Electronic Supplementary Information (ESI)

Enzymatic Cleavage of Uracil-Containing Single-Stranded DNA Linkers for the Efficient Release of Affinity-selected Circulating Tumor Cells

Soumya V. Nair,[a] Małgorzata A. Witek,[a] Joshua M. Jackson,[b] Maria A.M. Lindell,[b] Sally A. Hunsucker,[c] Travis Sapp,[d] Caroline E. Perry,[b] Mateusz L. Hupert [a],Victoria Bae-Jump,[c] Paola A. Gehrig,[c] Weiya Z. Wysham,[c] Paul M. Armistead,[c] Peter Voorhees,[c] and Steven A. Soper[a,b,e,f]

[a] Department of Biomedical Engineering, UNC-Chapel Hill, NC
[b] Department of Chemistry, UNC-Chapel Hill, NC
[c] UNC Lineberger Comprehensive Cancer Center, UNC-Chapel Hill, NC and School of Medicine Chapel Hill, UNC-Chapel Hill, NC
[d] Department of Pathology and Laboratory Medicine, UNC-Chapel Hill, NC
[e] Department of Chemistry, LSU-Baton Rouge, LA
[f] Ulsan National Institute of Science and Technology, Ulsan, Republic of Korea

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Experimental

Reagents and materials. COC surface modification included the following materials: Reagent-grade isopropyl alcohol, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 2-(4-morpholino)-ethane sulfonic acid (MES), PBS buffer (pH 7.4), and bovine serum albumin (BSA 7.5%) in PBS buffer, pH 7.4 (Sigma-Aldrich). The monoclonal antibodies used for these studies included rat anti-human anti-CD138 (clone 359103, R&D Systems), mouse anti-human anti-CD34 (clone 561, class III epitope from Biolegend), mouse anti-human Fibroblast Activation Protein, FAPα (clone 427819, R&D Systems), mouse anti-human EpCAM/TROP-1 (clone 158210, R&D Systems), and IgG (R&D Systems). CTCs were stained with anti-CD45-FITC (clone HI30, eBioscience), anti-cytokeratin mAbs (CK-PAN-eFluor®615, clone LP3K, BA17), (eBioscience,) to provide immunophenotyping distinction from infiltrating leukocytes.

Other reagents used for these studies included sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC, No-Weigh Format) (Thermo-Pierce), Zeba spin desalting columns (7K MWCO) from Thermo Scientific, Uracil Specific Excision Reagent USER™ enzyme from New England Biosciences, and the Live/Dead™ cell viability kit from Life Technologies. All reagents were nuclease-free. Nuclease-free microfuge tubes were purchased from Ambion and were used for preparation and storage of all samples and reagents.

Fabrication of the cell selection device. Hot embossing was used to fabricate the thermoplastic cell selection device as described previously (Figure S1).[1] Mold masters for hot embossing were prepared in brass using high precision-micromilling (KERN 44, KERN Micro- und Feinwerktechnik GmbH & Co.KG; Murnau, Germany) and carbide bits (Performance Micro Tool, Janesville, WI).[1] Hot embossing of the cell selection devices was performed using a HEX03 embossing machine (Jenoptik Optical Systems GmbH, Jena, Germany). The embossing conditions consisted of using a temperature of 155°C and 30 kN force for 120 s for the substrate material, which was cyclic olefin copolymer, COC; we have shown that COC produces high loads of functional groups to its surface following UV/O_3 activation with minimal amounts of non-specific adsorption to its surface.[2]

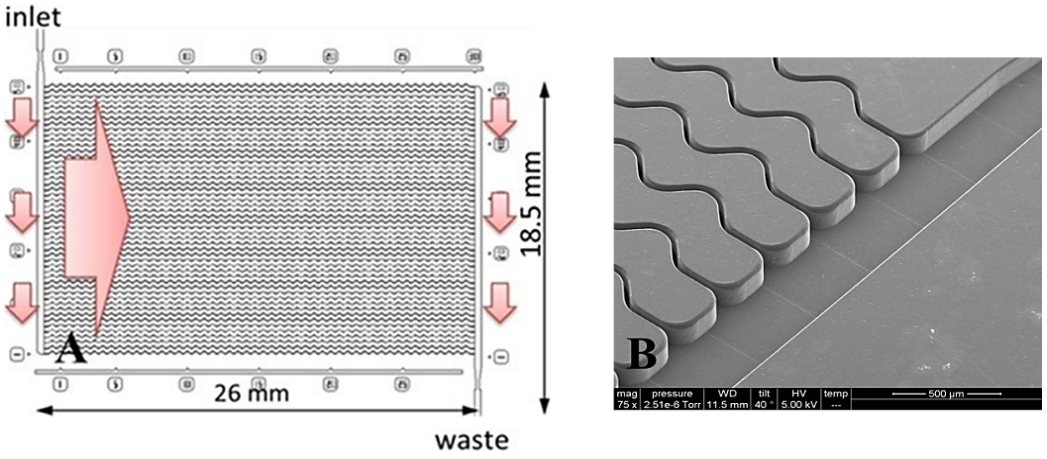


Figure S1. (A) AutoCAD drawing of the z-configuration cell selection device, which in this format contains 50 high aspect ratio parallel, sinusoidal channels with dimensions of 25 x 150 μm (w x h). The large arrow indicates the sample flow direction through the microchannels. (B) SEM image of the cell selection bed showing the input channel and the associated sinusoidal channels. The sinusoidal channels contained the mAbs, which were covalently anchored to the polymer surface via an amide bond formed between the polymer's surface confined carboxylic acid groups and a primary amine group resident within the mAb primary structure (direct attachment) or to a 5' primary amine group resident on the ssDNA linker that contained a uracil residue for clipping via USER™ (see Scheme S1).

