SUPPORTING INFORMATION

Ascites analysis by a microfluidic chip allows tumor cell profiling

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Fig. S1. Profiling of cancer cell lines. Twelve different ovarian cancer (OvCA) cell lines, and six benign cell lines (two mesothelial cell lines (LP9, LP3); two benign ovarian cell lines (TIOSE4,TIOSE6); primary human lymphocytes/neutrophils) were tested for their expression levels (λ=signal/background-1) of putative diagnostic protein markers using flow cytometry. For each marker the frequency of cell lines with λ >1.5 (red) are shown on right hand side of the heat map (grey bar) and are rank-ordered by abundance of positive cell lines. The data is categorized into 4 subgroups: i) markers present in malignant cells (Unique Malignant; top left), ii) markers in malignant and benign cells (Ubiquitous, right), iii) markers in benign cells only (Benign; middle left), and iv) markers absent in both cell types (Absent; bottom left). This dataset was used to identify markers for subsequent analysis of primary human samples (**Fig. 2**). Parenthesis represents different antibodies used for the same marker (**Table S2**).

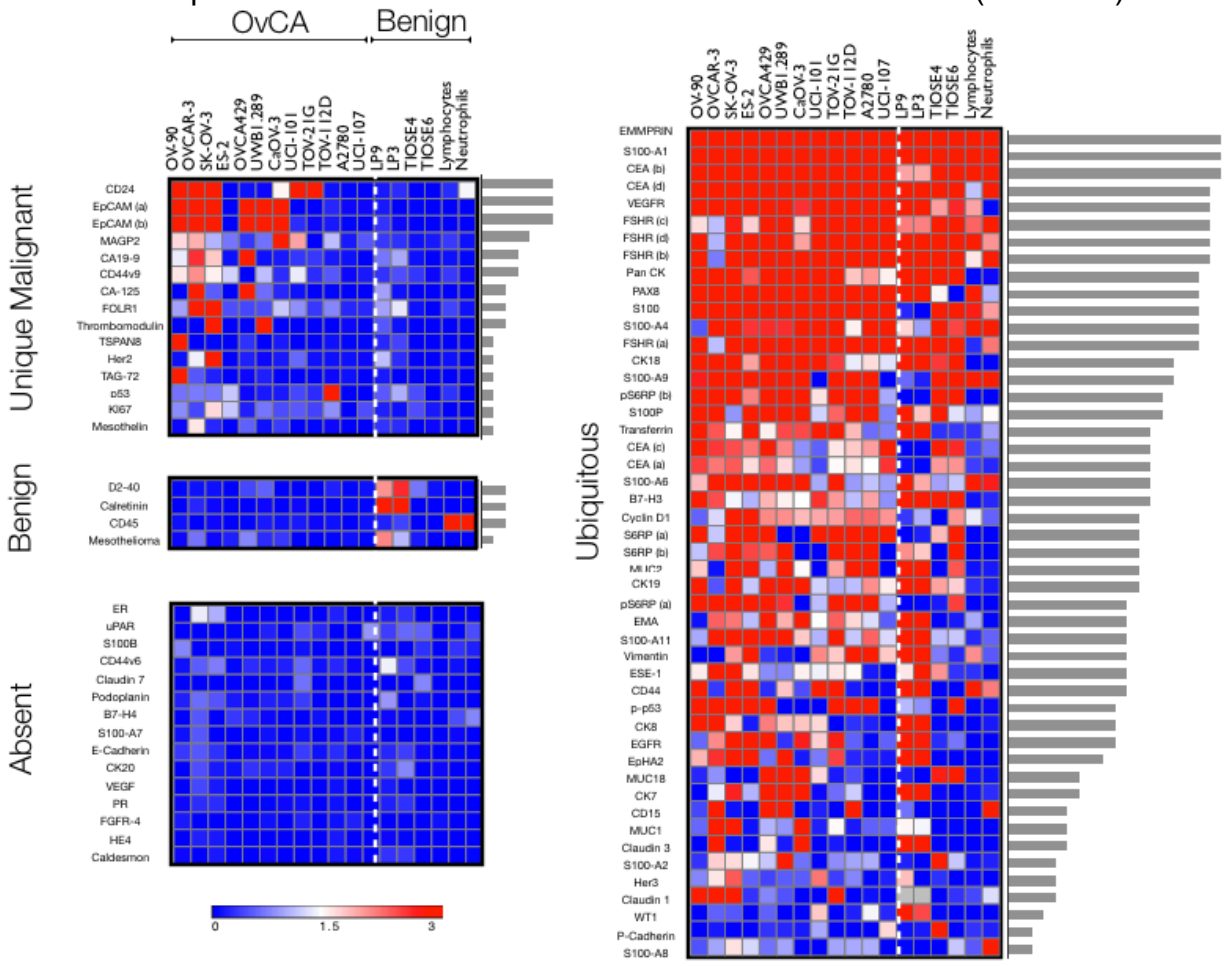


Fig. S2. Shows representative histograms of a reduced set of markers and cell lines from the large scale cell profiling data shown in **Fig. S1**. The reduced set includes 7 different markers(EpCAM, CD24, TAG-72, CA19-9, Vimentin, Calretinin, and CD45) measured across 12 cell lines (8 OvCA, 4 Ctrl) using flow cytometry. The fluorescent signal of each marker is shown in blue and the control is shown in red. The control in this example refers to the secondary antibody without the primary antibody.

