Supplementary Figures and Tables

Novel platform for detection of CK+ and CK- CTCs

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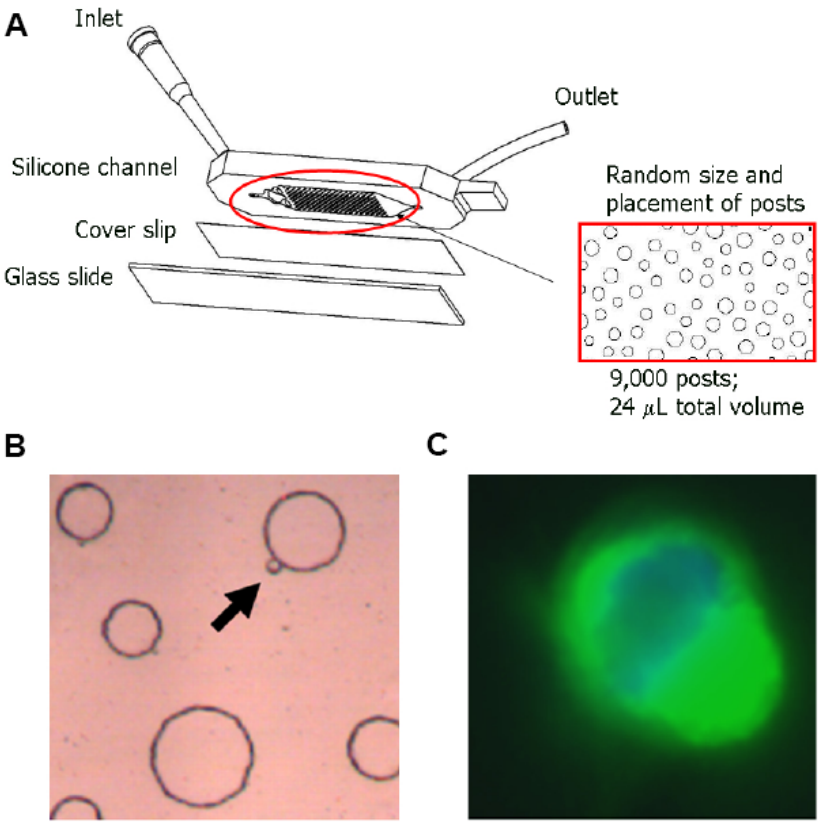
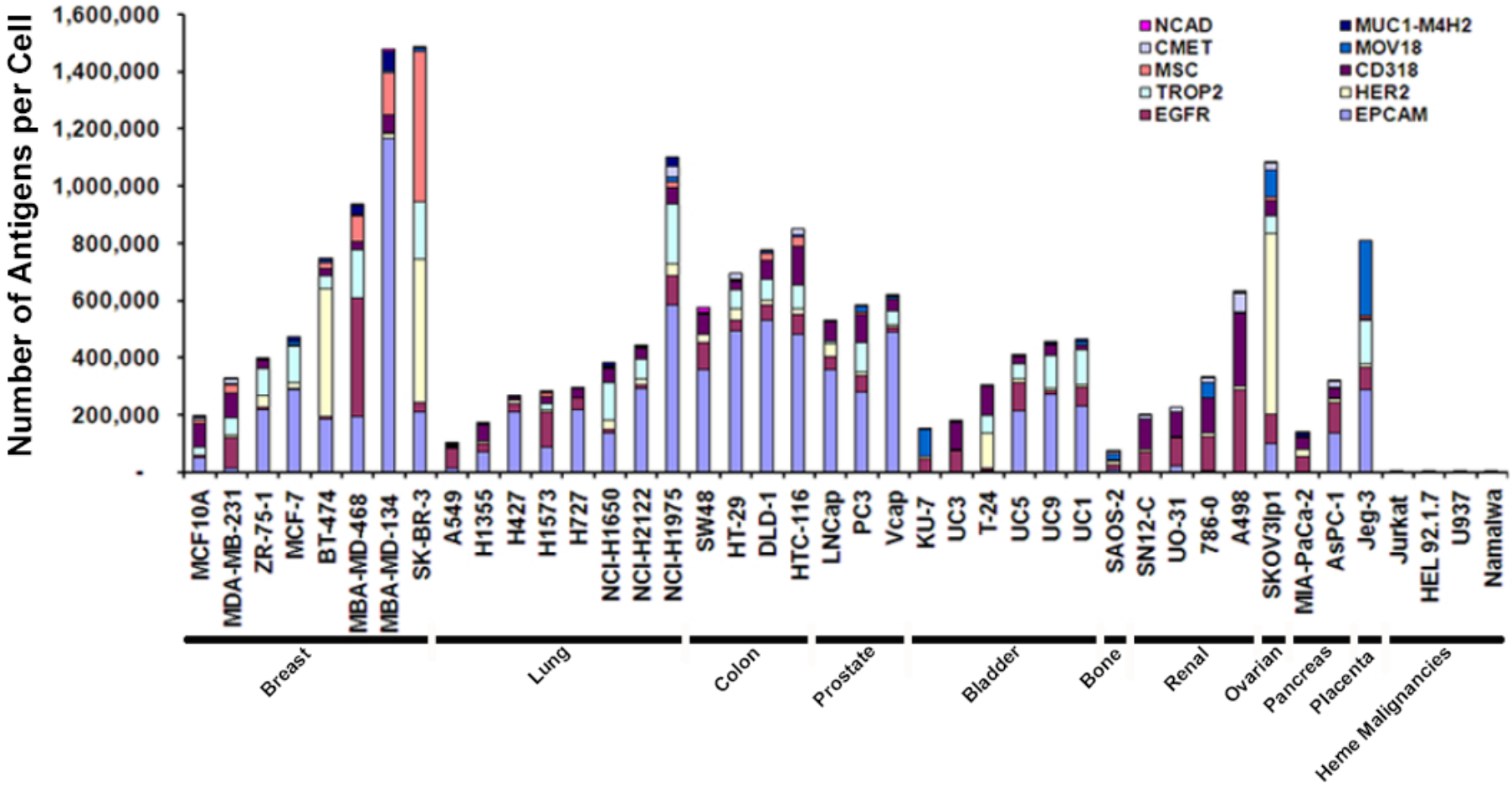
Supplementary Figure 1



