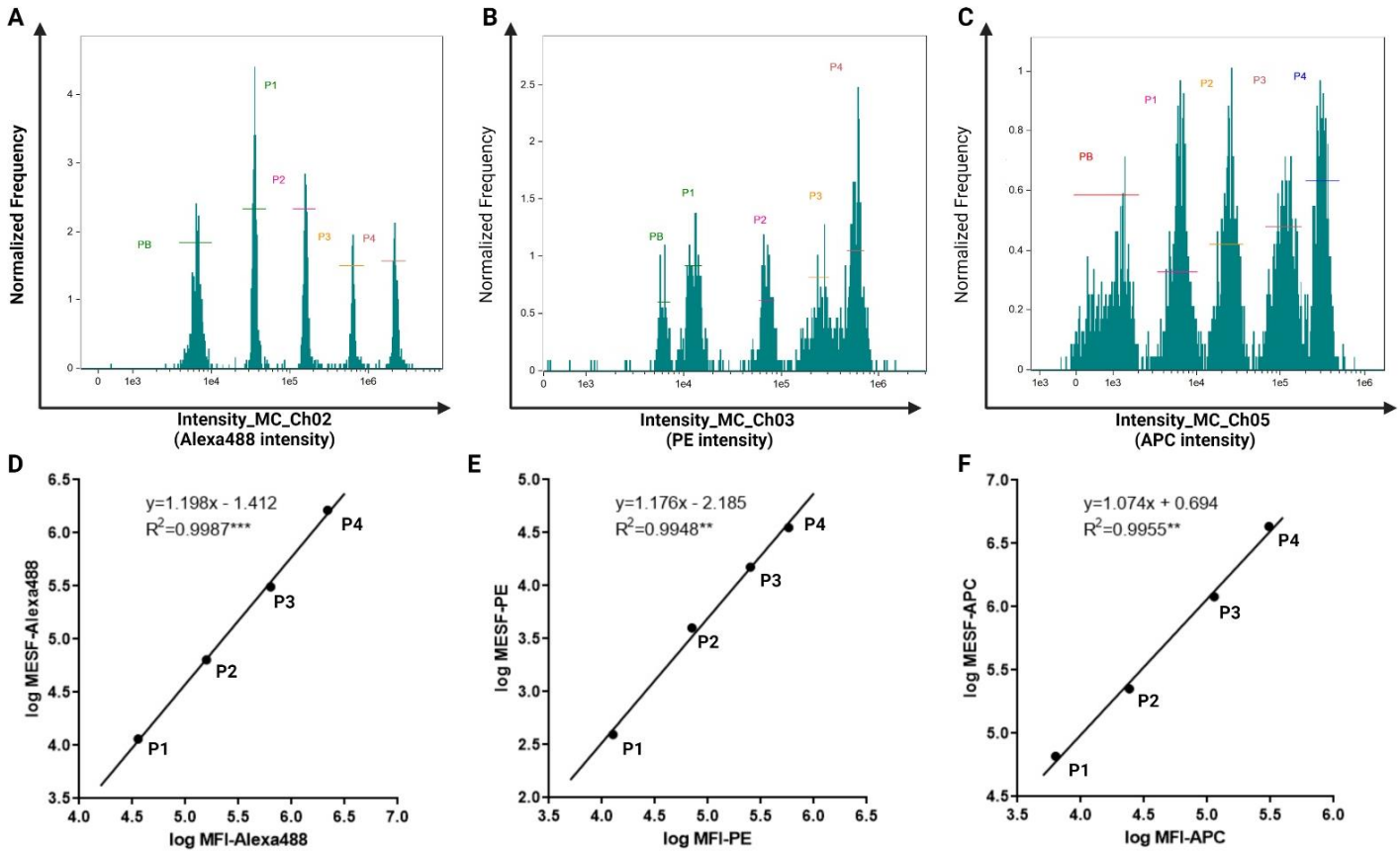
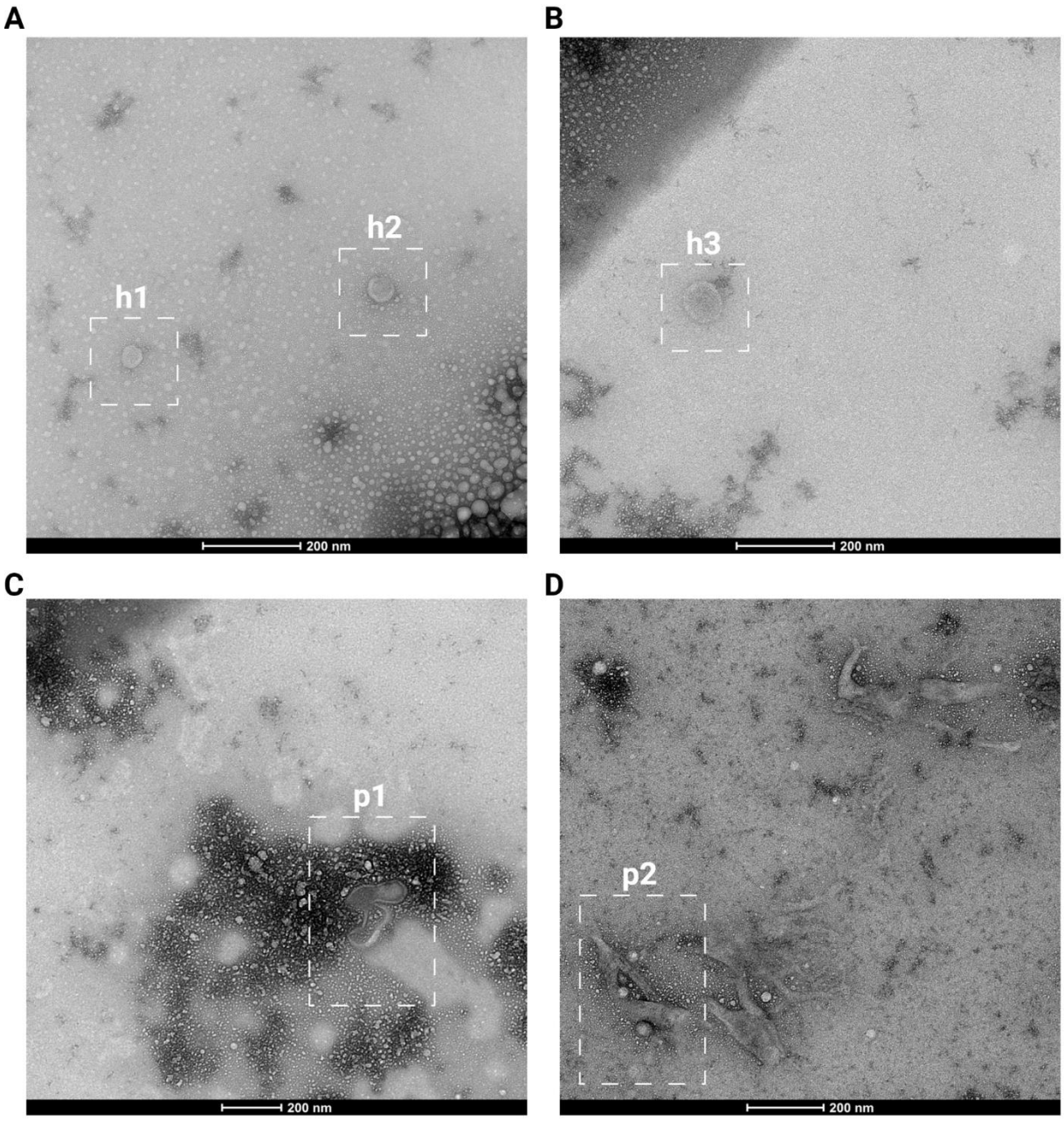
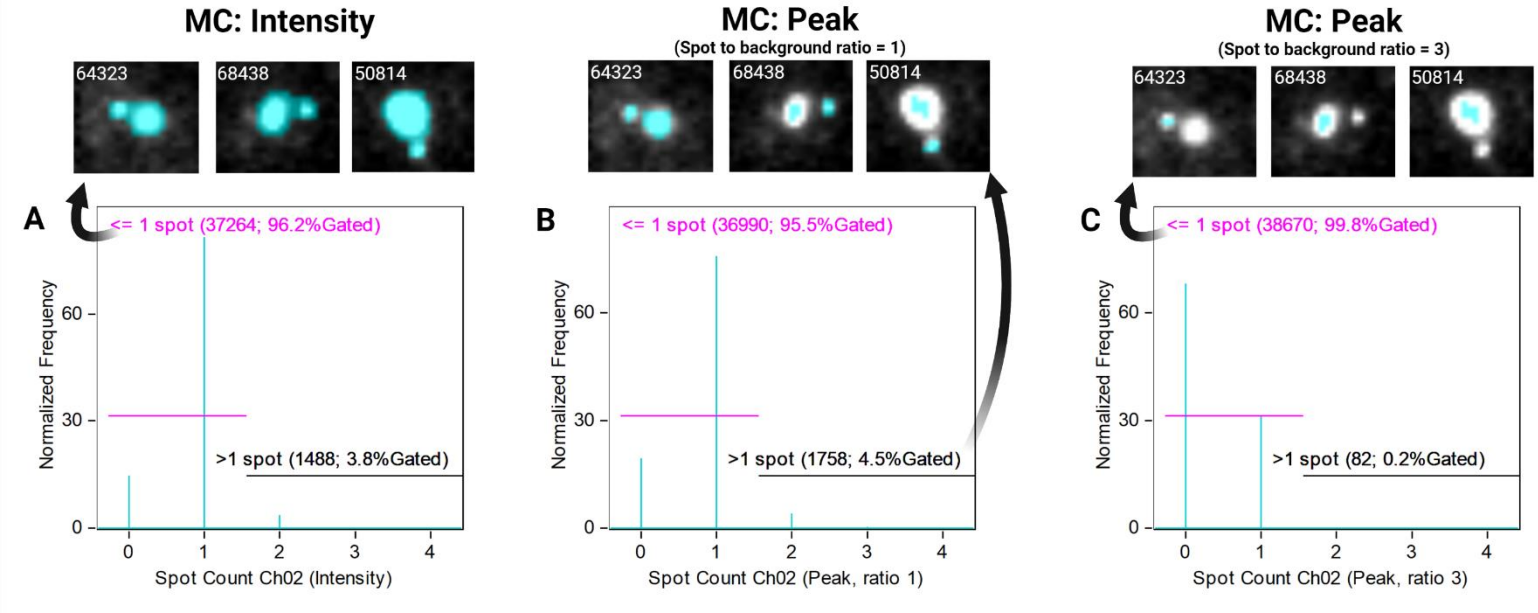