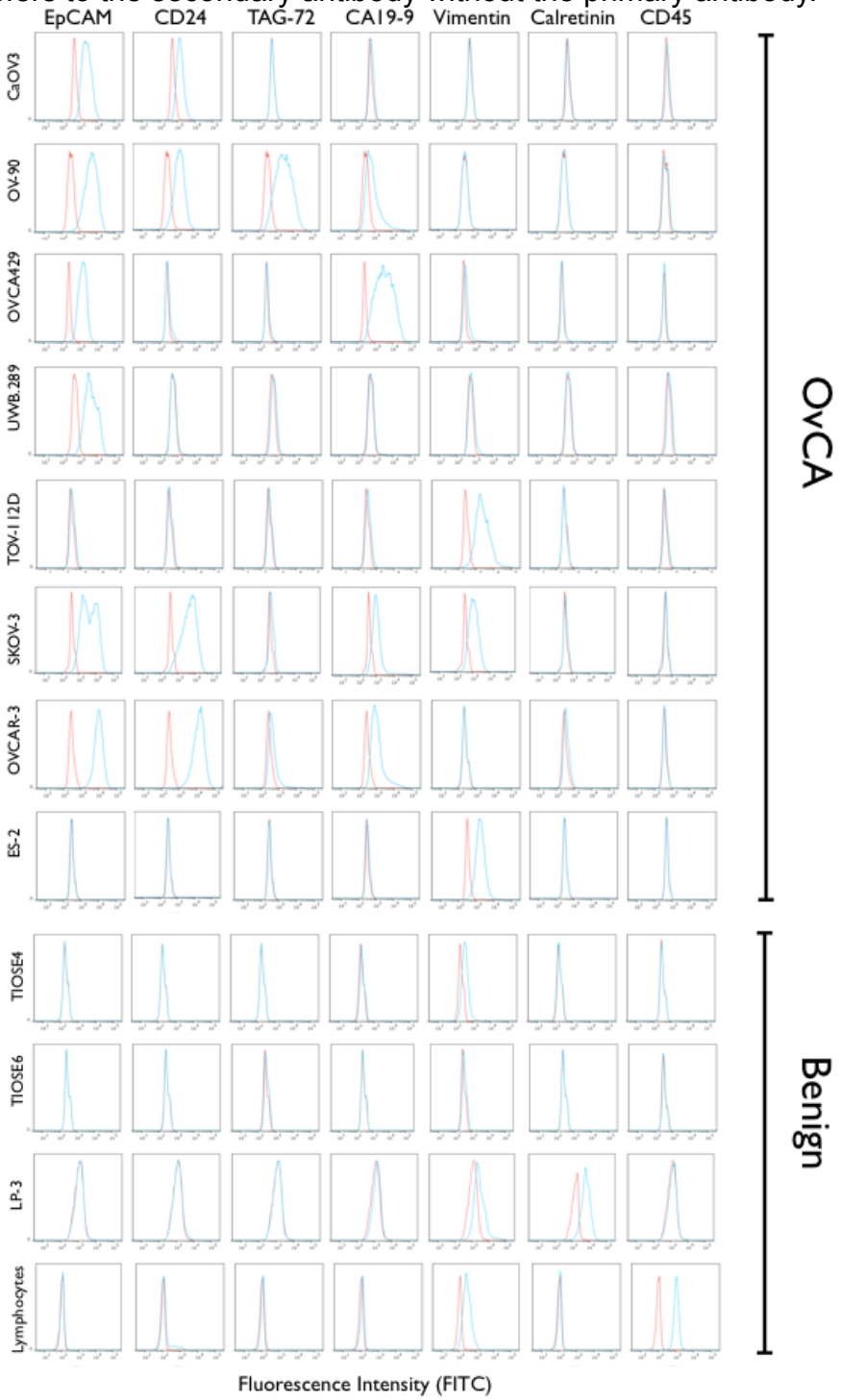


Fig. S3. Flow cytometry data of 8 representative clinical samples from the training set (**Fig. 2**). Histograms for 6 different markers (EpCAM, CD24, TAG-72, CA19-9, Vimentin, or Calretinin) are shown for four OvCA populations (Calretinin-/CD45-) and four mesothelial cell populations (Calretinin+). The primary antibody cocktail consisted of CD45 rat antibody, calretinin rabbit antibody, and mouse antibody for the marker of interest (e.g. either EpCAM, CD24, TAG-72, CA19-9, Calretinin, or Vimentin). The secondary antibody cocktail consisted of three fluorophores: Anti-mouse FITC, Anti-Rabbit AF647, and Anti-Rat PeCY7. Calretinin-AF647/CD45-PECy7 negative cells or Calretinin-AF647 positive cells were then gated on (see **Fig. 2** for details). The signal over noise ratio (SNR) was determined by dividing the geometric mean of the signal (blue) over that of the ctrl (red) (λ=sig/ctrl-1). Flow cytometry data was analyzed using FlowJo 7.6.3.

