Journal of Visualized Experiments

Decentralized (ex vivo) Murine Bladder Model with Detrusor Muscle Removed: A Novel Approach for Direct Access to Suburothelium during Bladder Filling

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60344R2
Full Title:	Decentralized (ex vivo) Murine Bladder Model with Detrusor Muscle Removed: A Novel Approach for Direct Access to Suburothelium during Bladder Filling
Section/Category:	JoVE Medicine
Keywords:	Urothelium; urinary bladder; lower urinary tract; suburothelium; lamina propria; detrusor; purines; bladder filling
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

A Decentralized (*ex vivo*) Murine Bladder Model with the Detrusor Muscle Removed for Direct Access to the Suburothelium during Bladder Filling

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KEYWORDS:

lower urinary tract, urinary bladder, suburothelium, lamina propria, detrusor, purines, bladder filling

SUMMARY:

The detrusor-free bladder model enables direct access to the suburothelium to study local mechanisms for regulation of biologically active mediator availability in suburothelium/lamina propria during storage and voiding of urine. The preparation closely resembles filling of an intact bladder and allows pressure-volume studies to be performed without systemic influences.

ABSTRACT:

Previous studies have established the release of chemical substances from flat bladder mucosa sheets affixed in Ussing chambers and exposed to changes in hydrostatic pressure or mechanical stretch and from cultured urothelial cells upon hydrostatic pressure changes, stretch, cell swelling, or drag forces, and in bladder lumen at end of filling. Such findings led to the assumption that these mediators are also released in suburothelium (SubU)/lamina propria (LP) during bladder filling, where they affect cells deep in the bladder wall to ultimately regulate bladder excitability. There are at least two obvious limitations in such studies: 1) none of these approaches provide direct information about the presence of mediators in SubU/LP, and 2) the stimuli used are not physiological and do not recapitulate authentic filling of the bladder. Here, we discuss a procedure that enables direct access to the suburothelial surface of the bladder mucosa in the course of bladder filling. The murine detrusor-free preparation created closely

resembles filling of the intact bladder and allows pressure-volume studies to be performed on the bladder in the absence of confounding signaling from spinal reflexes and detrusor smooth muscle. Using the novel detrusor-free bladder model, we recently demonstrated that intravesical measurements of mediators cannot be used as a proxy to what has been released or present in the SubU/LP during bladder filling. The model enables examination of urothelium-derived signaling molecules that are released, generated by metabolism and/or transported into the SubU/LP during the course of bladder filling to transmit information to neurons and smooth muscle of the bladder and regulate its excitability during continence and micturition.

INTRODUCTION:

The purpose of this model is to enable direct access to the submucosal side of the bladder mucosa during different phases of bladder filling.

The bladder must refrain from premature contraction during filling and empty when critical volume and pressure are reached. Abnormal continence or voiding of urine are frequently associated with abnormal excitability of the detrusor smooth muscle (DSM) in the course of bladder filling. Excitability of DSM is determined by factors intrinsic to the smooth muscle cells and by influences generated by different cell types within the bladder wall. The wall of the urinary bladder consists of urothelium (mucosa), suburothelium (SubU)/lamina propria (LP), detrusor smooth muscle (DSM) and serosa (**Figure 1A**). The urothelium consists of umbrella cells (i.e., the outermost layer of the urothelium), intermediate cells, and basal cells (i.e., the innermost layer of the urothelium). Various types of cells, including interstitial cells, fibroblasts, afferent nerve terminals, small blood vessels, and immune cells reside in the SubU/LP. It is widely assumed that the bladder urothelium is a sensory organ that initiates reflex micturition and continence by releasing mediators into the submucosa that affect cells in the SubU/ LP and the DSM[1-3]. For the most part, such assumptions are based on studies that have demonstrated release of mediators: from pieces of mucosa exposed to changes in hydrostatic pressure[4,5]; from cultured urothelial cells exposed to stretch[6,7], hypotonicity-induced cell swelling[7] or drag forces[8]; from isolated bladder wall strips upon receptor or nerve activation[9-14]; and in bladder lumen at end of bladder filling[15-19]. While such studies were instrumental to demonstrate release of mediators upon mechanical stimulation of bladder wall segments or cultured urothelial cells, they need to be supported by direct evidence for release of mediators in the submucosa that is elicited by physiological stimuli that reproduce bladder filling. This is a challenging task given that the SubU/LP is located deep in the bladder wall hampering the straightforward access to the vicinity of SubU/LP during bladder filling.

Here, we illustrate a decentralized (ex vivo) murine bladder model with the detrusor muscle removed[13] that was developed to facilitate studies on **local** mechanisms of mechanotransduction that participate in the signaling between the bladder urothelium, DSM and other cell types in the bladder wall. This approach is superior to using flat bladder wall sheets, bladder wall strips or cultured urothelial cells because it allows direct measurements in the vicinity of SubU/LP of urothelium-derived mediators that are released or formed in response to physiological pressures and volumes in the bladder and avoids potential phenotypic changes in cell culture. It can be used to measure availability, release, metabolism and transurothelial transport of mediators in

SubU/LP at different stages of bladder filling (**Figure 1B**). The preparation can also be used to examine urothelial signaling and mechanotransduction in models of overactive and underactive bladder syndromes.

PROTOCOL:

All procedures involving animals described in this manuscript were conducted according the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the Institutional Animal Use and Care Committee at the University of Nevada.

NOTE: The model presented here consists of the removal of the detrusor muscle while the urothelium and SubU/LP remain intact (**Figure 1B**) to enable investigators direct access to SubU/LP in the course of bladder filling.

1. **Dissection of the detrusor-free bladder preparation**

1.1. Place the isolated bladder in a dissecting dish filled with cold (10 °C) and oxygenated with 5% CO_2/95 % O_2 Krebs bicarbonate solution (KBS) with the following composition (mM): 118.5 NaCl, 4.2 KCl, 1.2 $MgCl_2$, 23.8 $NaHCO_3$, 1.2 KH_2PO_4, 11.0 dextrose, 1.8 $CaCl_2$ (pH 7.4)[13].

1.2. Pin a small portion of the dome of the isolated bladder to a Sylgard-covered dissecting dish filled with KBS. Make sure that the pin goes through a piece of the serosa or the outermost edge of the detrusor muscle far from the innermost edge of the muscle that faces the SubU/LP.

