Defining protein expression in the urothelium: a problem of more than transitional interest

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Yu W, Hill WG. Defining protein expression in the urothelium: a problem of more than transitional interest. Am J Physiol Renal Physiol 301: F932–F942, 2011. First published August 31, 2011; doi:10.1152/ajprenal.00334.2011.—The transitional epithelium of the bladder, the urothelium, is a challenging tissue to study due to its fragility, complex cellular makeup, stratified composition, and intimate connections to both neural and connective tissue elements. With the increasing focus on the urothelium as a mechanosensory tissue with complex autocrine and paracrine signaling activities, there have arisen a number of unresolved controversies in the urothelial literature regarding whether certain important sensory and signaling proteins are expressed by the urothelium. Prominent examples of this include the transient receptor potential (TRP) family member TRPV1 and the purinergic receptor P2X₃. The problem is more than one of scientific bookkeeping since studies utilizing genetic models (primarily knockout mice) claim additional credibility for urothelial functions when phenotypes are discovered. Furthermore, both of the above-mentioned receptors are important therapeutic targets for various bladder disorders including inflammatory and neuropathic pain. The reasons for the confusion about urothelial expression are manifold, but they likely include low expression levels in some cases, poor specificity of antibodies (sometimes lacking adequate controls), the presence of nonurothelial cells resident within the urothelium, and the fact that the urothelium is particularly prone to aspecific adsorption of antibodies. In this review, we attempt to summarize some of the pitfalls with currently accepted practices in this regard, as well as to describe a set of guidelines which will improve the reliability of conclusions related to urothelial expression. It is hoped that this will be of value to investigators studying the urothelium, to those attempting to interpret conflicts in the literature, and hopefully also those charged with reviewing unpublished work. These recommendations will outline a set of “baseline” and “best practice” guidelines by which both researchers and reviewers will be able to evaluate the evidence presented.

function; localization; sensory; signaling; umbrella cell

Function of the Urothelium

The urothelium is a highly specialized layer of stacked epithelial cells which lines the inner surface of the mammalian urinary bladder. The most obvious function of this layer is to permit the accommodation and expulsion of large volumes of urine without allowing the components of that urine to diffuse across during prolonged storage (26). This sounds simple enough in principle and is nothing that a plastic bag cannot do. However, implicit in this one sentence description are a number of daunting physiological challenges. First, these cells must not leak, but at the same time they must be distensible enough to allow a major expansion in overall bladder surface area. To “not leak,” the urothelium must maintain a permeability barrier to osmotic and chemical gradients which can be extremely large. For example in humans, urine osmolality can be two to four times higher than that of blood, creating osmotic pressures of 5–20 atmospheres (3,800 –15,200 mmHg), while urine concentrations of acid and urea can be orders of magnitude higher than that of isotonic blood plasma (25). The geometry of the bladder as a sac-shaped organ means that when filled it has a stretched and flattened urothelium with blood capillaries which infiltrate the lamina propria only a few micrometers away from the urine (25, 29). These cells must maintain a permeability barrier and tight junction integrity at the same time as they are enduring large mechanically deforming forces such as stretch (during filling and storage) and compression (during voiding).

The second aforementioned urothelial attribute, namely, an ability to undergo surface area expansion, requires consideration of the geometry of the bladder as well as the unique abilities of the cells themselves. In its empty state the inner surface of the bladder is highly folded into deep rugae, which disappear as the bladder fills and unfolds. An example of this foldability when empty can be seen in Fig. 4A. The urothelium by necessity is highly distensible so that in addition to the unfolding which takes place initially, it gradually becomes...
thinner. Indeed, the number of cell layers which can be discriminated in tissue sections, diminishes as well (see Fig. 1). Even in a semistretched bladder with infoldings, there are regions of thickened urothelium with multiple cell layers and plump cuboidal superficial cells (Fig. 1, inset i), as well as flattened stretched urothelium apparently only two cell layers deep (Fig. 1, inset ii). Even the nucleus (indicated by the arrowhead in Fig. 1) is flattened and curves to accommodate the overall shape change of the umbrella cell.

