Site-Specific Antibody Modification and Immobilization on a Microfluidic Chip to Promote Capture of Circulating Tumor Cells and Microemboli

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Table of contant

Experimental section:

Materials and Instruments:

All chemicals were obtained from commercial sources and without further purification. Sulfosuccinimidyl-6-(biotin-amido) hexanoate (Sulfo-NHS-LC-biotin **1,** Pierce), biotin-PEG7-amine (**2A**, Bioscience), *N*-biotinyl-3,6-dioxaoctane-1,8-diamine trifluoroacetate salt solution (**2B**, Sigma-Aldrich), biotin ethylenediamine hydrobromide (**2C**, Sigma-Aldrich), (+)-biotinamidohexanoic acid hydrazide (**2D**, Sigma-Aldrich), biotin hydrazide (**2E**, Sigma-Aldrich), EZ-Link™ alkoxyamine-PEG12-biotin (**2F**, ThermoFisher Scientific), EZ-Link™ alkoxyamine-PEG4-Biotin (**2G**, ThermoFisher Scientific), sodium (meta) periodate ($NaIO_4$, Sigma-Aldrich), sodium cyanoborohydride ($NaBH_3CN$, Sigma-Aldrich), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti Polar Lipids) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-cap-biotinyl (b-PE, Avanti Polar Lipids), neutravidin (NA, Life Technologies), Triton X-100 (Sigma-Aldrich) and paraformaldehyde (PFA, Sigma-Aldrich), were used as received. Monoclonal mouse anti-human antibody EpAb4-1 (anti-EpCAM mAb) were generated as previously reported.[R1] The recombinant human EpCAM/Fc chimera (Sino Biological) was used as antigen in ELISA and QCM assays.

Protein concentration was determined by Nanodrop 1000 spectrophotometer (Thermo Scientific). The UV absorption in 96-well plate was determined by SpectraMax $M2^e$ microplate reader (Molecular Devices). Syringe pump (PHD 2000, Harvard Apparatus) was used in microfluidic chip processing. Cell images were taken with a Nikon-Ti Eclipse microscope at 100 x magnification, and analyzed with NIS-Elements AR Analysis software (Nikon).

Synthesis of biotinylated anti-EpCAM

*Random biotinyl anti-EpCAM (**Ab-1**)*

The synthesis of random biotinyl anti-EpCAM (**Ab-1**) was similar with our previously described protocol.[R1a] Freshly prepared 130 μL of 10 mM (2.8 mg in 500 μL dd-H_2O) sulfosuccinimidyl-6-(biotin-amido) hexanoate (Sulfo-NHS-LC-biotin **1**) was dissolved in double-distilled water, which was then added to 2 mL antibody solution (2.0 mg/mL) in PBS buffer (10 mM PBS + 150 mM NaCl, pH 7.4). The mixture was allowed to react at room temperature for 1 h to form random biotinyl anti-EpCAM (**Ab-1**). Excess biotin ligands were removed by dialysis in PBS buffer at 4 ^{o}C for 24 h with buffer solution changes for three times. The final concentration of **Ab-1** was determined by Nanodrop 1000 spectrophotometer.

*Site-specific biotinylation anti-EpCAM (**Ab-2A** to **Ab-2G**)*

Site-specific biotinylation anti-EpCAM (**Ab-2A** to **Ab-2G**) were prepared in following procedure. Briefly, sodium (meta) periodate ($NaIO_4$, 3 mg, 14 μmole) in an eppendorf tube was added with 1 mL of 2.5 mg/mL monoclonal antibody EpAb4-1 (anti-EpCAM mAb) in PBS buffer (10 mM PBS + 150 mM NaCl, pH 7.4). The reaction tube was mixed gently on a rotator at room temperature for 30 min in dark condition. The excess sodium periodate was then removed by a filtration using a 30 K molecular-weight-cut-off centrifuge filter (Microcon® Centrifugal Filters, Merck Millipore) at 4 ^{o}C, centrifuged at 13500 rpm for 5 min and then washed with PBS twice. The retained oxidized antibody was resuspended in 2 mL of PBS (pH 7.4). The individual biotin-ligands **2A**, **2B**, **2C**, **2D**, **2E**, **2F** and **2G** were prepared as stock solutions in a concentration of 25 mM in PBS. The 0.5 mL oxidized IgG antibody was added separately to 50 μL of 25 mM biotin ligand (**2A** to **2G**) stock solution, followed by incubation at room temperature for 30 mins, and then 5 μL of freshly prepared 5 M $NaBH_3CN$ in PBS was subsequently added into each of reaction mixtures at 4 ^{o}C for 16 hr . After the conjugation reactions, the remained aldehyde groups from each of the biotinyl antibody IgG were quenched with 50 μL of 1M tris. HCl (pH 7.4) at room temperature for another 30 mins. Excess biotin ligands, $NaBH_3CN$ and tris-HCl were removed by dialysis in PBS buffer at 4 ^{o}C for 24 hr with buffer solutions change for three times. The final concentrations of each biotinyl anti-EpCAM antibody (**2A** to **2G)** were determined by Nanodrop 1000 spectrophotometer.

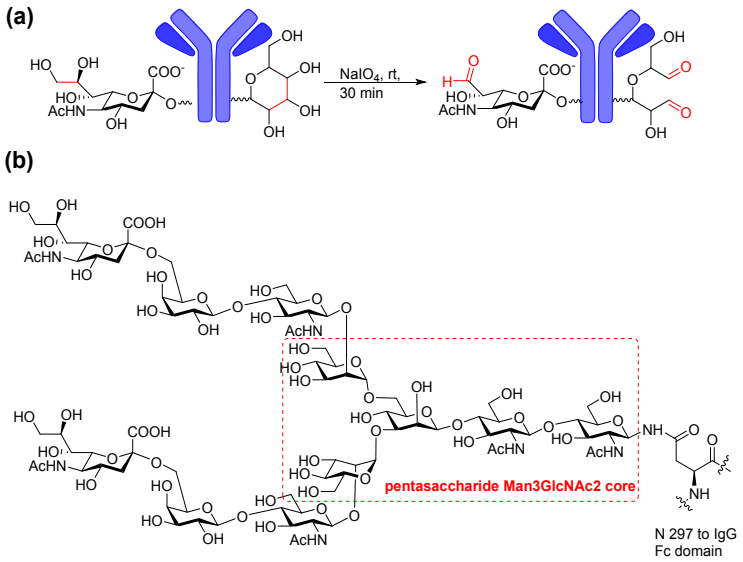


Figure S1. (a) Schematic illustration showing exocyclic sialic acid diols or endocyclic cis-diols of mono-sugar on *N*-glycan moiety of the antibody, which can be oxidized by excess sodium *m*-periodate ($NaIO_4$) to produce reactive aldehyde groups. (b) one of typical *N*-glycans types in mammals. Note: sialic acid is not always – but less commonly present on mammalian IgGs Fc *N*-glycans. Values from~ 6% to 26% for monosialylated glycans and 1% to 13% for disialylated glycans in human IgG were reported. The Asn N297-linked glycan is a complex, usually core-fucosylated, biantennary glycan containing a pentasaccharide Man3GlcNAc2 core, which can be modified by addition of terminal galactose or sialic acid.[R2] (Man, mannose; GlcNAc, *N*-acetylglucosamine)

