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(54) **Title:** DEVICES AND METHODS FOR DETECTING CELLS AND OTHER ANALYTES

(57) **Abstract:** The invention features methods, devices, and kits for the isolation of analytes (e.g., a cell). A sample containing a desired analyte is introduced into a microfluidic device containing moieties that bind the desired analyte. A shear stress is applied that is great enough to prevent binding of undesired analytes and low enough to allow binding of the analyte of interest. Once bound, the desired analytes can be analyzed (e.g., counted). The invention also features methods for determining a shear stress for isolating a desired analyte.

DEVICES AND METHODS FOR DETECTING CELLS AND OTHER ANALYTES

Cross-Reference to Related Applications

5   This application claims benefit of U.S. Provisional Application No.

60/782,470, filed March 15, 2006, which is hereby incorporated by reference in its

entirety.

Background of the Invention

10   This invention relates to the fields of medical devices, medical diagnostics,

and cell counting.

Of the 40.4 million people infected with HIV globally, more than 35 million

live in developing countries with significant resource limitations, many of whom are

in urgent need of diagnosis, monitoring and antiretroviral therapy. In the process of

15   managing HIV-infected subjects, counts of a specific white blood cell population,

CD4+ T lymphocytes, have proven to be essential biological indicators. In adults, the

absolute number of CD4+ T cells per microliter of blood has critical prognostic and

therapeutic implications and is used for both HIV staging and treatment decisions.

Regular monitoring of CD4 counts—two to four times per year—is recommended for

20   all stages of infection. Clinically, a CD4 count below 200 cells mL^{-1} establishes the

diagnosis of AIDS and in most settings is used as a marker to initiate antiretroviral

treatment (ART) and prophylaxis against opportunistic infections. Higher CD4 count

thresholds of 350 and 500 cells mL^{-1} are widely used as markers to increase the

intensity of monitoring, and in some settings, to initiate ART. However, affordable

and appropriate laboratory monitoring tools to determine CD4 counts have little

penetration in resource-limited settings, despite ongoing international efforts to extend

the availability of ART to these areas.

5   Currently, the gold standard for CD4+ T cell enumeration is flow cytometric

counting of lymphocyte subpopulations using monoclonal antibodies and commercial

multi-purpose flow cytometers or single-purpose CD4-counting flow cytometers.

Although these instruments are high throughput and accurate, their cost and technical

requirements for operation and maintenance have limited their reach and significantly

10   delayed the implementation of HIV treatment programs in resource-limited areas

worldwide. Smaller instruments like the Guava EasyCD4 offer limited improvements

and have not been widely adopted. Non-cytofluorographic methods, including ELISA

and bead format assays have been suggested as useful alternatives for CD4+ T

lymphocyte quantification, since these methods require less equipment and have

15   lower reagent costs than flow cytometry. However, they have much lower

throughput, are more labor intensive and less accurate, and are not widely used or

recommended by World Health Organization guidelines.

          In addition, improvements on the back-end aspects of CD4 counting—such as

miniaturization of equipment—do not address the most problematic issue for

20   resource-limited settings, which is sample preparation. The requirements to collect

blood by venipuncture, to lyse erythrocytes, to centrifuge samples, or to use pipettes

for any step in the diagnostic assay are extremely problematic in these settings.

Accordingly there is still a need for low cost methods for the diagnosis and monitoring of CD4 cell populations.

Summary of the Invention

5   The invention features a method for isolating a desired cell from a sample by introducing the sample into a microfluidic device containing a binding moiety specific for the desired cell, allowing desired cells in the sample to bind to the binding moiety, and applying a shear stress to the microfluidic device so that desired cells remain bound while undesired cells do not. In this embodiment, the step of allowing the

10   desired cells to bind to the binding moiety and the step of applying a shear stress, can occur simultaneously.

The invention also features a method for isolating a desired cell from a sample by introducing the sample into a microfluidic device containing a first binding moiety specific for a first desired cell disposed in a first chamber. This method includes

15   allowing the first desired cell in the sample to bind to the first binding moiety, applying a first shear stress to the microfluidic device so the first desired cells remain bound while other cells do not, and allowing the remaining sample to flow into a second chamber of the microfluidic device containing a binding moiety for a second desired cell. This method further includes allowing second desired cells in the sample

20   to bind to the second binding moiety and applying a second shear stress to the second chamber so the second desired cells remain bound while undesired cells do not. In this embodiment the first shear stress and the second shear stress can be the same or different. Also in this embodiment, the first binding moiety and the second binding

moiety can be the same or different. As above, the stress may be applied at the same
time as binding occurs.

The invention further features a kit for isolating a desired cell. This kit
includes a device including a chamber containing a binding moiety specific for the
5   desired cell, and a pump for producing a shear stress so that the desired cells bind
preferentially compared to undesired cells. This kit can also include a labeling
reagent specific for the desired cell. Optionally, this kit can also include instructions
for AIDS diagnosis.

In another aspect, the invention features a method of determining a shear
10   stress for isolating a desired cell on a device. This method includes introducing a
sample containing the desired cell into a chamber of a microfluidic device, the
chamber containing a binding moiety specific for the desired cell, allowing desired
cells in the sample to bind to the binding moiety, and applying shear stress to the
microfluidic device. In this method the shear stress is varied along the length of the
15   chamber and a shear stress at which the desired cell binds to the binding moiety
preferentially compared to another cell is identified. In this aspect, the shear stress
may be applied at the same time as binding occurs

In any of the forgoing aspects, the binding moieties can be selected from
antibodies, antibody fragments, oligo- or polypeptides, nucleic acids, cellular
20   receptors, ligands, aptamers, MHC-peptide monomers or oligomers, biotin, avidin,
oligonucleotides, coordination complexes, synthetic polymers, and carbohydrates.

Also in any of the forgoing aspects, the sample can be a blood sample, the
binding moiety can bind to CD66, CD14, CD4, CD8, EpCAM, E-Selectin, or P-

Selectin, and the desired cell can be selected from neutrophils, monocytes,

lymphocytes, circulating tumor cells, HIV infected CD8 lymphocytes, circulating

endothelial cells, and platelets.

In a preferred embodiment, the desired cells are CD4+ lymphocytes. In this

5   embodiment, the sample may be obtained from a patient at risk of developing AIDS.

Methods of the invention can also include analyzing at least one property (e.g.,

biological property) of the desired cells (e.g., mRNA expression, protein expression,

DNA quantification, DNA sequence, and chromosomal abnormalities); counting the

desired cells (e.g., CD4+ lymphocytes), e.g., to diagnose a disease state such as AIDS.

10   Although described in terms of cells, the methods, devices, and kits of the

invention may be employed in conjugation with other analytes, as described herein.

In preferred embodiments, desired cells bind preferentially compared to other

cells capable of binding to the binding moiety in the absence of shear stress.

By a "patient" is meant a living multicellular organism. The term "patient" is

15   meant to include humans, mice, dogs, cats, cows, sheep, horses, non-human primates,

and fish.

By "binding moieties" is meant a molecule that specifically binds to an analyte

(e.g., a cell). Binding moieties include, for example, antibodies, aptamers, receptors,

ligands, antigens, biotin/avidin, metal ions, chelating agents, nucleic acids, MHC-

20   peptide monomers, tetramers, pentamers or other oligomers.

By "cell surface marker" is meant a molecule bound to a cell that is exposed to

the extracellular environment. The cell surface marker can be a protein, lipid,

carbohydrate, or some combination of the three. The term "cell surface marker"

includes naturally occurring molecules, molecules that are aberrantly present as the result of some disease condition, or a molecule that is attached to the surface of the cell.

By "lysis" is meant disruption of the cellular membrane. For the purposes of

5   this invention, the term "lysis" is meant to include complete disruption of the cellular membrane ("complete lysis"), partial disruption of the cellular membrane ("partial lysis"), and permeabilization of the cellular membrane.

By "binding moiety" is meant a chemical species to which a cell binds. A binding moiety may be a compound coupled to a surface or the material making up

10   the surface. Exemplary binding moieties include antibodies, antibody fragments (e.g., Fc fragments), oligo- or polypeptides, nucleic acids, cellular receptors, ligands, aptamers, MHC-peptide monomers or oligomers, biotin, avidin, oligonucleotides, coordination complexes, synthetic polymers, and carbohydrates.

By "permeabilization" is meant the disruption of the cellular membrane such

15   that certain intracellular components are able to escape the cell, while other components remain inside the cell.

The term "chamber" is meant to include any designated portion of a microfluidic channel, e.g., where the cross-sectional area is greater, less than, or the same as channels entering and exiting the chamber.

20   Other features and advantages will be apparent from the following description, the figures, and the claims.

Brief Description of the Drawings

Fig. 1A is a diagram showing the operating procedure of a CD4 counting device.

Fig. 1B is a photograph of a cell counting device. Microfabricated PDMS

5   devices with one inlet and one outlet were bound to glass slides to form closed chambers.

Fig. 1C is a diagram of the geometry of a Hele-Shaw device. The Hele-Shaw device offers linear variation of shear along its central line.

Fig. 1D is a diagram of the cell counting device of 1B. This device has a

10   volume of 10 µL for sample volume metering.

Fig. 2A is a series of photomicrographs of cells captured in a Hele–Shaw chamber at locations corresponding to shear stresses of 0.4 dyn cm^{-2} (left), 1.7 dyn cm^{-2} (middle) and 5 dyn cm^{-2} (right). The image was created by overlapping a phase contrast photograph and the corresponding fluorescence photograph.

15   Fig. 2B is a series of photomicrographs of captured cells after CD4 (arrows) and CD14 staining at the shear stresses conditions as described above. Both lymphocytes (CD4+CD14-) and monocytes (CD4+CD14+) were captured at the shear stress of 0.5 dyn cm^{-2}, but pure lymphocytes were captured at two higher shear stresses. (Bar: 100 mm)

20   Fig. 2C is a graph showing adhesion of CD4+ T cells (solid circles), monocytes (empty circles) and other cells (solid triangles) in response to shear stress. Differentiated capture of monocytes and lymphocytes in response to shear was observed. A shear stress window between 1 and 3 dyn cm^{-2} was optimal for CD4+ T

cell adhesion, while monocytes adhesion drops significantly above 0.7 dyn cm^{-2} (inset). The adhesion of other cells is minimal in the whole range of tested shear stress. Each data point was repeated in 3 devices spanning different shear stress ranges; error bars represent standard deviations in measurements within each

5   experiment.

Fig. 2D is a graph showing composition of the surface captured cells as a function of shear stress. When shear stress is above 0.7 dyn cm^{-2}, 95% of the surface captured cells are CD4+ T cells (solid circles). The purity of these cells drops quickly to less than 50% when shear stress drops below 0.7 dyn cm^{-2}. Each data point was

10   repeated in 3 devices spanning different shear stress ranges; error bars represent standard deviations in measurements within each experiment.

