Supplemental Information

Discrete Microfluidics for the Isolation of Circulating Tumor Cell Subpopulations Targeting Fibroblast Activation Protein alpha and Epithelial Cell Adhesion Molecule

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Experimental Methods

Study design. This work was instigated based upon the hypothesis: *FAPα, which is expressed on cells comprising the tumor microenvironment, can be used as an additional marker for selecting a phenotypically distinct CTC subpopulation with respect to a CTC subpopulation that expresses EpCAM*. To test this hypothesis, we conducted a prospective trial with patients diagnosed with five different malignancies: CRPC, CRC, BC, PDAC, and EOC. We evaluated the following: Correlation of CTC numbers from each subpopulation with (i) disease burden, (ii) surveillance of recurrence, and (iii) ability to monitor treatment response. Longitudinal studies in PDAC patients was possible during a post-surgical follow-up. The individuals analyzing samples knew from what patient group blood was collected, but were blinded to the distinction between patients' type of treatment, disease stage, type of tumor, benign condition, etc. After completion of the study, patients' cancer characteristics (*i.e.,* histologic/pathologic type, stage/grade, disease recurrence via CT scans), history and prior treatments were obtained from the study coordinator and cohorts were established.

Cell culture and flow cytometry of model cell lines (Hs578T and SKBR3). The Hs578T cell line was cultivated in 1× MEM, 1× NEAA and 10% FBS. SKBR3 cells were grown in 1× McCoy Medium and 10% FBS. Cultures were incubated at 37° C with 5% CO_2. Cells were released from the flask surface with TrypLE™ reagent (Gibco). Flow cytometry was performed with a CyAn flow cytometer (Beckman-Coulter) equipped with a 25 mW 488 nm laser. Ten thousand events were counted for all samples. Data acquisition and analysis was performed using Summit software (Dako, Carpineteria, CA). Prior to staining, the cells' surface were blocked with human IgG and incubated at 4º C. The following samples were prepared for analysis: (i) Unstained cells serving as an autofluorescence control; (ii) cells incubated with propidium iodide (PI); (iii) cells stained with 10 µl of 0.1 mg/ml isotype control, IgG_1-FITC mAbs (R&D Systems, Minneapolis, MN) for Hs578T and IgG_2 for SKBR3; (iv) cells incubated with goat anti-mouse secondary IgG-FITC; (v) cells stained with mouse anti-human EpCAM-FITC Ab and incubated in the dark at 4º C for 30 min; and (vi) cells stained with 10 µl of 0.1 mg/ml CD4-FITC mAb (BD Biosciences, Franklin Lakes, NJ) and incubated in the dark at 4º C for 30 min. Cells were washed three times with 1 ml of cold PBS/0.5% BSA. PI staining (0.5 µg/ml) was performed on all of the samples for determining cell viability.

Self-referencing method. Quantifying the number of selected cells recovered is typically accomplished using seeding experiments in which a known number of target cells are introduced into a suspension. Unfortunately, this technique does not allow one to determine recovery of target cells present in clinical samples because the cell frequency is unknown. We developed a "self-referencing" method, where prior knowledge of the number of target cells is not required. The self-referencing method uses multiple cell selection devices connected in series. The number of cells isolated in the first device divided by the total cell count from all devices in the series quantifies recovery. An error in the quantification is low (<7%) at high device recovery (70-100%), requiring only two devices in series; the error can be minimized for this measurement scheme with three devices in series when the recovery is <60%. Thus, the self-referencing method can accurately measure a device's recovery from samples and with low standard deviations. Using this method, we calculated the recovery from following equation:

$$\frac{CTC_{chip\,1}}{CTC_{total}} = \frac{CTC_{chip\,1}}{CTC_{chip\,1} + CTC_{chip\,2} + \cdots CTC_{chip\,N}}$$

Patient Derived Xenograft (PDX) models. All animal procedures were done under protocols approved by the University of North Carolina Institutional Animal Care and Use Committee. PDX tumors were obtained from the Washington University School of Medicine in St. Louis (http://digitalcommons.wustl.edu/hamlet/). Two PDX models (WHIM2 and WHIM30) corresponding to the basal-like breast cancer subtype were used. Tumors were established in NOD *scid* gamma (NSG) (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice. The mice were briefly anesthetized with 2% isoflurane and ~2 million tumor cells in 50% Matrigel and 50% Hanks Balanced Salt Solution were subcutaneously injected into the fat pad of the fourth (inguinal) mammary gland. Mice were euthanized and the PDX tumors were removed when their size reached ~12 mm × 12 mm. Mouse terminal bleeds were performed via cardiac puncture. Blood was collected into 1 ml EDTA tubes and processed within 2 h of collection. Both CTC$^{FAP\alpha}$ and CTC^{EpCAM} were collected using the sinusoidal microfluidic chip. Genomic DNA (gDNA) was isolated and whole-genome amplification (WGA) was performed using the Illustra Single Cell GenomiPhi DNA Amplification Kit (GE Healthcare) according to the manufacturer's protocol. Exon 6 of the *TP53* was amplified via PCR with the forward primer – 5'CCTCTGATTCCTCACTGATTGCTCTTA3'; and a reverse primer with sequence – 5'GGCCACTGACAACCACCCTTAAC3'. Twenty ng of template per PCR was used with the following thermal cycling steps: Denaturation at 94˚C for 2.5 min followed by 40 cycles of denaturation at 94˚ C for 15 s; annealing for 30 s at 58˚ C and extension at 72˚ C for 30 s. A final extension at 72˚ C for 7 min was followed by a cooling step at 4˚ C. PCR products (199 bp) were electrophoresed at 8.3 V/cm in 1X TBE (Tris–boric acid/EDTA, Bio-Rad Laboratories) on a 4% agarose gel with ethidium bromide (Lonza) staining. Amplicons were excised from the gel, purified, treated with ExoSAP-IT reagent (Affymetrix) and Sanger sequencing performed.

Tumor tissue staining. Tissues were fixed in 1× Zn fixative (formalin free, BD Pharmingen™ IHC Zinc Fixative) for 48 h at room temperature followed by 24-48 h storage in 70% ethanol. Three mm thick blocks obtained from PDX primary tumor were embedded in paraffin. Four µm thick sections were cut from the block and placed on slides with the slides incubated at 70º C for 25 min. Staining was performed for immunohistochemical analysis using: (1) Monoclonal pan-CK antibody (MA5-13203, Life Technologies, 1:100, no antigen retrieval); (ii) monoclonal anti-CK19 antibody (MA512319, Life Technologies, 1:500, no antigen retrieval); (iii) monoclonal anti-EpCAM antibody (clone#158210, R&D, 1:100, antigen retrieval using Ventana's CC1 (pH 8.5), for 16 min at 100º C,); and (iv) monoclonal anti-Vimentin antibody (ab92547, Abcam, 1:250, antigen retrieval using Ventana's CC1 (pH 8.5) for 64 min at 100º C). Antibody dilutions were prepared using the Dako ARK Kit (Animal Research Kit, K3954). The dilutions were incubated in a biotinylated reagent for 15 min at room temperature, followed by addition of a blocking solution and incubation for 5 min at room temperature. The samples were given a hydrogen peroxide block for 8 min at room temperature and then, incubated in the primary Ab for 1 h at room temperature, followed by the secondary Ab (Dako Ark Kit Streptavidin HRP solution) for 16 - 32 min at 35º C. The samples were treated with DAB, Hematoxylin II for 8 min, and then Bluing Reagent for 4 min followed by monoclonal anti-FAPα antibody (427819, R&D, 1:200, antigen retrieval using Ventana's CC1 (pH 8.5), 72 min at 100º C). The slides were incubated in avidin and biotin (Ventana's A/B Block, 760-050) for 8 min each, followed by a hydrogen peroxide block for 8 min at room temperature and then, incubated in the primary Ab for 6 h at room temperature. The slides were incubated in the secondary Ab (Anti-IgG1 + IgG2a + IgG3 antibody, Abcam, ab133469, 1:500) for 1 h, followed by a tertiary Ab (Ventana Omap OmniMap anti Rabbit HRP, 760-4311, Ready to Use) for 32 min at room temperature. The

samples were treated with DAB, Hematoxylin II for 8 min and then, Bluing Reagent for 4 min. All slides were visualized using Ventana's Discovery Ultra Automated IHC staining system.

