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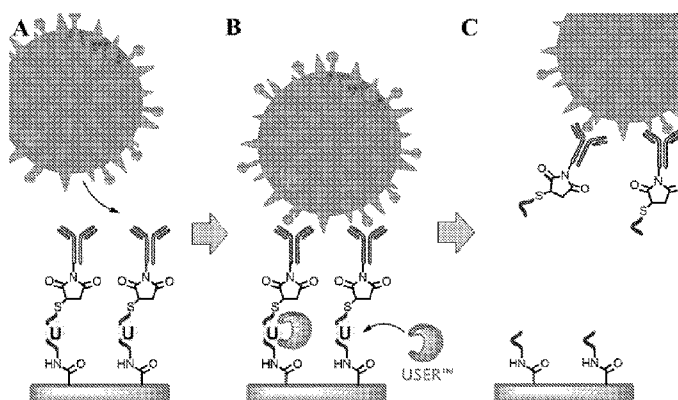


Figure 1

(57) Abstract: A device useful for collecting a target cell or compound from a composition containing the same is formed from the coupling of a construct having a functional group to a substrate. The construct includes: (a) a functional group for attachment to the substrate; (b) a specific binding agent that specifically binds to said target cell or compound; and (c) a single stranded polynucleotide linker coupling the affinity binding group to the substrate, the oligonucleotide having a cleavable site therein.

SIMPLE RELEASE STRATEGY FOR SURFACE CAPTURED CELLS AND COMPOUNDS

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Background of the Invention

10 The ability to release affinity-selected cells, such as circulating tumor cells (CTCs), from surfaces containing selection antibodies (Ab) without perturbing the cells' morphology, viability, molecular content and phenotype has been a major challenge. In spite of the challenge, compelling applications would result from the ability to release the selected cells such as securing molecular information from the cells that can be used for basic discovery
15 and/or molecular diagnostics.

The challenges associated with current release strategies include inefficient release or damage imposed on the cell by the release process. For example, cell release by shear-based methods¹⁻² are highly dependent on the number of attachment points between the cells' antigens (Ags) and surface immobilized Abs. Excessively high shear rates are required to
20 release cells with multiple attachment points, which can damage them, especially fragile CTCs.³ Synthesis or graft methods are incompatible or difficult to apply in microfluidic devices,⁴ which have proven to be an attractive platform for CTC selection due to the high recovery they offer with minimal damage imposed on the cells during selection.

Thermally responsive materials⁵⁻⁷ have been used to release selected cells with an
25 efficiency of ~59% and viability of 90%.⁵ Polymer brushes on nanostructured surfaces were capable of isolating cells at 37°C and releasing 90% of the selected cells after 30 min at 4°C, but the fabrication of the cell selection device and its functionalization was a challenge.⁸ Functionalized alginate hydrogels were able to isolate and release cells with 90% viability, but the purity of the isolated population was low due to non-specific cell adsorption to the
30 hydrogels.⁹⁻¹⁰ Lectins were reported to isolate and release lymphocytes; however, the specificity resulted in low purity of the selected cell population with ~50% release efficiency.¹¹ DNA aptamers have also been used for cell isolation; cells could be released

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5 using DNase (68%) with 66% of the released cells remaining viable.¹² Proteolytic digestion of selection Abs has been reported, for example using trypsin.¹³⁻¹⁶ While efficient cell release was demonstrated, the damage of extracellular domains of membrane Ags is possible, thereby limiting the ability to immunostain the cells, which is required for phenotypic analysis of selected cells. In addition, trypsin can induce cell death and also, affect cell integrity. Light-
10 triggered cell release using photocleavable linkers attached to quartz surfaces, which achieved 85% release efficiency, has been reported.¹⁷ Light-based cell release methods, however, can induce DNA damage in cells that can confound diagnostic information.¹⁸

Summary of the Invention

15 A first aspect of the present invention is a construct useful for collecting a target cell or compound from a composition containing the same. The construct comprises, consists of, or consists essentially of: (a) a functional group for attachment to a substrate; (b) a specific binding agent (or affinity binding group) that specifically binds to the target cell or compound; and (c) a single stranded polynucleotide linker coupling the affinity binding group
20 to the functional group and substrate, the polynucleotide having a cleavable site therein.

A second aspect of the present invention is a device comprising a construct as described above, and in further detail below, formed the coupling (*e.g.*, covalent coupling) of the functional group to a substrate.

A third aspect of the invention is a method of collecting a target cell or compound of
25 interest, comprising the steps of: (a) contacting a composition containing the target cell or compound to the specific binding agent in a device as described herein; (b) separating the composition from the device; and then (c) cleaving the polynucleotide linker in the device to thereby collect the target cell or compound.

The foregoing and other objects and aspects of the present invention are explained in
30 greater detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1. Schematic illustration of one embodiment of a method of the present invention.

35 **Figure 2.** Fluorescence images of sinusoidal microchannels (A) before and (B) after 5 min enzymatic cleavage of a 3'-Cy3 modified 25 nt oligonucleotide containing a dU residue.

5 The microchannel surfaces were activated using UV/O₃ exposure and functionalized with 20 mg/mL EDC and 2 mg/mL NHS in 0.1 M MES (pH 4.8). After 20 min of the EDC/NHS reaction, a 40 μ M solution containing the 3'-Cy3 oligonucleotide was infused into the device. Before imaging, the device was rinsed with 0.1% SDS and nuclease-free water.

Figure 3. Release of KG-1 cells isolated using the 40dT linker. Cells stained with (A) 10 Live/Dead™ kit were released after (B) 15 min and (C) 30 min USER™ incubation. (D) Cells (eluted at 10 μ L/min) were enumerated. Live cells generate esterase-dependent Calcein, fluorescing green (ex/em 488/515 nm). Dead cells were susceptible to a cell-impermeable, DNA-intercalating dye fluorescing red (ex/em 570 nm/605 nm).

Figure 4. Fluorescence images used to quantify cell viability (A, B) after isolation 15 and (C-E) after release. Images C-E share the same scale bar.

Figure 5. Flow cytometry analysis of KG-1 cells isolated, stained, and released from the cell selection device.

Figure 6. FISH images with a 13q14 probe of patient circulating multiple myeloma cells (CMMCs). Green signal indicates the presence of chromosome 13 and red signal 20 indicates absence of the chromosome 13 deletion. The patient sample contained a mixture of CMMCs: (A) Cell shows the presence of the deletion and (B) polyploidy cell without the deletion. Cells were isolated using anti-CD138 antibody modified microfluidic chip.

Figure 7. (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 μ m (w x h). The large arrow indicates the sample flow direction through the 25 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 30 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 Figure 8).

Figure 8. Schematic of mAb immobilization onto UV/O₃-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 35 with slight modifications of the immobilization chemistry.

Figure 9. 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

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5 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.

Figure 10. 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.

Detailed Description of Illustrative Embodiments

15 The present invention is now described more fully hereinafter with reference to the accompanying drawings, in which embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather these embodiments are provided so that this disclosure will be thorough and complete and will fully convey the scope of the invention to those skilled in the art.

20 The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a," "an" and "the" are intended to include plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms "comprises" or "comprising," when used in this specification, specify the presence of stated features, integers, steps, operations, elements components and/or groups or combinations thereof, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components and/or groups or combinations thereof.

25 As used herein, the term "and/or" includes any and all possible combinations or one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

30 Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the specification and claims and should not be interpreted in an idealized or overly formal sense unless expressly so defined herein. Well-known functions or constructions may not be described in detail for brevity and/or clarity.

5 It will be understood that when an element is referred to as being “on,” “attached” to, “connected” to, “coupled” with, “contacting,” etc., another element, it can be directly on, attached to, connected to, coupled with and/or contacting the other element or intervening elements can also be present. In contrast, when an element is referred to as being, for example, “directly on,” “directly attached” to, “directly connected” to, “directly coupled”
10 with or “directly contacting” another element, there are no intervening elements present. It will also be appreciated by those of skill in the art that references to a structure or feature that is disposed “adjacent” another feature can have portions that overlap or underlie the adjacent feature.

15 **1. Definitions.**

“Target cell” as used herein may be any cell, including but not limited to microbial, bacterial, and animal cells. Examples include but are not limited to pathological and infectious agents, circulating tumor cells (CTCs), leukocytes and leukocyte subpopulations, etc.

20 “Target compound” as used herein may be any molecule, macromolecule or compositions, including vesicles, microvesicles, exosomes, liposomes, proteins, peptides, antibodies, drugs and toxins, etc.

“Biological fluid” as used herein may be any biological fluid, including but not limited to blood, blood fractions, cerebrospinal fluid, sputum, urine, etc. The biological fluid
25 may be concentrated or diluted, have fractions removed, and may have additives or stabilizers added thereto, such as in accordance with typical laboratory procedures for handling the same.

“Substrate” as used herein may be any suitable substrate for a binding procedure, including fluidic and microfluidic devices. Materials may be organic (*e.g.*, polymers such as polycarbonate), inorganic (*e.g.*, silica, glass, quartz, silicon dioxide, etc.), and composites
30 thereof.

“Specific binding agent” as used herein may be any suitable binding agent that specifically binds to a target cell or compound, including, but not limited to, antibodies, peptides, aptamers, peptoids, lectins, carbohydrates, etc.

35 “Antibody” as used herein includes natural, synthetic, and recombinant antibodies, as well as specific binding fragments thereof. Both monoclonal and polyclonal antibodies may be used, with monoclonal antibodies generally preferred.