Fig. 3A is a graph showing flow cytometric analysis of a blood sample before CD4+ T cell isolation. The CD4+ T cells (CD3+CD4+) compose 29.67% of all lymphocytes.

15   Fig. 3B is a graph showing flow cytometric analysis of the same blood sample after CD4+ T cell capture in the device. The composition of the target cells in the sample flow through dropped to 2.13% of all lymphocyte population after device capture. Ten microlitres of whole blood were injected into the linear device at a shear stress of 1.7 dyn cm^{-2}. Cells were acquired in the gated lymphocyte population, and

20   the quadrants were set up with an isotype matched control.

Fig. 3C is a graph showing percent yield of captured cells as a function of shear stress calculated from flow cytometric analysis. Nearly 95% of the target cells can be isolated from whole blood using shear stress in the range of 1 to 3 dyn cm^{-2}.

The yield quickly drops to less than 85% out of this range. Each data point was repeated in at least 3 devices. The error bars represent standard deviations in measurements within each experiment.

5   Fig. 4 is a graph showing capture cell density as a function of distance from inlet using the linear cell capture chamber at two shear stresses. At 1.7 dyn cm^{-2} (solid circle, yield nearly 95%), captured cell density reaches maximum near the sample inlet. By contrast, at 7 dyn cm^{-2} (empty circle, yield 75%), the distribution of cells is fairly uniform along the device. The experiments were performed using 10 µL of whole blood from healthy subjects. Each data point was repeated in at least 3 devices.

10   The error bars represent standard deviations in measurements within each experiment.

          Fig. 5 is a graph showing cells counted on the microfluidic chip as a function of CD4+ T cell count by flow cytometer using whole blood from 13 HIV+ adult subjects. A linear regression of the experimental data for absolute CD4 counts under 800 cells mL^{-1} (n = 11) indicates good correlation between the two measurements

15   (dash–dot line). The dashline represents an ideal 1 : 1 correlation between the two.

          Fig. 6A is a graph showing purity of the surface captured CD4+ T cells as a function of the absolute CD4 counts. Purity was above 60% and fairly consistent for absolute CD4 counts greater than 200 cells µL^{-1}.

          Fig. 6B is a graph showing yield of CD4+ T cells within the linear device as a

20   function of the absolute CD4 counts. Fairly consistent yield was observed for absolute CD4 counts up to 800 cells µL^{-1}. The dashed lines are drawn as a visual guide.

          Fig. 7A is a schematic of the microfluidic device with multiple parrellel chambers for cell isolation.

Fig. 7B and 7C are photographs of examples of devices connected to a syringe

pump.

Fig. 7D is a photograph of a microfluidic station using pressure driven flow to

automate the isolation of cells from whole blood.

5   Fig. 8 is a photograph of a Microfluidic Cell Isolation Device with two cell-

capture chambers in series. The first chamber (top) captures and depletes

contaminating cells, increasing the purity of the cells captured inside the second

chamber.

Fig. 9 is a photomicrograph of captured CD66b+ granulocytes treated with

10   Wright-Giemsa stain. No significant platelet contamination is observed.

Fig. 10 is a graph showing typical electrophoresis of the RNA isolated from

neutrophils isolated using the microfluidic chambers. A total of 33 ng total RNA was

isolated from neutrophils isolated from 100 µL of blood. The quality of RNA is very

good indicting the compatibility of cell capture with RNA extraction techniques. Fig.

15   11 is a schematic illustrating coupling of the pMHC pentamer molecules to a

microfluidic channel.

Fig. 12 is a schematic illustrating pMHC pentamers loaded with A2-SL9

antigenic peptide are specifically recognized by CD8+ T cells with receptors for this

antigen, while non-specific CD8+ T cells (and all other cell populations) will not bind

20   to the biotinylated pentamer.

Figs. 13A and 13B are photomicrographs showing capture of pure clonal

populations of (A) A2-SL9 CD8+ T cells and (B) A2-IV9 CD8+ T cells using a

PDMS microfluidic device coated with pMHC pentamers loaded with A2-SL9 antigenic peptide.

Figs. 14A-14C are photomicrographs of captured cells in whole blood.

Fig. 14D is a schematic illustrating a channel from which the cells depicted in

5   Figs. 14 A-C were obtained.

Fig. 14E is a graph showing shear stress as a function of distance from the results of a concordance experiment to test experimental variability. Two experiments were run using the same sample under identical conditions.

Fig. 14F is a graph showing attached cells as a function of shear stress in a

10   comparison of capture efficiency between whole blood and lysed RBC blood.

Detailed Description of the Invention

In general the invention features methods, devices, and kits for the isolation of analytes (e.g., a cell). A sample containing a desired analyte is introduced into a

15   microfluidic device containing moieties that bind the desired analyte. A shear stress is applied that is great enough to prevent binding of undesired analytes and low enough to allow binding of the analyte of interest. Once bound, the desired analytes can be analyzed (e.g., counted). The invention also features methods for determining a shear stress for isolating a desired analyte.

20   An exemplary embodiment of the invention provides a whole blood CD4+ T lymphocyte count assay using cell affinity chromatography in a microfluidic format. The device was functionalized with a specific antibody for affinity selection of target cells. Controlled shear stress applied in the microfluidic channel allows specific and

efficient selection of CD4+ T cells versus monocyte and other white blood cells from

a small volume sample, compatible with fingerprick collection. To perform CD4

counts, 10 μL of unprocessed whole blood is injected into the microfluidic channel at

a controlled flow rate, and CD4+ T cell counts are determined as the number of all

5   captured cells using a light microscope. The total assay time is less than 10 minutes.

One critical factor for accurate CD4 counting using this approach is the

specificity of cell capture. To achieve this, we used cell affinity chromatography with

immobilized antibodies, which are further blocked with BSA to reduce non-specific

binding (Amiji and Park. J. Biomater. Sci. Polym. Ed. 4:217 (1993)). Because CD4

10   is also expressed on monocytes, we used shear stress as a secondary selection step to

exclude monocytes. We found that CD4+ T lymphocytes and monocytes respond

differently to shear stress on the functionalized device surface, as preferential binding

of lymphocytes occurs in a window of 1–3 dyn cm^{-2}; by contrast, monocytes bind

optimally at lower shear stresses. Selective binding within this window (1–3 dyn cm^{-2}

15   ) may be accounted for by two reasons. First, compared to lymphocytes, monocytes

express about an order of magnitude less surface CD4, which reduces the chance of

antibody–antigen interaction, especially under dynamic flow conditions (Lee et al.

Proc. Natl. Acad. Sci. U. S. A. 96:5215 (1999)). Second, the larger size of

monocytes increases the shear force exerted on individual cells (which is roughly

20   proportional to the square of cell diameter), resulting in decreased binding efficacy.

These two factors allow for differential binding of lymphocytes relative to monocytes.

Controlled shear stress is also shown to be critical for efficient cell capture. The

capture efficiency is further promoted by the elongated channel geometry, which

increases the interaction time between target cells and the active surface area.

Within the optimal lymphocyte-binding window (1–3 dyn cm^{-2}), the shear

force exerted on a cell 10 μm in diameter is ~8–25 pN. This is of the same order as

5   the binding force of a single antibody–antigen pair. (Harada et al. Langmuir 16:708

(2000) and Hinterdorfer et al. Proc. Natl. Acad. Sci. U. S. A. 93:3477 (1996)) When

the shear force is above this level, up to two orders of magnitude drop in cell adhesion

is observed. This observation implies that when target cells come into contact with the

surface, cell–substrate attachment is initiated by the formation of a single antibody–

10   antigen interaction (Tissot et al. Biophys. J. 61:204 (1992)), and high membrane

antigen density will favor the opportunity of such interaction. A somewhat surprising

result was that the number of adherent cells also decreases when the shear is dropped

below 1 dyn cm^{-2}. This decrease occurred when experiments were performed with

whole blood, but not with lysed blood (data not shown). We believe that erythrocytes

15   play an important role in the reduction of target cell binding at the low shear stress

range. Erythrocytes have been known to induce margination of leukocyte flow in

capillaries above certain flow rates. (Goldsmith and Spain. Microvasc. Res. 27:204

(1984) and Shevkoplyas et al. Anal. Chem. 77:933 (2005)) In our devices,

margination is analogous to pushing the leukocytes to the floor and roof of the

20   chamber. At low flow rates, the margination effect is not favored and, the erythrocytes

in whole blood could occupy most of the functional surface and prevent antibody–

antigen interactions. In lysed blood, cell–cell interaction is greatly reduced, and

leukocyte settling is driven mainly by sedimentation, which does not decrease with

reduced flow.

After the single channel device was optimized with blood from healthy donors

(95%+ purity and 90%+ yield), its performance was tested with whole blood from

5   HIV-infected subjects. We found a slight decrease in the device performance with

samples from these subjects, which may be accounted for by several reasons.

Interfering soluble factors in the serum of HIV-infected patients, such as soluble CD4,

(Peakman et al. J. Infect. Dis. 165:799 (1992)) may compromise the yield of

captured cells. Other soluble factors including cytokines, chemokines and immune

10   complexes (Trial et al. J. Clin. Invest. 95:1690 (1995)) may influence behavior of

blood cells. (Polo et al. AIDS. 13:447 (1999), Vonsydow et al. Aids Res. Hum.

Retrov. 7:375 (1991), and Clerici et al. J. Clin. Invest. 91:759 (1993)). Leukocyte

surface adhesion molecules may also be altered during HIV disease progression,

(Trial et al. J. Clin. Invest. 95:1690 (1995) and Trial et al. J. Immunol. 173:2190

15   (2004)) which may lead to elevated non-specific binding in our device.

Changes on CD4+ T cell surfaces in HIV-infected subjects, such as down-

regulation of CD4 receptors (Anderson et al. J. Virol. 67: 4923 (1993)) and binding

of gp120, (Thali et al. J. Virol. 66:5516 (1992)) might also reduce the receptor–

antibody interaction and decrease yield. Thus, it was not surprising to see both purity

20   and yield drop when samples from HIV-infected subjects were tested in the linear

chamber device. Nonetheless, a linear relationship was observed for CD4 counts

obtained by microchip and by flow cytometer in the clinically relevant range between

200 and 800 cells mL (Sato et al. Adv. Drug Deliv. Rev. 55:379 (2003)). Thus, the

microfluidic device is useful for clinical decision making and disease monitoring in resource-limited settings. Further optimization of purity and yield should lead to even higher levels of accuracy.

The application of a microfluidic device for CD4 counting offers the

5   advantages of reduced sample volume, decreased reagent consumption, low fabrication cost, and portability over conventional flow cytometric equipment. It uses a direct volumetric method and functions as a single platform. Moreover, no reagents need to be added to the assay, as required in single-platform flow cytometry and other proposed methods. Compared to the labor-intensive bead format assay and previously

10   described miniaturized flow cell design, (Rodriguez et al. PLoS Med. 2:663 (2005)), the present invention this microfluidic CD4 counting device directly addresses the sample preparation challenge faced in most resource-poor settings. No sample preparation, such as lysing erythrocytes, pipetting, or mixing with antibody reagents is required, so the device serves as a self-contained system. No differentiation of

15   monocytes from CD4+ T lymphocytes needs to be made during microscope-based counting.

