Automated method for the isolation of collecting ducts

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Automated method for the isolation of collecting ducts. Am J Physiol Renal Physiol 291: F236–F245, 2006. First published February 7, 2006; doi:10.1152/ajprenal.00273.2005.—Structural and functional heterogeneity of the collecting duct present a tremendous experimental challenge requiring manual microdissection, which is time-consuming, labor intensive, and not amenable to high throughput. To overcome these limitations, we developed a novel approach combining the use of transgenic mice expressing green fluorescent protein (GFP) in the collecting duct with large-particle-based flow cytometry to isolate pure populations of tubular fragments from the whole collecting duct (CD), or inner medullary (IMCD), outer medullary (OMCD), or connecting segment/medullary collecting duct (CNT/CCD). Kidneys were enzymatically dispersed into tubular fragments and sorted based on tubular length and GFP intensity using large-particle-based flow cytometry or a complex object parametric analyzer and sorter (COPAS). A LIVE/DEAD assay demonstrates that the tubules were >90% viable. Tubules were collected as a function of fluorescent intensity and analyzed by epifluorescence and phase microscopy for count accuracy, GFP positivity, average tubule length, and time required to collect 100 tubules. Similarly, mRNA and protein from sorted tubules were analyzed for expression of tubule segment-specific genes using quantitative real-time RT-PCR and immunoblotting. The purity and yield of sorted tubules were related to sort stringency. Four to six replicates of 100 collecting ducts (9.6 ± 0.44–14.5 ± 0.66 cm or 9.2 ± 0.7 mg tubular protein) were routinely obtained from a single mouse in under 1 h. In conclusion, large-particle-based flow cytometry is fast, reproducible, and generates sufficient amounts of highly pure and viable collecting ducts from single or replicate animals for gene expression and proteomic analysis.

COPAS; real-time PCR; fluorescent protein; transgenic mouse

The collecting duct plays a critical role in the regulation of salt, water, potassium, and acid-base balance. It consists of three structurally and functionally heterogeneous segments, i.e., the cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and the inner medullary collecting duct (IMCD), that connect to the tubule via the connecting segment (CNT). Each segment is composed of two major cell types: principal and intercalated cells. Principal cells are involved in salt and water regulation and are characterized by the expression of the vacuolar proton ATPase (V-ATPase) (4, 5) and basolateral localization of AE1 (1) and pendrin (27). Intercalated cells are characterized by the apical localization of V-ATPase (4, 5) and pendrin (27). These cells are responsible for the secretion of hydrogen ions. β-Intercalated cells are characterized by basolateral localization of V-ATPase and apical expression of pendrin (27) and reside in the CCD (36). These cells are responsible for the secretion of bicarbonate. Non-α/β-intercalated cells are characterized by the expression of V-ATPase in apical and basolateral plasma membranes and pendrin in the apical plasma membrane, but they do not express AE1 (36). They reside in the CCD only. As many as 36–38% of the total cells in the collecting duct and connecting segment are intercalated cells in mice (14). Although there are intercalated cells located in the distal convoluted tubule (DCT) (5), they make up only 4% of the total DCT cells in mice (14). In addition, there are segment-specific variations in principal cells. Whereas AQP2 is expressed in all principal cells, it is most abundant in principal cells of the OMCD (16). In contrast, expression of AQP3 (10) is most abundant in the basolateral membrane of principal cells throughout the collecting duct (10), whereas AQP4 is expressed only in the basolateral membrane of principal cells within the IMCD (37). Similarly, ENaC is found in all collecting duct principal cells (7); however, under normal physiological conditions it is restricted primarily to the CNT, CCD, and OMCD (9). Finally, the urea transporter is highly expressed in principal cells within the IMCD (31).