Supplementary Figure 1. Cell enrichment and extraction (CEE^{TM}) platform. (**a**) The CEE^{TM} platform is centered on the principles of microfluidics to selectively capture and enrich target cells, including CTCs. The inclusion of a glass coverslip as part of the CEE^{TM} device allows direct and immediate visual assessment of captured cells, in addition to immunochemical and genetic analysis, using standard microscopy. Furthermore, the polymer chip holder, which forms the basis of the micro-channel consists of ~9000 posts of variable size and diameter. (**b**) Demonstration of the ability of the CEE platform to directly perform molecular characterization within the microchannel. (**c**) Representative image of a CK+/CD45-/DAPI+ CTC within the microchannel.

Supplementary Figure 2



Supplementary Figure 2. Expression of antibodies (antigens per cell) included in the AC15 cocktail in 43 cancer cell lines analyzed by flow-assisted cell sorting.

Supplementary Table 1

	EpCAM Only	Cocktail
CRC	31	28
Lung	12	37
Breast	4	4
CRC	4	10
CRC	42	45
Breast	11	19
Breast	4	3
Lung	5	68
Lung	3	9
Prostate	4	14
Breast	17	13
Breast	3	1
CRC	0	6
Lung	0	6
Breast	2	4
Breast	18	26
Skin	1	11
Breast	4	4
Lung	6	4
Prostate	74	62
Breast	13	24

Supplementary Table 1. Comparison of CTC capture between EpCAM only and the antibody cocktail using OncoCEE^{TM} CTC detection based on standard stain criteria (CK+/CD45-/DAPI+) using 8.5 mL blood from patients with advanced stage cancer.