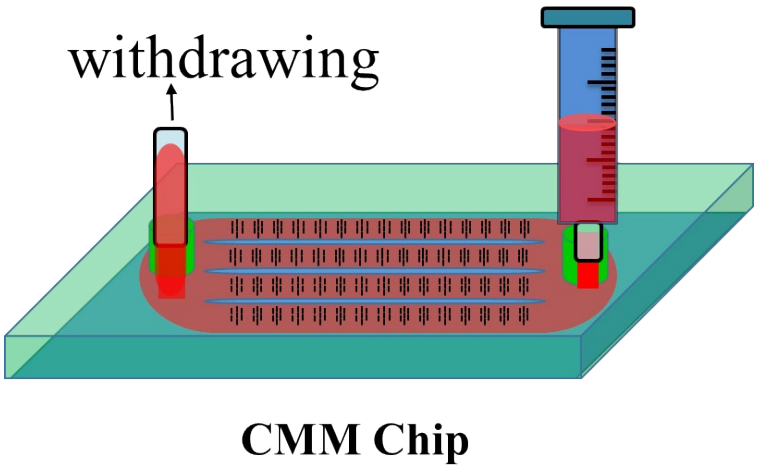


Figure S2. An illustration of chaotic mixing microfluidic (CMM) chip. Syringe loaded with 2 mL blood and connected at the inlet of the chip. The blood sample will then be processed through the microfluidic channel on the CMM chip by syringe pump withdrawal. CMM chip has slight modification from CMx chip. [R3-4]

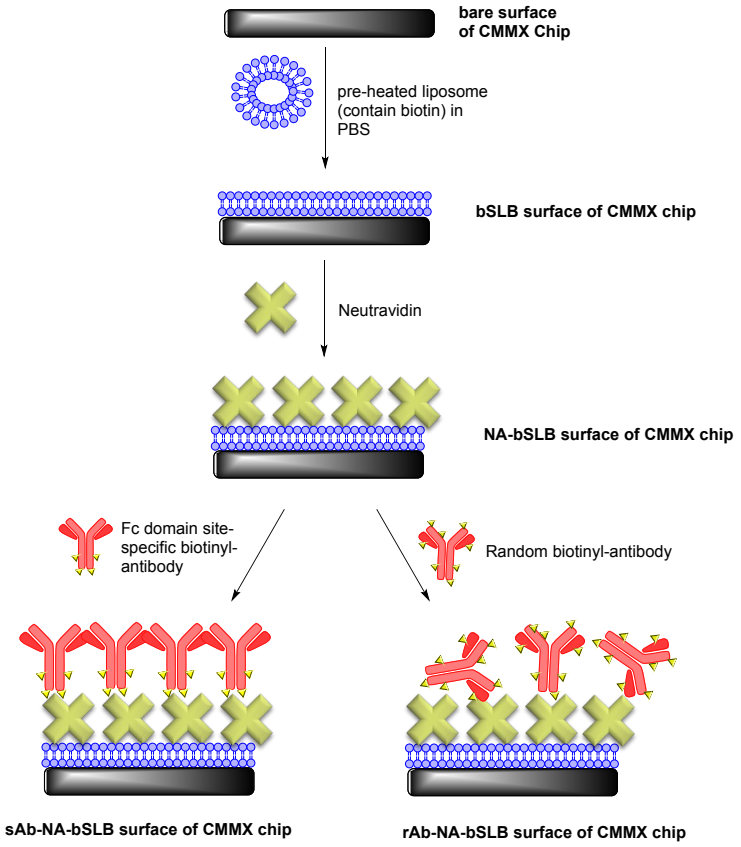


Figure S3. Schematic illustration showing reaction steps on formation of anti-EpCAM surface on the SLB of CMM chip.

The biotin number of biotinylated anti-EpCAM mAb assay

Event of biotinylation on each anti-EpCAM can be determined by HABA kit (Pierce). The number of each biotinylation antibody was shown in **Table S1**.

Table S1. The biotin number of each biotinylated anti-EpCAM antibody.

Ligand	Biotinylated anti-EpCAM antibody	Biotin number
1' (batch1)	**Ab-1'**	13.0
1 (batch 2)	**Ab-1**	10.6

2A	**Ab-2A**	4.3
2B	**Ab-2B**	4.8
2C	**Ab-2C**	2.2
2D	**Ab-2D**	5.4
2E	**Ab-2E**	3.4
2F	**Ab-2F**	9.2
2G	**Ab-2G**	9.1

Enzyme-linked immunosorbent assay (ELISA) for the reactivity of different biotinyl anti-EpCAMs to its antigen.

The principle of the ELISA was shown in **Figure S4**. A 96-well NeutrAvidin™ coated plate (Pierce) was washed twice with PBS and blocked with 1% BSA for 30 min. The different biotinylated Anti-EpCAM mAbs (**Ab-1**, **Ab-2A**, **Ab-2B**, **Ab-2C**, **Ab-2D**, **Ab-2E**, **Ab-2F** and **Ab-2G**) and a non-biotinylated anti-EpCAM mAb (as control) in concentration of 3.0, 0.3 and 0.03 uf/mL, respectively, in 1% BSA and in PBS, were added at 100 uL to wells of the plate and incubated at 4^{o}C overnight. The plate was then washed with 100 µL of PBS containing 0.1% w/v Tween 20 (PBST) once and PBS twice. Recombinant human EpCAM/Fc chimera as antigen (100 µL, 0.1 µg/mL) in PBS was added and incubated at room temperature for 2 h. After washing with PBST once and PBS twice, HRP conjugated goat anti-human IgG secondary antibody (1:30,000 dilution, 0.033 µg/mL, Sino Biological) in 1% BSA in PBS were added and incubated at room temperature for another 1h. After washing with PBST once and PBS three times, the plate was incubated with 100 µL of substrate solution 3,3',5,5'-Tetramethylbenzidine (TMB, eBioscience) for ~ 10 mins. The reaction was stopped by adding 50 µL of 0.16 M H_2SO_4, and the plate was readout using a microplate reader at 450 nm.