The investigation of collecting duct heterogeneity presents a tremendous experimental challenge. Such investigation includes the analysis of segment-specific gene and protein expression. Such experimentation requires the “skilled art” of manual microdissection, originally described by Burg and Orloff in 1962 (6), where individual nephron segments are isolated based on morphological criteria. This was extended by Schafer et al. in 1997 (32), where tubular segments from rat and rabbit were manually sorted under a microscope using a 30-gauge needle; ~50 mm of a given nephron could be isolated in 1 h. While effective, manual microdissection and the approach described by Schafer et al. 1997 are time-consuming, labor intensive, and require a high level of skill for the identification of tubular segments. Therefore, use of these methods is currently limited to a few laboratories and is not amenable to high throughput and automation.

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In this study, we establish and describe a novel approach combining the use of transgenic mice expressing green fluorescent protein (GFP) transgenic mice (19) or their wild-type littermates. The mice were maintained on a standard rodent diet with free access to water. The use of animals conformed to protocols approved by the University of Utah Institution Animal and Care and Use Committee.

Preparation of single tubular fragments. Mice were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ) and then euthanized by cervical dislocation. The kidneys were removed and placed in ice-cold Krebs (145 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM NaH2PO4, 2.5 mM CaCl2, 1.8 mM MgSO4, 5 mM glucose, pH 7.3). Samples were finely minced and digested in 10 ml Krebs digestion media containing 3 mg/ml collagenase IV (Worthington Biochemical, Lakewood, NJ), 2 mg/ml hyaluronidase, and 0.1 mg/ml Dnase I (Sigma, St. Louis, MO) at 37°C for 20 min. The tubular digest was filtered with a 100-µm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ), the flowthrough was discarded, whereas the larger tubules retained by the filter were filtered through 250- and 212-µm sieves. The flowthrough was retained and diluted with ice-cold Krebs containing 0.5% BSA (Sigma-Aldrich) to a total volume of 40 ml.

Isolation of single tubular fragments using the complex object parametric analyzer biosorter. All sorting was performed with a complex object parametric analyzer and sorter (COPAS) instrument (Union Biometrica, Somerville, MA). The following instrument settings were used: delay 8, width 5, photomultiplier tube (PMT) 700, and sheath fluid pressure 3.9 – 4.1. The sample fluid pressure was set to 102 copies/µl. PCR reactions were considered valid only if the amplification was linear and efficient, i.e., r² ≥0.95 and slope of the curve was −0.3025 ± 0.010. Products were analyzed by agarose gel electrophoresis. All amplicons were verified by sequencing; specificity was verified by the lack of amplification with nontemplate controls.

Immunoblotting. Tubules were collected directly into cold PBS, centrifuged at 12,000 g for 10 min and resuspended in 100 µl of TRIzol (Invitrogen, Carlsbad, CA); RNA was isolated according to the manufacturer’s recommendations and dissolved in 15 µl of water. cdNA synthesis was performed by reverse transcription of 15 µl of RNA using oligo-dT priming and Superscript Reverse Transcriptase II (Invitrogen).

Table 1. RT-PCR primer pairs

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>GAPDH</td>
<td>5′-TCTAGATGTTCCAGATGACTCTCA-3′</td>
<td>5′-AGTCGAGGTCAAGTCGACCCC-3′</td>
</tr>
<tr>
<td>AQP2</td>
<td>5′-CCACGGCGGACACTTGAGG-3′</td>
<td>5′-CTACTGCTGTTCCCGTGGACACC-3′</td>
</tr>
<tr>
<td>B1</td>
<td>5′-ATCAATGTTGCTGCCATCTCCT-3′</td>
<td>5′-GAAAGAGATGCAAGGCCATT-3′</td>
</tr>
<tr>
<td>AQP1</td>
<td>5′-TCCCTTAACATTTCCTCTGTC-3′</td>
<td>5′-TGGCAAGAGAAGAGTATGTT-3′</td>
</tr>
<tr>
<td>KAP</td>
<td>5′-CTCTGGTGTGGATCGGCTGCG-3′</td>
<td>5′-GTTCAATGATGCACCCACC-3′</td>
</tr>
<tr>
<td>NCC</td>
<td>5′-CTCCCGGAGATCAACGAA-3′</td>
<td>5′-CACCCAGTGCTCTCGTCC-3′</td>
</tr>
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</table>