Operation of the cell selection device. Prior to blood sample infusion and following mAb attachment, the selection device was thoroughly washed with 1 mL of 0.5% BSA/PBS buffer at a flow rate of 40 µL/min to remove unbound mAb from the microchannel walls. Blood specimens collected into BD Vacutainer®

tubes were placed on a nutator until the blood sample was processed. Two mL of patient blood was transferred into a disposable Luer Lock™ syringe (BD Biosciences, Franklin Lakes, NJ) using a BD vacutainer female luer transfer adapter. Immediately after transfer, blood samples were processed through the cell selection device. A PHD2000 syringe pump (Harvard Apparatus, Holliston, MA) was used to hydrodynamically drive the whole blood sample through the selection device at the appropriate volumetric flow rate to attain an average linear velocity of sample through the sinusoidal microchannels (1.1 mm/s for CMMCs, 2 mm/s for CTCs and KG-1 model leukemic cells). Finally, the cell selection device was flushed with 2.5 mL of 0.5% BSA/PBS at a linear velocity of 4 mm/s to remove any nonspecifically bound cells.

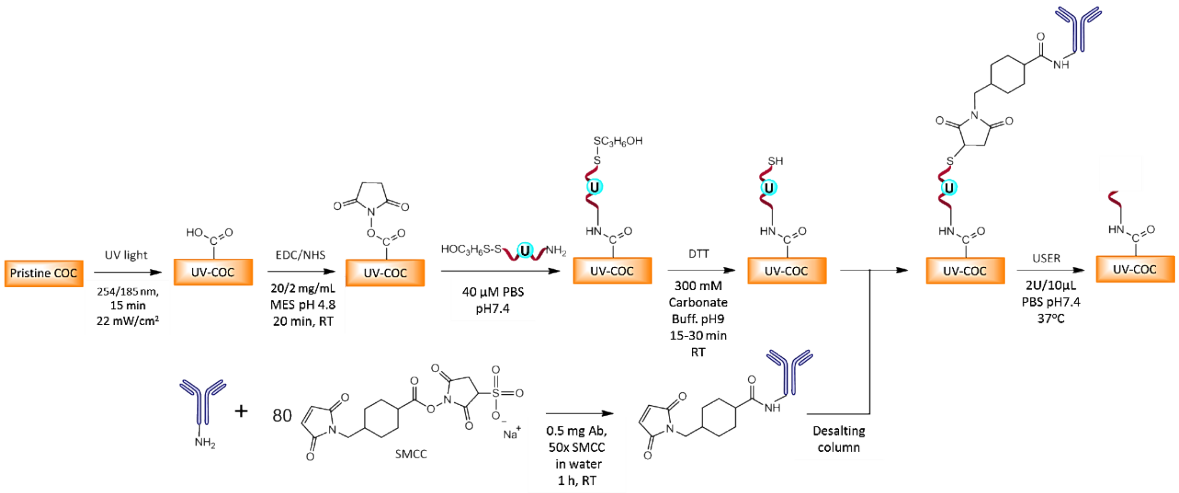
Heating system. Temperatures were maintained on-chip using thin film resistive heaters (KHLV-101/10, Omega Engineering, Inc., Stamford, CT) under closed-loop PID control (CN77R340, Omega Engineering, Inc., Stamford, CT). Temperature feedback was provided through Type K thermocouples (5TC-TT-K-36-36, Omega Engineering, Inc., Stamford, CT) mounted between the cover plate and heaters. Double-sided thermal tape (Digi-Key) was attached to the cover plate of the cell selection device and the copper block.

Oligonucleotide linker sequences. Single-stranded oligonucleotide linkers with an internal dU residue were obtained from Integrated DNA Technologies (Coralville, IA). The sequences of oligonucleotides that were used as cleavable linkers (ssDNA linkers) are summarized in Table S1. In principle, the ssDNA linker can contain any sequence, however, a string of dTs at the 3' and 5' ends were used to eliminate multiple points of attachment to the solid surface as dTs do not contain primary amines.

The effective footprint for each ssDNA linker can be calculated from the radius of gyration R_g for a coil-like ssDNA, where $R_g = 0.38N^{1/2}$ nm and N is the number of nucleotides in the strand.[3] It has been reported that the surface coverage decreases as N increases; ~6 x 10^{12} probes/cm^2 for an 8mer and ~7 × 10^{11} probes/cm^2 for a 48mer.[4]

Table S1. Sequences of the ssDNA linkers investigated in this study.

5'-3' Oligonucleotide sequence (length)	ID	Length (nm)
NH_2-C_6-T_{10} GCT ATA TUT T_6-C_3-SS-C_3OH (25 nt)	25dX	9.9
NH_2- C_6-T_8 UTT TTT TCC GAC ACT TAC GT_8-C_3-SS-C_3OH (34 nt)	34dX	12.9
NH_2-C_{12}-T_8 CCC TTC CTC CTC ACT TCC CTT TU T_9-C_3-SS-C_3OH (40 nt)	40dX	15.8
NH_2-C_6-$(C_2H_6O_2)_6$-T_{10} U T_{10}-C_3-SS-C_3OH (20 nt)	20dT	10.9
NH_2-C_6-$(C_2H_6O_2)_6$-T_{30} U T_{10}-C_3-SS-C_3OH (40 nt)	40dT	17.5



Scheme S1. Schematic of mAb immobilization onto UV/O_3-activated COC thermoplastic substrate using cleavable ssDNA linkers. While a COC substrate is shown here, any surface containing functional groups can be used for the ssDNA linker attachment with slight modifications of the immobilization chemistry.

Monoclonal antibody (mAb) labeling with sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC). mAb labeling involved addition of 6 µL (50x excess) of maleimide-crosslinker sulfo-SMCC (10 mg/mL in nuclease free water) to 0.5 mg mAb in 500 µL of water followed by incubation for 1.5 h at room temperature on a rocker. Following reaction, the mAb was purified using a Zeba column (with exchanged buffer for PBS pH 7.4) to remove excess non-reacted sulfo-SMCC. mAb-SMCC in PBS pH 7.4 was stored up to 3 d at 4°C for cell selection device modification. When non-lyophilized mAbs were used, which contained sodium azide, the mAb was purified using a Zeba column prior to SMCC labeling or direct attachment.