1.3. Using a microscope, identify the urethra and ureters and pin each of them to the bottom of the dissecting dish.

1.4. Remove the excess adipose and connective tissues so that the entire main body of the bladder, the urethra and both ureters are displayed.

1.5. Tie the ureters with 6-0 nylon sutures. Then, pin the open ends of ureters towards the bottom of the dissecting dish to secure the preparation.

1.6. Using fine-tip forceps, gently pull a piece of the serosa at the corner between the ureter and the bladder body.

1.7. Adjust the light of the microscope to increase transparency and distinguish the margin of the submucosa underneath the detrusor muscle.

1.8. Start cutting (**not peeling!**) the bladder wall with fine-tip scissors along the inner surface of the detrusor muscle layer while gently pulling the cut segment away from the preparation. At all times, ensure that the lateral edge of mucosa can be seen and avoid touching it.

1.9. Remove the detrusor muscle entirely by turning around the dissecting dish so that the

position of the preparation is comfortable to continue dissecting out the detrusor muscle.

1.10. Leave a small piece of detrusor muscle on the top of the bladder dome to ensure ability to immobilize the preparation during the remaining steps of the protocol.

1.11. Make a double loop of 6-0 nylon thread, place it around the neck of the bladder preparation, and leave the loop loose.

1.12. Add a second double loop of 6-0 silk thread, place it around the neck of the bladder preparation, and leave the loop lose. Having two sutures prevents leaks around the sutures.

1.13. Cut about 2 cm of 20 PE tubing (catheter), flare up the tip by moving slowly the tip close to a flame.

1.14. Fill the catheter with warm (37 °C) oxygenated KBS.

1.15. Insert the catheter in the orifice of the bladder urethra and gently push the catheter until the catheter tip reaches approximately the middle of the bladder.

1.16. Tie the suture around the catheter and the surrounding tissue of the bladder neck.

1.17. Slowly fill the bladder with about 50-100 µL of warm (37 °C) oxygenated KBS, lift it briefly (<10 s) above the surface of KBS, and monitor for leaks at the sutures and bladder body.

1.18. If no leak is observed, the preparation is ready for the experiment. If a leak around the suture is observed, remove the suture and replace it. If a leak from a hole in the bladder body is noticed, discard the preparation.

2. Filling of the denuded bladder preparation

2.1. Perfuse KBS (37 °C) into a 3 mL chamber of a water (37 °C) jacketed organ dish with a Sylgard bottom.

2.2. Adjust the oxygen and suction lines.

2.3. Place the denuded bladder preparation in the chamber.

2.4. Secure the catheter to the side of the chamber so that the preparation does not float above the surface of the perfusion solution.

2.5. Connect the bladder catheter to a longer PE20 tubing (infusion line) connected to the three way stopcock using same size fitting.

2.6. Make sure that the lines between the infusion pump, the pressure transducer and the

bladder are open.

2.7. Fill the infusion syringe with fresh, warm (37 °C) and oxygenated KBS.

2.8. Adjust the pump parameters: type/volume of syringe (i.e., 1 mL), operation (i.e. Infuse), flow (i.e., constant), and flow rate (i.e., 15 mL/min).

2.9. Press the **Start** button on the syringe pump to fill the bladder.

2.10. Monitor filling volume and intravesical pressure during bladder filling.

3. **Detection of mediators in the SubU/LP aspect of the denuded bladder preparation**

3.1. Collect aliquots of the bath solution into ice-cold microcentrifuge tubes or high-performance liquid chromatography (HPLC) inserts.

3.2. Prepare and process the samples according to the appropriate detection application. In the case of detecting purine availability, process the samples by HPLC with fluorescence detection[13, 18].

REPRESENTATIVE RESULTS:

The wall of murine detrusor-free bladder preparation is intact and contains all layers except the DSM and serosa. Proof-of-principle studies demonstrated that the DSM-free bladder wall includes urothelium and SubU/LP while the *tunica muscularis* and the serosa are absent (**Figure 2**)[13].

Filling of the detrusor-free bladder approximates normal bladder filling. **Figure 3** shows schematics of the experimental setup for filling ex vivo bladder preparations at different filling rates, volumes and intraluminal pressures. The murine ex vivo intact and denuded bladder preparations require a broad range of filling volumes to reach voiding pressure[13]. The pressure-volume relationships are remarkably similar in the intact and denuded preparations (**Movie 1, Movie 2**, and **Figure 4).** Therefore, the DSM-free preparation is suitable for functional studies of the role of urothelium and SubU/LP in bladder mechanosensation and mechanotransduction.

Possible use of the detrusor-free bladder model

Measure availability of mediators in bladder lumen and SubU/LP during bladder filling

The experimental setup for collecting extraluminal (EL; e.g., bathing SubU/LP) and intraluminal (IL) samples during filling of bladder preparations while monitoring bladder pressure is illustrated in **Figure 5**. Suitability of the model for measuring urothelium-derived mediators that are released in the SubU/LP side during filling was tested by measuring the release of purine mediators (e.g., adenosine 5'-triphosphate, ATP; adenosine 5'-diphosphate, ADP; nicotinamide adenine dinucleotide, NAD; adenosine 5'-monophosphate, AMP; and adenosine, ADO) in the solution bathing the SubU/LP of the denuded preparation. As demonstrated in **Figure 6A**,

negligible amounts of purines were detected in the bath containing an isolated bladder preparation with intact DSM whereas the amounts of these purines were significantly higher in samples collected from the bath containing a denuded bladder preparation (**Figure 6B**). Notably, the distribution of purines and metabolites in samples collected from the lumen and the SubU/LP at the end of filling differed significantly (**Figure 6C**).

Examine extracellular metabolism of mediators in SubU/LP during bladder filling

Addition of the highly-fluorescent analogue of ATP, 1,N^6-etheno-ATP (εATP), to the suburothelial side of the detrusor-free preparation resulted in a decrease of εATP and an increase in the εATP products εADP, εAMP, and εADO (**Figure 7Aa** and **Figure 7Ab**). Likewise, addition of εATP to the preparation lumen resulted in a decrease of εATP and an increase in εADP, εAMP, and εADO in the lumen (**Figure 7Ba** and **Figure 7Bb**). Therefore, the model is suitable for studies of metabolism of bioactive mediators on both sides of the urothelium during bladder filling.