Supplementary Table 2

Age (years)	Total CK+	Pathology	Stage	CA-125
55	0	Endometrioid ovarian adenocarcinoma	IV	8
41	7	Granulosa cell tumor of the ovary	Recurrent	13
59	0	Granulosa cell tumor of the ovary	Recurrent	14
31	1	High-grade adenocarcinoma + sarcomatoid features	IIb	9
62	0	High-grade endometrioid adenocarcinoma	Recurrent	37
49	0	High-grade mucinous adenocarcinoma	IIIc	210
73	4	High-grade mullerian adenocarcinoma	IV	76
66	0	High-grade papillary adenocarcinoma	IV	261
59	0	High-grade papillary serous adenocarcinoma	IIIc	26
69	0	High-grade papillary serous adenocarcinoma	IV	22
55	14	High-grade papillary serous adenocarcinoma	Recurrent	104
66	0	High-grade serous adenocarcinoma	Recurrent	----
66	1	High-grade serous adenocarcinoma	Recurrent	184
66	0	High-grade serous adenocarcinoma	Recurrent	15
70	0	High-grade serous adenocarcinoma	IIIc	295
54	0	High-grade serous adenocarcinoma	IIIc	9
69	1	High-grade serous adenocarcinoma	IIIc	5,505
69	0	High-grade serous adenocarcinoma	IIIc	273
45	0	High-grade serous adenocarcinoma	IIIc	116
78	0	High-grade serous adenocarcinoma	IV	213
74	0	High-grade serous adenocarcinoma	IV	44
60	1	High-grade serous adenocarcinoma	IV	67
58	0	Low-grade mixed endometrioid adenocarcinoma	Recurrent	1,147
59	11	Low-grade papillary serous adenocarcinoma	Recurrent	578
83	0	Metastatic malignant mixed mullerian tumor	IIIc	83
65	0	Peritoneal serous adenocarcinoma	Recurrent	26
53	21	Poorly differentiated ovarian adenocarcinoma	IV	18

Supplementary Table 2. CK+ cells captured with antibody cocktail in blood from women with ovarian cancer (cells per 17 mL of whole blood, total of two tubes).

Supplementary Table 3

Total	Complex Aneuploidy		Trisomy and Complex Aneuploidy				
CK+ Cells	CK+	CK–	CK+	CK–	Pathology	CA-125	Chemo
7	0	0	1	2	Granulosa cell tumor of the ovary	13	Yes
1	0	1	0	1	High-grade adenocarcinoma with sarcomatoid features	9	Yes
0	0	0	0	0	High-grade endometrioid adenocarcinoma	37	Yes
0	0	0	0	0	High-grade mucinous adenocarcinoma	210	Yes
4	2	0	2	2	High-grade mullerian adenocarcinoma	76	Yes
0	0	0	0	3	High-grade papillary adenocarcinoma	261	Yes
0	0	0	0	0	High-grade papillary serous adenocarcinoma	26	Yes
14	0	2	1	5	High-grade papillary serous adenocarcinoma	104	Yes
0	0	0	0	2	High-grade serous adenocarcinoma	295	No
0	0	0	0	1	High-grade serous adenocarcinoma	9	Yes
0	0	2	0	3	High-grade serous adenocarcinoma	213	Yes
0	0	0	0	3	High-grade serous adenocarcinoma	----	Yes
1	0	0	0	2	High-grade serous adenocarcinoma	184	Yes
11	1	0	1	1	Low-grade papillary serous adenocarcinoma	578	No
0	0	0	0	1	Metastatic malignant mixed mullerian tumor	83	No
0	0	0	0	2	Peritoneal serous adenocarcinoma	26	Yes
21	2	0	2	0	Poorly differentiated ovarian adenocarcinoma	18	Yes

Supplementary Table 3. Yield of ovarian circulating tumor cells when looking at complex aneuploidy (defined as at least one locus gain and a gain or loss at a separate locus) or at least trisomy in one locus (cells per 8.5 mL of whole blood, average scored, 2,137 cells/patient).

