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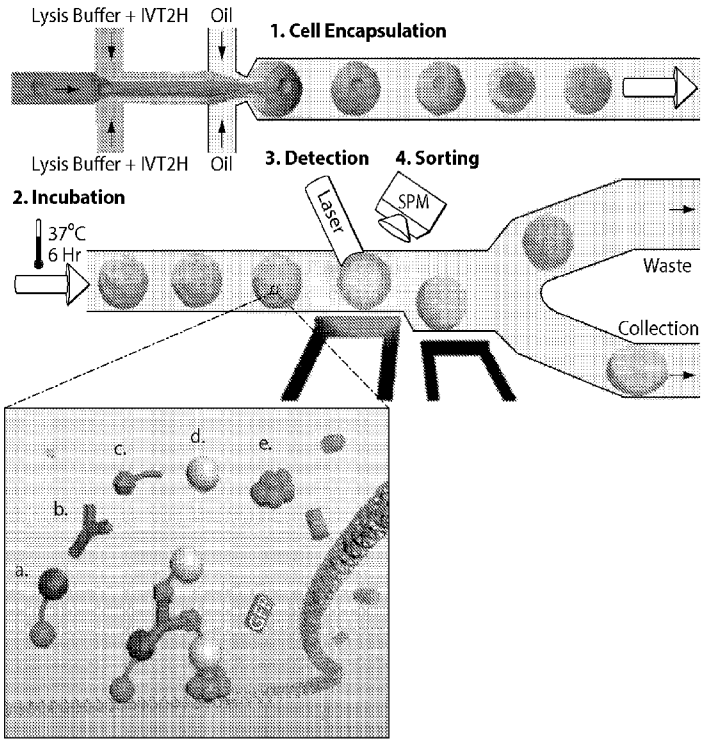

Fig. 1

(57) Abstract: The present invention generally relates to systems and methods for determining proteins such as antibodies, or other targets. In one aspect, fusion proteins are exposed to a target, such as an antibody, such that binding of the fusion proteins to the targets produces a complex that is able to produce a nucleic acid. The nucleic acid may be expressed to produce a protein. The protein may be fluorescent or otherwise determinable, such that determination of the protein may be used to determine or identify the target. In some cases, this may be performed within a microfluidic droplet. As an example, a single cell contained within a droplet may produce a target, and determination of the target within the droplet may then be associated with that particular cell.

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PROTEIN ANALYSIS ASSAY SYSTEM

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/008,341, filed June 5, 2014, entitled "Protein Analysis Assay System," by Weitz, *et al.*, incorporated herein by reference in its entirety.

GOVERNMENT FUNDING

This invention was made with government support under Grant No. HR0011-11-C-0093 awarded by the Defense Advanced Research Project Agency. The government has certain rights in the invention.

FIELD

The present invention generally relates to systems and methods for determining proteins such as antibodies, or other targets.

BACKGROUND

Cell behaviors in multicellular organisms can be assessed by studying cellular heterogeneity in genetic pattern (DNA), expression pattern (RNA) and protein profile at a single cell level. Within these three cellular components, proteins are main functional molecules and it is important to precisely quantify them and measure their activity. Characterizing the quantity and activity of proteins is important, for example, to understanding the mechanisms of cellular processes including those involved in disease progression, cell differentiation and fate, and discovery of novel therapeutics and vaccines. Cells are heterogeneous in nature and hence, population-averaged data can mask the underlying molecular mechanisms, therefore it is important to obtain data at the level of single cells. For example, one of the unanswered questions in cancer therapy has been why essentially identical cells respond differently to a drug. Single-cell level measurement of proteins has provided valuable insight into mechanisms that dictate heterogeneity in cellular response to drugs and other internal and external stimuli. As another example, in stem cell research, studying the different level of Sca-1 protein in individual cells revealed that the abundance of Sca-1 protein determines the timing and type of differentiation. Screening T Cells at single cell level revealed that they contain subpopulation with different cytokine profiles, and these differences may serve to predict patient immune response to therapeutics.