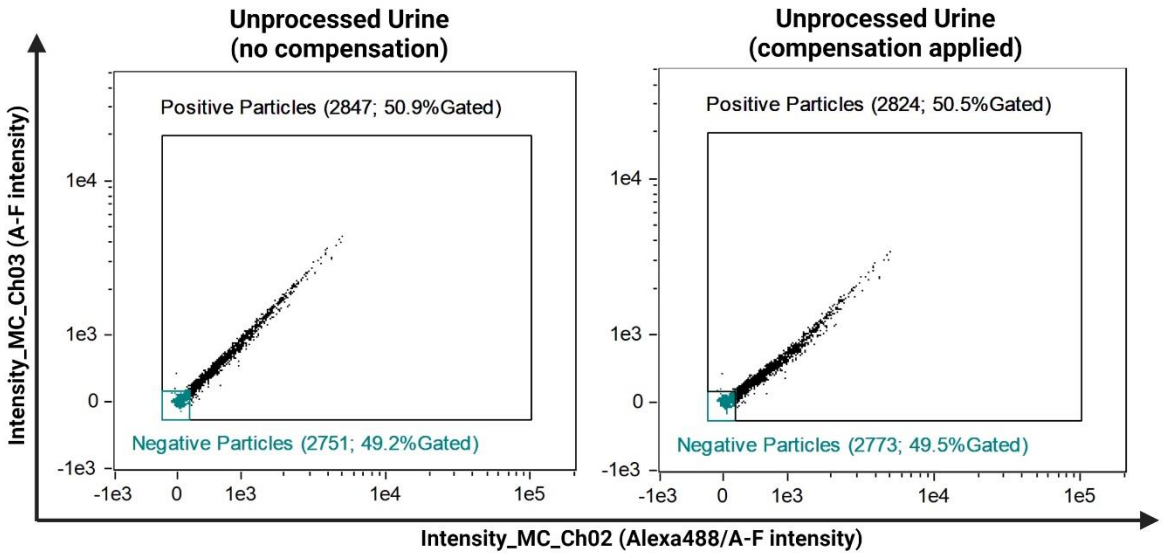
Supplementary Figure S1 Calibration of MESF detection of IFCM based on standardized fluorescent beads. (**A** ~ **C**) IFCM measured 5 populations of beads per kit with standardized MESF-Alexa488 (**A**), MESF-PE (**B**), or MESF-APC (**C**). (**D** ~ **F**) The regression analysis between log MESF and log MFI for Alexa488 (**D**), PE (**E**), or APC (**F**). Regression formulas were calculated based on non-blank populations (P1-4), and the R^2 denoted the regression coefficient. Marks: ***p < 0.001; **p < 0.01. **Abbreviations:** MESF, molecules of equivalent soluble fluorochrome; MFI, median fluorescent intensity; IFCM, imaging flow cytometry; PB: blank population.