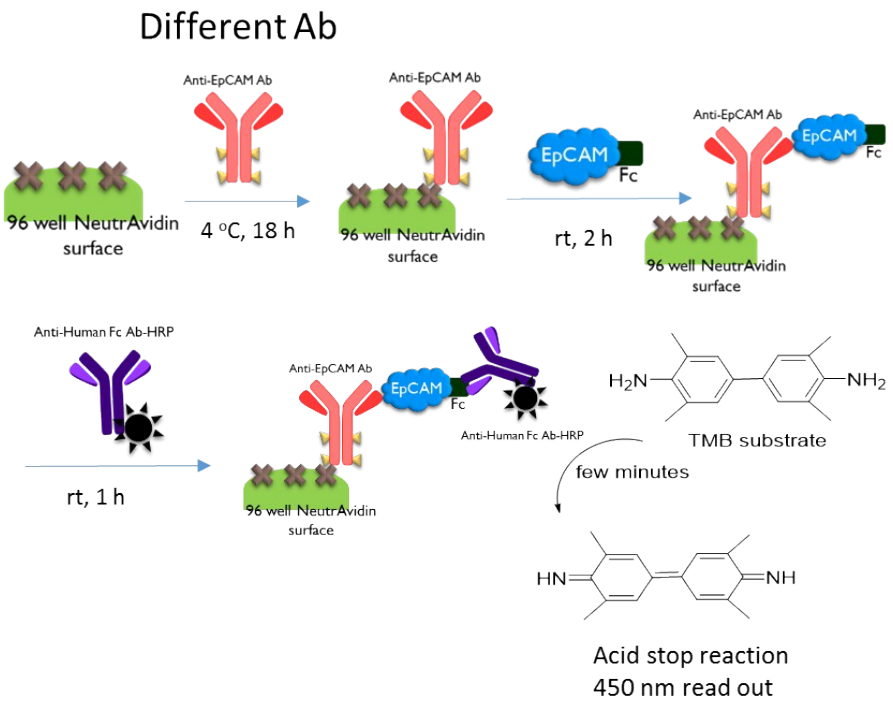


Figure S4. Schematic illustration of ELISA assay principal for the ability of diverse biotinyl anti-EpCAM in recognizing its antigen.

QCM measurements

The silicon oxide (SiO_2) -coated Quartz Crystal Microbalance (QCM) crystal chips (AT-cut quartz crystals, f_0 = 5 MHz) (Q-Sense AB) were cleaned in 0.1 M sodium dodecyl sulfate, followed by rinsing with Milli-Q water, then dried under nitrogen stream, and finally exposed to oxygen plasma for 20 sec. The concentration and washing conditions of each coating step in the QCM-D chamber were identical to those performed for CMM platforms. Preparation of biotin-doped vesicles and formation of supported lipid bilayer (SLB) were described previously.[R4] Briefly, as shown in **Figure S3**, the cleaned QCM crystal chip was exposed to POPC/b-PE (85/15, molar percentage) vesicle mixture with 0.15 mg/mL lipid concentration to form biotinylated SLB (bSLB), followed by extensive rinsing with PBS buffer to remove excess vesicles. Next the bSLB-coated substrate was incubated with a 0.125 mg/mL solution of NA for 60 mins, followed by rinsing extensively with PBS buffer to remove excess NA, forming NA-bSLB. Finally, immobilized anti-EpCAM surface was formed by introducing 0.03 mg/mL solution of biotinylated antibody to NA-SLB surface, forming anti-EpCAM-NA-bSLB. To study binding ability of anti-EpCAM on supported lipid bilayer (SLB) to EpCAM antigen, two concentrations of EpCAM antigen (0.01 and

0.03 mg/mL) were subsequently introduced to test its specific recognition on anti-EpCAM-NA-bSLB surface. For QCM-D measurement, the chamber was temperature-stabilized to 24.98 °C. All measurements were recorded at the third overtone (15 MHz), and the data shown here for calculations were normalized to fundamental frequency (5 MHz) by dividing the overtone number.[R5]

Calculations[R5]*:*

A linear relationship has been observed between the mass adsorbed Δm and the frequency change:

$$\Delta m = \frac{C\Delta f_z}{z}$$

where, C is the Sauerbrey mass sensitivity constant (around −17.7 ng/cm^2 Hz for a 5 MHz crystal in water) and Δf_z is the frequency shift measured at the zth overtone. The antibody molecular weight was used as a typical IgG as 150 KDa. The diameter of silicon oxide crystal chip is 1 cm.

Table S2. QCM calculation.

	Ab-1	**Ab-2F**	**Ab-2G**
Δf Ab loading	31	42	39
Δm (ng/cm^2) Ab loading	186	247	232
Δf Antigen	24	45	47
Δm (ng/cm^2) Antigen	144	266	276

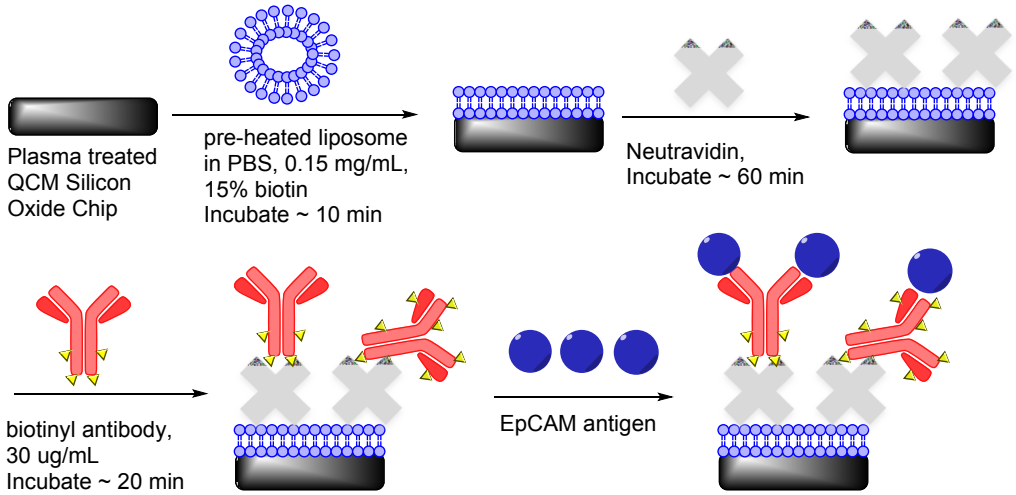


Figure S5. Schematic illustration of Quartz Crystal Microgravimetry (QCM) assay including steps on formation and coating of each later.