AQ1P1 and AQP2, aquaporin-1 and -2, respectively; KAP, kidney androgen-regulated protein; NCC, Na-Cl-cotransporter; Kik1, kallikrein-1.
followed by 150 V for 60 min in SDS running buffer. Proteins were transferred to a polyvinylidene difluoride 0.45-µm membrane (Immobilon-P, Millipore) using a semidry transfer protocol (Bio-Rad, Hercules, CA). Membranes were blocked with 5% dry milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.01% vol/vol Tween 20). Blots were incubated for 1 h at room temperature with either a rabbit anti-AQP2 polyclonal or goat anti-actin polyclonal antibody at 1:500 dilution and then washed with TBST for 5 min (5×). Membranes were incubated with either a goat anti-rabbit or rabbit anti-goat secondary antibody at 1:2,000 dilution for 1 h at room temperature. The affinity-purified rabbit anti-AQP2 polyclonal antibody was a generous gift from Dr. Bellamkonda Kishore at the University of Utah (15); all other primary and secondary antibodies were purchase from Zymed (Carlsbad, CA).

**Statistical analysis.** The means ± SE were calculated while statistical comparisons were performed using a two-sample t-test. \( P < 0.05 \) was considered significant.

**RESULTS**

**B1-EGFP transgenic mouse.** We used B1-EGFP transgenic mice for all experiments (19). In B1-EGFP transgenic mice, EGFP expression is driven by 6.5 kb of human V-ATPase B1-subunit promoter. The B1 subunit and EGFP are expressed exclusively in intercalated cells throughout the length of the renal collecting duct. Figure 1A shows representative expression of GFP in a 200-µm vibratome section of kidney from these animals. Figure 1B shows GFP-positive and -negative tubules produced by mincing, collagenase digestion, and sieving of B1-EGFP transgenic kidney as described in **METHODS**.

**Large-particle flow cytometry.** GFP-positive or -negative tubules were isolated using a large-particle flow cytometer, which has been termed COPAS (Union Biometrica). Using large-bore fluids, this instrument automatically analyzes, sorts, and dispenses particles (≥250 µm) based on size, optical density, and fluorescence (Fig. 2).

We used this technology to isolate GFP-positive tubules from B1-EGFP transgenic mouse kidneys. Figure 3 shows a representative dot plots and histograms from B1-EGFP transgenic mice (Fig. 3, A and B) and their wild-type (Fig. 3, C and D) littermates. Dot plots show GFP fluorescence vs. time of flight (particle length), whereas histograms show frequency vs. GFP fluorescence. Results showed that the majority of events were characterized by a fluorescence intensity of ≤50, i.e., 90 ± 1.5% for transgenic mice and 98 ± 1.2% for their wild-type littermates; these events represent autofluorescence. Intensities between 50 and 250 were observed for 4.7 ± 2.3% for transgenic animals and 0.5 ± 0.27% for control littermates and represent both autofluorescent and GFP fluorescent tubules. Fluorescence intensities between 250 and 1,000 were observed only in transgenic animals and represent GFP-positive tubules.

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Fig. 1. Kidney from B1-enhanced green fluorescent protein (EGFP) transgenic mouse. A: 200-µm vibratome section shows the expression of GFP in the connecting tubule (CNT) and collecting duct (CD). B and C: examples of a GFP-positive collecting duct (B) and GFP-negative non-collecting duct tubule. Magnification: ×4.5 (A) and ×20 (B and C).
Fig. 2. Schematic of complex object parametric analyzer and sorter (COPAS). Particles (tubules) suspended in solution flow continuously from a pressurized sample chamber into the flow cell, which is then surrounded by a sheath of fluid that focuses the particle in the center of the flow stream. Two lasers are used to measure 5 optical parameters, a red diode laser (670 nm) for axial length and optical density, and a multiline argon laser (488 and 514 nm) for fluorescence emission at 610 (red), 545 (yellow), and 510 nm (green). As the sample fluid exits the flow cell, it is diverted by a constant stream of air. Rapidly switching the air diverter off and on generates a 0.7-μl slice of liquid containing the fluorescent or nonfluorescent particle (tubule), which is then collected in a microwell or bulk container. (Used with permission from Union Biometrica.)

