Ex Vivo Expansion of Circulating Lung Tumor Cells Based on a One-Step Microfluidics-Based Immunomagnetic Isolation

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Supplementary Methods

1. Fabrication of microfluidic herringbone chip

The photoresist mold of microfluidic herringbone chip was fabricated by a two-step photolithographic process. In the first step, a layer (30 μm) of negative photoresist (SU8-2050, Microchem) was spin-coated onto a 4-inch silicon wafer. After UV exposure, another layer (70 μm) of SU8-2050 was spin-coated on the same wafer with good alignment between the previous pattern and the pattern to be fabricated. The pattern was then UV exposed and developed. A serpentine fluidic channel with rectangular shape in the cross section and herringbone structures was obtained. The photoresist mold was treated by trimethylchlorosilane (TMCS, Sigma-Aldrich) to facilitate the separation between the mold and replicate PDMS component. The PDMS pre-polymer (Sylgard 184) was mixed in a ratio of 10:1, and subsequently casted on this lithographically patterned mold. After curing at 80°C for 2 h, the PDMS device was separated from the mold, followed by trimming and hole introduction before bonding to a glass slide to form a chip.

2. Isolation of CTCs from whole blood samples

In this study, blood samples from lung cancer patients were obtained from Longhua Hospital (Shanghai, China) with informed consent, which was approved by the Ethics and Scientific Committee of the hospital. According to a standard protocol, blood samples were obtained at the middle of vein puncture after first 3 mL of blood was discarded, then collected into EDTA-contained vacutainer tubes and processed immediately. A volume of 4 mL peripheral blood from the patient was used in this study, and equally split in two samples with one for *ex vivo* culture and another sample for molecular analysis. Briefly, 2 mL of whole blood sample was firstly centrifuged at 200 g for 5 min to remove the platelet-rich plasma. The cell pellet was re-suspended in Hank's balanced salt solution (HBSS) to generate a new 2 mL cell suspension, and then actively incubated with a mixture of EpCAM-coated and EGFR-coated immunomagnetic microbeads for 1 h. The antibody-coated magnetic microbeads were prepared by incubating biotinylated antibodies (anti-EpCAM, anti-EGFR) with streptavidin-coated magnetic beads (0.8 μm, Solulink) for 30 min and washed twice with PBS. The cell suspension was further diluted with HBSS to 10 mL, and then processed through a herringbone chip pre-blocked with 3% BSA and 10% normal goat serum at the flow rate of 20 mL/h. An array of magnets was placed on the chip to capture magnetic microbead-coated CTCs. After a wash (20 mL/h, 2 min) with CTC culture medium containing RPMI1640 medium, EGF (20 ng/mL), FGF2 (20 ng/mL) and B27 supplement to remove nonspecifically bound blood cells, magnets were retracted and CTCs were therefore released and collected in a ~200 μL of solution. The cell suspension was added into a 96-well non-adherent plate.

3. *Ex vivo* expansion of isolated CTCs

Isolated CTCs were incubated under hypoxic conditions (3% O_2, 5% CO_2) at 37°C in a 96-well non adherent plate in the CTC culture medium containing RPMI1640 medium (Life Technologies), EGF (20 ng/mL, Life Technologies), FGF2 (20 ng/mL, Life Technologies) and B27 supplement (1X,, Life Technologies). CTCs started proliferate after two weeks of cell culture and expanded slowly. The culture condition was then

switched to normoxic condition (5% CO_2). After six months we obtained tens of millions of tumor cells.

4. Detection of genetic mutations on isolated CTCs

Isolated CTCs in the 96-well plate was stained with PE-conjuaged anti-EpCAM and anti-EGFR, and FITC-conjugated CD45 in 1% BSA/PBS. Ten CTCs were retrieved by a micromanipulator based on the criteria of EpCAM/EGFR+, CD45-, and magnetic microbeads+. The genome amplification of CTCs was conducted with REPLI-g Single Cell Kit (QIAGEN), which is specially designed for highly uniform whole genome amplification (WGA) from single cells. Amplified genomic DNA was then purified by phenol-chloroform extraction and ethanol precipitation, followed by determination of concentration by a Nanodrop (Thermo Scientific). PCR for the target regions with potential mutation sites was conducted with the primers listed in Table S1. The amplified PCR fragments were purified with AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), which were further subjected to Sanger sequencing (Life Technologies).

