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# A single cell dissociation approach for molecular analysis of urinary bladder in the mouse following spinal cord injury

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Cover Letter

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April 17th, 2020

Dear Dr. D'Souza,

Please find enclosed our revised manuscript entitled "A single cell dissociation approach for molecular analysis of urinary bladder in the mouse following spinal cord injury" by Atta, Hashemi Gheinani, *et al*. We thank the reviewers and editor for their constructive critiques. We have modified the manuscript along the lines suggested in the critiques and have attempted to respond to the comments raised by the editor and reviewers in a point-by-point rebuttal included with the revised manuscript. We have provided a clean version of the manuscript as well as a version incorporating tracked changes to illustrate the revisions made to the text. We hope these modifications render the article acceptable for publication. The manuscript is not under consideration at other journals, nor has it been submitted or published elsewhere. All authors have contributed significantly, are in agreement with the content of the article and have no conflicts to declare. In recognition of his substantial contributions I have included Dr. Hashemi Gheinani as co-corresponding author for the revised manuscript.

Thank you for consideration of our work.

Yours sincerely,

Rosalyn Adam

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**TITLE:**

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

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**KEYWORDS:**

Spinal cord, injury, single cell, dissociation, flow cytometry, murine

**SUMMARY:**

The goal of this protocol is to apply an optimized tissue dissociation protocol to a mouse model of spinal cord injury and validate the approach for single cell analysis by flow cytometry.

**ABSTRACT:**

We describe the implementation of spinal cord injury in mice to elicit detrusor-sphincter dyssynergia, a functional bladder outlet obstruction, and subsequent bladder wall remodeling. To facilitate assessment of the cellular composition of the bladder wall in non-injured control and

spinal cord injured mice, we developed an optimized dissociation protocol that supports high cell viability and enables the detection of discrete subpopulations by flow cytometry.

Spinal cord injury is created by complete transection of the thoracic spinal cord. At the time of tissue harvest, the animal is perfused with phosphate-buffered saline under deep anesthesia and bladders are harvested into Tyrode's buffer. Tissues are minced prior to incubation in digestion buffer that has been optimized based on the collagen content of mouse bladder as determined by interrogation of publicly available gene expression databases. Following generation of a single cell suspension, material is analyzed by flow cytometry for assessment of cell viability, cell number and specific subpopulations. We demonstrate that the method yields cell populations with greater than 90% viability, and robust representation of cells of mesenchymal and epithelial origin. This method will enable accurate downstream analysis of discrete cell types in mouse bladder and potentially other organs.

**INTRODUCTION:**

Perturbations of normal urinary bladder function can lead to decreased quality of life for many individuals. In order to gain a better understanding of how injury or disease derails normal bladder function, it is important to probe the normal biological state of cells within the bladder and how they change under experimental perturbation. To date, however, the specific cell populations that reside within the urinary bladder, and how they change with injury, have been incompletely characterized.

Single cell profiling methods such as flow cytometry or single cell RNA sequencing (scRNA-seq) have the potential to shed light on specific cell types within the bladder. However, for these approaches to be informative tissue must be digested in a manner that does not affect viability, gene expression, and representative cell population percentages of the harvested tissue. Protocols that employ enzymatic disaggregation can impact surface marker expression through indiscriminate protease activity[1], thereby impacting cell identification by flow cytometry, whereas the dissociation process itself can lead to the induction of immediate early genes, as described recently by Van den Brink and colleagues[2]. The authors showed that although the dissociation-affected subpopulation was small, it could trigger a strong contaminating signal in bulk expression studies due to the high expression levels of immediate early genes. In addition, the duration of the dissociation protocol affected detected bulk expression levels of genes shown to be unique to some subpopulations. Thus, single cell datasets generated without accounting for the impact of the dissociation protocol may yield gene expression changes arising from the dissociation method, as opposed to underlying biology. These observations suggest that published single cell transcriptomics data should be interpreted with caution, and that results should be validated by independent methods.

Although, harsh and lengthy dissociation methods may alter gene expression in cells[2]; effective isolation of cells is essential to obtain accurate representation of the cell types present. Since the bladder is a complex organ comprising multiple cell types, some populations such as urothelial or stromal cells may be relatively under-represented whereas other cell types such as fibroblasts exist within extracellular matrix and can be challenging to isolate. Dissociation becomes even more challenging if the bladder has undergone significant remodeling and fibrosis such as that observed in spinal cord injury[3,4] or bladder outlet obstruction[5,6].

Here, we describe an optimized tissue dissociation method for downstream single cell analysis in the spinal cord injured mouse bladder. Using flow cytometry, we compared four enzymatic digestion protocols for their ability to yield a single cell suspension, support cell viability and maintain the correct proportion of cell populations. Based on this analysis, we conclude that minimizing cell death, cellular aggregates, non-cellular nucleic acids and potential inhibitors of downstream analysis are critical to achieving high quality data.

**PROTOCOL:**

The procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were approved by the Animal Care and Use Committee of Boston Children's Hospital.

NOTE: Mice were housed in an AAALAC-accredited animal facility with ad libitum access to food and water. Female mice at 8–12 weeks of age were used for these experiments. Given the nature of the injury, additional nutritional enrichment was provided to mice to ensure their wellbeing.

**1. Low-thoracic spinal cord transection in mice**

**1.1. Preparation before spinal cord transection**

NOTE: The surgical instruments required for this procedure are micro dissecting spring scissors, micro dissecting forceps, micro suturing needle driver, hemostats, and 7-0 polyglactin 910 sutures. Other surgical supplies required are surgical drapes, sterile sheets for surgical field, gauze sponges, cotton-tip applicators, and 1 mL syringes with 25 G needles.

**1.1.1.** Autoclave the surgical instruments and supplies prior to the surgery.

**1.1.2.** Clean the surgical area and the heating pads with alcohol wipes. One heating pad will be used during the surgery and the other for the immediate postoperative period to maintain the animal's body temperature until regaining full activity.

**1.1.3.** Use magnifying loupes (2.5x or more) to perform the surgical procedure.

**1.1.4.** Switch on the heating pads, the light source, and the glass bead sterilizer to be ready for use during the procedure.

**1.1.5.** Open the surgical drapes and the instruments. Use sterile gloves to drape the surgical field and place the instruments in the surgical field.

**1.2. Preparation of the animals**

**1.2.1.** Bring the mice to the procedure room and also bring a clean cage for the spinal cord injured mice.

**1.2.2.** Administer anesthesia by placing the mouse in the induction chamber with isoflurane flow set at 3.0%, oxygen flow at 1 L/min, and suction at 20 mmHg until there is no paw-pinch response.

**1.2.3.** Immediately weigh the animal and then place the animal in the prone position on the heating pad.

**1.2.4.** Place the anesthetic cone snugly over the mouse's nose, switch the gas flow from the induction chamber to the nose cone, and set the isoflurane flow to 2% and oxygen flow to 1 L/min.

**1.2.5.** Confirm that the animal is adequately anesthetized with the absence of response to paw-pinch. Tape the animal limbs to the heating pad. Place a rolled piece of gauze sponge under the lower chest to elevate and flex open the lower thoracic vertebrae.

**1.2.6.** Apply ophthalmic lubricant to both eyes. Administer pain medication (Meloxicam, 10 mg/kg, subcutaneously) and antibiotic (Enrofloxacin, 5 mg/kg, subcutaneously).

NOTE: End users should use the pain medication and antibiotics recommended by their local animal care and use committee.

**1.2.7.** Palpate the most prominent spinous process in the thoracic spine which typically corresponds with T13 spinous process[7]. Shave a longitudinal rectangle area on the back of the mouse from the lower neck to just below the most prominent spinous process (T13) and for 1 cm on each side of the midline.

**1.3. Surgical procedure**

**1.3.1.** Prep the shaved area with 10% povidone iodine solution and 70% alcohol three times alternatively in a circular fashion starting from the incision site working outward and then cover the animal with sterile 4 x 4 gauze sponge with a window in the center overlying the surgical field.

**1.3.2.** Make 1.5 cm incision in the midline of the back using fine scissors that ends at the most prominent spinous process (T13). The incision should include the skin and superficial fascia. Using scissors separate the skin and superficial fascia on either side to expose the spinous processes and the surrounding paraspinous muscles.

**1.3.3.** Using sharp and blunt dissection separate the muscles from the spinous processes and the laminae of T9, T10, and T11 vertebrae.

**1.3.4.** Sharply divide the interspinous ligaments between T9 and T10 and between T10 and T11 using fine scissors and then excise the spinous process of T10 and carefully perform T10 laminectomy bilaterally to expose the spinal cord. Ensure that the laminae are completely excised.