We demonstrate a simple, quick, and inexpensive CD4+ T cell counting device based on microfluidic cell affinity chromatography operated under controlled shear stress. To our knowledge, this is the first device with which CD4 counts can be

20   performed from a fingerprick sample of whole blood, without either sample processing or the addition of reagents. Minimum handling procedures, rapid operation, simple device device and potential high-throughput detection makes this strategy a promising candidate for managing HIV patients in resource limited settings.

We also note that CD4 counting is just one application for devices of this type. The demonstration of specific cell isolation with high efficiency using shear stress combined with cell affinity chromatography could be applied to a number of applications where specific and efficient cell isolation is required. In addition,

5 applications of the invention are not limited to cells but are applicable to any analyte which can associate with a binding moiety and is subject to an appropriate amount of shear force. Such analytes include particles, e.g., magnetic, ceramic, or plastic, viruses, and molecular complexes, e.g., organelles and lipoprotein complexes.

10 **I. Methods of isolating an analyte**

The invention features methods and devices for isolating cells and other analytes. The devices of the invention are microfluidic devices with at least one chamber, which may be a portion of a designated portion of a microfluidic channel or an entire channel. This chamber contains binding moieties specific for a desired

15 analyte. Typically, the binding moieties are disposed on the walls of the channel, although additional structures, e.g., posts, may be included in the channel to increase the surface area. A sample containing the desired analyte is applied at a shear stress preferably low enough to allow binding of the desired analyte, but preferably great enough to prevent binding of undesired analytes, as described above for CD4+ cells.

20 In one embodiment, the chamber is coated with binding moieties that bind to a cell surface marker of a desired cell population. Through application of an appropriate shear stress, the methods of the invention result in the selective isolation of cells expressing these cell surface markers at a specific concentration. The applied

shear stress is preferably great enough to prevent binding of undesired cells that

contain the cell surface marker at a concentration lower than the desired population of

cells and other non-specific binding interactions.

The methods of the invention result, for example, in the isolation of 50%,

5   60%, 70%, 80%, 90%, 95%, 99%, or 100% of the desired analyte, e.g., cells, in a

sample while retaining, for example, less than 20%, 10%, 5%, or 1% of undesired

analytes. In addition, while analytes that bind are described as being "desired" and

analytes that do not bind are described as being "undesired," either type or both types

of analyte may be of actual interest in a particular experiment. For example, the

10   methods of the invention may be used to isolated analytes that either bind to the

device or flow through the device.

At least two variables can be manipulated to control the shear stress applied to

the channel: the cross sectional area of the chamber and the fluid pressure applied to

the chamber. Other factors may be manipulated to control the amount of shear stress

15   necessary to allow binding of desired analytes and to prevent binding of undesired

analytes, e.g., the binding moiety employed and the density of the binding moiety in

the channel.

The chamber may include multiple types of binding moieties (e.g., 1, 2, 3, 4,

5, or more). Multiple binding moieties may bind to the same or different analytes,

20   and may be placed in the same or different chambers. For example, binding moieties

to multiple cell surface markers that occur on a desired cell may be disposed in one

chamber.

In another embodiment, the invention features chambers arranged in series, (e.g., 2, 3, 4, 5, or more chambers). In this embodiment, each chamber isolates one or more types of cells, which may or may not be the cells of interest. When multiple chambers are arranged in series, the shear stress applied to each of the chambers can

5 be different (achieved for example by varying the cross sectional area of the chambers) or the shear stress can be the same. Also, when multiple chambers are arranged in series, each chamber can contain binding moieties that bind to different cell surface markers or the same cell surface markers. When the same binding moiety in employed in different chambers, the methods may be used to isolate, in series,

10 analytes that have progressively lower amounts of substance to which the binding moiety binds.

The methods may also be employed to isolate various types of analytes in parallel, e.g., by passing aliquots of the same sample through separate devices or one device including multiple chambers in parallel. Different samples may also be

15 assayed in parallel.

Devices used in the methods of the invention may be simply a microfluidic channel to which binding moieties are attached and which is capable of supporting fluid flow at the desired shear stress. The device geometry will be determined based on the assay. Devices may, or may not, include regions that allow for optical or

20 visual inspection of the chambers. Fluid pumps capable of producing desired shear stress are also known in the art. Example of pumps include syringe pumps, peristaltic pumps, vacuum. Methods for coupling pumps to devices are known in the art. The

device may be configured for substantially constant shear stress in any given chamber or variable shear stress in a given chamber. Exemplary devices are described herein.

Devices of the invention may be fabricated using techniques known in the art. The fabrication techniques employed will depend on the material used to make the

5   device. Examples of fabrication techniques include molding, photolithography, electroforming, and machining. Exemplary materials include glass, polymers (e.g., polystyrene, silicones such as polydimethylsiloxane, epoxy, and urethanes), silicon and other semiconductors, and metals.

Binding moieties may be attached to chambers using methods known in the

10   art. The method employed will depend on the binding moiety and the material used to construct the device. Examples of attachment methods include non-specific adsorption to the surface, either of the binding moiety or a compound to which the binding moiety is attached or chemical binding, e.g., through self assembled monolayers or silane chemistry.

15   Devices of the invention may be combined with fluids, pumps, and/or detectors. Devices may also be combined with reagents, e.g., lysis reagent, labeling reagents, and instructions for use, e.g., for disease diagnosis.

II. Target analytes

20   Table 1 provides exemplary cell populations, cell surface markers appropriate for the methods and devices of the invention, and the corresponding shear stresses necessary to specifically isolate the indicated cells from a blood sample.

Table 1. Blood cells isolated from whole blood

	Optimal Shear [dyne/cm^2]	Wash [dyne/cm^2]	Capture molecule	Purity*	Yield
Neutrophil	0.4	1.5	Anti-CD66	93%	80%
Monocyte	0.3	0.7	Anti-CD14	93%	
Lymphocyte	1.3-1.7	3	Anti-CD4	99%	80%
			Anti-CD8		
Circulating tumor cells	8-9	35	Anti-EpCAM	80%#	60%
Neutrophils	1-7	-	E, P Selectins	70%	80%
HIV-specific T cell	0.082	N/A	HLA A2-SL9 pentamer	>99%	N/A
Any disease specific T cell	0.07-0.1		Pentamer		

* blood from healthy donor
# blood from patients with cancer stage III – IV

Additional exemplary cell surface markers appropriate for the methods and devices of the invention are set forth in Table 2. Cell types also include disease-specific T cells, either CD4+ or CD8+, which can be isolated using MHC-peptide monomers, tetramers, or pentamers as the binding moiety. Shear stresses appropriate for isolating cells expressing the cell surface markers in Table 2 can be determined as described. In addition the cells set forth in Table 2, the invention is also useful, for example, for isolating human and animal pathogens (e.g., protists, bacteria, and fungi), fetal cells (e.g., nucleated red blood cells, amniocytes, and trophoblasts), stem cells (embryonic or adult), sickle cell red blood cells, and white blood cells.

Table 2. Cell Surface Markers

Desired Cell	Binding Moiety	Cross reactivity
Dendritic cells	Anti-CD83	Weak cross reactivity with lymphoblastoid cell lines and with some germinal center B cells
Monocyte	Anti-CD14 antibody	weakly on the surface of granulocytes; also expressed by most tissue macrophages
Tuberculosis sensitive CD8 T cell	P MHC Pentamer A2-SL9	Weak cross reactivity with other MHC class II molecules
Platelets	Anti-CD41	Less abundant expression on Megakaryocytes
Endothelial progenitor cells	Anti-CD34	Less expressed on small-vessel endothelial cells, embryonic fibroblasts
Epithelial progenitor cells	Anti-CD133	Hematopoietic cells
Mesenchimal progenitor cells	Bone morphogenetic protein receptor (BMPR)	Stromal cells, fibroblasts

In order to determine the optimum shear stress to isolate cells containing a

5   desired cell surface marker, a sample of cells may be applied to a Hele–Shaw

chamber (e.g., the device set forth in Fig. 1C) designed based on the equations derived

by Usami et al. (Murthy et al. Langmuir. 20:11649 (2004) and Usamiet al. Ann.

Biomed. Eng. 21:77 (1993)). The shape of the chamber of this device is such that the

shear stress along the axis of the chamber decreases linearly along the chamber

10   length. The Hele–Shaw devices can be used to determine the dynamics of cell

attachment to microfluidic devices because multiple shear rates can be obtained in

each flow chamber without changing the inlet flow rates. By identifying the

narrowest location along the axis of the Hele-Shaw chamber where the desired cells

bind, the corresponding shear rate can be calculate using the equations cited above.

III. Binding moieties

Binding moieties useful in the devices and methods of the invention include antibodies, antibody fragments (e.g., Fc fragments), oligo- or polypeptides, nucleic acids, cellular receptors, ligands, aptamers, MHC-peptide monomers or oligomers,

5   biotin, avidin, oligonucleotides, coordination complexes, synthetic polymers, and carbohydrates.

IV. Methods of Use

In addition to methods of isolating various analytes from a sample, the

10   invention provides methods in which the analyte isolated may be used to provide additional information. In particular, cells isolated using the methods and devices of the invention can be further assayed using additional methods of the invention. In one embodiment, cells that are isolated using the methods and devices of the invention are counted. Cells can be counting by any method known in the art, including optical,

15   e.g., visual inspection, automated counting, microscopy based detection, and FACS, and electrical detection, e.g., Coulter counters. Counting of the cells, or other analytes, isolated using the methods and devices of the invention can be useful for diagnosing diseases, monitoring the progress of disease, and monitoring or determining the efficacy of a treatment. Cell, or other analyte, counting may also be

20   of use in non-medical applications, e.g., for determination of the amount, presence, or type of contaminants in environmental samples (e.g., water, air, and soil), pharmaceuticals, food, or cosmetics.

Many diseases are characterized by abnormal levels of cells containing certain

cell surface markers (e.g., see Table 2). Also cells infected with certain pathogens

often express unique cell surface markers, unique combinations of cell surface

markers, or express cell surface markers at abnormal levels.

5   In another embodiment, cells isolated using the methods and devices of the

invention can be lysed, and one or more properties of the cells, or portions thereof,

can be measured. Examples of biological properties that can be measured in isolated

cells include mRNA expression, protein expression, and DNA quantification.

Additionally, the DNA of cells isolated by the methods of the invention can be

10   sequenced, or certain sequence characteristics (e.g., polymorphisms and chromosomal

abnormalities) can be identified using standard techniques, e.g., FISH or PCR. The

chemical components of cells, and other analytes, may also be assayed after isolation.