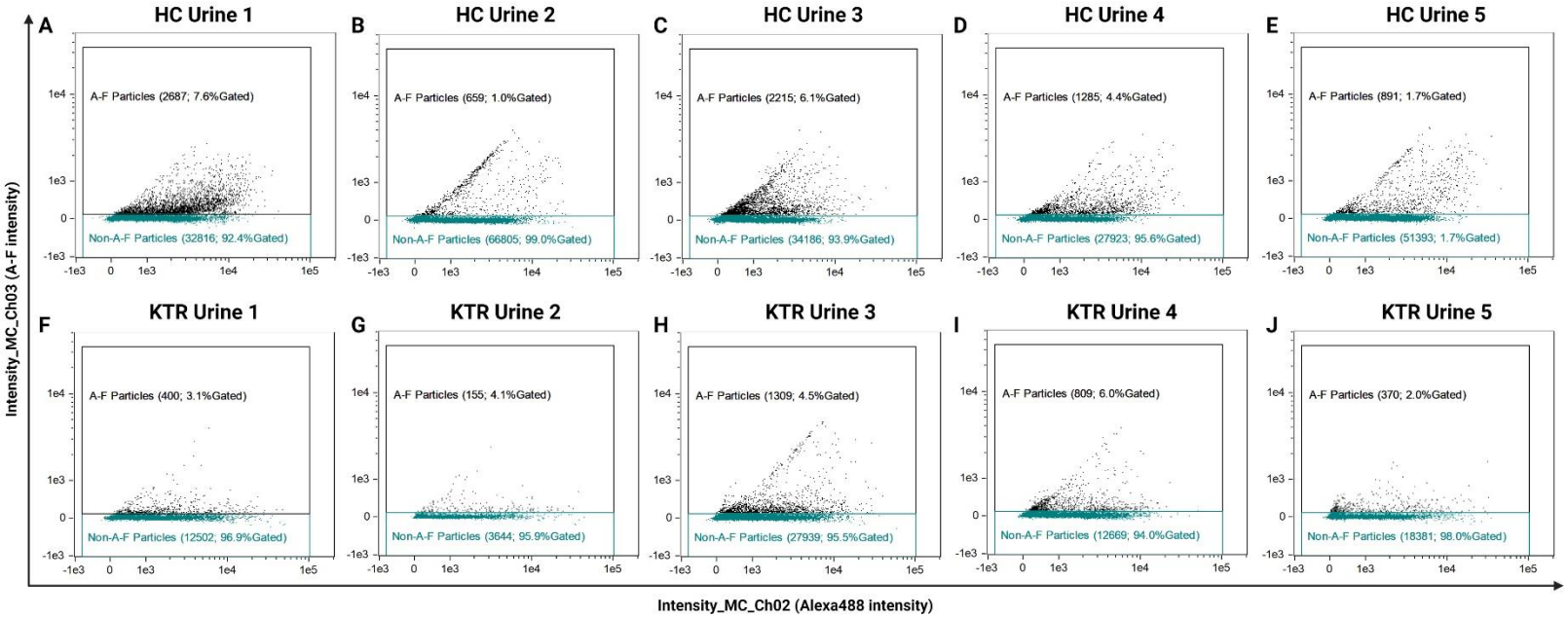
Supplementary Figure S2 The large-area pictures obtained from transmission electron microscopy showing urinary extracellular vesicles from healthy individuals (**A** & **B**) and kidney transplant recipients (**C** & **D**).



