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RESEARCH ARTICLE

A method to study bladder urothelial cellular function *in situ*

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ABSTRACT

OBJECTIVE: The bladder urothelium is comprised of basal, intermediate, and apical urothelial cells. Ability to functionally study an individual urothelial cell while preserving its in situ location would represent an advance in bladder urothelial biology.

MATERIALS AND METHODS: Mice were euthanized and cardiac perfused with phosphate-buffered saline. Bladders were then excised. Urothelial sheets were dissected off the lamina propria using forceps with a microscope (5 × magnification). Sheets were stained with H&E. In separate experiments, cell-attached electrophysiology was performed by placing urothelial sheets in Ringer's bath solution, with either basal or apical surface down. Using 40 × magnification, individual urothelial cells from different layers were identified. Potassium currents on these cells were measured in situ using single channel patch-clamp technique.

RESULTS: Histology revealed that urothelial sheets were free of lamina propria and smooth muscles. The proportion of apical cells, compared to proportion of intermediate and basal cells, with measurable potassium currents was considerably higher in apical cells (69% of apical versus 16% of intermediate and 18% of basal cells). Of the active patches detected in apical cells, 100% of these patches showed a 43 pS current conductance. For intermediate and basal cells, 75-83% demonstrated a 43 pS current and 17-25% demonstrated a 22 pS current. Single cells, from all 3 layers, could also be individually microdissected completely off the urothelium.

CONCLUSIONS: A novel approach was developed in which individual urothelial cells within the multi-layered urothelium were identified and functionally studied *in situ*. Electrophysiologic characterization revealed different phenotype between the cells from different layers. Single cells from an identified layer can be harvested off the urothelium allowing for other studies. This technique allows investigators to study various cellular functions while preserving cellular location within the urothelium.

Keywords: bladder, urothelium, urothelial cells, potassium current

INTRODUCTION

The bladder urothelium is a multi-layered epithelium, with the apical cells lining the bladder lumen, the intermediate cells just below, and the basal cells abutting the basement membrane. The apical urothelial cells have specialized function that maintain bladder-blood impermeability [1] via expression of uroplakin plaques [2] and tight junction proteins [3]. Because the urothelium is theorized to have additional properties, such as urothelial-afferent signaling [4] and immune surveillance [5], the ability to dissect out individual cells from the multi-layered urothelium would represent an advance in the field of urothelial biology. Physiologic studies on bladder urothelial function (such as patch clamp electrophysiology and calcium microfluorimetry) have often utilized primary dissociated cell cultures which loses cellular specificity. Cultured urothelial cells undergo changes that de-differentiate from their *in situ* phenotype [6].

A new technique was developed in which we were able to dissect a urothelial sheet of pure urothelial cells without lamina propria nor smooth muscle and to patch-clamp a single urothelial cell, *in situ* within the urothelium, all the while, knowing which specific layer this cell resided. Furthermore, this technique allows the complete removal of a single cell off the urothelium entirely; thus allowing possibility of other single cell studies. This technique will increase the granularity of our understanding of urothelial cellular biology, and allow the ability to measure potential differential function between the cells from different layers of the urothelium.

METHODS

Dissection of Urothelial Sheet from Mouse Bladder

This protocol was approved by Yale's Institutional Animal Care and Use Committee. Male C57BL/6 WT mice, 10 weeks old, were purchased from Jackson Labs and housed in animal facility on normal

diet for one week. After the mouse was anesthetized with intraperitoneal pentobarbital sodium (0.07 mg/mg body weight), animal was cardiac (left ventricle puncture, right atriotomy) perfused with PBS solution at 0.2 mg/ml for 5 minutes at a perfusion speed of 10 ml/min. Then, the whole bladder was excised and placed into PBS. The bladder was initially cross-sectioned into three pieces. Each cross-sectional piece (which was a horizontal slice giving a ring configuration to the section) was opened up and then cut into four equal pieces. Each of these pieces was used for urothelial sheet dissection. The urothelium was peeled off under microscope (5x magnification) with microforceps, preserving the orientation of the apical surface (meaning that the apical side of the urothelium was identified and maintained at all times). The urothelial sheet was then bathed in a solution containing 0.9 mM $CaCl_2$, 0.4 mM $MgCl_2$, 2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl and 8.1 mM Na_2HPO_4.

