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Atomic Force Microscopy of Mammalian Urothelial Surface

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SUMMARY

The mammalian urothelium apical surface plays important roles in bladder physiology and diseases, and it provides a unique morphology for ultrastructural studies. Atomic force microscopy (AFM) is an emerging tool for studying the architecture and dynamic properties of biomolecular structures in near physiological conditions. But AFM imaging of soft tissues remains a challenge due to the lack of efficient methods for sample stabilization. Using a porous nitrocellulose membrane as the support, we were able to immobilize large pieces of soft mouse bladder tissue thus enabling us to carry out the first AFM investigation of the mouse urothelial surface. The sub-micron resolution AFM images revealed many details of the surface features, including the geometry of the urothelial plaques that cover the entire surface and the membrane interdigitation at the cell borders. This interdigitation creates a membrane-zipper, likely contributing to the barrier function of the urothelium. In addition, we were able to image the intracellular bacterial communities (IBCs) of type 1-fimbriated bacteria grown between the intermediate filament bundles of the umbrella cells, shedding light on the bacterial colonization of urothelium.

Keywords

Atomic force microscopy; urothelium; tissue; surface; cell junction

INTRODUCTION

Mammalian urothelium, that lines the urinary bladder as well as other parts of the lower urinary tract, functions as the urine-blood barrier.¹ The urothelium usually consists of 3 distinct cell layers: the basal layer of small cells, the intermediate layer of moderately sized cells, and the apical layer of very large umbrella cells.² The apical surface of the umbrella cells is almost completely covered by unique urothelial membrane plaques (also known as asymmetric unit membrane, or AUM; see below) made of crystalline arrays of 16 nm hexagon-shaped protein particles consisting of 4 major integral membrane proteins called uroplakins (UPs).^{3–8} It was suggested that there are two physical barriers in the urothelium;¹ the urothelial plaques, covering about 90% of the apical cell surface, serve as the transcellular barrier, while tight junctions between the umbrella cells contribute to the paracellular barrier.^{9–11} The mammalian urothelium is not just a passive barrier because many dynamic processes can happen at the apical surface.¹² The surface of the urothelium changes greatly by folding and

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unfolding during the contraction and distension of the micturition cycle.^{4,13} Extended stretching has been proposed to induce the fusion of numerous subapical fusiform vesicles, of the same AUM structure, with the plasma membrane - that may again be retrieved back into the cytoplasm when the bladder contracts.^{14–16} Stretch may also induce endocytosis of the apical membrane as shown by Ussing chamber experiments using rabbit bladders.¹⁷

Urothelium is also the major site for uropathogenic *E. coli* (UPEC) colonization to cause urinary tract infection (UTI). UTI is one of the most common infectious diseases that affects a large proportion of the world population and accounts for significant morbidity and high medical costs.^{18–19} One of the uroplakins, UP Ia, has been shown to be the urothelial receptor for type 1-fimbriated *E. coli*, the primary agent of UTI.^{20–22} The attachment of UPECs to the urothelial surface through the adhesin FimH at the distal end of the fimbria is a critical first step in the establishment of this infection. It can trigger a signaling cascade leading to the urothelial engulfment of the bacteria.^{23–24} The invaded bacteria can then rapidly multiply inside the umbrella cells and form the biofilm-like intracellular bacterial communities (IBC).^{25–26}

The apical surface of the mammalian urothelium has a unique morphology especially suitable for ultrastructural studies. First, the urothelial plaques of the plasma membrane and the cytoplasmic fusiform vesicles exhibit an asymmetric membrane structure when viewed by thin section electron microscopy (EM), i.e., the outer leaflet of the membrane is twice as thick as the inner leaflet, hence the name AUM.^{9,13,15} The urothelial plaques of the plasma membrane are interconnected by symmetric membrane regions called “hinges”,^{3,4,9,15} and linked to cytoskeletal intermediate filaments by small bridges of unknown molecular composition.⁴ It was also observed that these plaques exhibit a concave scalloped shape,¹⁵ although the curvature of the concavity was never systematically measured. Second, the urothelial plaques can be readily isolated in milligram quantities from mammalian urothelia,^{27–28} hence they are suitable for high resolution structural studies using electron crystallography as these plaques are two-dimensional (2D) crystals of 16 nm uroplakin particles.^{8,21,29–32} These 2D protein crystals exhibit good order and sufficient size (up to 1 μ m) and have allowed atomic force microscopy studies²¹ as well as three-dimensional (3D) reconstructions at molecular resolution to be performed.³³ In addition, other microscopy techniques, such as freeze-fracture,^{3–4} scanning EM,^{10,12,34–37} have also been applied to visualize the urothelial surface. However, all these ultrastructural techniques only provided static images of the urothelial surface of fixed samples. Some light microscopy studies of the urothelium have been reported recently,^{26,38–40} which can potentially allow the low resolution visualization the dynamics of the urothelial surface directly. But we still lack tools that can observe the dynamics of urothelium at the resolution range that bridging the EM and light microscopy.

