Supporting Information

Bead-based extracellular vesicle analysis using flow cytometry

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Supporting experimental section

Cell Culture: AsPC-1, BxPC-3, Capan-2, and MIA PaCa-2 cells were obtained from the American Type Culture Collection (ATCC) and used without any modification. AsPC-1 and BxPC-3 cells were cultured in RPMI 1640 media (Thermo Scientific, 11875119), Capan-2 cells in McCoy's 5a media (Thermo Scientific, 16600108), and MIA PaCa-2 cells in Dulbecco's Modified Eagle's medium (Mediatech, 10-013-CV). All media were supplemented with penicillin (10,000 IU)/streptomycin (10,000 µg/ml, Mediatech 30-002-CI) and 10% fetal bovine serum (Atlanta Biologics, S12450). 1617 cells are a patient-derived xenograft cell line (kind gift from Drs. Carlos Fernandez del Castillo and Andrew Liss, Massachusetts General Hospital) and were cultured in a 50:50 mix of DMEM/F-12 media (Thermo Scientific, 10565042), supplemented as above.

EV Isolation: We isolated EVs using size-exclusion columns for human plasma samples as described by Lobb et al.[Lobb et al., 2015, J Extracell Vesicles, 4, 27031] and ultracentrifugation for in vitro cell culture[Théry et al., 2006, Curr Protoc Cell Biol, Chapter 3, Unit 3.22]. *EVs from human plasma.* Plasma samples were collected from patients who provided informed written consent under a sample collection protocol approved by the Dana-Farber/Harvard Cancer Center IRB. PDAC patients had locally advanced disease, with samples collected at baseline prior to initiation of systemic treatment. All experiments were performed in accordance with relevant guidelines and regulations. Whole blood was collected in one 10 mL purple-top EDTA tube, mixed by inverting 10 times, and then centrifuged for 10 min at 400 × g (4°C). The plasma layer was collected in a 15 mL conical tube without disturbing the buffy coat and was then centrifuged for 10 min at 1100 × g (4°C). The plasma layer was pipetted into a 15 mL tube and stored at -80°C until processing for EVs. qEV size-exclusion columns (qEVoriginal/70nm, iZON Science, SP1) were used for EV isolation and were first washed with 10 mL PBS (0.22µm-filtered). During column washing, 500 µl plasma was cleared by centrifugation at 1500 × g for 10 min (4°C). The supernatant was then spun again at 10,000 × g for 20 min (4°C). 500 µl cleared supernatant was loaded onto the qEV column and 0.5 mL fractions were immediately collected. As soon as the sample completely entered the resin, PBS was added. The first six fractions were discarded (3 mL, column void volume). Fractions 7-9 (1.5 mL total) were pooled and filtered through an Ultrafree 0.22 µm centrifugal filter at 12,000 × g for 1 min (Millipore, UFC40GV0S). The pooled fractions were then concentrated using an Amicon Ultra-4 10kDa filter by centrifugation at 3200 × g for 15 min (Millipore, UFC801024). Concentrated EVs were stored at -80°C. *EVs from cell culture.* Cells were trypsinized and split into 8 x 15-cm dishes in 20 mL appropriate media containing 5% exosome-depleted FBS (Thermo Scientific, A272801) and grown for 48-72 hours. Media containing released EVs (160 mL total) was collected and centrifuged at 300 × g for 10 min, followed by filtration through a 0.22 µm cellulose-acetate vacuum filter (Fisher Scientific, 09-761-1). Conditioned media was aliquoted to ultracentrifuge tubes (Beckman Coulter, 344058) and then underwent ultracentrifugation at 100,000 × g for 70 min (4°C). Supernatant was removed, EV pellets resuspended in a single tube in PBS and centrifuged a second time at 100,000 × g for 70 min. Supernatant was again removed and the EV pellet resuspended in ~100 µl PBS. EVs were stored at -80°C until use. The size of EVs were measured by dynamic light scattering (DLS) using a Malvern Zetasizer APS (**Figure S10**). EV solutions were diluted by 10-100 fold (depending on the sample) in 100µl total volume of

PBS. Measurement settings were as follows: material refractive index: 0.01, dispersant name: water, dispersant refractive index: 1.33, viscosity (cP): 0.8872, temperature: 25°C, duration used (s): 60. A total of 3 measurements per sample was recorded.