Examine transurothelial transport of mediators during bladder filling

Addition of εATP to the SubU/LP side of the denuded preparation resulted in the appearance of εAMP, εADO and some εADP in the lumen, suggesting that purines can be transported from the SubU/LP to the lumen[13] (**Figure 7Ac**). Likewise, adding εATP in the lumen resulted in the appearance of εAMP and εADO in SubU/LP[13] (**Figure 7Db**). Note that no εATP was observed on the opposite side of εATP application. Together, these observations strongly suggest that the detrusor-free bladder preparation is suitable for studies of bilateral transurothelial transport of mediators during filling.

FIGURE AND TABLE LEGENDS:

Figure 1. Principle of the detrusor-free bladder model. (**A**) The bladder wall is composed of urothelium, suburothelium/lamina propria (SubU/LP), detrusor muscle and serosa. Each of these layers contain various cell types that are important for bladder functions during storage and voiding of urine. During bladder filling, biologically active mediators are released from the urothelium into the bladder lumen and in the SubU/LP to affect cells deep in the bladder wall, including the detrusor muscle. While the access to the bladder lumen is relatively straightforward, there is no direct access to the SubU/LP during filling to detect urothelium-derived mediators that might affect cells in the bladder wall and control detrusor muscle excitability. (**B**) Removal of the detrusor muscle layers along with the serosa exposes the entire surface of SubU/LP enabling direct access to the SubU/LP where urothelium-derived signaling molecules can be measured at different phases of bladder filling.

Figure 2. Histology of murine intact and detrusor-free bladder walls. Masson's trichrome staining of filled intact (**A**) and detrusor-free (**B**) bladder walls demonstrates that the denuded preparation contains intact urothelium (U) and SubU/LP, but not the detrusor muscle (D) and serosa (S). L, lumen. This figure has been reproduced from a previous publication[13].

Figure 3. Schematic representation of experimental setup used in filling of ex vivo bladder preparations. The ex vivo intact or denuded urinary bladder (UB) preparation is placed in a warm

(37 °C) water-jacketed organ chamber that is perfused with oxygenated Krebs-bicarbonate solution (KBS, 37 °C, pH 7.4). The bladder preparation is infused with KBS at different filling rates and volumes and the intraluminal bladder pressure (BP) is recorded throughout the experiment This figure has been reproduced from a previous publication[13].

Figure 4. **Pressure-volume relationships in intact and detrusor-free preparations.** (**A**) and (**B**) are representative recordings of intravesical volume and pressure of ex vivo intact and denuded bladder preparations that are filled with Krebs-bicarbonate solution at 15 μL/min. As anticipated, the intact preparation developed transient contractions (TCs) due to the presence of the detrusor. In contrast, the denuded preparation lacked TCs. (**C**) and (**D**) show summarized data of pressure-volume relationships of intact and denuded bladder preparations that accommodated >250 μL of solution. Note that the filling volumes and intravesical pressures were remarkably similar in the intact and denuded bladder preparations. This figure has been reproduced from a previous publication[13].

Figure 5. Schematic diagram of the isolated bladder model utilized to evaluate availability of urothelium-derived mediators in SubU/LP and lumen during filling. The bladder preparation is placed in a water-jacketed chamber and superfused with oxygenated Krebs bicarbonate solution (KBS). The urinary bladder (UB) preparation is filled with warm oxygenated KBS via a catheter in the urethra connected to an infusion pump. Bladder pressure (BP) is monitored via a three-way connector through the infusion line during bladder filling. Samples from extraluminal (EL, organ bath) and intraluminal (IL) solutions are processed for detection of mediators (m) according to detection applications. This figure has been reproduced from a previous publication[13].

Figure 6. The detrusor-free bladder preparation is suitable for measuring availability of mediators in SubU/LP during filling. Representative chromatograms demonstrating availability of purines in the extraluminal samples in intact (**A**) and detrusor-free (**B**) bladder preparations. Note that purine mediators are better detected in the denuded preparation than in the intact preparation. (**C**) The relative contribution of individual purines to the purine pools detected in the lumen and in SubU/LP of the denuded preparation is significantly different. This figure has been reproduced from a previous publication[13].

Figure 7. The detrusor-free bladder preparation is suitable for examining metabolism and transurothelial transport of mediators during bladder filling. (**A**, **B**) Representative chromatograms showing εATP substrate (**Aa**, **Ba**). When the substrate is applied to either the SubU/LP (**Ab**) or the lumen (**Bb**) εATP was decreased and the products εADP, εAMP, and εADO were increased. Therefore, εATP is degraded at either side of application; however, formation of εATP products is asymmetrical in SubU/LP and lumen. Note that the εATP products εAMP and εADO, but not the substrate εATP, appeared on the opposite side of εATP application (**Ac**, **Bc**). Therefore, purines appear to be transported through the wall of the detrusor-free preparation during filing. This figure has been modified from[13].

Video 1. Representative recording of intact bladder filling. The bladder was filled at 15 μL/min. Video was recorded using a zoom stereomicroscope with a charged coupled device (CCD) camera

at 5 Hz; recording was stopped upon reaching 25 mmHg intraluminal pressure. The full duration of the image is 64x real-time. This video has been reproduced from[13].

Video 2. Representative recording of detrusor-free bladder filling. Bladder was filled at 15 μl/min. Video was recorded using a zoom stereomicroscope with a CCD camera at 5 Hz; recording was stopped upon reaching 25 mm Hg intraluminal pressure. The full duration of the image is 64 times real-time. This video has been reproduced from[13].

DISCUSSION:

The bladder has two functions: storage and voiding of urine. Normal operation of these functions requires proper mechanical sensing of intraluminal volume and pressure and transduction of signals through cells in the bladder wall to regulate detrusor muscle excitability. The bladder mucosa (urothelium) is believed to regulate bladder excitability by releasing a variety of signaling molecules in the SubU/LP that affect numerous cell types in the bladder wall. Currently, most attempts at characterization of urothelium-derived mediators involve the use of bladder preparations (e.g., flat bladder wall sheets, bladder wall strips, or cultured cells) that do not reproduce physiological bladder filling. Measurements of mediators that are released in the bladder lumen at the end of bladder filling are frequently used as indication for release of these mediators from the opposite side of the urothelium. However, recent studies suggest that intraluminal content of mediators is not representative of what is available deep in the bladder wall[13]. Novel experimental approaches enabling access to SubU/LP during bladder filling are needed to further our understanding of local mechanisms of signaling between bladder urothelium, SubU/LP and DSM.