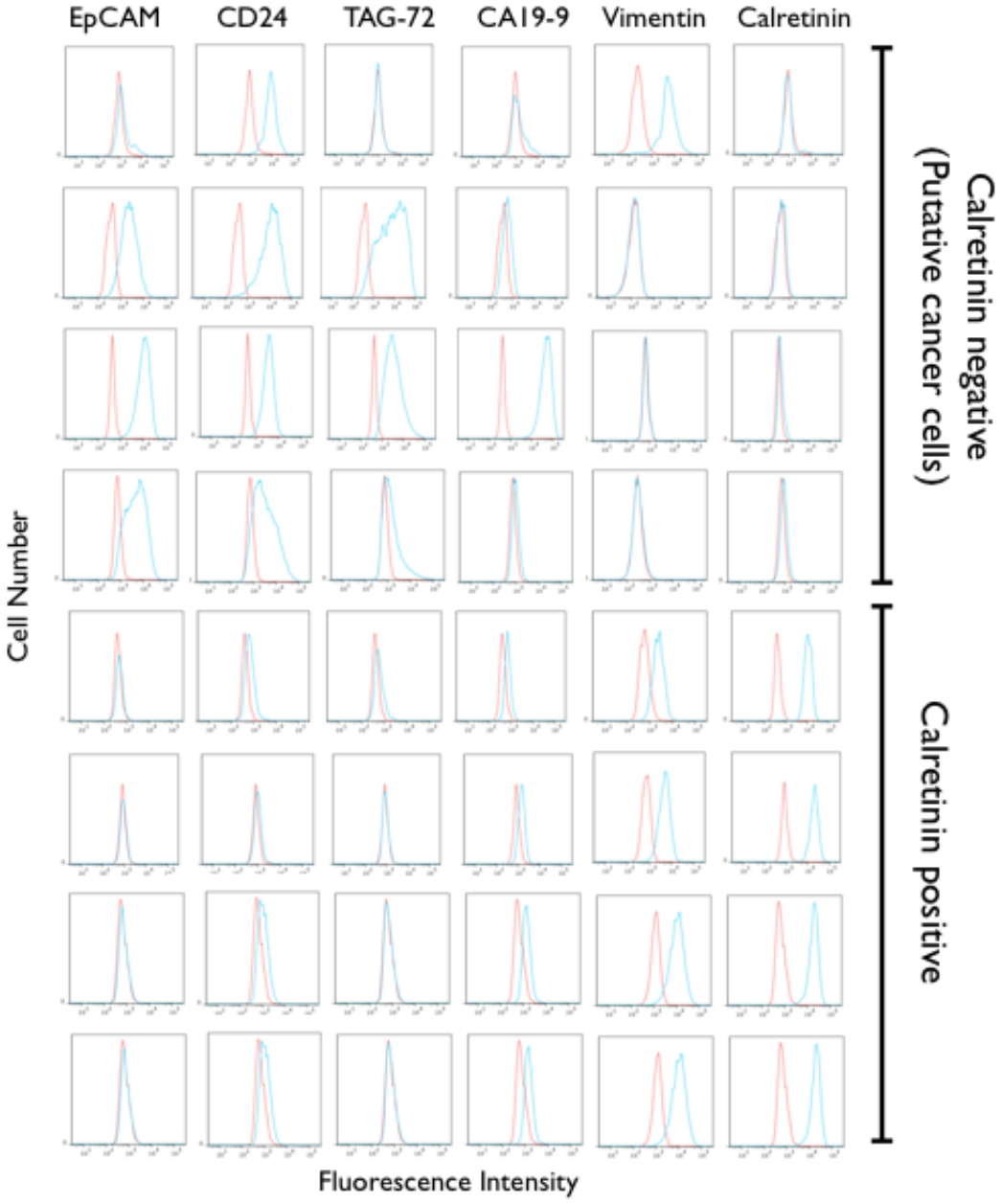
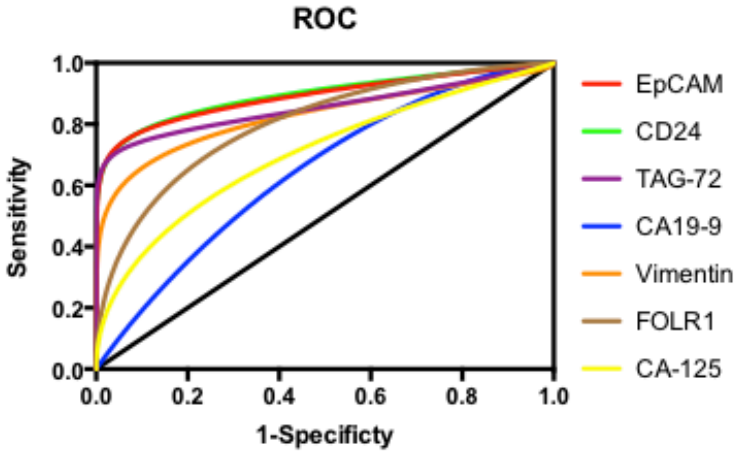


Fig. S4. ROC analyses of training set. ROC curves were plotted for individual markers using the 18 patient samples in the training set (top). The area under the curve (AUC) and the optimal cutoff level were calculated and are summarized in the bottom table. The cutoff values were then used to determine the sensitivity, specificity, accuracy of each individual marker and the V3 and ATC_{dx} panel (**Table 3, Table S1**).



Markers	AUC	Optimal Cutoff level	SE	95% CL
EpCAM	0.92	0.40	0.074	0.778 to 1.068
CA19-9	0.59	1.57	0.121	0.355 to 0.828
CD24	0.92	0.81	0.074	0.778 to 1.068
TAG-72	0.82	0.49	0.096	0.635 to 1.010
FOLR1	0.75	1.65	0.099	0.551 to 0.940
CA-125	0.61	10.47	0.123	0.369 to 0.850
Vimentin	0.86	2.20	0.078	0.705 to 1.011

Fig. S5. Ascites cellular composition and volume. Ascites samples from 65 patients with (blue; n=46) or without (green; n=19) ovarian cancer were analyzed for total cell number (top left), malignant cell number (bottom left; ATCs), cell volume (top right) and fraction of malignant cells compared to total cells (bottom right). Viable cells were counted using trypan blue staining and the Countess cell counter (Invitrogen). Malignant cell number were determined using ATC_{dx} via flow cytometry (see Methods for more details). Data are plotted as waterfall plots.