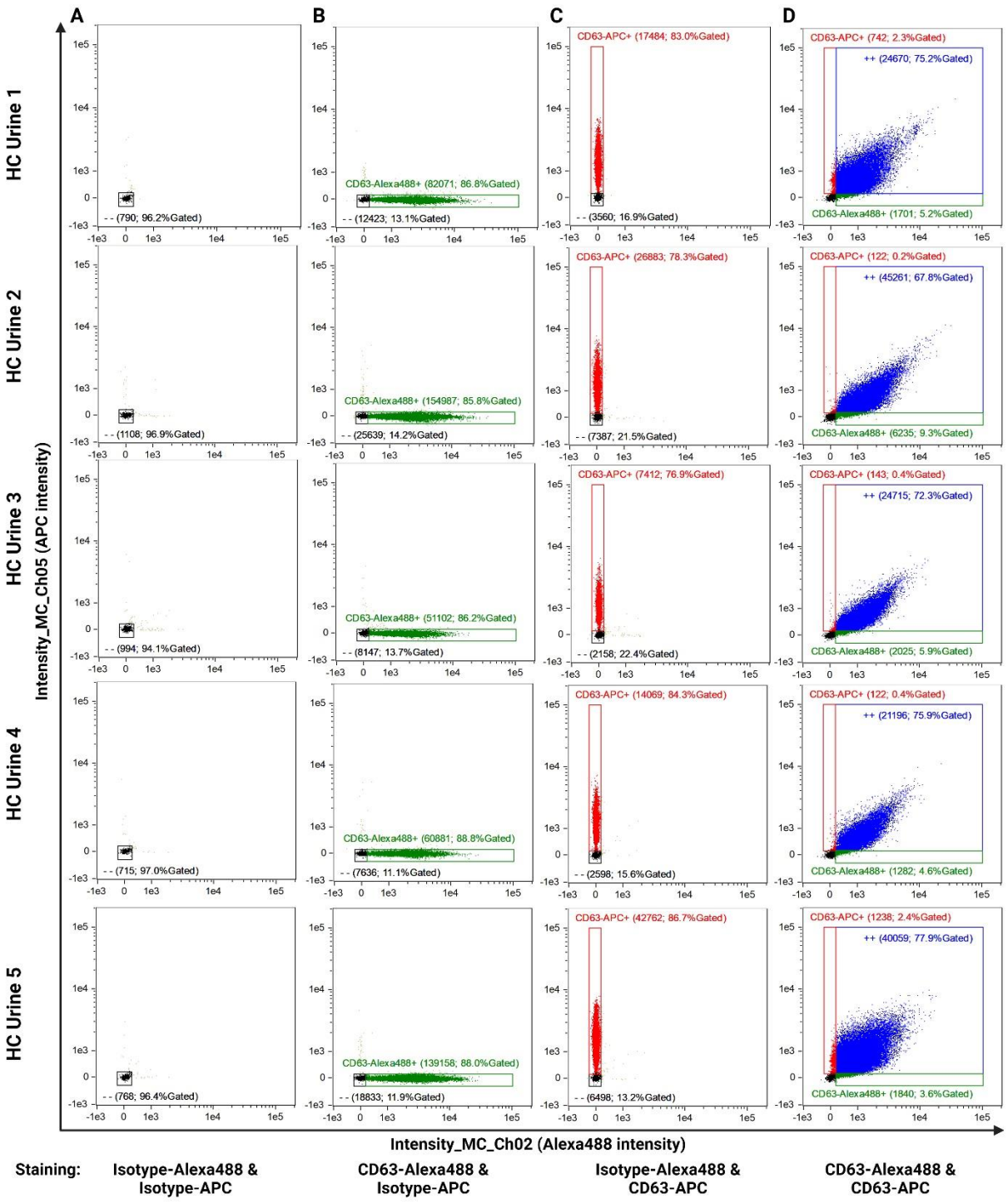
Supplementary Figure S3 Distinguish singlets from multiplets by calculating spot count based on different MCs: Intensity MC (**A**) or Peak MC (**B** and **C**). (**C**) used a higher spot-to-background ratio than (**B**). At the top of (**A** ~ **C**), typical images of Ch02-positive doublets and corresponding MC (blue area) were shown. Object numbers were presented at the top left corner of each image. Black arrows denoted in which gate these events were included. Each gate's name gave the counts and percentage of gated events (Counts; %Gated). **Abbreviations:** MC, masks combined.



