Video Article

Implementing Patch Clamp and Live Fluorescence Microscopy to Monitor Functional Properties of Freshly Isolated PKD Epithelium

Tengis S. Pavlov[1], Daria V. Ilatovskaya[1], Oleg Palygin[1], Vladislav Levchenko[1], Oleh Pochynyuk[2], Alexander Staruschenko[1]

[1]Department of Physiology, Medical College of Wisconsin

[2]Department of Integrative Biology & Pharmacology, University of Texas Health Science Center at Houston

Correspondence to: Alexander Staruschenko at staruschenko@mcw.edu

URL: http://www.jove.com/video/53035
DOI: doi:10.3791/53035

Keywords: Medicine, Issue 103, Patch-clamp, polycystic kidney disease, ARPKD, ADPKD, kidney, intracellular calcium, Fura-2 AM, nephron, cyst development, polycystin

Date Published: 9/1/2015

Citation: Pavlov, T.S., Ilatovskaya, D.V., Palygin, O., Levchenko, V., Pochynyuk, O., Staruschenko, A. Implementing Patch Clamp and Live Fluorescence Microscopy to Monitor Functional Properties of Freshly Isolated PKD Epithelium. *J. Vis. Exp.* (103), e53035, doi:10.3791/53035 (2015).

Abstract

Cyst initiation and expansion during polycystic kidney disease is a complex process characterized by abnormalities in tubular cell proliferation, luminal fluid accumulation and extracellular matrix formation. Activity of ion channels and intracellular calcium signaling are key physiologic parameters which determine functions of tubular epithelium. We developed a method suitable for real-time observation of ion channels activity with patch-clamp technique and registration of intracellular Ca^{2+} level in epithelial monolayers freshly isolated from renal cysts. PCK rats, a genetic model of autosomal recessive polycystic kidney disease (ARPKD), were used here for *ex vivo* analysis of ion channels and calcium flux. Described here is a detailed step-by-step procedure designed to isolate cystic monolayers and non-dilated tubules from PCK or normal Sprague Dawley (SD) rats, and monitor single channel activity and intracellular Ca^{2+} dynamics. This method does not require enzymatic processing and allows analysis in a native setting of freshly isolated epithelial monolayer. Moreover, this technique is very sensitive to intracellular calcium changes and generates high resolution images for precise measurements. Finally, isolated cystic epithelium can be further used for staining with antibodies or dyes, preparation of primary cultures and purification for various biochemical assays.

Video Link

The video component of this article can be found at http://www.jove.com/video/53035/

Introduction

Ion channels play a significant role in many physiological functions, including cell growth and differentiation. Autosomal dominant and recessive polycystic kidney diseases (ADPKD and ARPKD, respectively) are genetic disorders characterized by the development of renal fluid-filled cysts of the tubular epithelial cell origin. ADPKD is caused by mutations of PKD1 or PKD2 genes encoding polycystins 1 and 2, membrane proteins involved in the regulation of cell proliferation and differentiation. PKD2 by itself or as a complex with PKD1 also function as a Ca^{2+}-permeable cation channel[1]. Mutations of the PKHD1 gene encoding fibrocystin (a cilia-associated receptor-like protein involved in the tubulogenesis and/or maintenance of polarity of epithelium) are the genetic impetus of ARPKD[2]. Cyst growth is a complex phenomenon accompanied with disturbed proliferation[3,4], angiogenesis[5], dedifferentiation and loss of polarity of tubular cells[6-8].

Defective reabsorption and augmented secretion in cystic epithelium contribute to fluid accumulation in the lumen and cyst expansion[9,10]. Impaired flow-dependent $[Ca^{2+}]_i$ signaling has been also linked to cystogenesis during PKD[11-15].

Here, we describe a method suitable for patch-clamp measurements of single channel activity and intracellular Ca^{2+} levels in cystic epithelial monolayers isolated from PCK rats. This method was successfully applied by us to characterize of activity of the epithelial Na^+ channel (ENaC)[10] and $[Ca^{2+}]_i$-dependent processes induced by Ca^{2+}-permeable TRPV4 and purinergic signaling cascade[13].