H&E Staining

To assess the quality for the above described dissection, urothelial sheets were fixed in 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer for 10 min before placing in 30% sucrose in 0.01 M phosphate buffer saline (PBS) for overnight. Then the urothelial sheets were paraffin embedded and sectioned at 8 µm and mounted on Fischer Superfrost Plus glass slides. Standard H&E staining was used to assess the architecture and anatomy of the dissected urothelial sheet. A full thickness bladder wall was also sectioned and H&E stained for comparison to the dissected urothelial sheet.

Localization and microdissection of apical, intermediate, and basal cells within the urothelial sheet

A urothelial sheet was transferred to a Cell-Tak (Sigma) coated cover glass (5 × 5 mm) to immobilize it for cell identification during patch clamping which was performed under an inverted microscope with 40 × magnifications. Basal and apical layer cells were directly patched because we oriented either apical or basal side of the urothelial face up (closer to eyepiece). For localization of intermediate cells, another glass micropipette (Sutter Model P-97 micropuller to a tip diameter of 20 µm), controlled with a micromanipulator (Narshige), was used to physically remove either basal or apical cells (based on orientation of the sheet) to expose underlying intermediate cells. Removal of cells was facilitated by applying suction through the micropipette in addition to applying physical motion to the micropipette (see **Movie S1**: removal of apical cell, **Movie S2**: removal of intermediate cell, and **Movie S3**: removal of basal cell).

Electrophysiologic Protocols and Solutions

The experiments were conducted at room temperature (22-24°C) on an inverted microscope (IMT-2, Olympus). Electrodes were pulled from borosilicate glass capillaries on a Sutter Instrument Co (Model P-97, USA) and polished on a MF-830 microforge (Narishige, Tokyo). Patch electrodes had resistances of 6-7 MΩ when filled with pipette solution (in mM: 140 KCl, 1.8 $MgCl_2$, 10 HEPES, pH 7.4 with KOH). Single channel currents were recorded by using Axopatch 200B and digitized by Digidata 1322A with pCLAMP10.2. All the chemicals were purchased from Sigma.

Single channel patch clamp recording were performed in cell-attached configuration to measure potassium currents. Urothelial sheet were bathed in solution contained of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4.