**1.3.5.** Transect the spinal cord using fine scissors. Minimal bleeding usually occurs at this point due to transection of the spinal cord vessels. Compress the bleeding area with a sterile cotton tipped applicator to achieve hemostasis. After confirming complete hemostasis, close the skin with 7-0 polyglactin 910 continuous sutures.

**1.3.6.** Administer 1 mL of saline solution subcutaneously to prevent postoperative dehydration.

**1.4. Postoperative care**

**1.4.1.** Place the animal on a heating pad until full recovery has occurred, then transfer to a cage for only spinal cord injured-mice.

**1.4.2.** Postoperative care includes observing and weighing the animals daily, and monitoring of the incision site for signs of infection for up to 7 days. Administer 1 mL saline solution, analgesic (meloxicam 10 mg/kg), and antibiotic (enrofloxacin 5 mg/kg) all subcutaneously daily for 3 days.

NOTE: End users should use the pain medication and antibiotics recommended by their local animal care and use committee.

**1.4.3.** Perform manual bladder expression (Credé maneuver) every 12 h until the animal is able to urinate on its own (usually in 10 to 14 days). Hold the animal with one hand and massage the lower abdomen with the other hand, then feel and gently compress the distended urinary bladder with the index finger and thumb. Gentle transient compression should alternate with relaxation. Following manual expression, wash the lower abdomen with tap water and dry gently with paper towel without excessive rubbing.

NOTE: A small size of the bladder before the beginning of the expression and wetting of the lower abdomen with urine are indications that the animal has gained the ability to void on its own.

**1.4.4.** To minimize weight loss, provide nutritional enrichment to mice in the form of nutritive gel and other nutritious treats (bacon softies, fruit crunchies and veggie bites) placed on the floor of the cage for easy access.

**1.4.5.** Euthanize mice at desired times after injury using compressed $CO_2$ or other approved method, following perfusion tissue as described below.

NOTE: In this study, mice were euthanized at 8 weeks following SCI.

**1.5. Postoperative complications**

**1.5.1.** Minimize the potential for rupture of the bladder due to overzealous manual bladder expression by not fully expressing the bladder.

**1.5.2.** Prevent excoriation of the perineal skin from continuous exposure to urine dribbling from incompetent sphincter through washing of the perineal region with tap water. Minimize inflammation through application of triple antibiotic ointment.

NOTE: Urethral obstruction due to blood clot during the period of transient hematuria or from semen coagulum due to retrograde ejaculation in male mice may occur following spinal cord injury. Complete urethral obstruction in male mice frequently culminates in bladder rupture and death. In our experience the frequency of urethral obstruction in male mice that led to death was 10%.

**2. Perfusion and tissue procurement**

NOTE: For downstream analyses of certain cell types such as immune cells in peripheral tissues it is beneficial to remove blood by perfusion at the time of tissue harvest, as described below.

**2.1.** Administer anesthesia as mentioned in the surgical procedure (step 3.2) and confirm that the animal is adequately anesthetized with no forepaw-pinch response (the animal is paraplegic, therefore the hindlimbs have diminished sensation and the hindpaw-pinch response becomes irrelevant).

**2.2.** Place the animal in the supine position and swab the abdomen and chest with 70% ethanol to wet the fur to prevent it from getting into the operating site.

**2.3.** Perform a midline laparotomy from the pelvis to the diaphragm. Cut the diaphragm away from the ribs.

NOTE: Following this step, speed is important since the thoracic pressure differential no longer exists and the lungs cannot inflate, so the animal begins to suffocate.

**2.4.** Cut the thorax open along the ribs on the left and right side following the bone-cartilage border on a line parallel to the sternum, commencing at the diaphragm and proceeding as far as the first rib.

**2.5.** Place the complete anterior thoracic wall over the animal's head and fix it in this position using towel clamps. Do not cut off the anterior thoracic wall as this will cause severe bleeding from the two internal thoracic arteries.

**2.6.** Cut away the pericardium using fine scissors.

**2.7.** Connect a 23 G needle to the perfusion apparatus, then insert it into the left ventricle and slowly into the aorta, taking care not to puncture it.

NOTE: The perfusion apparatus comprises a perfusion pump and 50 mL syringe connected to intravenous tubing.

**2.8.** Start the perfusion and quickly make a small cut with the tip of fine scissors in the right atrium for drainage. Take care not to introduce air bubbles during fluid infusion.

**2.9.** Perform perfusion with phosphate buffered saline (PBS) solution run at 15 mL/min. Perfusion is complete when drainage is clear and lightened color of the liver is achieved (**Figure 1**).

NOTE: The average time for perfusion was 3.5–4 min. Inadequate perfusion is manifested as slow progression of blanching of tissues and usually is due to incorrect positioning of the needle in the left ventricle. Adjusting the needle and extending the duration of perfusion for 1 to 2 min will ensure adequate perfusion of tissues.

**2.10.** Discontinue the perfusion and dissect the bladder free from the vascular pedicles and urethra and place it in a microcentrifuge tube containing ice-cold Tyrode's solution.

**3. Digestion of urinary bladder in control and spinal cord-injured mice**

NOTE: In order to formulate an efficient digestion mixture which is tailored for mouse urinary bladder we sought to adjust the unit of enzymes used to degrade the predominant extracellular matrix components such as collagens and hyaluronic acid. Therefore, we used publicly available RNA sequencing data generated by the Mouse ENCODE project (BioProject: PRJNA66167) to extract reads per kilobase per million (RPKM) and Tabula Muris[8] for assessment of spatial expression within bladder. Collagens 1, 3 and 6 were the three most highly expressed genes among 42 different collagens (**Figure 2A**). The expression of those collagens and hyaluronan synthase 1 (Has1) were mostly observed in muscle cells and fibroblasts of the bladder wall (**Figure 2B**).

**3.1. Preparation of buffers and solutions**

**3.1.1.** Prepare sodium Tyrode solution as per **Table 1** in a clean 500 mL bottle. Add 300 mL of dd$H_2O$. The solution is acidic after preparation. Adjust pH to 7.4 using NaOH. Bring volume to 500 mL using double distilled $H_2O$, then aliquot and store at -20 °C.

NOTE: This buffer maintains the pH and osmotic balance in the digestion buffer and provides the cells with water and essential inorganic ions. It contains magnesium, as well as glucose as an energy source. The potassium in the solution provides protective effects on electromechanical activity in the isolated cell solution. Powdered salts are hygroscopic and should be protected from moisture. The entire contents of the mix should be used immediately after preparation. Preparing a concentrated salt solution is not recommended as precipitates may form.

Sterilization using filtration (0.22 µm filter) can be performed if the cells are going to be cultured after analysis.

**3.1.2.** Prepare enzymatic digestion solution in a 15 mL conical tube by adding recommended volumes and amounts for each component (**Table 2**). Add sodium Tyrode solution up to 2.5 mL. Vortex thoroughly to dissolve.

NOTE: Papain is a sulfhydryl protease from Carica papaya latex. Papain has broad specificity and it will degrade most protein substrates[9]. Papain has proved less damaging and more effective than other proteases in cell dissociation protocols[10]. We provide details on the four dissociation protocols in **Table 2**; we observed protocol section 3 to support highest viability (93%) of cell suspensions prepared from mouse bladder.

**3.2. Dissociation procedure and preparation of cell suspension**

**3.2.1.** Collect the bladder from mice post-perfusion.

**3.2.2.** Puncture the bladder to release contents, if any.

**3.2.3.** Add 100 µL of Tyrode's solution to an empty 1.5 mL centrifuge tube and tare. Place the bladder in the tube and weigh again to determine an accurate bladder weight.

**3.2.4.** Place bladder on a 10 cm Petri dish on ice and add 100 µL of Tyrode's solution for mincing.

**3.2.5.** Using surgical scissors, cut pieces as small as possible while minimizing the mincing time to no more than 2–3 min per bladder. If pooling bladder tissue from multiple animals, mince the bladders all at once.

**3.2.6.** Transfer the minced bladder tissue using a wide-bore pipet tip into 2.5 mL of digestion buffer for each bladder. Adjust the volume if multiple bladders are pooled. Incubate the tissue in digestion solution at 37 °C in an incubator on a nutator mixer for 40 min.

**3.2.7.** At the end of the incubation period, remove digestion tube from the incubator. Triturate (pipet up and down) digestion solution using a 5 mL pipette for 1 min.

**3.2.8.** Centrifuge for 10 min at 350 x *g* at 4 °C. Remove the supernatant and resuspend the pellet in 1 mL of cell detachment solution. Place in a 37 °C incubator on nutator mixer for 10 min.

**3.2.9.** Centrifuge for 10 min at 350 x *g* at 4 °C. Remove the supernatant and resuspend the pellet in 1 mL of RBC lysis buffer (1x). Incubate for 1 min.

