tissue fragments into 2.5 milliliters of digestion buffer per bladder **[2-TXT]**.

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5.2.1. Talent mincing bladder *Videographer: Important step*

5.2.2. Tissue being added to tube, with buffer container visible in frame *Videographer: Important step* **TEXT: See text for all buffer and solution preparation details**

5.3. Incubate the tissue for 40 minutes at 37 degrees Celsius on a nutator mixer **[1]** and use a 5-milliliter pipette to triturate the sample for 1 minute **[2]** before collecting the dissociated cells and tissue by centrifugation **[3-TXT]**.

5.3.1. Talent placing tube onto mixer *Videographer: Important step*

5.3.2. Tissue being triturated *Videographer: Important step*

5.3.3. Talent placing tube into centrifuge *Videographer: Important step* **TEXT: 10 min, 350 x g, 4 °C**

5.4. Resuspend the pellet in 1 milliliter of cell detachment solution **[1]** and place the cells on the nutator mixer for an additional 10 minutes **[2]**.

5.4.1. Shot of pellet if visible, then solution being added to cells, with solution container visible in frame

5.4.2. Talent placing cells onto mixer

5.5. At the end of the incubation, collect the cells with another centrifugation **[1]** and resuspend the pellet in 1 milliliter of red cell lysis buffer **[2]**.

5.5.1. Talent placing tube into centrifuge

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

5.6. After 1 minute, stop the lysis with the addition of 9 milliliters of PBS **[1]** and filter the cells through a 70-micrometer strainer into a 50-milliliter tube **[2]**.

5.6.1. Talent adding PBS to tube, with PBS container visible in frame

5.6.2. Talent filtering cells

5.7. Then centrifuge the cells again **[1]** and resuspend the pellet in 200 microliters of staining buffer supplemented with Fc (F-C) block **[2]**.

5.7.1. Shot of pellet if visible, then buffer being added to tube, with buffer and Fc block containers visible in frame

6. Cell Staining

6.1. After a 10-minute incubation on ice, collect the cells by centrifugation **[1]** and resuspend the pellet in the appropriate volume of fluorophore-conjugated antibody master mix for 20 minutes on ice protected from light **[2]**.

6.1.1. WIDE: Talent adding tube(s) to centrifuge

6.1.2. Shot of pellet, then antibody being added to cells, with antibody container(s) visible in frame

6.2. At the end of the incubation, wash the cells with 1 milliliter of cell staining buffer **[1-TXT]** and resuspend the pellet in 100 microliters of fresh cell staining buffer supplemented with 5 microliters or FITC (FIT-sea)-Annexin five and 1 microliter of propidum iodide **[2-TXT]**.

6.2.1. Talent adding buffer to tube, with buffer container visible in frame **TEXT: 5 min, 350 x g, 10 °C**

6.2.2. Shot of pellet if visible, then buffer being added to tube, with buffer container visible in frame **TEXT: FITC: Fluorescein isothiocyanate**

6.3. After 15 minutes at room temperature, add 400 microliters of Annexin-binding buffer to the cells **[1]** and mix by inversion **[2-TXT]**.

6.3.1. Talent adding buffer to tube, with buffer container visible in frame

6.3.2. Talent inverting tube **TEXT: Hold cells on ice until analysis**

7. Flow Cytometry Analysis

7.1. To analyze the cells by flow cytometry, first use an unstained cell sample **[1]** to set the side and forward scatter parameters of the cells **[2]**.

7.1.1. WIDE: Talent loading tube onto cytometer

7.1.2. SCREEN: 61455_screenshot_1. 0:00 – 0:20.

7.2. Then measure the fluorescence emission at 530 nanometers and greater than 575 nanometers to exclude the dead cells **[1]**.

7.2.1. SCREEN: 61455_screenshot_2. 0:00 – 0:28.

7.3. Use the grids on each dot plot to define the negative population in the first decade **[1]** and use the fluorescence minus one controls **[2]** to correct the spectral overlap until the negative and positive population medians are aligned **[3]**.