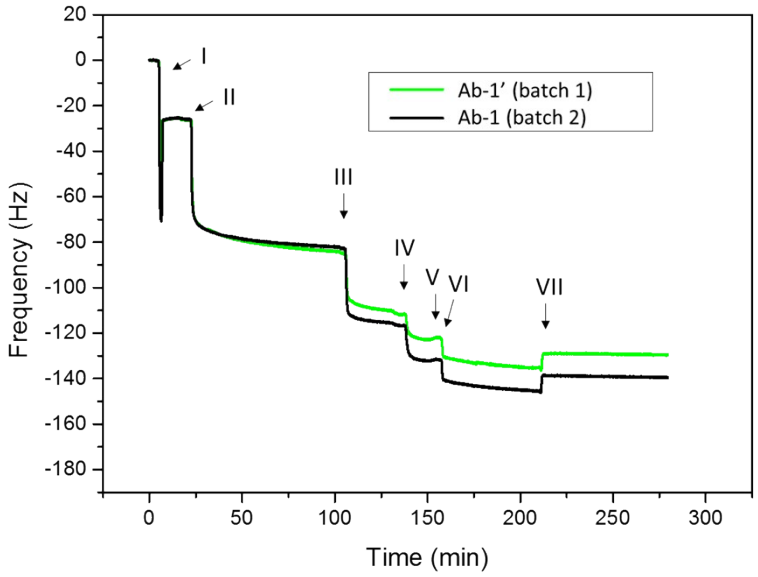


Figure S6. The *in situ* QCM kinetic monitoring data for two batches of random biotinylation anti-EpCAM antibodies (**Ab-1** and **Ab-1'**) that has shown different anti-EpCAM loading amount and corresponding EpCAM antigen recognition ability.

Biotin-lipid containing lipid vesicles were introduced at point (I) to form bSLB on silicon oxide chip. NA solution was injected at point (II) for specific binding on SLB, forming NA-bSLB. Random biotinylation anti-EpCAM antibodies (bench 1 and bench 2) solutions were injected at point (III) to form Ab-NA-bSLB, respectively. EpCAM antigen (10 μg/mL) was injected at point (IV), incubated for 20 min and then PBS was introduced at point (V) to wash excess of antigen. EpCAM antigen (30 μg/mL) was

injected again at point (VI), incubated for 50 mins and then PBS was introduced at point (VII) to wash excess antigen and finally forming saturated AT-Ab-NA-bSLB layers on silicon oxide chip.

Cell Culture

Human cancer cell lines were purchased from Bioresource Collection and Research Center (BCRC, Taiwan). HCT-116, PANC1 or ASPC-1 were incubated and maintained with Dulbecco's modified Eagle's medium (DMEM, Life Technologies) for HCT-116 and PANC1, and RPMI1640 (Life Technologies) for ASPC-1, respectively, supplemented with 10% fetal bovine serum (FBS, Life Technologies), and 1% antibiotic-antimycotic solution (ThermoFisher Scientific, Corning, NY) in a humidified incubator with 5% CO_2 atmosphere at 37 ^{o}C. Cells were gently harvested by using accutase (Merck Millipore) to ensure the cell surface antigen would not be degraded.

CMM Chip Platform Sample Processing

The preparation of anti-EpCAM surface coated microfluidic chip

The detailed fabrication and surface modification processes of CMM chips were similar as previously reported. [R1a, R3-4, R6-7] Briefly, the CMM chips were 76 mm long, 26 mm wide. The CMM chips were comprised of an oxygen-plasma-treated glass at the slide bottom and a PMMA top plate with a 60-μm-deep line groove that was bounded by a 60-μm-thick, double-coated, acrylic adhesive. For the chip surface coating, 200 μL of biotin-containing lipid vesicles (0.15 mg/mL) consisting of POPC/b-PE (85/15, mole percentage) and syringe pump will be used to withdraw the samples to fill the microfluidic channels. This will then be incubated at room temperature for 30 min to form biotinylated supported lipid bilayer (bSLB). The coating chip was subsequently washed with 600 μL PBS to remove excess vesicles and unbound lipids. The biotinylated supported lipid bilayer (bSLB)-coated chip was then filled with 200 μL Neutravidin (NA, 0.125 mg/mL) solution in PBS with the aid of syringe pump and incubated at 4 ^{o}C for 16 h to form NA-bSLB on chip surface. After incubation, the excess NA was rinsed with 600 μL of PBS. Finally, site-specific or random biotinylated anti-EpCAM antibody (200 μL, 0.03 mg/mL) was injected into the micro-channel and

left for 1hr at room temperature followed by PBS rinse to remove excess biotinylated anti-EpCAM before sample loading.

The microfluidic chip process

The sample loading and processing in the chip was similar as previously reported. [R1a, R3-4, R6] 2 mL samples (culture medium or whole blood) were flowed through the microfluidic chip at 1.8 mL/h flow rate. Once this 2 mL samples have filled up the microfluidic chip and the syringe pump has come to a stop, the device was rinsed with 0.4 mL PBS at 1.8 mL/h flow rate following by addition of 1.5 mL PBS at 3 mL/h flow rate. Captured circulating cancer cells were then released by air foam generated from 5% bovine serum albumin (BSA, Millipore). [R4, R7] The released cells will be contained in foam solution in 1.5 mL eppendorf at volume approximately 300 µL. A 100 µL solution of 16% paraformaldehyde (PFA, pH = 7.4) in PBS was added, gently mixed by pipetting, and incubated for 10 min in the Eppendorf tube at 4^{o}C.

The process of spiking cancer cells in cell culture medium or whole blood samples.

Cell lines (HCT-116, ASPC-1 or PANC-1) were detached with an accutase, a cell detachment solution, following by addition of fresh media. The cells were then pre-stained with CellTracker green CMFDA (Life Technologies) at 37 ^{o}C for 20 min, followed by centrifugation (300g, 3 mins) and then re-suspended in culture medium. The number of detached cells in suspension was determined by an automated hemocytometer (Millipore). The desired concentration of cells (cell number/mL) was obtained by serial dilution of the detached cells in culture medium. The prepared cell solution (100 µL consisted of ~ 200 fluorescence-labeled cells) was spiked into 2 mL culture medium DMEM or whole blood from a healthy individual donor for the purpose of chip capture efficiency experiments. To ensure accurate spiking cell number in the cell capture efficiency experiments, three portions of the prepared 100 µL pre-stained cell solution were loaded separately into glass-bottomed wells (diameter: 6 mm, height: 5 mm); the exact spiking cell number was obtained by counting on the average number of spiked cells from the three glass-wells by microscopy. After chip processing, the total amount of cancer cells captured by the chip were enumerated under a fluorescence microscope. The capture efficiency performance was defined as the ratio of number of cells bound on the chip to the total number of cells spiked into the chip.

Immunofluorescence Staining for clinical sample.