Single EV Flow Cytometry Staining and Analysis: 2 µg 1617 EVs were incubated with isotype control-AF488 or EGFR-FITC antibodies (1 µl, 1:50 dilution in a total volume of 50µl PBS/0.5% BSA) overnight with rotation/mixing at 4°C. Excess antibody was removed by diluting samples to 500µl volume with PBS/0.5% BSA and purifying EVs using the qEV size exclusion columns, as described above for EV purification from patient plasma. Fractions 7-9 from iZON columns were concentrated using Amicon Ultra-4 10kDa centrifugal filter units. Samples were diluted to 200 µl final volume in PBS/0.5% BSA and run on the CytoFlex flow cytometer (Beckman Coulter, C09752, B2-R0-V2 configuration) using Violet SSC to resolve smaller particles and the following settings: FSC 201V, SSC 30V, violet SSC (VSSC) 22V (threshold using VSSC, 2000 on area), FITC 20V, and width set on FSC. The 525/40 nm bandpass filter (Beckman, A01-1-0051) was used for FITC measurement. Mix and backflush options were not used and samples were run at a 10µl/min flow rate. 100nm dragon green fluorescent microspheres were used to identify smaller particles on the flow cytometer (Bangs Labs, FC02F). Controls used in the flow cytometer measurements include: PBS/0.5% BSA buffer only (no EVs) and rat IgG2ak-AF488 isotype control antibody. Given the sample loss during processing and technical nature of single EV flow cytometry, as many events as possible were collected (typically between 1000 to 5000 events). Post-measurement analysis was done using FlowJo (v10), with gating first done using the 100 nm dragon green beads to identify small particles. Subsequent gating on FITC negative vs positive signal was done using the isotype control treated sample and the percentage of EGFR-positive EV was identified.

Scanning electron microscopy: After dehydration in a series of increasing ethanol concentrations, samples were transferred for critical drying and subsequently coated with 2 nm platinum/palladium using a sputter coater (EMS 150T S Metal Sputter Coater), before imaging with a field-emission scanning electron microscope (Ultra Plus, Zeiss).

EV adsorption onto latex beads and flow cytometry: EV adsorption onto latex beads for flow cytometry followed the protocol of Théry et al[Théry et al., 2006, Curr Protoc Cell Biol, Chapter 3, Unit 3.22]. Briefly, 0.5 µg 1617 EV per 10 µl 3.9µm (Thermo Scientific, A37304, 4% w/v, 1.3 x 10^9 particles/mL, 1.3 x 10^7 particles in 10 µl) or 5µm (Thermo Scientific, A37306, 4% w/v, 4.9 x 10^8 particles/mL, 4.9 x 10^6 particles in 10 µl) aldehyde/sulfate latex beads were mixed. This step was done in bulk, with 6µg 1617 EV diluted in 120µl latex beads. Samples were diluted to a final volume of 1mL in PBS and adsorption proceeded overnight at 4°C on a rotator wheel. To stop the reaction, 110µl of 1M glycine (in PBS) was added and incubated for 30 min at room temperature on a nutating mixer. Samples were centrifuged at 4000 rpm for 3 min and supernatant was removed/discarded. Latex beads were then washed twice with 1mL PBS/1% BSA. After the final wash, samples were resuspended in 1.2 mL PBS/1% BSA and 100µl per well were aliquoted to a u-bottom 96-well plate. Staining for flow cytometry was done using identical conditions and flow cytometer setting as described above for streptavidin-polystyrene beads.

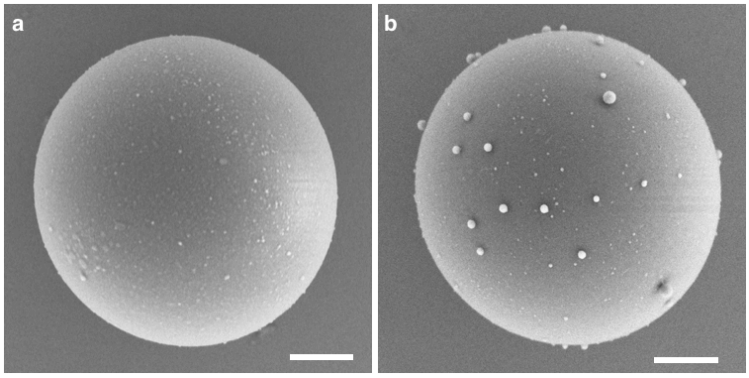

Figure S1. Scanning electron micrographs (SEMs) showing (**a**) PS bead used for EV capture and (**b**) EVs captured on the PS beads.