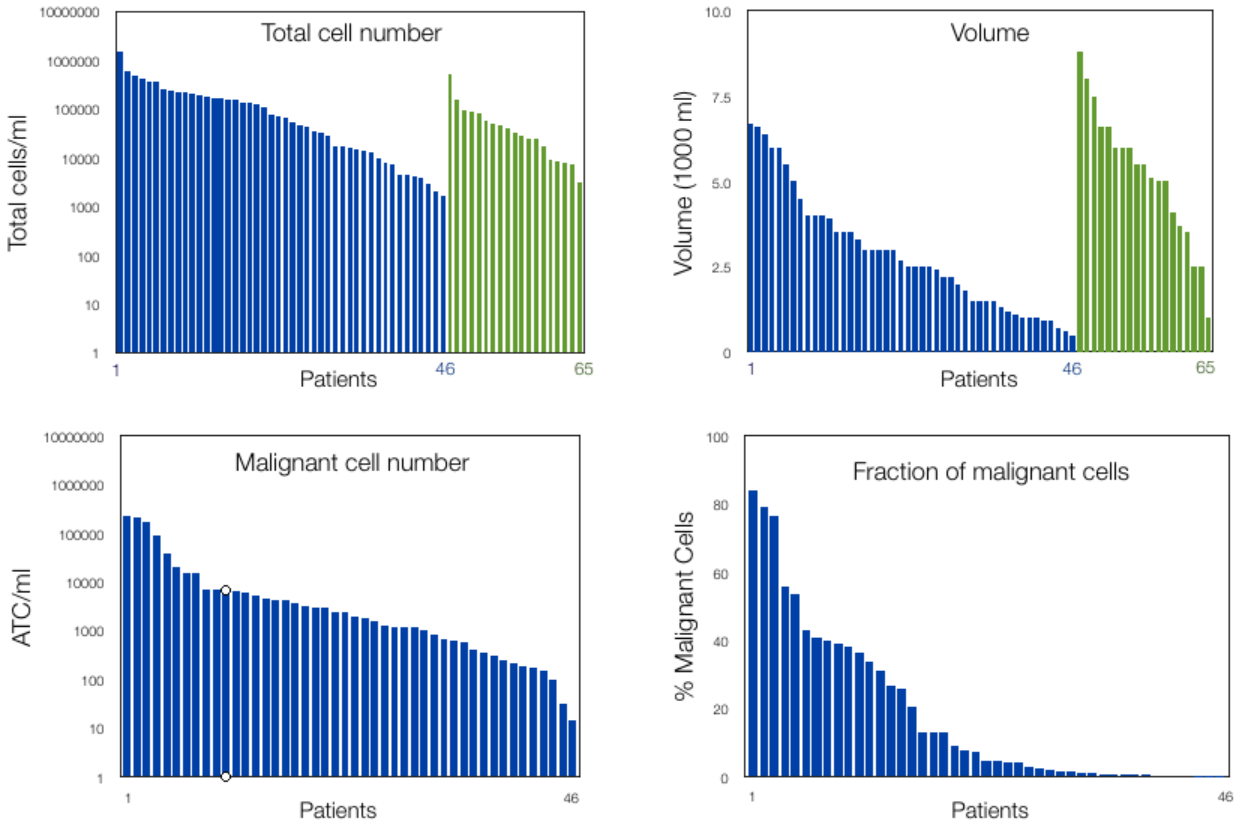


Fig. S6. ATC chip design and measurements. A representative example of an ascites sample before purification (A) and after on-chip purification (B). Captured cells (green represents DAPI staining) in the 20 μm capture sites of the ATC chip.

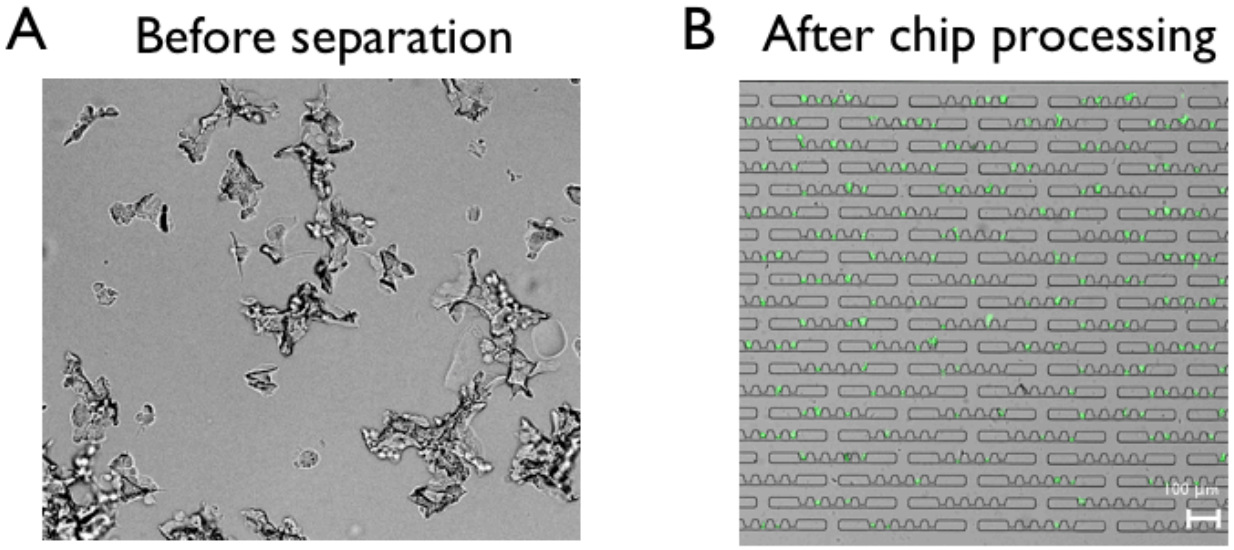
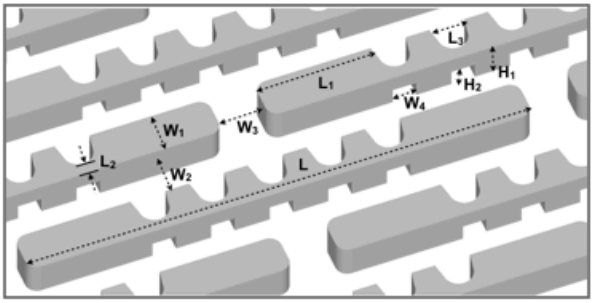