In this study, we introduce the AFM as a novel tool to investigate the morphology and the dynamics of the native urothelial apical surface at a spatial resolution between 50 and 100 nm. To achieve this, we had to overcome the challenging task of stabilizing the extreme soft tissue of the mouse urothelium and develop a simple protocol to immobilize freshly dissected tissues on porous nitrocellulose membranes, thereby enabling us to image unfixed tissues by AFM. More specifically, we have obtained AFM images of the distinct morphology of the urothelial surface and, for the first time, the fine details of the urothelial cell boundaries. Moreover, we have been able to image clusters of type 1-fimbriated *E. coli* grown between bundles of intermediate filaments within the umbrella cells, thus shedding new light on the development of intracellular bacterial communities of uropathogenic *E. coli* in urothelial tissues.

RESULTS

Immobilization of mouse urothelial tissues

As a first attempt to immobilize bladder tissue for AFM imaging, we used the device developed by Reichlin and coworkers to look at the surface features of coronary arteries.⁴¹ In this method, a piece of tissue is placed on top of a metal screw and pressed against an EM grid. For a rigid sample, the grid prevents any movement and enables reliable imaging. In contrast, a soft and easily extensible tissue, such as the urinary bladder, was not stable on top of the screw and pressing it against a grid simply pushed the tissue through the grid holes. Hence, we tried to rigidify the bladder using glutaraldehyde fixation and osmium staining. In that case, pressing the tissue against an EM grid allowed stable immobilization of the apical bladder surface so that images could be recorded (see below).

For native, unfixed bladder tissue, we have developed a novel immobilization protocol involving a porous nitrocellulose membrane commonly used for Western blotting (Supplementary Data, Figure 1). This method takes advantage of both the nitrocellulose affinity for protein and the porous nature of the membrane. We achieved a uniform adhesion to the nitrocellulose by removing the interstitial liquid between the tissue and the membrane using filter paper.

AFM visualization of the mouse urothelial surface

The first step in this study was to obtain AFM images of the apical surface of glutaraldehyde-fixed and osmium-stained mouse urothelium since this is the standard preparation protocol for tissue in transmission EM.⁴² By AFM, the apical bladder surface appears folded with deep crevasses (Supplementary Data, Figure 2), consistent with that observed by scanning electron microscopy (SEM).³⁶ At higher magnification, the whole surface is packed with urothelial plaques exhibiting a concave shape (Supplementary Data, Figure 2). The concave shape of the plaques causes the hinges between individual plaques to protrude into the lumen by ~50 nm relative to the center of the plaques. Notice that close packing of the plaques made them appear as irregular hexagons. Even the arrays of the 16 nm uroplakin particles were visible on the urothelial plaques in close-up images, but the hexagonal shape of the particles was not clear.

To obtain a closer-to-native look at the apical urothelial surface, we adsorbed a piece of bladder tissue to a nitrocellulose membrane, as described in the Materials and Methods section, and fixed the sample briefly by placing a drop of 8% glutaraldehyde solution on the tissue for ten minutes. The sample was then washed with PBS and imaged by AFM in the same buffer. As with osmium-stained samples, the concave urothelial plaques cover the entire apical cell surface all the way to the cell borders (Figure 1(a) and (b)).⁴ The urothelial plaques had an average hinge-to-hinge diameter of 600 ± 100 nm ($n=30$) and a depth of 110 ± 50 nm ($n=30$). This corresponded to an average radius of curvature of 460 ± 215 nm. The concavity of the plaques may be caused by the density difference between the two leaflets of the lipid bilayer as suggested for plasma membrane rafts containing high concentration of cholesterol and sphingolipid (cerebroside in the case of urothelial plaques).⁴³ Compared to the osmium-stained samples, the 10 minutes glutaraldehyde fixation preserved better the apical urothelial surface. For example, the junctions between the hexagonally shaped umbrella cells appear rather straight (Figure 1(a)), while after osmium staining the junctions were much wavier (data not shown). The umbrella cell borders appeared to be a long ridge, consistent with previous ultrastructural studies.^{34,36} This ridge was ~500 nm wide and ~400 nm in height (Figure 1(c)). A fine interdigitated boundary traversing the whole ridge is visible on the AFM deflection image (Figure 1(d), arrowheads). This interdigitation of membrane at the cell borders has not been reported earlier, and it creates a zipper-like structure along the ridge. With consecutive thin sections of better-preserved samples using high-pressure freeze fixation, it can be seen

that the interdigitation is created by the membrane overlap at the cell edges (Figure 1 (e) and (f)). Hence the interdigitation does not include the tight junction itself.