CTC lysis and molecular profiling on CRPC samples. On-chip CTC lysis and total RNA extraction were performed using a protocol adapted from RNeasy micro kit (Catalog no. 74004, Qiagen). Briefly, following CTC selection, the chips were flushed with 1 ml of cold 1× PBS at 60 µl/min. Three-hundred µl of RLT buffer was flowed through the CTC microfluidic chip at a flow rate of 60 µl/min. Twenty ng of carrier RNA was added to the eluent and RTL buffer to obtain 350 µl as the final volume of the lysate. Then, 350 µl of 70% ethanol was added to the lysate and mixed by pipetting. The sample was transferred to an RNeasy MinElute spin column placed in a 2 ml collection tube and centrifuged for 30 s at 10,000× g. The column was washed with 350 µl Buffer RW1 (centrifuged at 10,000× for 30 s). Eighty µl of DNase I was added to the RNeasy MinElute spin column membrane and incubated at room temperature for 15 min. RNeasy MinElute spin column was then washed with 350 µl Buffer RW1 and 500 µl Buffer RPE (10,000x for 30 s). After that, the column was washed using 500 µl of 80% ethanol and dried by centrifuging 5 min at 18,000×. Finally, RNA was eluted using 14 µl RNase-free water (1 min centrifuge at 18,000× g). Reverse transcription was performed using SuperScript III reverse transcriptase (Catalog no. 18080, Invitrogen) and 8 µl of the extracted RNA. PCR was performed using primers targeting *GAPDH* (housekeeping control), *PSMA, PSA, AR, EpCAM, FAPα1*, and *FAPα2*.

SUPPLEMENTARY FIGURES

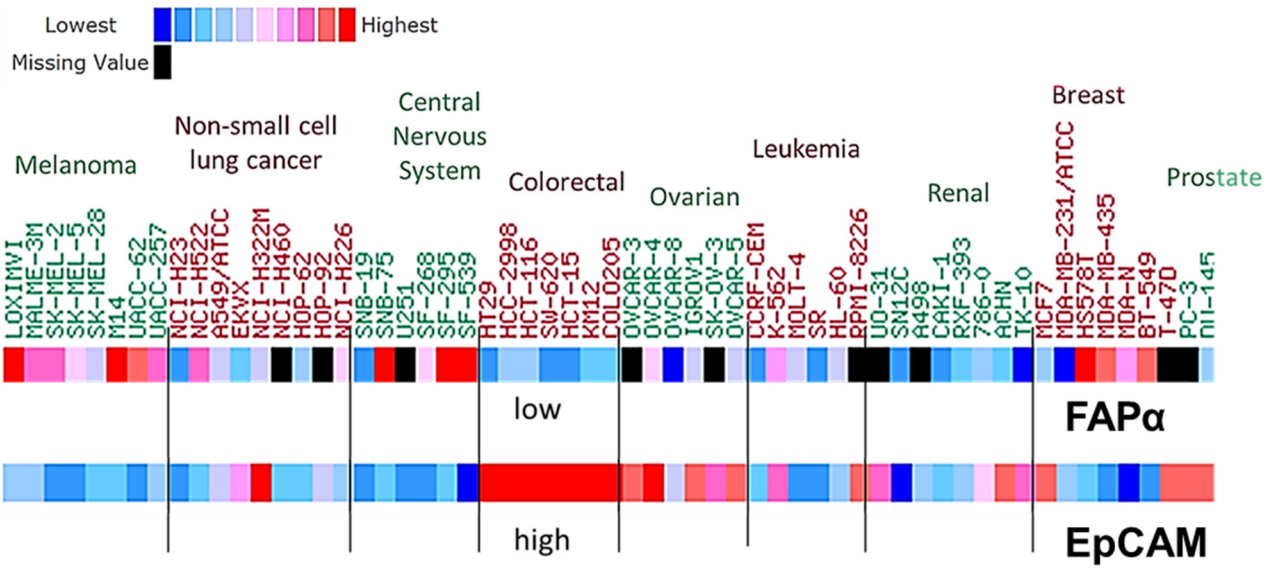


Figure S1. Expression of FAPα and EpCAM in various cancer cell lines. Data was secured from the NIH cancer genome atlas project as of 2013 (http://cgap.nci.nih.gov). Similar data can be now secured at http://www.ebi.ac.uk/.

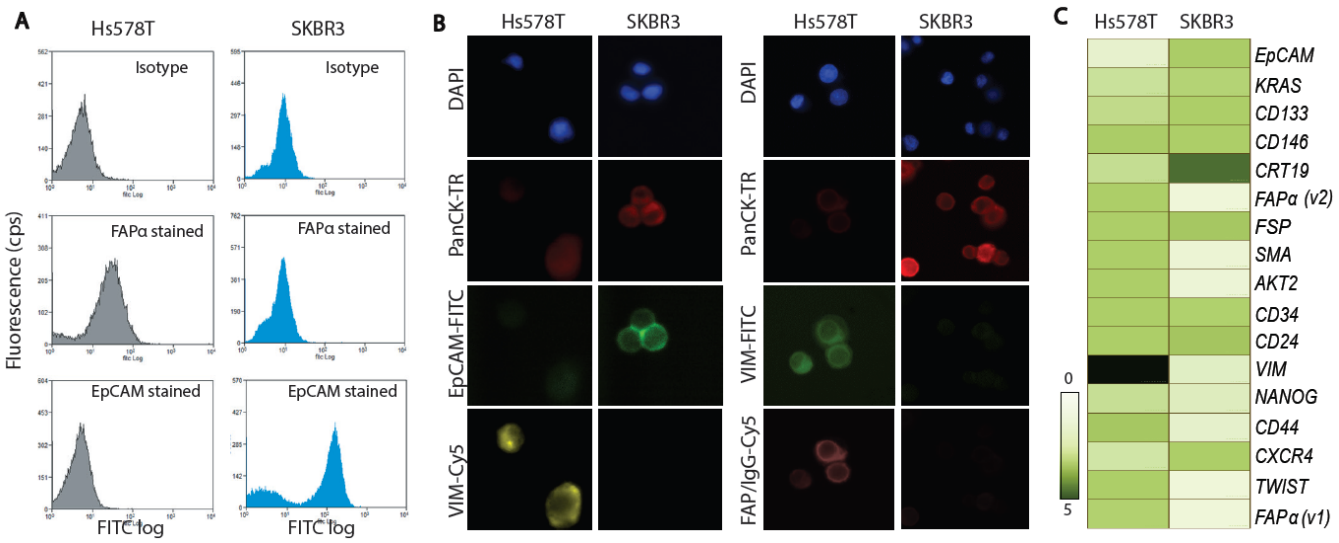


Figure S2. Characterization of model cell lines. **(A)** Multi-parameter flow cytometry (MFC), **(B)** immunostaining, and **(C)** RT-qPCR. **(A)** Histograms show MFC results for stained Hs578T and SKBR3 cells with the IgG isotype, FAPα via a secondary mAb, and anti-EpCAM mAb. **(B)** Immunostaining with epithelial and mesenchymal markers showing images of breast cancer cell lines stained with DAPI, Pan-CK, anti-EpCAM, and anti-Vim mAbs. Hs578T cell line (Claudin-like) is derived from a mammary gland of a carcinosarcoma. These cells showed mesenchymal and luminal morphology. SKBR3 cell line was derived from a metastatic site of a mammary gland adenocarcinoma. mAbs were labeled with Cy5, FITC, or Texas Red (TR). **(C)** RT-qPCR mRNA expression profiles for Hs578T and SKBR3 cell lines relative to *GAPDH* expression.

