Where Light Meets Life®

Delaware Flow NanoCytometer®

Application Note: Urinary EVs as Biomarkers for ADPKD

Interest in Extracellular Vesicles (EVs) is growing rapidly as they are increasingly recognized as ubiquitous carriers of valuable biological information. EVs, such as exosomes and microvesicles, are nanoscale subcellular particles secreted by cells and used for intercellular signaling and transport; they typically range in size from about 50 to 200 nm. One important new application of EVs is **noninvasive diagnostics**. In particular, EVs are abundant in urine, making them an ideal vehicle of information about the kidneys.

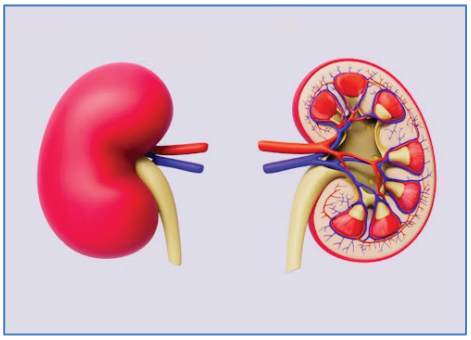

Fig. 1. ADPKD affects 417,000 people in the US alone. EVs, abundant in urine, are showing extraordinary promise for rapid, accessible, and accurate kidney disease diagnosis and prognosis. Image by Point Normal on Unsplash

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is an inherited genetic pathology leading to the development of fluid-filled cysts in the collecting ducts of the kidney. About 8M people worldwide suffer from the disorder, which can lead to renal failure. Current methods of diagnosis are expensive (MRI), confounded by false positives (genetic screening), or slow and labor-intensive (Western blotting). Additionally, there currently isn't a simple way to determine disease severity and provide a reliable prognosis. There is a need for a better, faster, and/or cheaper diagnostic–and prognostic–tool.

The *Delaware* Flow NanoCytometer is a particle analyzer with extraordinary nanoparticle resolution (6 nm), high sensitivity (68 nm in liposomes), and the unequaled ability to easily measure both EVs and cells without clogging. The *Delaware* offers up to three channels of light scattering and up to five channels of simultaneous fluorescence detection for multiparametric phenotyping.

Analysis of Urinary EVs on the *Delaware* Flow NanoCytometer. We have been fortunate to collaborate with Dr. Christopher Ward at the University of Kansas Medical Center in developing assays aimed at making ADPKD diagnostics and prognostics simpler, more accurate, and more actionable. Dr. Ward has dedicated his entire professional life to ADPKD: He was involved in isolating the genes responsible for it, and has developed a number of monoclonal antibodies specific to ADPKD. In particular, he has created clones for polycystin-1 (PC1), polycystin-2 (PC2), exosomal polycystin-1 interacting protein (EPCIP), and a fibrocystin homolog (TMEM2). Thanks to the *Delaware*'s sensitivity and its unique ability, among research flow cytometers, to perform absolute volumetric measurements; and to Dr. Ward's highly specific antibody conjugates, we have developed several noninvasive assays that can be used, with minimal preparation (no ultracentrifugation involved), to measure ADPKD-specific biomarkers. Below are two such assays.

For more information:
Kinetic River Corp.
+1 (650) 439-7413
info@KineticRiver.com
www.KineticRiver.com
897 Independence Ave. #4A
Mountain View, CA 94043 USA

Sample preparation

NOTE: All steps of the procedure outlined here to be performed only by trained personnel in accordance with applicable regulations and institutional rules concerning collection and analysis of biological specimens and operation of the Delaware Flow NanoCytometer.

Fig. 2. The Delaware Flow Nanocytometer®.

- collect urine samples (2nd morning void 10 mL) and gently spin at 4000g for 5 minutes
- aspirate the supernatant
  - typical EV concentration: ~ 0.3–1x10^7 EV/µL
- if needed, dilute supernatant to intermediate concentration ~ 5x10^6 EV/µL
- prepare stained samples in duplicate: for each sample, add into a microcentrifuge tube the desired conjugated monoclonal antibodies, add dilution buffer to reach 30 µL, then add 20 µL of supernatant; vortex mix
  - example 1 (double stain):
    - 1 uL of PC1-PE† at 1 mg/mL (FL3)
    - 5 µL of ThermoFisher CD133-AlexaFluor488 (#53-1331-80) at 100 µg/mL (FL2)
    - 24 µL of dilution buffer
    - 20 µL of supernatant
  - example 2 (triple stain):
    - 1 µL of PC1-PE† at 1 mg/mL (FL3)
    - 5 µL of PC2-AlexaFluor488† at 1 mg/mL (FL2)
    - 5 µL of 1:100 dilution of Biotium CellBrite Steady 650 (#30108-T) membrane stain (FL5)
    - dilute 1 µL of CBS650 in 99 µL of HyPure water
    - use 5 µL of the resulting solution
    - 19 µL of dilution buffer
    - 20 µL of supernatant