AFM imaging in PBS of unfixed urothelial tissue adsorbed to a nitrocellulose membrane was also achieved despite the softness of the sample and the high number of folds on the surface. The numerous urothelial plaques covering the apical surface and their cup-like appearance are clearly depicted in large flat areas (Figure 2(a)). However, regions with deep membrane folds were more difficult to image in the absence of glutaraldehyde-fixation especially because the highest features could be perturbed by the movement of the tip (see the numerous streaks in Figure 2(b)). Infusion of glutaraldehyde into the fluid cell of the AFM yielded rapid hardening of the surface, similar to what has been reported for cell monolayers cultured on a petri dish.⁴⁴ The urothelial plaques were then visible (Figure 2(c)) and contrast enhancement of the images revealed the hexagonal packing of the plaques (Figure 2(d), arrowheads).

AFM visualization of bacteria grown in the umbrella cells

Finally, we investigated the interaction between the type 1-fimbriated *E. coli* bacteria and the mouse urothelial apical surface. Since the adhesin FimH is sufficient to mediate the bacterial invasion, we used the recombinant *E. coli* strain SH48 that express type 1-fimbriae in this experiment. To visualize the intracellular bacteria communities, we first facilitated a fast bacterial invasion by washing the apical membrane for 5 min with 0.1% Triton X-100, as detergents are often used to enhance the invasion of pathogens,⁴⁵ followed by the incubation of the urothelium with overlaid type 1-fimbriated *E. coli* for 2.5 hours. We then removed the plasma membrane already treated with detergent by repeatedly pipetting PBS over the sample surface. Instead of a typical apical surface image, we observed an extensive network of fine, flexible filaments within which single bacteria and bacterial communities were nesting (Figure 3(a) and (b)). The wavy appearance of the filaments and their tendency to form large bundles (Figure 3(b)) suggest that they are intermediate filaments.⁴⁰

DISCUSSION

Nitrocellulose membrane provides a simple, effective way for immobilizing soft tissues

Over the last decade, AFM has become a common tool for investigating protein assemblies and cultured cells in their near-physiological environment.⁴⁶ For tissue samples, most AFM studies were reported for fairly stiff tissues, such as cartilage,⁴⁷ cornea⁴⁸ and coronary arteries.^{41,49} It remains very difficult to image unfixed tissue, especially soft ones.⁵⁰ This is largely due to the lack of an adequate method to immobilize soft tissue without fixation. Nevertheless, efforts have been made to overcome the difficulty of immobilization. For example, aorta tissue which is relatively stiff was successfully imaged by AFM by immobilizing it either with fine pins⁴⁹ or by pressing the sample against an EM grid with a screw.⁴¹ We now report a rather simple tissue immobilization protocol particularly useful for stabilizing soft tissue by using porous nitrocellulose membranes (Supplementary Data, Figure 1). The nitrocellulose membrane is a high-affinity substrate for protein materials; it allows the freshly dissected tissue sample to adsorb by simply overlaying it onto the membrane. The porous membrane allows easy removal of interstitial liquid from the backside, thereby yielding an effective suction and a uniform adhesion to the membrane. It also harbors the as yet unexplored potential to introduce small molecules from the backside for pharmacological manipulations of the sample. In addition, the nitrocellulose membrane-immobilized sample is extremely simple to be mounted on any AFM stage, thereby rendering further manipulations of the sample straightforward. These may be mechanical, for example by the cantilever/mechanical probes, or chemical, for example by introducing reagents to the sample surface, such as fixatives or bacteria as we have documented (see Figures 1–3). Our immobilization method has enabled us to produce the first

AFM images of the extremely soft mouse urothelial tissue (Figures 1 and 2), and we expect this method to be more generally applicable to soft tissues.

Membrane interdigitation at the apical cell junctions may contribute to the permeability barrier function