The most established single-cell protein analysis is flow cytometry, which mainly relies on the use of fluorescent conjugated antibodies. Except analyzing cell surface proteins, this technique requires pretreatments of cells. Pretreatments includes fixation and permeablization; this results in stressing cells prior analyzing proteins exist inside the cell. In addition, this technique lacks precision to measure and characterize secreted proteins. Moreover, it is not trivial to find antibody for all specific protein targets. Another important method to analyze proteins at single cell level is mass spectrometry (MS). This technique provides quantitative analysis of the entire proteome of a single cell. Although in MS there is no need to use molecular labels, due to the low signal to noise ratio, this technique is not used to detect small amounts of proteins typically found in single cells.

SUMMARY

The present invention generally relates to systems and methods for determining proteins such as antibodies, or other targets. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In one aspect, the present invention is generally directed to a method of determining a target antibody. In one set of embodiments, the method comprises acts of producing a first fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising a binding domain; producing a second fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising an activation domain; binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target antibody within a compartment having a volume of less than about 1 microliter; binding an RNA polymerase to the activation domain; binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein; and determining expression of the protein, thereby determining the target antibody.

The method, in another set of embodiments, includes acts of producing a first fusion protein within a microfluidic droplet, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising a binding domain; producing a second fusion protein within the microfluidic droplet, wherein the

first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising an activation domain; binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target antibody; binding an RNA polymerase to the activation domain; binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein; determining expression of the protein, thereby determining the target antibody; and sorting the microfluidic droplet based on the expression of the protein.

According to yet another set of embodiments, the method includes acts of producing a first fusion protein *in situ*; producing a second fusion protein *in situ*; binding the first fusion protein and the second fusion protein to the target and to an RNA polymerase to form a complex within a compartment having a volume of less than about 1 microliter; binding the complex to a nucleic acid to express a protein encoded by the nucleic acid; and determining expression of the protein, thereby determining the target.

In still another set of embodiments, the method includes acts of producing a first fusion protein *in situ*; producing a second fusion protein *in situ*; binding the first fusion protein and the second fusion protein to a target antibody and to an RNA polymerase to form a complex within a compartment having a volume of less than about 1 microliter; and binding the complex to a nucleic acid to express a protein encoded by the nucleic acid; and determining expression of the protein, thereby determining the target antibody.

The method, in another set of embodiments, includes acts of producing a first fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target and a second portion comprising a binding domain; producing a second fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target and a second portion comprising an activation domain; binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target within a compartment having a volume of less than about 1 microliter; binding an RNA polymerase to the activation domain; binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein; and determining expression of the protein, thereby determining the target.

In still another set of embodiments, the method comprises acts of producing a first fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising a binding domain; producing a second fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising an activation domain; binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target antibody; binding an RNA polymerase to the activation domain; binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein; and determining expression of the protein, thereby determining the target antibody. In some cases, one or more of these may occur within a compartment having a volume of less than about 1 microliter.

According to another set of embodiments, includes acts of producing a first fusion protein within a microfluidic droplet, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising a binding domain; producing a second fusion protein within the microfluidic droplet, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising an activation domain; binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target antibody; binding an RNA polymerase to the activation domain; binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein; determining expression of the protein, thereby determining the target antibody; and sorting the microfluidic droplet based on the expression of the protein. In some embodiments, one or more of these may occur within a compartment having a volume of less than about 1 microliter.

In yet another set of embodiments, the method includes acts of producing a first fusion protein *in situ*; producing a second fusion protein *in situ*; binding the first fusion protein and the second fusion protein to the target and to an RNA polymerase to form a complex; binding the complex to a nucleic acid to express a protein encoded by the nucleic acid; and determining expression of the protein, thereby determining the target. In some cases, one or more of these may occur within a compartment having a volume of less than about 1 microliter.

The method, in still another set of embodiments, comprises acts of producing a first fusion protein *in situ*; producing a second fusion protein *in situ*; binding the first fusion protein and the second fusion protein to a target antibody and to an RNA polymerase to form a complex; and binding the complex to a nucleic acid to express a protein encoded by the nucleic acid; and determining expression of the protein, thereby determining the target antibody. In certain embodiments, one or more of these may occur within a compartment having a volume of less than about 1 microliter.

The method, in yet another set of embodiments, includes acts of producing a first fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target and a second portion comprising a binding domain; producing a second fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target and a second portion comprising an activation domain; binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target; binding an RNA polymerase to the activation domain; binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein; and determining expression of the protein, thereby determining the target. In some embodiments, one or more of these may occur within a compartment having a volume of less than about 1 microliter.