Fig. S7. ATC chip design and measurements. (A) The layout of the ATC chip consists of four different sized capture sites (15, 20, 30 and 40 μm; n = 4,925). Capture site dimensions are μm scale. (B) The capture site in the device has a bowl-like structure with an underpass gap that allows smaller cells to pass through. The length of Path A is considerably shorter than Path B resulting in less flow resistance. Due to the flow resistance difference, most of the fluid flows through path A rather than Path B. Cells that attempt to pass through Path A that are larger than the capture site will be trapped while smaller cells will pass through.

A

Capture Sites Size (μm)	# of capture sites	L	L_1	L_2	L_3	W_1	W_2	W_3	W_4	H_1	H_2
40 μm	495	580	100	5	60	60	60	120	40	60	30
30 μm	1190	480	100	10	40	45	50	100	25	60	30
20 μm	1490	430	100	10	30	35	40	40	20	30	15
15 μm	1750	340	100	5	20	25	25	50	7	30	15



B

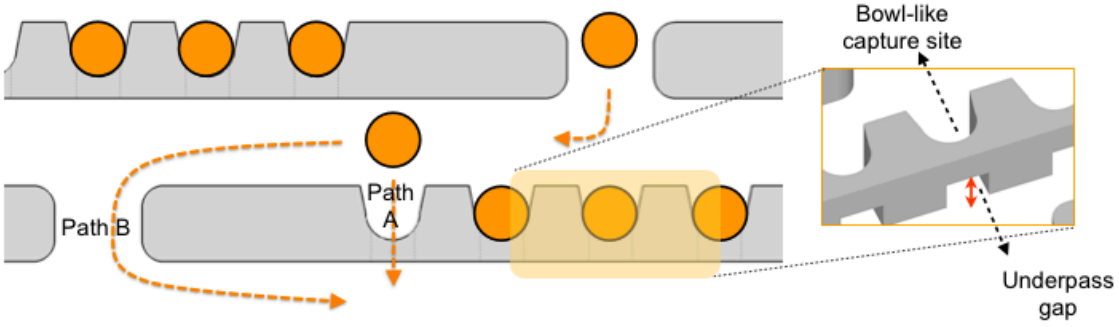


Fig. S8. Schematic of on-chip labeling and purification. First, ascites fluid is collected from the patient which contains malignant cells (red) amongst an anti-inflammatory milieu of host cells (green). Ascites cells are added to the chip followed by an antibody cocktail (EpCAM-FITC, Vimentin-PE/Cy7 Calretinin-Biotin/AF647, CD45-Biotin/AF647, Marker-AF555). Streptavidin-coated magnetic particles then bind to the benign mesothelial cells (Calretinin+) and leukocytes (CD45+). A magnet under the inlet captures the benign cells (red) while the malignant cells (green) pass freely through the microchip. The four different size microwells (40, 30, 20,15 μm) allow for capture of the malignant cells while allowing for the typically smaller leukocytes to pass through the device. The ATC_{dx} signature (EpCAM+ and/or Vimentin+/Calretinin-/CD45-) can then be imaged to determine number of ATCs.