The immunofluorescence staining was performed on a 10 mm diameter collection membrane (Millipore) with 2 μm pore size. Collection membrane was first rinsed with PBS three times. Cells were then transferred to the collection membrane and fixed with 4% PFA for 10 mins, following by addition of PBS for three times to remove any PFA remained on membrane. The cells were then permeabilized with 0.1% Triton X-100 (Sigma Aldrich) for 10 mins, washed with PBS three times, and then treated with 10% normal goat serum (NGS, Abcam), which functions as blocking agent to avoid non-specific binding, at room temperature for 1 h. The primary antibodies rabbit anti-human CK-20 (for colon cancer patients, Abcam, dilution factor 1:200), were diluted in 1% NGS, which were then added to the collection membranes and incubated at 4 °C overnight. Next, the collected cells on the membrane were then rinsed with PBS for three repeats, with PBS stays on the membrane for 5 mins in each repeat. Next, samples will be incubated with Alexa Fluor-647 conjugate goat anti-rabbit IgG secondary antibody (Invitrogen, dilute factor 1:500) and fluorescein isothiocyanate (FITC) pre-conjugated mouse anti-human CD45 antibody (DAKO, dilute factor 1:10), as for white blood cell (WBC) staining, at room temperature for 1h. After incubation, the cells were then rinsed three times with PBS and the collection membranes were then mounted on glass slides with ProLong Gold Antifade Mountant with DAPI (Life Technologies). Images were taken with Nikon-Ti Eclipse microscope at 10-fold magnification, and analyzed with NIS-Elements AR Analysis software (Nikon).

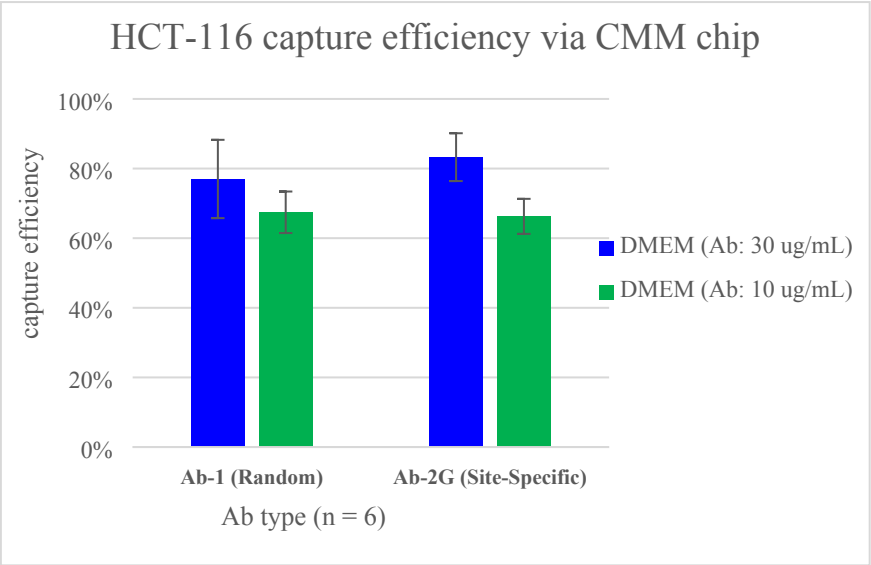


Figure S7. The comparison of cell capture efficiency in DMEM with two concentrations (10 or 30 μg/mL) of two antibodies (**Ab-1** and **Ab-2G**) coated on microfluidic chip surface. The result suggested the concentration 30 μg/mL of antibody was needed to inject onto CMM chip to fill the antibody-biotin-NA binding vacancy on SLB.

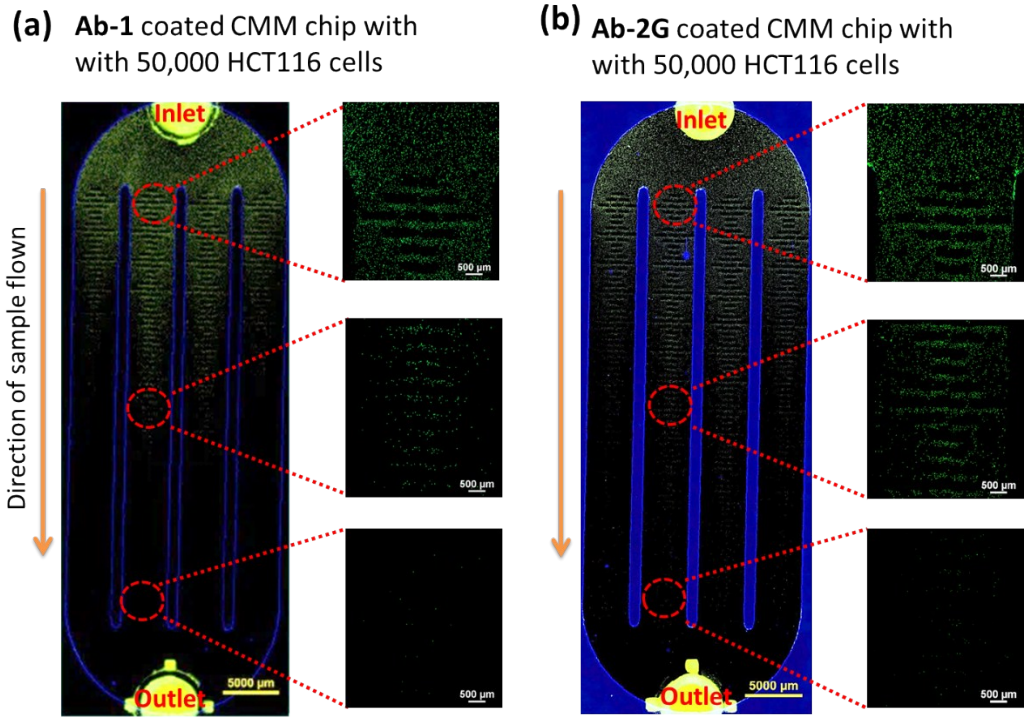


Figure S8. The density and distribution of the capture HCT-116 cells within the CMM chip. In the condition of 50,000 number cells spiking experiment, we can see clear that the capture cells in the **Ab-2G** coated chip having wider distribution than **Ab-1** coated chip. The Ab-2G coated chip can keep the binding cells ability in the later route in the chip (close to outlet side), but not happen the in the case of **Ab-1** coated chip. The result could contribute by the heterogeneity of HCT-116 cells, although HCT-116 is usually consider as high EpCAM antigen expression cell line. In the bigger population such as 50,000 cells, small portion of the cell could express little EpCAM that **Ab-1** coated chip cannot capture, but **Ab-2G** coated chip can capture. The data is consistent with result of Figure 3.

The experiment procedure: The experiment is conducted by using CMM chips coated with antibodies, **Ab-1** or **Ab-2G**. After washing with filtered PBS solution, 0.15 mL at flow rate 3 ml/h, for three times, we add diluted antibody to the chip at flow rate 1.8 ml/h and stay for 1hr. After 1 hr antibody coating process, we washed away the free antibody in the chip with filtered PBS solution, 0.15 mL at flow rate 3 ml/h, for three times. At the same time, we prepared cell for the experiment and detached HCT-116 cell line by treating with accutase for 5 minutes. The detached cell was centrifuged in 300 g for 3 minutes. Neutralizing the accutase activity by using DMEM and washing away the remaining accutase, then we optimizing the cell concentration and spike with 50000 cells in 2mL DMEM in the chip with flow rate 1.8 ml/h. After washing with 1 mL DMEM at flow rate 3 ml/h, we stained live HCT-116 cells captured in the chip with calcein AM (LIVE/DEAD Cell Viability Assay Kits, Cat. L3224, dilution rate: 1:200) and stained nuclei with Hoechst 33342 (dilution rate: 1:1000) for ten minutes. Then the images of whole chip were taken by Nikon ECLEPSE Ti-ESN 636901 and merged by Nikon NIS Analysis software.