While hydrostatic pressure imparted by slowly accumulating urine increases membrane tension making this layer thinner, it also has the effect of initiating a biochemical response in the superficial cells of the urothelium, which results in a net wave of exocytosis, causing large numbers of intracellular vesicles to traffic to and fuse with the apical membrane (31, 63, 80). This has the consequence of increasing the overall surface area of the urothelium in contact with urine and therefore the volume which can be accommodated.

The urothelium must also communicate information to the underlying sensory nerve fibers to reflect its degree of physical distension, so that both sympathetic and parasympathetic neurons can coordinate their activities to ensure synchronized detrusor smooth muscle contraction and urethral relaxation (17). One of the key neurotransmitters released by the urothelium in response to hydrostatic pressure induced stretch is ATP (21, 42, 80). Since there are multiple purinergic receptors expressed throughout the bladder wall, secreted ATP can elicit membrane depolarization (via P2X receptors) or G protein-coupled signaling via P2Y receptors on multiple cell types. Furthermore, metabolites of ATP including adenosine can bind to cognate receptors and initiate intracellular responses necessary for an appropriate voiding reflex (40, 82). The sensory and signal transduction functions of the urothelium are rapidly becoming hot areas for investigation as they carry the promise of providing explanations for certain syndromes of bladder pain and disease.

**Structure of the Urothelium**

To fulfill the many functions alluded to, mammals have evolved a highly specialized transitional epithelium which features stratified layers. Effectively, the layers on top are created by a transformation of the cells from the layers which lie below. The urothelium is therefore composed of several layers of cells which differ markedly in their morphology, function, and repertoire of expressed proteins (72).

Figure 2 summarizes schematically the major cell types present in the bladder and their locations relative to the urothelium, stroma, and smooth muscle. Interfacing with the urine and sitting atop the urothelium is a single layer of highly differentiated superficial cells known as umbrella cells. These cells are large, hexagonal in shape, often multinucleated, and express a specialized multiprotein complex which forms an entirely unique paracrystalline array of particles inserted at high density into the apical membrane and covering a large proportion of its surface area (25, 72). These particles, composed of uroplakin proteins, are thought to contribute to the permeability barrier of the bladder (28, 73–74, 78) as well as to the differentiation program which creates the umbrella cell (34). Umbrella cells express high concentrations of uroplakins both in the apical membrane and within the richly abundant subapical fusiform and/or discoidal vesicles which are available for exocytosis (72).

Lying beneath the umbrella cells are two or more layers of much smaller epithelial cells known as intermediate and basal cells, which appear to be undifferentiated precursors able to undergo programmed differentiation into umbrella cells when required. Urothelial stem cells reside within this niche also and constitute ~9% of basal cells (36). Uroplakins are expressed within all urothelial cell layers in rodents but appear to be primarily expressed in the umbrella cells of large mammals like cows and humans (72), indicating some species specificity to expression patterns. Another example of differentiation-dependent expression occurs with cytokeratins. Basal/intermediate cells express cytokeratin 17, but this protein is completely absent in umbrella cells. Conversely, umbrella cells exhibit robust expression of cytokeratin 20, which is entirely lacking in the underlying epithelium (54). An experimental model of urothelial regeneration in rats exposed to high dietary sodium saccharin showed that a differentiation-dependent switch occurs in cytokeratin expression during the process of umbrella cell renewal (54). This renewal can take place rapidly. In a model of protamine sulfate-mediated umbrella cell destruction, a functional reconstitution of the umbrella cell layer occurred within 7 days (37). Remarkably, barrier function as assessed by transepithelial resistance, and urea and water permeability was restored within 72 h, thus emphasizing its critical importance to maintaining homeostasis.

Immediately subjacent to the basal cells is the lamina propria containing basement membrane and connective tissue elements, blood vessels, and a diversity of cell types including fibroblasts, myofibroblasts, interstitial cells of Cajal, afferent nerve fibers, and immune cells (30, 49, 51, 69, 79, 81). However, some of these cells are found within the urothelium also. In particular, afferent neurons intercalate between basal
and intermediate epithelial cells and reach up and appear to end in close proximity to the basolateral membrane of umbrella cells. Also, immune system cells like mast and dendritic cells can be found within the epithelium. (14, 22, 35). Thus the urothelial layer is a complex mixture of transitional epithelium, neurons, and immune surveillance myeloid cells.