Our AFM data revealed for the first time the membrane interdigitation of the umbrella cell borders (Figure 1). Previously, various groups had visualized the umbrella cell borders by scanning EM and thin section transmission EM.^{10,14–15,34–36,51} In scanning EM images, the cell borders appear as straight ridges slightly raised along the cell borders,^{34–36,51} consistent with our findings (Figure 1(a)). Although it was generally assumed the ridge is the cell boundaries, scanning EM never visualized the details of where the cells actually divide due to the low-resolution limitation of the technique. The details of the cell junction regions can be visualized by thin section transmission EM, and the dimension of the raised ridge can be estimated from the micrographs to be ~ 300–400 nm wide and ~200 nm tall (Figure 1(e) and (f)), consistent with our AFM measurements. However, it is hard to visualize the interdigitation of the membrane along the cell borders from thin section EM. Only when consecutive thin sections were checked under EM, we can catch membrane overlay at the cell borders (Figure 1(e) and (f)). From our thin section transmission EM images (Figure 1(e) and (f)), one sees that the interdigitation is made of only the plasma membrane of the apical cell surface at the cell edges, i.e., the cell membrane in the border ridge forms many membrane “tongues” that stick out to the other side of the border. Since the membrane interdigitation does not include the junctional regions (containing the tight junctions) below the cell surface, it does not increase the circumferences of the cells and it is unlikely that it will be affected by surface stretching during the micturition cycles.

This membrane interdigitation at the umbrella cell borders may contribute to the blood-urine barrier of the bladder epithelium. Mammalian urothelium, with a transepithelial electrical resistance much higher than that of other epithelia, is the tightest epithelium known.^{2,52–53} As mentioned, urothelium has two physical barriers: the transcellular one formed by the highly specialized plasma membrane of the apical cells, and the paracellular one mainly consists the tight junctions at the cell borders. On the one hand, we know quite well the structure of the transcellular barrier due to the recent 3D cryo-EM structures of the urothelial plaques.^{30,33} The lipid bilayer of urothelial apical membrane contains a very high fraction of sphingolipids (mainly cerebroside) and cholesterol,^{54–55} which favor ordered lipid structures that can reduce the permeability of the membrane. The hexagonal close packing of the 16 nm uroplakin particles further organizes the lipid domains and reduces the fluidity of the lipids; hence enhance the permeability barrier function of the membrane.^{1,30} On the other hand, we know much less about the structure of the tight junctions and the related cell border region. Freeze fracture studies showed that the urothelial tight junction constitutes of only 3–6 junctional strands.^{34,56} Since the number of the junctional strands can be proportional to the paracellular electrical resistance,⁵⁷ a junctional strands of 3–6 should yield only a “intermediate tight” junction,^{57–58} but mammalian urothelium has the highest recorded electrical resistance.^{2,52} In addition to that the urothelium tight junctions contain claudin molecules that are associated with junctions of higher resistance,³⁸ the morphological details at the apical cell junction revealed by our AFM study may further improve the barrier function of the urothelium. The zipper-like interdigitation of the membrane at the cell border can i) better fasten the two neighboring cells, and ii) provide additional surface contacts of the apical membranes along the borders, hence further increase the electrical resistance of the junction. This membrane-zipper may also help stabilizing the tight junctions of the umbrella cells when the bladder is stretched during the micturition cycles. If membrane-zipper exists in other epithelial tissues remains to be seen.

Intermediate filament bundles of the umbrella cells provide niches for bacterial colonization of the urothelium

Our immobilization protocol has allowed us to image bacteria that replicated in the cytoplasm by AFM after removing the apical membrane by detergent washes. It has been shown in a mouse model of UPEC infection that the internalized bacteria replicate to form IBC, which can go through several stages of maturation.^{26,59–60} In the early hours of the IBC, the bacteria form loosely organized colonies with regular shape. But after 6 to 8 hours, the bacteria mature into tightly packed, biofilm-like clusters. The bacteria then can further change into filamentous, motile ones that can flux out from the IBC to enter a new cycle of infection. It appears from light microscopy and EM data that the IBC closely associates with keratin intermediate filaments.^{59,61} It was suggested that the intermediate filaments may provide scaffolds for the development of intracellular biofilms of UPEC.⁶¹ The bacteria we visualized may reflect the early stage of the IBC development. They seemed to form clusters right in the cavities between the bundles of intermediate filaments. It was found that as the urothelial cells mature into umbrella cells, the keratin filaments become dominant,^{62–63} the cavities between the boundless of these filaments may provide the right niches for the bacteria to replicate.⁶¹

MATERIALS AND METHODS

Immobilization and fixation of mouse bladder tissue

Urinary bladders were obtained from common laboratory mice of 3–6 month old. All animals were fed with a standard diet with free access to water before euthanization. Mice were euthanized by inhalation of CO₂, and bladders were rapidly excised and kept in phosphate buffered saline (PBS). For osmium staining, 50 μ l of 2% glutaraldehyde was introduced in the mouse bladder chamber while the ureters and urethra were blocked with tightened threads. The whole bladder was then immersed in 2% glutaraldehyde for 2 hours, and then cut into halves and stained with 1% OsO₄ overnight and washed with water before imaged by AFM.