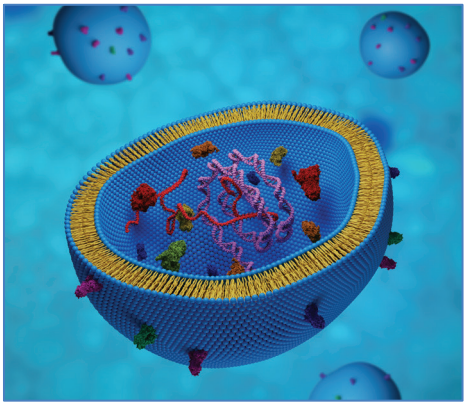

Fig. 3. EVs are abundant in urine, making them great candidates for noninvasive diagnostics.

NOTE: Reagents marked with (†) are produced by and are available from Dr. Ward, KUMC.

- prepare appropriate control samples
  - unstained (same as example 1 above, replacing antibody conjugates with 6 µL of buffer; or example 2, replacing antibody conjugates with 11 µL of buffer)
  - negative control (unstained Cellarcus LIPO100)
  - stain-only (same as 1 or 2 above, replacing 20 µL of supernatant with 20 µL of buffer)
  - single-stains (same as 1 or 2 above, replacing in turn two of the conjugates with the same volume of buffer)
  - recommended if possible: Fluorescence-Minus-Ones (same as 1 or 2 above, replacing in turn one of the conjugates with the same volume of buffer)
- incubate in the dark at RT for 1 hr
- dilute stained samples ~ 1:1000; vortex mix
  - typical concentration used for analysis: ~ 5x10^3/µL
  - no wash step needed

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Delaware setup

NOTE: All these (*) steps need only be performed once for an entire series of experiments.meter.

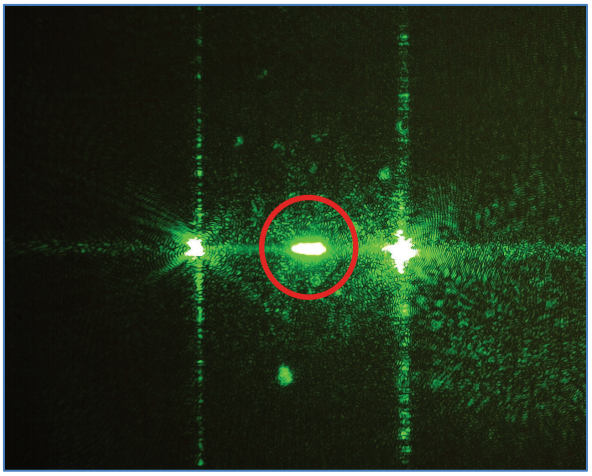

Fig. 4. Reference beads showing up brightly under 561-nm illumination on the Cavour flowcell monitoring module.

- set up optics*
  - o verify lasers (as needed for the selected assay) are at maximum power: 375 nm → 70 mW, 405 nm → 300 mW, 488 nm → 200 mW, 561 nm → 150 mW, 642 nm → 150 mW
  - o using the Cavour flowcell monitoring module, verify laser alignment (see at right)
  - o run reference 100-nm polystyrene beads (e.g., ThermoFisher 3100A) to set SSC gains
  - o run reference capture beads (Cellarcus vCal nanoCAL Antibody Capture Beads) stained with the same antibody conjugates as in the panel above to set relevant FL gains (example 1: FL2, FL3; example 2: FL2, FL3, FL5)

  NOTE: It is recommended to run a voltration series when running the first sample of its kind in order to optimize gains and signals
- set up fluidics*
  - o using a blank (buffer) sample, verify on the flow monitoring utility that flows are stable (consistent sample flow rate; no jumps in flow traces)
  - o using PS beads above, verify transit time ≥ 5 µs; adjust sheath pressure if needed
  - o using PS beads above, verify sample flow rate ≤ 10 µL/min; adjust sample pressure if needed
- set up acquisition parameters*
  - o SSC1, SSC2: set input voltage range to 0.2 V, offset to 0.095 V
  - o FL2, FL3, FL5 (as needed): set input voltage range to 1 V
  - o set SSC2 as the trigger channel (AC coupling)
  - o set trigger threshold between around 20 mV (for SSC2 gain of 600 V)
  - o set acquisition gate 1: SSC2 width from 0.5 to 15 µs
  - o set up scattergrams/dot plots:
    - SSC1 vs. SSC2 (front page)
    - FL2 vs. FL3 (front page)
    - other combinations as desired (second page): FL3 vs. SSC2, FL5 vs. FL3, etc.