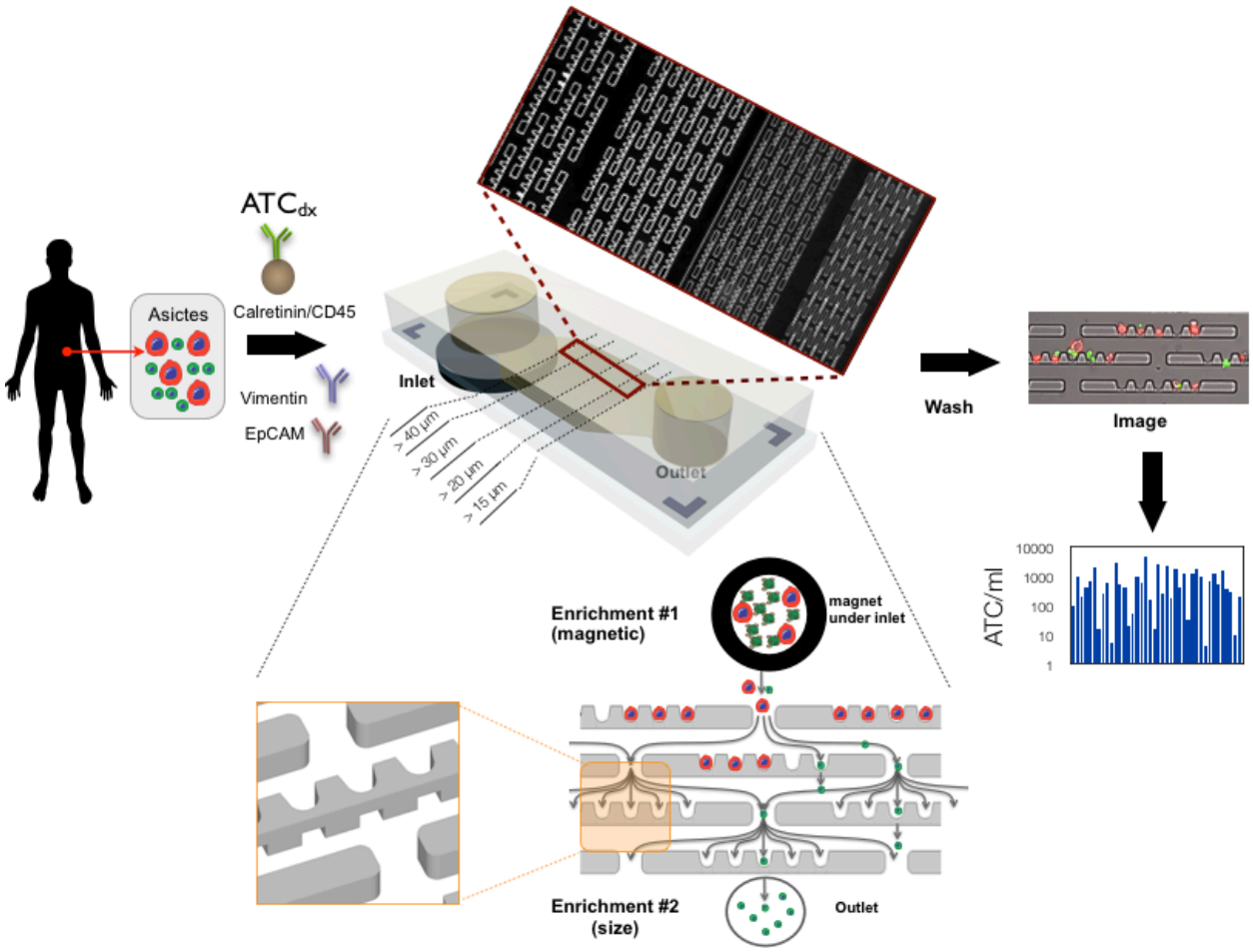


Fig. S9. Predictive ATC markers of treatment response. Key treatment response markers are plotted for 6 patients who were analyzed serially and either responded to treatment (left) or progressed (right). Responders typically have proliferation (Ki67, p-Histone 3, p-CyclinD), mRNA translation (p-4E-BP1) and protein translation (p-S6RP) markers downregulated compared to the non-responders. Each marker was measured in duplicate for each time point and the error bars represent the SEM.

