MIFlowCyt-List

Requirement	Please Include Requested Information
1.1. Purpose	We intend to provide a step-by-step approach to evaluate the performance of a high sensitivity flow cytometer (Apogee A60-MicroPlus). We want to perform calibration of nanoscale flow cytometer and transform side-scatter intensities and fluorescence intensities in standardized units by standardization of acquisition parameters for both label-free side-scatter/fluorescence detection of particles and EVs from platelet-free plasma and cell-free urine samples. Next, we decided optimal pre-acquisition and data acquisition settings such as sample dilution, illumination wavelength power, triggering threshold, and flow rates. We also intend to apply our optimized protocol for nanoscale flow cytometric analysis of particles and EVs from plasma and urine of healthy individuals and prostate cancer patients.
1.2. Keywords	Clinical extracellular vesicles, nanoscale flow cytometry, standardization, calibration, side-scatter intensity, label-free EV detection, rigor, reproducibility, antibody-labeled prostate specific EV detection.
1.3. Experiment variables	Acquisition parameters (e.g. illumination wavelength power, triggering threshold, flow rate), sample type (e.g. urine or plasma), patient type (e.g. localized prostate cancer and metastatic castration resistant prostate cancer patients and benign prostate hyperplasia patients)
1.4. Organization name and address	Mayo Clinic Guggenheim 4-06 55901 Rochester, Minnesota USA
1.5. Primary contact name and email address	Fabrice Lucien-Matteoni, Lucien-matteoni.fabrice@mayo.edu
1.6. Date or time period of experiment	January 2021 – November 2021
1.7. Conclusions	A60MP flow cytometer can allow rigorous submicron particle quantification from platelet-free plasma and cell-free urine samples with linear particle detection range up to $5.8x10^8$ particles per mL. Optimization of illumination wavelength power resulted in improved sensitivity of smaller particle detection and higher particle counts. Optimization of triggering threshold also improved particle counts from PFP, but changes in triggering threshold had minimal effect on particle counts from urine. Optimization of flow rate allowed to determine optimal flow rate that allows stable particle counting and less variation. Lastly, we applied standardized and calibrated EV-FCM to compare prostate-derived EV concentration in biofluids from men with benign prostatic hyperplasia, localized prostate cancer, and metastatic castration resistant prostate cancer. Here we found a significant elevation of $PSMA^+$-EVs and $STEAP1^+$-EVs in metastatic castration-refractory prostate cancer (mCRPC) compared to localized prostate cancer and

	BPH. Our data suggest that blood levels of $PSMA^{+}$-EVs and $STEAP1^{+}$-EVs may serve as prognostic biomarkers in prostate cancer. our work can serve as a framework for inter-lab comparison studies and further investigate the clinical utility of prostate-derived EVs as liquid biomarkers for the management of prostate cancer.
1.8. Quality control measures	Side-scatter (LALS) intensities in arbitrary unit were converted to standardized unit in nm with the use of Rosetta Calibration (#Cal002, Exometry, Amsterdam, The Netherlands). Fluorescence intensities in arbitrary unit were converted to standardized unit in MESF with the use of AF488 and 647 calibration beads (Bangs Laboratory Inc., Fishers, Indiana).
1.9 Other relevant experiment information	N/A
2.1 Sample description	Plasma and urine from localized prostate cancer patients (n=85), and benign prostate hyperplasia patients (n=35). Plasma from metastatic prostate cancer patients (n=20).
2.1.1 Biological sample source description	Both platelet-free plasma and urine samples were collected from localized prostate cancer patients and benign prostate hyperplasia patients under approved Mayo Clinic IRB#19-006675). Platelet-free plasma samples were collected from metastatic castration resistant prostate cancer patients under approved Mayo Clinic IRB#21-004451).
2.1.2 Biological sample source organism description	See table S1 for demographics and clinicopathological characteristics of patients.
2.2 Sample characteristics	We anticipate that platelet-free plasma samples contain EVs, lipoproteins, and proteins. We expect that urine samples contain EVs, lipoproteins, proteins, and salts.
2.3. Sample treatment description	See 'Blood and urine collection' in the Materials and Methods section of the manuscript.
2.4. Fluorescence reagent(s) description	See 'Cell Lines and Transfection and Antibody labeling and preparation' in the Materials and Methods section of the manuscript. PSMA (3E7, Creative Biolabs) and STEAP1 (SMC1, Mayo Clinic Hybridoma Core) antibodies were labeled with Alexa Fluor 647 (AF647) and 488 (AF488) antibody labelling kits (Thermo Fisher Scientifics, Waltham, MA) respectively. Degree of antibody labeling (DOL) was measured using a Nanodrop One C spectrophotometer (Fisher Scientific). Degree of labeling (DOL) for PSMA and STEAP1 was 3.2 and 3.6 respectively. Final concentration of each antibody is 6 μg/mL.
3.1. Instrument manufacturer	Apogee, Northwood, UK
3.2. Instrument model	A60-Micro Plus
3.3. Instrument configuration and settings	Samples were analyzed at a flow rate of 0.75, 1.5 and 3.01 μl/min on an A60-Micro Plus, equipped with a 405 nm laser (70, 100, 150, 200 mW), 488 nm laser (70 mW), 647 nm laser (70 mW). Samples were measured for 1 minute with 405-nm side scatter using triggering thresholds of

	1800, 2000 and 2300 a.u. GFP fluorescence was collected in the 488-green channel (525/50 nm band pass filter). PMT voltages were set to 320 V for SALS, 305 V for LALS, and 500 V for 488-green and 647-red.
3.4 Instrument light scatter detection limits	We used triggering threshold on side scatter (LALS) only because of the higher sensitivity for small particles. It was set at 2300 arbitrary units which corresponds to a scattering cross section of 19 nm^2 and an EV diameter of 188 nm, given the assumed refractive index distribution of an EV by Mie theory modeling (Line 425). The upper limit is 4.1 x 10^6 arbitrary units corresponds to a scattering cross section of 40,000 nm^2 and an EV diameter of 4600 nm.
4.1. List-mode data files	All raw flow cytometry data files are available via Figshare: https://figshare.com/projects/Kim_Y_et_al_2022_Calibration_and_Standardization_of_Extracellular_Vesicle_Measurements_by_Flow_Cytometry_for_Translational_Prostate_Cancer_Research/138135 Additional requests can be made to the corresponding author Lucien-matteoni.fabrice@mayo.edu
4.2. Compensation description	We do not have any compensation for the experiment because we used Alexa fluor 488 and 647, which does not cause any overlap in excitation and emission.
4.3. Data transformation details	FlowJo (v10.6.2; FlowJo, Ashland, OR) and Rosetta Calibration software (Purchased license, Exometry, Amsterdam, The Netherlands) were used to transform Data acquired from Apogee Micro 60 Plus. We also used Flow-SR determine the size and refractive index (RI) of particles and improve specificity by enabling label-free differentiation between EVs and lipoprotein particle. Flow-SR was performed as previously described.