7.3.1. SCREEN: 61455_screenshot_3. 0:00 – 1:12.

7.3.2. Talent loading tube

7.3.3. SCREEN: 61455_screenshot_4. 0:00 – 0:16.

7.4. Then measure 100,000 events of the cells with the specific markers of interest **[1]**, creating gates for the cell populations of interest as necessary **[2]**.

7.4.1. SCREEN: 61455_screenshot_5. 0:00 – 0:59.

7.4.2. SCREEN: 61455_screenshot_6. 0:00 – 0:56.

7.5. When all of the samples have been run **[1]**, analyze the data using the appropriate flow cytometric analysis software **[2]**.

7.5.1. Talent at computer, opening files, with monitor visible in frame

7.5.2. SCREEN: 61455_screenshot_7. 0:00 – 0:19.

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Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

3.2.-3.4., 5.1.-5.3.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.2. To ensure success make sure that division of the spinal cord is complete by ensuring that the lateral column of the spinal cord on both sides are completely divided. This is accomplished by delicately sweeping the tip of the fine scissors on both sides to completely cut the lateral columns. It is advisable to practice the SCI surgery on a cadaver mouse prior to implementation of the procedure.

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Results

8. Results: Representative Mouse Bladder Collagen Expression and Immune Cell Population Analyses

8.1. Collagen 1A1 **[1]**, collagen 3A1 **[2]**, collagen 1A2 **[3]**, and collagen 6A1 **[4]** are the most abundant collagen types found within the mouse bladder **[5]**.

8.1.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize Col1a1 data bar*
8.1.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize Col3a1 data bar*
8.1.3. LAB MEDIA: Figure 2A *Video Editor: please emphasize Col1a2 data bar*
8.1.4. LAB MEDIA: Figure 2A *Video Editor: please emphasize Col6a1 data bar*
8.1.5. LAB MEDIA: Figure 2A

8.2. Tabula Muris analysis to determine the mRNA expression level of collagens 1, 3, 6, and hyaluronan **[1]** reveals that the expression of these extracellular matrix components is more prevalent in mesenchymal cell populations **[2]** than in the urothelium **[3]**.

8.2.1. LAB MEDIA: Figure 2B
8.2.2. LAB MEDIA: Figure 2B *Video Editor: please emphasize purple data clusters*
8.2.3. LAB MEDIA: Figure 2B *Video Editor: please emphasize pink data clusters*

8.3. Flow cytometric analysis indicates that multiple different enzymatic digestion protocols yield highly viable cell populations **[1]**, with the third digestion protocol used in this analysis determined to be the most effective at cell preservation **[2]**.

8.3.1. LAB MEDIA: Figure 4A *Video Editor: please emphasize cells in bottom right quadrants*
8.3.2. LAB MEDIA: Figure 4A *Video Editor: please emphasize bottom right quadrant in Protocol 3 graph*

8.4. Using this method, a significant increase in the total number of cells **[1]** isolated from the bladders of spinal cord injury mice **[2]** can be obtained compared to control bladders **[3]**.

8.4.1. LAB MEDIA: Figure 4C first two columns
8.4.2. LAB MEDIA: Figure 4C first two columns *Video Editor: please emphasize SCI plots*
8.4.3. LAB MEDIA: Figure 4C first two columns *Video Editor: please emphasize Control plots*

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8.5. Compared to controls **[1]**, the bladders from spinal cord injury animals also display a significant increase in CD45 (C-D-forty-five)-positive cells **[2]**.

8.5.1. LAB MEDIA: Figure 4C last two columns *Video Editor: please emphasize right Control plots*

8.5.2. LAB MEDIA: Figure 4C last two columns *Video Editor: please emphasize right SCI plots*

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Conclusion

9. Conclusion Interview Statements

9.1. **Yaser Heshmati**: The bladders from SCI animals have distinct cell populations. For correct identification of the populations of interest, the gating strategy should be informed by the cells of interest **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (7.1)

9.2. **Ali Hashemi Gheinani**: This protocol can be used for cell sorting, primary cell culture, single cell sequencing, and other downstream molecular and cellular assays that require viable single cells **[1]**.

9.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

9.3. **Ali Hashemi Gheinani**: This protocol facilitates the acquisition of a highly viable cell population with little cell loss, which is essential for single cell analysis techniques such as single cell RNA sequencing **[1]**.

9.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

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