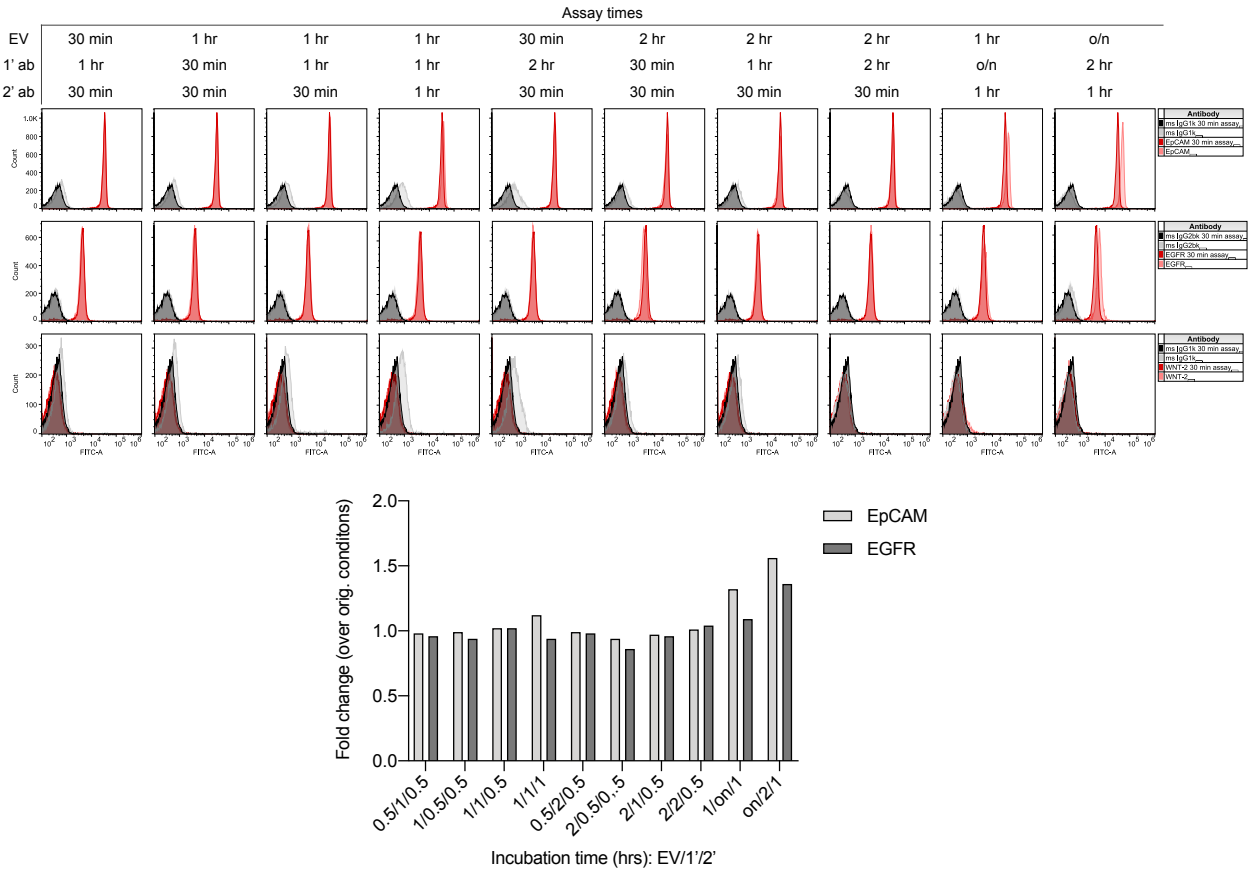

Figure S2. Effect of varying assay time on BEAD-flow signal. Biotinylated 1617 EV were incubated with streptavidin polysytrene beads for 30 min to overnight (o/n). Primary antibody incubation time was also varied from 30 min to o/n and secondary antibody staining varied from 30 min to 1 hr. 1617 EV were evaluated for EpCAM (high expression), EGFR (med expression), or WNT-2 (no expression) median fluorescent intensity signal (pink hisotgrams) over isotype control background (gray histograms). The bar graph shows the fold change in median fluorescent intensity of new assay times over the median fluorescent intensity of the original assay conditions (30 min EV incubation with beads, 30 min primary antibody, 30 min secondary antibody). Note that longer incubation times do not lead to a significant increase in signal.