Here, we demonstrate a novel animal bladder model in which the detrusor muscle is removed to provide direct access to mediators released from the urothelium in SubU/LP during filling[13]. The denuded preparation closely resembles filling of intact bladder[13,18], suggesting that the lack of the detrusor muscle does not change the mechanosensitive properties of the urothelium during bladder filling. The preparation allows pressure-volume studies to be performed on the bladder in the absence of confounding signaling from spinal reflexes and detrusor muscle. Therefore, mediators released in SubU/LP can be measured without systemic influences or contamination from other sources. The ability to directly access the vicinity of SubU/LP at different volumes and pressures during bladder filling makes the model suitable to study release, metabolism and transurothelial transport of biologically active mediators during the storage and pre-voiding stages of bladder filling.

The most critical step in this protocol is the removal of the detrusor smooth muscle while keeping the urothelium and SubU/LP intact. The procedure is particularly straightforward in the mouse bladder due to the loose connection between the detrusor muscle and SubU/LP. The preparations showed excellent reproducibility with remarkably similar pressure–volume characteristics to intact bladders[13]. The model is also feasible in bladders from larger animals in which the detrusor muscle can be partially or completely removed. For example, we have previously demonstrated that the model can be reproduced in bladder from the Cynomolgus monkey *Macaca fascicularis* and demonstrated that mediators can be measured in SubU/LP

during preparation filling[13].

The potential limitation of this ex vivo model is the very issue that is the strength of the preparation, in essence, the lack of systemic effects of the central nervous system and circulation allows thorough examination of local mechanisms of mucosa–detrusor connectivity during bladder **filling**. Lack of systemic effects are shared with numerous ex vivo approaches, including isolated bladder wall sheets or strips or cultured urothelial cells. The detrusor-free bladder model, however, is superior to the aforementioned approaches in urothelial research in that it allows direct access to the SubU/LP in the course of bladder filling. Therefore, use of this approach will enhance understanding of mechanosensitive mechanotransduction mechanisms that originate in the urothelium during filling of the bladder.

ACKNOWLEDGMENTS:

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant DK41315.

DISCLOSURES:

Parts of this work was previously published in the Journal of Physiology (PMCID: PMC6418748; DOI:10.1113/JP276924[13]). Permission has been granted by Wiley and Sons, Inc. for the use of materials from this publication. The authors have no financial or other conflicts to disclose.

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Figure 1

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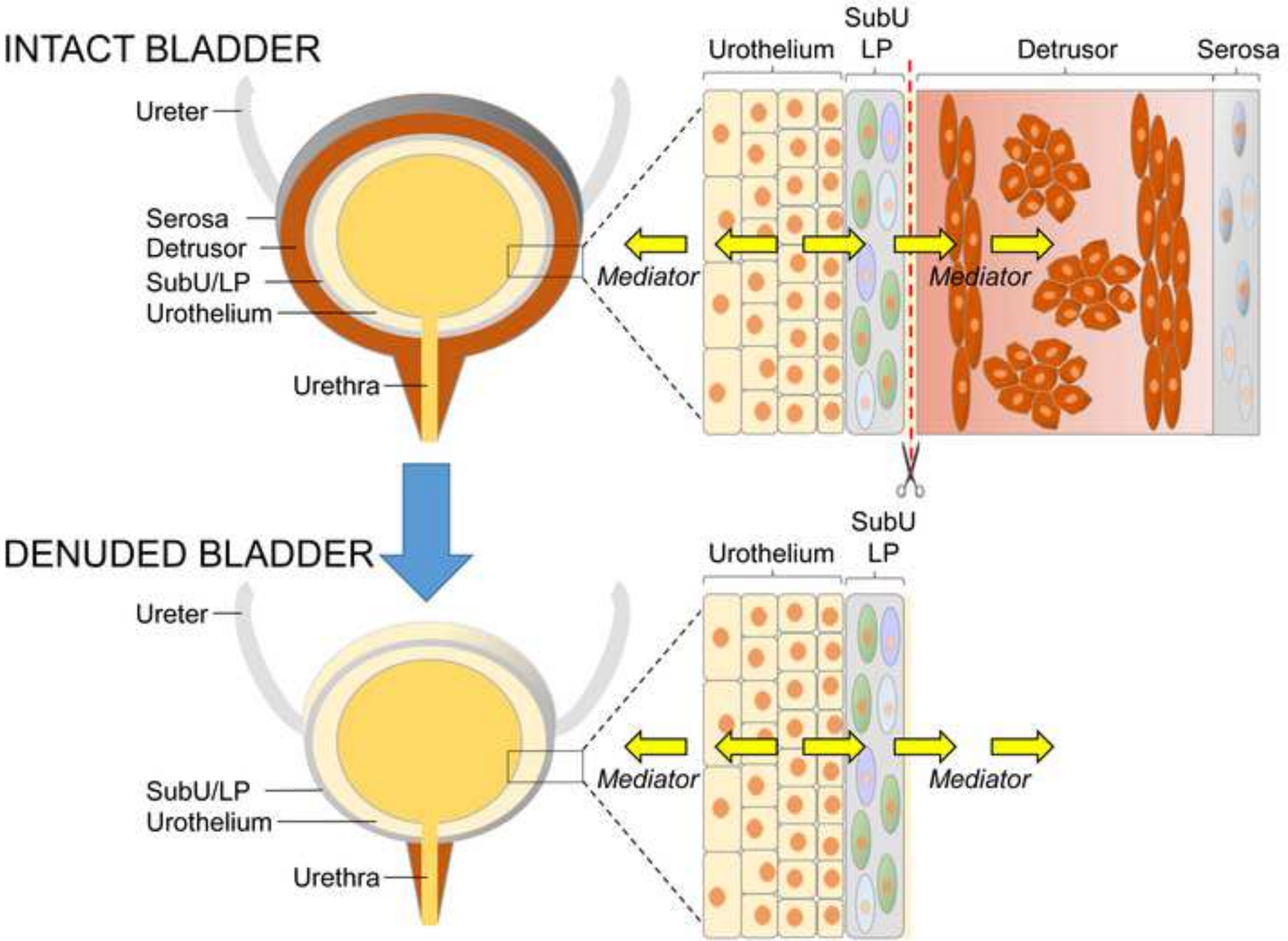