In another aspect, the present invention encompasses methods of making one or more of the embodiments described herein. In still another aspect, the present invention encompasses methods of using one or more of the embodiments described herein.

Other advantages and novel features of the present invention will become apparent from the following detailed description of various non-limiting embodiments of the invention when considered in conjunction with the accompanying figures. In cases where the present specification and a document incorporated by reference include conflicting and/or inconsistent disclosure, the present specification shall control. If two or more documents incorporated by reference include conflicting and/or inconsistent disclosure with respect to each other, then the document having the later effective date shall control.

BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not

intended to be drawn to scale. In the figures, each identical or nearly identical component illustrated is typically represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. In the figures:

Fig. 1 illustrates determination of antibodies, according to one set of embodiments;

Figs. 2A-2B illustrate certain embodiments of the invention;

Figs. 3A-3D illustrate determination of an antibody according to one set of embodiments;

Figs. 4A-4F illustrate determination of kinase activity according to another set of embodiments;

Fig. 5 illustrates determination of anti-Myc in yet another set of embodiments;

Fig. 6 illustrates amplification products produced in one set of embodiments;

Fig. 7 illustrate an assay in accordance with another set of embodiments;

Figs. 8A-8C illustrate assays for detecting kinase activity, according to certain embodiments;

Fig. 9 illustrates cells undergoing apoptosis and necrosis during drug treatment, in still another set of embodiments; and

Fig. 10 illustrates a procedure for experimental steps in accordance with one embodiment of the invention.

DETAILED DESCRIPTION

The present invention generally relates to systems and methods for determining proteins such as antibodies, or other targets. In one aspect, fusion proteins are exposed to a target, such as an antibody, such that binding of the fusion proteins to the targets produces a complex that is able to produce a nucleic acid. The nucleic acid may be expressed to produce a protein. The protein may be fluorescent or otherwise determinable, such that determination of the protein may be used to determine or identify the target. In some cases, this may be performed within a microfluidic droplet. As an example, a single cell contained within a droplet may produce a target, and determination of the target within the droplet may then be associated with that particular cell.

One aspect of the present invention is generally directed to systems and methods of determining a target, such as a target antibody, a protein, or other species. One example embodiment is now described with respect to Fig. 2A. However, other configurations may be used as well. In Fig. 2A, a target 15 is to be determined. In this non-limiting example, target 15 is an antibody. However, it should be understood that this is by way of example only, and in other embodiments, other targets (e.g., kinases, enzymes, small molecules, etc.) may be used.

In this figure, target 15 may be determined by allowing it to interact with two fusion proteins 21 and 22 that, when assembled, are able to cause a nucleic acid to be produced or expressed, e.g., to produce a protein, such as a fluorescent protein, that is relatively easily determined. More than two fusion proteins may also be used in some instances. If target 15 is present, the fusion proteins can assemble into a complex that is able to cause expression of the protein to occur as discussed below. However, if target 15 is not present, then the fusion proteins are not able to sufficiently assemble or interact with each other to produce such a complex, and thus, no expression of the protein occurs. Accordingly, by determining expression of the protein, the presence of target 15 can similarly be determined. In addition, in some cases, this may be quantified, e.g., such that the concentration of protein can be used to determine the concentration of target 15.

In Fig. 2A, target 15 is an antibody having Fab ("antigen-binding") and Fc ("crystallizable") regions (11 and 13, respectively). Fusion protein 20 is able to bind or otherwise interact with region 11 of the target antibody, while fusion protein 30 is able to bind to or otherwise interact with region 13 of the target antibody. For example, fusion protein 20 may contain an antigen region 21, which Fab region 11 of the antibody can specifically bind. Similarly, fusion protein 30 may contain a Protein A/G region 31, which contains one or more Fc binding domains that can bind to Fc region 13 of the antibody. Thus, in this example, each of fusion proteins 20 and 30 are able to interact with target 15.