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Sample analysis on the *Delaware*

NOTE: All sample, control, and background runs must be acquired with the same fixed sample volume setting to ensure accurate background subtraction.

- set acquisition mode to Fixed Volume:
  100 µL (see at right)
- run controls
  - o unstained (1 tube per sample)
  - o negative control (1 tube)
  - o stain-only (1 tube)
  - o single stains (example 1: 2 tubes per sample; example 2: 3 tubes per sample)
  - o recommended if possible: Fluorescence-Minus-One (example 2: 3 tubes)
- run samples
  - o recommended: 3 replicates if possible
- run backgrounds
  - o always run filtered buffer between sample and/or control runs
- during sample acquisition:
  - o monitor event pulses and verify lack of substantial outliers
  - o monitor fluorescence histograms and dot plots: stained sample signals > stain-only baseline
- perform Probe Wash after every sample and control run
  - o important to minimize carryover and ensure controls are valid

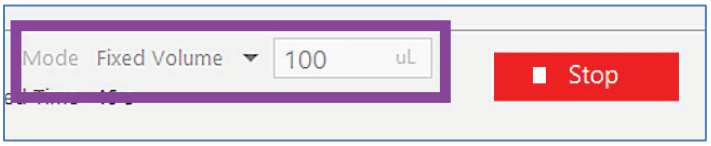

Fig. 5. Acquisition mode selection on the Panama Flow Cytometry Software.

The sample and control data is saved for further offline analysis (e.g., using FlowJo, FCS Express, or equivalent utility), where cross-channel compensation is calculated and applied as customary and gates are set, based on the acquired control runs, to quantify the subpopulations of interest.

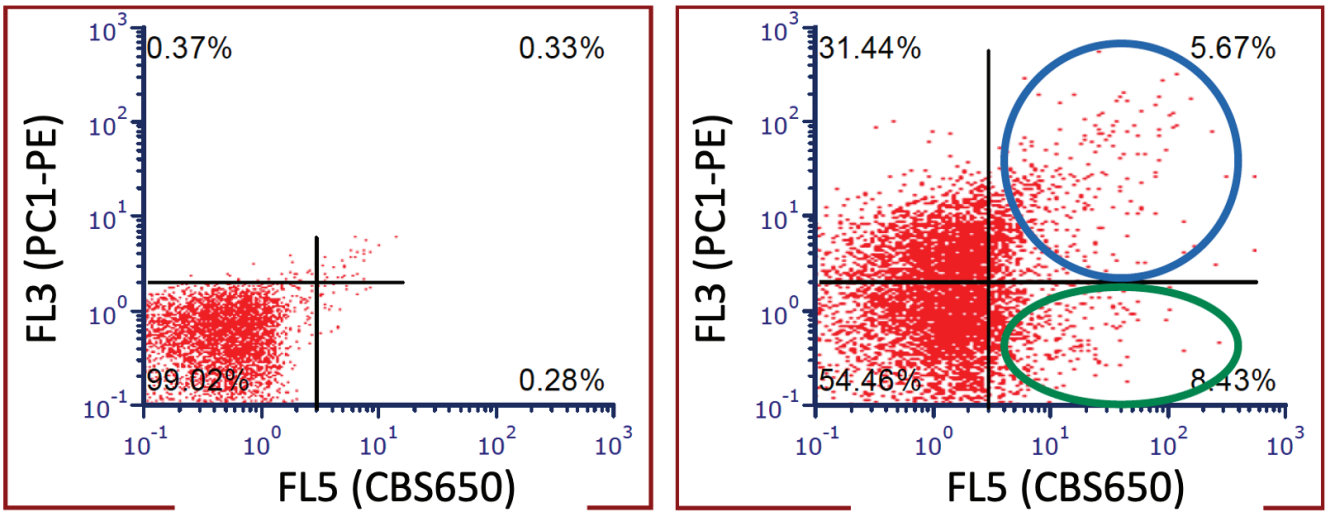

Fig. 6. An example of the use of dot plos during data analysis to identify background and control levels, set gates, and quantify subpopulations of interest. (Left) unstained sample; (right) normal urinary EVs; the blue oval shows PC1-positive urinary EVs.

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Results

The following graphs show data obtained on the ***Delaware*** from normal and urine from a patient with advanced ADPKD. In this case, samples were stained with PC1-PE and CD133-AlexaFluor488 (example 1 in the protocol above). The ADPKD patient results indicate a level of PC1⁺/CD133⁺ EVs drastically suppressed as compared to the normal donor, and indistinguishable from background. This lines up with findings, by Dr. Ward and others, established using independent techniques, such as Western blotting; however, the technique described here is much faster, has greater specificity, and can readily be expanded to include additional markers if desired.