5. Whole exome sequencing of *ex vivo* expanded CTCs

Whole-exome enrichment and sequencing library construction were performed by Agilent SureSelect Human All Exon kit V5. The whole exome sequencing was conducted on the Illumina HiSeq™ X Ten platform. Briefly, the products of genome amplification were quantitated by nanodrop-1000 (Nanodrop Technologies) and then qualified by Agilent Bioanalyzer 2100 (Agilent Technologies). Genomic DNA was fragmented by Covaris sonicator into target peak size of 180-280 base pairs (bp). The fragmented genomic DNA was end repaired and an "A" base was added to the 3' ends. Paired end DNA adaptors (Illumina) with a single "T" base overhang at the 3' end were ligated and the resulting constructs were purified using AMPure XP beads from Agencourt. The adapter-modified DNA fragments were enriched by PCR using PE 1.0 forward and PE 2.0 reverse (Illumina) primers. Whole exome capture was carried out using the protocol from Agilent's SureSelect Human All Exon kit V5. The prepped library was incubated with whole exome biotinylated RNA capture baits and the captured DNA: RNA hybrids were recovered using Dynabeads MyOne Streptavidin T1. Captured libraries were then

sequenced on Illumina HiSeq™ X Ten plateform as 150-bp paired-end reads, following the manufacturer's protocols. Sequencing reads were processed, mapped to the human genome (hg19). BWA was used to allow gapped alignment to the reference genome (1). Picard, GATK and SAMtools were used to performed duplicate removal, local realignment, base quality recalibration, call targeted bases and any base call that deviates from reference base was regarded as a potential variation(2,3) mutation genes annotation of identified variants was done using ANNOVAR (4).

Whole exome sequencing generated 15.06 Gb of sequence with 175X mean target coverage (Table S2). More than 96.0% of the target regions were covered sufficiently for confident variant calling (>10X). A total of 134749 single nucleotide mutations were found in the ex vivo expanded CTCs. These SNVs are distributed in various genomic regions including exonic (13.1%), splicing (0.3%), 5'-untranslated regions (1.4%), 3'-untranslated regions (2.4%), intronic (53.2%), upstream (1.3%), downstream (0.7%), intergenic regions (22.5%) and ncRNA (5.1%), as shown in Table S3. Among all exonic mutations, 8256 (missense) substitutions led to amino acid changes; 9023 mutations were synonymous; 96 nonsense mutations led to truncated proteins; 9 sites lost termination codon and led to abnormal proteins extension; 424 splicing sites mutations were detected. There were also 110 insertions and deletions (Indels) causing exonic frameshifts and 235 causing non-frameshifts (Table S4).

6. Glucose uptake measurement of *ex vivo* expanded CTCs

Ex vivo expanded CTCs were deprived of glucose for 10 min and then exposed to 0.3 mM 2-NBDG (ThermoFisher Scientific) for 20 min. After extensive washing with cold PBS, CTCs were recorded using an ANDOR™ Zyla sCMOS CCD camera for imaging of 2-NBDG uptake. The excitation was at 488 nm, and the emission was collected between 515 and 585 nm.

7. Computational modeling

A commercial computational fluid dynamics software package ANSYS FLUENT 15.0 based on Finite Volume Method was utilized to solve the fluid flow of the model. For all of the simulations, an array of streamlines was employed which were defined in a

uniform block with 100 launching points from the inlet surface. The post-processing was conducted using a custom numerical MATLAB code to analyze these streamlines. The specifics of the surface binding process were neglected.

Supplementary Tables

Table S1. Primers used in this study. All primers were synthesized by Life Technologies.