Results indicated that Hs578T cells were EpCAM-, with the FAPα expression 6-fold greater than the IgG control (**Fig. S2A**). The SKBR3 cell line showed high expression of EpCAM (15-fold IgG control) and no expression of FAPα. Hs578T weakly expressed pan-CK and strongly expressed vimentin (VIM), while SKBR3 cells demonstrated strong pan-CK and no VIM expression (**Fig. S2B**). RT-qPCR revealed high expression of *EpCAM* and *CRT19* in the SKBR3 cell line, but not in the Hs578T cell line (**Fig. S2C**). As expected, SKBR3 *VIM* mRNA expression was low.

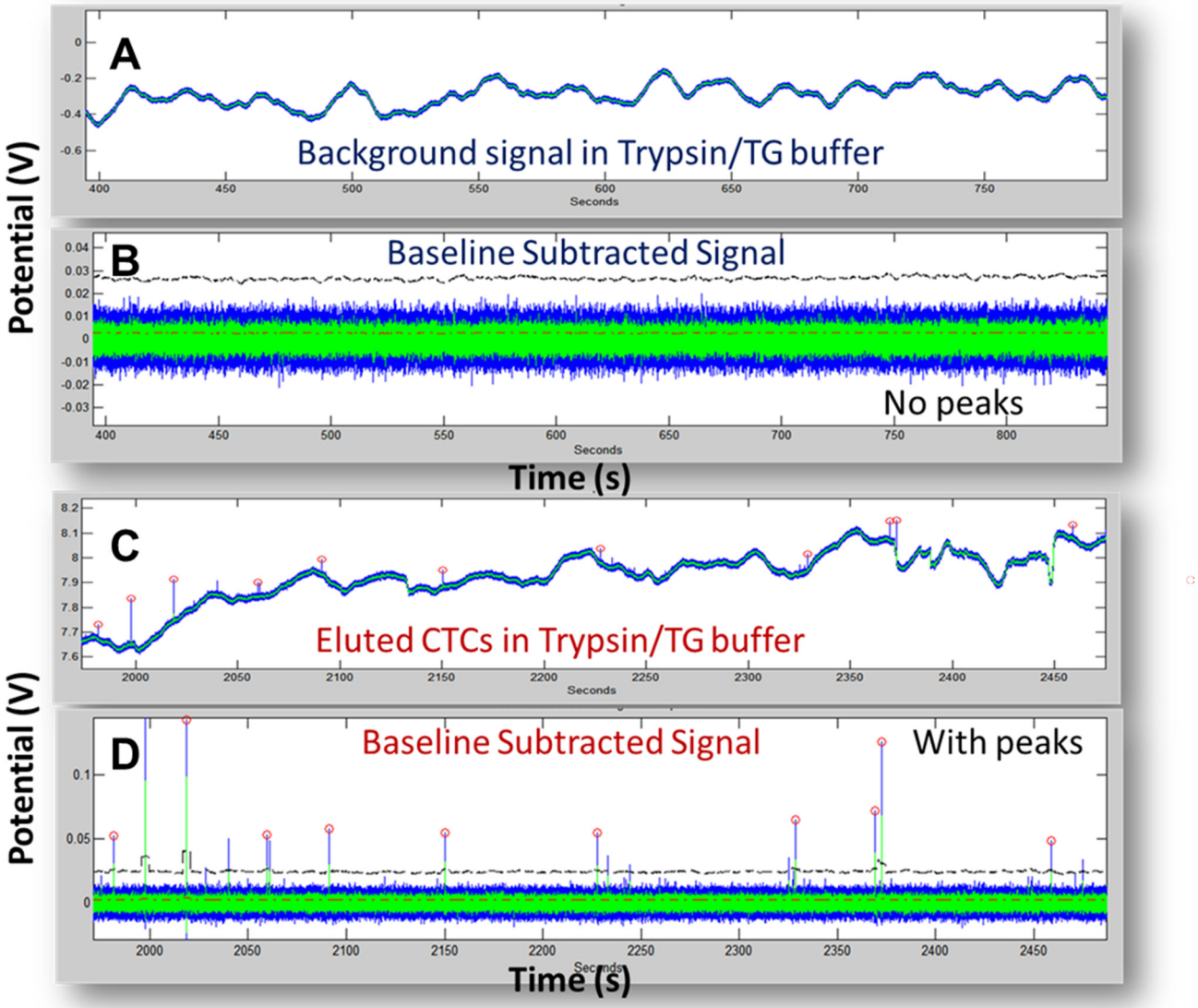
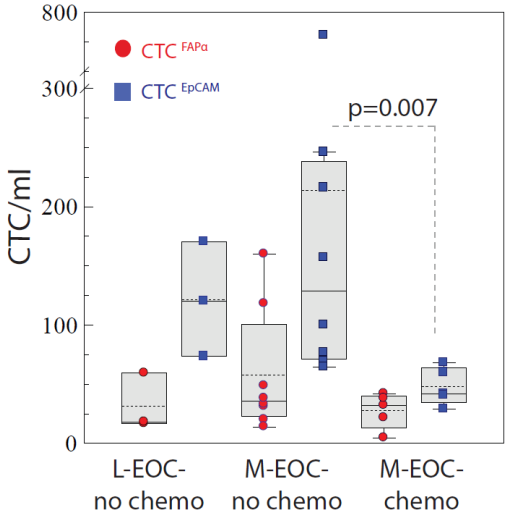


Figure S3. Cell counting via impedance detection. (A) Raw and (B) baseline subtracted signals for the enzyme/buffer system before cells are eluted, and (C) raw and (D) baseline subtracted signals for CTCs released from the sinusoidal CTC isolation device. Peaks observed in the traces in (C) and (D) correspond to single CTCs. Data analysis was performed with Matlab, and peak events were classified as CTCs when the signal magnitude was higher than 10x SD. The sensor operated at 40 kHz frequency; the flow rate was 25 µl/min; and the data collection frequency was 3 kHz. The Pt electrodes (75 µm) were separated by 50 μm producing a cell constant, K, equal to 0.01 μm^{-1} (defined as the ratio of electrode gap to the electrode area). K was scaled to specifically detect CTCs due to their larger size with respect to leukocytes or erythrocytes. As shown in our previous work, the conductivity sensor with K = 0.01 μm^{-1} provided near 100% CTC counting and does not transduce signals from leukocytes or erythrocytes due to their smaller size.[1,2]

A



B

Ovarian Cancer Stage and Treatment	$CTC^{FAP\alpha}$/ml avg (median)	CTC^{EpCAM} /ml avg (median)
Localized chemo naïve (n=3)	31.3 (18.0)	121.3 (120.0)
Metastatic, chemo naïve (n=8)	57.8 (35.5)	213.7 (128.5)
Metastatic, chemotherapy (n=5)	27.6 (32.0)	47.9 (42.0)

Figure S4. Enumeration data for CTCs in EOC patients**. (A)** Box plot for $CTC^{FAP\alpha}$ and CTC^{EpCAM} isolated from the blood of EOC patients: Non-metastatic chemo naïve (L-EOC-no chemo); metastatic chemo naïve (M-EOC-no chemo); and metastatic with chemotherapy treatment (M-EOC-chemo). **(B)** Tabulated average and median CTC enumeration data. The solid lines in the box plots represent the median and the dotted line is the mean for the data shown.