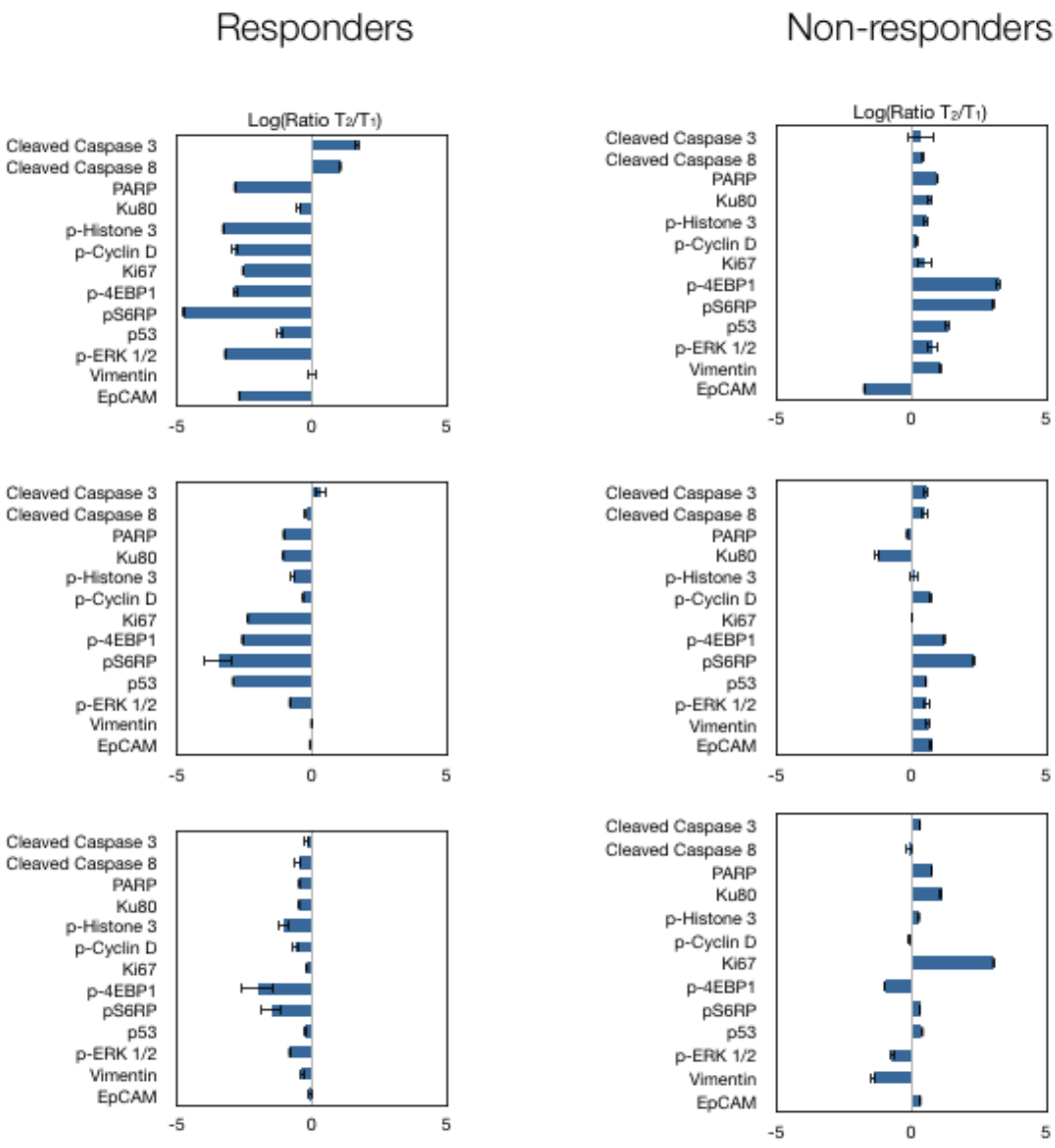


Table S1. Sensitivity, specificity, and accuracy of different protein markers in the test set.

	Test set (n = 47)					
Marker	**EpCAM**	**CA19-9**	**CD24**	**TAG-72**	**V3**	**ATC_{dx}**
Sensitivity	93.9	35.7	85.7	78.6	15.2	100.0
Specificity	100.0	100.0	100.0	100.0	100.0	100.0
Accuracy	95.7	53.8	89.7	84.6	40.4	100.0

Table S2. List of antibodies used in the study.

Number	Biomarker	Clone	Company	Species	Dilution
1	53BP1	Polyclonal	Cell Signaling	Rabbit	1 to 100
2	B7-H3	185504	R&D	Mouse IgG1	1 to 100
3	B7-H4	MIH43	Pierce	Mouse IgG1	1 to 100
4	CA-125	X75	Abcam	Mouse IgG1	1 to 100
5	CA-19-9	SPM110	Abcam	Mouse IgG1	1 to 20
6	Caldesmon	h-CD	Dako	Mouse IgG1	1 to 30
7	Calretinin	DC8	Invitrogen	Rabbit	1 to 100
8	Calretinin	DAK-Calret 1	Dako	Mouse IgG1	1 to 50
9	CD15	28	AbCam	Mouse IgM	1 to 100
10	CD24	SN3 A5-2H10	eBioscience	Mouse IgG1	1 to 50
11	CD44	IM7	Biolegend	Rat IgG2b	1 to 50
12	CD44v6	2F10	R&D	Mouse IgG1	1 to 100
13	CD44v9	RV3	Dr. Hideyuki Saya (Keio University)	Rat IgG2a	1 to 250
14	CD45	YTH24.5	Abcam	Rat IgG2b	1 to 100
15	CD45	H130	Biolegend	Mouse IgG1	1 to 50
16	CD45-Alexa Fluor 647	H130	Biolegend	Mouse IgG1	1 to 100
17	CD45	Polyclonal	Abcam	Rabbit	1 to 100
18	CEA (a)	M111146	Fitzgerald	Mouse IgG1	1 to 100
19	CEA (b)	M85151A	Fitzgerald	Mouse IgG1	1 to 100
20	CEA (c)	M111147	Fitzgerald	Mouse IgG1	1 to100
21	CEA (d)	487618	R&D	Mouse IgG1	1 to 100
22	CK18	DA-7	EXBIO	Mouse IgG1	1 to 100
23	CK19	A53-B/A2.26	Neomarkers (Pierce)	Mouse IgG2a	1 to 20
24	CK20	Q2	Thermo Scientific	Mouse IgG1	1 to 20
25	CK7	OV-TL 12/30	Neomarker	Mouse IgG1	1 to 20
26	CK8	C-43	Affinity Bioreagents	Mouse IgG1	1 to 100
27	Claudin 3	385021	R&D	Mouse IgG2a	1 to 100
28	Claudin 7	4D4	Abnova	Mouse IgG2a	1 to 50
29	Cleaved Caspase 3	5A1E	Cell Signaling	Rabbit	1 to 100
30	Cleaved Caspase 8	18C8	Cell Signaling	Rabbit	1 to 100
31	Cyclin D1	CD1.1	GeneTex	Mouse IgG1	1 to 100
32	D2-40	D2-40	Abcam	Mouse IgG1	1 to 50
33	E-Cadherin	HECD-1	Life Technologies	Mouse IgG1	1 to 30
34	EGFR	F4	Abcam	Mouse IgG1	1 to 100
35	EMA	E29	Dako	Mouse IgG2a	1 to 30
36	EMMPRIN	109403	R&D	Mouse IgG2b	1 to 100
37	EpCAM (a)	MOC-31	Dako	Mouse IgG1	1 to 30
38	EpCAM (b)	BerEP4	Dako	Mouse IgG1	1 to 100
39	EpCAM-FITC	BerEP4	Dako	Mouse IgG1	1 to 100
40	EpHA2	371805	R&D	Mouse IgG2a	1 to 100
41	ER (Estrogen Receptor alpha)	6F11	Abcam	Mouse IgG1	1 to 100
42	ESE-1	Polyclonal	Abcam	Rabbit	1 to 100
43	FGFR-4	4FR6D3	Biolegend	Mouse IgG1	1 to 50
44	FOLR1	548908	R&D	Mouse IgG1	1 to 100
45	FSHR (a)	Polyclonal	GeneTex	Rabbit	1 to 100
46	FSHR (b)	H-190	Santa Cruz	Rabbit	1 to 20
47	FSHR (c)	Polyclonal	Novus Biologicals	Rabbit	1 to 100
48	FSHR (d)	Polyclonal	Genetex	Rabbit	1 to 100