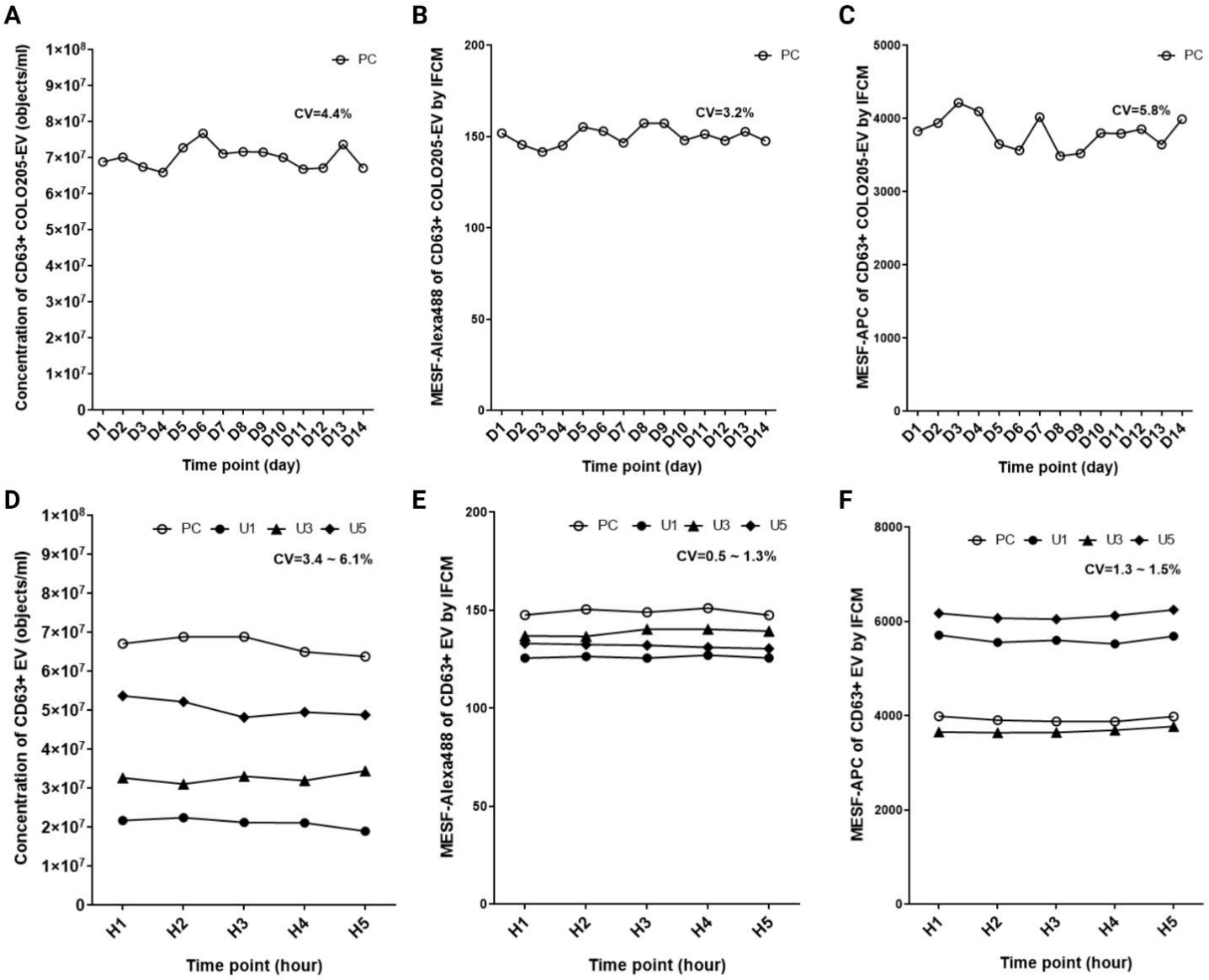
Supplementary Figure S4 The fluorescent properties of autofluorescence particles in the unprocessed and unlabeled urine with or without compensation applied. The compensation matrix is the same as in Figures 3G & 3H in the results.