5 “Polynucleotide” as used herein refers to any natural or synthetic polynucleotide, including both ribonucleic acids and deoxyribonucleic acids. While the length of the polynucleotide is not critical, lengths of 10 to 100 bases are typical. Nucleotide bases may be natural or synthetic and common or rare. The nucleotides may be modified, particularly end-terminal modified, to facilitate coupling thereof to substrates and specific binding groups,
10 *e.g.*, in accordance with known techniques.

1. Constructs and Devices.

As noted above, the present invention provides constructs useful for collecting a target cell or compound from a composition containing the same. The construct comprises,
15 consists of, or consists essentially of: (a) a functional group for attachment to a substrate; (b) a specific binding agent that specifically binds to the target cell or compound; and (c) a single stranded polynucleotide linker coupling the affinity binding group to the functional group or substrate, the polynucleotide having a cleavable site therein. Such constructs are generally synthetic constructs and can be produced by any suitable technique, including recombinant
20 synthesis, covalent coupling of the specified elements, or combinations thereof.

Suitable functional groups include, but are not limited to, amine, carboxylic acid, aldehyde, thiol, alcohol, azide and imine.

Suitable binding agents include, but are not limited to, antibodies, peptides, aptamers, peptoids, lectins, and carbohydrates.

25 The polynucleotide may be of any suitable length, but in typical embodiments is from 10 to 100 nucleotide bases in length.

The polynucleotide is, in general, single stranded, and may be DNA or RNA.

Where the polynucleotide is a DNA, the cleavable site may comprise, for example:
(a) a target nucleotide (*e.g.*, a deoxyuridine (U), an 8-oxo-guanine, or a deoxyinosine), (b) an
30 abasic site, (c) a photocleavable nucleotide; (d) a restriction endonuclease cleavage sequence;
(e) a phosphorothioated or methylated DNA; or (f) an allyl-modified nucleoside.

Where the polynucleotide is an RNA, the cleavable site may comprise, for example:
(a) an unpaired purine and unpaired pyrimidine base (b) a ribozyme cleavage site; or (c) a
Cas9 cleavage site.

35 Devices of the present invention may be produced by coupling (*e.g.*, covalent coupling) a construct as described above to a substrate. Any suitable substrate may be

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5 employed including an organic substrate (e.g., an organic polymer such as polycarbonate), an inorganic substrate (e.g., glass), or a composite thereof.)

In some embodiments, the device is a microfluidic device, with the polynucleotide and associated binding agent covalently coupled to the capture zone or capture bed therein. Examples of suitable microfluidic devices include, but are not limited to, those described in
10 S. Soper, M. Murphy et al., *Microfluidic Isolation of Tumor Cells or Other Rare Cells from Whole Blood or Other Liquids*, US Patent Pub. No. US 2012/0100521 (April 26, 2012).

2. Methods of use.

As noted above, devices of the present invention may be used to collect a target cell
15 or compound of interest. Such a method generally comprises steps of: (a) contacting a composition (e.g., a liquid such as a biological fluid) containing the target cell or compound to the specific binding agent in a device as described herein above and below; (b) separating the composition from the device; and then (c) cleaving the polynucleotide linker in the device to thereby collect the target cell or compound.

20 Examples of suitable target cells or compounds include, but are not limited to, a biological cell (e.g., a tumor cell such as a circulating tumor cell), microvesicle, exosome, small molecule (e.g., nucleotide, amino acid, peptide, synthetic drug) or large molecule (e.g., protein, DNA, RNA).

Contacting and separating steps will depend upon the particular physical structure of
25 the device, but are typically carried out in accordance with known techniques. See, e.g., S. Soper, M. Murphy et al., *Microfluidic Isolation of Tumor Cells or Other Rare Cells from Whole Blood or Other Liquids*, US Patent Pub. No. US 2012/0100521 (April 26, 2012).

Cleaving of single-stranded DNAs may be carried out by any suitable technique, including but not limited to: (a) cleaving a target nucleotide such as uracil with a nicking
30 agent, such as described in US Patent No. 7,435,572 (one embodiment of which is commercially available as USER™; (b) cleaving an abasic site using ENDONUCLEASE VIII; (c) cleaving photocleavable nucleotides using near-UV light (e.g., ~365 nm); (d) cleaving sequence-specific cleavage sites using restriction endonucleases; (e) cleavage phosphorothioated or methylated DNA using McrA enzyme in the presence of Mn⁺² (Liu,
35 Ou, Wang, Li, Tan, Zhou, Rajakumar, Deng and He, PLOS Genetics (December 23, 2010); and (f) cleaving allyl-modified nucleosides in the polynucleotide by self-alkylating and

- 5 depurinating, that will cleave when placed in iodine and heat (*See, e.g.,* Gupta and Kool, Chem. Comm. (1997) 1425-1426).

Cleaving of single-stranded RNAs may likewise be carried out by any suitable technique, including but not limited to (a) *via* a DNA enzyme – cleaves any RNA substrate between an unpaired purine and unpaired pyrimidine base (Santorio and Joyce; PNAS, 94
10 (1997) 4262-4266); (b) ribozyme cleavage of RNAs; and (c) Cas9 cleavage of RNA (Ghodsizadeh, Nature Methods, 11 (2014) October, 2014).

In one preferred embodiment, cleaving of the DNA is carried out by including a modified nucleotide in the nucleotide, and contacting the nucleotide to a nicking agent as described in J. Bitinaite, US Patent No. 7,435,572. In some embodiments, such a nicking
15 agent, comprises: an artificial mixture of two or more enzymes, wherein at least one of the enzymes is a DNA glycosylase and at least one of the enzymes is a cleaving enzyme. In the presence of the polynucleotide containing the modified nucleotide, the mixture cleaves at the modified nucleotide. In some embodiments, the DNA glycosylase and the cleaving enzyme having an activity ratio of at least 2:1, the activity ratio being the ratio of a first rate of
20 forming an abasic site by excision of the modified nucleotide from the polynucleotide by DNA glycosylase to a second rate of cleaving at the abasic site in the polynucleotide by the cleaving enzyme, the first and second rates determined for a single predetermined molar amount of the modified nucleotide in the polynucleotide. In some embodiments, the cleaving enzyme generates a 5' phosphate in the polynucleotide molecule after excision of the
25 modified nucleotide. In some embodiments, the cleaving enzyme is selected from a FPG glycosylase/AP lyase and a EndoVIII glycosylase/AP lyase. In some embodiments, the at least one cleaving enzyme generates a 3' OH in the polynucleotide molecule after excision of the modified nucleotide. In some embodiments, the cleaving enzyme is EndoIV endonuclease. In some embodiments, the two or more enzymes generate a 5' phosphate and a
30 3' OH in the polynucleotide molecule after excision of the modified nucleotide. In some embodiments, the two or more enzymes are selected from; (i) EndoIV endonuclease and EndoVIII glycosylase/AP lyase, and (ii) EndoIV endonuclease and FPG glycosylase/AP lyase. In some embodiments, the modified nucleotide is deoxyuridine (U); 8-oxo-guanine; or deoxyinosine. In some embodiments, the two or more enzymes are UDG glycosylase and
35 EndoIV endonuclease; UDG glycosylase and FPG glycosylase/AP lyase AlkA glycosylase and EndoIV endonuclease; UDG glycosylase and EndoVIII glycosylase/AP lyase; UDG glycosylase, EndoIV endonuclease and EndoVIII glycosylase/AP lyase; UDG glycosylase,

- 5 EndoIV endonuclease and FPG glycosylase/AP lyase; AlkA glycosylase and EndoVIII glycosylase/AP lyase; or AlkA glycosylase and FPG glycosylase/AP lyase.

Collecting of the cleaved compound with its linker can be carried out by any suitable technique, including washing, rinsing, eluting, etc., and combinations thereof, again in accordance with known techniques.

- 10 The present invention is explained in greater detail in the non-limiting examples set forth below.

Experimental

- The invention reported herein describes a unique assay (**Figure 1**) for the positive selection of rare cells (see **Figure 7** for description of cell selection device used in this work) with their subsequent release for post-selection applications, such as the analysis of clinical CTCs, flow cytometry (FC) and fluorescence *in situ* hybridization (FISH). Heterobifunctional linkers (**Table 1**) were used to immobilize monoclonal Abs (mAbs) to a UV/O₃-activated fluidic surface presenting carboxylic acids (-COOH; **Figure 8**). mAbs were reacted with a sulfo-NHS ester of succinimidyl trans-4 (maleimidylmethyl) cyclohexane-1-carboxylate (SMCC), yielding a maleimide-labeled mAb (SMCC-mAb). Once purified, the SMCC-mAb could be covalently attached to the reduced 3'-disulfide group (sulfhydryl) of a single-stranded oligonucleotide (ssDNA) linker that was immobilized to the activated surface using EDC/NHS coupling of the ssDNA linker's 5'-amino group to the surface-confined COOHs.
- 25 Incorporating uracil (dU) into the ssDNA linker enabled enzymatic nicking by the USER™ (Uracil-Specific Excision Reagent) system and thus, cell release (**Figure 1**). USER™ consists of a mixture of Uracil DNA glycosylase (UDG) and DNA glycosylase-lyase Endonuclease VIII. UDG catalyzes the excision of dU forming an abasic site. Endonuclease VIII breaks the phosphodiester bond of the abasic site, cleaving the ssDNA linker and releasing the selected cell from the capture surface. The advantage of ssDNA linkers lies in their stability, low cost, and ease of covalent and ordered attachment to a variety of surfaces with a high load.¹⁹⁻²³ Moreover, USER™ is active at physiological temperatures and in a variety of buffers, such as PBS, both of which can maintain cell viability.²⁴⁻²⁵

- 35 The efficiency and specificity of USER™ cleavage was tested by nicking a 34 nt ssDNA substrate in a microtube at 37°C and monitoring the appearance of a 26 nt product electrophoretically (**Figure 9**). Electropherograms indicated that after a 5 min reaction in

5 PBS (pH 7.4), most of the 34 nt substrate was converted into the 26 nt product as a result of enzymatic nicking. Reaction times of 15 min completely nicked the substrate (**Figure 9**), demonstrating that USER™ could efficiently and specifically cleave the oligonucleotide at only the dU residue because only the 26 nt product was observed (**Figure 9**).