In these studies we used PCK rats, a model of ARPKD caused by a spontaneous mutation in the PKHD1 gene. The PCK strain was originally derived from Sprague-Dawley (SD) rats[16] thereby SD rats are used as an appropriate control for comparison with the PCK strain. As a result, both SD rat nephron segments and non-dilated collecting ducts isolated from same PCK rats can serve as two different comparison groups for experiments on cystic epithelium.

Protocol

The experimental procedures described below were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin and University of Texas Health Science Center at Houston and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. **Figure 1** demonstrates main steps of the tissue isolation and processing procedure. Briefly, kidneys from

PCK or SD rats are used for manual isolation of epithelial monolayers of collecting ducts either from healthy non-dialed tubules or cysts. Here we studied kidneys from 4-16 weeks old PCK rats[10,13].

1. Isolation of Renal Cysts and Connecting Tubules (CNT)/Collecting Ducts (CD) Segments

1. Anesthetize experimental animal with isoflurane (5% induction, 1.5 to 2.5% maintenance)/medical grade O_2 or another approved method. Animals must be continually monitored to ensure adequate level of anesthesia. Stable respiratory rate and toe pinch reaction are used to confirm proper anesthesia.
2. Perform laparotomy and flush the kidneys with phosphate buffered saline (PBS) through the abdominal aorta[17].
    1. Prepare a polyethylene tubing catheter (PE50) and connect it to a syringe pump filled with PBS. Cut the skin and abdominal wall along the linea alba, then make a transverse abdominal incision across the abdomen from side to side and shift the abdominal organs with cotton swabs to get access to the descending aorta with branching mesenteric and celiac arteries. Moisten the internal organs with warm saline during further procedures to prevent their dryness.
    2. Place one ligature (#1) around the mesenteric and celiac arteries and another ligature (#2) around the aorta under the diaphragm (do not tie). Gently separate *a. abdominalis* by blunt dissecting connective tissues around it with thin forceps and place two ligatures ~3 mm below the left renal artery (#3) and above the iliac arteries bifurcation (#4).
    3. Tie ligature #4 and attach a vessel clamp around the aorta between the left renal artery and ligature #3. Make an incision between the clamp and ligature #4 to catheterize the aorta, use ligature #3 to firmly fix the catheter.
    4. Release the clamp and make sure that the blood pulse is visible in the catheter to ensure proper installation. Start perfusion at a rate of 6 ml/min, tie ligatures #1 and #2, and cut the left renal vein. Continue flushing for 1-2 min until the organs are completely blanched.
    5. Cut renal blood vessels, urethra and surrounding connective and adipose tissues with scissors to collect the kidneys. Then make a short tear in kidney capsule and peel the kidneys to decapsulate them. Place the kidneys into ice-cold PBS. Euthanasia is confirmed by a thoracotomy.
3. Prepare 5 x 5 mm cover glass chips coated with Poly-L-Lysine. Cut a cover glass with a diamond pencil and place approximately 20 µl of 0.01% sterile filtered Poly-L-Lysine solution onto each glass chip. Prepare saline and two pairs of watchmaker forceps for dissection.
4. Cut the kidneys with a razor blade along the frontal plane into slices of ~1-2 mm thickness. Place one of the slices under a stereomicroscope. Isolation of tissues should be done in ice-cold saline.
5. Locate cysts under a stereomicroscope as round-shape cavities (**Figure 2A**); their walls often contain prominent network of proliferated blood vessels. Using fine-tipped forceps dissect the internal epithelial layer of a cyst as thin as possible to obtain a monolayer area. Attach it to a glass chip covered with Poly-L-Lysine. Sticky Poly-L-Lysine surface allows the researcher to firmly position the internal cyst layer on glass. Expose the cyst with the apical side up to provide access for the pipettes (**Figure 2B**).
6. Use PCK or SD rat kidney central slices containing papilla for isolation of non-dilated CNT/CCDsegments[18-21].
NOTE: Papillary tissues are more durable than cortex and by holding papillary tubular bands with forceps and tearing along radial axis the slice can be cleaved into thin sectors. Individual tubules are visible and can be pulled out to be placed on cover glass chips.
7. Identify the CNT/CCD segments by bifurcations, higher transparency than proximal tubules and large prominently visible cells (**Figure 2C)**. Place the cover glass chip with attached tubules under a microscope equipped with micromanipulators suitable for driving micropipettes. Using sharp micropipettes split-open tubules and attach their edges to the glass to make the apical surface accessible (**Figure 2D**).