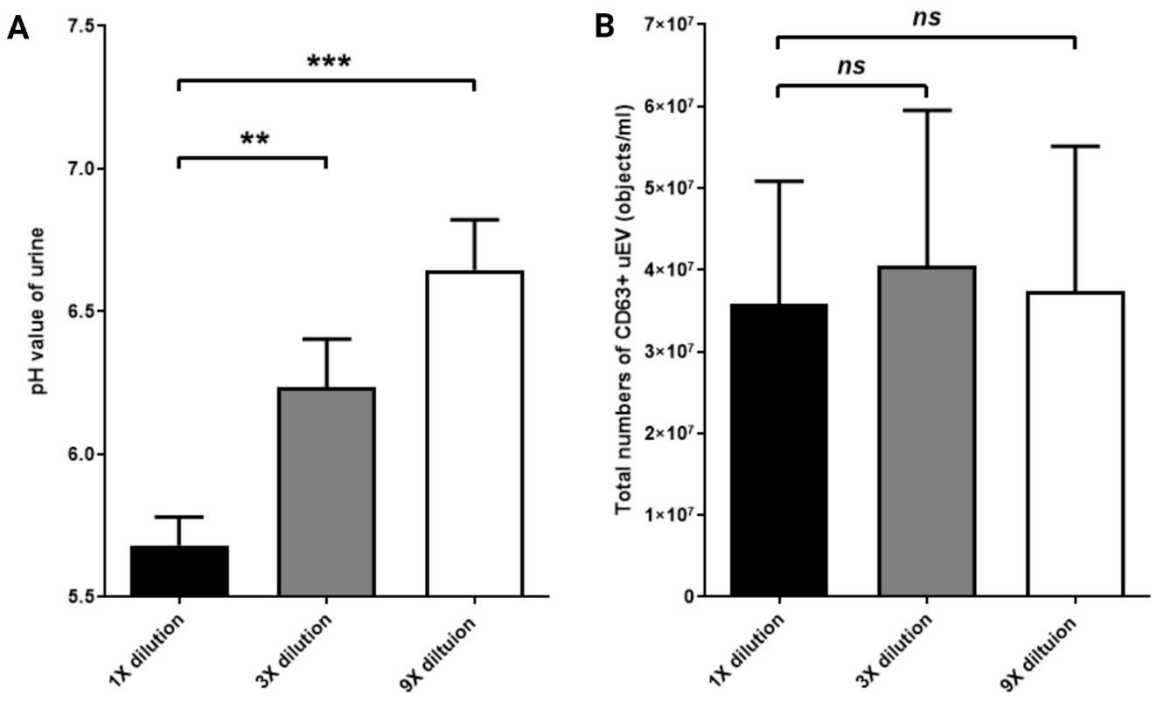
Supplementary Figure S5 The "Non-A-F Particles" gate and compensation matrix obtained from Figure 3G can be applied to distinguish uEVs from A-F particles in other healthy control (HC) or kidney transplant recipient (KTR) urine samples. All urine samples were stained by CD63-Alexa488 and CD63-APC without any further treatments.