To evaluate the ability to select cells and then release the selected cells using ssDNA
10 linkers containing a dU residue, a microfluidic device used for cell selection fabricated in cyclic olefin copolymer (COC) consisting of an array of sinusoidal channels (25 µm wide, 150 µm deep and 3 cm in length; see **Figure 7**) was used as the model system. Using this microfluidic device and direct attachment of the selection mAbs to the activated COC surface (*i.e.*, no ssDNA linker used), CTC recoveries of 98% have been noted.^{13,15} COC was used as
15 the material of choice in this study because of its low propensity for showing non-specific adsorption artifacts and its high loads of COOH groups following UV/O₃ activation.²⁶ The USER™ reaction was performed by attaching a heater to the microfluidic device.

To initially evaluate the ability to cleave a ssDNA linker when immobilized to the surface of the COC microchannels, a dye-labelled oligonucleotide containing an internal dU
20 residue was immobilized to the microchannel surfaces, visualized (**Figure 2A**), and nicked by infusing a solution of USER™ (2 U/10 µL) into the device followed by visualization again of the same surfaces. Successful oligonucleotide nicking was confirmed by microscopy, which indicated a loss of fluorescence within the microchannels (**Figure 2B**) and the presence of fluorescence in the effluent. Fluorescence measurements indicated 5.2 ± 0.4 pmol/cm² of the
25 oligonucleotide was cleaved from the surfaces (1.9×10^{13} linkers/device). Based on the size of a 25 nt ssDNA with a random sequence,²⁷⁻²⁸ the theoretical oligonucleotide surface density was estimated to be 13.2 pmol/cm², which is close to the measured load of the ssDNA linker. It is important to note that during immobilization, ssDNA linkers can cross-link to the surface with multiple points of attachment due to the presence of primary amines on some of the
30 DNA bases. To mitigate this, ssDNA linkers were designed (see **Table 1**) containing poly dT sequences at the 3' and 5' ends to eliminate crosslinking. A 20dT and 40dT sequence possessing only dT residues lack primary amines that could potentially compete with the 5'-amino group of the ssDNA linker during immobilization to the microchannel surfaces. These linkers were compared to 34 nt and 40 nt linker sequences with mixed bases and minimal
35 secondary structures (34dX and 40dX).

5 We used three cancer cell lines to evaluate the efficiency of cell isolation and release, SKBR3 and Hs578T (both adherent), which express EpCAM and FAP α (fibroblast activation protein alpha, FAP α), respectively, and acute myeloid leukemia KG-1 cells (non-adherent) expressing CD34. mAbs directed against these Ags were used for cell isolation. Cell recoveries were determined using a “self-referencing method” (**Figure 10**). The self-referencing method uses multiple cell selection devices connected in series to deplete the input cells with the recovery determined from the number of cells collected by the first device with respect to the total number of cells collected in the series. We found that the self-referencing method yielded similar calculated recovery values to seeding experiments, but the relative standard deviation in the measurements were significantly lower (35% versus 6%).

10 Recoveries using the cleavable ssDNA linkers were compared to a previously reported direct attachment approach,¹⁵ where mAbs were covalently attached to the UV/O₃ activated microchannel surfaces that bear COOH groups using EDC/NHS coupling chemistry. The recoveries for direct attachment were compared to the recoveries in which the surface attachment of the mAb was accomplished using the ssDNA linker. These results are

15 summarized in **Table 2**.

The ssDNA linkers demonstrated similar recoveries for three cell lines investigated when compared to the direct attachment protocol. Recoveries were normalized with respect to the anti-EpCAM mAb recovery of the SKBR3 cells isolated via the 40dX linkers. Statistically similar results were observed for cell recovery via direct attachment when compared to

25 attachment using the ssDNA linkers for SKBR3 cells, $96 \pm 12\%$ (n=4). FAP α Hs578T cells were recovered with slightly higher efficiency when mAbs were directly attached to the surface, $90 \pm 9\%$ (n=8), compared to the 34dX or 40dX linkers, $74 \pm 7\%$ (n=3) and $80 \pm 6\%$ (n=5), respectively. Between the ssDNA linkers tested, the data did not indicate a strong dependence of recovery on linker length, sequence, or the nature of the chemical group

30 between the 5'-amino group and the ssDNA linker (C₆ for 34dX and C₁₂ for 40dX). The recovery of CD34 KG-1 cells did not statistically differ between direct attachment, $81 \pm 6\%$ (n=7), and attachment using 40dX, 40dT, or 20dT linkers, $76 \pm 5\%$ (n=5), $74 \pm 7\%$ (n=9), and $77 \pm 5\%$ (n=5), respectively. We concluded that the linkers used in this study were able to generate accessible mAbs on the microchannel surfaces, irrespective of the linker length and

35 sequence.

5 The efficiency of cell release following affinity selection for the three cell lines tested are summarized in **Table 3**. We also evaluated two formats for the cell release process within the cell selection device: (i) continuous-flow (CF) of USERTM into the cell selection device; and (ii) incubation for a fixed time period after flooding the cell selection device with USERTM. Cells were stained after isolation using a LIVE/DEADTM assay to determine
10 viability post-selection. Affinity-selected cells were then released with the USERTM system to determine release efficiency. To determine the viability following release, unstained cells were released and then stained with the LIVE/DEADTM assay. Lastly, the release efficiency of fixed and DAPI stained cells was evaluated as well.

We determined that the incubation format provided more efficient release of selected
15 cells using the microfluidic device compared to the CF reaction. In the CF reaction, 60 min was required to release ~84% of the selected cells, while the same release efficiency was achieved within 15 min using the incubation format; the release efficiency was $89 \pm 3\%$ ($n=5$) for SKBR3 cells and $86 \pm 4\%$ for the Hs578T cells when the selection surface was modified with mAb and the 40dX linker. Additional advantages of the incubation format is low
20 enzyme consumption and maintaining the enrichment factor because the cells were eluted into a smaller volume; ~10 μ L incubation reaction format. Enzymatic release of KG-1 cells with USERTM was carried out on live and fixed cells with mAb attached via 20dT and 40dT ssDNA linkers. The release was monitored by enumerating the cells in the effluents with the data collected every 15 min (**Table 3**). Within 30 min, >80% release efficiency was observed
25 with no significant increases in the release efficiency observed for longer incubation times.

Figure 3 presents a set of images illustrating the release of viable KG-1 cells. High release efficiency was achieved for both viable and fixed cells. Proteolytic (*i.e.*, trypsin) release was not possible for paraformaldehyde fixed cells following isolation for direct mAb attachment to the device surface due to Ag-mAb crosslinking. Interestingly, the rate of
30 release was faster for the Hs578T and SKBR3 cells compared to the KG-1 cells; longer incubation times (30 min) were required for KG-1 cells to achieve the same release efficiency ($84 \pm 4\%$, see **Table 3**). We believe this result may reflect differences in the number of attachment points created by the mAb/Ag associations. The density of CD34 antigens on KG-1 cells is significantly higher (89x isotype, see **Table 4**) compared to FAP α on Hs578T cells
35 (6x isotype) and EpCAM on SKBR3 cells (15x isotype). Thus, the rate of release depends on the number of mAbs/Ag associations (*i.e.*, the adhesion force).

5 Cell viability was analysed following isolation and also after release. Hs578T, SKBR3, and KG-1 cells freshly harvested from culture demonstrated a viability of $93 \pm 3\%$. Following selection, $89 \pm 5\%$, $92 \pm 4\%$, and $92 \pm 3\%$ viability was found for Hs578T, SKBR3, and KG-1 cells, respectively, which was similar to the viability found from culture (**Figure 4A-B**). Post-release viabilities of cell lines were also high, although slightly lower than post-
10 isolation values. For example, $80 \pm 3\%$, $82 \pm 5\%$ and $84 \pm 4\%$ viabilities for Hs578T and SKBR3 and KG-1 cells, respectively, were found following USERTM release. The fluorescence images of released SKBR3 cells in a titer well are shown in **Figure 4C-E**. In a control experiment, KG-1 cells were suspended in PBS (pH 7.4) within a fluidic device containing no mAb or subjected to release; the viability was $85 \pm 7\%$, very similar to
15 viabilities observed after enzymatic release. Therefore, the slight decrease in cell viability after release does not necessarily reflect enzymatic incubation or fluidic pressures, but is partly caused by natural cell viability loss. Clearly, the majority of cells were not damaged by the shear forces observed in the cell selection device²⁶ and/or treatment with USERTM.