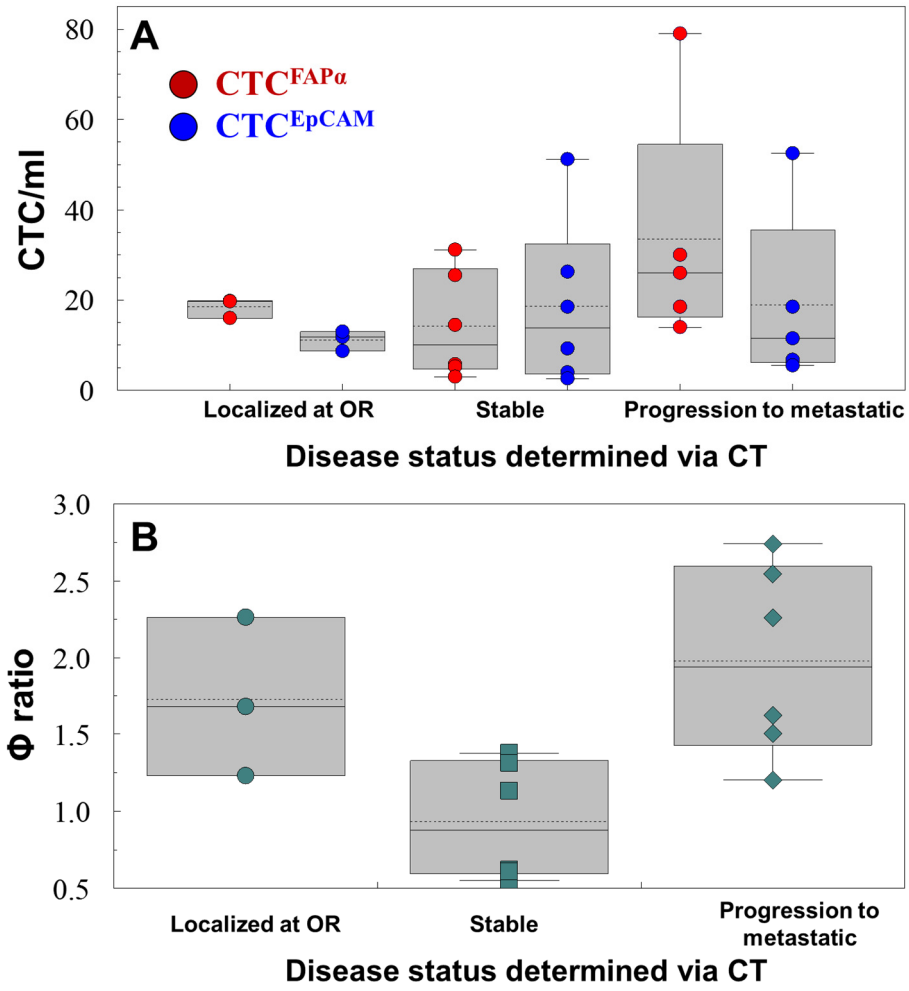


Figure S5. Summary of longitudinal tracking data in L/M-PDAC patients. (**A**) Box plot for $CTC^{FAP\alpha}$ and CTC^{EpCAM} counts isolated from the blood of L/M-PDAC patients, and (B) $CTC^{FAP\alpha}/CTC^{EpCAM}$ ratio (*i.e.*, ϕ ratio) calculated for the same patients at different stages of disease, as determined by CT imaging. Box plots represent upper and lower quartiles, dashed line is average and solid line is median. Localized at OR represents localized disease at time of surgical resection of tumor.

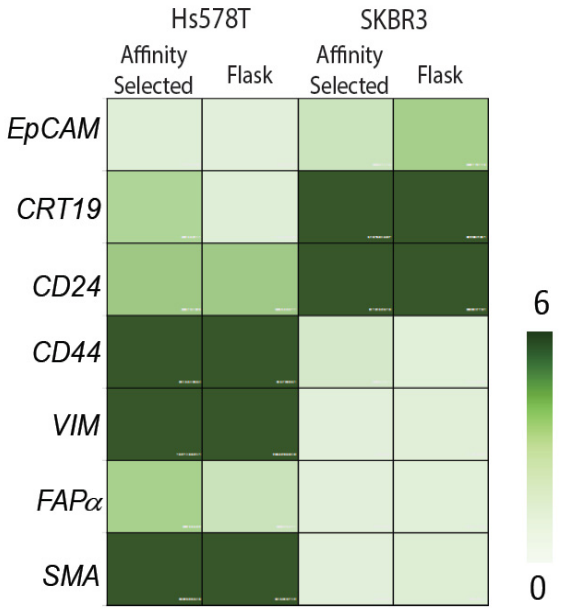


Figure S6. Relative expressions of mRNA for selected genes evaluated for Hs578T and SKBR3 cell lines for cells harvested from the culture flask and cells affinity isolated using the sinusoidal microfluidic chip. Approximately 200 cells were used in both experiments. Relative mRNA expression levels were calculated by the comparative CT method using *GAPDH* as an endogenous control.

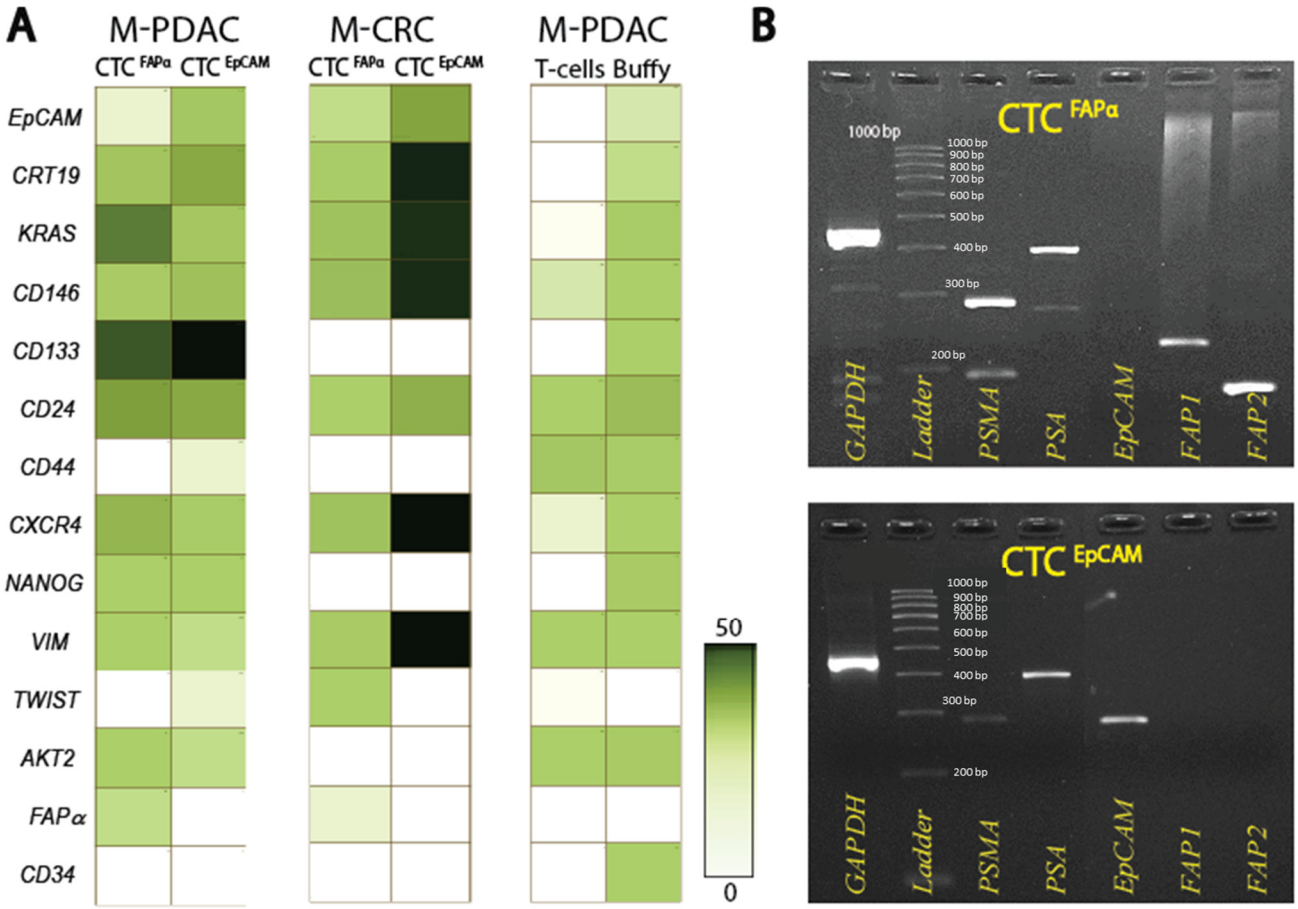


Figure S7. Average relative expressions of mRNA for selected genes from $CTC^{FAP\alpha}$ and CTC^{EpCAM}. **(A)** mRNA expression heat plots for 5 M-PDAC, 2 M-CRC, T-Cells, and M-PDAC-buffy coat. Relative mRNA expression levels were calculated by the comparative CT method using *GAPDH* as an endogenous control. The average results ($2^{\Delta Ct}$) obtained from RT-qPCR for all samples are summarized. Variations of *GAPDH* between samples were not evaluated. **(B)** Agarose gel fluorescence images for gene amplification after reverse transcription of mRNA taken from $CTC^{FAP\alpha}$ and CTC^{EpCAM} affinity selected from a CRPC patient. The samples shown here were derived from the same experiment and processed in parallel.