2. Single Channel Patch-clamp Electrophysiology

1. Fill the patch-clamp chamber with bath solution and transfer the cover glass chip with isolated tissues to the chamber. Ensure that the patch-clamp micropipettes have resistance of 7-10 MΩ for reliable on-cell (cell-attached) measurements.
2. For the cell-attached measurements, set the amplifier gain ratio to 20x and low-pass the currents at 300 Hz by an eight-pole Bessel filter. For ENaC channels monitoring, use a bath solution, in mM: 150 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES (pH 7.4); pipette: 140 LiCl, 2 $MgCl_2$ and 10 HEPES (pH 7.4).
3. Conduct a conventional patch-clamp experiment in a cell-attached mode[10]. Select a cell in the epithelial monolayer and approach the pipette to the apical membrane. Form a high-resistance seal between a pipette and cell membrane by applying gentle suction. Once a high resistant gigaOhm seal is formed, a gap-free protocol at a holding potential should be started for monitoring activity of the channel of interest.
4. Store gap-free single channel current data from gigaOhm seals for subsequent analysis. Analyze the channel events using a software package generally supplied with patch-clamp setups[18]. Calculate channel activity as *NPo* where N refers to the number of active channels in the patch and P_o is average open probability of the channels.
NOTE: Use isolated cysts or tubules in patch-clamp experiments for no more than 30 min.

3. Ratiometric Epifluorescence Measurements of Intracellular Calcium Concentration in the Epithelial Cells

1. Use 5 mM Fura-2AM dissolved in DMSO. Aliquot stock solution into individual 500 µl conical tubes (approximately 10 µl). Protect from light and store in the freezer at -20 ˚C for up to six months.
2. Incubate isolated cysts and split-open tubules for 30-40 min in PSS (in mM: 145 NaCl, 4.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$ and 10 HEPES at pH 7.35) containing 5 µM Fura-2 AM dye and 0.05% pluronic acid to help disperse the acetoxymethyl esters. For that, place tissues in the 3.5 cm dish containing loading cocktail, protect from light and incubate on a slow shaker at room temperature.
3. Change Fura-2 AM containing media to clean PSS after incubation and place the tissue under a microscope to further perform epifluorescence imaging.
4. Turn on CCD camera and stable light source (monochromator system) equipped with filter wheel (each filter position can be associated with its own attenuation level, selected every time the filter is called).

5. Find the tissue in bright field. Switch to detection of the fluorescence signal and adjust the intensity of the light source using neutral density filters and lamp power to avoid saturation of the signal.
6. Monitor Fura-2 AM fluorescence in the tissue sample with ratiometric excitation at 340/380 nm with frequency of 0.125 Hz or higher. Use a 40×/NA 1.3 or similar objective lens for proper resolution image.
7. For image processing and calculations import the image sequence. Make sure to split the channels and use a hyperstack grayscale mode. Select several regions of interest (single cyst cells or selected areas of cystic tissue) and calculate intensity values for each channel (340 and 380 nm) into preferred data analysis software; subtract background intensity values from each data point.
8. For each time point calculate the ratio of intensities of the Fura-2 AM 340 to 380 channels. Plot scatter/line point-time changes of Ca^{2+} transient for each region and calculate mean/SE values.

Representative Results

Potential ENaC involvement in cystogenesis has been demonstrated by several studies that observed disrupted epidermal growth factor (EGF) signaling in PKD progression[22-25] and abnormal sodium reabsorption in ARPKD murine models and tissue cultures[26-28]. For example, Veizis *et al.* showed that amiloride-sensitive Na^+ absorption is decreased in CD cells from the non-orthologous BPK mouse model of ARPKD[29]. We recently demonstrated that impaired sodium and water reabsorption in cysts is an important factor in aggravating cystogenesis[10]. Specifically, we employed electrophysiological and immunohistochemical analysis and found that cysts exert blunted sodium reabsorption. A pharmacological approach demonstrated that selective ENaC blockade markedly exacerbates cyst progression[10].