**3.2.10.** Add 9 mL PBS to dilute the RBC buffer and stop the RBC lysis.

**3.2.11.** Pass cells through 70 µm cell strainer into 50 mL conical tube, using the plunger from a syringe to lightly scrape the cell strainer to ensure full cell passage. Make sure to collect the liquid that passes through strainer but might be caught on underside of strainer.

**3.2.12.** Centrifuge for 10 min at 350 x *g* at 4 °C. Remove the supernatant and resuspend the pellet in 200 µL of cell staining buffer (PBS with 2% FBS).

**3.2.13.** Count the cells.

**3.3. Immunolabeling of specific cells for flow cytometry**

NOTE: To detect different types of cells in the bladder, we designed a multi-color flow cytometry panel. To perform compensation and devise an appropriate gating strategy, we include unstained and fluorescence minus one (FMO) controls. FMO controls are important to gate positive population, particularly when the positive fraction is dim. The staining procedure is as follows.

**3.3.1. Blocking FcγRII/III receptors on cells**

NOTE: We recommend to blocking nonspecific binding of monoclonal antibodies by pre-incubation of cells with monoclonal anti-Fc receptor antibodies, or recombinant Fc protein.

3.3.1.1. Wash the cells by centrifugation at 350 x *g* for 5 min at 4 °C and add cell staining buffer.

3.3.1.2. Discard supernatant and block FcγRII/III receptors on cells to prevent non-specific antibody staining by adding CD16 and CD32 antibodies in cell staining buffer at dilution of 1:100.

3.3.1.3. Incubate on ice for 10 min.

NOTE: There is no need to wash the cells; cells can be stained directly after this stage.

**3.3.2. Staining for FMOs**

NOTE: A Fluorescence Minus One (FMO) control is a tube of all fluorochromes used in the experiment that contains all the fluorochromes except one.

3.3.2.1. For example, if one has 4 different fluorochromes (A, B C and D + Annexin V and propidium iodide (PI)), prepare the FMO tubes as following. FMO Tube 1: Antibodies conjugated with B, C, D fluorochromes + (Annexin V and PI); FMO Tube 2: Antibodies conjugated with A, C, D fluorochromes + (Annexin V and PI); FMO Tube 3: Antibodies conjugated with A, B, C fluorochromes + (Annexin V and PI); FMO Tube 4: Antibodies conjugated with A, B, C, D fluorochromes + (Annexin V); FMO Tube 5: Antibodies conjugated with A, B, C, D fluorochromes + (PI).

3.3.2.1. Consider the nature of the conjugated fluorochrome for the Annexin V antibody.

### 3.3.3. Staining the blocked cells with desired antibodies

3.3.3.1. Incubate blocked cells with appropriate master mixes of fluorophore-conjugated antibodies against the desired proteins for 20 min on ice protected from light. Remember to include FMOs.

3.3.3.2. Wash cells with 1 mL of cell staining buffer added to each tube and then centrifuge again at 350 x *g* for 5 min at 10 °C.

3.3.3.3. Discard the supernatant and resuspend the cell pellet in 200 µL of cell staining buffer. Keep on ice until fluorescence data can be acquired using a flow cytometer.

### 3.3.4. Apply Annexin V/ PI stain.

3.3.4.1. Prepare a working solution of PI (100 µg/mL) in 1x Annexin-binding buffer as described in the manufacturer's protocol for the dead cell apoptosis kit.

3.3.4.2. Determine the cell density and note the buffer and volume in which they are stored.

3.3.4.3. Centrifuge samples at 350 x *g* for 5 min, discard the supernatant and resuspend cells in 1x Annexin-binding buffer to a density of ~1 x $10^6$ cells/mL in a volume of 100 µL.

3.3.4.4. Add FITC-Annexin V (5 µL) and PI working solution (1 µL) to each sample (100 µL), as described in the manufacturer's protocol, and incubate at room temperature for 15 min.

3.3.4.5. Add 400 µL of 1x Annexin-binding buffer to samples, mix by inversion and keep on ice until flow cytometry.

## 3.4. FACS calibration

### 3.4.1. Flow cytometry and purity control

3.4.1.1. Start flow cytometry analysis by measuring the unstained cells to delineate cell morphology and the troughs of the fluorochromes.

3.4.1.2. Adjust the side scatter (SSC) and forward scatter (FSC) by modifying the voltages of each fluorescence parameter. Measure the fluorescence emission at 530 nm (Annexin V) and >575 nm (PI).

3.4.1.3. Define the negative population in the first decade by using the grids on each dot plot. Place each FMO control in the cytometer and correct the spectral overlap until the negative and positive population medians are aligned.

3.4.1.4. Measure 100,000 events. Measure cells stained with specific markers and create gates for cell populations of interest.

**3.5. Data analysis**

**3.5.1.** Collect the data from the flow cytometer. Open the software to visualize the workspace for analysis.

**3.5.2. Creating a workspace**

3.5.2.1. Import FCS files by dragging them into the workspace. Files will be visible in the sample and group section of the workspace. Double-click in the sample name to open the file.

3.5.2.2. Use side scatter area (SSC-A) for the y-axis and forward scatter area (FSC-A) for the x-axis (**Figure 3A**). Click the icon for polygon gating.

3.5.2.3. Create a gate around the cell population of interest in the dot plot by clicking to make a gate node and then continue clicking around the cell population until complete; double click to close the gate.

3.5.2.4. Name the gate according to the population captured (e.g., “All cells”) and click **OK**.

NOTE: Double clicking within the “All cells” gate will open a new graph window showing only the events contained in “All cells”.

3.5.2.5. Adjust the y-axis of the new dot plot to SSC-H (side scatter height) by clicking in the black arrow and select to change the y-axis.

NOTE: This gates for single cells (singlets) and excludes doublets or larger aggregates (**Figure 3B**). Since single cells have proportional width and length, they should be represented as a population on the diagonal. Cells falling outside of this diagonal gate are doublets or larger aggregates.

3.5.2.6. Double click on the gate to analyze necrotic (PI-positive), early apoptotic (Annexin V-positive, PI-negative) and late apoptotic (Annexin V-positive, PI-negative) cells (**Figure 3C**).

3.5.2.7. Label the x-axis as Annexin V and the y-axis as PI.

NOTE: In some cases, where signal intensity is low, cell populations may appear to have negative fluorescence values, as a result of correction for background. In this case, it is recommended to perform a bi-exponential transformation. To do this, click on the **T** next to the y-axis and choose **Customize Axis**. In the new window change the scale to bi-exponential (Biex), add negative values to the axes by increasing the width basis and click **Apply**. This will improve the resolution of events with low signal intensity.

3.5.2.8. Show data as a count plot. Use the **Option** tab just below the x-axis and select **counter plot** from the menu.

3.5.2.9. Draw a **Quat** gate on the plot to define 4 discrete target populations.

3.5.2.10. Click at the top of the window to open the layout editor, by clicking in **Layout editor** and drag populations into each separate area.

3.5.2.11. Place plots into the layout editor by dragging and dropping populations from the workspace into the layout editor window.

**3.5.3. Visualizing using histograms**

3.5.3.1. Choose histogram from the **Options** tab.

3.5.3.2. Apply a gate to select Annexin V-positive cells; alternatively, positive and negative populations can be defined by using the **bisector** tool. The sample section must now show the different populations that have been form and their hierarchy.

3.5.3.3. To compare samples, drag all the histograms on top of each other; right click on the histogram and from **histogram** choose **Stagger Offset**.

**3.5.4. Add statistical analyses**

3.5.4.1. Open the **Statistic** tab by double clicking on the population of interest. Select the function to apply and the parameter involved.

3.5.4.2. Repeat with other populations, by dragging the **Sigma** icon into the population name.

3.5.4.3. Apply the analysis to all samples by selecting the gating strategy from the sample of interest and dragging it into the group defined by the marker of interest, e.g. Annexin V.

3.5.4.4. Create gates in the FMO control samples and define negative and positive populations; this gating strategy will be applied to the entire experiment (**Figure 3D–F**).
NOTE: Check each sample individually to ensure gating is correct and modify where necessary.

3.5.4.5. If cells have been stained with marker antibody (e.g., CD45) use the corresponding FMO and gate accordingly.

3.5.4.6. To export the layout, click on **File | Export Image | Select file format** (e.g., jpg, pdf).

3.5.4.7. Click **Create Table** to open a window with the final version of the table.

3.5.4.8. Export the table by selecting **File | Save As | Filename**.

## REPRESENTATIVE RESULTS:

### Surgical procedure

The success of thoracic spinal cord transection is determined by assessment of a number of parameters, the most obvious of which is hindlimb paralysis. The animal moves only using its forelimbs, dragging its hindlimbs. Otherwise activity levels, including feeding, grooming and alertness are typically normal. In addition, animals lose volitional bladder control resulting in the need for manual bladder expression by the investigator every 12 h until reflex voiding returns at 10 to 14 days after injury. Following euthanasia, additional signs of the success of the injury relate primarily to the increase in bladder-to-body weight ratio, indicative of tissue remodeling. Histological analysis reveals hyperplasia within both the urothelial and smooth muscle compartments[3].