SUPPLEMENTARY TABLES

Table S1. Variations in CTC counts when the order of the FAPα and EpCAM devices was changed.

Order 1st chip→2nd chip	CRC #116		PDAC#46		PDAC pt #66	
	$CTC^{FAPα}$ /ml	CTC^{EpCAM}/ml	$CTC^{FAPα}$ /ml	CTC^{EpCAM}/ml	$CTC^{FAPα}$ /ml	CTC^{EpCAM}/ml
FAPα → EpCAM	16	14	17.0	20.5	99.3	55
EpCAM → FAPα	11	12	12.0	32.0	58.7	50
% RSD*	26.2	10.9	24.4	30.9	36.3	6.7

*%RSD=(SD/Avg)x100

Table S2. CTC enumeration in healthy donors. Blood samples from 11 healthy donors were collected and between 1 and 5 ml of blood was processed using the dual selection assay.

ID	Clinical Sample	Analysis date	Blood volume processed (ml)	$CTC^{FAPα}$ /ml (WBC/ml)	CTC^{EpCAM} /ml (WBC/ml)
HD1	Healthy Donor	09/05/12	1.0	1 (3)	0 (1)
HD2	Healthy Donor	09/05/12	2.0	0.5 (1.5)	0 (0.5)
HD4	Healthy Donor	03/02/12	1.0	0 (3)	0 (1)
HD5	Healthy Donor	04/12/13	2.0	0 (2)	0.5 (0.5)
HD3	Healthy Donor	02/05/14	1.0	0 (3)	0 (1)
HW16	Healthy Donor	07/05/12	2.5	-	0.4 (4.8)
HW1	Healthy Donor	07/12/12	2.5	-	0 (2)
HW2	Healthy Donor	07/12/12	2.5	-	0 (4)
HW3	Healthy Donor	08/16/12	5.0	-	0 (2.2)
HW4	Healthy Donor	08/30/12	1.5	-	0 (8.0)
HW5	Healthy Donor	08/30/12	1.5	-	0 (5.3)

Table S3. CTC enumeration in non-cancer patients. Six patients with non-cancer diseases were enrolled in the study. Two ml of blood was processed using the dual selection strategy.

Patient ID	Diagnosis	Surgery date	Blood volume processed (ml)	$CTC^{FAPα}$ /ml (WBC/ml)	CTC^{EpCAM} /ml (WBC/ml)
44	Benign pancreas	09/06/12	2	0 (0.5)	0.5 (2)
69	Goiter	12/11/12	2	1.5 (0)	3.5 (0)
71	benign pancreas	12/13/12	2	4.5 (2)	4 (1)
75	adrenal mass	12/19/12	2	1.5 (1)	1 (0)
81	pancreatitis	03/27/13	2	3.5 (0)	4 (1)
85	benign pancreas	03/22/13	2	0 (2)	2.5 (2)

Table S4. Metastatic breast cancer patients enrolled in the study.

Pt ID	Age	Race	Date of primary diagnosis	Date of metastatic diagnosis	Metastatic sites	Receptor status		Therapy at time of blood draw		
						Primary	Metastatic	Endocrine	Chemo-therapy	Comments
#01	70	C	1981	Dec 2008	sternum, lungs, mediastinal lymph nodes	unknown	ER+/PR-/HER2-	Yes	No	biologic
#02	69	C	first presented with stage IV disease, Nov 2008		left axilla, breast, liver, bone, lung	unknown	ER+/PR+/ HER2+	yes	No	
#03	65	C	2007	Jun 2013	lung, liver, bone	ER-/PR-/ HER2-	ER-/PR-/HER2-	No	Yes	
#04	62	C	2007	Oct 2009	liver, skin	ER+/PR+/ HER2+	ER+/PR+/ HER2-	Yes	No	
#05	51	C	2010	May 2012	right axilla, mediastinum, left adrenal	ER+/PR-/ HER2-	ER-/PR-/HER2-	No	yes	PD-L1 clinical trial
#06	56	C	2003	Oct 2009	bone, liver	ER+/HER2-	ER+/PR+/ HER2-	Yes	No	
#07	46	C	first presented with stage IV disease, Jan 2013		bone, sacrum, lungs, brain	unknown	ER+/PR+/ HER2-	yes	No	
#08	36	C	2006	July 2007	left submandibular LN and mediastinum, right parietal	ER-/PR-/ HER2-	ER-/PR-/ HER2-	No	No	
#09	58	AA	2004	Feb 2013	bone, lung	ER+/PR+/ HER2-	ER+/PR+/ HER2+	Yes	No	
#10	61	C	2000	Aug 2008	lung	ER+/PR+/ HER2-	ER+/PR+/ HER2-	No	Yes	

Table S5. PDAC patients enrolled in the study.

Patient ID	Cancer stage at enrollment	Surgery	Status at Last Follow up
2	Metastatic	N	Deceased
24	Metastatic	N	Deceased
25	Metastatic	N	Deceased
40	Metastatic	N	Deceased
41	Metastatic	N	Deceased
42	Metastatic	N	Deceased
45	local resectable	Y	Deceased
46	local resectable	Y	Alive, disease free
47	Metastatic	N	Deceased
48	local, resectable	Y	Deceased
66	local resectable	Y	Deceased
67	locally advanced unresectable	N	Deceased
68	Metastatic	N	Deceased
89	Metastatic	N	Deceased
102	Metastatic	N	Deceased

Table S6. PDAC patients' disease progression and treatment.

Pt ID	Age	Cancer type	Time to progression (months)	CA9-19 (U/ml)	Preoperative chemotherapy/ radiation	Postoperative chemotherapy/ radiation	Metastatic sites	Status at last follow up
45	63	Local, resected	9.2	1764	no/no	yes/yes	liver, lung	deceased
46	66	local, resected	--	--	no/no	yes/yes	--	alive, disease free
48	44	local, resected	4.6	25	no/no	yes/no	liver	deceased
66	60	local, resected	19.5	1	yes/no	---	liver	deceased
67	50	locally advanced unresectable	4.9	141	yes/no	---	liver, peritoneal	deceased
25	59	metastatic	0.0	7	---	---	liver	deceased
41	65	metastatic	16.7	22	yes/yes	no	liver	deceased

Table S7. Epithelial ovarian cancer (EOC) patients enrolled in the study. na not applicable