Supplementary Table 4

Total CK+ Cells	Complex Aneuploidy		Trisomy and Complex Aneuploidy				
	CK+	CK-	CK+	CK-	Pathology	Stage	CEA
1	0	2	1	3	Well-differentiated	I	2
6	0	0	1	1	Moderately differentiated	IIa	1
5	0	0	0	0	Poorly differentiated	IIa	6
5	0	0	0	2	Moderately differentiated	IIIb	2
1	1	1	1	2	Moderately differentiated	IIIb	1
0	0	0	0	0	Colon adenocarcinoma	Metastatic	158
0	0	0	0	0	Colon adenocarcinoma	Metastatic	57
0	0	0	0	1	Colon adenocarcinoma	Metastatic	2
0	0	0	0	0	Colon adenocarcinoma	Metastatic	17
11	0	1	0	4	Colon adenocarcinoma	Metastatic	62
5	0	0	1	8	Colon adenocarcinoma	Metastatic	787
5	1	1	2	2	Colon adenocarcinoma	Metastatic	4
0	0	0	0	0	Rectal adenocarcinoma	Metastatic	39
0	0	0	0	3	Rectal adenocarcinoma	Metastatic	59
12	0	0	0	0	Rectal adenocarcinoma	Metastatic	4

Supplementary Table 4. Cytokeratin and aneuploidy results for resected colon carcinoma (collected after resection) and metastatic colorectal carcinomas (cells per 8.5 mL of whole blood, average scored, 2,855 cells/sample). CEA, carcinoembryonic antigen.

SUPPLEMENTARY METHODS

CTC Capture and Detection. Blood samples were initially processed for recovery of peripheral blood mononuclear cells by using a Percoll density gradient method and Leucosep tubes (Greiner Bio-One, Monroe,NC). Each Leucosep tube was pre-filled with Percoll Plus (GE Healthcare, Piscataway, NJ) at a density of 1.083 g/mL (adjusted using normal saline) and stored at RT. Each 10-mL blood sample was diluted three-fold with a 1× phosphate-buffered saline (PBS) containing 1 mg/mL casein and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer and poured directly into a Leucosep tube. Samples were centrifuged for 15 minutes at 1000 *g* at RT in swinging bucket rotors (Allegra X-12R centrifuge; Beckman Coulter, Brea, CA), with breaks set to their lowest setting. After separation, the upper layer (above the separation barrier) was recovered by decanting into a 50-mL conical tube through a 70-μm cell strainer (BD). The decanted sample volume was adjusted to 45 mL with PBS/casein/EDTA and then centrifuged for 10 minutes at 400 *g*. Supernatant was removed by aspiration with use of a vacuum wand. The pellet was then resuspended and incubated with Fc blocker (100 μg/mL human IgG) and capture antibody cocktail (each antibody adjusted to 1 μg/mL) for 30 minutes at RT. The antibody cocktail consisted of: EpCAM, Trop-2, (BD Biosiceinces, San Diego, CA); c-Met and Folate binding protein receptor, (R&D Systems, Minneapolis, MN); N-Cadherin, (Sigma-Aldrich, St Louis, MO); CD318, MSC (Mesenchymal Stem Cell), Her2, (Biolegend, San Diego, CA); Muc-1 (Fitzgerald, Acton, MA); EGFR (Santa Cruz Biotechnology, Santa Cruz, CA). Each of the antibodies used in the cocktail has been tested one by one and as a mixture and was specifically chosen due to lack of capture of “unwanted” leukocytes from normal blood in our microfluidic device. This technology allows us to target several antigens simultaneously on tumor cells, and we use a cocktail of capture antibodies to target many possible tumor phenotypes. After incubation, the pellet was washed by adjusting the volume to 45 mL with PBS/casein/EDTA and centrifuging for 10 minutes at 400 *g* at RT. Biotinylated anti-mouse secondary antibody was added to the pellet and after mixing, was incubated for 30 minutes at

RT. The resulting pellet was washed three times with PBS/casein/EDTA. Each wash step consisted of centrifugation for 10 minutes at 400 *g*, followed by supernatant aspiration. The final pellet was suspended in 1 mL PBS/BSA/EDTA and subjected to capture and staining on the CEE^{TM} microchannel (manufactured at Biocept, Inc, San Diego).