V. Examples

15   **Example 1: Isolation of CD4+ T lymphocytes**

          Materials

          3-Mercaptopropyl trimethoxysilane was purchased from Gelest (Morrisville,

PA). Ethanol (200 proof), glass coverslips (35 x 60 mm, no. 1), hemacytometer and

microslide fieldfinder were obtained from Fisher Scientific (Fair Lawn, NJ). For

20   chamber fabrication, SU-8 photoresist and developer were obtained from MicroChem

(Newton, MA); silicone elastomer and curing agent were obtained from Dow Corning

(Midland, MI). Phosphate buffered saline (PBS) was obtained from Mediatech

(Herndon, VA). Lyophilized bovine serum albumin (BSA) was obtained from Aldrich

Chemical Co. (Milwaukee, WI). The coupling agent GMBS (N-γ-

maleimidobutyryloxy succinimide ester) and NeutrAvidin were obtained from Pierce

Biotechnology (Rockford, IL). Biotinylated mouse anti-human anti-CD4 (clone

13b8.2) was purchased from Beckman Coulter (Somerset, NJ). Biotinylated mouse

5   antihuman anti-CD36 (clone SMO) was obtained from Ancell (Bayport, MN). Alexa

Fluor1 488-conjugated mouse antibody to human CD4 (AF488-anti-CD4, clone 289-

14120), Alexa Fluor1 647-conjugated mouse antibody to human CD3 (AF647-anti-

CD3, clone 289-13801) and 4'-6-diamidino-2-phenylindole (DAPI) were obtained

from Molecular Probes (Eugene, OR). Phycoerythrin (PE)-conjugated mouse

10   antihuman CD14 monoclonal antibody (PE-anti-CD14, clone

M5E2) was purchased from BD Bioscience (San Diego, CA). Paraformaldehyde was

obtained from Electron Microscopy Sciences (Hatfield, PA).

Chamber design and fabrication

15   Two types of microfluidic devices were used in this work. The first one was

the Hele–Shaw chamber (Fig. 1C) designed based on the equations derived by Usami

et al. (Murthy et al. Langmuir. 20:11649 (2004) and Usami et al. Ann. Biomed. Eng.

21:77 (1993)). The shape of the chambers is such that the shear stress along the axis

of the chamber decreases linearly along the chamber length. The fabricated flow

20   chambers were 43 ± 1 μm in height with inlet width and total length of 5 mm and 50

mm, respectively. The Hele–Shaw devices were used to study the dynamics of

lymphocyte attachment to microdevices using healthy donor blood, since multiple

shear rates can be obtained in each flow chamber without changing the inlet flow

rates. The second type of device was a straight flow channel (Fig. 1d), which provides

a constant shear stress along the channel length and has a footprint of 2 cm^2. The

width, length and height of the channel were 4 mm, 51 mm and 50 ± 1 μm

respectively. The straight channel device was used for actual cell capture and

5   counting experiments, using the operating shear stress determined from the Hele–

Shaw experimental data. The devices were fabricated in PDMS and bonded

permanently to clean glass cover slips using standard clean room techniques. (Murthy

et al. Langmuir. 20:11649 (2004) and Usamiet al. Ann. Biomed. Eng. 21:77 (1993))

10   **Surface modification**

Freshly fabricated devices were modified using the method described

previously. (Murthy et al. Langmuir. 20:11649 (2004) and Usamiet al. Ann.

Biomed. Eng. 21:77 (1993)) Briefly, the chambers were pretreated with 4% (v/v)

solution of 3-mercaptopropyl trimethoxysilane in ethanol for 30 min at room

15   temperature, followed with incubating the chambers with 0.01 mmol mL (Sato et al.

Adv. Drug Deliv. Rev. 55:379 (2003)) GMBS in ethanol for 15 min at room

temperature. Afterwards, NeutrAvidin was immobilized to GMBS by incubating the

chamber surfaces with 10 mg ml^{-1} NeutrAvidin solution in PBS for at least 1 h at 4°

C. Finally, 10 mg mL^{-1} (ibid) biotinylated anti-CD4 solution in PBS containing 1%

20   (w/v) BSA and 0.09% (w/v) sodium azide was injected to react at room temperature

for 15 min. After each step, the surfaces were rinsed with either ethanol or PBS,

depending on the solvent used in the previous step, to flush away unreacted

molecules.

Study subjects and blood preparation

Healthy HIV-negative and HIV-infected subjects were recruited from the

Massachusetts General Hospital (MGH) in Boston. All subjects provided written

5   informed consent. Samples of 5 mL of peripheral blood were collected by

venipuncture in either heparin or EDTA Vacutainer collection tubes (Becton

Dickinson). All samples were run on the microfluidic devices on the day of blood

collection. Parallel measurement of CD4 counts of the HIV infected subjects were

taken through standard clinical laboratory operating procedures, as described

10   previously. (Rodriguez et al. PLoS Med. 2:663 (2005)) Briefly, patient samples

were processed in the hospital clinical laboratory using standard 4-color flow

cytometry on a Becton Dickinson FACSCalibur, using the MultiTEST reagents and

TruCOUNT beads and analyzed using MultiSET software to obtain CD4 counts.

15   **Microfluidic flow experiments**

In experiments performed to develop the prototype microdevice, 300 µl of

unprocessed whole blood from healthy donors was introduced into the Hele–Shaw

chambers at the desired shear rates (5–40 µl min^{-1}) using a syringe pump (Harvard

Apparatus PHD 2000, Holliston, MA). The volume of whole blood was chosen such

20   that cell adhesion on the surfaces reached a pseudo-steady state, where no significant

increase of captured cells was observed as determined by both examining the number

of cells adhered on the surfaces and the blood samples collected from the device

outlet. Immediately after sample delivery, PBS containing 1% BSA (w/v) and 1 mM

EDTA was flowed through the chamber at 40 μL min^{-1} for 5 min to rinse off the

unbound cells. The cells were then fixed on the surfaces by incubating with 1%

paraformaldehyde, followed with incubating with an antibody mixture containing

AF647-anti-CD3/AF488-anti-CD4/PE-anti-CD14 for 15 min. After rinsing off the

5   unbound antibody with PBS containing 1% BSA (w/v) and 1 mM EDTA, the number

of adhered cells were counted by placing a field finder under the chambers and

counting cells at select points along the device axis using an inverted microscope

(Nikon Eclipse TE2000, Nikon, Japan). Monocytes were identified by staining with

antibody to CD14, CD4+ T cells were recognized by CD3+/CD4+/CD14- staining,

10   and the total number of adherent nucleated cells were determined by staining with

DAPI or direct observation under the phase contrast microscope. For each point, three

measurements were made, corresponding to three 1 mm^2 squares in that vicinity, and

averaged. Images were obtained at 10^6 magnification using fluorescein, rhodamine,

and Cy5 excitation/emission filters. DAPI staining was performed afterwards by

15   incubating the surface-attached cells with 300 nM DAPI in PBS at room temperature

for 5 min and rinsing with PBS. The cells were counted either manually or using

Image J software (http://rsb.info.nih.gov/ij/). To avoid competitive binding between

the capture antibody and the labelling antibody, CD4 antibodies were selected to bind

to different epitopes.

20   In experiments performed to test the CD4 cell counting device, 10 μl of whole

blood from healthy donors or HIV infected subjects was flowed into linear chambers

at the desired flow rates (1–20 μl min^{-1}). After rinsing at a flow rate of 20 μl min^{-1},

cells adhered to the surface were stained and counted using similar procedures to the

ones described above for Hele–Shaw devices. Sample flow-through and rinse buffer were collected from the outlet of the device into Eppendorf tubes and centrifuged to concentrate the cells for flow cytometry.

5   **Flow cytometry analysis**

In order to confirm the efficiency of the devices in depleting target cells from whole blood, aliquots of samples before and after passage through the linear chamber devices were collected and analysed using standard flow cytometry to quantify the percentage of CD4+ T cells. The flow cytometric measurements were performed on a

10   FACSCalibur (Beckton Dickinson Immunocytometry System (BDIS), San Jose, CA) instrument using BD CellQuest Pro Software. The capture efficiency, or yield of the device was estimated from the ratio of the percentage of CD3+ CD4+ T cells in samples collected before and after passing through the microfluidic device.

15   **Statistics and data analysis**

The experiments performed with blood from healthy HIV negative subjects were repeated in at least 3 different devices at each condition. Data shown in Figs. 2, 3 and 4 represent cell counts or flow cytometric measurements averaged over these devices, and each error bar represents the standard error of the mean.

20

Results

Development of a simple device for CD4+ cell separation from whole blood using affinity isolation chemistry

Using device simplicity and accuracy as our key objectives, we identified two

5   key factors in our design criteria: the specificity (purity) and efficiency (yield) of

CD4+ T cell capture in a label-free microfluidic device. We first tested how specific

an anti-CD4-immobilized, BSA-blocked surface is in capturing CD4-presenting cells.

Fig. 2A shows representative overlapped phase contrast images and anti-CD4 stained

fluorescent images of cells from whole blood obtained from a healthy, HIV-negative

10   subject and adherent to anti-CD4 functionalized surfaces. As shown, almost all

captured cells (density ~50–500 cells mm^{-2}) stained positively for surface CD4

antigen. A control device lacking a specific cell capture antibody demonstrated 1–2

orders of magnitude lower cell attachment from whole blood (density, 5 cells mm^{-2}).

Thus, a surface functionalization scheme appeared successful in exclusively capturing

15   CD4-presenting cells from unlabeled whole blood samples.

Monocyte versus CD4+ T lymphocyte adhesion from whole blood

In circulating cells, CD4 molecules are present on both lymphocytes and

monocytes, which cannot be differentiated by immobilized anti-CD4 alone. This can

20   be observed in Fig. 2B, where some of the CD4-presenting cells (stained green) also

stain with the monocyte marker CD14. Thus, for the purpose of enumerating CD4+ T

cells alone, a secondary selection mechanism has to be used to exclude monocyte

binding. We used shear stress for this purpose, considering the lower CD4 expression

29

level on monocytes relative to CD4+ T cells, as well as their size differences. To

study the effect of shear stress on monocyte and lymphocyte adhesion, we used

antibody-functionalized Hele–Shaw devices (Fig. 1C), which allows for an analysis of

cell adhesion over a range of shear stresses in a single experiment. (Murthy et al.

5   Langmuir. 20:11649 (2004) and Usamiet al. Ann. Biomed. Eng. 21:77 (1993))

Fig. 2C compares the adhesion profiles of monocytes (empty circles) and

CD4+ lymphocytes (solid circles) within a shear stress range of 0.15 to 5 dyn cm^{-2}.