Pt ID	Age	Date diagnosed	Date of the last chemo regimen	Analysis date	Initial treatment/ chemo regimen	CA125 at diagnosis	Preop. CA125	Stage/ Grade	Histology	Metastasis site
L-EOC-no chemo										
#2	41	01/22/2014	na	01/22/14	debulking	123	123	IA/3	Endometrioid adenocarcinoma of the ovary	None
#6	61	05/12/2014	na	05/12/14	debulking	835	835	IA/2	Endometrioid adenocarcinoma of the ovary	None
#17	60	03/2315	na	04/24/15	debulking	12	12.3	IC2/3	Papillary serous carcinoma of the ovary	None
M-EOC-chemo										
#4	71	12/19/2013	02/01/14	03/11/14	neoadjuvant chemotherapy: Carboplatin + paclitaxel, 3 cycles/ interval debulking	5900	437	IIIC/3	Papillary serous carcinoma of the ovary	Omentum, fallopian tube
#10	68	6/25/2014	08/25/14	09/26/14	Neoadjuvant chemotherapy: Carboplatin + paclitaxel, 3 cycles/ interval debulking	3580	495	IIIC/3	Serous carcinoma of the peritoneum	Omentum, ovaries, uterus, sigmoid colon, appendix
#12	53	04/22/2014	10/01/14	10.22/14	Neoadjuvant chemotherapy: Carboplatin + paclitaxel, 6 cycles/ interval debulking	1423	290	IV/3	Mixed serous, clear cell, and endometrioid adenocarcinoma of the ovary	Omentum, fallopian tube
#14	45	12/09/14	02/25/15	03/27/15	Neoadjuvant chemotherapy: Carboplatin + paclitaxel, 4 cycles/interval debulking	1340	1030	IVB/3	Serous carcinoma of the ovary	Fallopian tubes, uterus, cervix, appendix
#19	58	12/04/14	03/30/15	05/22/15	Neoadjuvant chemotherapy: Carboplatin + paclitaxel, 6 cycles/interval debulking	2390	18.3	IVB/3	Papillary serous carcinoma of the ovary	Uterus, fallopian tubes, cervix, omentum, anterior abdominal wall
M-EOC-no chemo										
#1	65	01/14/2014	na	01/17/14	debulking	na	na	IIIC/ Low	Serous carcinoma of the ovary	Omentum, peritoneum, fallopian tube, lymph nodes
#5	68	03/12/2014	na	03/12/14	debulking	281	281	IIA/3	Carcinosarcoma of the ovary	Fallopian tube
#8	63	07/25/2014	na	07/25/14	debulking	1230	1230	IIIC/3	Serous carcinoma of the peritoneum	Omentum, pelvis, uterus, fallopian tube, ovary, sigmoid colon
#9	49	10/01/2009	09/01/11	09/03/14	debulking/ Carboplatin + taxotere + Avastin, 6 cycles, then PARP inhibitor trial x 15 courses (recurrence 7/8/14)/ secondary debulking	355	4.5	IIIC/3	Papillary serous carcinoma of the ovary	Sigmoid colon
#11	62	10/03/2014	na	10/03/14	debulking	154	154	IIIC/3	Papillary serous carcinoma of the fallopian tube	Omentum, ovaries
#13	66	11/06/2014	na	11/06/14	debulking	915	915	IIIC/3	Papillary serous carcinoma of the ovary	Omentum, fallopian tube, peritoneum, spleen
#18	66	04/24/15	na	05/01/15	debulking	1500	1500	IIIC/3	Papillary serous carcinoma of the ovary	Omentum, small bowel mesentery, appendix, rectum
#20	70	05/14/15	na	05/28/15	debulking	296	296	IIIC/3	Papillary serous carcinoma of the ovary	terus, fallopian tubes, omentum, peritoneum, colon, small bowel mesentery, appendix, abdominal wall

Table S8. CRPC patients enrolled in the study.

Patient ID	Metastatic sites	Treatment	PSA (ng/ml)
1	none	ADT*, bicalutamide, abiraterone	0.6
2	bone, liver	ADT, abiraterone, docetaxel	363
3	bone	ADT, ketoconazole, sipuleucel-T	6.4
4	bone	ADT, docetaxel, abiraterone	0.7
5	bone	ADT, bicalutamide, docetaxel	1920

*ADT- androgen deprivation therapy

Table S9. Comparison of CTC numbers secured via phenotyping (stain) and impedance sensing (e-count).

Pt ID	FAPα Selection Chip				EpCAM Selection Chip			
	WBC/ml via stain	**$CTC^{FAPα}$ /ml via stain**	**$CTC^{FAPα}$ /ml via e-count**	**%RSD between staining and e-count**	**WBC/ml via stain**	**CTC EpCAM /ml via stain**	**CTC EpCAM /ml via e-count**	**%RSD between staining and e-count**
#24	2	21	24.5	10.9	3	23.5	n/a	n/a
#25	3	9.5	6.5	26.5	2	23.0	21.0	8.3
#46	1	7.5	4.0	43.0	2	4.0	4.5	12.5
#66	3	25.0	34.5	22.5	5	52.5	44.0	29.9
#68	4	16.5	26.0	31.6	2	10.0	6.5	6.4

*%RSD=(SD/Avg)x100. n/a = Not applicable. In this case, data was not collected.

Table S10. CTC enumeration data from all patients. Summary of the enumeration data for $CTC^{FAP\alpha}$ and CTC^{EpCAM} isolated from patients on serially connected CTC selection microfluidic devices.

Samples (n-number of samples, m-number of measurements)	CTC $^{FAP\alpha}$ /ml			CTC ^{EpCAM} /ml		
	Average	Median	Range	Average	Median	Range
Healthy Donor (n=11, m=11)	0.3	0.0	0-1.0	0.1	0.0	0.0-0.5
Non-Cancer (n=6, m=6)	1.8±1.8*	1.5	0.0-4.5	2.6±1.5*	3.0	0.5-4.0
L-PDAC (n=5, m=17)	29.9	26.0	3.0-79.0	23.1	22.0	2.7-52.5
L-PDAC at OR (n=3, m=6)	31.7	19.7	16.0-59.5	19.1	13.0	8.7-35.5
M-PDAC (n=10, m=25)	22.1	17.5	6.5-83.3	27.3	20.5	3.5-105.4
L-CRC (n=3, m=5)	66.6	15.0	10.0-280.0	16.1	13.0	7.0-34.0
M-CRC (n=3, m=4)	34.4	32.8	26.0-48.5	47.8	31.5	17.0-111.0
M-BC (n=10, m=12)	41.8	24.0	0.5-179.0	88.7	47.8	1.0-278.0
M-EOC-no-chemo (n=8, m=12)	57.8	35.5	14.0-160.0	213.7	128.5	64.5-680.0
M-EOC-chemo (n=5, m=10)	27.6	32.0	4.5-42.0	47.9	42.0	29.0-68.0
L- EOC -no chemo (n=3, m=3)	31.3	18.0	16.5-59.3	121.3	120.0	73.3-170.5
CRPC (n=5, m=5)	19.5	18.0	12.7-27.3	12.7	9.3	2.0 – 39.3

*-SD used for determination of clinical sensitivity and selectivity, n-number of samples, m-number of measurements

Table S11. Pairwise statistical analyses of CTC counts.

Clinical Samples	p-value*	
	CTC^{FAP}	**CTC^{EpCAM}**
L-PDAC vs. HD (n=5, m=17)	18.8×10^{-4}	0.1×10^{-4}
L-PDAC at OR vs. HD (n=3, m=6)	80.4×10^{-4}	22.2×10^{-4}
M-PDAC vs. HD (n=10, m=25)	0.47×10^{-4}	0.12×10^{-4}
L-CRC vs. HD (n=3, m=5)	120.8×10^{-4}	22.2×10^{-4}
M-CRC vs. HD (n=3, m=4)	not determined**	not determined**
M-BC vs. HD (n=10, m=12)	26.6×10^{-4}	6.7×10^{-4}
M-EOC-no-chemo vs. HD (n=8, m=12)	43.8×10^{-4}	3.4×10^{-4}
M-EOC-chemo vs. HD (n=5, m=10)	120.8×10^{-4}	22.2×10^{-4}
L- EOC -no chemo vs. HD (n=3, m=3)	not determined**	not determined**
CRPC (n=5, m=5)	79.4×10^{-4}	79.4×10^{-4}
L-PDAC vs. NC (n=5, m=17)	13.2×10^{-4}	7.8×10^{-4}
L-PDAC at OR vs. NC (n=3, m=6)	38.0×10^{-4}	48.0×10^{-4}
M-PDAC vs. NC (n=10, m=25)	0.030×10^{-4}	0.008×10^{-4}
L-CRC vs. NC (n=3, m=5)	57.8×10^{-4}	43.8×10^{-4}
M-CRC vs. NC (n=3, m=4)	not determined**	not determined**
M-BC vs. NC (n=10, m=12)	67.8×10^{-4}	117.0×10^{-4}
M-EOC-no-chemo vs. NC (n=8, m=12)	14.8×10^{-4}	9.4×10^{-4}
M-EOC-chemo vs. NC (n=5, m=10)	73.6×10^{-4}	43.8×10^{-4}
L-EOC -no chemo vs. NC (n=3, m=3)	not determined**	not determined**
CRPC (n=5, m=5)	25.2×10^{-4}	31.0×10^{-4}

*- $p < 500 \times 10^{-4}$ (<0.05) considered statistically significant
**-not enough data points for Mann-Whitney U-Test
HD- healthy donor
NC-non-cancer

Table S12. NGS for CTC subpopulations isolated from an ovarian cancer patient. Mutations were detected using the TruSight Tumor Sequencing Panel. Q for all = 100, GQX = 100. *- het- heterozygous, hom- homozygous. NGS data were processed using VariantStudio (Illumina).