Gene	Primer sequence(5'-3')
BRAF-15F	CTCATCCTAACACATTTCAAGCCC
BRAF-15R	CAGCATCTCAGGGCCAA
EGFR-18F	TGGAGAAGCTCCCAACCAA
EGFR-18R	TTCCCAAACACTCAGTGAAACA
EGFR-Del19-F	GTGGCACCATCTCACAATT
EGFR-Del19-R	ATGCTCCAGGCTCACCAAG
EGFR-T790M-F	CTTTATCCAATGTGCTCCTC
EGFR-T790M-R	TCTCCCTTCCCTGATTACCT
EGFR-L858R-F	TTCGCCAGCCATAAGTCCT
EGFR-L858R-R	TCATTCACTGTCCCAGCAAG
KRAS-2F	TGAGAGCCTTTAGCCGCC
KRAS-2R	TACCCTCTCACGAAACTCTG
PIK3CA-9F	GGGAAAAATATGACAAAGAAAGC
PIK3CA-9R	CTGAGATCAGCCAAATTCAGTT
PIK3CA-20F	CTCAATGATGCTTGGCTCTG
PIK3CA-20R	TGGAATCCAGAGTGAGCTTTC
TP53-(5+6)F	CAACTCTGTCTCCTTCCTCTTC
TP53-(5+6)R	GCACATCTCATGGGGTTATAGG
TP53-(7+8)F	ACAGGTCTCCCCAAGGC
TP53-(7+8)R	CCTCGCTTAGTGCTCCCTG

Table S2. Summary of mutations in the CTC whole exome sequencing.

Total mutations	99659626 (100%)
Duplicate	8966020 (9.04%)
Mapped	99187587 (99.53%)
Properly mapped	97429294 (97.76%)
Total_effective_reads	100493259
Total_effective_yield (Mb)	14671.62
Effective_sequences_on_target (Mb)	8801.27 (60.0%)
Average_sequencing_depth_on_target	174.66
Fraction_of_target_covered_with_at_least_4x	98.3%
Fraction_of_target_covered_with_at_least_10x	96.2%
Fraction_of_target_covered_with_at_least_20x	92.3%

Table S3. SNV of CTC whole exome sequencing.

Total SNV	134749
UTR3	3250 (2.4%)
UTR5	1871 (1.4%)
exonic_missense	8256 (6.1%)
exonic_stopgain	96*
exonic_stoploss	9*
exonic_synonymous	9023 (6.7%)
exonic_unknown	249 (0.2%)
intronic	71707 (53.2%)
upstream	1795 (1.3%)
downstream	880 (0.7%)
splicing	424 (0.3%)
intergenic	30294 (22.5%)
ncRNA	6895 (5.1%)

Table S4. Indel of CTC whole exome sequencing.

Total Indel	18098
UTR3	462 (2.6%)
UTR5	320 (1.8%)
exonic_frameshift_deletion	59 (0.3%)
exonic_frameshift_insertion	51 (0.3%)
exonic_nonframeshift_deletion	117 (0.7%)
exonic_nonframeshift_insertion	118 (0.7%)
exonic_stopgain	9*
exonic_stoploss	0
exonic_unknown	71 (0.4%)
intronic	10208 (56.4%)
splicing	69 (0.4%)
upstream	275 (1.5%)
downstream	122 (0.7%)
ncRNA	1038 (5.7%)

Table S5. Summary of mutations in 60 driver genes.

CHROM	POS	REF	ALT	QUAL	Gene Name	Func	Exonic Func
9	133761001	A	G	228	ABL1	exonic	synonymous SNV
14	105239894	C	T	181.5	AKT1	exonic	synonymous SNV
2	29416572	T	C	228	ALK	exonic	missense SNV
2	29449819	C	T	221.5	ALK	exonic	synonymous SNV
2	29455267	A	G	228	ALK	exonic	synonymous SNV
2	29543663	T	C	228	ALK	exonic	synonymous SNV
2	29940529	A	T	228	ALK	exonic	synonymous SNV
2	30143499	G	C	228	ALK	exonic	synonymous SNV
5	112162854	T	C	221	APC	exonic	synonymous SNV
5	112164561	G	A	193	APC	exonic	synonymous SNV
5	112176325	G	A	80	APC	exonic	synonymous SNV