The ability to culture selected and released cells was evaluated as well.
20 Approximately 110 SKBR3 cells were collected into the well of a 24-well titer plate following selection and release. Media reagents were introduced and microscopic observations of cell growth were made in ~24 h intervals. Cells divided and grew in culture; after 5 d the number of cells doubled. The low seeding density caused a slower growth rate of these cells; at higher seeding densities, released SKBR3 cells divided more rapidly.

25 We performed flow cytometry of KG-1 cells to evaluate the ability to detect and quantify expression of immunostained cells following selection, on-chip staining and USERTM release. Histograms in **Figure 5** are shown for 3 antigens, CD33, CD34, and CD117. Cells selected and stained on-chip had fluorescence values of $54 \pm 7\%$ compared to the KG-1 cells labelled in solution for all antigens indicating that approximately half of the
30 cell's surface Ags were inaccessible, possibly due to attachment and masking by the channel surface. Only for the weakly expressed CD117 was it difficult to discern these cells from an unstained population (see **Table 4**). Staining of surface attached cells resulted in slightly lower fluorescence signal, however, solid-phase staining provides advantages in terms of minimizing reagent consumption, easy washing of cells and eliminating cell loss or damage
35 during washing steps.

To demonstrate the utility of our assay for the selection and release of clinical CTCs, metastatic ovarian cancer (M-OVC) blood samples were processed using anti-EpCAM modified cell selection devices with the mAb attached to the surface via uracil containing ssDNA linkers. Isolated CTCs were stained with CD45, DAPI, and CK-Pan and enumerated. **Table 5** summarizes results secured from M-OVC patient blood samples. Similar cell counts from these samples were found using the ssDNA linker and direct attachment (**Table 5**). The average viability of CTCs isolated from M-OVC blood samples was $93 \pm 5\%$ (n=2) before release. Upon release, the viability was $87 \pm 6\%$ (n=5).

The assay was also evaluated by selecting circulating multiple myeloma cells (CMMCs) using anti-CD138 mAbs from multiple myeloma (MM) patients. The selected cells were isolated using the device shown in **Figure 7** with the selection mAbs attached to the substrate using the ssDNA linkers. Following selection, the CMMCs were released and subsequently tested by FISH for a possible deletion in chromosome 13, which is found in ~86% of MM patients. The presence of the deletion is typically associated with poor prognosis and the propensity of the disease to progress from an asymptomatic to symptomatic stage.²⁹

Both mutated (**Figure 6A**) and wild type (**Figure 6B**) CMMCs were observed in patient's blood. The deletion identified in chromosome 13 in the isolated CMMCs was also present in bone marrow from the same patient. Chromosomal analysis of CMMCs selected from whole blood and released intact allows for conventional cytogenetic analysis without the need for requiring the patient to undergo a bone marrow biopsy, which can provide the ability for more frequent testing.

Conclusions. We have successfully demonstrated the use of ssDNA linkers engineered with a dU residue for attaching mAbs to surfaces for selecting clinically relevant cells with high recovery and when using the USER™ enzyme system, release the cells both efficiently (~90%) and rapidly (15-30 min) while maintaining their viability. Clinical CTCs and CMMCs were selected directly from patients' peripheral blood then released for integration of the cell selection/release assay into existing clinical processing pipelines while obviating the need for invasive biopsy procedures. We have demonstrated that the selected cells can be fixed, stained, released, and subjected to conventional flow cytometry. The selected/released cells could also be successfully subjected to FISH analysis to determine chromosomal abnormalities using conventional cytogenetic assays. The USER™ release

strategy preserved cell viability making them available for cultivation as well. An additional benefit of the ssDNA linkers and the USER™ enzyme system was the seamless integration with existing microfluidic devices used for rare cell selection and the general attachment chemistry, which can enable the application of these ssDNA linkers to a variety of solid-phase supports and rare cell assays that require post-capture analysis.

Materials and Methods.

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 7**) (M. Hupert et al., *Microfluid. Nanofluid.*, 2007, **3**, 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). 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

5 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/O₃ activation with minimal amounts of non-specific adsorption to its surface (J. M. Jackson et al., *Lab Chip*, 2014, **14**, 106).

Operation of the cell selection device. Prior to blood sample infusion and following
 10 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
 15 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
 20 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
 25 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
 30 sequences of oligonucleotides that were used as cleavable linkers (ssDNA linkers) are summarized in **Table 1**. 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
 35 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 (B. Tinland et al., *Macromolecules*, 1997, **30**, 5763). It has been reported that

- 5 the surface coverage decreases as N increases; $\sim 6 \times 10^{12}$ probes/cm² for an 8mer and $\sim 7 \times 10^{11}$ probes/cm² for a 48mer (A. A. Adams et al., *J. Am. Chem. Soc.*, 2008, **130**, 8633).

Table 1. 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 ₃ OH (25 nt)	25dX	9.9
NH ₂ -C ₆ -T ₈ UTT TTT TCC GAC ACT TAC GT ₈ -C ₃ -SS-C ₃ OH (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 ₃ OH (40 nt)	40dX	15.8
NH ₂ -C ₆ -(C ₂ H ₆ O ₂) ₆ -T ₁₀ U T ₁₀ -C ₃ -SS-C ₃ OH (20 nt)	20dT	10.9
NH ₂ -C ₆ -(C ₂ H ₆ O ₂) ₆ -T ₃₀ U T ₁₀ -C ₃ -SS-C ₃ OH (40 nt)	40dT	17.5

- 10 **Monoclonal antibody (mAb) labeling with sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC).** mAb labeling involved addition of 6 μ L (50x excess) of maleimide-crosslinker sulfo-SMCC (10 mg/mL in nuclease free water) to 0.5 mg mAb in 500 μ L 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
- 15 (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/O₃-
- 20 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 μ M 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
- 25 5'- terminus to the activated COC surface. After the reaction was complete, the microfluidic chip was rinsed with 100 μ L PBS (pH 7.4) at 40 μ L/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 μ L PBS (pH 7.4) at 50 μ L/min, and immediately an aliquot of the modified mAb-
- 30 SMCC was introduced (~ 0.5 mg/mL). The reaction proceeded for 2 h on ice or overnight at 4°C (**Figure 8**).

5 **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
10 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
15 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% CO₂
20 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
25 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
30 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
35 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 μ L 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 SSC / 0.1% NP-40 at room temperature for 1 min. Slides were air-dried and 10 μ L (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 USERTM. Enzymatic cleavage was evaluated using a bench top experiment with a thermo-cycler, ssDNA linkers designed with a single dU residue and the USERTM system. The definition of 1 unit of USERTM 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 μ L 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 USERTM kinetics and specificity (*i.e.*, cleavage of the 34mer at only the dU residue should generate a 26 nt fragment). **Figure 9** 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 9A**) 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).

Electropherograms revealed that the single-stranded oligonucleotides (**Figure 9B**) 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 USERTM cleavage at the dU residue. Reaction times ≥ 15 min allowed the enzyme to completely nick the substrate (**Figure 9E**). These experiments demonstrated that the USERTM 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 10**). As shown in **Figure 10**, 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**:

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

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

5 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 7** can have an influence on recovery when using surface
10 immobilized mAbs (A. A. Adams et al., *J. Am. Chem. Soc.*, 2008, **130**, 8633). 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
15 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,
20 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 (K.-C. Chang and D. A. Hammer, *Biophys. J.*, 1999, **76**, 1280).

25 We were interested in comparing the recovery for the case where the mAb is directly attached to the surface as we have shown previously (J. M. Jackson et al., *Lab Chip*, 2014, **14**, 106; J. W. Kamande et al., *Anal. Chem.*, 2013, **85**, 9092), 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 2** for three cases: (i) Direct attachment of the mAb to the activated
30 COC surface using our reported methods ((J. M. Jackson et al., *Lab Chip*, 2014, **14**, 106; J. W. Kamande et al., *Anal. Chem.*, 2013, **85**, 9092),); (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
35 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

5 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% (A. A. Adams et al., *J. Am. Chem. Soc.*, 2008, **130**, 8633).

10 **Table 2. 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.¹**

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

15 ¹The 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-FAP α 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 $\pm$ 4%) using the ssDNA linker, 40dX (see Table 1 for sequence of this linker). nd = not determined.

20 **Cell enzymatic release efficiency.** The release efficiency of selected cells are given in Table 3 as a function of USERTM 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. When using the direct attachment method and trypsin as the release reagent, the selected cells could not
25 be released from the surface when the cells were fixed (data not shown).

Cell cultivation after release with USERTM. SKBR3 cells upon release were collected into a titer well (2 cm²) of a 24-well plate and culturing medium was introduced. Observations of cell culturing using a microscope were made in 24 h intervals. Micrographs (not shown) collected during a 5 d period for 5 SKBR3 cells (out of 113 present in the well).
30 After 16 h following release, a group of cells was identified (not shown). After 30 min, we observed morphological changes occurring in the largest cell of the group (not shown). After an additional 30 min, the same cell divided (not shown). The area was further observed

5 during a 67 h time period and showed 2 cells migrating out of the field of view (not shown). 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
10 capable of dividing after selection and the subsequent release from the selection microfluidic chip. Another example of SKBR3 dividing was seen, where from an initial 7 cells there were 15 cells after 5 d of cultivation (data not shown).

15 **Table 3. Enzymatic release efficiency of viable and fixed KG-1, SKBR3 and Hs578T cells isolated as a function of cell incubation time with USER™ (n ≥ 3).**

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; nd = not determined.

20 **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.