Maximum adhesion of CD4+ lymphocytes occurs in a shear stress window between 1

and 3 dyn cm^{-2}. Within this region, roughly 500 cells adhered per square millimeter of

10   area. The adhesion of CD4+ T cells decreases rapidly outside of this shear stress

window. In contrast to lymphocyte adhesion on the anti-CD4 surface, monocytes have

a different dependence on shear stress (inset in Fig. 2C). Monocyte adhesion drops

from about 40 cells mm^{-2} to around 5 cells mm^{-2} when the shear stress increases from

0.3 to 0.7 dyn cm^{-2}, and remains below 5 cells mm^{-2} when the shear stress is above

15   0.7 dyn cm^{-2}. We also plotted the number of other cells (the total number of adherent

cells minus monocytes and CD4+ T cells) adherent to the anti-CD4 surface (solid

triangles in Fig. 2C). The non-specific cell number remained at a constant low level

(<5 cells mm^{-2}) throughout the tested shear stress range.

When the cell composition on the device surface is plotted (Fig. 2D), we

20   observe that purity of surface captured CD4+ T lymphocytes is more than 95% when

the shear stress is above 0.7 dyn cm (Chovan and Guttman. Trends Biotechnol.

20:116 (2002)). Purity drops at shear stress below 0.7 dyn cm^{-2}, due mainly to the

adhesion of monocytes under the low shear condition; purity also drops slightly above

4 dyn cm^{-2}. Thus, shear stress is a powerful tool to differentiate specific adhesion of CD4+ T lymphocytes and monocytes.

Development of a cell counting device and determination of capture efficiency

5   In the initial experiments, we demonstrated highly selective capture of CD4+ T cells using a monoclonal antibody functionalized surface operated under differential shear stress. Next, we designed a straight channel device for efficient isolation of CD4+ T lymphocytes under fixed shear stress within the shear stress range optimized for pure CD4+ T cell capture without contaminating monocytes (Fig. 1D). This

10   simple device had an internal volume of 10 µL, which serves as a sample volume metering mechanism. The 10 µL volume allows for convenient delivery of a small-volume sample obtained from a study subject, and sufficient sample size for statistically valid cell counts. The elongated chamber design increases the interaction time of blood with the functional surface.

15   We injected 10 µL of whole blood at shear stresses ranging from 0.2–7 dyn cm^{-2} into the linear device, collected samples before and after flow through the chamber, and analyzed them by flow cytometry to study the capture efficiency within this device. Representative quadrant analysis data from the lymphocyte window of blood samples before and after passage through the device at a shear stress of 1.7 dyn

20   cm^{-2} are shown in Figs. 3A and 3B. In this representative experiment, CD4+ T lymphocytes (CD3+ CD4+) comprised 29.7% of all lymphocytes entering the microfluidic channel (Fig. 3A); after selective capture, this fraction dropped to 2.1% of lymphocytes exiting the device (Fig. 3B), indicating retention of more than 90% of

the target CD4+ T cells within the device at this shear stress. Similar experiments

were performed at various shear stresses to study the effect of shear stress on capture

efficiency (or yield) of CD4+ T cells in the linear device (Fig. 3C). We observed that

a shear stress window of 1–3 dyn cm^{-2} was optimal for efficient CD4+ T cell capture

5   in the linear chamber, matching the results obtained using the Hele–Shaw chamber

(Fig. 2C). Within this shear stress window, nearly 95% of the CD4+ T lymphocytes

were isolated with purity greater than 95%. Outside of this window, capture efficiency

quickly dropped to 70–80%, along with a decrease in purity (Fig. 3C). When we

evaluated cell distribution in devices operated at two shear stresses in and out of the

10   optimal window, we observed differences that help to explain the mechanism for their

distinctive capture efficiency (Fig. 4). At a shear of 1.7 dyn cm^{-2}, which yielded 95%

target cells, a narrow cell density peak of around 200 adherent cells mm^{-2} was seen

within 10 mm from the device inlet; this density quickly dropped below 20 cells mm^{-2}

at greater distances from the inlet. In contrast, at a less efficient shear of 7 dyn cm^{-2},

15   surface-captured cells remain at a relatively constant, low density throughout the

length of the chamber. Thus, controlled shear flow in a simple, anti-CD4

functionalized device promotes efficient and specific CD4+ T cell capture.

CD4 counts from HIV-infected subjects using optimized, simple microfluidic

20   **devices**

        After determining the optimal conditions for the device using blood from

healthy donors, we next tested the devices using samples obtained from HIV+ adult

subjects. A ten-microliter sample of blood was introduced for 2 min at 5 μL min^{-2},

which corresponds to a shear stress of 1.7 dyn cm^{-2}. Next, buffer was introduced at 20

μL min^{-2}, which corresponds to a shear stress of ~7 dyn cm^{-2}, to remove monocytes

and non-specific cells. CD4 counts were then determined from the total number of

adherent cells, counted manually under a phase contrast microscope; total assay time

5   was under 10 min. We compared these CD4 counts from our microdevice with results

obtained from samples processed in parallel by flow cytometry (Fig. 5). For 13 adult

study subjects with CD4 counts ranging from 26 to 1428 cells μL^{-1} by flow

cytometry, our results show a close correlation between the two methods at CD4

counts up to 800 cells μL^{-1} ($n = 11$, $R^2 = 0.93$) respectively. At CD4 counts above 800

10   cells μL^{-1}, the microdevice cell counts are significantly lower than those obtained by

flow cytometry, which likely reflects saturation of cell binding within the chamber.

To confirm these findings, we assessed the purity and yield of CD4+ T cells in

the linear device using whole blood from HIV-infected subjects (Fig. 6A and B).

Purity (or capture specificity) was calculated by taking the ratio of CD4+ T cells

15   (CD3+CD4+) to the total number of captured cells (DAPI+); yield (or capture

efficiency) was defined as the ratio of captured CD4+ T cells to the sum of captured

CD4+ T cells plus those lost in the flow through. For the 13 study subjects, a

consistent yield (>75%) was observed for CD4 counts up to 800 cells μL^{-1}; the yield

started to drop for subjects with higher absolute CD4 counts. Purity ranged from 60–

20   90% for CD4 counts above 200 cells μL^{-1}. When the absolute CD4 count is below

200 cells μL^{-1}, poorer capture specificity was observed (20–50%). Nevertheless, a

clear cutoff was observed for CD4 counts around 200 cells μL^{-1}, which is used

clinically to discriminate relevant CD4 count thresholds. We also observed that >90%

of the non-target cells are monocytes (data not shown), which in the context of HIV

infection may be more adherent to an anti-CD4 functionalized surface than monocytes

from uninfected blood.

5   **Example 2: Arrangements of capture chambers**

A flat microfluidic chamber (Fig. 7) has been used for isolating various target

cells from whole blood (Table 1). The principle for specific cell capture is the

combination of specific antibodies, well controlled shear stress conditions inside the

device, and efficient passivation of the surfaces to prevent nonspecific binding of

10   unwanted cells. Following optimization, the purity of cell capture can be as high as

99% and the yield of capture as high as 80%. One approach for increasing the purity

in unfavorable situations relies on the use of isolation chambers in series (Fig. 8),

where the first chamber depletes the cells that would otherwise contaminate the

sample of interest in the second chamber. The captured cells can be imaged using

15   standard microscope (Fig. 9), counted, and RNA and protein extracted (Fig. 10) for

further analysis.

Example 3: HIV–specific CD8 T cell isolation

We developed a PDMS microfluidic device coated with pMHC class I

20   pentamers for the capture of disease-specific CD8+ T cells (Fig. 11). In our

experiments, we used soft lithography and SU-8 fabrication techniques to create

microchannels within a PDMS mold. The PDMS mold and a silica substrate were

then exposed to oxygen plasma treatment before being irreversibly sealed together.

The hydroxyl groups on the PDMS and silica surfaces were then treated with 3-

mercaptotrimethoxysilane in anhydrous ethanol, resulting in the formation of thiol-

terminal groups. After washing off unreacted silane solution with anhydrous ethanol,

the PDMS device was flushed with the heterobifunctional crosslinker GMBS in

5   anhydrous ethanol, during which the thiol group on the silane reacts specifically and

covalently with the maleimide region of GMBS. This leaves the succinimide residue

of the GMBS available for protein attachment, and after flushing unreacted GMBS

with 1x PBS (pH 7.4), NeutrAvidin solution (in PBS) was introduced into the

channel, resulting in the binding of the GMBS succinimide group to the terminal

10   amino group of NeutrAvidin. After flushing unreacted NeutrAvidin with 1x PBS (pH

7.4), biotinylated pMHC class I pentamers loaded with A2-SL9 antigenic peptide (a

dominant HIV gag epitope) were introduced into the device. The device was then

flushed with 1x PBS (pH 7.4) with 1% BSA (w/v) for the purpose of flushing out

unreacted pMHC pentamer and for minimizing non-specific cell interactions with the

15   channel surface (Fig. 12).

        With the device properly equipped for cell capture experiments, we cultured

two clonal CD8+ T cell lines - one specific for the HIV A2-SL9 (SLYNTVATL)

peptide, and one specific for an unrelated A2-IV9 (ILKEPVHGV) peptide. T cells

that recognize these peptides in the context of the MHC class I molecule are present

20   in most individuals infected with HIV.

        We introduced 500,000 A2-SL9 CD8+ T cells (125 microliters at a

concentration of 4 X 10^6 cells/ml) into a PDMS microfluidic device functionalized

with A2-SL9-loaded pMHC pentamers, at a flow rate of 2 μl/min. As a negative

control, T cells specific for recognizing A2-IV9 were introduced into an identical

device with the same specifications. The devices were flushed with 1x PBS to

remove unattached cells from the channel surface, and then fixed with 1% PFA

solution and fluorescently stained using DAPI solution before being imaged (Fig. 13).

5   We observed a high degree of specific capture in the device with A2-SL9 CD8+ T

cells, while a low level of non-specific T cell capture is seen in the device into which

A2-IV9-specific CD8+ T cells were introduced. Specific T cell binding was efficient,

as evidenced by a lack of a monolayer of cell coverage in the channel. This set of

experiments established that disease-specific T cells can be isolated in microfluidic

10   channels using pMHC complexes as capture agents. These devices have applications

for disease diagnosis, or for studies where identification of disease-specific T cells are

desirable, such as monitoring of vaccine efficacy.

Example 4: Capture of tumor cells using anti-EpCAM antibodies

15   Shear stress plays an important role in cell capture. An optimum shear stress

should be applied such that one can capture a maximum number of cancer cells at

high enough flow rates. To find optimal flow rate, we studied the effect of shear stress

on the cell capture using flat microfluidic chambers with variable width and constant

height along the chamber. The geometry of these chambers shown in Fig. 14 is such

20   that the shear stress varies linearly along the chamber length (Fig. 14E), permitting

the study of a wide range of shear stresses for a given flow rate. Cultured lung cancer

cells were spiked into PBS solution, and then passed through the Hele-Shaw

chambers functionalized with EpCAM Ab at a constant flow rate. As the shear stress

decreases along the channel, the density of the cells adhered to the surface increases

(Figs. 14A-14C). The effect of shear stress on the cell adhesion through EpCAM

antibody-antigen binding is plotted in Fig. 14F show 8 dyn/cm^2 is the optimum shear

rate, resulting in the capture of 200 cells/mm^2 of functionalized capture surface.