Samples were pulled through microchannels with syringe pumps (manufactured at Biocept Inc, San Diego) at a volumetric flow rate of 18 µL/min. After the entire sample was processed through the microchannels, cells were cross-linked by using an NHS homobifunctional protein cross-linker and fixed with 80% MeOH. Cells were stained with a mixture of antibodies for cytokeratins 7/17, 18, 19, (BioLegend, San Diego, CA) and a pan-cytokeratin antibody targeting CK 4, 5, 6, 8, 13, 18 (BioLegend, San Diego, CA) labeled with AlexaFluor-488 (green), CD45 antibody (BioLegend, San Diego, CA) labeled with AlexaFluor-594 for 30 minutes, washed with PBS and stained with DAPI III counterstain. Channels were stored at +8°C until microscopic analysis.

Microscopic CTC enumeration was performed to detect CTC-based CK+/CD45-/DAPI+ stain criteria. The precise location (X- and Y-stage coordinates) of each CTC was recorded, permitting re-localization of cells after FISH for nuclear interrogation. Visualization was achieved and images captured with use of the Olympus BX51 fluorescence microscope (Olympus America Inc, Center Valley, PA) at 200× magnification. After enumeration, each microchannel was processed for multicolor FISH by using direct-labeled probes (Abbott Molecular, Des Plaines, IL) specific to the centromeres of chromosomes 8 (CEP 8-SpectrumAqua) and 11 (CEP 11-SpectrumGreen) and the locus-specific region on chromosome 20 (LSI 20q12-SpectrumOrange) for the ovarian cancer cases. Centromere-specific probes for chromosomes 8 (SpectrumAqua), 11 (SpectrumOrange), and 17 (SpectrumGreen) were used for colorectal cancer cases. For the breast cancer cohort, direct labeled probes specific to Her2/centromere

17 were employed (Abbott Molecular, Des Plaines, IL). Each FISH probe was initially validated for chromosomal localization and hybridization efficiency (sensitivity and specificity) on metaphases and 500 normal interphase cells from five independent donors within microchannels. For each test case reported here, the FISH scoring strategy involved two steps. First, each CTC identified during the enumeration step was relocated and scored for the number of signals present for each of the probes directly within the microchannel. Next, all remaining CK-/CD45-/DAPI+ cells captured within the microchannel were scored for the number of FISH signals present for each probe; this permitted identification of CTCs that are cytokeratin-negative and complex aneuploid (trisomic for one probe and monosomic or trisomic for another locus/probe). Visualization was achieved and images captured with use of the Olympus BX51 fluorescence microscope (Olympus America Inc) at 400× and 600× magnification.

Scoring criteria were based on observed hybridization efficiencies for each probe as evaluated on a total of 2500 normal peripheral blood lymphocytes (500 nuclei from each of five donor blood samples) and cultured tumor cells (500 each MCF7 and SKBr3 cells) captured within the microchannel. As expected, monosomy (presence of only one FISH probe signal) alone for any probe was observed in 5-10% of nuclei scored. Trisomy alone for any probe was observed in less than 0.2% of nuclei scored. Therefore, monosomy alone in a single cell was not sufficient to deem a cell as abnormal by FISH. However complex aneuploidy, as defined by the detection of at least one trisomy for one probe with at least one additional abnormality, monosomy or trisomy (i.e. combined aneuploidy at two loci with at least one locus being trisomic), for another probe was not observed in any of 2500 normal nuclei scored. Thus, these criteria were used to classify CK- and CK+ cells as complex aneuploidy.