5	112177171	G	A	95.5	APC	exonic	synonymous SNV
5	112176756	T	A	178	APC	exonic	missense SNV
16	396264	A	G	228	AXIN1	exonic	synonymous SNV
X	39933339	A	G	228	BCOR	exonic	synonymous SNV
17	41223094	T	C	110	BRCA1	exonic	missense SNV
17	41234470	A	G	157.5	BRCA1	exonic	synonymous SNV
17	41244339	G	T	175	BRCA1	exonic	missense SNV
17	41244435	T	C	157	BRCA1	exonic	missense SNV
17	41246481	T	C	221	BRCA1	exonic	missense SNV
13	32906729	A	C	228	BRCA2	exonic	missense SNV
13	32913055	A	G	228	BRCA2	exonic	synonymous SNV
13	32915005	G	C	228	BRCA2	exonic	synonymous SNV
13	32929387	T	C	228	BRCA2	exonic	missense SNV
7	2946365	C	T	222	CARD11	exonic	missense SNV
7	2946461	T	C	228	CARD11	exonic	synonymous SNV
7	2957005	T	C	142.5	CARD11	exonic	synonymous SNV
7	2952942	C	T	43.68	CARD11	exonic	missense SNV
2	202122995	A	G	228	CASP8	exonic	missense SNV
11	119156088	C	T	228	CBL	exonic	missense SNV
16	68857441	T	C	211	CDH1	exonic	synonymous SNV
19	33792631	C	A	120.5	CEBPA	exonic	synonymous SNV
19	42799049	C	T	220.5	CIC	exonic	synonymous SNV
19	42791974	C	A	105	CIC	exonic	missense SNV
16	3820692	G	T	221.5	CREBBP	exonic	missense SNV
5	149457678	G	A	221	CSF1R	exonic	synonymous SNV
5	149460553	A	G	221.5	CSF1R	exonic	synonymous SNV
6	33288271	A	G	228	DAXX	exonic	synonymous SNV
19	10265312	T	C	228	DNMT1	exonic	synonymous SNV
19	10267077	T	C	228	DNMT1	exonic	synonymous SNV
7	55214348	C	T	183	EGFR	exonic	synonymous SNV
7	55229255	G	A	202.5	EGFR	exonic	missense SNV
7	55238874	T	A	221.5	EGFR	exonic	synonymous SNV

7	55249063	G	A	228	EGFR	exonic	synonymous SNV
7	55266417	T	C	228	EGFR	exonic	synonymous SNV
17	37884037	C	G	221	ERBB2	exonic	missense SNV
10	123298158	T	C	228	FGFR2	exonic	synonymous SNV
4	1807894	G	A	228	FGFR3	exonic	synonymous SNV
13	28623588	G	T	222	FLT3	exonic	missense SNV
13	28636084	G	A	228	FLT3	exonic	synonymous SNV
5	180030313	C	A	211.5	FLT4	exonic	missense SNV
3	128200072	C	T	228	GATA2	exonic	synonymous SNV
3	128205860	G	C	228	GATA2	exonic	synonymous SNV
19	3119239	C	T	210	GNA11	exonic	synonymous SNV
12	121435342	C	T	228	HNF1A	exonic	synonymous SNV
12	121437382	A	G	228	HNF1A	exonic	missense SNV
12	121416622	C	G	228	HNF1A	exonic	synonymous SNV
11	534242	A	G	104.5	HRAS	exonic	synonymous SNV
2	209108317	C	T	222	IDH1	exonic	missense SNV
2	209113192	G	A	222	IDH1	exonic	synonymous SNV
15	90628591	G	A	222	IDH2	exonic	synonymous SNV
7	50436033	A	G	221.5	IKZF1	exonic	missense SNV
9	5050706	C	T	228	JAK2	exonic	synonymous SNV
19	17950462	C	T	129	JAK3	exonic	missense SNV
12	25368462	C	T	228	KRAS	exonic	synonymous SNV
12	25398285	C	T	228	KRAS	exonic	missense SNV
11	8251921	G	C	228	LMO1	exonic	synonymous SNV
11	65380916	G	A	222	MAP3K11	exonic	synonymous SNV
X	70349947	A	C	228	MED12	exonic	synonymous SNV
11	64572018	T	C	228	MEN1	exonic	missense SNV
11	64572557	A	G	228	MEN1	exonic	synonymous SNV
11	64572602	G	A	222	MEN1	exonic	synonymous SNV
7	116415142	A	G	221	MET	exonic	missense SNV
7	116435768	C	T	221.5	MET	exonic	synonymous SNV
7	116436022	G	A	221.5	MET	exonic	synonymous SNV