Figure 2

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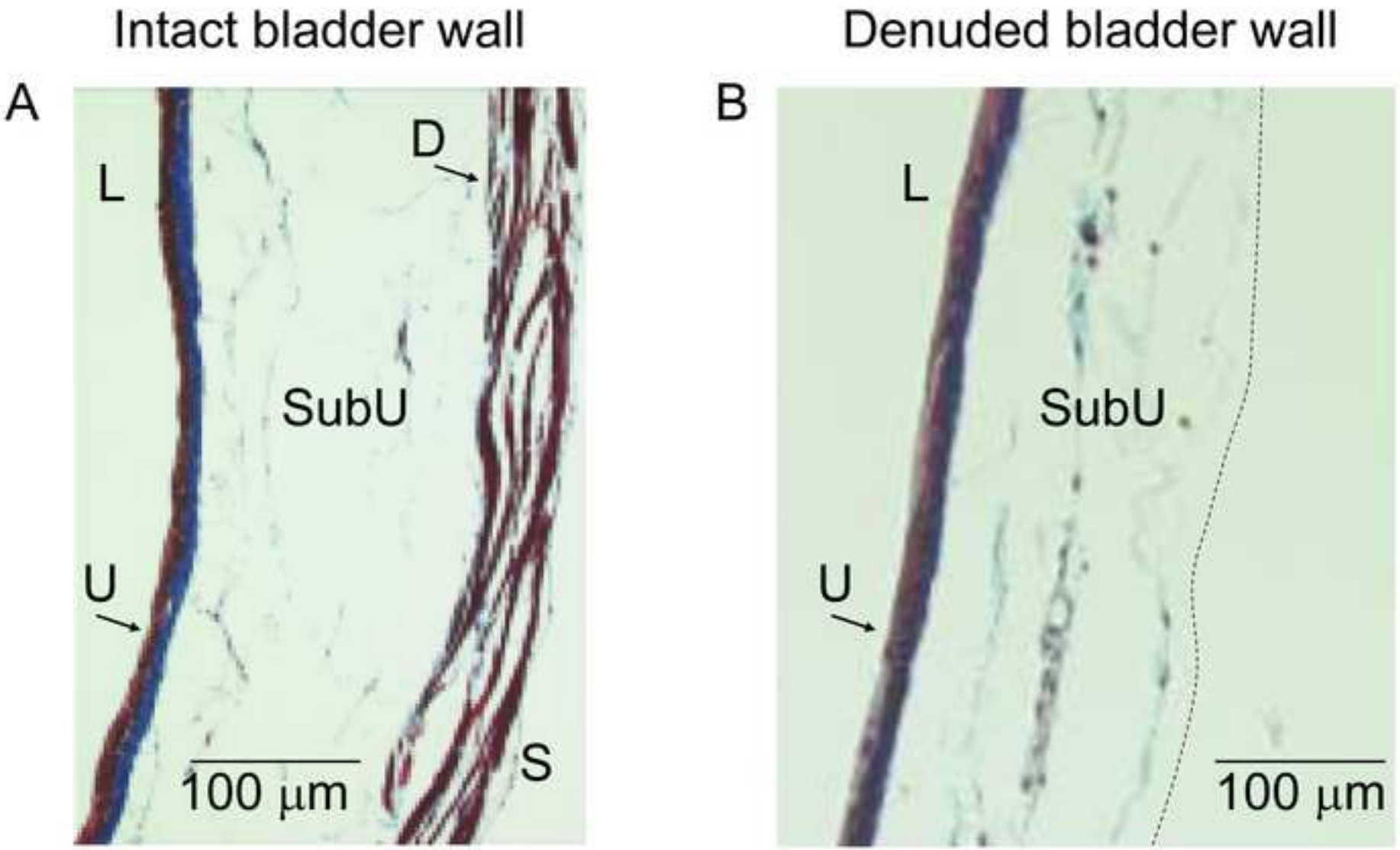

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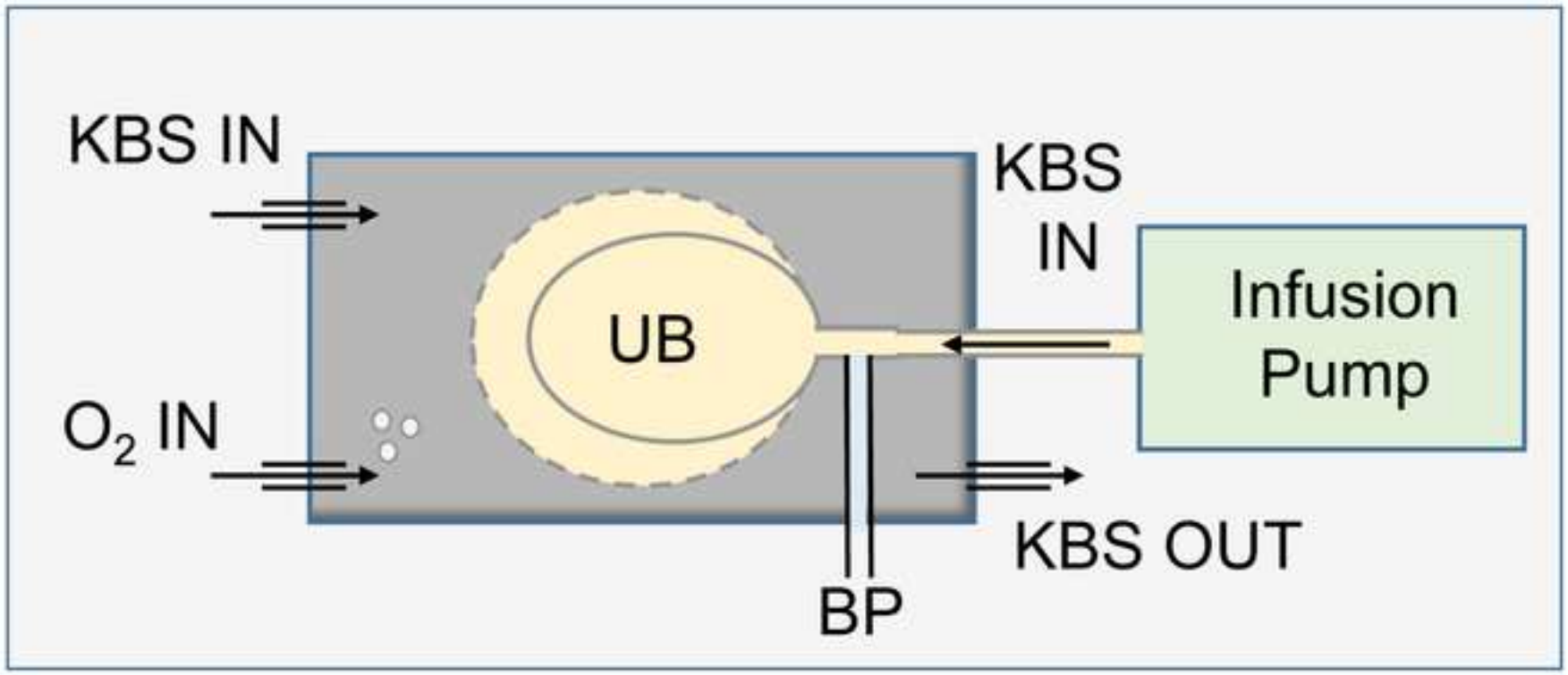