5 The following samples were prepared as controls: Unstained KG-1 cells serving as an autofluorescence control (not shown); isotype controls where cells were stained with a cocktail of isotype mAbs consisting of 5 μ L of 0.2 mg/mL IgG1-PE, 2.5 μ L of 0.2 mg/mL IgG1-APC and 4 μ L of 0.5 mg/mL IgG2a-FITC mAbs in 200 μ L PBS (not shown); positive controls with the cells stained using a cocktail of mAbs containing 5 μ L each of 0.2 mg/mL CD33-PE, 0.1 mg/mL CD117-APC and 0.4 mg/mL CD34-FITC in 200 μ L PBS (not shown); and cells stained on-chip following selection (not shown) 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 μ L 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 (not shown) was performed using FlowJo software (FlowJo LLC, Ashland, OR). Data from these experiments are summarized in **Table 4.**

Table 4. 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.

25 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.

5 For all antigens, the released KG-1 cells showed an average MFI that was $54 \pm 7\%$ 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
10 MFI, this did not significantly affect our ability to discern these cells from unstained cells (see **Table 4**). 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.

Isolation of CTCs from metastatic ovarian cancer (M-OVC) patient blood. We
15 performed clinical experiments by processing 2 M-OVC blood samples. We evaluated the number of selected EpCAM+ CTCs upon USERTM release into wells of a 96 well plate. Cells were stained with CD45, DAPI, and CK-Pan. **Table 5** summarizes the results for these samples.

The results suggested similar cell numbers isolated using microfluidic chips modified via
20 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 USERTM, cells were released following selection and eluted into titer wells where they were stained with the LIVE/DEADTM 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
25 $\pm 5\%$ (n=5) for EpCAM+ CTCs. Upon release with USERTM and based on evaluation of multiple areas of the wells in which cells were collected, the viability was determined to be $87 \pm 6\%$ for EpCAM+ CTCs (data not shown).

30 **Table 5. 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	mAb attachment via linker
		CTC/mL	CTC/mL
M-OVC1	anti-EpCAM	397	331
M-OVC2	anti-EpCAM	680	717

5

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- 28 -

5 The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

10

THAT WHICH IS CLAIMED IS:

1. A construct useful for collecting a target cell or compound from a composition containing the same, comprising:

(a) a functional group for attachment to a substrate;

(b) a specific binding agent that specifically binds to said target cell or compound;

and

(c) a single stranded polynucleotide linker coupling said specific binding agent to said functional group and substrate, said polynucleotide having a cleavable site therein.

2. The construct of claim 1, wherein said functional group is an amine, carboxylic acid, aldehyde, thiol, alcohol, azide or imine.

3. The construct of claim 1 to 2, wherein said specific binding agent is selected from the group consisting of antibodies, peptides, aptamers, peptoids, lectins, and carbohydrates.

4. The construct of claim 1 to 3, wherein said polynucleotide is from 10 to 100 bases in length.

5. The construct of claim 1 to 4, wherein said polynucleotide is a single-stranded DNA.

6. The construct of claim 1 to 5, wherein said cleavable site comprises: (a) a target nucleotide, (b) an abasic site, (c) a photocleavable nucleotide; (d) a restriction endonuclease cleavage sequence; (e) a phosphorothioated or methylated DNA; or (f) an allyl-modified nucleoside.

7. The construct of claim 1 to 4, wherein said polynucleotide is a single-stranded RNA.

8. The construct of claim 7, wherein said cleavable site comprises (a) an unpaired purine and unpaired pyrimidine base (b) a ribozyme cleavage site; or (c) a Cas9 cleavage site.

9. A device comprising a construct of any preceding claim formed from the coupling of said functional group to a substrate.

10. The device of claim 9, wherein said substrate comprises an organic substrate, an inorganic substrate, or a composite thereof.

11. The device of claim 9 or 10, wherein said substrate comprises a microfluidic device.

12. A method of collecting a target cell or compound of interest, comprising the steps of:

(a) contacting a composition containing said target cell or compound to said specific binding agent in a device of claim 9 to 11;

(b) separating said composition from said device; and then

(c) cleaving said polynucleotide linker in said device to thereby collect said target cell or compound.

13. The method of claim 12, wherein said composition comprises a biological fluid.

14. The method of claim 12, wherein said target cell or compound comprises a biological cell, microvessicle, exosome, small molecule or large molecule.

15. The method of claim 12, wherein said polynucleotide is a DNA and said cleaving step is carried out by: (a) cleaving a target nucleotide with a nicking agent; (b) cleaving an abasic site using ENDONUCLEASE VIII; (c) cleaving a photocleavable nucleotides using near-UV light; (d) cleaving sequence-specific cleavage sites using restriction endonucleases; (e) cleavage phosphorothioated or methylated DNA using McrA enzyme in the presence of Mn^{+2} ; or (f) cleaving allyl-modified nucleosides in the polynucleotide by self-alkylating and depurinating, in the presence of iodine and heat.

16. The method of claim 12, wherein said polynucleotide is an RNA and said cleaving step is carried out by (a) cleaving between an unpaired purine and unpaired pyrimidine base with a DNA enzyme; (b) ribozyme cleavage or (c) Cas9 cleavage.

17. The method of claim 12, wherein said polynucleotide is a DNA, said cleavable site comprises a modified nucleotide, and said cleaving step is carried out by contacting a nicking agent to said polynucleotide.

18. The method of claim 17, wherein said modified nucleotide is a deoxyuridine (U), an 8-oxo-guanine, or a deoxyinosine.

19. The method of claim 17 or 18, wherein said nicking agent comprises a mixture of two or more enzymes, wherein at least one of the enzymes is a DNA glycosylase and at least one of the enzymes is a cleaving enzyme.

20. The method of claim 19, wherein the cleaving enzyme is selected from a FPG glycosylase/AP lyase and a EndoVIII glycosylase/AP lyase.

21. The method of claim 19, wherein the cleaving enzyme is EndoIV endonuclease.

22. The method of claim 19, wherein the two or more enzymes are selected from; (i) EndoIV endonuclease and EndoVIII glycosylase/AP lyase, and (ii) EndoIV endonuclease and FPG glycosylase/AP lyase.

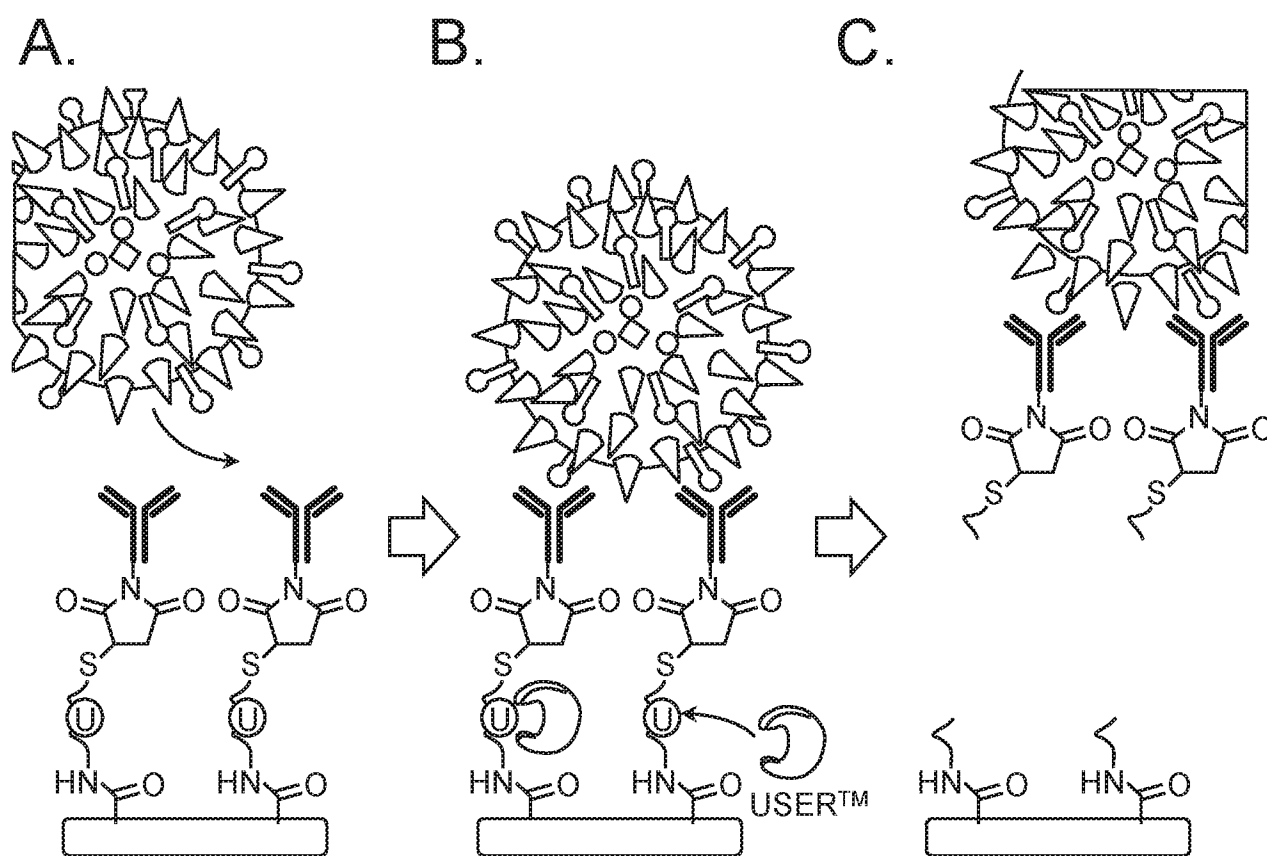


Figure 1.