How is Urothelial Protein Expression Defined?

The usual tools employed by investigators interested in studying the urothelium are immunoblotting of urothelial scrapings or enzymatic digests, RT-PCR, immunohistochemistry, in situ hybridization, fluorescently activated cell sorting
are TRPV1 and P2X3. There is little consensus on the subject of their urothelial expression. We will examine two examples where a consideration of TRPs with appropriate controls can be misleading. The urothelium appears to be a tissue which has a unique morphology, e.g., neurons. Examples of the utility and resolution of these techniques are shown in Fig. 3. The left panels show TRPV1 (Fig. 3, A and C) and TRPC4 (Fig. 3, B and D) immunolabeling of urothelium (U) and stroma in green. Figure 3, A and B, shows colocalization of TRPs with aquaporin-3 (AQP3), a useful robust marker of the intermediate and basal urothelial cell borders. There is clearly little to no colocalization of TRPV1 with AQP3 (Fig. 3A), while conversely there is extensive colocalization of TRPC4 with urothelial cell plasma membranes (Fig. 3B, yellow in merged panel). Furthermore, both TRPs can be seen in the stroma, labeling elongated fibrous structures (asterisks). Interestingly, fine green/yellow TRPV1-positive fibers can be seen within the urothelium (Fig. 3A, asterisks). TRPC4 is also highly likely to be expressed by urothelial cells in which it appears restricted to the basolateral membrane domain, since green staining of lateral borders (Fig. 3B, arrowheads) is readily apparent, while there is no staining associated with the apical membrane. Figure 3, A and B, thus allows an assessment of TRP staining in relation to the urothelium. In addition, the particular morphology of TRP-positive structures within the stroma (and within the urothelium for TRPV1; see asterisks in Fig. 3A) suggested the possibility that these proteins were on neurons. Colocalization with neuronal marker proteins like calcitonin gene-related peptide (CGRP) and protein gene product 9.5 (PGP9.5) (Fig. 3, C and D) confirms the likelihood that both TRPV1 and TRPC4 are expressed by sensory afferents in the bladder.

Therefore, IF can provide convincing information on urothelial expression and can sometimes allow conclusions to be drawn about the subcellular location, for example, whether the protein is on the cell surface, within subcellular vesicles, or in the nucleus, etc. However, antibodies are often not so clear in their staining specificity and certainly require adequate controls as well as care in interpretation. The staining pattern can be heavily influenced by the way in which the tissue is prepared and fixed (56). Therefore, even the experienced investigator can be misled. The urothelium appears to be a tissue which has a surprising number of conflicting reports related to protein expression. We will examine two examples where a considerable literature exists and yet the scientific community lacks consensus on the subject of their urothelial expression. These are TRPV1 and P2X3.

**TRPV1**

The first description of this protein in the bladder appears to have been published in 1993, when Szallasi et al. (60) showed that isotopic resiniferatoxin (RTX; a potent capsaicin analog and specific TRPV1 agonist) could bind to membranes from homogenized rat bladders. Since then it has become apparent that TRPV1, a member of the transient receptor potential family of nonselective cation channels, is activated by heat and protons and thereby integrates stimuli which contribute to transmission of pain sensation by C-type afferent fibers (9, 62). Its involvement in normal micturition reflexes has been convincingly shown in TRPV1−/− mice, which exhibit excessive contractile activity by detrusor and altered voiding intervals (6). What is not so clear, however, is the extent of its cellular expression within the urinary bladder. Initially, TRPV1 expression was thought to be restricted to small-diameter neurons within sensory ganglia, since Northern blotting failed to detect it in any other tissue including bladder (9, 62). The first explicit description of TRPV1 as not only present, but functional within the urothelium, was by Birder et al. (5). In addition to immunolocalizing TRPV1 in rat bladder, they showed that bladder strips and primary cultured urothelial cells from rats and mice released nitric oxide (NO) and intracellular Ca2+ in response to capsaicin stimulation. Since that study, others have provided evidence to support the claim that TRPV1 is present in human urothelium. Approaches used have included immunostaining of biopsy material (39) and functional studies, including patch clamping of human urothelial cells (11). In an interesting approach designed to differentiate the influence of TRPV1 on afferent neurons from other cell types, experiments were performed in spinal cord-injured (SCI) rats. When SCI bladders were stimulated by intravesical RTX, the amplitude and duration of bladder contractions were magnified compared with controls. This implied that some of the therapeutic effects noted for capsaicin and RTX may be through actions on autonomous bladder activity rather than neurogenic in origin (24). Changes in presumptive urothelial TRPV1 activity or expression level have now been reported in patients with idiopathic and neurogenic overactive bladder (3, 45) and in transitional cell carcinoma (38).