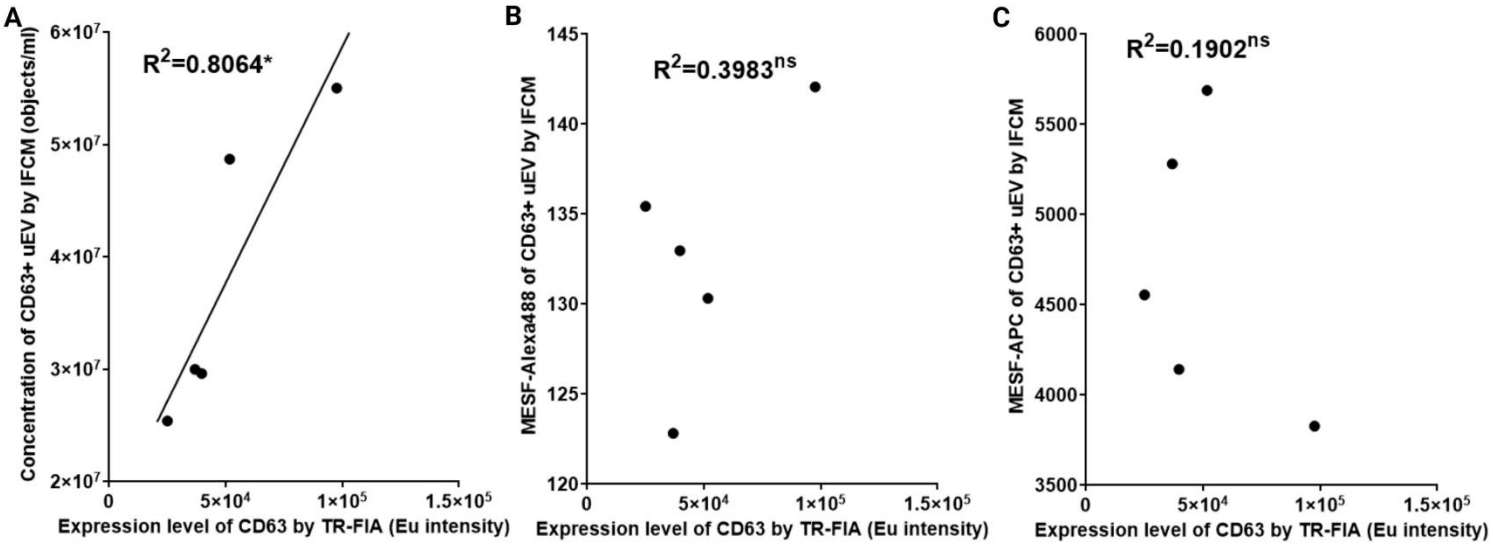
Supplementary Figure S6 Isotype and single staining set up the fluorescence thresholds. Data from all healthy control (HC) urine samples were presented here. Each gate's name showed the counts and percentage of gated events (Counts; %Gated).
Abbreviations: ++, double-positive.