5

Other Embodiments

All publications, patents, and patent applications mentioned in the above

specification are hereby incorporated by reference. Various modifications and

variations of the described method and system of the invention will be apparent to

10   those skilled in the art without departing from the scope and spirit of the invention.

Although the invention has been described in connection with specific embodiments,

it should be understood that the invention as claimed should not be unduly limited to

such specific embodiments. Indeed, various modifications of the described modes for

carrying out the invention that are obvious to those skilled in the art are intended to be

15   within the scope of the invention.

Other embodiments are in the claims.

What is claimed is:

Claims

1.   A method for isolating a desired cell from a sample comprising:

(a) introducing said sample into a microfluidic device containing a binding moiety specific for said desired cell;

(b) allowing desired cells in said sample to bind to said binding moiety; and

(c) applying a shear stress to said microfluidic device so that desired cells remain bound while undesired cells do not.

2.   The method of claim 1, wherein said binding moieties are selected from the group consisting of antibodies, antibody fragments, oligo- or polypeptides, nucleic acids, cellular receptors, ligands, aptamers, MHC-peptide monomers or oligomers, biotin, avidin, oligonucleotides, coordination complexes, synthetic polymers, and carbohydrates.

3.   The method of claim 1, wherein said sample is a blood sample.

4.   The method of claim 1, wherein said desired cell is selected from the group consisting of neutrophils, monocytes, lymphocytes, circulating tumor cells, HIV infected CD8 lymphocytes, circulating endothelial cells, and platelets.

5.   The method of claim 1, wherein said binding moiety binds to CD66, CD14, CD4, CD8, EpCAM, E-Selectin, or P-Selectin.

6. The method of claim 1, wherein said desired cells are CD4+ lymphocytes, and said binding moiety is an anti-CD4 antibody.

7. The method of claim 6, wherein said sample is obtained from a patient at risk of developing AIDS.

8. The method of claim 1, further comprising (d) analyzing at least one biological property of said desired cells.

9. The method of claim 8, wherein said biological property is selected from the group consisting of: mRNA expression, protein expression, DNA quantification, DNA sequence, and chromosomal abnormalities.

10. The method of claim 1, further comprising: (d) counting said desired cells.

11. The method of claim 6, further comprising: (d) counting said CD4+ lymphocytes.

12. The method of claim 11, using said count to diagnose a disease state.

13. The method of claim 1, wherein said step (b) and step (c) occur simultaneously.

14.   A method for isolating a desired cell from a sample comprising:

(a) introducing said sample into a microfluidic device containing a first

binding moiety specific for a first desired cell disposed in a first chamber;

(b) allowing said first desired cell in said sample to bind to said first binding

moiety;

(c) applying a first shear stress to said microfluidic device so said first desired

cells remain bound while other cells do not;

(d) allowing the remaining sample to flow into a second chamber of said

microfluidic device, wherein said second chamber contains a binding moiety for a

second desired cell;

(e) allowing said second desired cell in said sample to bind to said second

binding moiety; and

(f) applying a second shear stress to said second chamber so said second

desired cells remain bound while undesired cells do not.

15.   The method of claim 14, wherein said first binding moieties are

selected from the group consisting of antibodies, antibody fragments, oligo- or

polypeptides, nucleic acids, cellular receptors, ligands, aptamers, MHC-peptide

monomers or oligomers, biotin, avidin, oligonucleotides, coordination complexes,

synthetic polymers, and carbohydrates.

16.   The method of claim 14, wherein said second binding moieties are

selected from the group consisting of antibodies, antibody fragments, oligo- or

polypeptides, nucleic acids, cellular receptors, ligands, aptamers, MHC-peptide

monomers or oligomers, biotin, avidin, oligonucleotides, coordination complexes,

synthetic polymers, and carbohydrates.

17.   The method of claim 14, wherein said first shear stress and said second

shear stress are different.

18.   The method of claim 17, wherein said first binding moiety and said

second binding moiety are the same.

19.   A kit for isolating a desired cell comprising:

(a) a device comprising a chamber containing a binding moiety specific for

said desired cell; and

(b) a pump for producing a shear stress, so that said desired cells bind

preferentially compared to undesired cells.

20. The kit of claim 19, further comprising a labeling reagent specific for said

desired cell.

21. The kit of claim 19, further comprising instructions for AIDS diagnosis.

22. A method of determining a shear stress for isolating a desired cell on a device, said method comprising:

(a) introducing a sample containing said desired cell into a chamber of a microfluidic device, said chamber containing a binding moiety specific for said desired cell;

(b) allowing desired cells in said sample to bind to said binding moiety;

(c) applying shear stress to said microfluidic device, wherein said shear stress varies along the length of said chamber; and

(d) identifying a shear stress at which said desired cell binds to said binding moiety preferentially compared to another cell.