### Preparation of single cell suspension

Using publicly available expression data, the enrichment of bladder tissue for extracellular matrix proteins was determined (**Figure 2**) and used to inform the formulation of the digestion mix. Since collagens are key components of the bladder wall[11,12], first we sought to determine the most abundant collagen(s) in the mouse bladder using RNA profiling data sets generated by the Mouse ENCODE project[13]. Our analysis showed that Collagen 1A1, collagen 3A1, collagen 1A2 and collagen 6A1 are most abundant collagen types within mouse bladder (**Figure 2A**). We also used the Tabula Muris (a compendium of single cell transcriptome data from mouse (Mus musculus))[8] to determine the mRNA expression level of collagens 1, 3, 6 and hyaluronan. The data allow for direct and controlled comparison of gene expression in cell types shared between tissues. This analysis revealed that the expression of these extracellular matrix components is more prevalent in the mesenchymal cell types rather than urothelium (**Figure 2B**).

### Effect of dissociation on viability of isolated cells from bladder

Flow cytometry analysis demonstrated that enzymatic digestion using the 4 different protocols yielded viability of 83%, 86%, 93% or 90%, respectively. Thus, protocol section 3 was deemed most valuable for preservation of cell viability. We also observed that approximately 4% of the cells were necrotic ($PI^+$/Annexin $V^-$) (**Figure 4A**). These observations emphasize the efficiency of the digestion protocol and the ensuing benefit on cell viability.

### Effect of spinal cord injury on different populations of cells in the bladder

We observed a significant increase in the total cell number in bladders of SCI mice compared to controls. The pattern of the dot plots obtained from SCI bladders was also slightly different consistent with ongoing organ remodeling due to spinal cord injury (**Figure 4B**: first column). Compared to controls, the bladders from SCI animals displayed a significant increase in CD45-positive cells.

## FIGURE AND TABLE LEGENDS:

**Figure 1: Representative perfusion completion with lightened color of the liver.** (**A**) Demonstrates the liver color at the beginning of the perfusion. (**B**) Shows the lightened liver color at the end of perfusion. The mouse in (A) had spinal cord transection two weeks prior to perfusion resulting in bladder hypertrophy and its protrusion out of the pelvis unlike the mouse in (B) that did not have spinal cord injury; in this case the bladder is small and hidden in the pelvis.

**Figure 2: Transcriptomic expression of extracellular matrix (ECM) components in the mouse bladder.** (**A**) Bar chart of 43 different collagen types. The expression is stated by Reads Per Kilobase of transcript, per million mapped reads (RPKM) (data is collected from BioProject: PRJNA66167)[14]. (**B**) Violin plots of gene expression in cell types obtained from microfluidic droplet-based 3'-end counting in a pool of male and female dissociated urinary bladder samples (male and females). Counts were log-normalized for each cell using the natural logarithm of 1+ counts per million ln(CPM+1)[8]. A pseudocount of 1 CPM was added before taking logarithms.

**Figure 3: Gating strategy and FMO controls to determine fluorescence spread.** (**A**) Selection of cell population. (**B**) Gating strategy for singlets. (**C**) Gating for necrotic, and early and late apoptotic cells using PI and Annexin V antibody. (**D–F**) A schematic dot plot of multicolor flow cytometry (e.g., antibodies conjugated with A, B, C, D fluorochromes + (Annexin V and PI). This shows the fluorescence spread into the antibody with fluorochrome A channel shown by the FMO control compared to an unstained control. Orange dotted line represents FMO gating boundary compared to unstained boundary in red.

**Figure 4: Flow cytometry of different cell types in bladder.** (**A**) Annexin V/PI double staining flow charts. The different combinations of enzymes and chemical used for each protocol are represented in front of the corresponding viability plot. These data demonstrate highest viability was obtained with protocol section 3. (**B**) Representative histograms illustrating the intensity of Ly-6A/E (Sca-1) and CD326 (Ep-CAM) detected in single channels. (**C**) Effect of SCI on cell population of the mouse bladder. Upper panel shows results of staining on three dissociated bladders obtained from control non-surgical mice and lower panel shows the results of staining on three animals with SCI. The first column is the total cell population. The second column shows the singlet gating selection. The third column shows the subpopulation of live cells that are negative for B-cells, T-cells and NK cells. The fourth column shows the staining for live cells positive for CD45.

**Table 1: Components for preparation of Tyrode's solution.** The indicated components are for preparation of 500 mL Tyrode's solution.

**Table 2: Components for preparation of digestion buffer.** The indicated components are for preparation of 2.5 mL digestion mix (1 U catalyzes the hydrolysis of 1 µmol a substrate per minute at 37 °C. Refer to the product data sheet for definition of unit of each enzyme).

**DISCUSSION:**

The mouse spinal cord injury model described here provides a reproducible method to create a functional bladder outlet obstruction due to loss of coordination between bladder contraction and external urethral sphincter relaxation. This in turn evokes profound remodeling of the bladder wall as early as 2 weeks after injury characterized by expansion of urothelial and smooth muscle compartments. Critical steps in implementation of the SCI model in rodents include (i) rigorous attention to manual bladder expression during the period of spinal shock that ensues for 10–14 days after injury; (ii) nutritional enrichment to minimize weight loss; and (iii) mitigation of the potential for urine scalding particularly for experiments that extend beyond the return of reflex voiding. Limitations of the model include the potential for urethral occlusion in mice from blood clots during the period of transient hematuria, and additionally in male mice from semen coagulum following retrograde ejaculation following surgery.

The tissue dissociation approach described here illustrates the importance of considering structural changes in tissues that arise from the experimental insult, in this case significant tissue remodeling following SCI that may influence downstream analyses. With the increase in single cell analyses it is critical to ensure that differences observed in gene expression are not simply a result of dissociation-induced perturbations, but are truly representative of underlying biological changes relevant to the disease model. The use of publicly available expression data allowed us to modify the formulation of digestion buffers to ensure effective digestion of extracellular matrix while maximizing viability. Additional modifications that could be considered in future applications include the addition of actinomycin D, to halt transcription of immediate early genes that are sensitive to the dissociation protocol[15].

Pipetting technique is crucial when dissociating tissue or transferring cells that are already in suspension. To reduce physical damage to cells from shearing forces, it is important to pipette gently and slowly during cell resuspension. It is generally recommended to use wide-bore pipette tips. If using standard tips, it is particularly important to pipette cell suspensions gently to avoid shear forces that would otherwise damage cells. Using cell strainers is unavoidable in this protocol, however, the cell concentration can decline by 20% or more, accompanied by a volume loss of 100 µL or more. We recommend that cell concentration be determined after straining to ensure a precise cell count.

In flow cytometry, FMO controls provide a measure of background due to bleed-through of signal from overlapping emission peaks. They are not a measure of nonspecific antibody binding, or background staining that may be present when an antibody is included in that channel. To account for the nonspecific antibody binding, one has to include appropriate isotype controls; for the background staining, one needs to include negative controls. Taken together, these controls ensure accurate measurement of cell populations.

**ACKNOWLEDGMENTS:**
This work was supported by grants from the National Institutes of Health (R01 DK077195 to R.M.A, R01 DK104641 to R.M.A and D.R.B). We acknowledge valuable input from Dr. Stuart Orkin in the Division of Hematology/Oncology, Boston Children’s Hospital, Department of Pediatrics, Harvard Medical School and the Dana-Farber Cancer Institute. We also acknowledge support from Kyle Costa in post-operative care of mice and Mary Taglienti for technical assistance and helpful discussions.

**DISCLOSURES:**

No conflicts of interest declared.