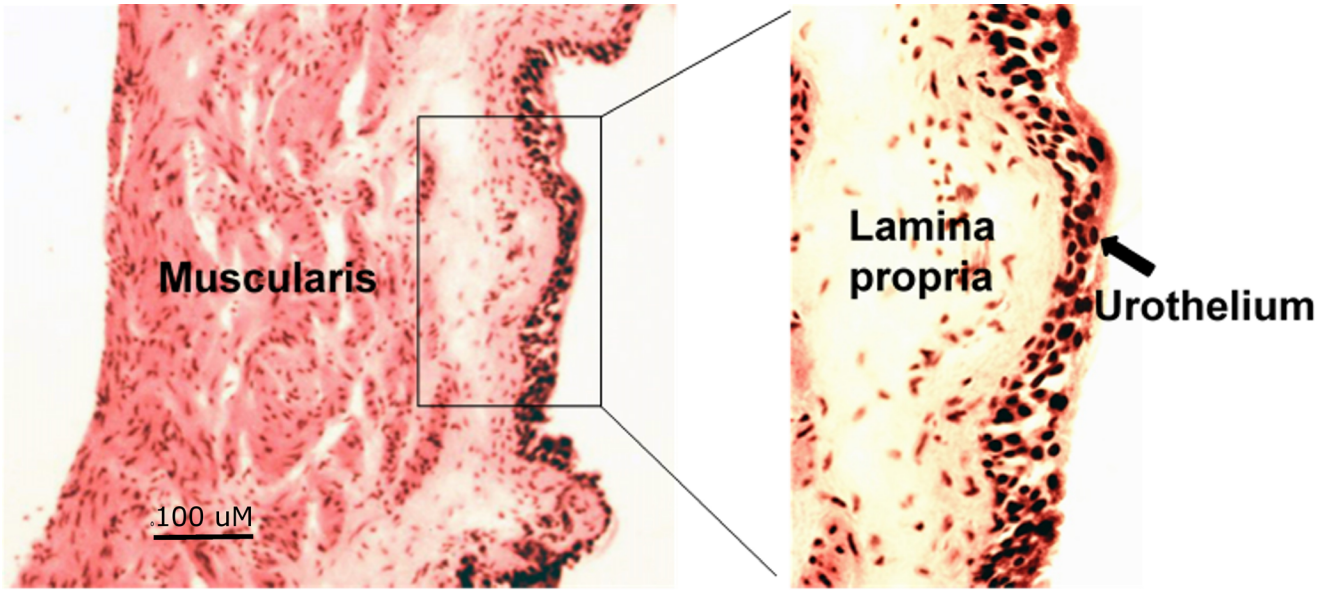

Figure 1. Mouse bladder wall cross-section in H&E staining showing urothelium, lamina propria and muscularis. The adventitia was not shown in this section. 5 × and 40 × magnifications.

Data Analysis

Signals were digitized at a sampling rate 4 kHz (DigiData 1322A, Axon Instruments) and saved in a PC for further analysis. Patch data were analyzed using Clampfit 10.2 at a digital filter frequency of 250 Hz. Channel activity was calculated by the following equation during sampling periods of 10-30 seconds. $NP_o = \Sigma(t_1 + t_2 + \ldots t_n)$, where N is the number of observed current levels in the patch, P_o is the single channel open probability; t_n is the fractional open time spent at each

of the current levels. Results are given as means ± SE. Channel conductance was calculated by using GraphPad Prism 5 software from linear regression analysis of single channel current-voltage curves. Voltage (V) applied to the pipette was referenced to the bath potential and reported as –V.

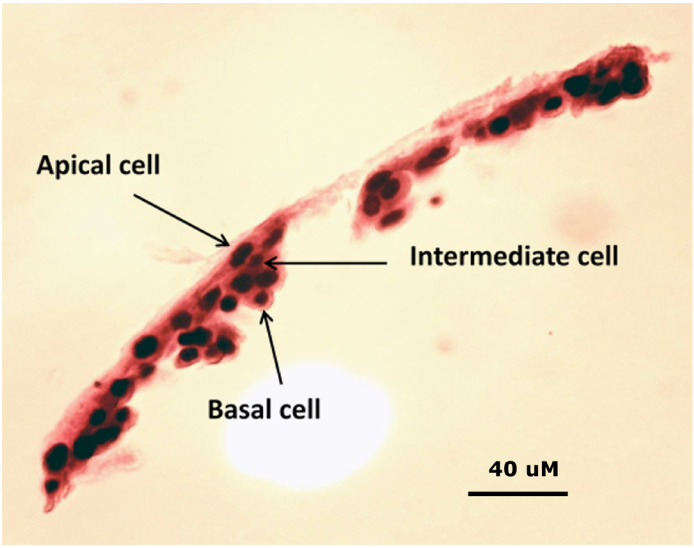

Figure 2. H&E staining of mouse bladder urothelial sheet cross-section cut showing urothelial purity of the dissection without lamina propria and without detrusor smooth muscle. Right panel is magnification (40 ×) showing details of the mouse bladder urothelium.

RESULTS

Dissected urothelial sheets are devoid of any lamina propria

From a full thickness section, the urothelium, lamina propria, and muscularis can be seen on H&E (**Fig. 1**). The urothelium was comprised of three layers of cells: a single layer of apical cells, two to three layers of intermediate cells and a single layer of basal cells.

A dissected mouse urothelial sheet was devoid of any lamina propria tissue contamination (**Fig. 2**). Urothelial sheets were harvested repetitively with the same results of having a non-contaminated multi-layered urothelium. **Figure 3** shows a urothelial sheet under a microscope, with 5x and 10x magnifications.

Visualization of apical, intermediate and basal cells

After harvesting urothelial sheets, and maintaining orientation of urothelium, the sheets were immobilized to the bottom of inverted microscope chamber bathed with perfusion solution. A glass micropipette was used to expose cells from different layers. Three cell layers are shown in **Figure 4**, right panel, at 40 × magnification. The basal cells faced closest to the eyepiece, and are indicated by "b". Between the basal cell and apical cell (indicated by "a"), are the two to three cells layer comprising the intermediate layer indicated by "i". Videos of the micropipette completely dissecting off an apical, intermediate and basal cells are included in the supplemental data uploaded on journal website (**Movie S1**： removal of apical cell, **Movie S2**： removal of intermediate cell, and **Movie S3**: removal of basal cell). A photomicrograph of a single apical cell, dissected off a urothelial sheet, is shown in **Figure 5**.