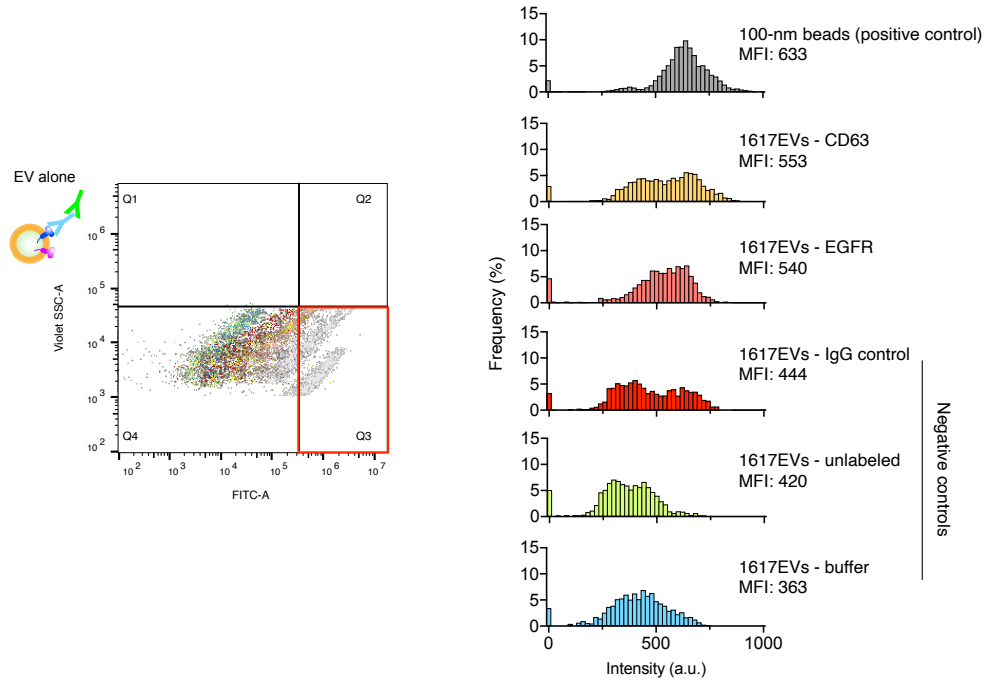

Figure S3. Single EV flow cytometry. Dot plot of EVs from a patient-derived xenograft cell line (1617 PDAC) stained with a FITC-EGFR antibody and measured using violet SSC on a CytoFlex flow cytometer. Q3 represents EVs positive for EGFR staining (gated on isotype stained EVs). Dot plot color coding matches the histograms shown on the right. EGFR positive EVs were identified using a combination of buffer only (blue), unlabeled EVs (green), isotype control antibody staining (red), and 100nm beads (gray). EVs were also positive for CD63, indicating the presence of CD63+ particles in our sample. MFI: Median fluorescence intensity.

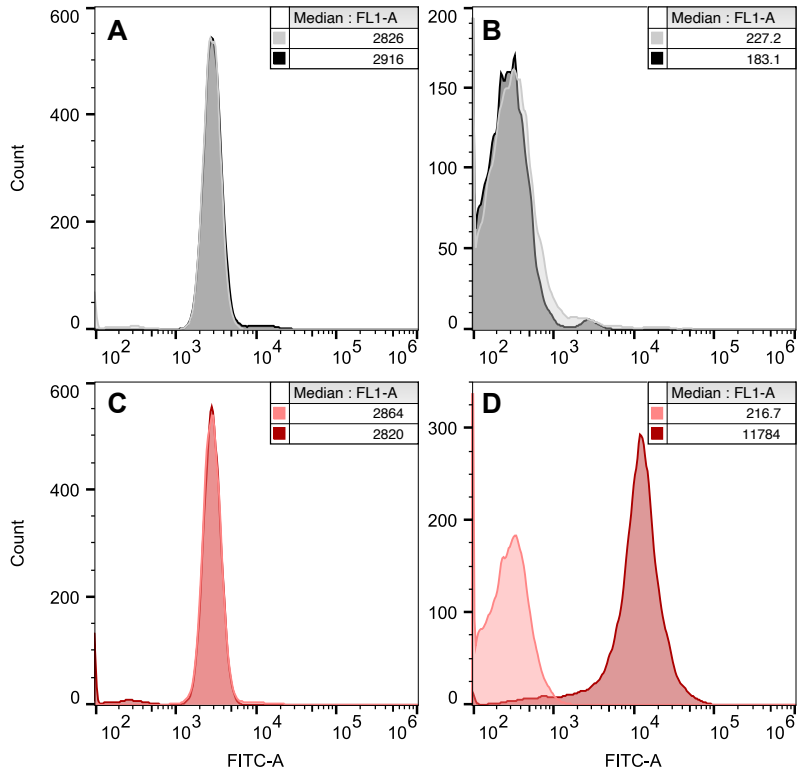

Figure S4. Effect of bead type and EV biotinylation on assay. Unmodified polystyrene (A, C) or streptavidin-labeled polystyrene beads (B, D) were incubated with unlabeled 1617 EV (A, B) or biotinylated 1617 EV (C, D). Beads were stained with mouse IgG1k isotype control (light gray or light pink) or EpCAM antibody (black or red). Note only the combination of biotinylated EV and streptavidin polystyrene beads results in an increase in fluorescent signal over background. Median fluorscence intensity is shown in the legend of each histogram.