Preparation of Ab-1@beads and Ab-2G@beads, and their application in HCT-116 cancer cell capture experiments.

*Preparation of **Ab-1@beads**, **Ab-2G@beads***

Two portions of 100 μL (1 mg) magnetic microbeads from commercial stock solution (10 mg/mL, Dynabeads® MyOne Streptavidin C1, in size of 1.05 μm, ThermoFisher Scientific) in 1.5 mL eppendorf tubes were washed three times with 1 mL of PBS, together with the use of magnetic stand separation. 20 μg (33 μL of 0.6 mg/mL) of **Ab-1** or **Ab-2G** was added into the 1 mg of streptavidin coated microbeads in 970 μL PBS in Eppendorf at room temperature and mixed on a rotator for 30 mins. After two

antibody immobilized on the microbeads through biotin-streptavidin affinity, the beads were washed with PBS three times. The resulting **Ab-1@beads** and **Ab-2G@beads** were suspended in 1 mL (1 mg/mL) of PBS and stored in 4 °C fridge.

*Capture cancer cell using **Ab-1@beads** or **Ab-2G@beads***

A 50 μg of **Ab-1@beads** or **Ab-2G@beads** was added to 1.5 mL Eppendorf tube containing approximately 200 cells (HCT-116) which has been pre-stained with CellTracker green CMFDA in DMEM. The resulting mixture solutions in the Eppendorf tubes were gently mixed on a rotator at room temperature for 1 h and then washed with 1 mL of PBS three times. The captured cells on beads were then fixed with 4% PFA for 10 mins and followed by washing with PBS. Finally, the cells were loaded on a 10 mm diameter collection membrane (Millipore) with 2 μm pore size and then mounted on glass slides with ProLong Gold Antifade Mountant with DAPI (Life Technologies). The cancer cells captured by the beads were enumerated under a fluorescence microscope. The capture efficiency performance was defined as the ratio of the number of captured cells to the total number of cells spiked into the eppendrof tube.

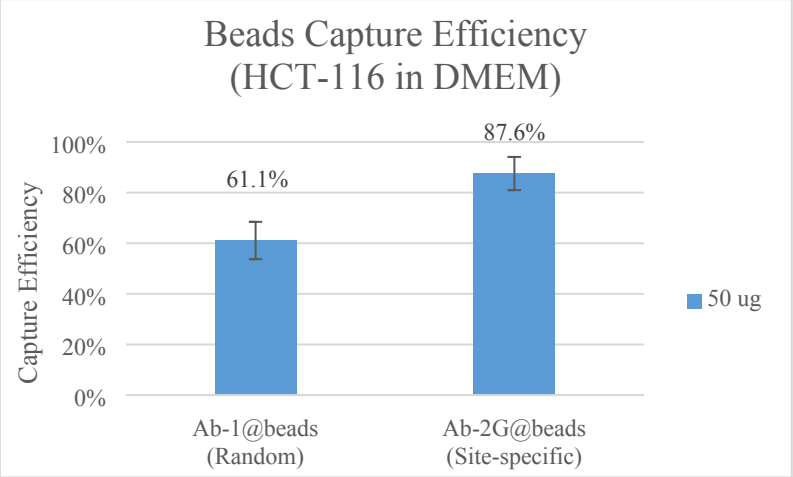


Figure S9. The comparison of HCT-116 cell capture efficiency *via* **Ab-1@beads** and **Ab-2G@beads** in DMEM (n = 6).

Table S3. The Clinic Capture CTCs and CTM numbers by using Ab-1 or Ab-2G onto CMM chips.

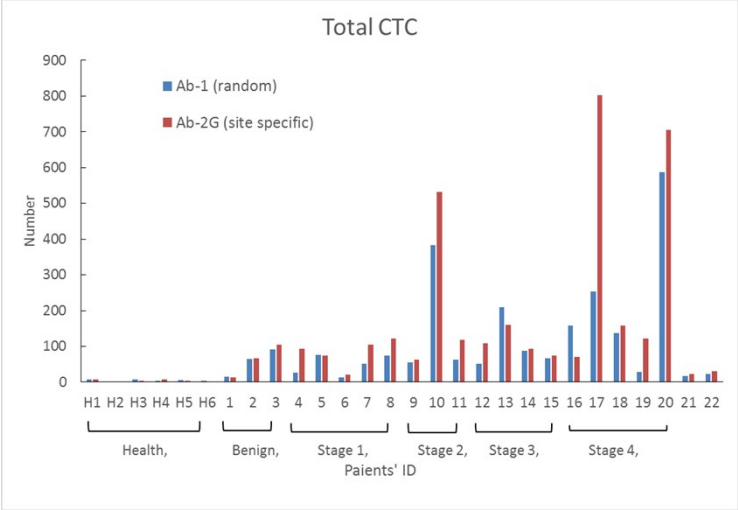
Sample number	Gender / Age	Clinical Condition	Tumor Size (cm)	Ab-1 (RANDOM)			Ab-2G (SITE-SPECIFIC)			RATIO (Ab-2G/Ab-1)		
				Total CTCs	Single CTCs	CTM clusters	Total CTCs	Single CTCs	CTM clusters	Total CTCs	Single CTCs	CTM clusters
HD 1	M,33	(No colon disease)		8	8	0	7	7	0	0.88	0.88	-
HD 2	F, 32	(No colon disease)		0	0	0	0	0	0	1.00	1.00	-
HD 3	M, 32	(No colon disease)		7	7	0	4	4	0	0.57	0.57	-
HD 4	M, 27	(No colon disease)		4	4	0	7	7	0	1.75	1.75	-
HD 5	M, 35	(No colon disease)		6	6	0	4	4	0	0.67	0.67	-
HD 6	M, 27	(No colon disease)		4	4	0	1	1	0	0.25	0.25	-
1	M, 63	colorectal cancer family history	Normal +Hemorrhoid	15	12	3	14	9	5	0.93	0.75	1.67
2	M, 57	OB +	Normal	65	63	2	67	67	0	1.03	1.06	0.00
3	M, 55	OB +	Adenomatous polyp	91	83	8	105	103	2	1.15	1.24	0.25
4	M, 53	CRC Stage 1	3 x 1.1	27	23	4	93	71	22	3.44	3.09	5.50
5	F, 62	CRC Stage 1	Non-measurable	76	62	14	74	65	9	0.97	1.05	0.64
6	M, 62	CRC Stage 1	1.6	13	13	0	20	20	0	1.54	1.54	-
7	M, 67	CRC Stage 1	Non-measurable	52	48	4	104	77	27	2.00	1.60	6.75
8	M, 72	CRC Stage 1	Non-measurable	74	69	5	122	85	37	1.65	1.23	7.40
9	M, 73	CRC Stage 2	2.2	55	45	10	63	57	6	1.15	1.27	0.60
10	F, 77	CRC Stage 2	5 x 5	384	320	64	532	237	295	1.39	0.74	4.61