In contrast to these studies, however, more recent ones have been unable to confirm the urothelial expression of TRPV1. Yamada et al. (76) demonstrated barely detectable PCR products for TRPV1 in isolated mouse urothelium, no specific TRPV1 antisense-cRNA hybridization to urothelium using in situ hybridization, and no IF staining of TRPV1 in the urothelium. However, IF was able to locate TRPV1-positive nerve fibers in wild-type mice which, significantly, were absent in TRPV1−/− knockout mice. In all cases, appropriate controls were included and confirmed the efficacy of the reagents and the assays. In further functional assays performed in primary cultured urothelial cells, capsaicin concentrations up to 10 μM were unable to elicit any release of intracellular Ca2+, suggesting an absence of TRPV1. Everaerts et al. (20) found similar results with no effect of 10 μM capsaicin on intracellular Ca2+ release in mouse urothelial cells and vanishingly small expression of TRPV1 by quantitative PCR. Using patch-clamp electrophysiology on freshly digested urothelial cells from guinea pig, Xu et al. (75) demonstrated TRPV4-mediated ion currents but no capsaicin-evoked currents, implying the cells lacked TRPV1. Recently, Yu et al. (79) failed to detect TRPV1 in urothelium by IF (Fig. 3, A and C), but CGRP-positive neurons were TRPV1 positive, attesting to the ability of the antibody to detect the protein. Most compellingly of all, data from a recent
Fig. 3. Immunofluorescent localization of TRP channels in and near urothelium (U). Multicolor confocal laser scanning immunofluorescence shows the cellular locations of transient receptor potential (TRP) family members TRPV1 (A and C) and TRPC4 (B and D). Antibodies to aquaporin-3 (AQP3) were used to define the intermediate and basal urothelial cell borders (red in A and B), while TRP immunostaining is green. TRP-positive fibers are indicated by asterisks, and the TRPC4-positive lateral boundaries of an umbrella cell are highlighted by arrowheads. Right panels (C and D) show colocalization of both TRPV1 and TRPC4 (green) with neuronal marker proteins calcitonin gene-related peptide (CGRP) and protein gene product 9.5 (PGP9.5; red), indicating that TRP-positive fibers are neurons. Scale bar = 10 μm. L, lumen; U, urothelium. Figure is modified from Ref. 79.
P2X3 is an ATP-gated ion channel normally associated with sensory innervation in nerve fibers and has been strongly associated with the neurology of pain (15, 68). In 2000 and 2001, a raft of papers were published on the location of P2X3 in the bladder as well as the effect on that organ, of knocking it out (16, 18, 40, 64, 77). The first immunolocalization studies by Burnstock’s group (40, 64) identified P2X3 in nerve bundles within the detrusor smooth muscle of rats and mice, but not in the urothelium. The importance of this particular purinoreceptor to overall bladder function was dramatically confirmed in a study employing highly innovative transgenic TRPV1 reporter mice found that TRPV1 expression was restricted to primary afferent neurons with very low-level expression in a few discrete brain areas and in a subset of arteriolar smooth muscle cells. These mice in which the TRPV1 promoter drove expression of placental alkaline phosphatase, lacZ and Cre were exquisitely specific in their ability to report low levels of TRPV1 expression (10). Furthermore, the authors confirmed the expression data using a range of approaches from calcium imaging and whole cell patch-clamp recordings to in situ hybridization. Despite the sensitivity of the reporters, they were unable to detect any expression of TRPV1 in urothelial cells.