Cell Lines and Flow Cytometry. MDA-MB-231 (ATCC, HTB-26), BT474 (ATCC, HTB-20), and SKBr3 (ATCC, HTB-30) breast cancer, T24 bladder, HeyA8 and SKOV3 ovarian cancer cell

lines were cultured according to ATCC recommendations, verified by morphology, growth curve analysis, and tested for mycoplasma. Experiments were performed within 6 months of receiving cell lines from an ATCC core facility and cell line identity was authenticated per ATCC standards. Measurement of surface antigens targeted by the capture cocktail was performed by incubating trypsin-detached, non-permeabilized breast cancer cells (listed above) with the indicated mouse anti-human IgG antibodies, followed by incubation with PE-labeled anti-mouse IgG (Sigma-Aldrich, St Louis, MO), according to a standard flow cytometry protocol. After additional washes to remove excess antibody, the cells were analyzed on the Accuri C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI). Flow cytometric estimation of the number of antibodies bound per cell was determined by using BD Quantibrite^{TM} PE beads (BD Biosciences), according to the manufacturer's instructions. By using known ratios of PE to antibodies, we converted PE molecules per cell to antibodies per cell.

Immunofluorescence staining on tissue. Both paraffin and frozen tissue sections of the primary tumors were blocked in 20% horse serum for 1 hour at RT before incubation with an anti-cytokeratin antibody mixture (see above) overnight at RT in a humidified chamber. After sufficient washing, binding of the secondary antibody linked to fluorescein isothiocyanate (FITC) was performed for 1 hour at RT. Slides were subsequently washed and mounted with use of DAPI mounting media (Vector Laboratories, Burlingame, CA). Visualization was achieved and images captured with use of the Olympus BX51 fluorescence microscope (Olympus America Inc) at 400× magnification.

Fluorescence *in-situ* hybridization. Formalin-fixed paraffin-embedded tumor sections were deparaffinized in xylene and rehydrated in an ethanol series. This was followed by pretreatment of the slides with a paraffin pretreatment kit III according to the manufacturer's recommendations (Abbott Laboratories, Abbott Park, IL). Slides were serially dehydrated and

allowed to air-dry. CEP8, CEP11, and 20q12 probes (Abbott Laboratories) were added to the dried slides and sealed with rubber cement. Cells captured in each of the microchannels were first dehydrated before the addition of the probe mixture. Co-denaturation of the probe mixture was performed on a ThermoBrite unit (Abbott Laboratories) at 75°C for 5 minutes (slides) or 95°C for 45 minutes (microchannels) followed by hybridization at 37°C overnight. Post-wash was performed at 74°C in 0.4× saline-sodium citrate (SSC) buffer containing 0.3% IPEGAL (Sigma-Aldrich, St. Louis, MO) followed by 2× SCC wash containing 0.1% IPEGAL and then counterstained with DAPI (blue). The slides and microchannels were imaged on the Olympus BX51 fluorescence microscope equipped with filters to view DAPI, SpectrumAqua, SpectrumOrange, and SpectrumGreen (Olympus America Inc). Images were analyzed with use of the ISIS imaging system v5.2 (Metasystems, GmbH, Germany). For slides, the number of fluorescent signals was counted in a minimum of 200 non-overlapping, intact nuclei. Abnormal FISH patterns were noted in percentages for each patient sample. Data are presented as the final percentage of normal and abnormal nuclei for each patient.

Matched blood and tissue collection. Blood samples were drawn before surgery from seven patients with ovarian cancer. Tumors removed during surgical resection were formalin-fixed and paraffin-embedded for pathologic review. Representative blocks confirming cancer by hematoxylin and eosin staining were assessed for complex aneuploidy and cytokeratin staining as described. Fresh frozen tissue was obtained from two patients for FISH quality control.