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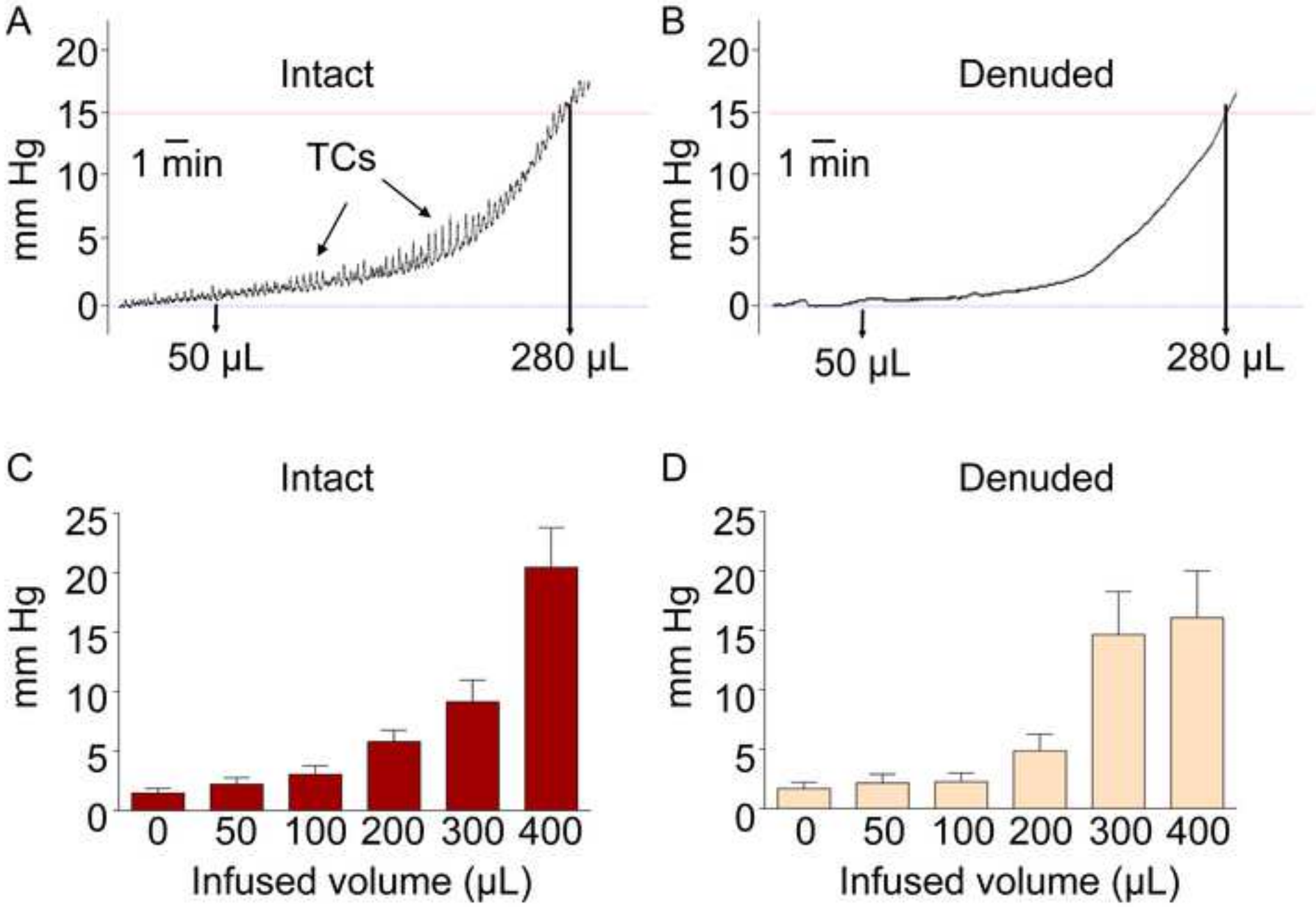
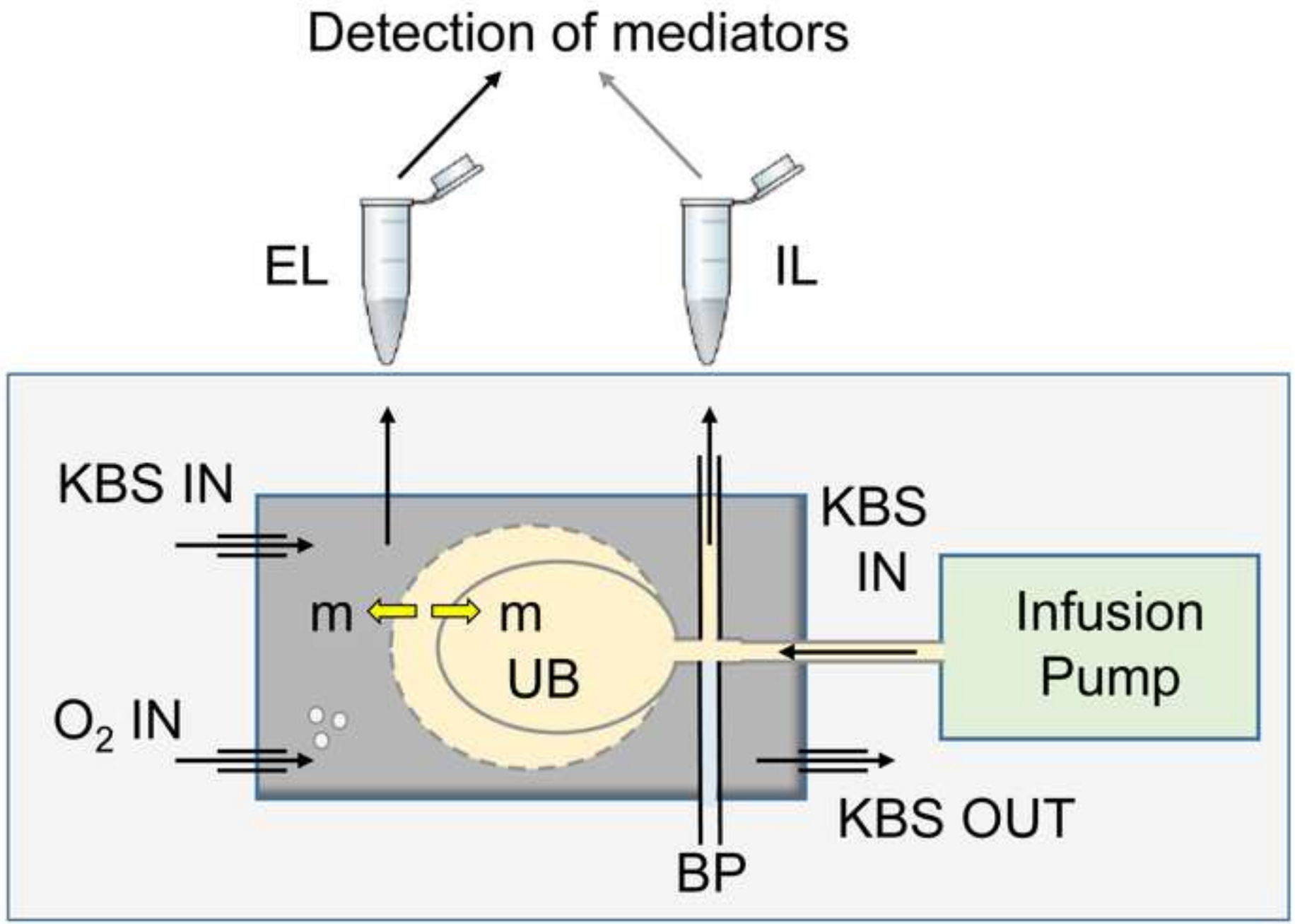