7	116436097	G	A	221.5	MET	exonic	synonymous SNV
2	47643457	G	A	221.5	MSH2	exonic	missense SNV
17	29483108	C	T	228	NF1	exonic	synonymous SNV
17	29508775	G	A	228	NF1	exonic	synonymous SNV
9	139391636	G	A	228	NOTCH1	exonic	synonymous SNV
9	139407932	A	G	207	NOTCH1	exonic	synonymous SNV
1	120458004	A	T	228	NOTCH2	exonic	synonymous SNV
13	25000617	C	G	225.5	PARP4	exonic	missense SNV
13	25008903	G	T	228	PARP4	exonic	missense SNV
13	25009099	A	C	228	PARP4	exonic	missense SNV
13	25009297	G	T	228	PARP4	exonic	missense SNV
13	25009441	C	G	228	PARP4	exonic	missense SNV
13	25009485	C	G	228	PARP4	exonic	missense SNV
13	25027744	A	G	228	PARP4	exonic	missense SNV
13	25029218	C	T	228	PARP4	exonic	missense SNV
13	25008630	A	G	228	PARP4	exonic	missense SNV
9	36840623	G	A	228	PAX5	exonic	missense SNV
3	52668638	G	A	212	PBRM1	exonic	synonymous SNV
3	52668776	A	G	228	PBRM1	exonic	synonymous SNV
4	55141055	A	G	228	PDGFRA	exonic	synonymous SNV
4	55161391	T	C	228	PDGFRA	exonic	synonymous SNV
5	67522722	C	T	204	PIK3R1	exonic	synonymous SNV
19	52729030	C	T	222	PPP2R1A	exonic	synonymous SNV
13	49047525	G	A	34.11	RB1	exonic	missense SNV
10	43595968	A	G	228	RET	exonic	synonymous SNV
10	43606687	A	G	221.5	RET	exonic	synonymous SNV
10	43610119	G	A	221	RET	exonic	missense SNV
10	43613843	G	T	228	RET	exonic	synonymous SNV
10	43615633	C	G	87	RET	exonic	synonymous SNV
17	56448297	C	T	221	RNF43	exonic	missense SNV
17	56492800	T	C	221	RNF43	exonic	missense SNV
18	42456653	G	A	222	SETBP1	exonic	missense SNV

18	42530472	T	G	221.5	SETBP1	exonic	synonymous SNV
18	42532606	G	A	222	SETBP1	exonic	missense SNV
18	42533130	A	G	228	SETBP1	exonic	synonymous SNV
3	47125385	G	A	228	SETD2	exonic	missense SNV
3	47162661	A	G	228	SETD2	exonic	synonymous SNV
2	198257795	T	C	228	SF3B1	exonic	synonymous SNV
2	198265526	A	G	228	SF3B1	exonic	synonymous SNV
2	198283305	T	C	228	SF3B1	exonic	synonymous SNV
19	11105608	T	C	228	SMARCA4	exonic	synonymous SNV
20	4164283	A	G	158.5	SMOX	exonic	synonymous SNV
17	74732507	A	G	228	SRSF2	exonic	synonymous SNV
17	74733099	G	A	228	SRSF2	exonic	synonymous SNV
19	1207021	C	T	228	STK11	exonic	stopgain
4	106156163	G	A	222	TET2	exonic	missense SNV
4	106196951	A	G	228	TET2	exonic	missense SNV
17	7579472	G	C	228	TP53	exonic	missense SNV
14	81574959	A	G	203.5	TSHR	exonic	synonymous SNV
14	81575005	C	A	218.5	TSHR	exonic	missense SNV
14	81610583	G	C	163	TSHR	exonic	missense SNV