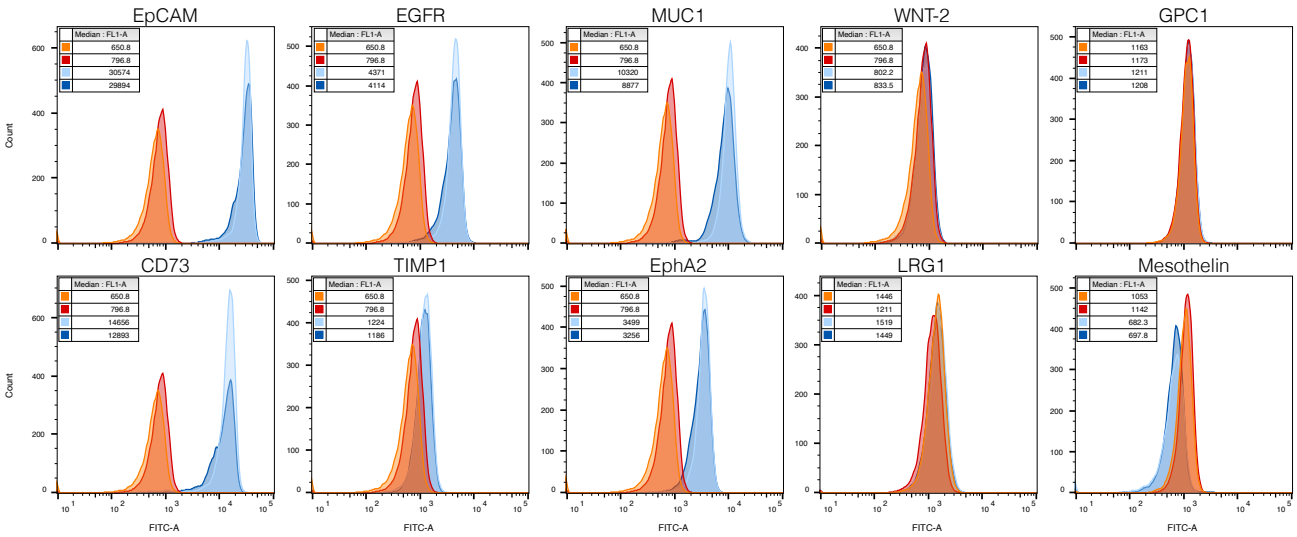

Figure S5. Bead-based flow reproducibility. 1617 PDAC EVs were isolated and biotinylated on different days. EVs were captured on streptavidin polystyrene beads and were stained with an isotype control (orange) or primary (blue) antibody for bead-based flow analysis on different days to assess assay reproducibility. Median fluorescence intensity is shown in the legend of each histogram.