For nitrocellulose immobilization, the bladder was cut into small pieces and the muscle side was slightly dissected to expose regions of underneath muscle for better adhesion to the cellulose membrane. Then the tissue was stretched slightly and flattened to be allowed to stick, with the urothelial side up, to a larger piece of nitrocellulose membrane (Bio-Rad, catalog number 162–0112). The excess PBS was blotted away with filter paper from the backside of the membrane to create a better adhesion (Supplementary Data, Figure 1). Then the whole piece was cut into a smaller square suitable for mounting onto an AFM sample stage (Supplementary Data, Figure 1).

Preparation of Bacteria

The type 1-fimbriated bacteria, *E. coli* strain SH48,⁶⁴ were first grown in Luria–Bertani (LB) broth overnight in tubes placed in a shaking incubator at 37°C. To induce expression of type 1 fimbria, the bacteria were then diluted 10-fold and subcultured overnight statically. Type 1 fimbria expression was confirmed by mannose-sensitive agglutination of 1% baker's yeast. The bacteria were then collected with centrifugation and re-suspended in the same amount of PBS before use.

Atomic force microscopy

The samples were either mounted on a pressing device,⁴¹ or double taped to a Teflon covered metal ring in the case of nitrocellulose membrane immobilization. All samples were scanned in PBS using cantilevers with oxide sharpened silicon nitride tips, which had a nominal spring constant of 0.06 N/m and nominal tip radii between 5 and 40 nm (type NP-S from Veeco, Santa Barbara, USA). AFM images were recorded in contact mode using a Nanoscope IIIa controller

running with software version 5.12r3 (Veeco, Santa Barbara, USA), operated at room temperature. 512×512 -pixel images were recorded with a scanner drive frequency of 1 to 5 Hz.

Electron microscopy

Mouse bladders were high pressure frozen and then freeze-substituted. Ultra-thin sections of 70 nm in thickness cut by using a Leica Ultracut UCT ultramicrotome (Leica, Deerfield, IL) were transferred onto Formvar-coated grids and double-stained with uranyl acetate and lead citrate. Images were recorded with CM200FEG electron microscope (FEI Corp., Eindhoven, Netherlands) equipped with a $1k \times 1k$ Multiscan 794 CCD camera (Gatan Corp., Pleasanton CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hicks RM. The mammalian urinary bladder: an accommodating organ. *Biol Rev Camb Philos Soc* 1975;50:215–46. [PubMed: 1100129]
2. Lewis SA. Everything you wanted to know about the bladder epithelium but were afraid to ask. *Am J Physiol Renal Physiol* 2000;278:F867–74. [PubMed: 10836974]
3. Kachar B, Liang F, Lins U, Ding M, Wu XR, Stoffler D, Aebi U, Sun TT. Three-dimensional analysis of the 16 nm urothelial plaque particle: luminal surface exposure, preferential head-to-head interaction, and hinge formation. *J Mol Biol* 1999;285:595–608. [PubMed: 9878432]
4. Staehelin LA, Chlapowski FJ, Bonneville MA. Luminal plasma membrane of the urinary bladder. I. Three-dimensional reconstruction from freeze-etch images. *J Cell Biol* 1972;53:73–91. [PubMed: 5013603]
5. Sun TT, Liang FX, Wu XR. Uroplakins as markers of urothelial differentiation. *Adv Exp Med Biol* 1999;462:7–18. [PubMed: 10599409]discussion 103–14
6. Hicks RM, Ketterer B. Hexagonal lattice of subunits in the thick luminal membrane of the rat urinary bladder. *Nature* 1969;224:1304–5. [PubMed: 4188048]
7. Vergara J, Longley W, Robertson JD. A hexagonal arrangement of subunits in membrane of mouse urinary bladder. *J Mol Biol* 1969;46:593–6. [PubMed: 5365961]
8. Walz T, Haner M, Wu XR, Henn C, Engel A, Sun TT, Aebi U. Towards the molecular architecture of the asymmetric unit membrane of the mammalian urinary bladder epithelium: a closed “twisted ribbon” structure. *J Mol Biol* 1995;248:887–900. [PubMed: 7760330]
9. Hicks RM. The fine structure of the transitional epithelium of rat ureter. *J Cell Biol* 1965;26:25–48. [PubMed: 5859020]
10. Hu P, Meyers S, Liang FX, Deng FM, Kachar B, Zeidel ML, Sun TT. Role of membrane proteins in permeability barrier function: uroplakin ablation elevates urothelial permeability. *Am J Physiol Renal Physiol* 2002;283:F1200–7. [PubMed: 12388410]
11. Negrete HO, Lavelle JP, Berg J, Lewis SA, Zeidel ML. Permeability properties of the intact mammalian bladder epithelium. *Am J Physiol* 1996;271:F886–94. [PubMed: 8898019]
12. Apodaca G. The uroepithelium: not just a passive barrier. *Traffic* 2004;5:117–28. [PubMed: 15086788]