Fusion protein 20 may also have a second, activation domain ("AD") or region 22, as is shown in Fig. 2A. The activation domain may be selected to bind to (or recruit) a polymerase, such as RNA polymerase 40, as is discussed below. The activation domain need not be related to antigen 21, e.g., where both are part of fusion protein 20. Thus, for example, the regions may come from different chromosomes, different cells,

different organisms, or even different species, and fusion protein 20 may comprise each of these regions (and optionally, other regions as well). As an example, activation domain 22 may include a protein such as TFIIIB150, which is able to recruit RNA polymerase, or fusion protein 20 may include YEEI peptide, which includes an activation domain.

Similarly, fusion protein 30 may also have a second domain of binding ("DB") or region 32. This region may be selected to be able to bind to nucleic acid 50, e.g., as discussed below. For instance, nucleic acid 50 may be reported nucleic acid that can be used to produce a nucleic acid, such as RNA. In some cases, the RNA may also be expressed as a protein. In some cases, the protein may be fluorescent, and/or the protein may have other characteristics that allow for easy determination of the protein. The domain of binding 32 need not be related to Protein A/G region 31, e.g., where both are part of fusion protein 30. For instance, these regions may come from different chromosomes, different cells, different organisms, or even different species, and fusion protein 30 may comprise each of these regions (and optionally, other regions as well). As a non-limiting example, in one embodiment, domain of binding 32 may comprise a sequence able to recognize a UAS (upstream activating sequence) region 51, or other region of nucleic acid 50 that can be specifically recognized by domain 32. For instance, domain 32 may comprise zinc finger DNA binding proteins, Cro protein, GAL4 protein, DAP I or DAP II (derepression activating protein), or portions thereof, or fusion protein 30 may comprise a SH2 fusion protein which contains a domain of binding.

In some embodiments, as mentioned, activation domain 22 may be selected to bind to (or recruit) a polymerase, such as RNA polymerase 40. RNA polymerase 40 may be any suitable polymerase that can be recruited, e.g., RNA polymerase I, II, or III. Recruitment of RNA polymerase 40 to activation domain 22 may occur before, during, or after binding of antigen 21 to region 11 of target antibody 15. The polymerase may also interact with nucleic acid 50 in some fashion. For example, RNA polymerase 40 may bind to or recognize a promoter site 52 on nucleic acid 50.

Similarly, domain of binding 32 may interact with a suitable nucleic acid 50 before, during, or after binding of Protein A/G region 31 to region 13 of target antibody 15. In some cases, nucleic acid 50 may include a UAS or upstream activating sequence, or other sequence that can be specifically recognized by domain of binding 32.

The above-described interactions may occur in any suitable order, and in some cases, may result in the formation of a complex that is able to express at least a portion of nucleic acid 50. The presence or concentration of the target may affect the expression of at least a portion of nucleic acid 50, e.g., to produce a protein, which can be determined to determine the target. For instance, as is shown in Fig. 2B, RNA polymerase 40 is able to interact with nucleic acid 50 to produce RNA 45. The RNA may be directly detected, and/or the RNA may be subsequently expressed, e.g., to produce a protein 60 that can be determined. For instance, the protein may be a fluorescent protein, such as GFP (green fluorescent protein).

In certain cases, the above-described system may include components able to cause *in situ* expression of proteins, e.g., where nucleic acids can be expressed to form proteins *in situ* or *in vitro*, rather than inside of living cells. Techniques for *in vitro* expression of proteins from nucleic acids are known, e.g., by using suitable transcription and translation proteins and other components *in vitro*. Kits for producing such *in vitro* expression of proteins are available commercially. In some cases, for example, nucleic acids encoding fusion proteins 30 and/or 40 may initially be present, such that fusion proteins 30 and 40 are produced *in vitro* in such an environment. Fusion proteins 30 and 40 can then react or proceed as discussed above. In some cases, RNA 45 may also be produced as discussed above, and then expressed *in vitro* into a protein 60. In some cases, the same molecular "machinery" may be involved in producing both fusion protein 20, fusion protein 30, and/or expressed protein 60. Once expressed, protein 60 can be detected using any suitable technique. In one set of embodiments, protein 60 is fluorescent. Thus, by determining fluorescence in a sample, the presence or concentration of a target in the sample can be determined.