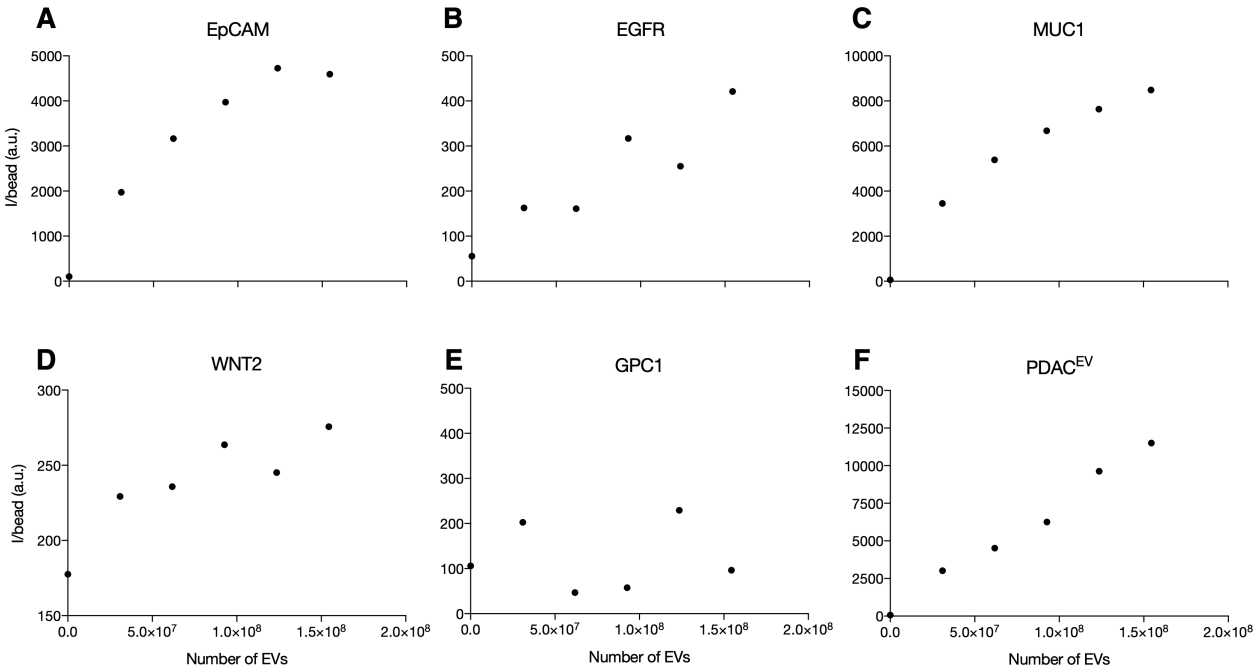

Figure S6. EV titration curves for limit of detection analysis of each antibody used in bead-based flow. Increasing numbers of biotinylated 1617 EVs were captured on streptavidin polystyrene beads and stained with antibodies against (A) EpCAM, (B) EGFR, (C) MUC1, (D) WNT2, (E) GPC1, and (F) PDAC^{EV} cocktail (mixture of all 5 antibodies). Signal intensity per bead was calculated, with isotype control signal subtracted, and plotted versus the number of EVs.

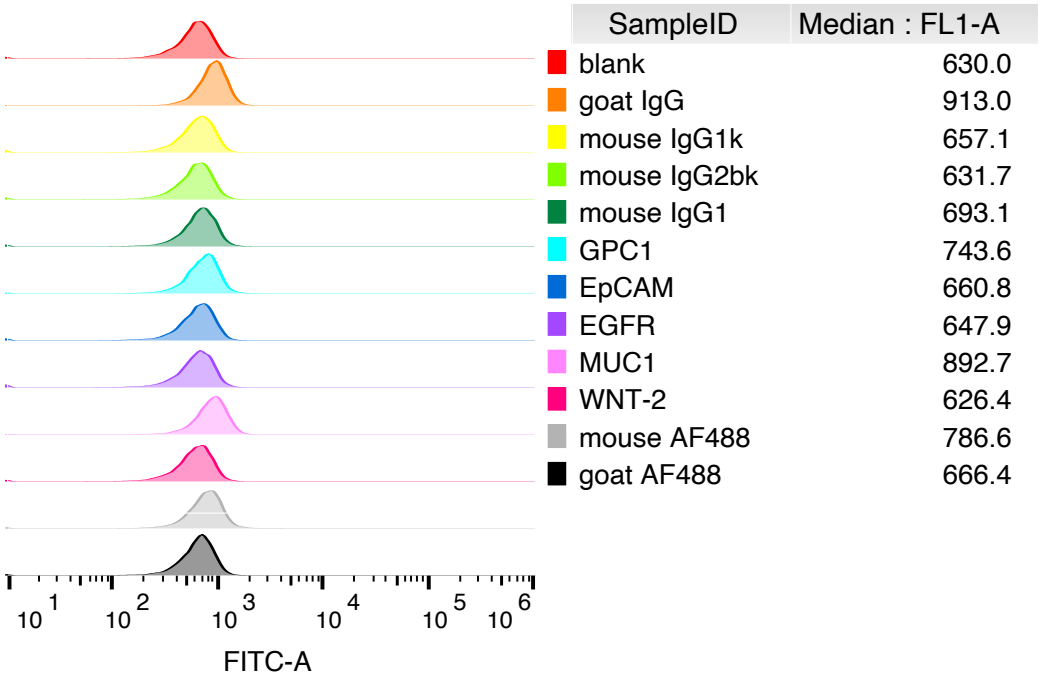

Figure S7. Background antibody binding to streptavidin-coated polystyrene (PS) beads in bead-based flow. 5 µm streptavidin-coated PS beads were stained with the indicated primary antibodies, followed by the indicated secondary antibodies to confirm that the antibodies used in this study do not bind to beads in the absence of EVs. Median fluorescence is shown in the legend for each antibody.