Fig. 1
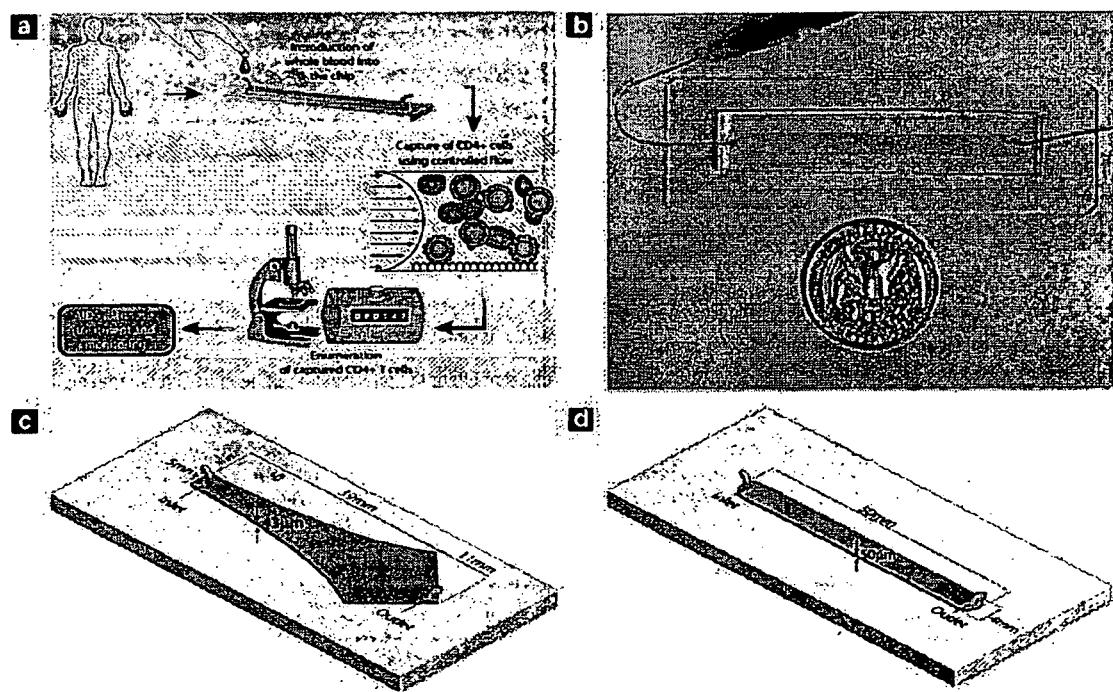
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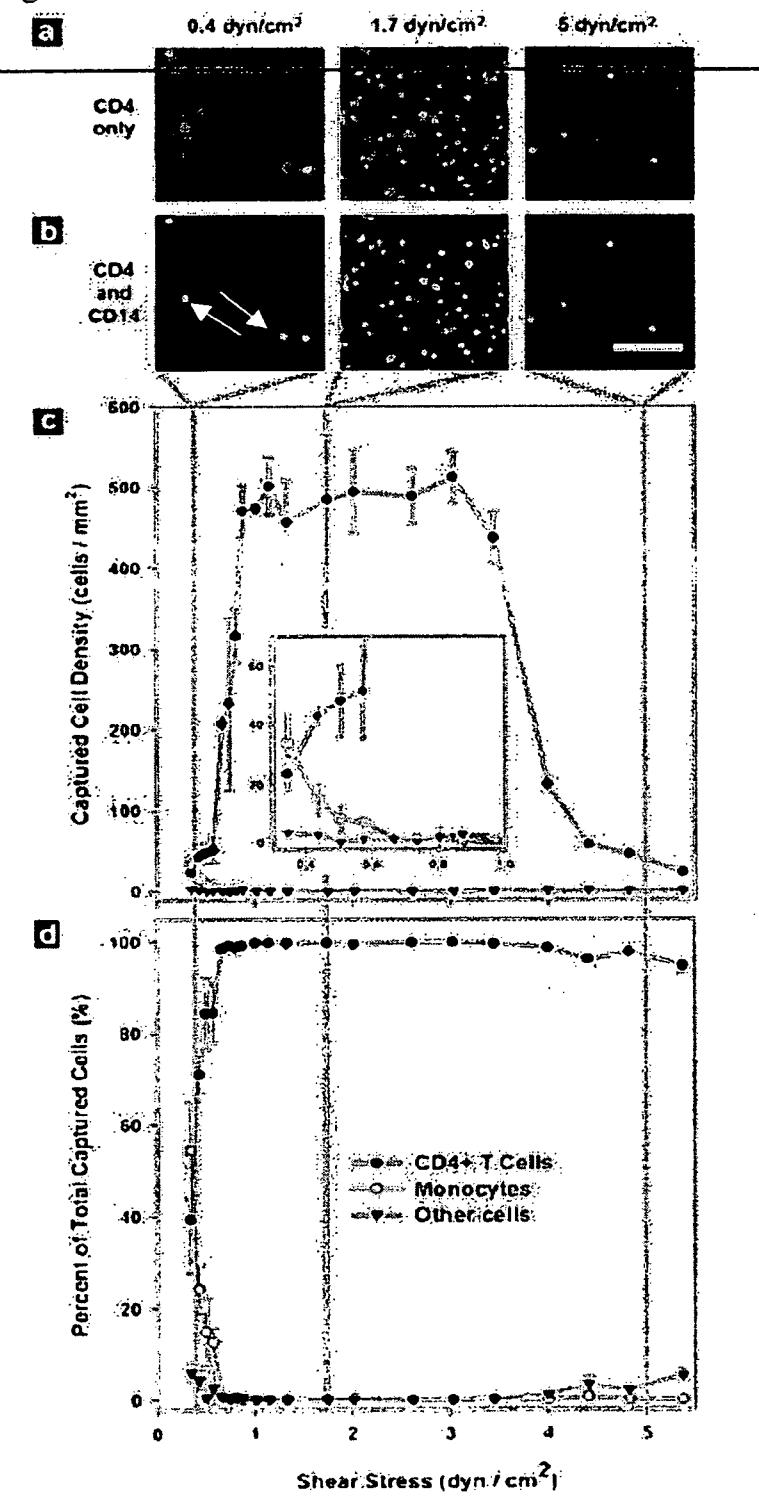
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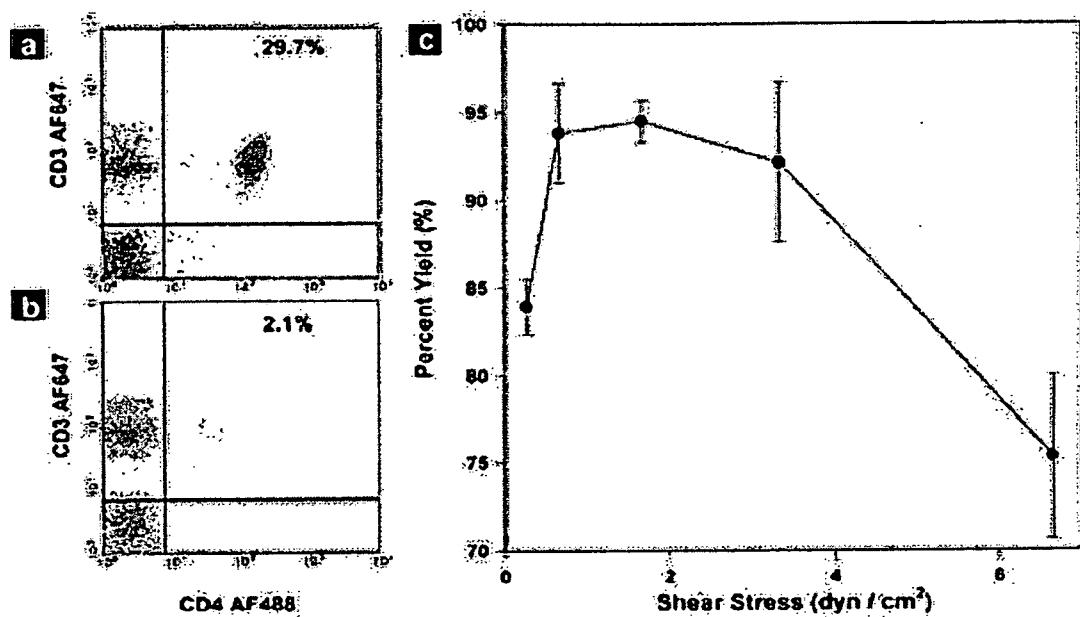
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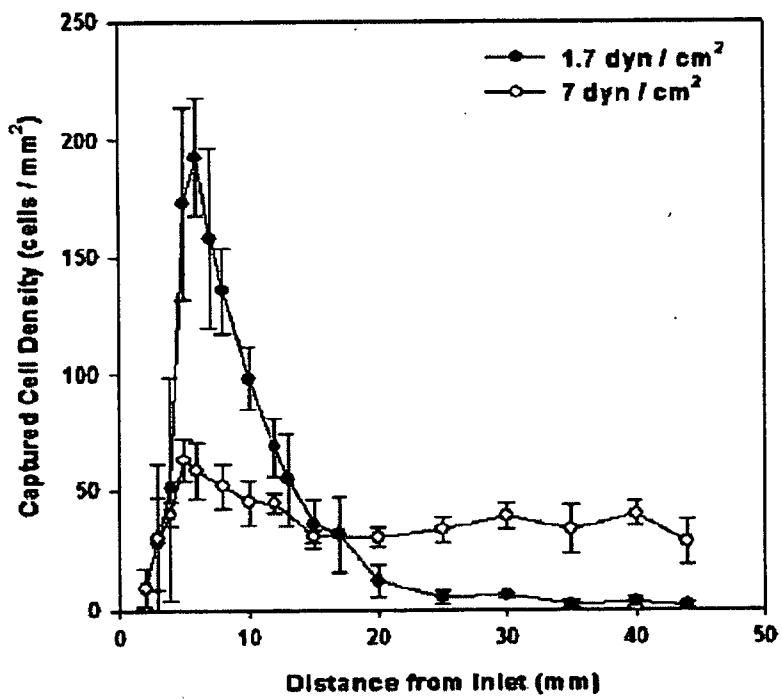
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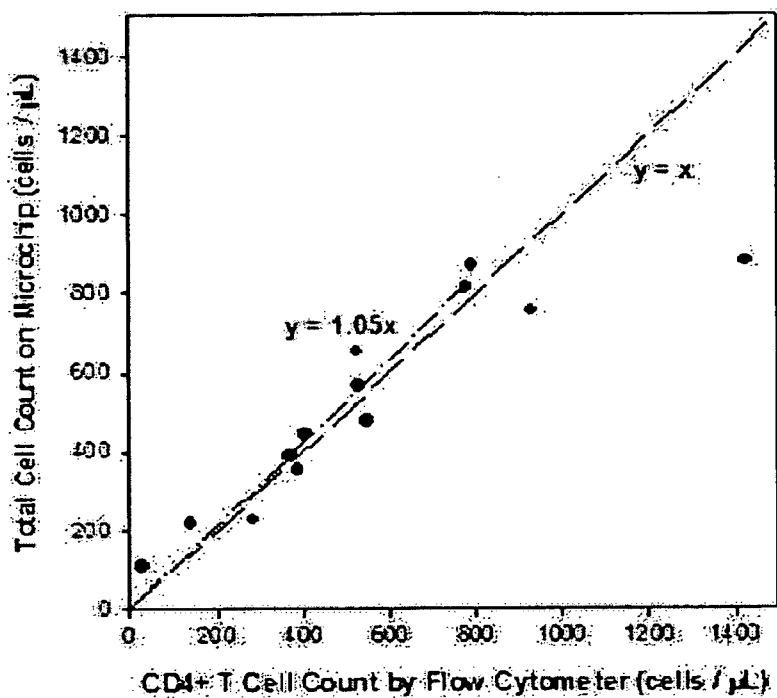
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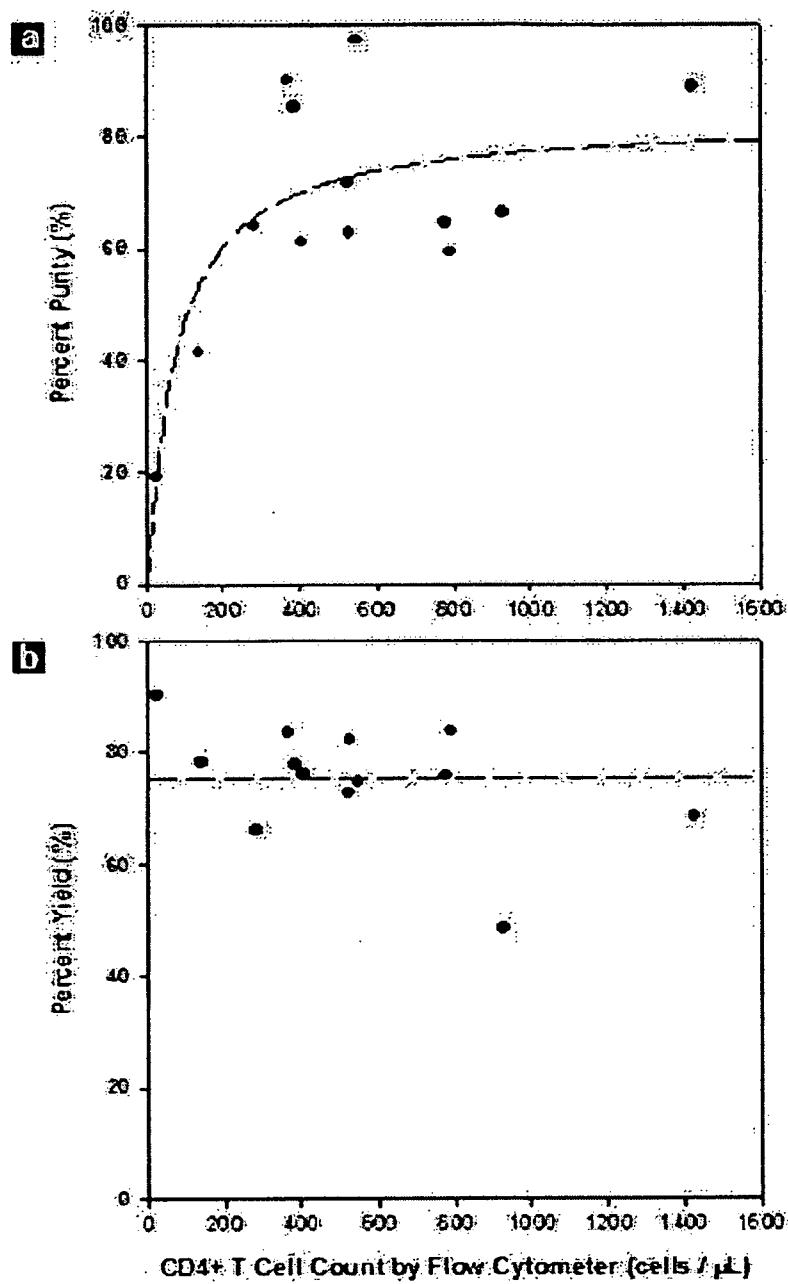
Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

pMHC Pentamer

NeutrAvidin

GMBS

3-Mercaptotrimethoxysilane

PDMS or Silica Substrate

Fig. 12

pMHC Pentamer
A2-SL9 Peptide
(Biotinylated)