Clearly, there are some peculiar difficulties in studying this tissue. In an illuminating comparison of the performance of three different commercial antibodies against TRPV1 in rat and mouse tissues, Everaerts et al. (19) discovered that all three reacted with the urothelium, while only two of three passed the “positive control test” by labeling trigeminal ganglia. Of most interest, however, was the finding that all three antibodies to TRPV1 labeled urothelium of bladders from TRPV1−/− mice, leading the authors to conclude there was significant aspecific staining. This suggests there is some molecular or structural element, perhaps unique to the urothelium, which contributes to high rates of false positives by antibody staining. In fact, the problem of apparent nonspecificity of antibody labeling of the urothelium for many proteins appears to be widespread. It is unclear why this is, but one explanation may be that the cells are unusually viscous. Caution is therefore necessary when the results of urothelial immunohistochemistry are interpreted. Nonetheless, while antibody labeling can be problematic, investigators using a variety of other techniques have also arrived at conflicting conclusions. This indicates that some of the experimental systems employed may be misleading.

P2X3

Since those early reports, there have been studies which claimed evidence for expression of P2X3 in urothelium of cats (7), mice (65), rabbits (67), rats (33), and humans (59, 61). Of particular interest was the apparent increase in urothelial P2X3 levels found in biopsies from patients with interstitial cystitis (59, 61). However, changes in P2X3 expression were not seen in the cat model of interstitial cystitis (7).

While the weight of published evidence would tend to favor the conclusion that P2X3 is expressed by the urothelium, the finding of significant nonspecific staining of the urothelium by antibodies adds a cautionary note (19). In all of the cited studies, IF, immunohistochemistry, or immunoblotting was used to anchor the conclusion about location. The purpose of this review is to suggest that a higher burden of evidentiary proof is required to help settle some of these debates and to suggest a number of combined experimental approaches which would help in meeting this greater burden.

Structural and Technical Difficulties in Studying the Urothelium

The urothelium is unique because it is a transitional epithelium with a specific set of functions not replicated elsewhere in the body. It consists, as noted, of a heterogeneous collection of epithelia at different stages of differentiation, which include umbrella cells, intermediate cells, and basal/stem cells which clearly differ from each other in their repertoire of expressed proteins. Thus the investigator is immediately confronted with a diversity which should be taken into account when describing a protein as urothelial. Imaging modalities of high resolution such as laser-scanning confocal microscopy can differentiate antibody staining which occurs in umbrella cells vs. suburothelium (see Fig. 3). Furthermore, they can locate proteins on particular membranes and within organelles (27, 79). This approach obviously requires that high-quality antibodies are used and preferably that the cognate peptide is also available which can be used to further ensure the specificity of the antibody.

Even good antibodies may not be sufficient if the protein is present in low abundance and/or its expression is developmentally regulated or is differentiation specific. In a study of P2Y receptor expression in the bladder, Chopra et al. (13) concluded that the P2Y4 purinergic receptor was expressed in the urothelium based on functional assays and pharmacological profiling performed with rat urothelial cells in culture and based on real-time PCR of mRNA extracted from native urothelium. However, immunofluorescence was negative and immunoblotting showed that only one bladder of three gave a positive band for the protein, clearly indicating the limits of antibodies for the visual identification of P2Y4 in the urothelium (13).

The presence of nonurothelial cells within the urothelium creates yet another challenge and may complicate the interpretation of immunohistochemistry. This is particularly true if images of low resolution are obtained, e.g., by epifluorescence. Furthermore, their presence makes it virtually impossible to isolate pure urothelium for immunoblotting if the usual techniques of scraping, dissection, or enzymatic digestion are employed.