49	HE4	3F9	Abnova	Mouse IgG2b	1 to 50
50	Her2	191924	R&D	Mouse IgG2b	1 to 100
51	Her3	RTJ2	Abcam	Mouse IgG1	1 to 100
52	Ki67	B56	BD Pharmingen	Mouse IgG1	1 to 50
53	Ki67-Alexa Fluor 555	B56	BD Pharmingen	Mouse IgG1	1 to 30
54	Ku80	C48E7	Cell Signaling	Rabbit	1 to 100
55	MAGP2	Polyclonal	Abnova	Rabbit	1 to 100
56	Mesothelin	K1	Abcam	Mouse IgG1	1 to 100
57	Mesothelioma	ME1	Thermo Scientific	Mouse IgG1	1 to 50
58	MUC1	M01102909	Fitzgerald	Mouse IgG1	1 to 100
59	MUC18	128018	R&D	Mouse IgG1	1 to 100
60	MUC2	M53	Neomarker (Pierce)	Mouse IgG2a	1 to 20
61	p-4E-BP1	17489	Cell Signaling	Rabbit	1 to 100
62	P-Cadherin	104805	R&D	Mouse IgG1	1 to 100
63	p-Cyclin D	D29B3	Cell Signaling	Rabbit	1 to 100
64	p-Histone 3	D2C8	Cell Signaling	Rabbit	1 to 100
65	p-p44/42 MAPK (p-ERK 1/2)	D13.14.4E	Cell Signaling	Rabbit	1 to 100
66	p53	1C12	Cell Signaling	Mouse IgG1	1 to 100
67	Pan Cytokeratin	C-11	Axxora	Mouse IgG1	1 to 100
68	PARP	46D11	Cell Signaling	Rabbit	1 to 100
69	PAX8	Polyclonal	Proteintech	Rabbit Poly	1 to 50
70	p-p53	FP3.2	Pierce	Mouse IgG1	1 to 100
71	Podoplanin	NZ-1.3	eBioscience	Rat IgG2a	1 to 100
72	PR (progesterone receptor)	1A6	Millipore	Mouse IgG1	1 to 30
73	p-S6RP (a)	D57.2.2E	Cell Signaling	Rabbit	1 to 50
74	p-S6RP (b)	2F9	Cell Signaling	Rabbit	1 to 50
75	S100	6G1	Fitzgerald	Mouse IgG1	1 to 200
76	S100-A1	1D5	Abgent	Mouse IgG1	1 to 100
77	S100-A11	2F4	Abgent	Mouse IgG2a	1 to 50
78	S100-A2	M2	Abgent	Mouse IgG2a	1 to 50
79	S100-A4	1F12-1G7	Abgent	Mouse IgG1	1 to 100
80	S100-A6	6B5	Abgent	Mouse IgG1	1 to 100
81	S100-A7	1A4	Abgent	Mouse IgG1	1 to 100
82	S100-A8	2H2	Abgent	Mouse IgG2a	1 to 100
83	S100-A9	1C10	Abgent	Mouse IgG	1 to 100
84	S100B	472806	R&D	Mouse IgG2a	1 to 100
85	S100P	4E7	Abnova	Mouse IgG2b	1 to 100
86	S6RP (a)	5G10	Cell Signaling	Rabbit	1 to 50
87	S6RP (b)	54D2	Cell Signaling	Mouse IgG1	1 to 50
88	TAG-72	CC49	Abcam	Mouse IgG1	1 to 30
89	Thrombomodulin	1009	Dako	Mouse IgG1	1 to 30
90	Transferrin	29806	R&D	Mouse IgG1	1 to 100
91	TSPAN8	458811	R&D	Rat IgG2b	1 to 100
92	uPAR	62022	R&D	Mouse IgG1	1 to 100
93	VEGF	VG1	Dako	Mouse IgG1	1 to 30
94	VEGFR 2	KDR/EIC	Abcam	Mouse IgG1	1 to 100
95	Vimentin	Vim 3B4	Abcam	Mouse IgG2a	1 to 100
96	Vimentin	280618	R&D	Rat IgG2a	1 to 100
97	WT1	6F-H2	Millipore	Mouse IgG1	1 to 100

Table S3. List of secondary antibodies used in the study.

Fluorophore	Biomarker	Clone	Company	Species	Dilution
Alexa 647	Anti Mouse	Polyclonal	Cell Signalling	Goat	1 to 900
FITC	Anti Mouse	Polyclonal	Abcam	Goat	1 to 300
Dylight 650	Anti Rabbit	Polyclonal	Abcam	Goat	1 to 300
PE/Cy7	Anti Rat	Poly4054	Biolegend	Goat	1 to 300
Dylight 488	Anti Rabbit	Poly4064	Biolegend	Donkey Ig	1 to 300
FITC	Anti Mouse IgM	II/41	eBioscience	Rat IgG2a	1 to 150
FITC	Anti Rat IgG2a	RG7/1.30	BD Pharmingen	Mouse IgG2b	1 to 150
FITC	Anti Rat IgG2b	G15-337	BD Pharmingen	Mouse IgG2b	1 to 150
FITC	Anti Rabbit	RG-96	Sigma	Mouse IgG1	1 to 150