13. Koss LG. The asymmetric unit membranes of the epithelium of the urinary bladder of the rat. An electron microscopic study of a mechanism of epithelial maturation and function. *Lab Invest* 1969;21:154–68. [PubMed: 5804627]
14. Minsky BD, Chlapowski FJ. Morphometric analysis of the translocation of luminal membrane between cytoplasm and cell surface of transitional epithelial cells during the expansion-contraction cycles of mammalian urinary bladder. *J Cell Biol* 1978;77:685–97. [PubMed: 681453]
15. Porter KR, Kenyon K, Badenhause S. Specializations of the unit membrane. *Protoplasma* 1967;63:262–74. [PubMed: 6037209]
16. Hicks RM. The function of the golgi complex in transitional epithelium. Synthesis of the thick cell membrane. *J Cell Biol* 1966;30:623–43. [PubMed: 5971009]
17. Truschel ST, Wang E, Ruiz WG, Leung SM, Rojas R, Lavelle J, Zeidel M, Stoffer D, Apodaca G. Stretch-regulated exocytosis/endocytosis in bladder umbrella cells. *Mol Biol Cell* 2002;13:830–46. [PubMed: 11907265]
18. Foxman B, Brown P. Epidemiology of urinary tract infections: transmission and risk factors, incidence, and costs. *Infect Dis Clin North Am* 2003;17:227–41. [PubMed: 12848468]
19. Litwin MS, Saigal CS, Yano EM, Avila C, Geschwind SA, Hanley JM, Joyce GF, Madison R, Pace J, Polich SM, Wang M. Urologic diseases in America Project: analytical methods and principal findings. *J Urol* 2005;173:933–7. [PubMed: 15711342]
20. Zhou G, Mo WJ, Sebbel P, Min G, Neubert TA, Glockshuber R, Wu XR, Sun TT, Kong XP. Uroplakin Ia is the urothelial receptor for uropathogenic *Escherichia coli*: evidence from in vitro FimH binding. *J Cell Sci* 2001;114:4095–103. [PubMed: 11739641]
21. Min G, Stolz M, Zhou G, Liang F, Sebbel P, Stoffler D, Glockshuber R, Sun TT, Aebi U, Kong XP. Localization of uroplakin Ia, the urothelial receptor for bacterial adhesin FimH, on the six inner domains of the 16 nm urothelial plaque particle. *J Mol Biol* 2002;317:697–706. [PubMed: 11955018]
22. Xie B, Zhou G, Chan SY, Shapiro E, Kong XP, Wu XR, Sun TT, Costello CE. Distinct glycan structures of uroplakins Ia and Ib: structural basis for the selective binding of FimH adhesin to uroplakin Ia. *J Biol Chem* 2006;281:14644–53. [PubMed: 16567801]
23. Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. *Science* 1998;282:1494–7. [PubMed: 9822381]
24. Martinez JJ, Mulvey MA, Schilling JD, Pinkner JS, Hultgren SJ. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *Embo J* 2000;19:2803–12. [PubMed: 10856226]
25. Anderson GG, Dodson KW, Hooton TM, Hultgren SJ. Intracellular bacterial communities of uropathogenic *Escherichia coli* in urinary tract pathogenesis. *Trends Microbiol* 2004;12:424–30. [PubMed: 15337164]
26. Justice SS, Hung C, Theriot JA, Fletcher DA, Anderson GG, Footer MJ, Hultgren SJ. Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. *Proc Natl Acad Sci U S A* 2004;101:1333–8. [PubMed: 14739341]
27. Liang FX, Riedel I, Deng FM, Zhou G, Xu C, Wu XR, Kong XP, Moll R, Sun TT. Organization of uroplakin subunits: transmembrane topology, pair formation and plaque composition. *Biochem J* 2001;355:13–8. [PubMed: 11256943]
28. Wu XR, Manabe M, Yu J, Sun TT. Large scale purification and immunolocalization of bovine uroplakins I, II, and III. Molecular markers of urothelial differentiation. *J Biol Chem* 1990;265:19170–9. [PubMed: 2229070]
29. Brisson A, Wade RH. Three-dimensional structure of luminal plasma membrane protein from urinary bladder. *J Mol Biol* 1983;166:21–36. [PubMed: 6854632]
30. Min G, Zhou G, Schapira M, Sun TT, Kong XP. Structural basis of urothelial permeability barrier function as revealed by Cryo-EM studies of the 16 nm uroplakin particle. *J Cell Sci* 2003;116:4087–94. [PubMed: 12972502]
31. Oostergetel GT, Keegstra W, Brisson A. Structure of the major membrane protein complex from urinary bladder epithelial cells by cryo-electron crystallography. *J Mol Biol* 2001;314:245–52. [PubMed: 11718558]
32. Taylor KA, Robertson JD. Analysis of the three-dimensional structure of the urinary bladder epithelial cell membranes. *J Ultrastruct Res* 1984;87:23–30. [PubMed: 6544865]