Modification of the cell selection chip with oligonucleotide linkers. A UV/O_3-activated device was flooded with a solution of 20 mg/mL EDC and 2 mg/mL NHS in 100 mM MES (pH 4.8) and incubated at room temperature. After 20 min, an air filled syringe was used to remove solution from the chip and immediately after that, a 40 µM solution of the ssDNA linker in PBS buffer (pH 7.4) was introduced into the device and allowed to incubate for 2 h at room temperature or overnight at 4°C to covalently attach the ssDNA linker at its 5'- terminus to the activated COC surface. After the reaction was complete, the microfluidic chip was rinsed with 100 µL PBS (pH 7.4) at 40 µL/min and 300 mM DTT in carbonate buffer (pH 9), which was infused into the microfluidic chip for 20 min to reduce the 3'-disulfide group into a reactive sulfhydryl moiety (-S-H). The microfluidic chip was rinsed with 100 µL PBS (pH 7.4) at 50 µL/min, and immediately an aliquot of the modified mAb-SMCC was introduced (~0.5 mg/mL). The reaction proceeded for 2 h on ice or overnight at 4°C (Scheme S1).

Cell release. The cell selection device was infused with USER™ enzyme (2U/10µL PBS pH 7.4) and incubated at 37°C. Immediately after incubation, the released cells were washed from the microfluidic chip at 10-25 µL/min and collected into a well of a titer plate. When cell staining was required, the microfluidic chips were viewed under a microscope before and after release for visual confirmation of the release and the released cells were identified in the wells of a titer plate.

Clinical samples. Patients with multiple myeloma and metastatic ovarian cancer were recruited according to a protocol approved by the University of North Carolina's IRB. Blood was collected into BD Vacutainer® (Becton-Dickinson, Franklin Lakes, NJ) tubes containing the anticoagulant EDTA and were processed within 6 h of the blood draw to obviate issues with blood coagulation.

Cell cultures. KG-1 (leukemic) cancer cell lines were cultured in RPMI 1640 with 2.5 mM L-glutamine supplemented with 10% FBS (GIBCO, Grand Island, NY). Hs578T and SKBR3 (breast cancer, adherent) cell lines were grown in 1x MEM/1x NEAA/10%FBS, and 1x McCoy/10%FBS, respectively. The cell lines were incubated at 37˚C under a 5% CO_2 atmosphere.

FISH analysis of CMMCs isolated from clinical samples. FISH analysis was performed on CMMCs selected from patient samples. CMMCs were selected from 1 mL of a multiple myeloma patient's blood. CD138 mAb was used for CMMC selection. The mAb-modified microfluidic chip was washed with PBS pH 7.4/0.5% BSA before analysis and whole blood was infused at 2 mm/s. Post-selection washing of the microfluidic chip removed remaining blood cells from the chip and was performed at 4 mm/s. The chip was then infused and incubated with USER™ enzyme at 37°C and immediately after incubation, cells were washed out and collected into a titer well with 1:1 (v/v) methanol:acetic acid. Chips were viewed under a microscope before and after release for visual confirmation of release. Cells were spun down for 7 min and supernatant was removed. A 3:1(v/v) methanol:acetic acid solution was added with this process repeated twice.

Cell suspensions in 1:1 (v/v) methanol:acetic acid solution were transferred onto a glass slide. Slides were immediately placed on a hot plate at 42°C and left to dry for ~15 min. Cells on the slide were treated with 0.05% NP-40 in 2X SSC (Sigma Aldrich, pH 7.3) at 37°C, dehydrated successively in 70%, 85% and 100% ethanol at room temperature for 2 min each and dried completely. The DLEU 13q14 Kreatech probe with 13qter control probe was used for the FISH assay. A 7.5 µL solution of the probe (DLEU 13q14 Kreatech probe) was applied to each slide and was covered with a coverslip and sealed with rubber cement.

Cells with probes were denatured at 75°C for 7 min and hybridized at 37°C overnight in a HYBrite oven. After removal of rubber cement and cover slips, the slides were washed in 0.4X SSC / 0.3% NP-40 at 73°C for 2 min and then in 2X SCC / 0.1% NP-40 at room temperature for 1 min. Slides were air-dried and 10 µL (0.1 ng/mL) of DAPI II counterstain (Vysis) was applied to each slide. The cells were analyzed with a Zeiss Axioplan 2 Microscope with a 63X or 100X Zeiss oil immersion objective.

Results and Discussion

Evaluation of the ssDNA enzymatic cleavage with USER™. Enzymatic cleavage was evaluated using a bench top experiment with a thermo-cycler, ssDNA linkers designed with a single dU residue and the USER™ system. The definition of 1 unit of USER™ enzyme is described as the amount of enzyme required to nick 10 pmol of a 34mer oligonucleotide containing a single uracil residue in a total reaction volume of 10 µL in 15 min at 37°C. For these initial experiments, we used 10 pmol of a Cy5-labeled single stranded 34 nt oligonucleotide with a dU incorporated into its structure as the model to evaluate the USER™ kinetics and specificity (*i.e.*, cleavage of the 34mer at only the dU residue should generate a 26 nt fragment). Figure S2 presents data of electrophoretic separations for the products produced from the enzymatic reaction (USERTM) of the Cy5-labeled oligonucleotide. Enzyme kinetics were monitored by observing the disappearance of the 34 nt substrate and appearance of a 26 nt product (Figure S2A) as a function of reaction time. Reactions were stopped with a formamide solution. The products were separated using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