Gene	Variant (Chr#)	HGVSc	Mut. Frequency (%)		Read Depth		Classification	Genotype	Consequences
			$CTC^{FAP\alpha}$	CTC^{EpCAM}	$CTC^{FAP\alpha}$	CTC^{EpCAM}			
PDGFRA	A>G/G (4)	c.1701A>G	99.8	99.9	14419	22908	Synonymous protein change	hom	synonymous variant
APC	G>G/A (5)	c.4479G>A	45.3	41.7	19408	25760	Synonymous protein change	het	synonymous variant
EGFR-AS1	G>G/A (7)	n.1201C>T	55.0	50.0	11545	21077	Synonymous protein change	het	non coding exon variant and transcript variant
EGFR-AS1	G>G/A (7)	c.2361G>A	55.0	50.0	11545	21077	Synonymous protein change	het	synonymous variant
MET	G>G/A (7)	c.4071G>A	46.3	50.3	17913	20455	Synonymous protein change	het	synonymous variant
MET	G>G/A (7)	c.4146G>A	55.2	54.0	9918	11573	Synonymous protein change	het	synonymous variant
CDH1	G>G/A (16)	c.1774G>A	58.6	49.2	46254	56978	variant of uncertain significance (VUS)	het	missense variant
TP53	G>G/C (17)	c.215 C>G	40.6	66.0	42310	64875	SNP (>1% of population)	het	missense variant

COSMIC Histology for all: carcinoma

Table S13. Primers sequences used for the ligase detection reactions. ph – phosphorylated

Mutation	Discriminating primer 5' - 3'	Common primer 5' – 3'	Ligation product size (nt)
35 WT	TTTTTTTAAACTTGTGGTAGTTGGAGCTGG (30 nt)	ph-TGGCGTAGGCAAGAGTGCCT-Cy5 (20 nt)	**50**
G35A	TAAACTTGTGGTAGTTGGAGCTGA (24 nt)		**44**
G35T	TTTTTTTTTTTTTAAACTTGTGGTAGTTGGAGCTGT (35 nt)		**55**
34 WT	TTTTTTTTTTTTTTTAAACTTGTGGTAGTTGGAGCTG (37 nt)	ph-GTGGCGTAGGCAAGAGTGCCTTGACGATAC-Cy5 (30nt)	**67**
G34C	TTTTTTTTTAAACTTGTGGTAGTTGGAGCTC (31 nt)		**61**

Table S14. The average number of $CTC^{FAP\alpha}$ and CTC^{EpCAM} isolated from the blood of metastatic breast ductal carcinoma patients (M-BC). n.a., not applicable, as cells were enumerated with the impedance detector. Also shown is the number of white blood cells enumerated for each assay.

Patient Id	Analysis date	Blood volume (ml)	Affinity bed	CTC/blood volume	WBC/blood volume	Purity (%)	CTC/ml
#1	11/14/13	2	FAPα	1	2	33.3	0.5
#1	11/14/13	2	FAPα	1	0	100	0.5
#1	11/14/13	2	EpCAM	3	0	100	1.5
#1	11/14/13	2	EpCAM	2	2	50	1
#1	11/14/13	2	IgG bed	0	2	0	0
#3	11/14/13	2	FAPα	6	2	75	3
#3	11/14/13	2	EpCAM	2	0	100	1
#4	11/21/13	2	FAPα	115	3	97.4	57.5
#4	11/21/13	2	EpCAM	468	2	99.6	234
#4	11/21/13	2	pristine COC	0	0	na	0
#5	12/05/13	2	FAPα	358	5	98.6	179
#5	12/05/13	2	FAPα	418	6	98.6	209
#5	12/05/13	2	EpCAM	556	3	99.5	278
#5	12/05/13	2	EpCAM	472	2	99.6	236
#2	12/12/13	2	FAPα	57	5	91.9	28.5
#2	12/12/13	2	EpCAM	81	4	95.3	40.5
#6	01/16/14	2	FAPα	12	na	na	6
#6	01/16/14	2	EpCAM	9	na	na	4.5
#7	01/16/14	2	FAPα	21	na	na	10.5
#7	01/16/14	2	EpCAM	7	na	na	3.5
#8	01/23/14	2	FAPα	124	na	na	62
#8	01/23/14	2	EpCAM	110	na	na	55
#9	01/23/14	2	FAPα	39	na	na	19.5
#9	01/23/14	2	EpCAM	190	na	na	95
#9	01/23/14	2	IgG	3	9	na	na
#10	01/23/14	2	FAPα	103	na	na	51.5
#10	01/23/14	2	EpCAM	347	na	na	173.5

Table S15. The average number of $CTC^{FAP\alpha}$ and CTC^{EpCAM} isolated per ml of blood for metastatic pancreatic ductal adenocarcinoma (PDAC) patients. n.a., not applicable, as cells were enumerated with the impedance detector. Also shown is the number of white blood cells (WBCs) enumerated per each assay as well as the calculated purity.

Patient ID	Analysis date	Affinity bed	Blood volume (ml)	CTC/ml	WBC/ml	Purity (%)
24	08/07/12	FAPα	2	21.0	2	91.3
		EpCAM	2	23.5	3	88.7
2	08/17/12	FAPα	2	8.5	0.5	94.4
		FAPα	2	14.5	3	82.8
		EpCAM	2	6.5	2	76.5
		EpCAM	2	3.5	3	53.8
42	08/27/12	FAPα	1.5	83.3	4	95.4
		EpCAM	2	49	2	96.1
40	08/31/12	FAPα	2	21	2.5	89.4
		FAPα	2	12.5	1	92.6
		EpCAM	2	31.5	1	96.9
		EpCAM	2	77.5	4	95.1
25	09/21/12	FAPα	3	29.3	na	na
		FAPα	3	30.7	na	na
		EpCAM	3	19	na	na
		EpCAM	3	18	na	na
47	09/28/12	FAPα	2	24.5	na	na
		FAPα	2	38.5	na	na
		EpCAM	2	20	na	na
		EpCAM	2	9.5	na	na
41	11/16/12	FAPα	2	25.5	na	na
		FAPα	2	15.5	na	na
		EpCAM	2	105	na	na
		EpCAM	2	60	na	na
25	11/29/12	FAPα	2	9.5	2	82.6
		FAPα	2	6.5	na	na
		EpCAM	2	23	3	88.5
		EpCAM	2	21	na	na
25	02/07/13	FAPα	2	10	3	76.9
		FAPα	2	7.5	2.5	75.0
		EpCAM	2	20	3	86.9
		EpCAM	2	34.5	2	94.5
25	03/21/13	FAPα	2	14	na	na
		EpCAM	2	5.5	na	na
41	03/22/13	FAPα	2	13.5	na	na
		EpCAM	2	16	na	na
		EpCAM	2	39	na	na
68	04/19/13	FAPα	2	26	na	na
		FAPα	2	16.5	4	80.5
		EpCAM	2	4.5	na	na
		EpCAM	2	6.5	na	na
		EpCAM	2	10	2	83.3
102	05/02/13	FAPα	2	40	na	na
		EpCAM	2	23.5	na	na
48	12/14/12	FAPα	2	19	na	na
		FAPα	2	34.5	na	na
		EpCAM	2	25	na	na
		EpCAM	2	19.5	na	na

Table S16. The average number of $CTC^{FAP\alpha}$ and CTC^{EpCAM} isolated per ml of blood for localized pancreatic ductal adenocarcinoma (PDAC) patients.