Fig. 3. COPAS biosorting of tubules from B1-EGFP transgenic and wild-type mouse kidneys. Tubules were sorted based on fluorescence intensity of GFP and time of flight (TOF). Representative dot plots for biosorting of tubules from a B1-EGFP transgenic (A) and wild-type (C) mouse. Representative frequency distributions for 5,000 events for a biosort of tubules from a B1-EGFP transgenic (B) and wild-type (D) mouse are also shown.
The viability of COPAS-isolated collecting duct fragments was determined using a LIVE/DEAD assay (Molecular Probes) that has been used to characterize islets isolated for transplantation (2). Enzymatically digested collecting ducts were stained with the Hoechst (blue) and Sytox (red) fluorescent stains; Hoechst stains the nuclei of all cells, whereas Sytox stains the nuclei of “dying” cells. Epifluorescence micrographs of non-COPAS- and COPAS-isolated tubules were obtained, and viability was determined by calculating the ratio of live to total cells (Fig. 4). Results show that the average percentage of live cells per tubule was greater in the COPAS-isolated sample compared with the non-COPAS-isolated sample, i.e., 91.8 ± 0.9 and 69.3 ± 3.7, respectively. Examination of the rate of cell death shows an increase of −5%/h following enzymatic digestion with no significant difference between COPAS- and non-COPAS isolated tubules.

The boundaries of the sort region were varied to demonstrate that the purity of the isolated tubules directly correlates with sort region or sort stringency. We observed an inverse relationship between the sort purity and the number of tubules obtained per collection time unit. Figure 5 shows representative dot plots and histograms for events collected from sort region 1 (Fig. 5, A and B), sort region 2 (Fig. 5, C and D), and region 3 (Fig. 5, E and F). Sorting events were evaluated for count accuracy, percent GFP-positive tubules, and total tubular length by imaging tubule suspensions using fluorescence confocal microscopy (Table 2). Results show that count accuracy and percent GFP-positive tubules increased with increased sort stringency. The collection time required to obtain 100 events increased with increasing stringency, whereas the average length of tubules remained constant. Therefore, 1.17 ± 0.06, 0.47 ± 0.03, and 0.25 ± 0.01 cm of collecting duct/min were obtained from region 1, 2, and 3, respectively. Four to six replicates of 100 collecting duct tubules were routinely obtained from a single mouse in under 1 h, which produced an estimated total length of 8.6–12.9 cm of collecting duct/mouse. The total length was calculated using the equation average length of tubules × total sorted events × percent GFP-positive tubules × percent accuracy × replicate number = total length.

Collecting duct-specific gene expression in COPAS-sorted cells. COPAS was used to isolate tubules from GFP-positive region 2 or a non-GFP region (Fig. 6, B and D). AQP2, B1, and AQP1 mRNA expression was then quantified. For tubules isolated from region 2, the expression of AQP2 and B1 mRNA was increased 44.3 ± 6.2- and 46.8 ± 3.3-fold, respectively, and AQP1 mRNA was decreased 0.5 ± 0.06-fold compared with whole kidney (Fig. 6A). In contrast, for tubules isolated from the non-GFP region, the expression of AQP2 and B1 mRNA was minimally increased 3.50 ± 0.78- and 2.89 ± 0.64-fold compared with whole kidney, whereas AQP1 mRNA was increased 34.8 ± 8.9-fold (Fig. 6C). These results demonstrate that COPAS can be used to isolate GFP-positive tubules that are highly enriched for collecting duct-specific mRNA (AQP2 and the B1 subunit of V-ATPase) (20, 23) but devoid of non-collecting duct tubule mRNA (AQP1) (8, 24, 30), whereas COPAS-sorted non-GFP tubules are enriched for non-collecting duct mRNA (AQP1). This indicates that the COPAS method can be used to isolate collecting ducts for the examination of gene expression by real-time RT-PCR and other gene profiling techniques.