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Figure 1



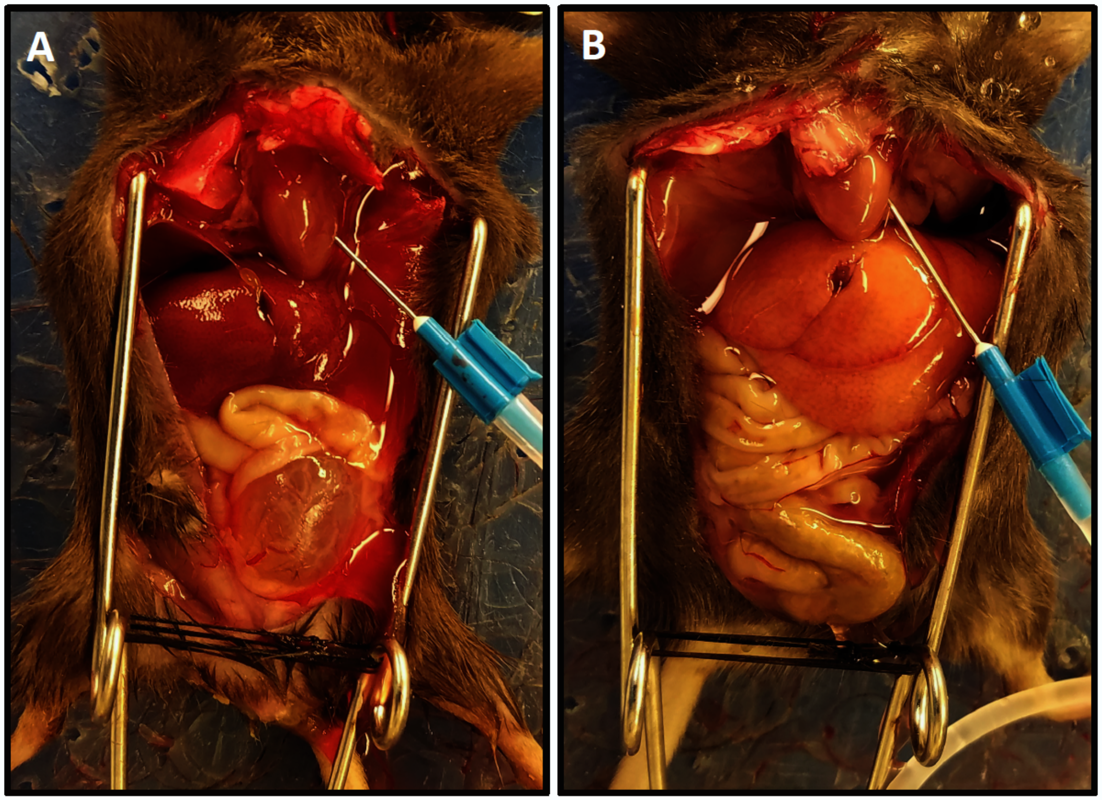



Figure 1

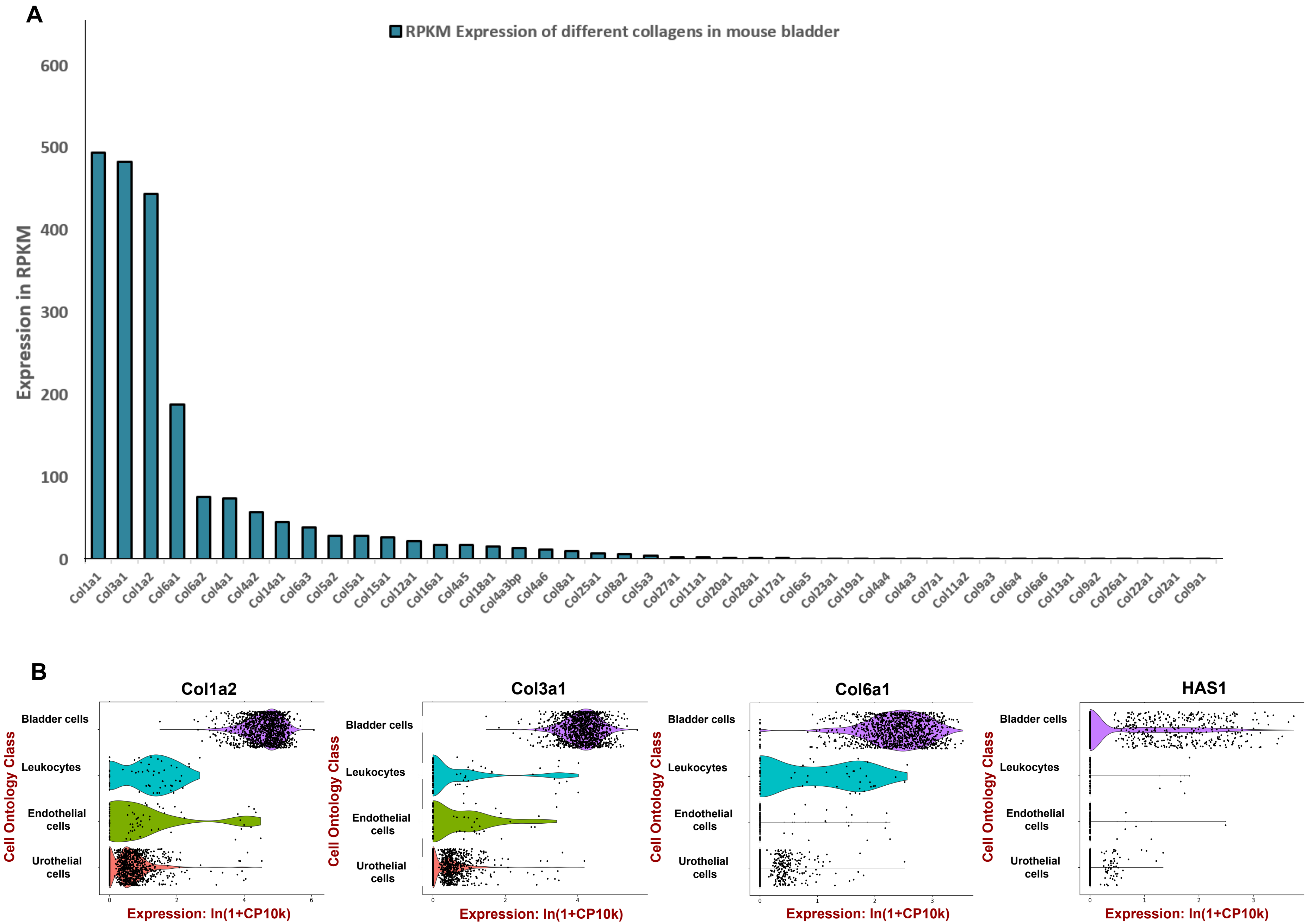

A
RPKM Expression of different collagens in mouse bladder
Expression in RPKM
600
500
400
300
200
100
0
Col1a1
Col3a1
Col1a2
Col6a1
Col6a2
Col4a1
Col4a2
Col14a1
Col6a3
Col5a2
Col5a1
Col15a1
Col12a1
Col16a1
Col4a5
Col18a1
Col4a3bp
Col4a6
Col8a1
Col25a1
Col8a2
Col5a3
Col27a1
Col11a1
Col20a1
Col28a1
Col17a1
Col6a5
Col23a1
Col19a1
Col4a4
Col4a3
Col7a1
Col11a2
Col9a3
Col6a4
Col6a6
Col13a1
Col9a2
Col26a1
Col22a1
Col2a1
Col9a1
B
Col1a2
Col3a1
Col6a1
HAS1
Cell Ontology Class
Bladder cells
Leukocytes
Endothelial cells
Urothelial cells
Expression: ln(1+CP10k)