Figure 5



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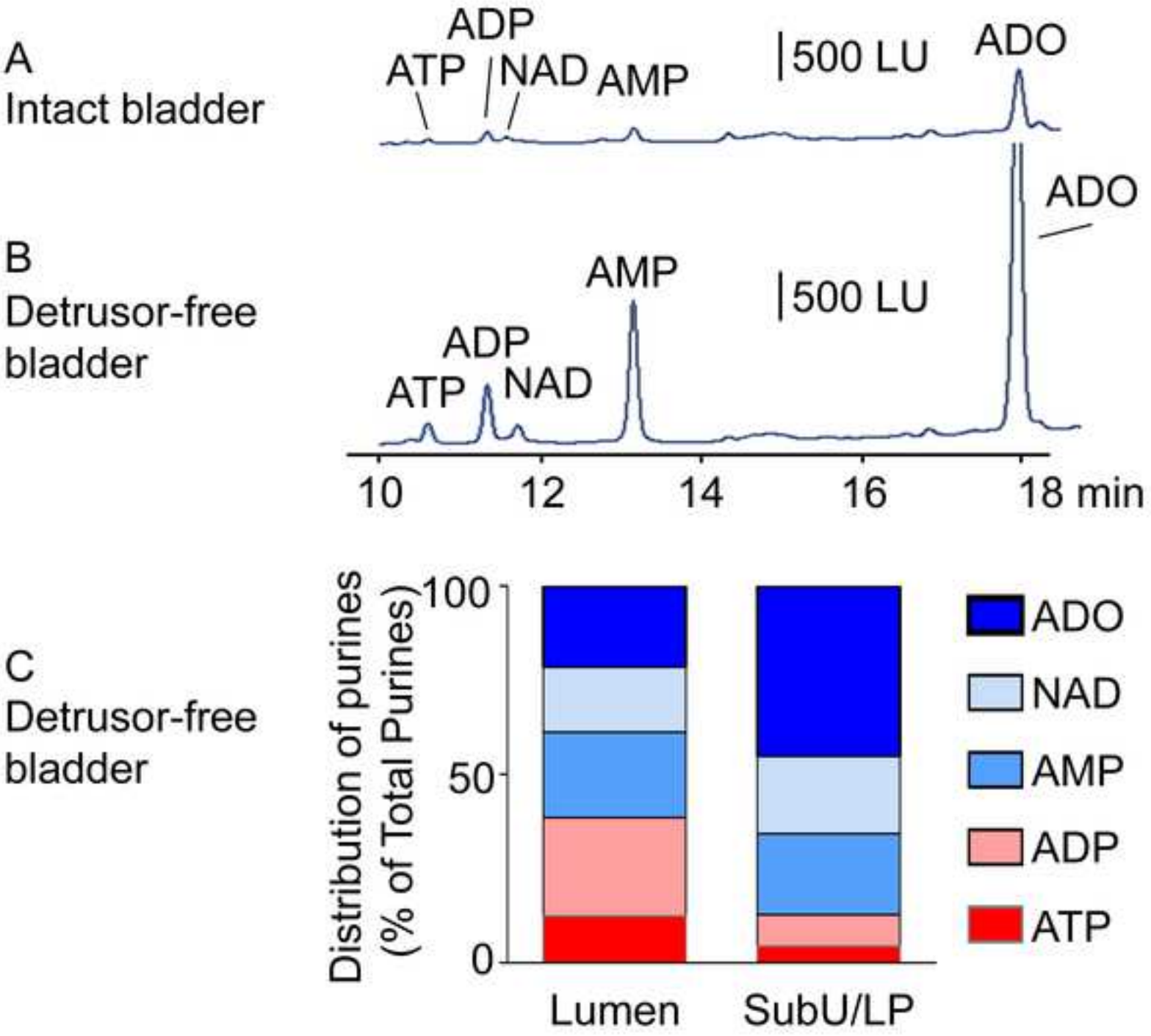
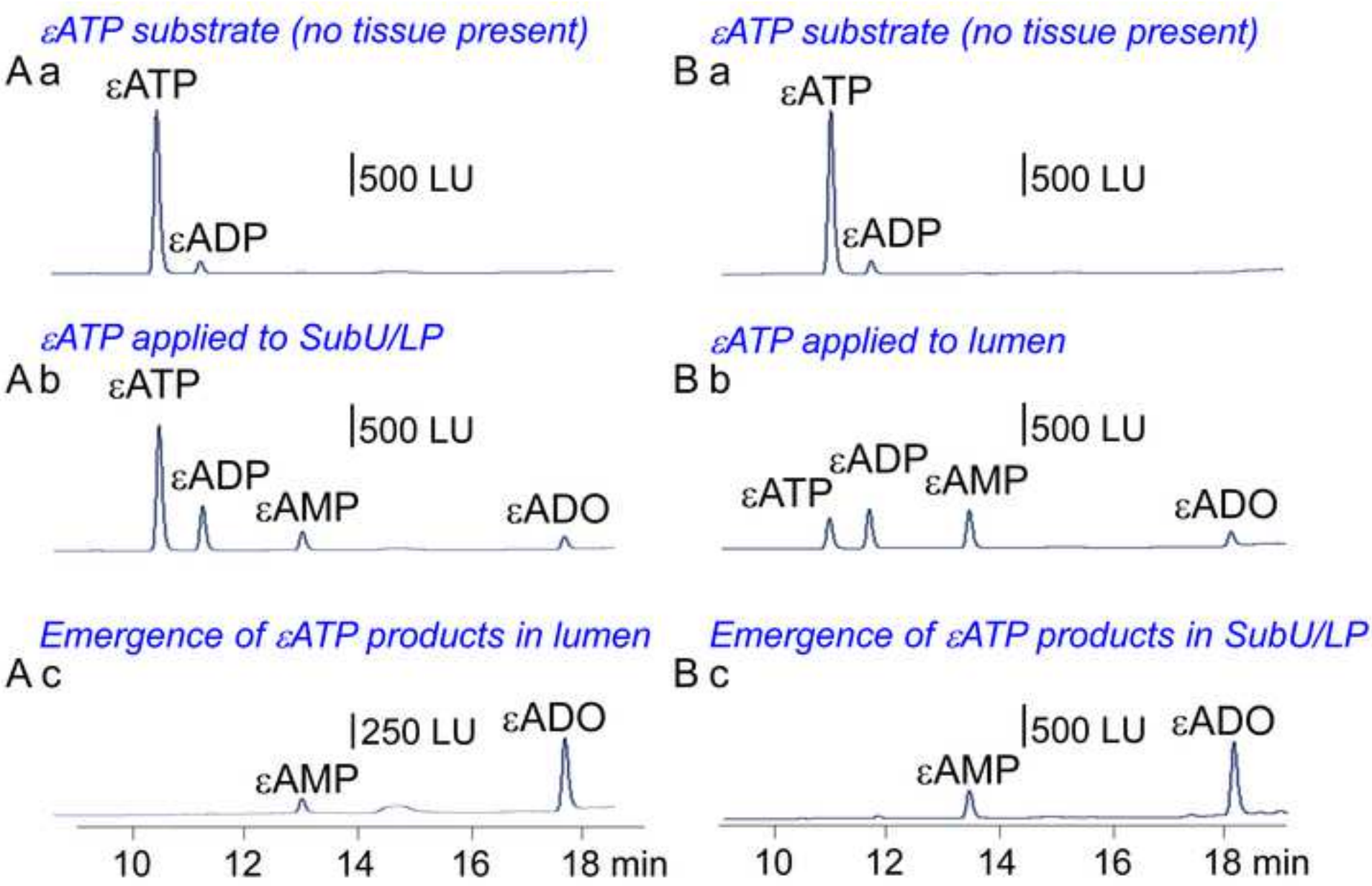