Quantitative real-time PCR. Total RNA was isolated by using a Qiagen RNeasy kit. Using 500 ng of RNA, cDNA was synthesized by using a Verso cDNA kit (Thermo Scientific) as per the manufacturer's instructions. Analysis of mRNA levels was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR Green-based real-time PCR, using primers designed with Primer Express (Applied Biosystems). Specific primers for vimentin (5′-

TCCAAGCCTGACCTCACTGC-3′ (forward) and 5′-TTCATACTGCTGGCGCACAT-3′ (reverse)), N-cadherin (5′-GCCATTGATGCGGATGATC-3′ (forward) and 5′-CCTGTACCGCAGCATTCCAT-3′ (reverse)), Twist (5′-TCGACTTCCTGTACCAGGTCCT-3′ (forward) and 5′-CCATCTTGGAGTCCAGCTCG-3′ (reverse)) and Snail (5′-CCCAAGGCCGTAGAGCTGA-3′ (forward) and 5′-GCTTTTGCCACTGTCCTCATC-3′ (reverse)) were used; 18S rRNA was used as a housekeeping gene. PCR was done with reverse-transcribed RNA and 100 ng/μL of sense and antisense primers in a total volume of 20 μL. Each cycle consisted of 15 seconds of denaturation at 95°C and 1 min of annealing and extension at 60°C (40 cycles).

***Ex vivo* spiking experiments.** To demonstrate precision and reproducibility in capture of tumor cells, cultured cells were spiked into normal peripheral blood from healthy donors. In demonstrating precision, ~10, ~25, ~50 T24, BT474, MDA-MB231, SKBr3, and SKOV3 cells were spiked into 10 mL of whole blood in triplicate. Cell spiking was performed by first diluting the cells into their corresponding media and counting the number of cells in a series of 10ul spots. Based on the number of cells counted, the average was calculated for the number of cells per microliter and then additional calculations were made to obtain the volume required for the number of cells needed to spike. Next, the amount spiked was determined by calculating the average of three spot counts. Due to potential for confounding with spike experiments, multiple replicates are often required. Therefore, given that the numbers plotted on the graph represent the average number of cells spiked, it is possible that a proportion of spiked samples had higher numbers of cells added, and the actual number recovered may be larger than the expected number spiked. A total of 30 tubes of blood (10 mL) were prepared and processed for these precision studies. For reproducibility experiments, each of the above tumor cells lines was spiked at ~150 tumor cells to 10 mL whole blood from normal donors in 10 independent replicates. Thus, a total of 50 tubes of blood (10 mL) were prepared and processed for

reproducibility as described for clinical samples. To determine the actual concentration and spiked number of tumor cells for each cell line was based on sequential spotting of equal volume cell suspensions onto glass slides. Spiked tumor cell and whole blood mixtures were allowed to incubate overnight at room temperature prior to processing as described for clinical samples. To demonstrate capture of post-EMT cell populations, SKOV3ip1 cells were grown in RPMI 1640 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). For EMT induction, cells were grown in RPMI 1640 without serum supplemented with recombinant human TGF-beta1 (R&D Systems, Minneapolis, MN) at a concentration of 10 ng/mL for 72 hours. Cells were trypsinized at 72 hours and spiked *ex vivo* into mouse blood that had been added to anticoagulant citrate dextrose (ACD) containing anti-clumping reagent (CEE-Sure^{TM}; Biocept, San Diego, CA) prior to entering a microchannel. For samples described here, the same antibody cocktail was used for capture followed by processing and capture within microchannels.

Orthotopic ovarian mouse experiment. Ten nude mice were injected with 2.5 x 10^5 HeyA8 ovarian cancer cells into the peritoneal cavity. Mice were monitored until the first mouse became moribund, which occurred 36 days after injection. All mice were killed on the same day and about 350 µL of blood per mouse was collected by cardiac puncture and added to pre-filled eppendorf tubes containing ACD. Blood samples were processed in microchannels as described.

Statistical analysis. Differences in continuous variables were analyzed using the Mann-Whitney rank sum or *t*-test. A P-value < 0.05 was considered statistically significant.