Supplementary Figure S7 Inter- and intra-assay reproducibility to detect CD63+ uEV by IFCM. (**A - C**) Individually repeated IFCM experiments measured the concentration, MESF-Alexa488, and MESF-APC of CD63+ EV from the positive control (COLO205 cell line supernatant) on 14 separate days. The CV represented the reproducibility of IFCM. (**D - F**) One IFCM experiment repeatedly measured the concentration, MESF-Alexa488, and MESF-APC of CD63+ EV from the positive control (n=1) and healthy urine sample (n =3) at each hour. The range of CV was shown (n =4). **Abbreviations:** CV, coefficient of variation; MESF, molecular equivalent soluble fluorochrome; MFI, median fluorescence intensity; PC, positive control.



Supplementary Figure S8 The influence of dilution on urinary pH and its effect on uEV detection by IFCM. (**A**) The effects of dilution on the total numbers of CD63+ uEV by IFCM. Marks: ***$p < 0.001$; **$p < 0.01$. (**B**) The total numbers of uEV were calculated by correcting the concentrations with dilution times. Marks: ns, no significance. **Abbreviations:** A-F, auto-fluorescence; IFCM, imaging flow cytometry.



Supplementary Figure S9 Comparison between IFCM and TR-FIA. (**A**) Correlation (linear regression) analysis between CD63+ uEV concentrations by IFCM and CD63 expression levels in healthy urine samples (n = 5). (**B** and **C**) Correlation (linear regression) analysis between the MESF of CD63+ uEV by IFCM and CD63 expression levels in healthy urine samples (n = 5). R^2 denoted the coefficient of regression. Marks: *p < 0.05; ns, no significance. **Abbreviations:** Eu, europium; IFCM, imaging flow cytometry; TR-FIA, time-resolved fluoroimmunoassay.

Supplementary Table S1 Clinical parameters of enrolled subjects

Clinical parameters	Healthy controls (n = 5)	Kidney transplant recipient with acute kidney injury in 2 weeks post-transplantation (n = 5)
Gender (male/female)	4/1	4/1
Pathology presented in biopsy	-	Rejection (n = 2); Acute tubular necrosis (n = 3)
Age (year)	27.80 ± 1.30	63.40 ± 7.73
Urine pH	5.68 ± 0.98	6.20 ± 0.45
Urinary total protein concentration (g/L)	0.07 ± 0.04	0.65 ± 0.30
Urinary creatinine concentration (mmol/L)	16.46 ± 7.76	8.80 ± 4.77

Supplementary Table S2 All used features with MCs in the IFCM analysis

Built-in or self-made	Feature	Mask
Built-in	Intensity_MC_Ch02	MC
Built-in	Intensity_MC_Ch03	MC
Built-in	Intensity_MC_Ch05	MC
Built-in	Intensity_MC_Ch06	MC
Self-made	Spot Count Ch02	Peak (M02, Ch02, Bright, 1)
Self-made	Spot Count Ch05	Peak (M05, Ch05, Bright, 1)
Self-made	Spot Distance Min (Ch02 & Ch05)	Peak (M02, Ch02, Bright, 1) Or Peak (M05, Ch05, Bright, 1)

Supplementary Table S3 Characteristics of MESF-standardized beads

Population	Beads-Alexa488				Beads-PE				Beads-APC			
	Median MESF	**Log MESF**	**Median MFI**	**Log MFI**	**Median MESF**	**Log MESF**	**Median MFI**	**Log MFI**	**Median MESF**	**Log MESF**	**Median MFI**	**Log MFI**
Blank population (PB)	NA	NA	6558.17	3.817	NA	NA	6227.22	3.794	NA	NA	1022.26	3.010
Population 1 (P1)	11489	4.060	35776.51	4.554	394	2.595	11489	4.10493	65829	4.818	11489	3.800
Population 2 (P2)	63949	4.806	157752.88	5.198	3997	3.602	63949	4.84879	224599	5.351	63949	4.385
Population 3 (P3)	311396	5.493	633621.63	5.802	14988	4.176	311396	5.400839	1200523	6.079	311396	5.055
Population 4 (P4)	1636893	6.214	2183260.06	6.339	35476	4.550	1636893	5.763067	4324094	6.636	1636893	5.488

Manufacturers provided the MESF values, and the MFI values were obtained in the detection by IFCM**.** The MFI values of non-blank populations (P1-4) were used to calculate the MESF. **Abbreviations:** MESF, molecules of equivalent soluble fluorochrome; MFI: median fluorescence intensity; NA: not available.