Figure 7



Video 1

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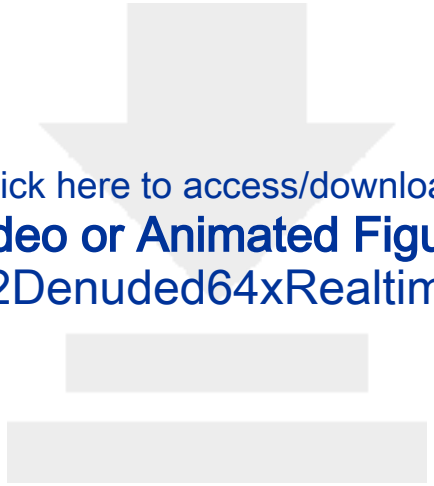
Movie1Intact64xRealtime.mov

Video 2



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Video or Animated Figure

Movie2Denuded64xRealtime.mov

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
CaCl2	Fisher	C79	Source flexible
Dextrose	Fisher	D16	Source flexible
Dissecting pins	Fine Science Tools	26002-20	Source flexible
Infusion Pump	Kent Scientific	GenieTouch	Source flexible
KCl	Fisher	P217	Source flexible
KH_2PO_4	Fisher	P284	Source flexible
Light source	SCHOTT ACEI		Source flexible
Microscope	Olympus SZX7		Flexible to use any scope
$MgCl_2$	Fisher	M33	Source flexible
NaCl	Fisher	S671	Source flexible
$NaHCO_3$	Fisher	S233	Source flexible
Needles 25G	Becton Dickinson	305122	Source flexible
Organ bath	Custom made		Flexible source; We made it from Radnoti dissecting dish
PE-20 tubing	Intramedic	427405	Source flexible
Pressure transducer	AD instrument		Source flexible
S&T Forceps	Fine Science Tools	00632-11	Source flexible
Software pressure-volume	AD Instruments	Power lab	
Suture Nylon, 6-0	AD surgical	S-N618R13	Source flexible
Suture Silk, 6-0	Deknatel via Braintree Scientific, Inc.	07J1500190	Source flexible
Syringes 1 ml	Becton Dickinson	309602	Source flexible
Vannas Spring Scissors	Fine Science Tools	15000-08	Source flexible
Water circulator	Baxter	K-MOD 100	Source flexible

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Author(s): LEONIE DURNIN, ROBERT D. CORRIGAN, KENTON M. SANDERS, VIOLETA N. MUTAFOVA-YAMBOLIEVA

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July 25, 2019

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Dear Editors,

In response to the editorial comments for 60334-R1, we have made the following revisions:

1. Revised the format of Reference List.
2. Numbered the references in order of appearance.
3. Defined all abbreviations before use.
4. Gave specific parameters in Step 2.8.
5. Proofread the revised manuscript.
6. Uploaded the revised manuscript with track changes.

In addition, we uploaded a revised Figure 1 in a psd format. The minor revision consists of adding designation of the Detrusor layer to the drawing of INTACT BLADDER.

Sincerely,

Prof. Violeta Mutafova-Yambolieva, MD, PhD
Corresponding author

Violeta N Mutafova-Yambolieva

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