Supplementary Figures

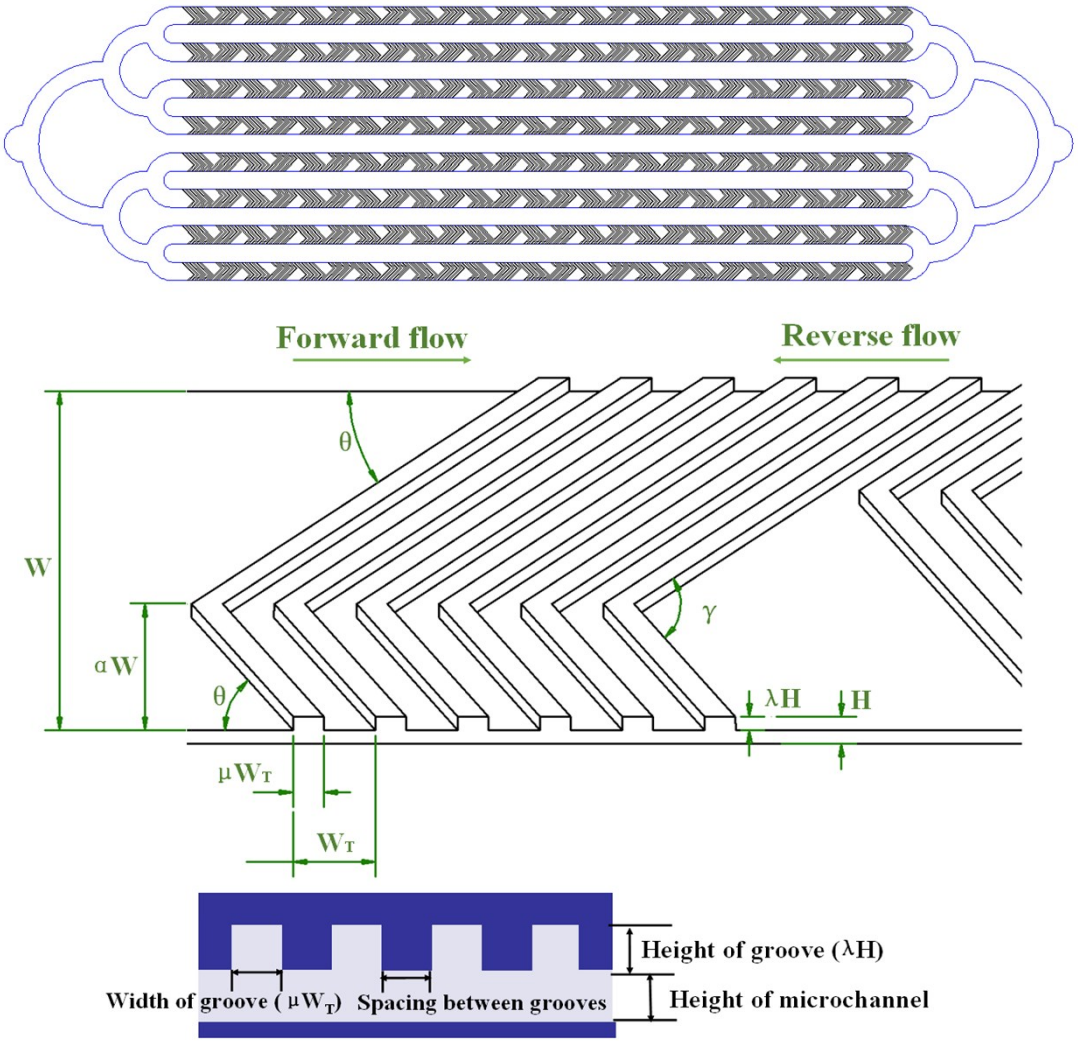

Figure S1. Design of the herringbone chip for capture of circulating tumor cells. (Top) The herringbone chip has eight 1 mm-wide microchannels with patterned chevrons on their upper surface. Each microchannel is ~40 mm long and has 13 unit cells consisting of two sequential regions of twelve chevrons shifted asymmetrically. (Bottom) Schematic illustration of the herringbone chip. The herringbone grooves are staggered periodically, with each unit cell defined by two sequential regions of twelve chevrons shifted assymmetrically. The geometrical parameters consist of 1 mm channel width (W), 100 μm total height (H) with herringbone groove height 70 μm (λH), 200 μm groove pitch (W_T) with herringbone groove width 150 μm (μW_T), 0.5 as herringbone groove asymmetry factor (α), 90° angle of the herringbone (γ), 45° angle with the channel wall (θ).