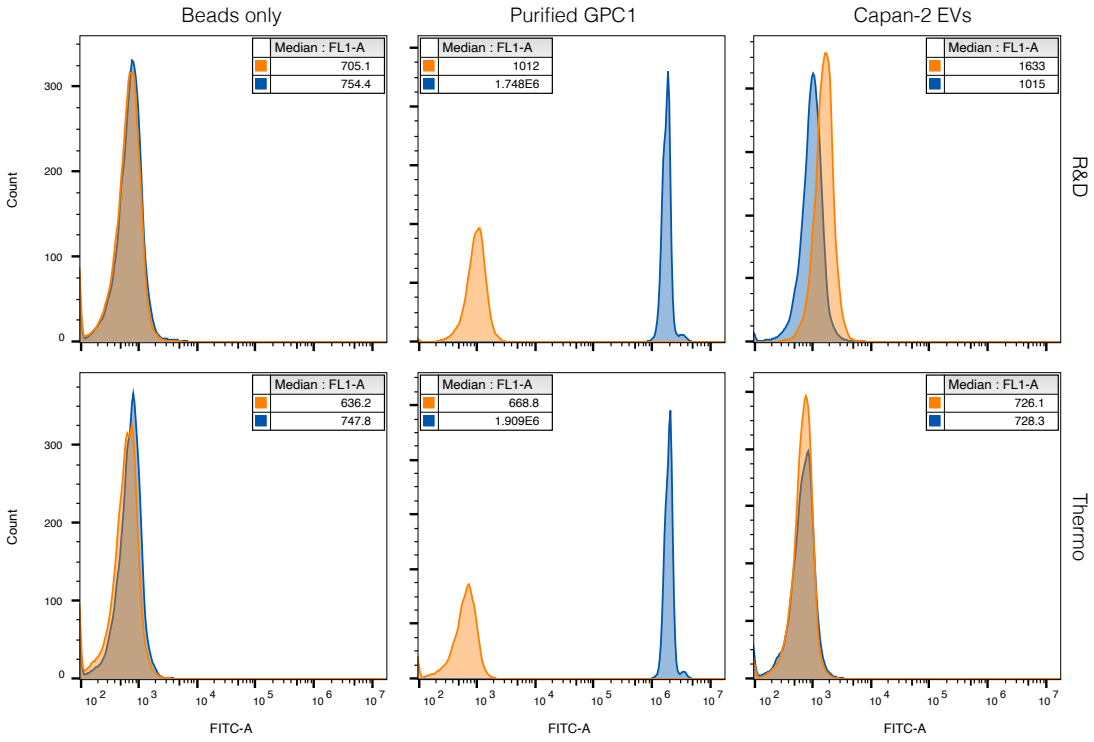

Figure S8. GPC1 antibody testing in bead-based flow. Streptavidin polystyrene beads alone (left), with biotinylated purified GPC1 protein (middle), or with biotinylated Capan-2 EVs (right) captured were stained with two commercially available GPC1 antibodies (blue) and compared to matching isotype control antibodies (orange). Median fluorescence intensity is shown in the legend for each hisotgram.

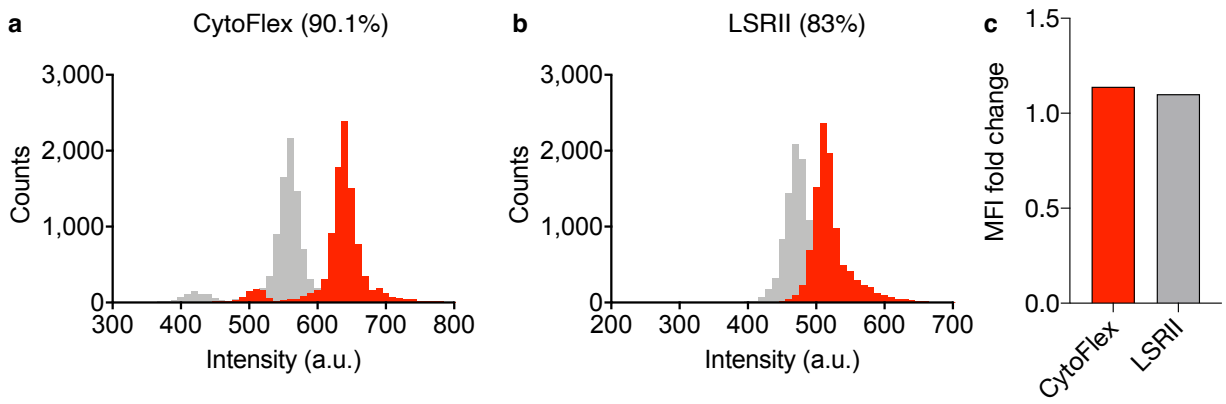

Figure S9. Variability tests of the assay using two flow cytometers in different laboratories. Aliquoted EV samples from patient 2 (P2) were processed and stained for MUC1 in two different laboratories and measured by two different flow cytometers **(a)** CytoFlex and **(b)** LSRII. (**c**) Both measurements show comparable median fluorescence intensity (MFI) fold change over isotype control. The variation could be further reduced by adjusting and optimizing gain settings in a new instrument (LSRII).