33. Min G, Wang H, Sun TT, Kong XP. Structural basis for tetraspanin functions as revealed by the cryo-EM structure of uroplakin complexes at 6-Å resolution. *J Cell Biol* 2006;173:975–83. [PubMed: 16785325]
34. Merk FB, Pauli BU, Jacobs JB, Alroy J, Friedell GH, Weinstein RS. Malignant transformation of urinary bladder in humans and in N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide-exposed Fischer rats: ultrastructure of the major components of the permeability barrier. *Cancer Res* 1977;37:2843–53. [PubMed: 872113]
35. Newman J, Hicks RM. Detection of neoplastic and preneoplastic urothelia by combined scanning and transmission electron microscopy of urinary surface of human and rat bladders. *Histopathology* 1977;1:125–35. [PubMed: 680648]
36. Riesco JM, Carretero J, Blanco E, Carbajo S, Sanchez F, Vazquez R. Three dimensional study of the rat urinary bladder in states of contraction and distension. *Anat Anz* 1989;168:245–53. [PubMed: 2764280]
37. Lavelle J, Meyers S, Ramage R, Bastacky S, Doty D, Apodaca G, Zeidel ML. Bladder permeability barrier: recovery from selective injury of surface epithelial cells. *Am J Physiol Renal Physiol* 2002;283:F242–53. [PubMed: 12110507]
38. Acharya P, Beckel J, Ruiz WG, Wang E, Rojas R, Birder L, Apodaca G. Distribution of the tight junction proteins ZO-1, occludin, and claudin-4, -8, and -12 in bladder epithelium. *Am J Physiol Renal Physiol* 2004;287:F305–18. [PubMed: 15068973]
39. Romih R, Veranic P, Jezernik K. Actin filaments during terminal differentiation of urothelial cells in the rat urinary bladder. *Histochem Cell Biol* 1999;112:375–80. [PubMed: 10603077]
40. Veranic P, Jezernik K. Trajectory organisation of cytokeratins within the subapical region of umbrella cells. *Cell Motil Cytoskeleton* 2002;53:317–25. [PubMed: 12378541]
41. Reichlin T, Wild A, Durrenberger M, Daniels AU, Aebi U, Hunziker PR, Stolz M. Investigating native coronary artery endothelium in situ and in cell culture by scanning force microscopy. *J Struct Biol* 2005;152:52–63. [PubMed: 16169249]
42. Sabatini DD, Bensch K, Barnett RJ. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J Cell Biol* 1963;17:19–58. [PubMed: 13975866]
43. Huttner WB, Zimmerberg J. Implications of lipid microdomains for membrane curvature, budding and fission. *Curr Opin Cell Biol* 2001;13:478–84. [PubMed: 11454455]
44. Hoh JH, Schoenenberger CA. Surface morphology and mechanical properties of MDCK monolayers by atomic force microscopy. *J Cell Sci* 1994;107(Pt 5):1105–14. [PubMed: 7929621]
45. Ramesh N, Memarzadeh B, Ge Y, Frey D, VanRoey M, Rojas V, Yu DC. Identification of pretreatment agents to enhance adenovirus infection of bladder epithelium. *Mol Ther* 2004;10:697–705. [PubMed: 15451454]
46. Frederix PL, Akiyama T, Staufer U, Gerber C, Fotiadis D, Muller DJ, Engel A. Atomic force bio-analytics. *Curr Opin Chem Biol* 2003;7:641–7. [PubMed: 14580570]
47. Stolz M, Raiteri R, Daniels AU, VanLandingham MR, Baschong W, Aebi U. Dynamic elastic modulus of porcine articular cartilage determined at two different levels of tissue organization by indentation-type atomic force microscopy. *Biophys J* 2004;86:3269–83. [PubMed: 15111440]
48. Tsilimbaris MK, Lesniewska E, Lydataki S, Le Grimellec C, Goudonnet JP, Pallikaris IG. The use of atomic force microscopy for the observation of corneal epithelium surface. *Invest Ophthalmol Vis Sci* 2000;41:680–6. [PubMed: 10711681]
49. Miyazaki H, Hayashi K. Atomic force microscopic measurement of the mechanical properties of intact endothelial cells in fresh arteries. *Med Biol Eng Comput* 1999;37:530–6. [PubMed: 10696714]
50. Braga, PC.; Ricci, D. Atomic force microscope. Biochemical methods and applications. In: Walker, JM., editor. *Methods in molecular biology*. 242. Humana; Totawa, NJ: 2004.
51. Hicks RM, Crhowanec J. Experimental induction, histology, and ultrastructure of hyperplasia and neoplasia of the urinary bladder epithelium. *Int Rev Exp Pathol* 1978;18:199–280. [PubMed: 350789]
52. Lewis SA, Diamond JM. Na⁺ transport by rabbit urinary bladder, a tight epithelium. *J Membr Biol* 1976;28:1–40. [PubMed: 9512]
53. Lewis SA, Eaton DC, Diamond JM. The mechanism of Na⁺ transport by rabbit urinary bladder. *J Membr Biol* 1976;28:41–70. [PubMed: 966267]