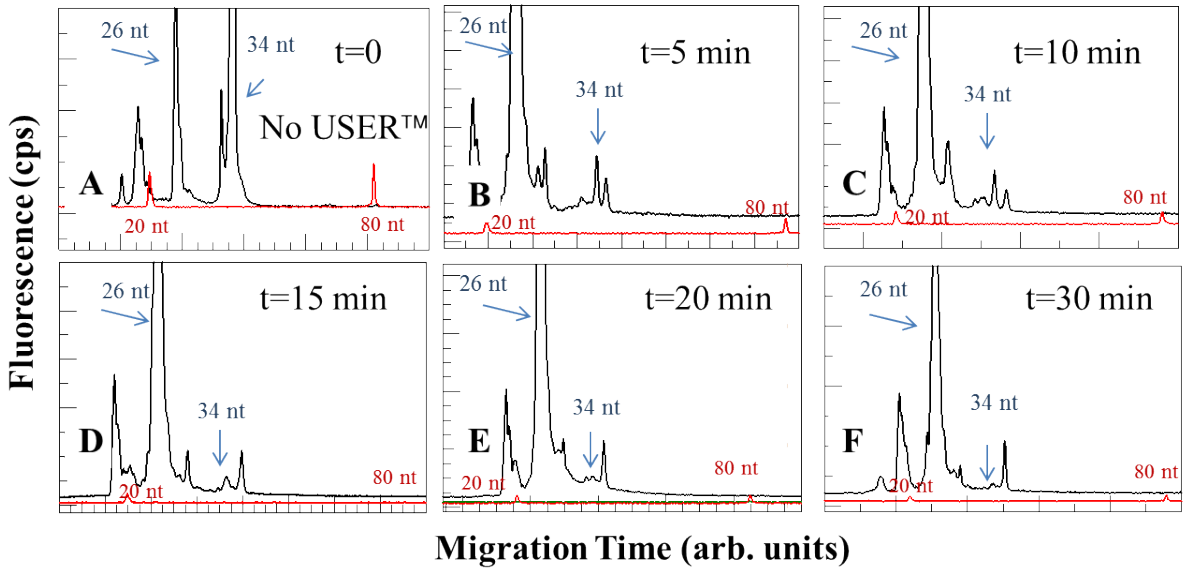
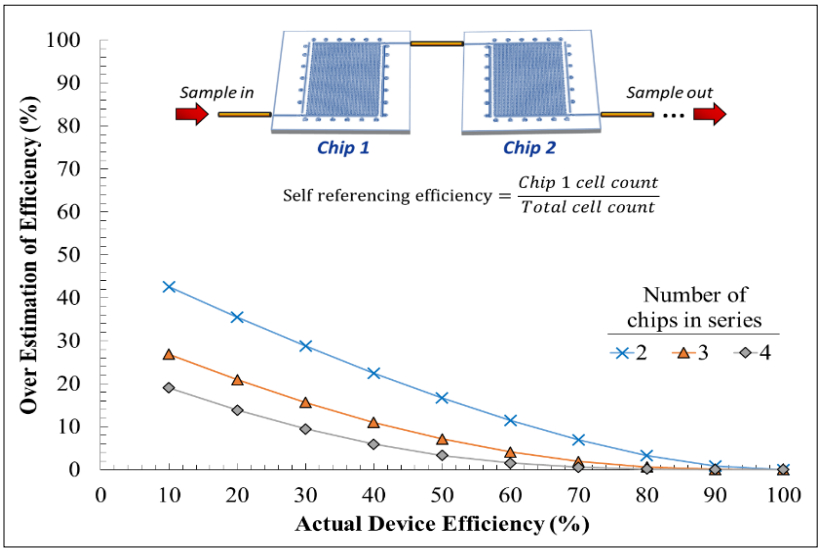


Figure S2. Electropherograms of the 26 nt (5'TT TTT TCC GAC ACT TAC GT8 Cy5-3') product generated from USER™ initiated cleavage of an oligonucleotide containing the following sequence; 5'- NH2- C6/T8 UTT TTT TCC GAC ACT TAC GT8 Cy5-3'(34 nt)**.** Reactions were terminated at the desired time using a formamide solution. The red traces represent the separation of standards (20 nt and 80 nt shown).

Electropherograms revealed that the single-stranded oligonucleotides (Figure S2B) contained 7-10% of the nicked oligonucleotide at the dU site prior to the enzymatic reaction as we observed both 26 and 34 nt fragments. After 5 min of the enzymatic reaction at 37°C, the majority of the 34 nt substrate was converted into a 26 nt product resulting from USER™ cleavage at the dU residue. Reaction times ≥15 min allowed the enzyme to completely nick the substrate (Figure S2E). These experiments demonstrated that the USER™ enzyme cleaved the oligonucleotide only at the dU residue as other products were not observed in the electropherograms.

Self-referencing method for cell recovery determinations. 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; the seeded cells are typically immortalized cell lines, which have a fixed and fairly constant expression level of the selection antigen. Unfortunately, this technique does not allow one to determine recovery of target cells present in clinical samples because the cell frequency is unknown. Clinical samples also contain target cells with a diverse array of selection antigen expression levels, which can dramatically affect cell recovery. In addition, seeded cells can undergo damage and/or loss before infusion into devices in an uncontrolled fashion, resulting in high levels of variances in the determinations.

In light of these issues, we developed a "self-referencing" method. Prior knowledge of the number of target cells is not required and also, is not affected by cell damage or loss prior to infusion of the sample into the selection device. 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 (Figure S3). As shown in Figure S3, 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% (<7% error). 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 Eq. 1:



$$\frac{CTC_{chip\,1}}{CTC_{total}} = \frac{CTC_{chip\,1}}{CTC_{chip\,1} + CTC_{chip\,2} + \ldots CTC_{chip\,N}} \quad \text{(Eq. 1)}$$

Figure S3. Accuracy of the self-referencing method for determining recovery of target cells from samples in which the expression level of the target would be highly variable and the input cell number is unknown or variable. Multiple devices in series deplete the sample of the target cells, allowing for quantification of the cell recovery. Over-estimation of recovery is minimized as more devices are used in the series.