COPAS sorting was limited to the outer cortex of kidney to demonstrate that CNT/CCD segments could be isolated. The outer cortex was dissected from B1-EGFP transgenic kidney and subject to digestion, sieving, and COPAS sorting, thus

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**Fig. 4.** Representative epifluorescence micrographs of enzymatically digested collecting duct fragments before (A) and after (B) COPAS. The blue stain (Hoechst) labels the nuclei of all cells, whereas the red stain (Sytox) labels the nuclei of dead cells (GFP fluorescence not shown). C: percentage of live cells from non-COPAS- and COPAS-isolated collecting duct fragments. Values are means ± SE of 15 tubules/condition.
enriching for CNT/CCD. RT-PCR analysis showed the presence of kallikrein, a marker of the CNT (12, 26) and AQP2 (Fig. 7). In contrast, kidney androgen-regulated protein, a marker for proximal tubules (3, 18), although present in the kidney starting material, was undetectable in CNT/CCD (Fig. 6). RT-PCR for GAPDH demonstrated that this was not due to lack of RNA in the sample. These results demonstrate that COPAS could be used to isolate tubules from renal cortex that expressed CNT/CCD-specific markers.

COPAS-isolated tubules from the renal cortex were examined for the expression of DCT- and CNT/CCD-specific markers. COPAS was used to isolate tubules that were +/−/H11/GFP (GFP fluorescence +/−/H13/200), whole (GFP fluorescence 0−/1,000) and +/−/H11/GFP (GFP fluorescence +/−/H11/50) (Fig. 8B). Expression of sodium chloride cotransporter (NCC), AQP2, and AQP1 was determined using real-time RT-PCR and was normalized to that from nondigested renal cortex and represented as percent of control (Fig. 8A). Results show that expression of NCC in the +GFP, whole, and −GFP tubules was 84.8 ± 8.7, 59.9 ± 16.8, and 84.9 ± 10.8% of control, respectively. Similarly, expression of AQP2 in the +GFP, whole, and −GFP tubules was 521.5 ± 58.7, 62.1 ± 15.4, and 0% of control, respectively. Expression of AQP1 in +GFP, whole, and −GFP tubules was 0, 157 ± 18.2, and 410 ± 53 of control, respectively. The enrichment of AQP2 in the +GFP sample

Table 2. Statistics on COPAS-sorted tubules from B1-EGFP transgenic mouse kidney

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Region 3</th>
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<tbody>
<tr>
<td>Count accuracy, %</td>
<td>76.6±1.6</td>
<td>86.6±2.8</td>
<td>93.3±2.4</td>
</tr>
<tr>
<td>GFP positive tubules, %</td>
<td>92.5±3.5</td>
<td>96.7±3.7</td>
<td>100</td>
</tr>
<tr>
<td>Collection time for 100</td>
<td>1.5±0.45</td>
<td>4.5±0.50</td>
<td>9.5±1.0</td>
</tr>
<tr>
<td>events, min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average tubular fragment</td>
<td>247.8±12.2</td>
<td>254.3±14.3</td>
<td>260.1±11.8</td>
</tr>
<tr>
<td>length, µm</td>
<td></td>
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COPAS, complex object parametric analyzer and sorter; EGFP, enhanced green fluorescent protein.
and its absence in the −GFP sample and, conversely, the lack of AQP1 in the +GFP and its enrichment in the −GFP sample clearly show that COPAS could differentiate between +GFP tubules, such as collecting duct, and and −GFP tubules, such as proximal tubule or thin limb of the loop of Henle. The reduction in NCC expression relative to AQP2, although not complete elimination, suggests that the enzymatically digested tubules from the B1-EGFP transgenic mouse consist of CNT/CCD with a small amount of late DCT that may be physically connected.