We further demonstrated the effect of administration of benzamil, an ENaC blocker, on activity of the channels in the cyst wall. 4-weeks old PCK rats were supplied with vehicle or drinking water containing benzamil (15 mg/ml) ad libitum. After 12 weeks of treatment the animals were processed according to the protocol described above. Patch-clamp was performed on the cystic monolayers isolated from vehicle and benzamil treated groups. **Figure 3** shows representative current traces of ENaC activity recorded from apical membranes. The summary graph demonstrates that average ENaC activity (NP_o) was 0.91±0.15[10] in the cysts of control group, whereas benzamil administration decreased the activity of the channel to 0.32±0.05. We conclude that in ARPKD (characterized by ecstatic distension of CDs to form numerous spindle-shaped renal cysts[30]) benzamil given in drinking water reaches renal cysts with urine flow[30]. This effect allows benzamil to decrease sodium reuptake from cyst fluid, contributing to cystogenesis[10].

In addition to the EGF pathway, adenosine triphosphate (ATP) and other purines were also identified as a critical paracrine signaling component that is inappropriately modulated in PKD models and in patients with this disease. It was reported that purinergic signaling plays an important role in cystogenesis during both ADPKD and ARPKD[31,32]. Cyst cells of PCK rats have previously been shown to exhibit low basal $[Ca^{2+}]_i$ and loss of flow-mediated $[Ca^{2+}]_i$ signaling compared to healthy non-dilated collecting ducts of SD rats[13]. P2 receptor agonists modulate the development of renal cysts in an in vitro model of cyst formation derived from the cpk/cpk mouse[33]. High ATP concentration was found in cystic liquid from ADPKD patients[34] and in media conditioned by cystic kidney epithelia cultured from cpk/cpk mice[35].

We tested calcium flux in response to exogenous ATP administration. PCK cysts and normal cortical ducts from SD rats were isolated for the calcium measurements before and after application of 10 μM ATP. Data illustrated in **Figure 4** reveal the effect of 10 μM ATP in the cystic tubules of PCK rats studied with two different approaches: **Figure 4A** shows measurements of Fura-2AM fluorescence intensity at 340 and 380 nM in an epifluorescence setup equipped with a monochromator, and **Figure 4B** represents registration of Fluo-8 dye staining with confocal microscopy. Both techniques demonstrate similar kinetics of calcium transient response to ATP application in cystic epithelium[10].