In addition to the ability to determine cell recovery from experiments in which the spiking level cannot be determined, the self-referencing approach also generates lower standard deviations for determinations of recoveries in spiking experiments. For example, in spiking experiments using KG-1 cells that were seeded into normal blood samples, the absolute KG-1 cell recoveries calculated using the self-referencing method (see Eq. 1) agreed favorably with the recoveries secured from the KG-1 seeding level. However, when relying on the targeted seeding level for efficiency measurements, the relative standard deviation for

the recovery efficiency was ~35% while that for the self-referencing method was only ~6% (data not shown).

Cell recovery using mAb-ssDNA linkers versus direct mAb surface attachment. We have noted that the size of the target cell with respect to the selection microchannels as used in the device shown in Figure S1 can have an influence on recovery when using surface immobilized mAbs.[5] For example, the average diameter of KG-1 cells is 12 μm compared to 16-19 μm for the Hs578T and SKBR3 model cells. Therefore, the 25 μm wide channel used in the current cell selection device would indicate that KG-1 cells would show lower recovery compared to the Hs578T and SKBR3 cells. Other factors affecting recovery include decreased centrifugal forces due to smaller cell size and the adherent nature of the cell, which can affect the extent of interaction of the solution-borne cells with the affinity decorated surface; KG-1 cells are non-adherent while the SKBR3 and Hs578T cells are adherent. Adherent cells would have a tendency to increase the interaction time with the mAb-decorated surface compared to non-adherent cells due to a higher tendency of these cell types to roll along the microchannel wall and thus, show higher recovery. Also, the expression level of the target antigen can influence the recovery. The recovery of cell selection is affected by encounter duration and strength of adhesion between a cell-bound receptor and tethered ligand, which can be controlled by the flow velocity as stipulated by the Chang-Hammer Model.[6]

We were interested in comparing the recovery for the case where the mAb is directly attached to the surface as we have shown previously,[2,7] to the case where the mAb was attached to the surface using the ssDNA linker. The relative recovery for the three model cell lines is shown in Table S2 for three cases: (i) Direct attachment of the mAb to the activated COC surface using our reported methods;[2, 7] (ii) direct attachment of an isotype control IgG mAb to the activated COC surface (the selection microfluidic chip was made from this thermoplastic); and (iii) attachment of the mAb to the activated COC surface using the ssDNA linker. As can be seen from this data, there was no statistical difference in the recovery of the adherent cells between direct attachment of the mAbs to the activated surface versus cases where the mAb was attached to the surface using the ssDNA linker. Even in the case of the non-adherent KG-1 cells, no difference was noted between direct surface attachment versus ssDNA linker attachment of the selection mAb to the microfluidic channel surface. As noted from our previous work, the absolute recovery of MCF-7 cells using anti-EpCAM monoclonal antibodies and the microfluidic device used herein was ~98%.[5]

Table S2. Relative recovery of SKBR3, Hs578T and KG-1 cell lines using direct mAb attachment to the activated thermoplastic (COC) surface and mAb attachment using the ssDNA linker. The number given in parentheses represents the number of experimental trials performed. In these experiments, anti-EpCAM mAbs were used for the selection of the SKBR3 cells, anti-FAPα mAbs for selection of the Hs578T cells, and anti-CD34 mAbs were used for selection of KG-1 cells. All recoveries were reference to the SKBR3 recovery (85 ±4%) using the ssDNA linker, 40dX (see Table S1 for sequence of this linker). nd = not determined. Note that for all cell lines, no statistically significant difference ($p > 0.05$) was observed between the Ab attachment methods.

Linker Name/Ab	Relative Cell Recovery (%) Mean ±SD		
	Hs578T	**SKBR3**	**KG-1**
No linker/direct attachment	90 ±9 (n=8)	96 ±12 (n=4)	81 ±6 (n=7)
No linker/IgG	1 ±0 (n=3)	1 ±0 (n=3)	nd
25dX	86	nd	nd
34dX	74 ±7 (n=3)	nd	nd
40dX	80 ±6 (n=5)	**100 ±5 (n=3)**	76 ±5 (n=5)
20dT	--	nd	74 ±7 (n=9)
40dT	--	nd	77 ±5 (n=6)

Cell enzymatic release efficiency. The release efficiency of selected cells are given in Table S3 as a function of USER™ reaction time. As can be seen from this data, the release efficiency was >85% for reaction times of 30 min with little difference seen for the 45 min reaction time irrespective of the sequence content and position of the dU residue. In addition, the release of both viable and fixed cells could be

achieved with high efficiency (Figure S4). When using the direct attachment method and trypsin as the release reagent, the selected cells could not be released from the surface when the cells were fixed (data not shown).

Table S3. Enzymatic release efficiency of viable and fixed KG-1, SKBR3 and Hs578T cells isolated as a function of cell incubation time with USER™ ($n \geq 3$). nd = not determined.

Cell Status	Linker Type	KG-1 Mean Release Efficiency ±SD (%)		
		15 min	30 min	45 min
Viable Cells	20dT	58 ±8†	83 ±5	86 ±3
	40dT	67 ±3†	84 ±4	88 ±2
	40dX	69 ±6†	87 ±3	89 ±3
Fixed Cells	20dT	62 ±7†	79 ±4	81 ±2
	40dT	59 ±6†	77 ±5	83 ±6
Cell Status	**Linker Type**	**SKBR3 Mean Release Efficiency ±SD (%)**		
		15 min	**30 min**	**45 min**
Viable Cells	40dX	89 ±3	93 ±4	nd
Fixed Cells	40dX	82 ±3	84 ±2	nd
Cell Status	**Linker Type**	**Hs578T Mean Release Efficiency ±SD (%)**		
		15 min	**30 min**	**60 min**
Fixed Cells/CF*	34dX	49 ±9†,‡	75 ±8	84 ±5
Viable Cells	34dX	83 ±6	nd	nd
Viable Cells	40dX	86 ±4	nd	nd
Fixed Cells	40dX	84 ±3	89 ±6	nd

CF*- continuous flow experiment

† The release efficiency after 15 min was statistically different ($p < 0.05$) from the efficiency observed after 30 min.