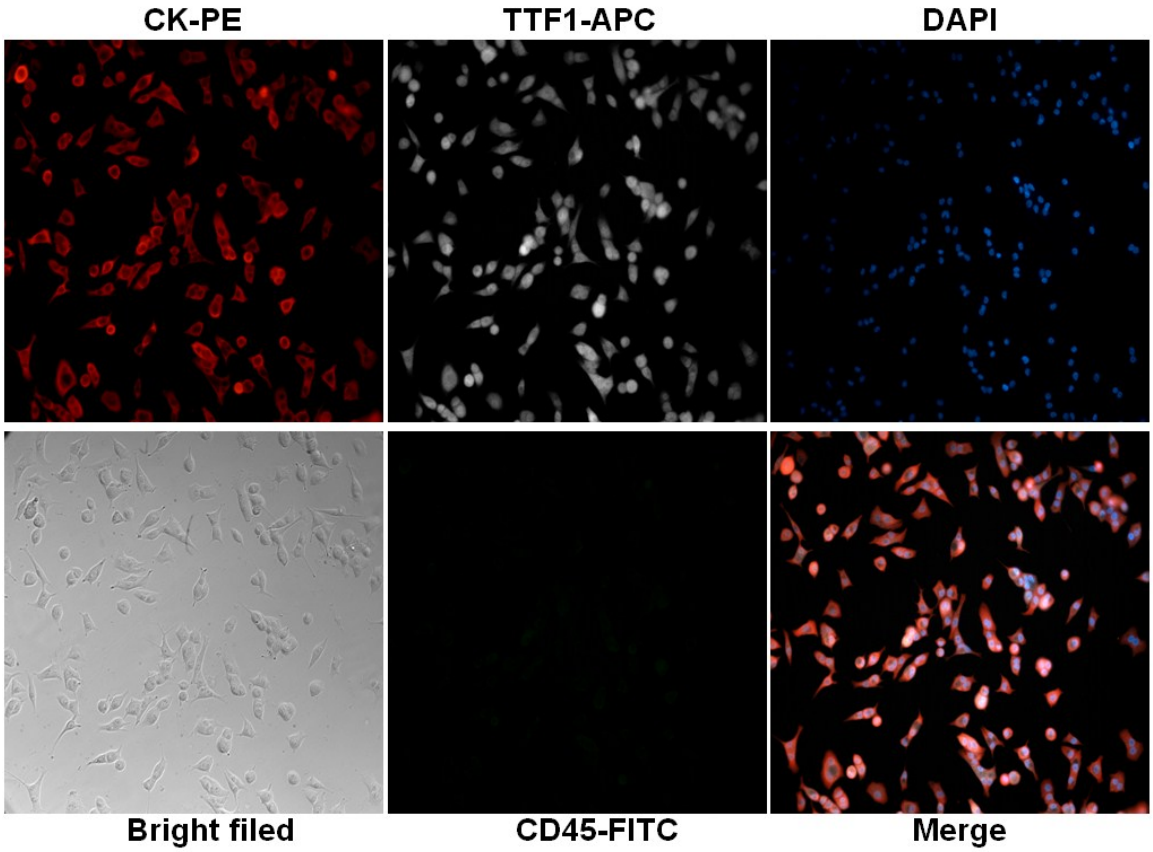

Figure S2. *Ex vivo* expanded CTCs were fixed with 4% paraformaldehye, permeabilized with 0.2% Triton X-100 in 1% BSA/PBS, and then immunostained with APC-conjugated TTF-1, FITC-conjugated anti-CD45 and PE-conjugated anti-pan CK overnight at 4^{o}C. Nuclei were stained with DAPI.