11	F, 61	CRC Stage 2	6	62	32	30	119	103	16	1.92	3.22	0.53
12	M, 54	CRC Stage 3	3.3	51	44	7	109	88	21	2.14	2.00	3.00
13	F, 56	CRC Stage 3	3.9 x 2.2	210	195	15	160	143	17	0.76	0.73	1.13
14	F, 57	CRC Stage 3	3.4	87	85	2	94	73	21	1.08	0.86	10.50
15	F, 48	CRC Stage 3	4 x4	66	58	8	75	68	7	1.14	1.17	0.88
16	M, 60	CRC Stage 4	8 x 8	159	140	19	70	6	64	0.44	0.04	3.37
17	M, 76	CRC Stage 4	7 x 7	254	229	25	803	729	74	3.16	3.18	2.96
18	F, 79	CRC Stage 4	5 x 5	137	107	30	158	114	44	1.15	1.07	1.47
19	M, 58	CRC Stage 4	6.6	29	20	9	121	79	42	4.17	3.95	4.67
20	M, 45	CRC Stage 4	5.4	588	554	34	706	682	24	1.20	1.23	0.71
21	F, 67	no operation	10 x 7	18	13	5	22	14	8	1.22	1.08	1.60
22	M, 50	no operation	5	23	18	5	30	28	2	1.30	1.56	0.40

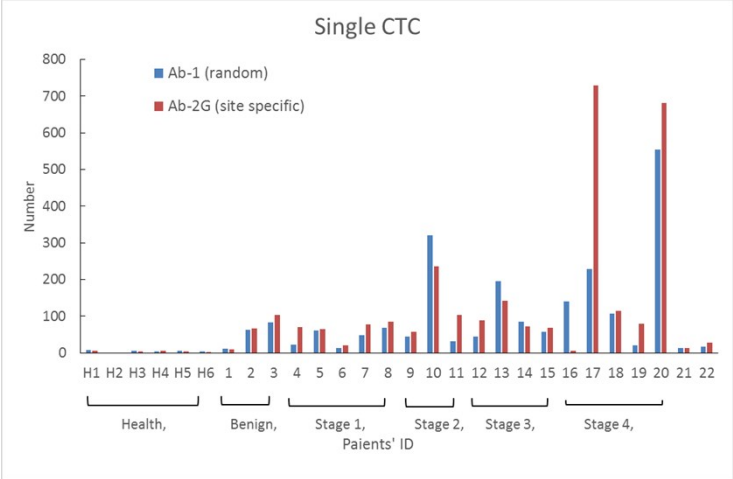
Statistical analysis

The clinic CTC capture number from **Ab-2G**/ or **Ab-1** coated CMM chips with two groups of total CTC and CTM were compared by an independent t-test, shown in Table S4. In the tail 1, type1 mode: the data was shown that 1) all cancer patient groups (N = 19) in total CTC and CTM were significant, 2) benign (N=3), healthy (N = 6) and all benign (N = 9) groups were no difference. Differences were considered significant at the 95% confidence level ($p < 0.05$).

(a)


Total CTC
Ab-1 (random)
Ab-2G (site specific)
Number
900
800
700
600
500
400
300
200
100
0
H1 H2 H3 H4 H5 H6 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Health,
Benign,
Stage 1,
Stage 2,
Stage 3,
Stage 4,
Paients' ID

(b)


Single CTC
Ab-1 (random)
Ab-2G (site specific)
Number
800
700
600
500
400
300
200
100
0
H1 H2 H3 H4 H5 H6 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Health,
Benign,
Stage 1,
Stage 2,
Stage 3,
Stage 4,
Paients' ID

(c)

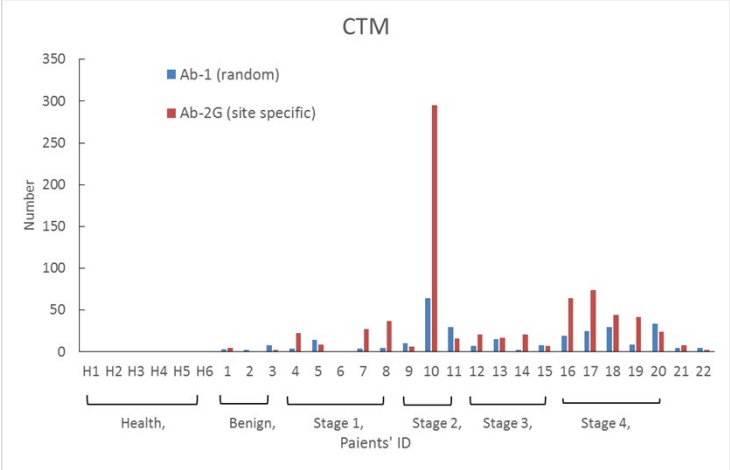

CTM
Ab-1 (random)
Ab-2G (site specific)
Number
350
300
250
200
150
100
50
0
H1 H2 H3 H4 H5 H6 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Health,
Benign,
Stage 1,
Stage 2,
Stage 3,
Stage 4,
Paients' ID

Figure S10. Graphic illustration of (a) Total CTCs, (b) single CTCs and (c) CTMs in CRCs patients' sample.

Table S4. Analysis of CTCs and CTMs by using Ab-1 or Ab-2G onto CMM chips: (a) based on number, (b) based on ratio of (Ab-2G/ Ab-1)

(a)

	Total CTC			CTM Average Number		
Patient type	**Ab-1** Average Capture Number	**Ab-2G** Average Capture Number	Student T-test (Tail 1, Type 1)	**Ab-1** Average Capture Number	**Ab-2G** Average Capture Number	Student T-test (Tail 1, Type 1)
Stage 1 (N = 6)	48 (28)	83 (39)		5 (5)	19 (15)	
Stage 2 (N = 3)	167 (188)	238 (256)		35 (27)	106 (164)	
Stage 3 (N = 4)	104 (73)	110 (36)		8 (5)	17 (7)	
Metastasis, Stage 4 (N = 5)	233 (214)	372 (353)		23 (10)	50 (20)	
Non-metastasis, Stage 1-3 (N = 12)	96 (103)	130 (131)		14 (18)	40 (81)	
All Cancer Patients (N = 19)	124 (147)	183 (229)	0.034*	15 (16)	39 (65)	0.036*
Healthy (N = 6)	5 (3)	4 (3)	0.166	0 (0)	0 (0)	-
Benign (N = 3)	57 (39)	62 (46)	0.195	4 (3)	2 (3)	0.239
All Benign (N = 9)	22 (33)	23 (37)	0.293	1 (3)	1 (2)	0.199