‡ For this cell line, this release format was statistically different ($p < 0.05$) from all other release formats at this time point.

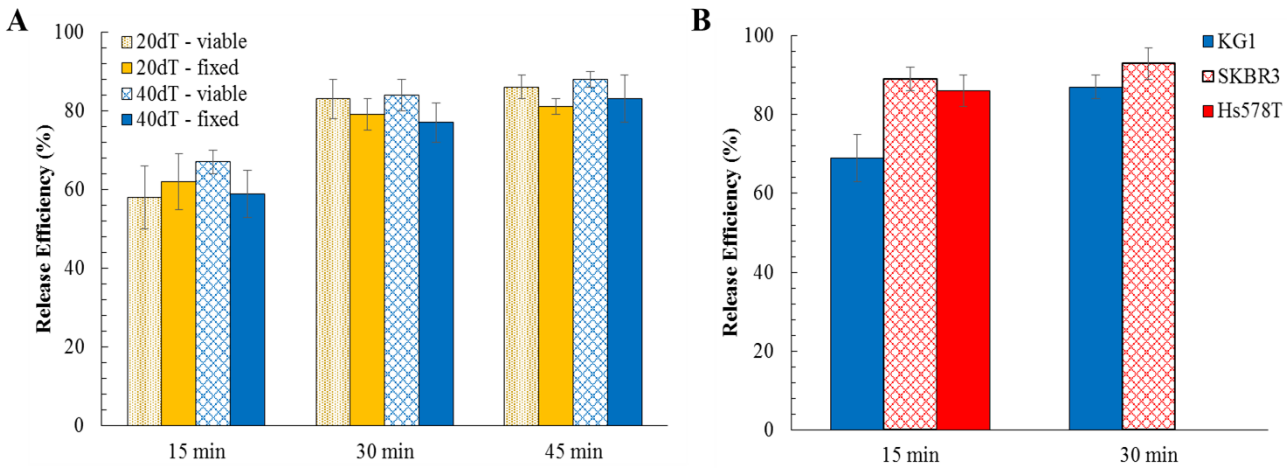


Figure S4. (A) Release efficiency of viable and fixed KG-1 cells isolated with the 20dT and 40dT linkers as a function of incubation time with USER™ enzyme. (B) Comparison of the release efficiency as a function of time for the USER™ enzymatic process for three cell lines isolated with 40dX linkers and released in a viable state.

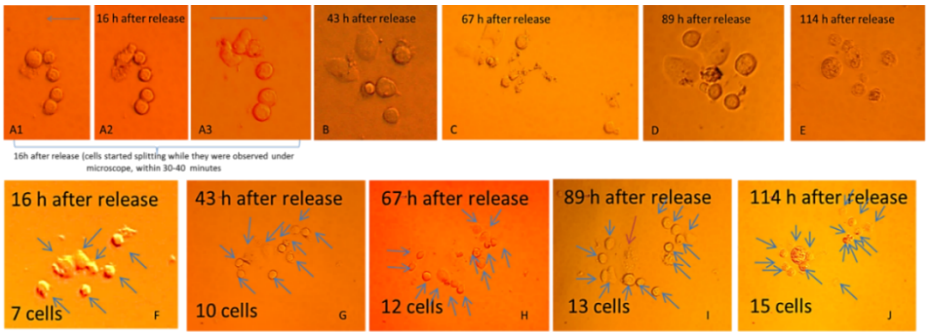


Figure S5. Micrographs of two SKBR3 cell groups (A-E group #1 and F-J group #2) identified on a culture plate after cells were isolated using the cell selection device, released from the device with USER™, collected into a well, and grown in McCoy medium supplemented with 10% FBS at 37°C, 5% CO_2 for 5 d.

Cell cultivation after release with USER™. SKBR3 cells upon release were collected into a titer well (2 cm^2) of a 24-well plate and culturing medium was introduced. Observations of cell culturing using a microscope were made in 24 h intervals. Figure S5A-E shows micrographs collected during a 5 d period for 5 SKBR3 cells (out of 113 present in the well). After 16 h following release, a group of cells was identified (Figure S5A1). After 30 min, we observed morphological changes occurring in the largest cell of the group (Figure S5A2). After an additional 30 min, the same cell divided (Figure S5A3). The area presented in Figure S5A1-A3, was further observed during a 67 h time period and showed 2 cells migrating out of the field of view (see Figure S5C). After 5 d, a new cell group of 8 cells was formed. In the 5th day, from the initial 113 cells, 203 cells were counted in the culture dish. Clearly, cells divided and grew in culture. We suspect the low seeding density caused a slow growth rate of the SKBR3 cells. By looking at individual cells (or a small group of cells) in the culture dish, we concluded that cells were capable of dividing after selection and the subsequent release from the selection microfluidic chip. Another example of SKBR3 dividing is shown in Figure S5F-J, where from an initial 7 cells there were 15 cells after 5 d of cultivation.

KG-1 cell staining and flow cytometry. KG-1 cells affinity selected using the cell selection device via anti-CD34 mAb isolation were stained with a cocktail of fluorescently conjugated antibodies, 20 µL each of 0.4 mg/mL CD34-FITC, 0.2 mg/mL CD33-PE and 0.1 mg/mL CD117-APC mAbs (BioLegend, San Diego, CA) and incubated in the dark at 4°C for 30 min. The cell selection device was then rinsed with 100 µL of PBS/0.5% BSA at 25 µL/min and stained with 10 ng/mL DAPI followed by post-rinsing with 100 µL of PBS/0.5% BSA at 25 µL/min. Cells were released via USER™ and collected into 250 µL of PBS.