54. Stubbs CD, Ketterer B, Hicks RM. The isolation and analysis of the luminal plasma membrane of calf urinary bladder epithelium. *Biochim Biophys Acta* 1979;558:58–72. [PubMed: 497198]
55. Vergara J, Zambrano F, Robertson JD, Elrod H. Isolation and characterization of luminal membranes from urinary bladder. *J Cell Biol* 1974;61:83–94.
56. Peter S. The junctional connections between the cells of the urinary bladder in the rat. *Cell Tissue Res* 1978;187:439–48. [PubMed: 639084]
57. Claude P. Morphological factors influencing transepithelial permeability: a model for the resistance of the zonula occludens. *J Membr Biol* 1978;39:219–32. [PubMed: 641977]
58. Claude P, Goodenough DA. Fracture faces of zonulae occludentes from “tight” and “leaky” epithelia. *J Cell Biol* 1973;58:390–400. [PubMed: 4199658]
59. Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ. Intracellular bacterial biofilm-like pods in urinary tract infections. *Science* 2003;301:105–7. [PubMed: 12843396]
60. Justice SS, Hunstad DA, Seed PC, Hultgren SJ. Filamentation by *Escherichia coli* subverts innate defenses during urinary tract infection. *Proc Natl Acad Sci U S A* 2006;103:19884–9. [PubMed: 17172451]
61. Eto DS, Sundsbak JL, Mulvey MA. Actin-gated intracellular growth and resurgence of uropathogenic *Escherichia coli*. *Cell Microbiol* 2006;8:704–17. [PubMed: 16548895]
62. Romih R, Korosec P, de Mello W Jr, Jezernik K. Differentiation of epithelial cells in the urinary tract. *Cell Tissue Res* 2005;320:259–68. [PubMed: 15778856]
63. Romih R, Veranic P, Jezernik K. Appraisal of differentiation markers in urothelial cells. *Appl Immunohistochem Mol Morphol* 2002;10:339–43. [PubMed: 12607602]
64. Wu XR, Sun TT, Medina JJ. In vitro binding of type 1-fimbriated *Escherichia coli* to uroplakins Ia and Ib: relation to urinary tract infections. *Proc Natl Acad Sci U S A* 1996;93:9630–5. [PubMed: 8790381]

Figure 1. AFM images of the apical surface of a mouse urothelium adsorbed to a cellulose membrane, and fixed in AFM mounting

The sample was mounted on nitrocellulose membrane and fixed briefly by laying a drop of 8% glutaraldehyde on the sample for 10 min and rinsed with PBS. (A) Overview of the apical surface showing a continuous coverage of urothelial plaques and the cell borders between three umbrella cells. (B) Typical image of the apical surface covered with urothelial plaques with deep infolds. (C) and (D) Height and deflection images, respectively, of the a border between two umbrella cells. (C) The cell border is a raised ridge with fine details. (D) The interdigitations between the two neighboring cells are highlighted by arrowheads. (E) and (F) Transmission EM images of 2 consecutive thin sections of the cell border. Note that the most apical membrane contact (indicated by arrowheads) changed orientation.

Figure 2. AFM images of the apical surface of unfixed mouse urothelium mounted on a nitrocellulose membrane

(A) A rather flat area where numerous concave urothelial plaques is visible. In general the folds on the surface and the softness of the tissue were preventing us from obtaining images of this quality. (B) Typical image of the apical surface, (C) Image obtained on the same sample after adding glutaraldehyde to a final concentration of 3%. The mechanical stability has been increased by the fixation and the plaques are clearly visible, even for such an extremely folded region. (D) High magnification of the area highlighted in (C), contrast enhancement reveals the contour of the plaques (arrowheads).

Figure 3. AFM images of bacteria growing among the intermediate filaments

(A) AFM image of bacterial clusters trapped in filamentous network in the cytoplasm of the urothelial cell. (B) Higher magnification image of the bacterial cluster. The filaments are difficult to image indicating that the network is still soft after the brief glutaraldehyde fixation.