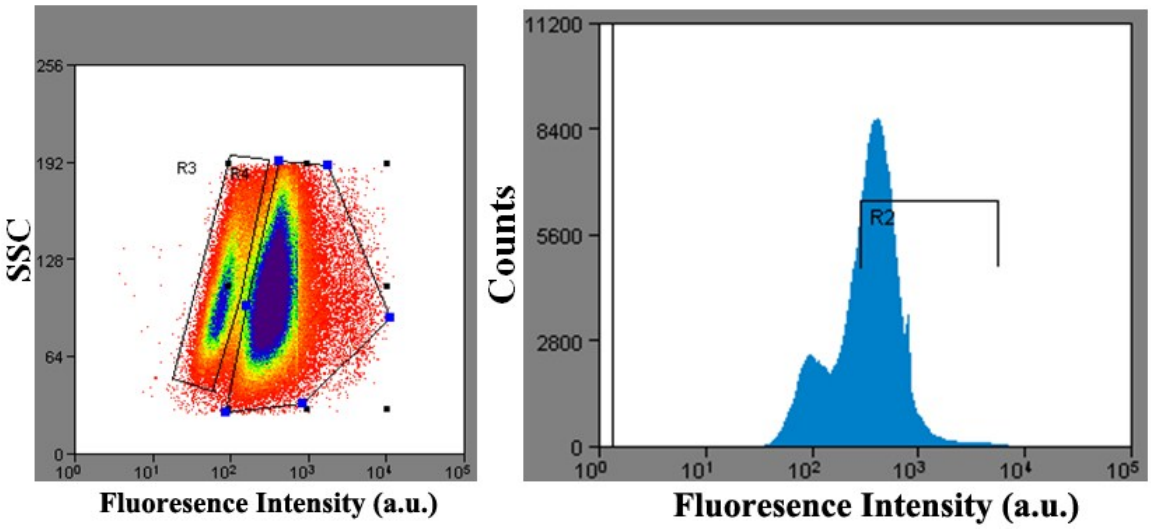

Figure S3. FACS-based cell sorting of *ex vivo* expanded CTCs using fluorescence intensity of 2-NBDG uptake. CTCs with high glucose uptake are around 8.6×10^5, and CTCs with low glucose uptake are around 1.1×10^5. These two subsets of expanded CTCs were cultured again with the same culture condition.

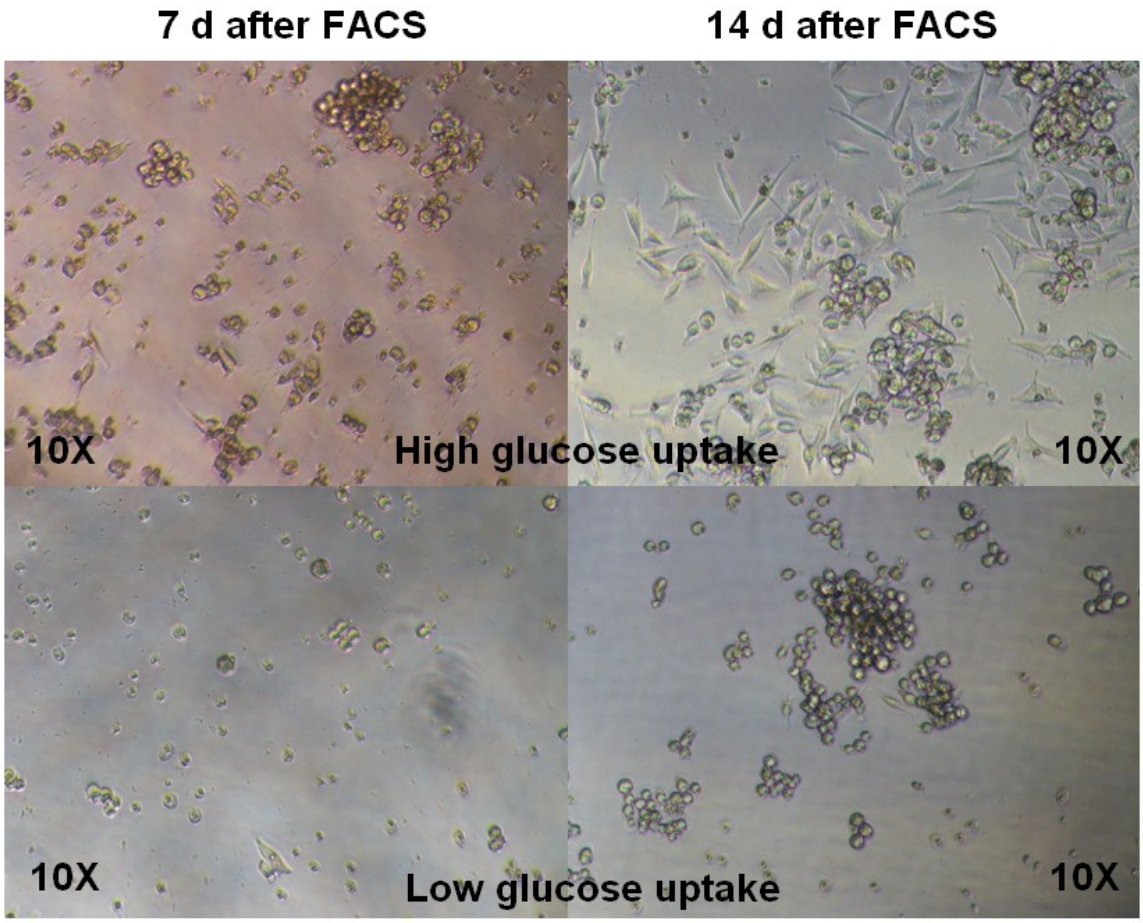

Figure S4. Microscopy images of two CTC subset (high glucose uptake and low glucose uptake) after 14-day *ex vivo* culture.

Supplementary References

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