The following samples were prepared as controls: Unstained KG-1 cells serving as an autofluorescence control (Figure S6A); isotype controls where cells were stained with a cocktail of isotype mAbs consisting of 5 µL of 0.2 mg/mL IgG1-PE, 2.5 µL of 0.2 mg/mL IgG1-APC and 4 µL of 0.5 mg/mL IgG2a-FITC mAbs in 200 µL PBS (Figure S6B); positive controls with the cells stained using a cocktail of mAbs containing 5 µL each of 0.2 mg/mL CD33-PE, 0.1 mg/mL CD117-APC and 0.4 mg/mL CD34-FITC in 200 µL PBS (Figure S6C); and cells stained on-chip following selection (Figure S6D) with the same concentration of mAbs used as the positive control cells. For the control cells, the samples were incubated in the dark at 4°C for 30 min following which the cells were washed three times with 1 mL of PBS/0.5% BSA and centrifuged. The supernatants were decanted and the cells were resuspended in 250 µL PBS/0.5% BSA. DAPI staining (1 ng/mL) was performed on the positive and negative controls just prior to the analysis for the determination of cell viability. Samples were analyzed on a MACSQuant Analyzer (Miltenyi Biotech, Inc., Bergisch Gladbach, Germany). Data acquisition and analysis (Figure S6) was performed using FlowJo software (FlowJo LLC, Ashland, OR). Data from these experiments are summarized in Table S4.

Table S4. Summary of flow cytometry results for the KG-1 cell line.

Antigen (Ag)	MFI vs. Isotype	% Stained Cells*,**		Change in Ag expression (positive control vs released cell)*
		Positive Control	Released Cells	
CD33	10x	94%	83%	47%
CD34	89x	96%	92%	54%
CD117	2x	77%	46%	61%

*- vs. unstained cells, **- Overton Method was used to calculate % stained cells.

The expression of CD33, CD34, and CD117 antigens on the KG-1 cells was evaluated by comparing the positive control's mean fluorescence intensity (MFI) to the isotype control, which takes into account nonspecific binding of the mAbs. The three antigens represent separate paradigms of antigen expression: (i) CD34 is highly expressed (89x isotype); (ii) CD33 is moderately expressed (10x isotype); and (iii) CD117 is weakly expressed (2x isotype). In order to assess the efficacy of staining on-chip then releasing, the released cells were compared to the positive control cells stained in solution by using the unstained cells as a reference point.

For all antigens, the released KG-1 cells showed an average MFI that was 54 ±7% of the positive control's fluorescence intensity. This data likely indicated that when the cells were affinity-selected and bound to the cell selection device surfaces during the staining process, only the cells' surfaces not attached to the surface mAb were sterically accessible (*i.e.,* roughly half the cell could be labelled). While this reduced the released cell's overall MFI, this did not significantly affect our ability to discern these cells from unstained cells (see Table S4). The obvious exception to this statement is when interrogating CD117 due to its low expression, which even in positive control only showed 77% positive with an MFI of 2x the isotype control.

Conducting experiments where cells were affinity-recovered from blood samples using the microfluidic chip and then released via USER™ and stained off-chip in solution was hypothesized as potentially improving the MFI signal compared to on-chip solid-surface staining. However, the rigorous purification steps, which includes multiple centrifugation steps, resulted in significant cell loss and compromised cell integrity and viability reducing the number of cells available for flow cytometric analysis from an already limited cell population. The solid-phase immunostaining and subsequent release of stained cells eliminated the purification steps significantly minimizing cell loss and/or cell damage making sufficient numbers of selected cells available for cytometric analysis in spite for the slightly lower MFI signal generated under these conditions. For the solid-phase immunostaining, the cells are stained, followed by a washing step and finally released with the released cells collected into a microfuge tube; this entire volume can be subjected to flow cytometric analysis with minimal cell loss and/or damage.