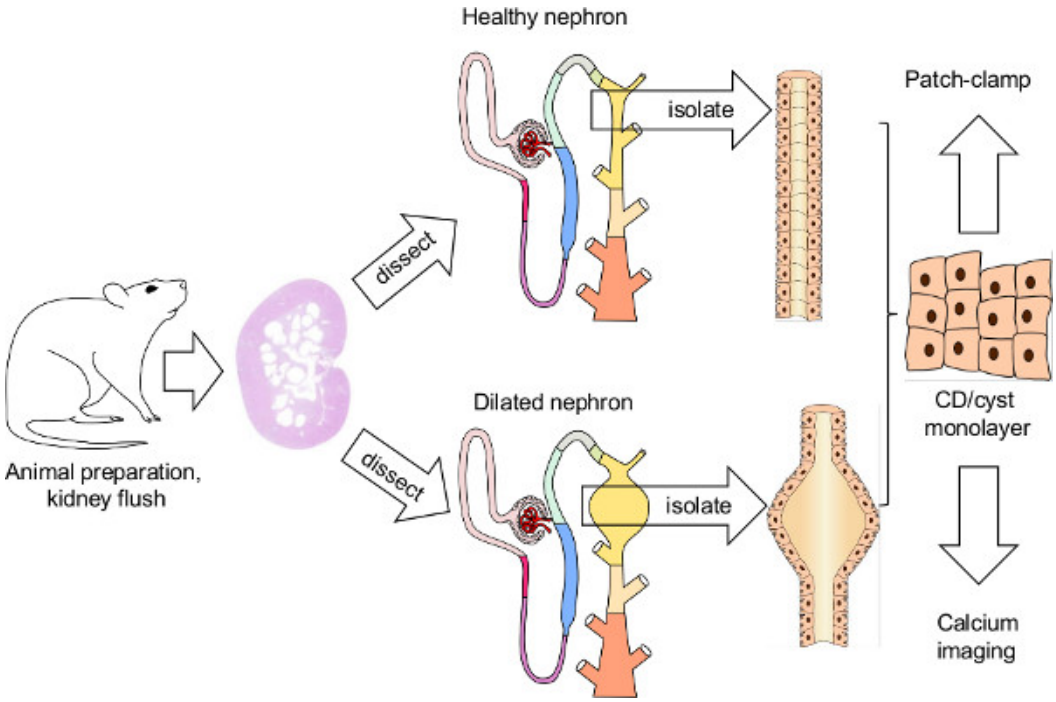

Figure 1: Schematic representation of the experimental protocol. After the animal is properly anaesthetized and prepared for the surgery, the kidneys are flushed with PBS to remove blood. Then, the kidneys are excised, decapsulated, and cystic monolayers or non-dilated tubules are isolated manually with forceps under a stereomicroscope. Non-dilated tubules are split-open with micropipettes driven by micromanipulators whereas cystic epithelium as internal surface of the cysts would be open to have access to the apical side.

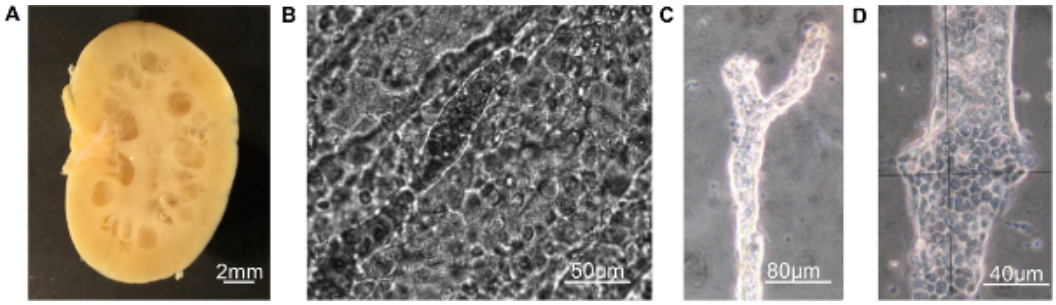

Figure 2: Isolation and preparation of cystic and collecting duct monolayers. (**A**) A representative kidney slice from 16 week old PCK rat. (**B**) A cystic monolayer on a cover glass chip transferred to a microscope for patch-clamp analysis or calcium imaging (60X). (**C**) A CNT/CCD segment with bifurcation isolated from an SD rat. (**D**) A CNT/CCD tubule split-open with micropipettes to gain access to the apical surface.

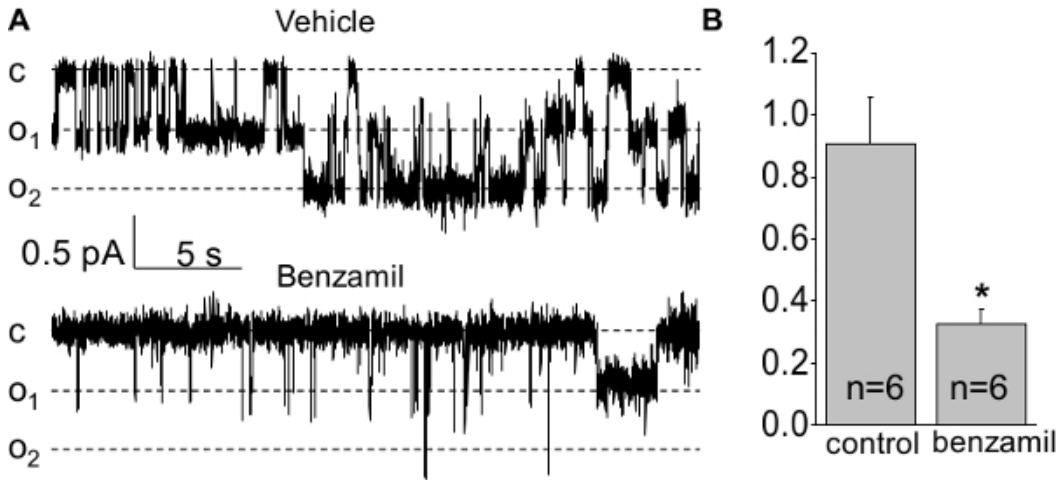

Figure 3: Effect of benzamil treatment on ENaC activity in cystic epithelium. (**A**) Representative current traces for ENaC activity measured in cell attached patches of cysts freshly isolated from 16 week old PCK rats administered 12 weeks of benzamil in drinking water. These patches were held at a test potential of $V_h = -V_p = -40$ mV. Inward Li^+ currents are downward. Dashed lines indicate the respective current state with a "c" and "o_n" denoting the closed and open states. (**B**) Summary graph of observed ENaC activity (NP_o) (partially reproduced from[10] with permission). **P* <0.05 versus vehicle.