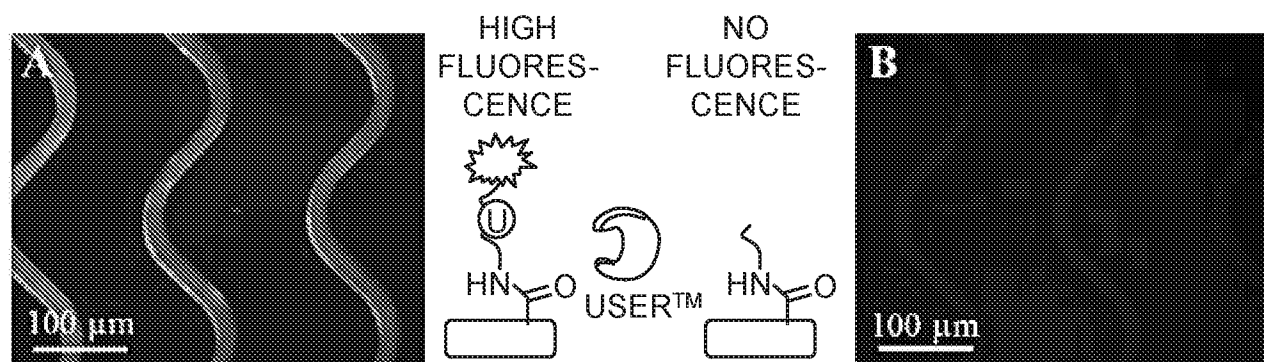


Figure 2.

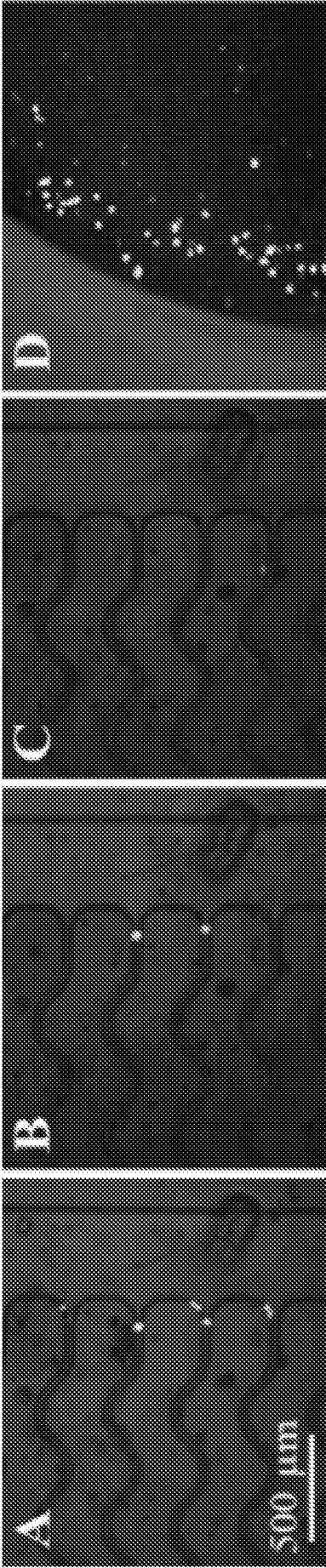


Figure 3.

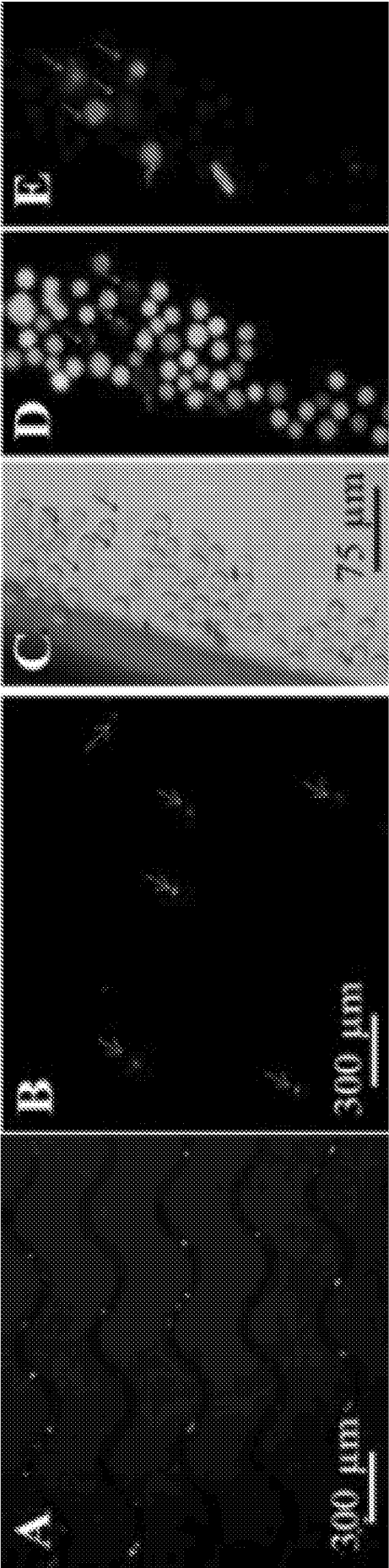


Figure 4.

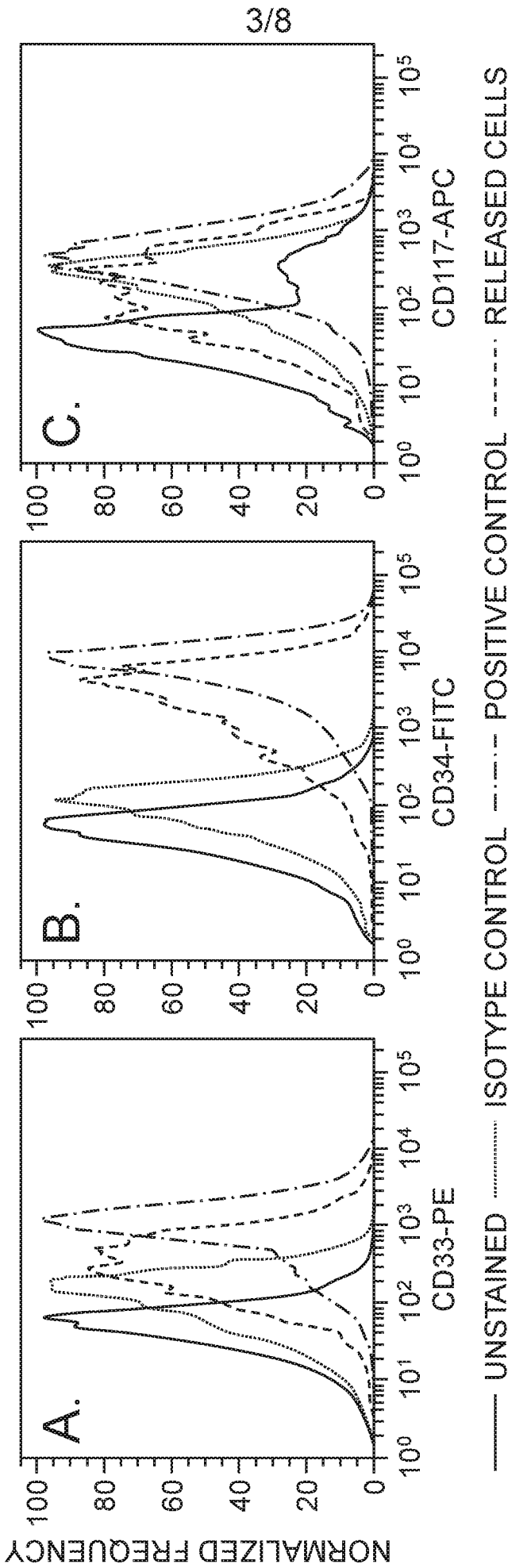


Figure 5.