Protein analysis in COPAS-isolated GFP-expressing tubules. COPAS was used to isolate tubules for protein analysis. Both kidneys from a single mouse will yield as much as 3,000 collecting duct tubules or 9.2 ± 0.7 mg total protein. Using immunoblotting analysis with a specific antibody against AQP2, we show the presence of AQP2 protein in COPAS-isolated GFP-positive tubules (Fig. 9, top). Densitometric analysis indicated a linear increase in AQP2 protein amount in samples from 50, 75, and 100 tubules. Furthermore, AQP2 was detected in as few as 50 tubules. In contrast, AQP2 protein was not detected in GFP-negative tubules. The presence of actin demonstrated that the lack of AQP2 in GFP negative tubules was not due the lack of tubular protein. (Fig. 9, bottom). These results demonstrated that the COPAS method could be used to isolate collecting ducts for immunoblotting, and potentially for other proteomics techniques.

**DISCUSSION**

The collecting duct consists of structurally and functionally heterogeneous segments; the CNT/CCD, OMCD, and IMCD. Each segment is composed of two major cell types, i.e., principal and intercalated cells, with three subtypes of intercalated cells. Furthermore, each segment is characterized by the expression of different genes, which have been analyzed using manual microdissection. However, this approach is time and labor intensive, requires specialized skills, and is not amenable to high throughput. The combination of transgenic mice expressing collecting duct-specific GFP, i.e., B1-EGFP (19), and large-particle-based flow cytometry now make it possible to study collecting duct-specific gene/protein expression.
We present a novel experimental approach for the automated isolation of large quantities of CNT/CCD, OMCD, and IMCD. This approach is based on enzymatic digestion of renal tubules (6, 32) and the use of a fluorescent collecting duct marker and large-particle flow cytometry. The success of this experimental approach is dependent on the intensity and collecting duct-specific expression of GFP. The B1-EGFP transgenic animals are well suited for this approach because GFP is expressed in all intercalated cells from the CNT to the papilla (Fig. 1A) (19) and is not affected by such perturbations as hydration/dehydration, acid/base loading, low/high-NaCl diet, and/or enzymatic digestion (Miller RL and Nelson RD, unpublished observations). In contrast, AQP2-EGFP transgenic mice show variegated GFP expression, which changes with hydration status (41). Similarly, GFP is not expressed in the CNT of Hoxb7-GFP transgenic animals (28, 33). In addition, attempts to COPAS isolate collecting ducts from wild-type mice labeled with DBA-lectin/fluorecin were not very successful (data not shown). Interestingly, COPAS isolation of double-positive AQP2-GFP and B1-GFP transgenic mouse increased the percentage of +GFP tubules to 25% of all sorting events compared with the maximum 7% seen in the B1-EGFP mouse alone (data not shown).

We show that the COPAS isolation procedure is capable of isolating large quantities of GFP-positive tubules, i.e., up to 12 cm of collecting duct or 9 mg of collecting duct protein/mouse, in under 1 h. In addition, we show that ≥90% of the cells in a given COPAS-isolated tubule were viable whereas 69% of the cells in a non-COPAS-isolated tubule were viable. We have observed that GFP leaks from dead/dying cells, which could account for this apparent bias.

Using collecting duct- and non-collecting duct-specific markers, we show that the overall purity of COPAS-isolated tubules is dependent on the sort stringency and chosen collecting duct segment. For example, COPAS could distinguish between collecting duct, and proximal tubule or loop of Henle. However, GFP-positive tubules isolated from the cortex of B1-GFP transgenic mouse expressed a low level of the distal convoluted tubule marker NCC. The lack of AQP1 expression in the GFP-positive tubules indicates that contamination did not arise from physically separate non-GFP nephron segments but rather from contiguous DCT fragments. Some DCT fragments may be physically connected to the GFP-positive CNT/CCD fragments and thus may be coisolated with CNT fragments. The DCT also contains some intercalated cells, although intercalated cells make up only ~4% of total cells in the mouse (5, 14). In contrast, intercalated cells in the connecting segment and collecting duct make up 36–38% of total cells (14). Thus there is a 10-fold increased abundance of intercalated cells in the CNT and CCD compared with the DCT. Contamination should be minimized by using a stringent fluorescence threshold, as was done in region 3 in Fig. 5. Regardless, such a limitation was easily overcome for OMCD and IMCD by isolating GFP-positive tubules from the outer and inner medulla of the B1-EGFP transgenic mouse (data not shown).