Cell culture models offer another way to study the urothelium. The urothelium is stratified and therefore has a complex multilayered phenotype. While some features of the urothelium...
are certainly retained in culture, the expression of differentiation markers like cytokeratins and uroplakins does not reproduce that seen in vivo (46, 58, 70). Depending on the culture conditions and the way in which cultures are established and maintained, urothelial cells can exist in a proliferative state or in a quiescent, differentiated state. Since the primary purpose of most cell culture is to facilitate cellular expansion, it is usually the former. This is clearly not the “normal” state for the urothelium. Sun has (58) proposed that typical culture conditions for the urothelium induce a phenotypic differentiation program which mimics wound healing. Further complicating interpretation of phenotype fidelity are different culture conditions used by different laboratories and the finding that urothelial lineages vary depending on the region of the bladder from which they are isolated (58). Furthermore, since primary cultured cells lack the appropriate interplay of molecular communication with cells of the mesenchyme, neurons, and smooth muscle, the repertoire of expressed proteins cannot be assumed to replicate with fidelity what is found in vivo. Cultured cells are, of course very useful for conducting functional assays, which provide information about responsiveness to various stimuli. These may be pharmacological or mechanical, for example. However, it would be best to regard most urothelial culture systems as having regenerative or myoeipithelial cell properties (70) and to be circumspect when drawing conclusions about expression.

Finally, the physical, chemical, and/or electrostatic properties of the urothelium may make it more viscid, thus facilitating nonspecific antibody adhesion in frozen and paraffin sections. As a result of these many experimental difficulties, we would like to suggest the following guidelines be adopted to reasonably justify a claim of urothelial expression. These are divided into two levels of stringency as recognition that sometimes antibodies can give clear unequivocal results and second, depending on the aims of the study and the relative importance of the urothelial assignation, different levels of proof can be justified. However, if a claim of urothelial expression is contested by other published studies or purports to define some new capability or function of the urothelium, then the second “best practice” level should be employed for greater certainty.

Recommended Experimental Approaches for Determining Urothelial Protein Expression

**Baseline.** The following techniques and controls should be used in conjunction with each other. Alone, they are insufficient for the reasons outlined earlier.

**RT-PCR.** RT-PCR is a simple but essential procedure for demonstrating that the urothelium is expressing an mRNA for the protein of interest. DNA products obtained by RT-PCR should then be sequenced to confirm that they are correct. If RT-PCR fails to generate a product of the correct size, then it is necessary to confirm that the primers and other experimental details are appropriate by running it again using a positive control tissue known to express the protein in question. If the PCR works in positive controls but does not amplify a mRNA in urothelial extracts, then it can be assumed the protein is not actively being expressed at that time or is not expressed at all.

**Immunoblotting.** Immunoblotting should be performed to confirm that a protein of the correct molecular weight can be detected in urothelial scrapings or enzymatic digestions. The appropriate control in this instance would be to preadsorb the antibody against its cognate peptide and thereby demonstrate that binding to the blot is abrogated. Assuming the same antibody is used for both Western blotting and immunostaining, an assessment can then be made as to the specificity of the antibody. If many other nonspecific protein bands are present in the blot, then there is a strong possibility for nonspecific interactions with frozen or paraffin sections in immunohistochemistry. An advantage of immunoblotting is that it has the potential to detect stable proteins with long half-lives which may be transcriptionally quiescent and therefore appear negative by RT-PCR. However, this may be considered a rare event.

**FACS or Magnetically Activated Cell Sorting (MACS).** FACS or MACS offers a way to isolate a specific population or subpopulation of urothelial cells (70). Utilizing epithelial-specific cell surface markers such as uroplakin, epithelial cell adhesion molecule (EpCAM), or E-cadherin, it is possible to obtain a highly enriched fraction of urothelial cells (23). These can be then be examined in a variety of ways from functional studies (if the cells are maintained/cultured) to molecular characterization with RT-PCR and/or immunoblotting (47). Analyzing sorted cells will offer greater confidence that contaminating cells are not the reason for any positive signal obtained and is especially useful when the target protein under investigation is also expressed by nonurothelial cells in close proximity.

**Functional Assays.** Functional assays are extremely useful and have been used to successfully define not just the expression but also important properties of proteins under investigation. The primary techniques used to obtain functional data on urothelial proteins are electrophysiological or flux measurements in Ussing chambers, patch-clamp electrophysiology on isolated urothelial sheets, or urothelial cell clumps and various divergent assays which can readily be applied to cultured urothelial cells.