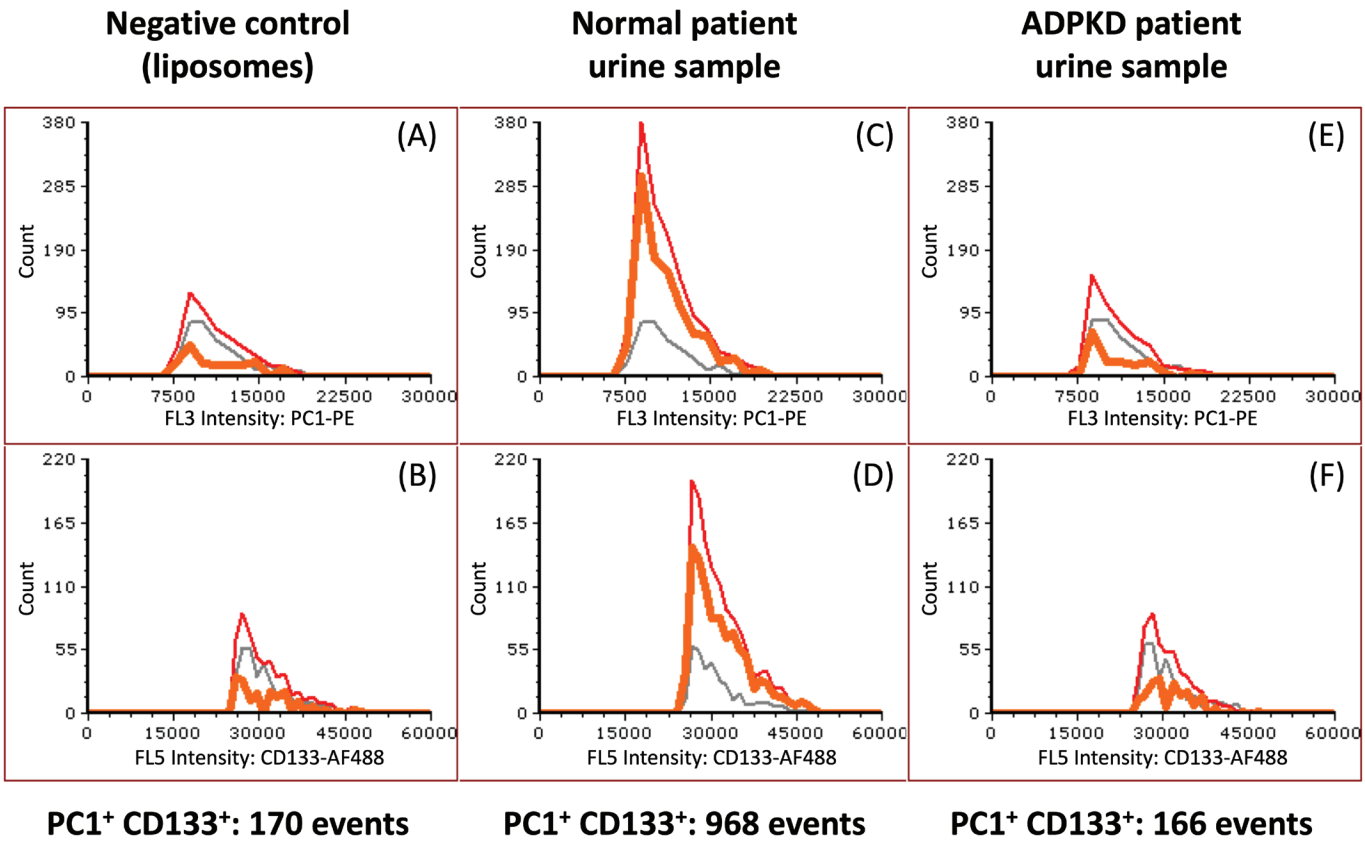

Fig. 7. **Delaware** *histogram data from samples stained with PC1-PE and CD133-AlexaFluor488. Thin red curves are from stained samples, thin grey curves are from stain-only control samples, and thick orange curves are the sample minus stain-only data. The event cound on the bottom for each sample represents the total under the orange curves. ADPKD-sample PC1⁺/CD133⁺ counts (E, F) are 83% lower than normal counts (C, D) and within measurement error from negative controls (liposomes; A, B). Anonymized samples from Dr. Ward / KUMC and KRC; PC1 conjugate provided by Dr. Ward.*

As this Application Note shows, the ***Delaware*** makes it possible to perform noninvasive, multiparametric assays on minimally processed urine samples. This paves the way toward simple, accessible, and accurate diagnostic and prognostic tests for ADPKD and other kidney disorders.

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