COPAS isolation has distinct advantages over the more conventional manual microdissection. First, it dramatically shortens the time required to isolate equal quantities of collecting duct, i.e., 10 cm of collecting duct can be isolated in 45–60 min using COPAS compared with 4–5 h required for manual microdissection. Second, it eliminates the need for a highly trained person, i.e., COPAS is suitable for internal core

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**Fig. 8.** Expression of distal convoluted tubule and CNT/CCD-specific markers in COPAS-isolated tubules. Real-time RT-PCR was used to quantify sodium-chloride cotransporter (NCC), AQP2, and AQP1 expression in tubules (A) collected from the GFP-positive (+GFP), GFP-negative (~GFP), and whole regions (B). Differences in cDNA concentration were normalized to GAPDH. Gene expression in ~GFP, ~GFP, and all (whole) tubules was normalized to that of the nonsorted and nondigested whole cortex. Values are means ± SE (n = 4).

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**Fig. 9.** Immunoblot analysis of AQP2 protein expression in COPAS-isolated tubules from B1-EGFP transgenic kidneys. In the analysis, 150, 100, 75, and 50 ~GFP and 150 –GFP tubules were collected from region 2 and the non-GFP region, respectively. Protein was isolated, separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed for AQP2 (top) or β-actin (bottom).
facilities, and others. Laboratory personnel trained in manual microdissection. This could be used to pool samples for genomic or proteomic analysis that require more starting material than one is able to obtain from manual microdissection.

COPAS could be used to isolate other nephrin segments using a variety of available transgenic mice expressing GFP or CRE recombinase in specific nephrin segments. Currently, the number of transgenic mice expressing GFP in single-nephron segments or cell types, including the podocytes in the glomerulus (13), thick ascending limb of the loop of Henle (42), collecting duct (33), principal cells in the collecting duct (41), and intercalated cells in the collecting duct (19). There are also a number of transgenic mouse expressing CRE recombinase in single-nephron segments or cell types: the podocytes in the glomerulus (11), the proximal tubule (29), thick ascending limb (TAL) (35), collecting duct (28), and principal cell (21). Double transgenic mice may be generated by mating of these CRE transgenic mice and mice containing a transgene that may be activated by CRE to cause the expression of GFP (25), YFP (34), or enhanced red fluorescence protein (RFP) (39). In either case, COPAS is capable of detecting and sorting particles labeled with GFP, YFP, and/or RFP. Furthermore, the combination of nephrin-specific CRE-activated RFP and nephrin segment-specific GFP would allow enhanced dual fluorescence sorting. For example, a DCT-CRE mouse mated with CRE-activated RFP mated on the background of the B1-EGFP would allow the isolation of both collecting duct and distal tubule while excluding the overlapping DCT/CNT (red/green). Future design of transgenic mice expressing GFP, RFP, and/or CRE in thin descending or ascending limb of the loop of Henle, DCT, connecting segment, and inner medulla would allow isolation of virtually any nephrin segment using COPAS.

There are several future applications that are currently being developed. First, COPAS could be used to isolate large quantities of CCD, OMCD, and IMCD for the generation of primary cultures. This would overcome limitations of immortalized cell lines, including dedifferentiation caused by excessive passaging of cells and the effects of an immortalizing gene. Second, COPAS could also be used to isolate CD suspensions for short-term studies of cell signaling or gene expression, for example, the differential effects of vasopressin on cAMP and intracellular calcium in CCD, OMCD, and IMCD or the regulation of ENaC by aldosterone in the CCD.

In conclusion, we show here that large-particle-based flow cytometry or COPAS along with the B1-EGFP transgenic mouse are an ideal combination for isolating collecting ducts for gene expression and/or proteomic analysis. The experimental approach is fast, reproducible, and generates starting material with high purity and sufficient amounts from single or replicate animals. Furthermore, preliminary results indicate that it can be used to generate collecting duct primary cultures and collecting duct suspensions to study cell signaling.

GRANTS
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DISCLOSURES
None of the authors has a financial conflict of interest regarding Union Biometrica.

REFERENCES