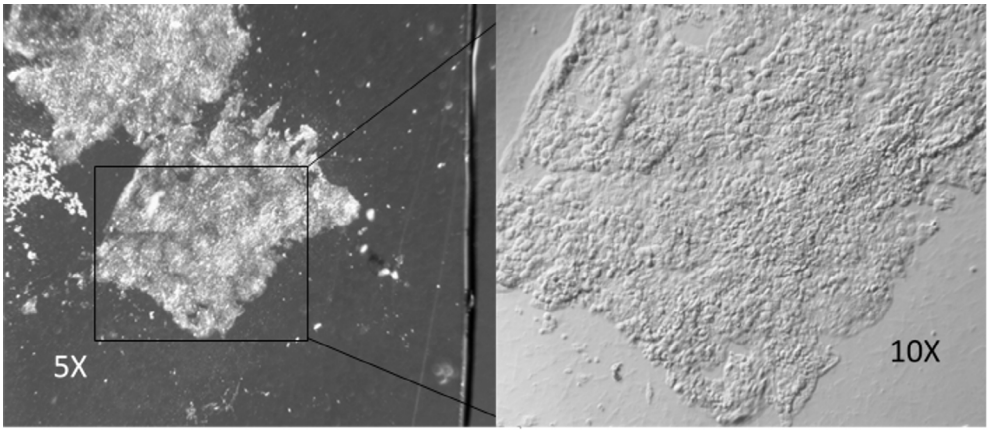

Figure 3. Shows a typical mouse bladder urothelial sheets under 5 × and 10 × magnifications.

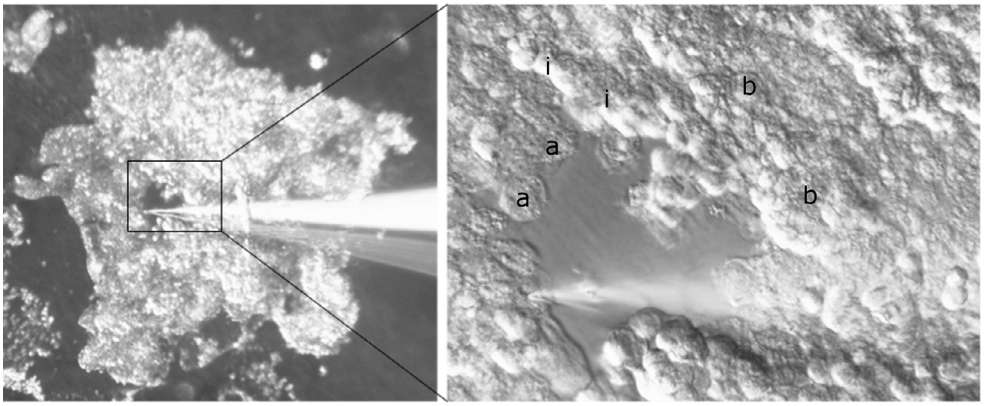

Figure 4. Mouse urothelial sheet under inverted microscope with basal urothelium facing up, towards eyepiece. Left panel is 4 x magnification and the patch clamp micropipette is seen. Right panel is 40 x magnification. Representative cells from the three layers of the urothelium can be seen after removing appropriate neighboring cells. a = apical cells, i = intermediate cells, b = basal cells.

Figure 5. Microphotograph image showing a single apical cell microdissected off the urothelial sheet. Videos showing removal of individual cells from the urothelium are at the journal website (Movie S1 = removal of apical cell, Movie S2 = removal of intermediate cell, Movie S3=removal of basal cell).

Electrophysiologic measurements of the different types of urothelial cells

From 14 mice, 168 total cells were patched; 64 cells (38%) showed active channel activities (**Table 1**). There were, proportionally, many more cells with active patches in the apical population (40/58, 69%), compared to intermediate (12/55, 22%) and basal (12/55, 22%) populations. The active patches were characterized by two different potassium conductances. Two representative tracings are shown in **Figure 6A** and 6B at holding voltages of -20 mV, -40 mV and -80 mV. The I-V (pA vs mV) curves are shown in **Figure 6A** and **6B**. These tracings reflected channel conductances of 43 pS and 22 pS, respectively. The channel open probabilities of these types of currents were different with 0.05 (for 43 pS conductance current) and 0.44 (for 22 pS conductance current). When active patches were detected, apical cells only expressed the 43 pS potassium current (100%), whereas intermediate and basal cells had similar dichotomy with ~80% expressing the 43 pS and ~20% expressing the 22 pS potassium current (**Table 1**).