Patient ID	Analysis date	Affinity bed	Blood volume (ml)	CTC/ml
45 OR	09/20/12	FAPα	3	16.3
		FAPα	3	23.3
		EpCAM	3	15.3
		EpCAM	3	8.3
45	12/13/12	FAPα	2	25.5
		EpCAM	2	18.5
45	02/28/13	FAPα	2	7.5
		FAPα	2	4.0
		EpCAM	2	9.0
		EpCAM	2	9.5
45	04/15/13	FAPα	2	17.5
		FAPα	2	19.5
		EpCAM	2	5
		EpCAM	2	8.5
46 at OR	09/20/12	FAPα	3	13.7
		FAPα	3	25. 7
		EpCAM	3	8. 7
		EpCAM	3	na
46	10/08/12	FAPα	2	3.5
		FAPα	2	7
		EpCAM	2	3
		EpCAM	2	5
46	11/12/12	FAPα	1.5	3.3
		FAPα	1.5	2. 7
		EpCAM	1.5	4.0
		EpCAM	1.5	1.3
46	06/03/13	FAPα	2	17
		FAPα	2	12
		EpCAM	2	20.5
		EpCAM	2	32
66 at OR	12/06/12	FAPα	2	23
		FAPα	2	9
		EpCAM	2	15.5
		EpCAM	2	10.5
66	03/11/13	FAPα	2	34.5
		FAPα	1	34
		FAPα	2	25
		EpCAM	2	44
		EpCAM	2	52.5
		EpCAM	1	57
66	06/17/13	FAPα	3	99.3
		FAPα	3	58.7
		EpCAM	3	55.0
		EpCAM	3	50.0
48	05/23/12	FAPα	2	26
		EpCAM	2	11.5
48	12/14/12	FAPα	2	19
		FAPα	2	34.5
		EpCAM	2	25
		EpCAM	2	19.5
67	03/22/13	FAPα	2	20
		EpCAM	2	28

Table S17. The average number of $CTC^{FAP\alpha}$ and CTC^{EpCAM} isolated from the blood of colorectal cancer patients. nd – not determined (impedance measurements). Also shown is the blood volume analyzed and the average number of white blood cells (WBCs) enumerated.

Patient ID	Cancer Type	Analysis date	Affinity bed	Blood volume processed (ml)	CTC/ml	WBCs/ml	Purity (%)
100	Colorectal, metastatic	04/24/12	FAPα	2	48.5	nd	nd
			FAPα	2	26	nd	nd
			EpCAM	2	18	nd	nd
			EpCAM	2	45	nd	nd
118	Colorectal, metastatic	05/02/13	FAPα	2	39.5	nd	nd
			EpCAM	2	111	nd	nd
125	Rectal, metastatic	05/03/13	FAPα	2	23.5	nd	nd
			EpCAM	2	17	nd	nd
116	Colorectal, non-metastatic	05/01/13	FAPα	2	16	nd	nd
			FAPα	2	12	nd	nd
			EpCAM	2	14	nd	nd
			EpCAM	2	11.5	nd	nd
122	Rectal, non-metastatic	05/03/13	FAPα	2	15	nd	nd
			FAPα	2	10	nd	nd
			EpCAM	2	13	nd	nd
			EpCAM	2	7	nd	nd
135	Colorectal, non-metastatic	06/14/13	FAPα	2.5	280	7.2	97.4
			EpCAM	2.5	34.8	4.8	87.9

Table S18. Average number of $CTC^{FAP\alpha}$ and CTC^{EpCAM} isolated from the blood of CRPC patients along with the purity of the selected fraction for CTC^{EpCAM}.

Patient ID	Analysis date	Blood volume processed (ml)	$CTC^{FAP\alpha}$/ml	CTC^{EpCAM}/ml	WBC/ml in EpCAM bed	Purity (%)
1	11/15/2012	1.5	12.7	39.3	4	90.8
2	11/08/2012	1.5	18.0	9.3	8	53.8
3	11/08/2012	1.5	27.3	33.3	8.7	79.3
4	11/15/2012	1.5	16.0	4.0	4.7	45.9
5	11/19/2012	1.5	23.3	20.0	2.7	88.1

Table S19. $CTC^{FAP\alpha}$ and CTC^{EpCAM} isolated from the blood of epithelial ovarian cancer (EOC) patients. The CTC number shown per ml represents the average. Also shown is the purity of the isolated fraction of CTCs and the average number of white blood cells (WBCs) enumerated per assay.

Patient Id	Disease/Treatment	Analysis date	Affinity bed	Blood volume (ml)	CTC/ml	WBC/ml	Purity (%)
#1	M-EOC-no chemo	01/17/14	FAPα	2	48.5	6	88.9
			EpCAM	2	157	16	90.8
#2	L-EOC-no chemo	01/22/14	FAPα	1.5	59.3	1.3	97.8
			EpCAM	1.5	73.3	2.7	96.5
#4	M-EOC-chemo	03/11/14	FAPα	2	4	na	
			FAPα	2	5	1	83.3
			EpCAM	2	45	na	
			EpCAM	2	40	2	95.2
#5	M-EOC-no chemo	03/12/14	FAPα	2	31	3.5	89.9
			EpCAM	2	246	4.5	97.6
#6	L-EOC-no chemo	05/12/14	FAPα	2	16.5	2.5	86.8
			EpCAM	2	170.5	8	95.5
#8	M-EOC-no chemo	07/25/14	FAPα	1	20	na	na
			EpCAM	1	100	na	na
#9	M-EOC-no chemo	09/03/14	FAPα	1.5	118	na	na
			EpCAM	1.5	76.7	na	na
#10	M-EOC-chemo	09/26/14	FAPα	1	42	na	na
			FAPα	1	68	na	na
			EpCAM	1	397	na	na
			EpCAM	1	476	na	na
#11	M-EOC-no chemo	10/03/14	FAPα	1	137	na	na
			EpCAM	1	680	na	na
			FAPα	1	94	na	na
			EpCAM	1	943	na	na
#12	M-EOC-chemo	10/22/14	FAPα	1	17	na	na
			EpCAM	1	54	na	na
			FAPα	1	26	na	na
			EpCAM	1	27	na	na
#13	M-EOC-no chemo	11/06/14	FAPα	2	32.5	na	na
			EpCAM	2	69	na	na
#14	M-EOC-chemo	03/27/15	FAPα	2	32	2	94.1
			EpCAM	2	29	3	90.6
#17	L-EOC-no chemo	04/27/15	FAPα	2	17.5	3	85.4
			EpCAM	2	119.5	5	96.0
#18	M-EOC-no chemo	05/01/15	FAPα	2	38	5	88.4
			EpCAM	2	64.5	3	95.6
#19	M-EOC-chemo	05/22/15	FAPα	2	37.8	3	92.6
			EpCAM	2	59.6	2	96.7
#20	M-EOC-no chemo	05/28/15	FAPα	2	13.5	3	81.8
			EpCAM	2	215.5	4	98.2

References

1 Adams, A. A. *et al.* Highly efficient circulating tumor cell isolation from whole blood and label-free enumeration using polymer-based microfluidics with an integrated conductivity sensor. *Journal of the American Chemical Society* **130**, 8633-8641, doi:10.1021/ja8015022 (2008).

2 Kamande, J. W. *et al.* Modular Microsystem for the Isolation, Enumeration, and Phenotyping of Circulating Tumor Cells in Patients with Pancreatic Cancer. *Analytical Chemistry* **85**, 9092-9100, doi:10.1021/ac401720k (2013).