In some cases, target antibody 15 (or another suitable target) may be produced by a cell. Thus, in one set of embodiments, systems such as those described herein may be used to identify or determine (e.g., qualitatively or quantitatively) cells that are able to produce the target. The cells may be intact or the cells may be lysed prior to determining the target. For instance, the target may be a target that is expressed or secreted by the cells, and/or the target may be contained within the cell, and the cell may be lysed to determine the target. Lysing of the cell may be performed using any suitable technique,

such as exposure to a suitable detergent (SDS, Triton X-100, etc.), or exposure to ultrasound or relatively high temperatures.

In one set of embodiments, the cells may be contained within one or more droplets or other compartments, such as those discussed below. For example, in one set of embodiments, a plurality of cells may be contained within a plurality of droplets. In some cases, the cell/droplet density may be low, e.g., there may be an average of one cell per droplet, or even less in some cases, such that the characteristics of each droplet is affected, at most, by one cell (or is not affected if no cell is present). The cells may secrete or otherwise produce a target. However, the target may remain contained within the droplet, whether the target leaves the cell or does not leave the cell. The target can then be determined in some fashion within the droplet, e.g., assayed as described herein. For example, the droplets may exhibit fluorescence if a certain target (such as a certain antibody) is present. Such droplets may be determined and sorted from droplets not exhibiting suitable fluorescence using techniques such as those described herein, or by techniques known to those of ordinary skill in the art. See, e.g., Fig. 1A, part 2, illustrating cells that are determined, e.g., for fluorescence, then sorted into a collection channel if the cells are sufficiently fluorescent or into a waste channel if the cells are not sufficiently fluorescent.

The above discussion is a non-limiting example of certain embodiments of the present invention. However, this is by way of explanation only, and other embodiments are also possible. Accordingly, more generally, various aspects of the invention are directed to various systems and methods for systems and methods for determining proteins, antibodies, or other targets.

For instance, it should be understood that a variety of targets may be determined, not only antibodies. More generally, any target to which two binding molecules, such as fusion proteins, are able to recognize, can be used. For example, the target may be a protein (e.g. an antibody), an enzyme (e.g., a kinase), a nucleic acid, a small molecule (e.g., less than about 1000 Da), or the like. In certain embodiments, fusion proteins or other binding molecules may include antibody portions or fragments able to specifically bind to such targets. Those of ordinary skill in the art will be familiar with antibodies and techniques for raising antibodies able to specifically bind a specific target (i.e., an antigen to the antibody). Thus, for example, one or more fusion proteins may comprise

an antibody or antibody fragment that can specifically bind a target. Systems and methods for producing antibodies are known to those of ordinary skill in the art, and are discussed in more detail herein.

In some embodiments, the target is one that is produced by a cell. However, it should be understood that the target need not be a target arising from a cell. In other cases, the target may be synthetically produced, or acquired from other sources. For instance, the target may arise from a natural source, such as water or soil, or the target may arise from a library of molecules. In some cases, the target may be encapsulated into droplets or other compartments as discussed herein (e.g., wells of a microwell plate, individual tubes or containers), using techniques known to those of ordinary skill in the art.

If the target is produced by a cell, the cell may be, for example, an isolated or single cell, a cell aggregate, a cell from a cell culture, from a tissue construct containing cells, or the like. Examples of cells include, but are not limited to, a bacterium or other single-cell organism, a eukaryotic cell, a plant cell, or an animal cell. If the cell is an animal cell, the cell may be, for example, an invertebrate cell (e.g., a cell from a fruit fly), a fish cell (e.g., a zebrafish cell), an amphibian cell (e.g., a frog cell), a reptile cell, a bird cell, or a human or non-human mammal, such as a monkey, ape, cow, sheep, goat, rabbit, pig, mouse, rat, guinea pig, hamster, dog, cat, etc. The cells may also be, in some embodiments, from a specific population of cells, such as from a certain organ or tissue (e.g., cardiac cells, immune cells, muscle cells, cancer cells, etc.), cells from a specific individual or species (e.g., human cells, mouse cells, bacteria, etc.), cells from different organisms, cells from a naturally-occurring sample (e.g., pond water, soil, etc.), or the like. In some cases, a target is secreted by the cell. In other cases, the target is not secreted by the cell, e.g., the target is contained internally of the cell.