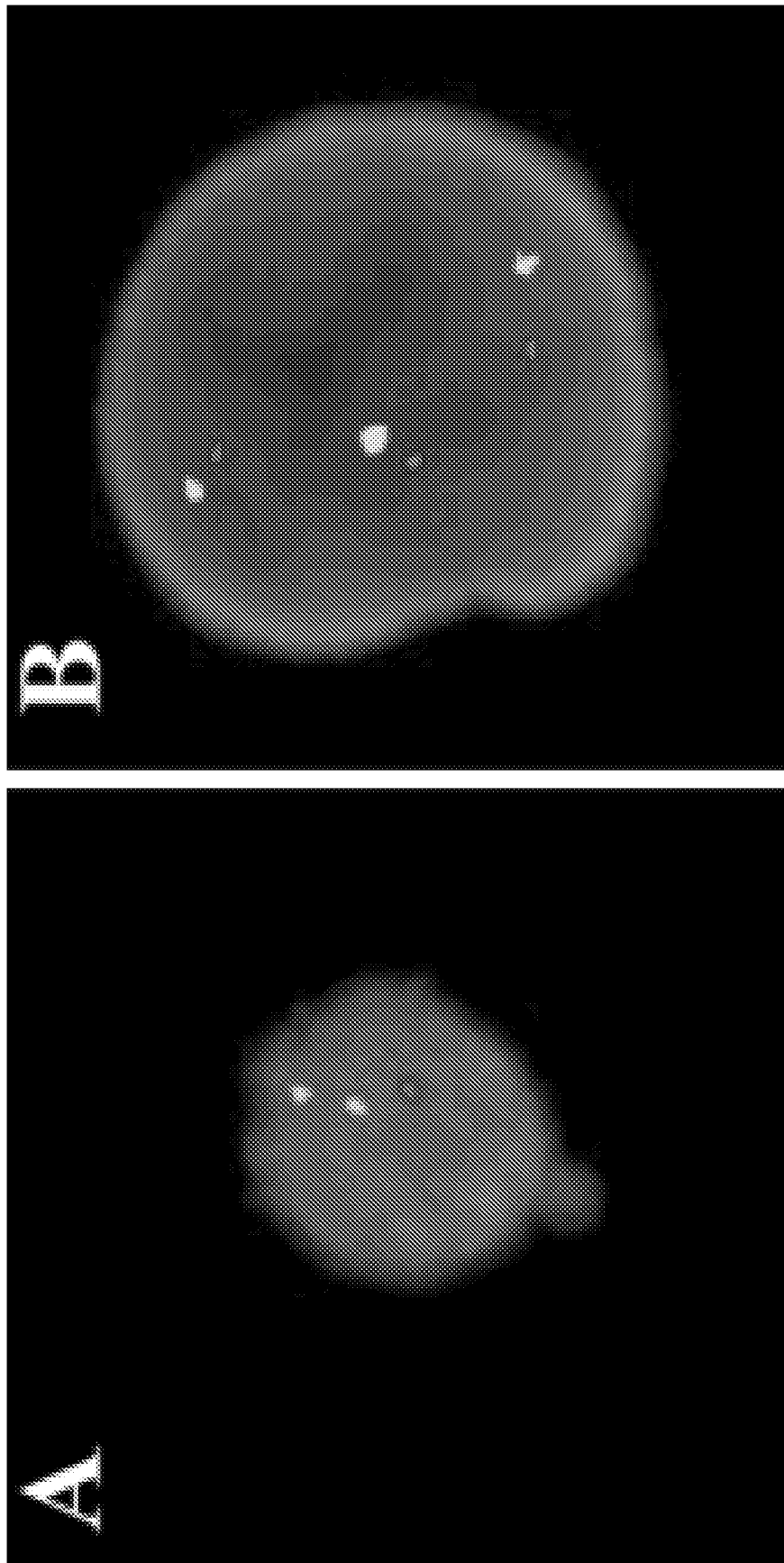
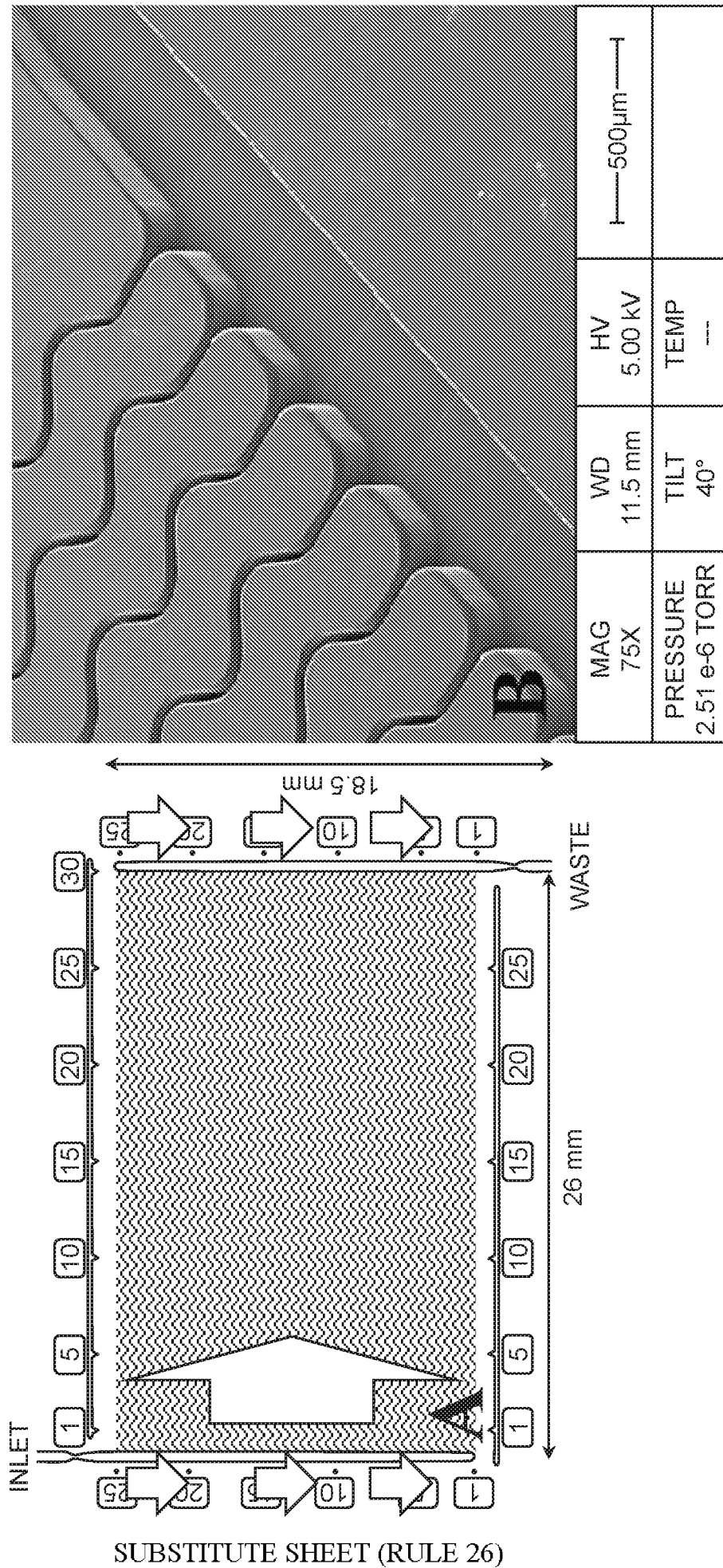
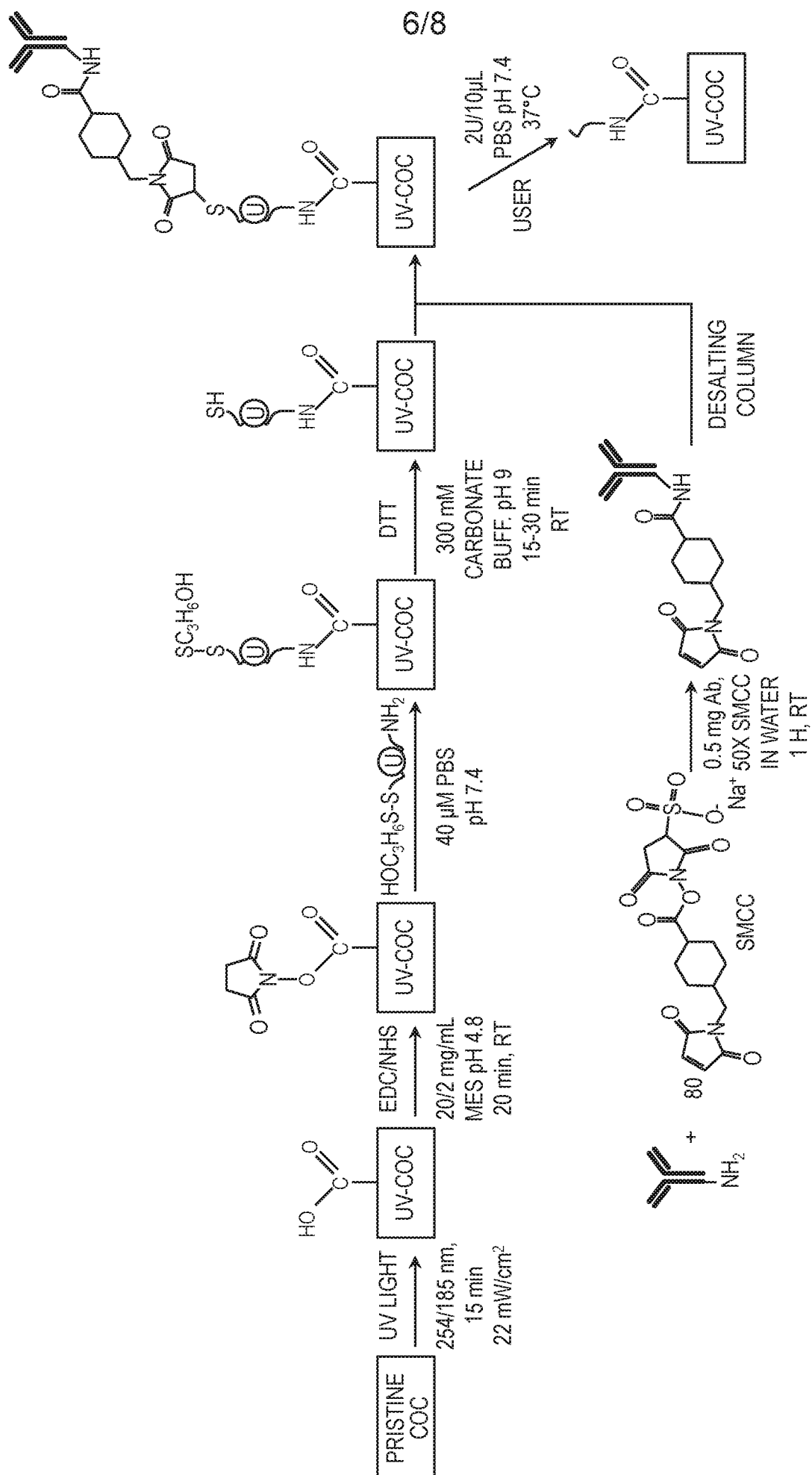
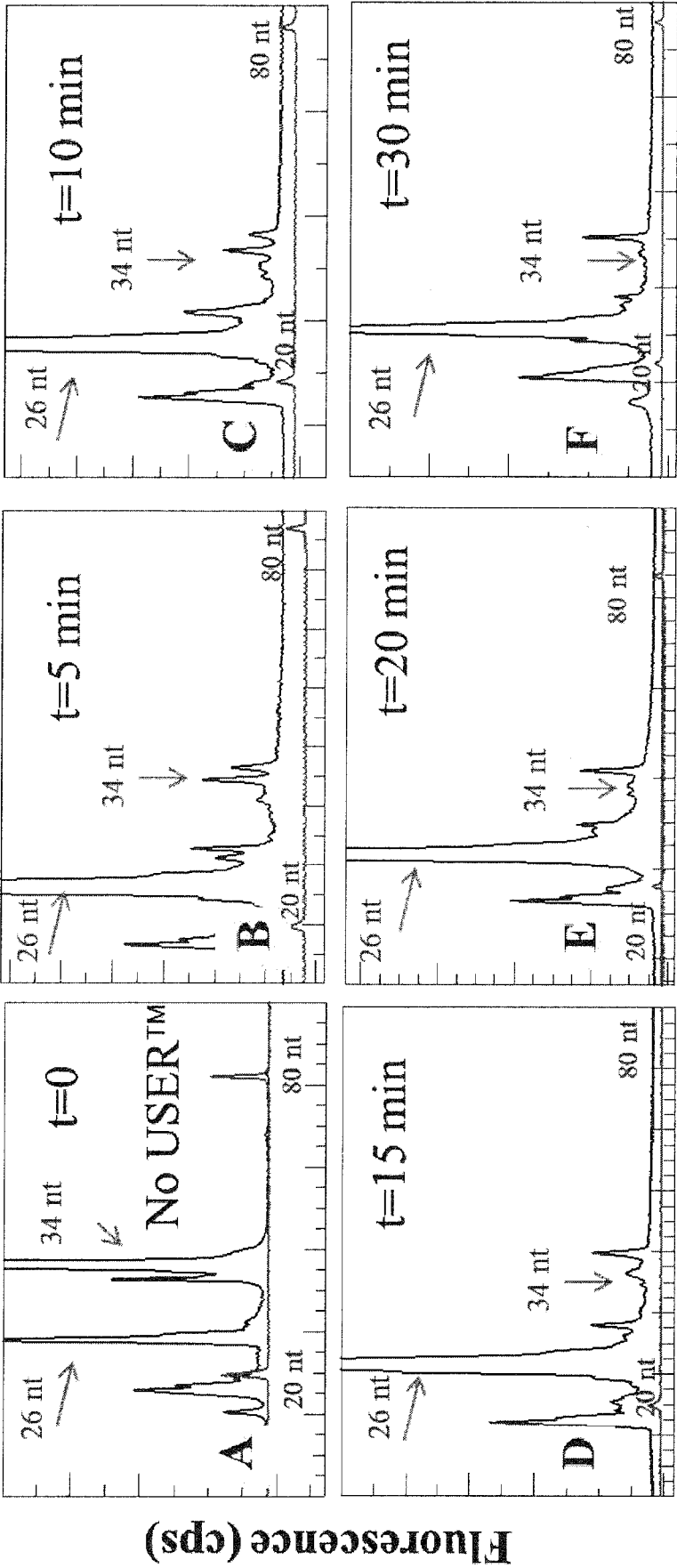