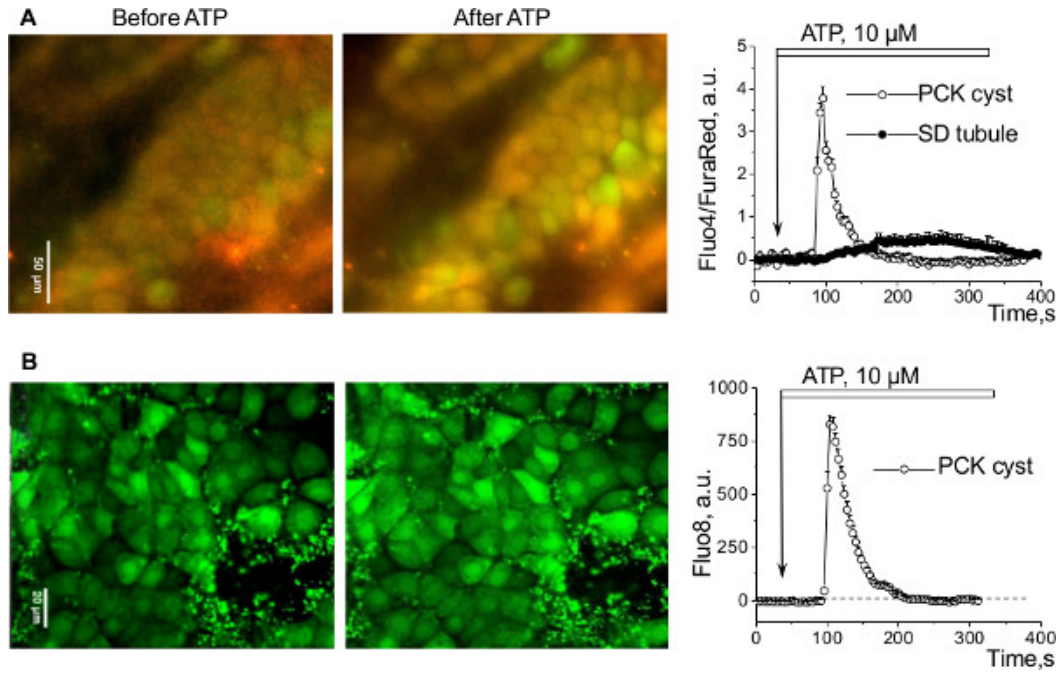

Figure 4: Effects of ATP on calcium influx in the cystic cells of the PCK rats. (**A**) Representative images of cystic monolayer loaded with Fura-2 AM before and after application of 10 μM ATP. Graph summarizing the effect of ATP on calcium levels in the cell monolayer of PCK rats and SD collecting ducts (N = 38 cells). **(B)** Cystic monolayer loaded with Fluo-8 dye before and after application of 10 μM ATP and summary graph of intracellular calcium response.

Discussion

We described here applications of conventional patch-clamp technique and epifluorescence calcium imaging to cystic epithelial monolayers derived from a murine genetic model of ARPKD. The protocol consist of three steps, of which the most attention should be paid to the isolation of the cysts (step 1.5 of the protocols section) and to the electrophysiological studies. These key procedures require extensive training and patience, and the reader should not be frustrated at once.

First of all, the most attention should be paid to the process of the cyst monolayer isolation. This part of preparation requires manual skills and significantly impacts further work, as thickness of the specimen and its even attachment to a glass chip is critical for the visibility of the cells. Thickness of isolated cyst walls varies in different parts of the specimen, and it is recommended to focus on larger cysts with bigger monolayer areas convenient for work. To help clean the specimen and access the monolayer, surrounding tissues should be peeled with forceps under a stereomicroscope. It should be emphasized that the technique implies utilization of a high quality stereomicroscope characterized by a significant field depth and a capability to change magnification in a wide range to accommodate varying sizes of objects during dissection. Another limitation

of the method is that such sophisticated techniques as patch-clamp and calcium imaging require personnel familiar with these methods. We suggest that initial experience can be obtained on collecting duct cell cultures such as commercially available cortical M1 and medullar IMCD cells as they form epithelial monolayer similar to cysts.