Figure 2

Figure 4

Click here to access/download;Figure;Figure 4_revised.pdf

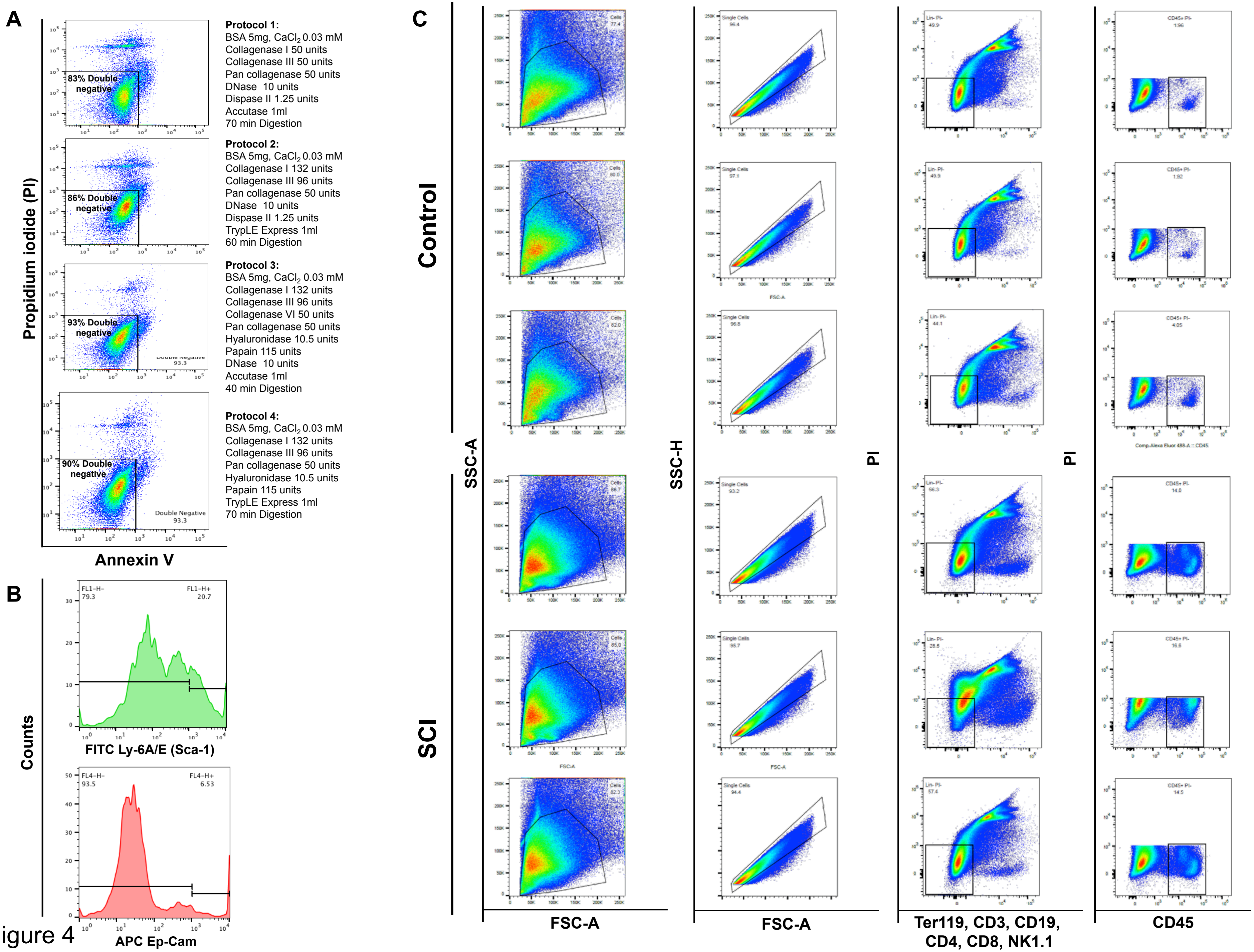



Figure 4

Table



| Component | Amount (for 500 mL) | Molarity |
|---|---|---|
| NaCl | 4.091 g | 140 mM |
| KCl | 0.186 g | 5 mM |
| $MgCl_2$ | 0.0476 g | 1 mM |
| D-Glucose | 0.9 g | 10 mM |
| HEPES | 1.19 g | 10 mM |

Table



| Component | Amount | Protocol section 1 | Protocol section 2 | Protocol section 3 | Protocol section 4 |
|---|---|---|---|---|---|
| BSA | 5 mg | yes | yes | yes | yes |
| $CaCl_2$ | 0.03 mM | yes | yes | yes | yes |
| Collagenase Type I | 132.5 units | yes | yes | yes | yes |
| Collagenase Type III | 96.4 units | yes | yes | yes | yes |
| Collagenase Type VI | 50 units | - | - | yes | - |
| DNase | 10 units | yes | yes | yes | - |
| Papain | 115 units | - | - | yes | yes |
| Pan Collagenase | 50 units | - | - | yes | yes |
| Hyaluronidase | 10.5 units | - | - | yes | yes |
| Dispase II | 1.25 units | yes | yes | - | - |
| Cell dissociation solution | 1 mL | yes | - | yes | - |
| Recombinant enzyme | 1 mL | - | yes | - | yes |

| Name of Material/Equipment |
| --- |
| 2.5 X Magnifying Loupes |
| 7-0 Vicryl suture, 6.5mm needle 3/8 circle |
| 70 µm Cell Strainer |
| Accutase in BPBS, 0.5mM EDTA |
| Aerosol Filter   Wide Orifice Pipettor Tips (1000 µL) |
| Aerosol Filter Wide Orifice Pipettor Tips (1000 µL) |
| APC anti-mouse CD326 (Ep-CAM), rat monoclonal, IgG2a, κ, affinity purified |
| BB515 Rat Anti-Mouse CD45, rat monoclonal, IgG2b, κ, Clone 30-F11 |
| BONN Micro Dissecting Forceps, Straight, 1x2 teeth, 3.75" length, 0.3mm tip width, 0.12mm teeth |
| Bovine Serum Albumin |
| CaCl2 |
| CASTROVIEJO Micro Suturing Needle Holder, Straight with lock, 5.75" length |
| Cell Counting Kit, 30 dual-chambered slides, 60 counts, with trypan blue |
| Cell Staining Buffer |
| Collagenase from Clostridium histolyticum |
| Collagenase Type I |
| Collagenase Type III |
| Collagenase, Type 6 |
| Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 & Propidium Iodide (PI) |
| Dispase II |
| DNase |
| Enrofloxacin (Baytril) |
| Falcon 15 ml conical centrifuge tubes |
| Falcon 50 ml conical centrifuge tubes |
| FITC anti-mouse Ly-6A/E (Sca-1) Antibody, rat monoclonal, IgG2a, κ, affinity purified |
| Hyaluronidase from sheep testes, Type II |
| MACS SmartStrainers (100 µm) |
| McPHERSON-VANNAS, Micro Dissecting Spring Scissors, Straight, 4" length, 0.15mm tip width |
| Meloxicam |

| |
|---|
| Papain |
| PE/Cy5 anti-mouse CD19 Antibody, rat monoclonal, IgG2a, κ, affinity purified |
| PE/Cy5 anti-mouse CD3ε Antibody, Armenian hamster monoclonal, IgG, affinity purified |
| PE/Cy5 anti-mouse CD4 Antibody, rat monoclonal, IgG2b, κ, affinity purified |
| PE/Cy5 anti-mouse CD8a Antibody, rat monoclonal, IgG2a, κ, affinity purified |
| PE/Cy5 anti-mouse NK-1.1 Antibody, mouse monoclonal, IgG2a, κ, affinity purified |
| PE/Cy5 anti-mouse TER-119/Erythroid Cells Antibody, IgG2b, κ, affinity purified |
| Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block), rat monoclonal, IgG2b, κ, Clone 2.4G2 |
| RBC Lysis Buffer (10X) |
| Red Blood Cell Lysis Buffer 1x |
| Screw-Cap microcentrifuge tubes, 1.5 ml |
| TC20 Automated Cell Counter |
| Triple antibiotic ointment (neomycin/polymyxin B/ bacitracin) |
| TrypLE Select Enzyme (10X), no phenol red |
| Vetropolycin eye ointment |

| Company | Catalog Number | Comments/Description |
|---|---|---|
| | | |
| ETHICON | J546 | |
| Thermofisher | 22363548 | |
| Millipore | SCR005 | |
| VWR | 89049-168 | |
| VWR | 89049-168 | |
| BioLegend | 118213 | |
| BD Biosciences | 564590 | |
| ROBOZ Surgical Instrument Company, Inc. | RS-5172 | OBOZ Surgical Instrument Company, Inc., Gaithersburg MD |
| Sigma | A9647-100G | |
| Sigma | 2115-250ML | |
| ROBOZ Surgical Instrument Company, Inc. | RS-6412 | MD |
| Biorad | 1450003 | |
| BioLegend | 420201 | |
| Sigma | C0130-1G | |
| Worthington Biochemical Corporation | LS004196 | |
| Worthington Biochemical Corporation | LS004182 | |
| Worthington Biochemical Corporation | LS005319 | |
| Thermofisher | V13241 | |
| Sigma | D4693-1G | |
| Sigma | DN25-1G | |
| Bayer Health Care LLC, | Approved by FDA. Lot | 2.27% Injectable Solution |
| Fisher Scientific | 352096 | |
| Fisher Scientific | 352070 | |
| BioLegend | 122505 | |
| Sigma | H2126 | |
| Miltenyi Biotec, Inc. | 130-110-917 | |
| ROBOZ Surgical Instrument Company, Inc. | RS-5630 | ROBOZ Surgical Instrument Company, Inc., Gaithersburg MD |
| Patterson Veterinary | 07-891-7959 | |

| | | |
|---|---|---|
| Worthington Biochemical Corporation | LS003119 | |
| BioLegend | 115509 | Dump Channel |
| BioLegend | 100309 | Dump Channel |
| BioLegend | 100409 | Dump Channel |
| BioLegend | 100709 | Dump Channel |
| BioLegend | 108715 | Dump Channel |
| BioLegend | 116209 | Dump Channel |
| BD Biosciences | 553141 | |
| BioLegend | 420301 | |
| Biolegend | 420201 | |
| VWR | 89004-290 | |
| Biorad | 1450102 | |
| Patterson Veterinary | 07-893-7216 | skin protectant |
| Thermofisher | A1217701 | |
| Dechra Veterinary Products | NADA # 065-016.<br>Approved by FDA. | protect eyes during anesthesia |

**JoVE61455**
**Response to Editor and Reviewers**

We thank the editor and reviewers for their comments on our manuscript. We have attempted to respond to the critiques below and/or in the body of the text. We have italicized editor and reviewer comments, and our comments are in standard font below.