In some cases, one, two, or more fusion proteins are used. The fusion proteins may include a first portion that is able to recognize the target, and a second portion that is able to participate in the production of a protein or a nucleic acid, e.g., from a reporter nucleic acid as discussed below. In some cases, other portions may also be present in a fusion protein as well. In one set of embodiments, a fusion protein may include a portion that is able to participate in a complex able to produce a nucleic acid or express a protein, and/or the portion may be able to recruit a component that is able to participate in a

complex able to produce a nucleic acid or express a protein. If more than one fusion protein is present, such portions may independently be the same or different. In an assay, the fusion proteins may be introduced as proteins, and/or introduced as nucleic acids which can be expressed (e.g., *in vitro*) to produce such proteins, using *in vitro* or *in situ* techniques known to those of ordinary skill in the art.

A fusion protein may include regions such as activation domains or domains of binding such as those described herein. In some cases, a region may be able to specifically bind a nucleic acid, or a specific region of the nucleic acid, such as a UAS (upstream activating sequence), an operon sequence, a promoter sequence, etc. As another example, a region may include a region able to recruit a component such as a polymerase. Specific examples include, but are not limited to, Protein A and/or Protein G, TFIIIB150, YEEI peptide, GAL4, DAP I, DAP II, SH2, Cro protein, zinc finger DNA binding proteins or the like.

If a polymerase is present, then the polymerase may be any suitable polymerase. For example, the polymerase may be a DNA polymerase (which produces DNA) or an RNA polymerase (which produces RNA). The polymerase may arise from any suitable organism (e.g., human, *E. coli*, etc.). One or more than one polymerase may be present. In some cases, the polymerase may be obtained commercially. Examples of DNA polymerases include, but are not limited to, DNA Polymerases beta, lambda, sigma, mu, alpha, delta, epsilon, I, II, III, IV, eta, iota, kappa, Rev1, zeta, gamma, theta, etc. Non-limiting examples of RNA polymerases include RNA polymerase I, II, III, IV, V, bacterial RNA polymerase, etc.

If the target is present, a complex can be formed that is able to produce or express a nucleic acid, e.g., using a reporter nucleic acid. The nucleic acid may include DNA and/or RNA. In some cases, the complex may produce DNA or RNA from the reporter nucleic acid (which, in some cases, may then be expressed as a protein), or the complex may itself be able to express the nucleic acid to produce a protein. However, if the target is not present (or if the target is not present in a sufficient amount or quantity), then the complex is not formed (or is formed, but not in a sufficient amount or quantity), and thus, little or no production or expression of the nucleic acid occurs.

In one set of embodiments, the nucleic acid encodes a protein that can be expressed, e.g., directly or indirectly. For example, the protein may be a fluorescent

protein, or the protein may be an enzyme. In one set of embodiments, for instance, the protein is an inherently fluorescent protein, such as GFP, or other similar proteins (e.g., blue fluorescent protein (EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein (ECFP, Cerulean, CyPet), yellow fluorescent protein (YFP, Citrine, Venus, YPet), etc. In another set of embodiments, the protein is an enzyme, such as horseradish peroxidase, whose activity can be readily determined, e.g., using spectrophotometric methods. In yet another set of embodiments, the protein is one that exhibits specific binding that may be assayable using other systems (e.g., streptavidin in a biotin-streptavidin assay).

The nucleic acid may also contain other regions, e.g., to facilitate binding by the complex, and/or production or expression. For example, in one set of embodiments, the nucleic acid may contain one or more promoter regions, operator regions, transcription start sites, upstream activating sequences, TATA elements, or the like. For instance, the nucleic acid may include one or more binding sites for RNA polymerase.

In one set of embodiments, RNA polymerase interacts with the nucleic acid to produce RNA. The RNA can itself be determined, and or the RNA may be expressed as protein, which is then determined, e.g., enzymatically or fluorescently, etc. In some cases, RNA expression may be occur *in vitro* or *in situ*, e.g., using *in situ* expression techniques. Many such kits may be readily obtained commercially. In some cases, the same systems may also be used to produce fusion proteins, for example, as discussed above.