DISCUSSION

The ability to characterize any function of an individual apical, intermediate or basal urothelial cell would significantly increase the details of our understanding of bladder urothelial biology. The orientation of the cells within the bladder urothelium is presumed to be related to differentiation, as the cells go through the layers, they become terminally differentiated in the apical layer. It was shown, using embryonic mice manipulations, that existence of a single layer of urothelial cells in the bladder expressing the terminally differentiated uroplakins III (UPIII) was possible in a p63 knock-out transgenic animal [7]. p63 is a protein that is a phenotypic marker for basal epithelial cells and is a master regulator for epithelial development. Because UPIII, a marker for apical (umbrella) cells was expressed in a single layer urothelium with no intermediate and no basal cells, the authors in reference 7 suggested that the apical cells arise from a certain stem cell whereas basal/intermediate urothelial cells arise from a different stem cell. However, others have shown that apical cells can arise from basal and intermediate cells [8, 9]. The biology of how the urothelium becomes stratified and whether specific functions are attributed to different layers remain complex and incompletely understood.

Most functional studies of bladder urothelial cells have used monolayer cell cultures derived from urothelial biopsies (numerous studies) or using chambers to measure permeability/transmembrane epithelial resistance of the entire thickness of the urothelium [10]. Antibody localization or antibody-related purification techniques have found certain proteins associated with certain layers, thus attributing function based on the specific proteins being differentially expressed (e.g. findings of tight junction proteins in the apical cells only [11], finding of uroplakins on apical cells [12]). It was suggested that the bladder urothelium adsorb proteins, including antibodies, in a non-specific manner which can give rise to inconsistent results [13]. None of these techniques permit layer-specific single cell functional studies. We described our technique of obtaining urothelial sheets devoid of lamina propria, by microforceps without any additional treatments or specialized techniques.

Table 1.Distribution of active potassium channels in mouse urothelial cells

n = 14 mice	Number of Cells Patched	Number of Cells with Active/ Inactive Patches	Number of Cells with 43 pS K channels (% of only active patches)	Number of Cells with 22 pS K channels (% of only active patches)
Apical cells	58	40/18	40 (100%)	0 (0%)
Intermediate cells	55	12/43	9 (75%)	3 (25%)
Basal cells	55	12/43	10 (83%)	2 (17%)
Total	168	64/104	59 (92%)	5 (8%)

Our data not only shows the feasibility of measuring electrophysiologic function of individual cells within the urothelial sheet (*in situ*), but the data suggested differential potassium ion conductance between the cells of the different urothelial layers. The specific type of potassium channels mediating these currents were not determined in this study because the primary intent of this work was to demonstrate the feasibility of this new technique. Certainly, use of specific pharmacologic blockers in the future can potentially identify specific channels mediating these potassium currents. However, the importance of this study was to demonstrate a technique which provided a novel approach to study urothelial cellular function *in situ* within the milieu of the urothelium.