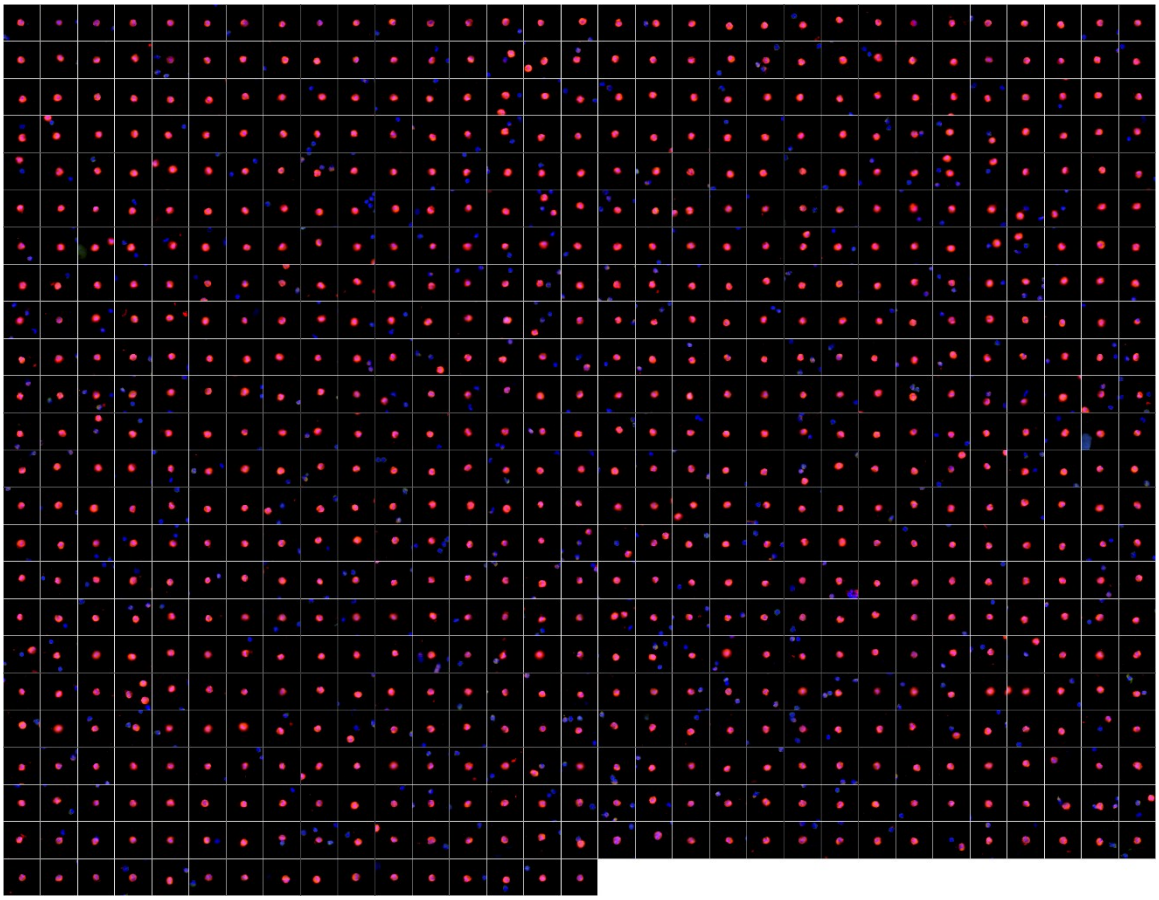
* Student T-test: P-values of less than 0.05 was considered statistically significant.

(b)

	AVERAGE RATIO (Ab-2G/ Ab-1)			**MEDIAN RATIO (Ab-2G/ Ab-1)**		
Patient type	Total	Single	CTM	Total	Single	CTM
Stage 1 (N = 5)	1.92 (0.93)	1.70 (0.81)	4.26 (3.21)	1.65	1.54	5.50
Stage 2 (N = 3)	1.48 (0.32)	1.74 (0.34)	1.91 (3.16)	1.39	1.27	0.60
Stage 3 (N = 4)	1.28 (0.60)	1.19 (0.57)	3.88 (4.52)	1.11	1.02	2.07
Metastasis, Stage 4 (N = 5)	2.03 (1.57)	1.89 (1.62)	2.63 (1.57)	1.20	1.23	2.96
Non-metastasis, Stage 1-3 (N = 12)	1.60 (0.73)	1.54 (0.84)	3.55 (3.36)	1.46	1.25	2.07
All Cancer Patients (N = 19)	1.68 (1.01)	1.61 (1.08)	3.04 (2.93)	1.30	1.23	1.60
Healthy (N = 6)	0.85 (0.51)	0.85 (0.51)	1.00 (0.00)	0.77	0.77	1.00

Benign (N = 3)	1.04 (0.11)	1.02 (0.25)	0.64 (0.90)	1.03	1.06	0.25
All Benign (N = 9)	0.91 (0.42)	0.91 (0.43)	0.88 (0.48)	0.93	0.88	1.00

(a) CTCs



(b) CTMs

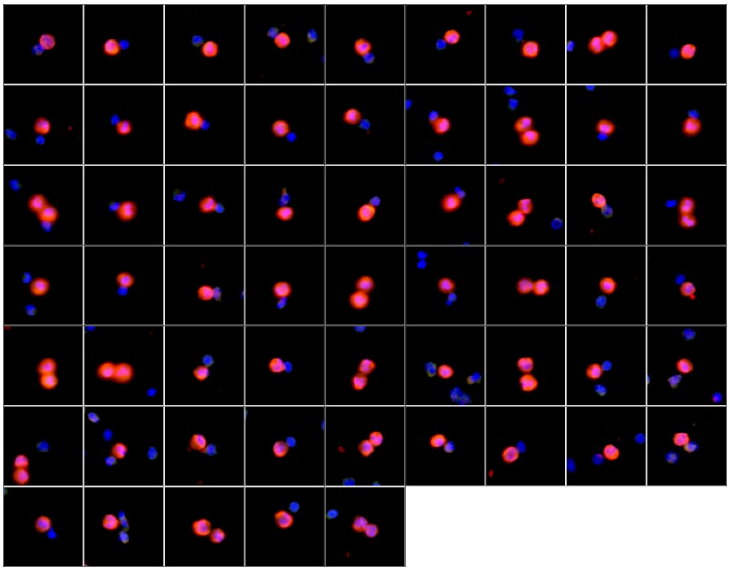


Figure S11. Example of whole images of CTCs and CTMs captured from CRC patient #17 by using Ab-2G coated CMM chip.

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