**Editorial Comments:**

• *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.*

We have proofread the manuscript to eliminate spelling and/or grammatical errors.

• ***Title:*** *Avoid superfluous language such as "An optimized".*

We have removed 'An optimized' from the title.

• ***Textual Overlap:*** *Significant portions show significant overlap with previously published work. Please re-write the text on lines 340-344, 350-354, 357-371, 378-391, 398-409, 415-417, 423-431, document to avoid this overlap.*

We have reworded the text in the indicated sections to avoid overlap with previously published work. We have also shortened some of these sections since they refer to instructions within commercial products and/or standard protocols.

• ***Protocol Language:*** *Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*
*1) Steps NOT in the imperative voice: 1.1-1.2 (you can move this to the start of the protocol instead), 2.1 etc*

We have revised the protocol text as requested.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added some additional detail related to use of software for flow cytometry, as requested.

• ***Protocol Numbering:*** *Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step. This should be adopted for the entire protocol.*

We have modified the numbering as requested and have added a one-line space between each protocol step.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3-page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have removed some extraneous text from the Discussion on alternative methods for creation of bladder outlet obstruction as this does not add to the manuscript.

• ***Figures:*** *Please increase font size in Fig 2B.*

We have increased the font size in Figure 2B.

• ***References:*** *Spell out journal names.*

We have reformatted the references to spell out journal names.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

**Comments from Peer-Reviewers:**

**Reviewer #1:**
*Manuscript Summary:*
*Atta et al provide a detailed dissocation protocol for the bladder following spinal cord injury. A comparison of four dissociation protocols is performed and it is suggested that the formula that results in the highest viability is superior. The manuscript is very detailed and clear and will be of keen interest to the community. However, a major flaw dampens enthusiasm.*

We thank the reviewer for their positive comments on the manuscript.

*Major Concerns:*
*The authors appropriately seek the literature for evidence of collagen production by specific cell types, but don't actually analyze the effect of the different dissociation protocols on the representation of the epithelial and stroma cell types that produce the collagen - only leukocytes. Instead of focusing on leukocyte populations, the protocols should be tested on their effect of the proportions of viable Leukocytes (CD45+), epithelia (CD326+), and stroma (CD326-/CD45-). If one wanted to get more detail, fibroblasts (CD326-/CD45-/PDPN+) and smooth muscle (CD326-/CD45-/PDPN-) should be further subdivided since it is well known that muscle populations are particularly difficult to salvage during dissociation. That would truly be beneficial for the community. As the protocol stands, there isn't really meaningful differences among the formulas in regards to cell viability. The real question is not about viability, but about bladder cell*

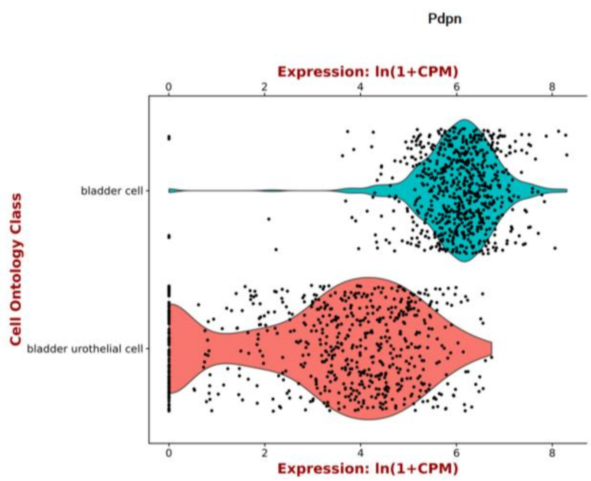
*populations. Leukocytes are the easiest population to dissociate so you could be underestimating the amount of time the tissue needs to be digested in order to free the epithelial and stromal populations.*

*Summary: repeat the experiments, but look at epithelial and stromal populations. It looks like epcam was already used (but not very well described in the figure legend) and Sca-1 is useless (just use the CD45-/CD326- gate as 'stroma' - this will contain fibroblasts, endothelia (CD31+ if you want to be accurate and remove them), and smooth muscle. If you added CD326 to all the experiments, then you don't even need to repeat. Just re-analyze the effects of the different dissociation formulas on epi and stroma proportions.*

We appreciate the reviewer raising these issues and respond to specific points below as follows.

1)…. *the protocols should be tested on their effect of the proportions of viable Leukocytes (CD45+), epithelia (CD326+), and stroma (CD326-/CD45-.*

The reviewer mentions that CD326-/CD45-/PDPN- identifies smooth muscle. Although smooth muscle doesn't express CD326 (Ep-CAM) and CD45 (PTPRC) they do express podoplanin abundantly, based on expression data from the Tabula Muris (see below). Thus, excluding cells that express PDPN will not yield the smooth muscle population.




| Tissue | Mean | Median | Median > 0 | # cells | % 0 | % > 0 |
|---|---|---|---|---|---|---|
| bladder cell | 6.03 | 6.11 | 6.11 | 695 | 0.58 | 99.42 |
| bladder urothelial cell | 3.24 | 3.70 | 4.02 | 683 | 17.42 | 82.58 |

Relative expression of Pdpn in mouse bladder using data from Tabula Muris. Bladder cell refers to smooth muscle cells.

2)…. *As the protocol stands, there isn't really meaningful differences among the formulas in regards to cell viability…. The real question is not about viability, but about bladder cell populations.*

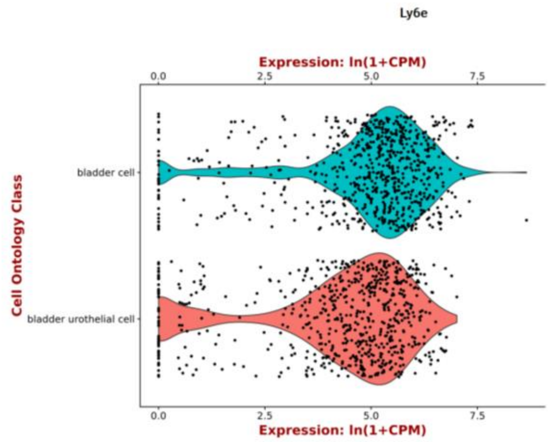
Based on new information added in this revised version of the manuscript, a comparison of the 4 protocols shows variation in viability based on the specific dissociation mix. Figure 4A now shows an increased in cell viability from 83% to 93% across the 4 protocols. This is a key conclusion of the protocol, particularly given the short duration of tissue digestion, highlighting the efficiency of the dissociation procedure.

We recognize the common belief that if a given protocol leads to a proportional loss of a particular cell type among the populations represented in a given sample, then viability is unimportant. On the contrary, however, data from us and others demonstrates the fact that low viability leads to unreliable cell recovery during flow cytometry, and for downstream analysis such as single cell RNA sequencing leads to missed sequencing targets and therefore suboptimal results. Furthermore, RNA released by dead or dying cells increases background noise, compromising data quality. To maximize the amount of useful sequencing data in single cell projects, the fraction of dead cells must be minimized. Therefore, it is recommended that cell viability exceeds 90%, emphasizing the importance of the dissociation protocol we describe here, in addition to the appropriate representation of different cell populations.

3)*….Sca-1 is useless….*

The focus of the current manuscript is on viability of the cells obtained from bladders that are dysfunctional as a consequence of functional bladder outlet obstruction, secondary to spinal cord injury. This injury results in severe structural and functional impairment, including persistent alterations to the tissue architecture of the bladder wall rendering it poorly compliant and with altered barrier function. Our rationale to use Sca-1 is as follows:

a) Sca-1 is expressed throughout the bladder wall and can provide a rough estimate of the performance of the dissociation protocol(s)(see figure below).




| Tissue | Mean | Median | Median > 0 | # cells | % 0 | % > 0 |
|---|---|---|---|---|---|---|
| bladder cell | 4.90 | 5.30 | 5.35 | 695 | 4.60 | 95.40 |
| bladder urothelial cell | 4.19 | 4.76 | 4.86 | 683 | 8.49 | 91.51 |

Relative expression of Pdpn in mouse bladder using data from Tabula Muris. Bladder cell refers to smooth muscle cells.