Our approach allows measuring ion channels activity and $[Ca^{2+}]_i$ levels in the native environment of freshly isolated tissues. The main advantage of this procedure is that preparation of specimens for single-channel analysis or dye loading does not require enzymatic treatment, damaging mechanical procedures or other potentially detrimental steps. It is performed manually with forceps in saline and provides large undamaged specimens. Such specimens can be used not only for the described techniques. In fact, isolated cystic epithelium represents thin film of pure tubular cells and maintains native monolayer properties, which makes it a valuable intact object for real-time experiments which produces physiologically relevant observations. If the isolation of cysts seems to hard a procedure for the researcher or a large amount of cystic tissue is required at once, enzymatic treatment (for instance, with dispase and collagenase, as described here[38] for the cortical collecting duct tubules) can be used to simplify the isolation process. However, the researcher should be very careful while performing this treatment, as enzymes, especially proteases, can significantly affect ion channels' activity or damage the membrane receptors. This is, for instance, very important for the studies of purinergic signaling, as these membrane proteins are very well known to quickly degrade under enzymatic treatment[39,40].

Additionally, cystic epithelium can be used for other purposes, such as immunohistochemistry or staining of a fixed monolayer with dyes (*e.g.* rhodamine phalloidin), or loading the fresh tissue with intracellular substance markers, such as DAF-FM for NO detection or various dyes to monitor reactive oxygen species production. It is suggested to use the pure fraction of isolated cystic epithelium for western blotting to eliminate impact of non-specific factors present in total kidney lysates. We also suggest that cystic epithelium can be similarly isolated from mice or other species that are available for the studies of various models of not only ARPKD, but also ADPKD. Freshly isolated cyst specimens have undeniable advantages for the molecular biology approaches compared to the cultured cystic cells, as they better sustain the properties of the cystic tissue within the organ, and undoubtedly provide more justified data regarding the *in vivo* disease processes.

One of the significant advantages of this technique is a possibility to use normal non-dilated collecting ducts from the same kidneys, or SD rats tubules (these rats share genetic background with PCK rats), as control tissues. Similar patch-clamp and calcium measurements in nephron segments are widely used on non-cystic kidneys in many laboratories[41-43]. Depending ion channels type, different modifications of patch-clamp method can be used (whole cell, inside-out, outside-out); for instance, ENaC and ROMK (renal outer medullary potassium channel) activity can be assessed by whole-cell configuration[44,45]. The proposed Fura-2 AM calcium imaging in wide-field epifluorescence with monochromator can be also performed with any other similar modification of calcium imaging such as analysis with confocal or two-photon microscopy with other calcium dyes. These microscope systems are less affordable but provide higher quality imaging and are more sensitive than wide-field microscopy. Similar approach was also applied to study TRPC channels activity and calcium signaling in podocytes of freshly isolated glomeruli[17,46,47]. Other calcium imaging that could be applied include utilization of Fluo-8 as demonstrated on **Figure 4B** or ratiometric confocal measurement with Fluo4/FuraRed fluorescent dyes as described in Ilatovskaya *et al.*[46] Technically, it is possible to perform both patch-clamp and calcium imaging simultaneously on the same cell if microscope is equipped with both setups. Thus, the described technique is a feasible approach for high-quality observations in the field of PKD, which can be flexibly modified according to the needs of your specific research and availability of the equipment. Furthermore, various modifications of calcium imaging can be used here, including ratiometric confocal measurements with Fluo4/FuraRed fluorescent dyes (as described in Ilatovskaya *et al.*[46]) or Fluo-8 as demonstrated on **Figure 4B**.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to thank Glen Slocum (Medical College of Wisconsin) and Colleen A. Lavin (Nikon Instruments Inc) for excellent technical assistance with microscopy experiments. This study was supported by the National Institutes of Health grants R01 HL108880 (to AS), R01 DK095029 (to OPo) and K99 HL116603 (to TSP), National Kidney Foundation IG1724 (to TSP), American Heart Association 13GRNT16220002 (to OPo) and the Ben J. Lipps Research Fellowship from the American Society of Nephrology (to DVI).

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