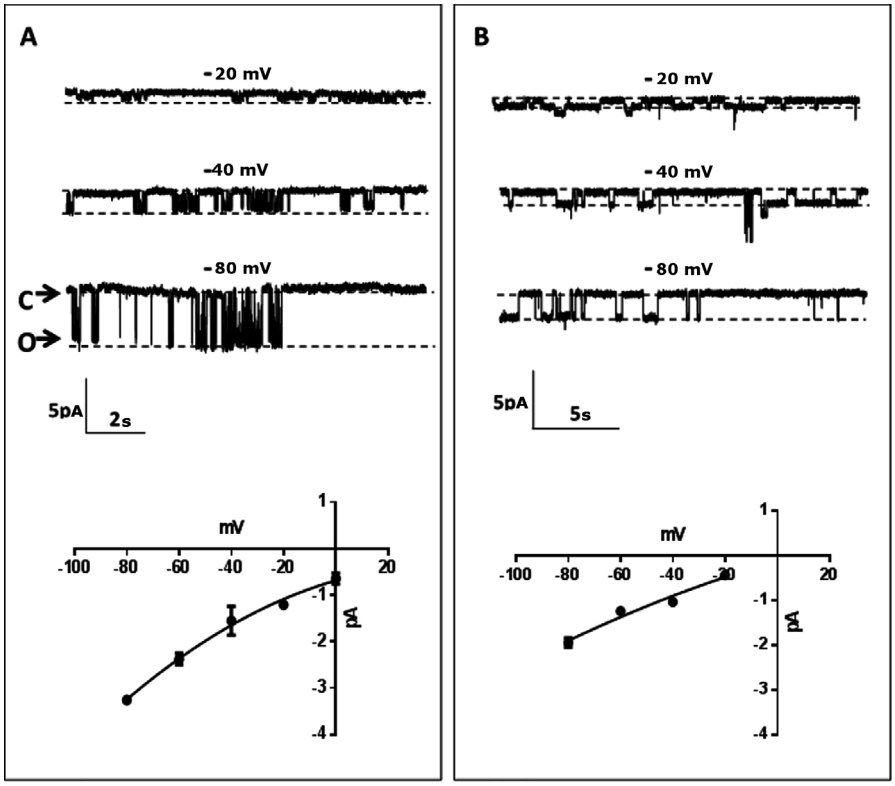

Figure 6. Two types of potassium channel activities were detected in the urothelial cells. Representative single channel recording traces were chosen at V = -20 mV, -40 mV and -80 mV in cell-attached configuration. "C" indicates channel closed state. "O" indicates channel open state. The I-V curves showed different channel conductance slopes

From these data, it appeared that the phenotype of potassium currents were different based on cellular location within the stratified urothelium. Compared to intermediate/basal cells, apical cells had a higher percentage of active patches (40 out of 58, 69%) and no expression of the 22pS potassium conductance current (**Table 1**). The possible reasons for lack of detecting active patches could be due to the fact that no potassium channels were present on the patched area or the channels were closed (silent). The role of potassium channel in the urothelial cells could be related to observation of potassium absorption across the urothelium [14]. As stated, the specific potassium ion channels mediating these currents were not addressed in this study. However, presence of active potassium channels, including Kir2.1 and BK, have been detected in cultured human bladder urothelial cells, with altered function of these channels in interstitial cystitis and overactive bladder [15, 16]. The conductance of Kir2.1 is small (~27 pS) [15], whereas conductance of BK is large (~180 pS) [16], so the 22 pS potassium current detected in this study might be Kir2.1. It should be noted that these channel activities in the prior urothelial studies were measured in cultured monolayer of cells from human subjects. Whereas in this study, patch clamp studies were performed on urothelial cells *in situ* and from a different animal species.

The other utility of this technique involve possibilities other functional studies on individual cells. Cells dissected completely free (see **Movie S1**, **Movie S2** and **Movie S3** on *Bladder* website) using this technique can also be functionally studied with calcium microfluorimetry. Primary cultured or dissociated urothelial cells have been shown to have increased intracellular calcium in response to capsaicin [17, 18], muscarinic agonists [19, 20, 21], ATP [22], stretch [23], and TRPV4 agonist [23]. This technique could allow investigators to be able to perform calcium imaging on single cells from identified layers of the urothelium.

It is recognized that heterogeneity of neighboring cells within the same tissue may be important in explaining the biology of certain diseases and embryonic development. For example, heterogeneity of neoplastic cells within the same tumor might drive the biologic behavior of the tumor. In embryogenesis, heterogeneity of neighboring cells is a necessity for normal development. Single cell analysis allows the genome sequence using the relatively new technique of amplification method-multiple annealing and looping-based amplification cycles (MALBAC) [24]. The ability to obtain viable single cells from the bladder urothelium would allow single cell genome sequencing which will increase our biologic understanding of the urothelium.

CONCLUSIONS

We described a microdissection technique enabling functional studies of individual urothelial cells *in situ* within the urothelium. We found that membrane potassium permeability properties were unique depending on the location of the cells. Basal and intermediate cells exhibited one type of potassium conductivity phenotype whereas apical cells exhibited another phenotype suggesting different membrane properties between cells in different layer of the urothelium. This technique can be further applied to study other single cell functional studies to increase our understanding of the complexity of the bladder urothelium.

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Supplementary information

Movie S1. Apical cell microdissection.
Movie S2. Intermediate cell microdissection.
Movie S3. Basal cell Microdissection.
Supplementary information of this article can be found online at http://www.bladderj.org.