(b) Some studies have attempted to characterize a progenitor cell population as a first step in understanding its role in bladder fibrosis and identified a Sca-1+/lin- (PECAM-/CD45-/Ter119-) population in the adult murine bladder. Therefore, we have used Sca-1 to show that samples dissociated using this protocol include this cell population (Lilly et al., 2015, PLoS ONE 10(11): e0141437).

4) Given the restrictions as a result of COVID-19, we are not in a position to repeat experiments at the current time.

**Reviewer #2:**

*Manuscript Summary:*

*The manuscript "An optimized dissociation approach for single cell analysis of urinary bladder in the mouse following spinal cord injury" , describes protocols for spinal cord injury, enzymatic dissociation of the bladder and flow cytometry. Given the many dissociation protocols used in the literature and many discrepancies in the results, this is an extremely important paper. The effort undertaken to establish a repeatable and reproducible protocol that could be used across different labs and would produce similar results is truly commendable. The manuscript is well organized and well presented. I have a number of suggestions.*

We thank the reviewer for their positive comments on the manuscript.

Major Concerns:

*One main concern is that it is not clear what the enzymatic protocols that were compared are and what is the final recommended protocol. In the introduction, the authors state "we describe an optimized tissue dissociation method for downstream single cell analysis in the spinal cord injured mouse bladder. Using flow cytometry we compared four enzymatic digestion protocols for their ability to yield a single cell suspension, support cell viability and maintain the correct proportion of cell populations." Where are the four protocols described? I suggest making a table to illustrate how they differ, and comment on which one is recommended to be used and why.*

We thank the reviewer for this point. We now include details on all 4 dissociation protocols in Table 2, as well as a discussion as to which is recommended and why.

Minor Concerns:

*1) There is significant remodeling in the bladder wall after SCI. Is the same enzymatic digestion protocol recommended for both control and SCI bladders?*

We thank the reviewer for this comment. The same enzymatic dissociation protocol was used for both control and SCI bladders with no detriment to the viability of cells isolated from less remodeled control samples. The key element in the success of dissociation is the relatively short time (1 hour or less) required for tissue digestion, which greatly enhances cell viability.

*2) There is also time dependent remodeling of the bladder wall. The authors do not specify what time points after SCI were the dissociations performed. Please add that information on the method section. Would there be a need for adjustments in the enzymatic digestion protocol based on the time post injury? Please comment.*

The representative data shown in this manuscript were from 8 weeks after SCI. This has now been added to the protocol (1.5.5.). We have applied the dissociation protocol described here to mice at different times of injury from as early as 2 weeks up to 16 weeks after injury (unpublished observations), with comparable results in terms of viability.

*3) The dissociation and staining procedures greatly depend on the quality and specificity of reagents and antibodies. In order to ensure reproducibility of the protocols, please add information for each enzyme (especially those used in Table 2), reagent and antibody used: e.g catalog number for enzymes and reagents, host, purification method and catalog # (polyclonal or mc if indicated) for antibodies. Whenever possible please use the RRIDs AB Registry ID found at [https://scicrunch.org/resources](https://scicrunch.org/resources).*

We fully agree with the reviewer's point about dissociation and staining being highly dependent on reagent and antibody quality. We have included the pertinent information for enzymes and antibodies in the Table of Materials that accompanies the manuscript.

*4) Lines 47-49: Animals are perfused at different times after injury with phosphate-buffered saline under deep anesthesia…What are these different times?*

The data shown here are from 8 weeks after SCI, therefore we have modified the text to indicate this.

*5) Line 154 section 4.5. It is recommended to place gelfoam as a hemostatic between the two segments of the transected cord (Pharmacia and Upjohn Company, Kalamazoo, MI).*

We thank the reviewer for this comment. In prior studies, we have used GelFoam as a hemostatic, but found that in mice, compression with a sterile cotton-tipped applicator was sufficient to stop the bleeding with no recurrence of bleeding or post-operative hematoma formation.

*6) Line 219 Part 2 Step 11. Add an average time for how long the perfusion should take. Mention what are the recommendations in case of inadequate perfusion.*

The average time for perfusion was 3.5 – 4 minutes. Inadequate perfusion is manifested as slow progression of blanching of tissues and usually is due to incorrect positioning of the needle in the left ventricle. Adjusting the needle and extending the duration of perfusion for 1 to 2 minutes will ensure adequate perfusion of tissues.

*7) Line 281 "Centrifuge for 10 minutes at 350 x g at 4C." Please add explanations for why choosing 4C. Please comment whether this may shock the cells by changing the temperature from 37 to 4C?*

We performed centrifugation at 4°C since one important application of the cell dissociation procedure would be to single cell RNA sequencing experiments. For such applications, it is critical that the dissociation procedure itself elicits minimal gene expression changes, hence the choice of 4°C for centrifugation experiments. In our experience, the use of 4°C as opposed to 37°C for centrifugation ensured high cell viability.

*8) Line 297 "Count the cells." Please elaborate. Please add what the yield should and advise what to do in* *cases where the cells are not completely dissociated, e.g. small aggregates are still present.*

The cell yield is likely to vary from experiment to experiment, but is expected to be on the order of 5 x $10^5$ cells from a single mouse bladder. We have used specific gating strategies during flow cytometry to exclude cell aggregates from analysis.

*9) Line 300 "In this study to detect different immune cells in the bladder" , do the authors mean different cell types?*

We thank the reviewer for pointing this out. We have corrected the text.

*10) Line 320, Describe the reason for staining for Annexin V and PI. Define PI = Propidium iodide when first used.*

Staining for Annexin V and PI enables the detection of apoptotic (Annexin V-positive) and necrotic (PI-positive) cells in populations subjected to flow cytometry.  The ability to detect and eliminate dead and dying cells from the analysis is important for determination of the efficacy of dissociation protocols at preserving cell viability. We have now defined PI as Propidium Iodide at first use.

*11) Lines 453 -455 Are there differences in collagen components between controls and SCI? Are data presented in Figures 2-4 from SCI mice? Please clarify in the figure legends.*

The graphs in Figure 2 are plotted using publicly available expression data from control mice. Figure 3 provides a schematic representation of different cell populations identified by flow cytometry, strategies for assessing cell viability using annexin V and PI staining and approaches for appropriate controls. Figure 4 shows representative data from both control (uninjured) and SCI mice. We have clarified these details in the figure legends.

*12) Table 1 and Table 2: please specify the volume of the solution, e.g. add 4.091 g NaCl in how much volume?*

We have included these details in the protocol.

**Reviewer #3:**
Manuscript Summary:
This is a manuscript that describes both a method to transect the spinal cord to create an animal model of neurogenic bladder and a cell dissociation method to efficiently achieve a high rate of living single cells appropriate for scRNA-seq.

Major Concerns:
*On lines 92-94 the authors describe a comparison of 4 different digestion methods in order to find the best approach, but later in the manuscript the idea of comparing different digestions is not clearly flushed out and I had to work hard to look for the details that supported the comparisons. The authors should decide if this is an important line of reasoning. If not remove the mention of comparing different methods of cell preparation.*

We apologize for the omission. We have now included the details of the 4 protocols in Table 2.

*In lines 354-435 there are detailed methods for FACS sorting and analysis. I believe that these methods are of general nature and don't add to the specific focus of the paper on bladder cell isolation. These methods could be found in any article on flow cytometry and this article is not about flow cytometry. So these sections should be reduced to sentences such as FACS sorting and controls are clearly described in the following article. FACS analysis using Flojo are described in the following article...*

While we agree that methods for FACS are somewhat standard, there are a variety of approaches to both data generation and data analysis. The major issue in the field is that investigators gate differently and obtain slightly different results. Thus there is no guarantee that users define their own settings and obtain the same results as reported here. The findings reported here result from the machine settings outlined in the protocol. For this reason we have chosen to include a detailed protocol here.

Minor Concerns:
*Line 137: please indicate that the pain medication drugs are the choice of the end user and that for example you can use the drugs you have cited.*

We have noted this in the text.

*Line 168: same comment as above.*

We have noted this in the text.

*Line 186: You comment "Complete urethral obstruction in male mice frequently culminates in bladder rupture and death." Please indicate the frequency that your lab has observed this in making neurogenic bladders. This is helpful in animal protocol applications.*

In our experience the frequency of urethral obstruction in male mice that led to death was 10%.

*Line 192: What is the vendor for caladryl?*

*Caladryl was added by mistake and has now been removed. Mice are treated with triple antibiotic ointment to minimize inflammation and skin breakdown.*

*Line 215: What are the details for the perfusion apparatus?*

The perfusion apparatus consists of infusion pump and 50 mL syringe connected to an intravenous tubing and 23-gauge needle. This has been added to the protocol.

*Line 238: should say in A clean 500mL bottle.*

We have corrected the text.