Fig. 13

A

B
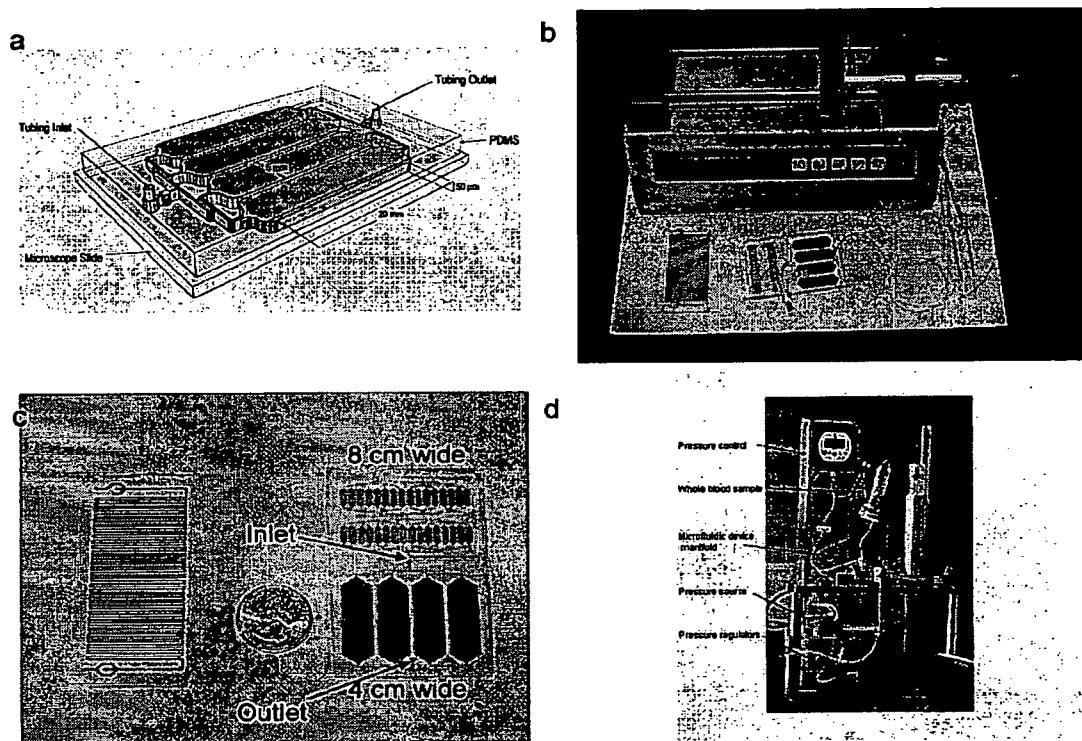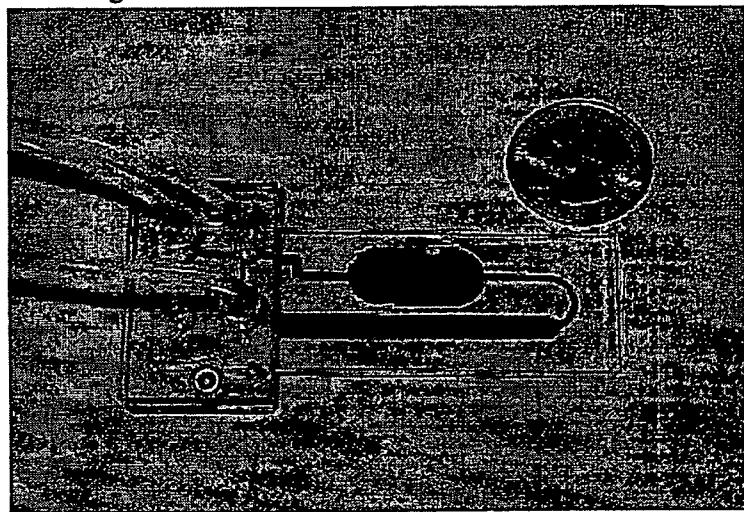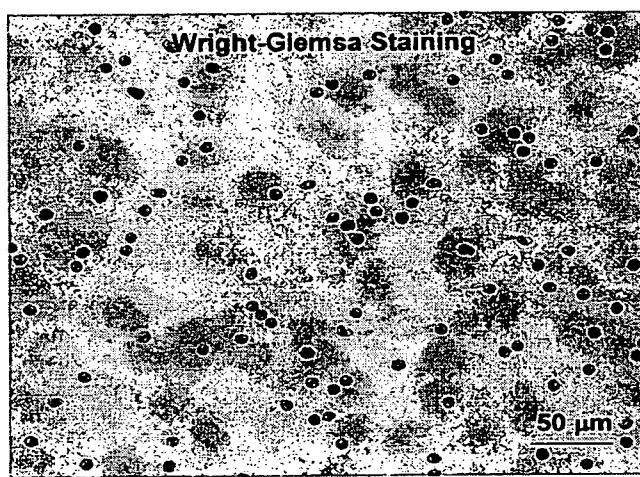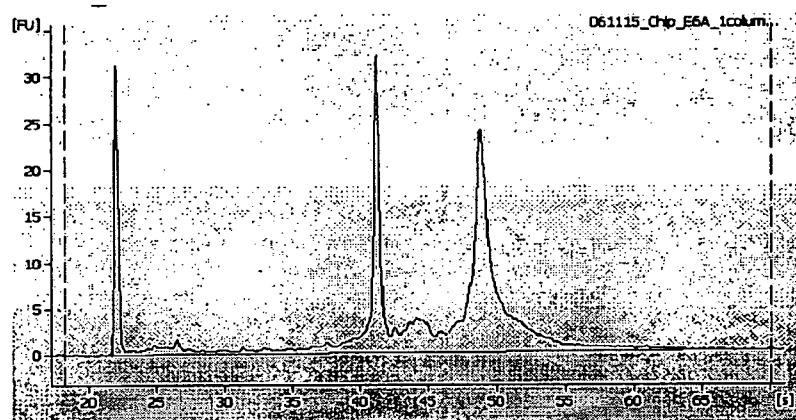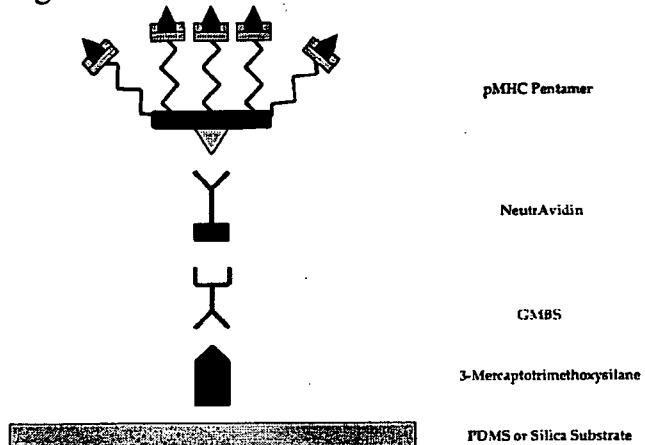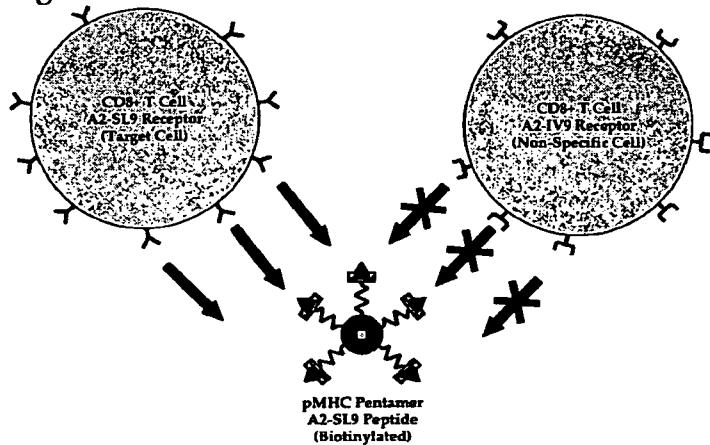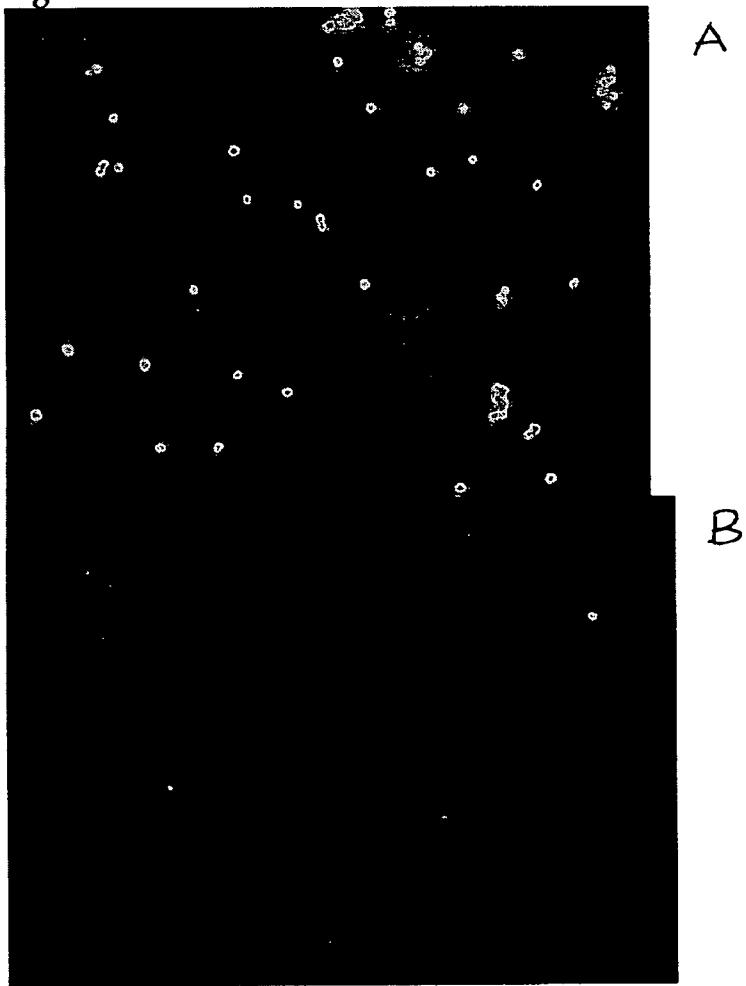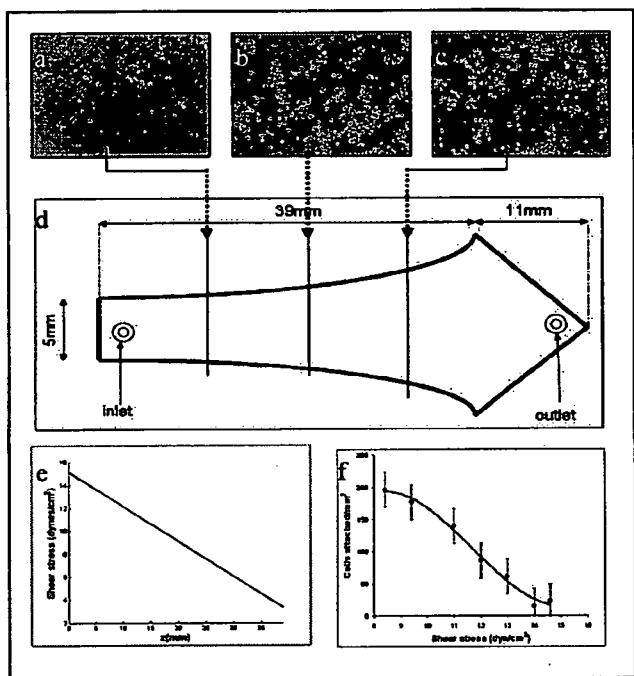
Fig. 14

专利名称(译)	用于检测细胞和其他分析物的装置和方法		
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申请(专利权)人(译)	总医院CORPORATION		
当前申请(专利权)人(译)	总医院CORPORATION		
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IPC分类号	G01N33/574 G01N33/53 B01L3/00 C12M1/00 C12M1/34 C12N5/00 C12Q1/68 G01N15/06 G01N33/543		
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其他公开文献	EP2002262A4 EP2002262B1		
外部链接	Espacenet		

摘要(译)

本发明的特征在于用于分离分析物（例如细胞）的方法，装置和试剂盒。将含有所需分析物的样品引入含有结合所需分析物的部分的微流体装置中。施加的剪切应力足以防止不需要的分析物的结合，并且足够低以允许目标分析物的结合。一旦结合，就可以分析（例如，计数）所需的分析物。本发明的特征还在于确定用于分离所需分析物的剪切应力的方法。