Figure 6.





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Migration Time (arb. units)

Figure 9

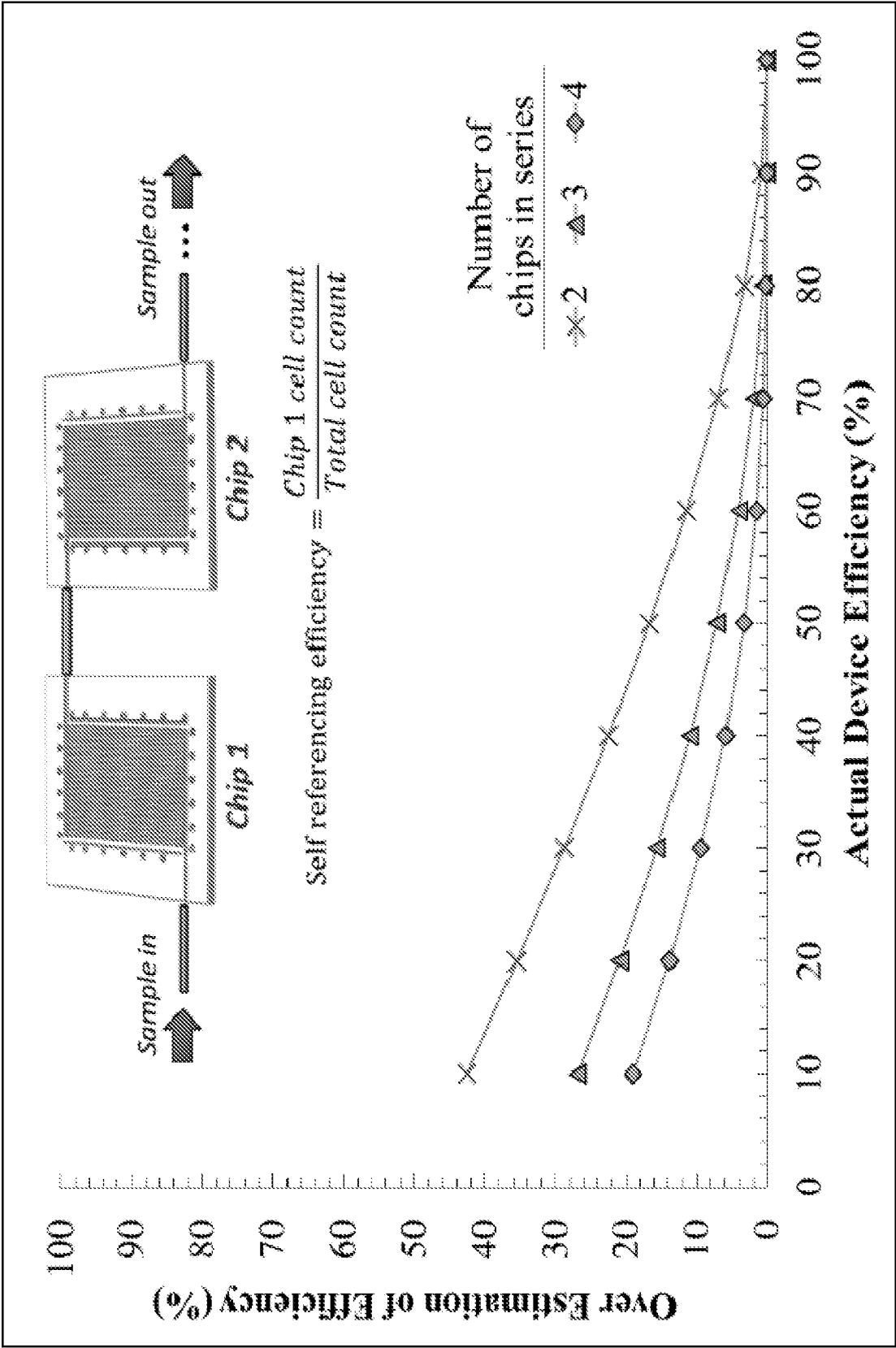


Figure 10.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/012065

A. CLASSIFICATION OF SUBJECT MATTER		
(see extra sheet)		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
C07K 2/00, 16/00, C12N 15/11, 15/115, C07K 14/42, C07C 321/00, B01L 99/00		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
PAJ, Espacenet, Patentscope, USPTO DB, RUPTO, EAPATIS, DEPATISnet, PatSearch (RUPTO internal)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	US 2014/0011192 A1 (SAMSUNG ELECTRONICS CO., LTD.) 09.01.2014, abstract, paragraph [0026], claims	1-3, 9-15 16-22
Y	US 2012/0220482 A1 (THE BOARD OF REGENTS OF THE UNIVERSITY OF TEXAS SYSTEM) 30.08.2012, paragraph [0059]	1-3, 9-15
Y	ZHENG T. et al. Single-stranded DNA as a Cleavable Linker for Bioorthogonal Click Chemistry-based Proteomics, Bioconjug Chem, 2013, Vol. 24, no.6, pp. 859-864, especially abstract	1-3, 9-15
Y	US 2012/0100521 A1 (STEVEN A. SOPER et al.) 26.04.2012, claims	9-15
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
28 April 2016 (28.04.2016)		05 May 2016 (05.05.2016)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37		Authorized officer E. Tereshkina Telephone No. (495)531-65-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/012065

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-8
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Classification of subject matter

International application No.

PCT/US 2016/012065

C07K 2/00 (2006.01)***C07K 16/00 (2006.01)******C12N 15/115 (2010.01)******C07K 14/42 (2006.01)******C07C 321/00 (2006.01)******C12N 15/11 (2006.01)******B01L 99/00 (2006.01)***