Perhaps the best example of functional characterization of a newly discovered protein in the urothelium was the amiloride-sensitive sodium channel, which is present in the luminal membrane (41, 43–44). Employing Ussing chambers and conventional as well as ion-sensitive microelectrodes to measure transepithelial resistance, conductance, short-circuit currents, and intracellular Na⁺ activity, the authors dissected the functional and regulatory signature of an ion channel which was ultimately cloned and shown to be the heteromeric epithelial sodium channel (ENaC) (8). Ussing chamber studies have also allowed dissection of other ion channel activities in the urothelium, including chloride and potassium fluxes in response to hydrostatically induced membrane stretch (66).

The patch-clamp technique for studying ion channels has also proven useful, but as noted in the earlier section on TRPV1 expression, contrasting conclusions have been arrived at even using this highly sensitive and discriminating approach. Charrua et al. (11) were able to demonstrate TRPV1 currents in human cultured urothelial cells while Xu et al. (75) did not see TRPV1 in freshly isolated guinea pig urothelial cells using the same technique.

Where there are known pharmacological reagents for affecting the activity of a target protein, it is always necessary to be mindful of the specificity (or relative lack of), the concentration at which drugs are used and the need for multiple drugs, if
possible, to obtain certainty in the conclusions. The potential for off-target effects if promiscuous reagents or inappropriately high concentrations are used cannot be overstated.

Functional assays are often performed in cultured cells and can be highly informative and confirmatory. However, due to the caveats already mentioned, any functional data obtained in isolated or cultured cells to support a claim of expression should be backed by other localization techniques performed on whole tissue.

Each of these techniques provides useful information and employed collectively can provide extremely strong evidence for urothelial expression. If antibody staining is diffuse or weak, however, as may be quite legitimately found for proteins that are predominantly cytoplasmic and/or weakly expressed, the degree of confidence is weakened. Therefore, where possible the following additional techniques or approaches should be employed.

**Best practice. Immunolocalization.** Immunolocalization will continue to be the major technique of choice for most investigators wishing to determine the expression of their protein in the bladder. For the results to be valid however, we recommend that the specificity of the antibody and technique be confirmed as follows. These recommendations are consistent with the more stringent requirements now being insisted upon by some scientific journals (53, 55–56). While some of the following suggestions are specific to IF, several apply also to immunoperoxidase approaches. IF does enjoy the distinct advantage that multiple markers can be immunostained simultaneously.

1) High-resolution microscopy is required; for IF, a confocal microscope should be used.

2) Dual-color colocalization with known urothelial marker proteins provides additional certainty about location; examples of proteins which can be used are uroplakins/cytokeratin 20 for umbrella cells and AQP3/cytokeratin 17 for intermediate and basal cells. This being said, it is worth noting that absolute certainty about umbrella cell plasma membrane expression can be difficult to arrive at, since it is difficult to distinguish whether a protein is in the basolateral membrane of the umbrella cell or is in the apical region of the intermediate cells which lie immediately below. Claudin 4 can be used to outline the cellular boundaries for all urothelial layers (1, 20, 54, 76, 79).

3) Antibodies should be preadsorbed with the cognate peptide before immunostaining to demonstrate that possible cellular staining is eliminated. While this is highly recommended, it is sometimes impossible in situations where a company will not provide a peptide or the amino acid sequence used to raise the antibody. Therefore, sources which can provide such controls are to be strongly preferred. Indeed, Saper editorializes (55) that such antibodies are “not fit for scientific work” and the policies of that journal prohibit publication of uncharacterized antibodies. It is always safer for the investigator to go with well-characterized and documented antibodies whose efficacy is ensured.

4) If antibody staining is diffuse or faint and does not clearly localize to defined structures (such as cell membranes or intracellular vesicles) or tissue layers, then it may be nonspecific. At a minimum, tissues/organisms which are known to express the protein should be obtained and the antibody tested on these to confirm its efficacy and specificity (positive control). In addition, and this point needs to be emphasized, urothelial staining should be specific; i.e., there should not be a generalized equivalent degree/intensity of staining in the lamina propria and/or smooth muscle. It is almost certain that even where a protein is expressed in multiple strata of the bladder wall, there will be differences in its concentration or distribution. Figure 3B illustrates this point since TRPC4 is clearly present in the plasma membrane of all three urothelial cell layers but in addition has a distinctive distribution within the stroma.