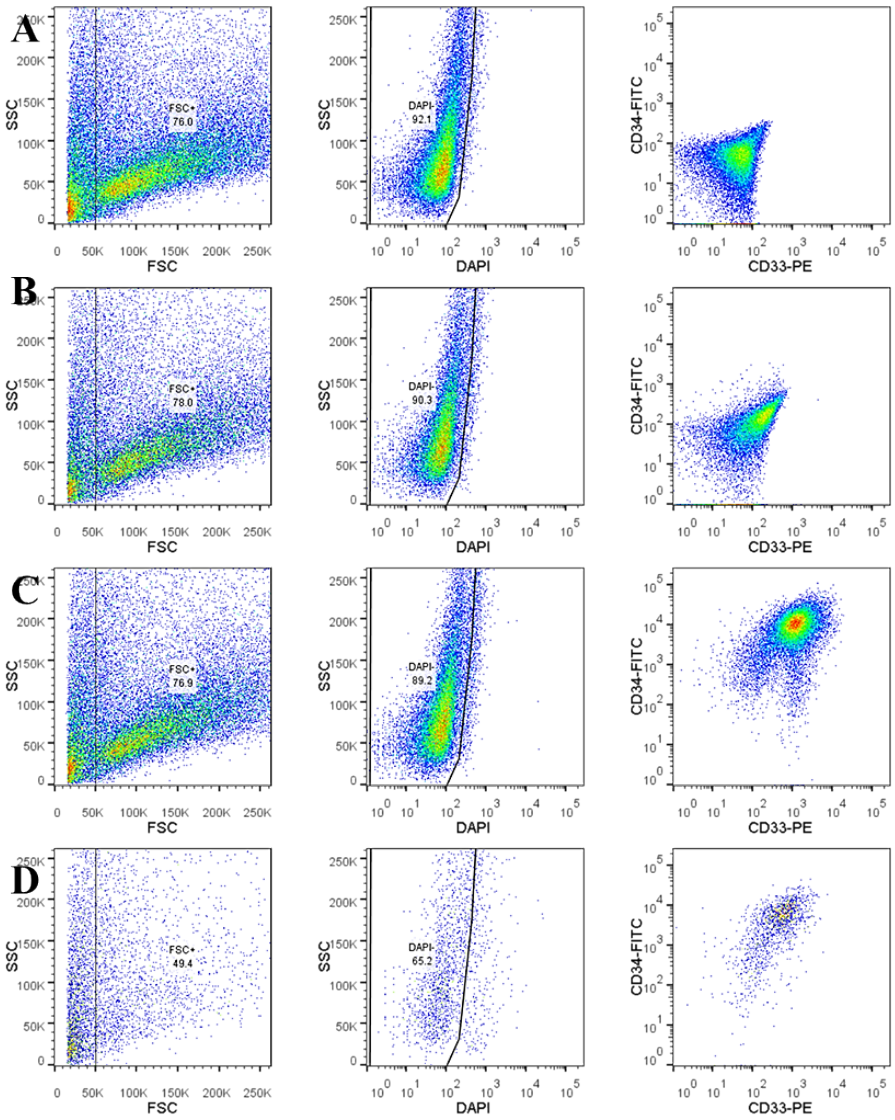


Figure S6. Flow Cytometry data for KG-1 cells. The following samples were prepared as controls for the analysis: (A) Unstained KG-1 cells serving as an autofluorescence control; (B) isotype controls; (C) positive controls with cells stained in suspension; and (D) cells stained on-chip then released by USER™ incubation. KG-1 cells were gated as DAPI-positive and forward scatter (FSC) positive before assessing antigen expression.

Isolation of CTCs from metastatic ovarian cancer (M-OVC) patient blood. We performed clinical experiments by processing 2 M-OVC blood samples. We evaluated the number of selected EpCAM+ CTCs upon USER™ release into wells of a 96 well plate. A composite micrograph of a 7 mm diameter well with DAPI-stained EpCAM+ CTCs isolated from blood of a M-OVC patient is shown in Figure S7. Cells were stained with CD45, DAPI, and CK-Pan (Fig. S6C-E). Table S5 summarizes the results for these samples.

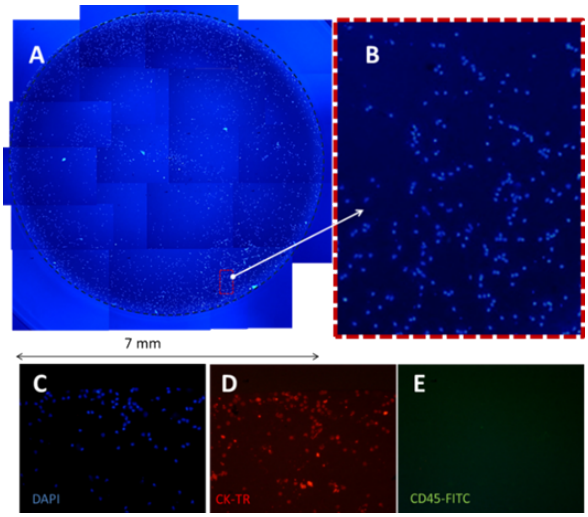


Figure S7. (A) Composite image of a 7 mm diameter well with DAPI-stained EpCAM+ CTCs isolated from a M-OVC patient. (B) Close-up image of a smaller area of the well. (C-E) Fluorescence images of CTCs stained with DAPI, CK-TR, and CD45.

Table S5. Clinical yields of CTCs from metastatic ovarian cancer patients (M-OV) using mAb directly attached to the selection surface versus attachment using the oligonucleotide linker.

Sample ID	Affinity Bed	mAb direct attachment CTC/mL	mAb attachment via linker CTC/mL
M-OVC1	anti-EpCAM	397	331
M-OVC2	anti-EpCAM	680	717

The results suggested similar cell numbers isolated using microfluidic chips modified via the ssDNA linker and direct attachment of the mAb to the activated surface, similar to what we observed for the cell line data. With the aid of USER™, cells were released following selection and eluted into titer wells where they were stained with the LIVE/DEAD™ kit and/or DAPI/CK/CD45 and visualized under a microscope. The average viability of CTCs after chip isolation and release was evaluated from M-OVC samples and determined to be 93 ±5% (n=5) for EpCAM+ CTCs. Upon release with USER™ and based on evaluation of multiple areas of the wells in which cells were collected, the viability was determined to be 87 ±6% for EpCAM+ CTCs. Figure S8 presents results for EpCAM+ CTCs secured from M-OVC (Figure S8A-C).

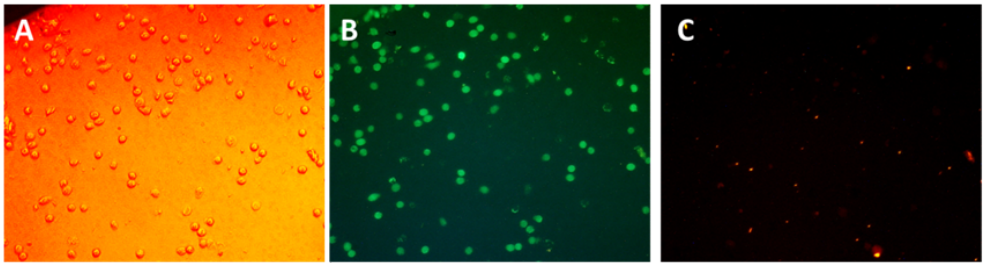


Figure S8. (A) DIC and fluorescence images of M-OVC CTCs isolated using anti-EpCAM antibodies and released into a titer well. (B-C) EpCAM+ CTCs were stained with LIVE/DEAD kit after release for viability determinations, (B) is green fluorescence signal for viable cells and (C) is red fluorescence signal from ethidium homodimer-1 in dead cells.

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