	AsPC-1	BxPC-3	Capan-2	Mia PaCa-2	1617	C1	C2	C3	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Size (d.nm)	40.5	90.6	94.7	97.7	93.6	93.6	99.1	150	155	117	88.2	112	88.9	63.0	139	81.9	84.5	95.8
SD (d.nm)	14.7	31.3	31.8	28.3	32.1	35.7	33.2	51.1	56.0	46.6	27.5	37.5	30.5	21.9	46.3	29.3	40.7	17.6

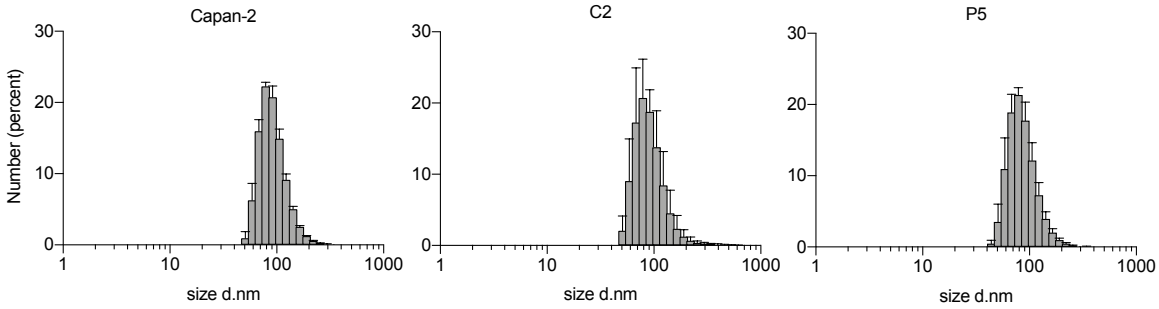

Figure S10. EV size measurements of cell line EVs and patient sample EVs by dynamic light scattering using a Malvern Zetasizer APS. Each sample represents the mean and standard deviation of at least two measurements.

Table S1. Minimum EV amounts needed in bead-based flow for antibody detection. EVs from 1617 PDAC cells were biotinylated and captured on streptavidin polystyrene beads, followed by staining with the indicated antibodies, or isotype control antibodies, for bead-based flow analysis. *ng: the total protein amount of biotinylated EVs required as measured using the Qubit protein assay.

$PDAC^{EV}$ Antibody	Limit of detection (ng)*	Limit of detection (# EVs)
Cocktail	41.34	1.28E+07
EGFR	48.02	1.48E+07
EpCAM	49.62	1.53E+07
MUC1	60.77	1.88E+07
WNT2	127.45	3.94E+07

Table S2. Antibodies used in this study.

	Antibody	Company	Catalog Number	Species	Clonality	Use
Isotype	Goat IgG	Invitrogen	02-6202	Goat		
	Mouse IgG1κ	BioLegend	400102	Mouse		FC, WB, IP, ICC, IF, IHC
	Mouse IgG2bκ	Abcam	ab18469	Mouse	Monoclonal	IHC, WB, FC, IP
	Mouse IgG1	R&D	MAB002	Mouse	Monoclonal	FC
	Rabbit IgG	Abcam	ab172730	Rabbit	Monoclonal	IF, IHC, FC, CHIPseq, IP
	Rat IgG2Aκ	BioLegend	400502	Rat		FC, IF, IHC, WB, IP
	Rat IgG2ak-AF488	BioLegend	400525	Rat		FC
Primary	Glypican 1 (GPC1)	R&D	AF4519	Goat IgG	Polyclonal	WB, FC, IF
	EpCAM	Abcam	ab20160	Mouse IgG1κ	Monoclonal	IF, FC, ELISA, IHC
	EGFR	Abcam	ab30	Mouse IgG2bκ	Monoclonal	IHC, IP, IF, FC
	MUC1	Fitzgerald	10-M93A	Mouse IgG1	Monoclonal	ELISA, IHC, WB
	WNT-2	Santa Cruz	sc-514382	Mouse IgG1κ	Monoclonal	WB, IP, IF, ELISA
	CD73	BD Pharmingen	550256	Mouse IgG1κ	Monoclonal	FC
	EphA2	R&D	MAB3035	Mouse IgG_{2A}	Monoclonal	WB, FC, IF
	Mesothelin	R&D	MAB32652	Rat IgG_{2A}	Monoclonal	FC
	LRG1	Invitrogen	PA5-25904	Rabbit IgG	Polyclonal	WB, IHC, FC
	TIMP1	Invitrogen	MS608PABX	Mouse IgG1	Monoclonal	FC, IF, IHC, WB
	EGFR-FITC	Abcam	ab11400	Rat IgG2a	Monoclonal	FC
Secondary AlexaFluor 488	Mouse IgG (H&L)	Abcam	ab150117	Mouse IgG	Polyclonal	IHC, IF, FC, ELISA
	Goat IgG (H&L)	Abcam	ab150133	Goat IgG	Polyclonal	IHC, IF, FC, ELISA
	Rabbit IgG (H&L)	Abcam	ab150073	Rabbit IgG	Polyclonal	IF, IC, IHC, ELISA
	Rat IgG (H&L)	Abcam	ab150153	Rat IgG	Polyclonal	IF, ELISA, FC, IHC