5) Tissue sections should be stained with secondary antibody alone to ensure it is not providing a nonspecific signal (secondary antibody control).

6) Sections should always be counterstained with a nuclear label (DAPI or TOPRO-3) and a cell border label such as rhodamine phalloidin for actin staining; this allows precise definition of the urothelium and its boundary (localization control).

**In situ hybridization.** In situ hybridization of labeled complementary probes to endogenously expressed cellular RNA allows precise definition of the tissue distribution, as well as the temporal expression, of any RNA species of interest. In this way it can become a powerful discriminatory tool. In situ hybridization makes it possible to examine the RNA transcripts within individual cells, such as the urothelium. In situ hybridization was used to demonstrate TRPV4 expression in basal urothelial cells and an apparent lack of urothelial expression for TRPV1 (76). Thus it is possible to use the technique to detect RNAs that are present in only a fraction of cells. Furthermore, it is a technique with extremely high specificity and does not rely on the use of antibodies for primary recognition. Indeed, it can be a powerful confirmatory assay to validate the specificity of antibody binding patterns (53). In some cases, it may be difficult to positively identify low-abundance transcripts.

**Knockout animals.** If available, the specificity of antibody staining in both immunoblots and immunofluorescence should be tested in animals engineered to lack the functional gene and therefore protein of interest. This provides an absolute reference and is incontrovertible evidence for the fidelity of antibody binding. In cases where a knockout animal has not been generated or is not viable, there are now available strategies based on the Cre/loxP system for producing urothelial-specific knockouts. Wu (50, 83) has utilized the unique specificity of uroplakin expression in the urothelium to create Cre-recombinase transgenic mice in which expression of Cre is driven by the uroplakin II promoter. The availability of these animals means that in theory any protein of interest expressed by the urothelium can be conditionally ablated. In cases where “floxed” mice are not available, this may require the generation of mice engineered to contain flanking loxP sites within critical regions of the gene of interest. However, such efforts would be well worth pursuing to settle some of the more controversial debates regarding sensory and neuronal properties suggested for the urothelium.

**Laser-capture microdissection.** The development of microscope-based laser-dissection systems has revolutionized our ability to remove whole cells and cell groupings intact from complex three-dimensional tissues composed of morphologically distinct cell types. Thus it presents itself as a powerful tool for the particular problem being discussed here. Figure 4 illustrates the potential for isolation of highly purified urothel-
Fig. 4. Laser-capture microdissection of urothelium from mouse bladder. Cryosections (10 μm) of normal mouse bladder were stained with Arcturus histogene stain, imaged (×20 objective) and laser microdissected on an ArcturusXT Laser Capture Microdissection System. A: intact bladder section before microdissection (L, lumen; U urothelium; LP, lamina propria; SM, smooth muscle). B: same bladder section showing laser outline before cells were removed. C: cells recovered. D: same section after urothelial cell removal. Scale bar = 100 μm.

... required. As with all antibody-based techniques, appropriate controls and specific labeling of cellular structures with gold particles are required.

Concluding Thoughts

Long dismissed as a simple inert barrier designed to hold urine for long periods, the urothelium has begun to surprise us with its dynamic properties and its complex interactions with other functional components of the bladder. It is an exciting time to be deciphering its mysteries since sensory and signaling features hint at fascinating neuroepithelial properties (2, 4). However, our full understanding of these functions is hampered by current uncertainties over whether it really expresses important molecules like TRPV1 and P2X3. Our recommendation is that multiple experimental approaches be employed, which when taken together, “build the case” for urothelial expression until the evidence becomes compelling. An overreliance on the fidelity of antibodies is perhaps the biggest common mistake and, in the absence of independent confirmatory assays, has led to much uncertainty in the field. Convergent data from other assays like in situ hybridization should become mandatory if knockouts are not available. It is hoped that these recommendations will adjust expectations to a higher threshold of proof and that they will help both investigators and reviewers decide whether claims of urothelial expression are reasonably warranted.

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