In addition, as mentioned, in some cases, such reactions may occur in discrete compartments. In some cases, the compartments are isolated from each other, such that target remains within the compartments and cannot move into adjacent compartments or diffuse away from the compartment. In one embodiment, the compartments are droplets. Systems and methods of manipulating droplets, e.g., for sorting purposes, are discussed in more detail below. In another embodiment, the compartments may be wells of a microwell plate (e.g., a 96-well, a 384-well, a 1536-well, a 3456-well microwell plate, etc.). In yet other embodiments, the compartments may be individual tubes or containers, test tubes, microfuge tubes, glass vials, bottles, petri dishes, or the like. In some cases, the compartments may have relatively small volumes (e.g., less than about 1 microliter, less than about 300 nl, less than about 100 nl, less than about 30 nl, less than about 10 nl, less than about 3 nl, less than about 1 nl, etc.), such that the target may be

present at a relatively high concentration within the compartment. In some cases, the compartments may be individually accessible.

In addition, as discussed, in some embodiments, the compartments may contain one or more cells. For example, the cells may produce a target that is to be assayed. In some embodiments, the density of cells/compartment may be kept relatively low, e.g., to prevent or at least reduce the possibility of multiple cells being present in a compartment, which may confound analysis. Thus, for example, the density of cells may be about 1 cell/compartment, or less than about 1 cell/compartment, less than about 0.9 cells/compartment, less than about 0.8 cells/compartment, less than about 0.7 cells/compartment, less than about 0.6 cells/compartment, less than about 0.5 cells/compartment, less than about 0.4 cells/compartment, less than about 0.3 cells/compartment, less than about 0.2 cells/compartment, less than about 0.1 cells/compartment, less than about 0.05 cells/compartment, less than about 0.01 cells/compartment, etc.

In some cases, the compartments may be analyzed, e.g., to determine a target. For example, compartments containing a target (or a suitable concentration of target) may be identified or distinguished from other compartments that do not. Thus, for example, if a target results in the expression of a fluorescent protein, then compartments that are fluorescent (or sufficiently fluorescent) may be identified or distinguished from other compartments. In addition, in some cases, sorting of compartments may occur. Thus, for example, a first group of compartments may be identified for subsequent processing or analysis, while a second group of compartments is not. For instance, if the compartments are droplets, then a first group of droplets may be retained for subsequent processing or analysis, while a second group of droplets is sent to waste (or, in some cases, retained for different processing or analysis, etc.). Systems and methods for manipulating droplets in such fashion are discussed in further detail below.

For example, as mentioned, various aspects of the invention relates to systems and methods for producing droplets of fluid surrounded by a liquid. Any technique may be used to make a droplet, including those described herein. The fluid and the liquid may be essentially immiscible in many cases, i.e., immiscible on a time scale of interest (e.g., the time it takes a fluidic droplet to be transported through a particular system or device). In certain cases, the droplets may each be substantially the same shape or size,

as described herein. The fluid may also contain other species, for example, certain molecular species (e.g., as discussed herein), cells, particles, etc.

In one set of embodiments, for example, electric charge may be created on a fluid surrounded by a liquid, which may cause the fluid to separate into individual droplets within the liquid. In some embodiments, the fluid and the liquid may be present in a channel, e.g., a microfluidic channel, or other constricted space that facilitates application of an electric field to the fluid (which may be "AC" or alternating current, "DC" or direct current etc.), for example, by limiting movement of the fluid with respect to the liquid. Thus, the fluid can be present as a series of individual charged and/or electrically inducible droplets within the liquid. In one embodiment, the electric force exerted on the fluidic droplet may be large enough to cause the droplet to move within the liquid. In some cases, the electric force exerted on the fluidic droplet may be used to direct a desired motion of the droplet within the liquid, for example, to or within a channel or a microfluidic channel (e.g., as further described herein), etc.

Electric charge may be created in the fluid within the liquid using any suitable technique, for example, by placing the fluid within an electric field (which may be AC, DC, etc.), and/or causing a reaction to occur that causes the fluid to have an electric charge, for example, a chemical reaction, an ionic reaction, a photocatalyzed reaction, etc. In one embodiment, the fluid is an electrical conductor. As used herein, a "conductor" is a material having a conductivity of at least about the conductivity of 18 megohm (MOhm or MΩ) water. The liquid surrounding the fluid may have a conductivity less than that of the fluid. For instance, the liquid may be an insulator, relative to the fluid, or at least a "leaky insulator," i.e., the liquid is able to at least partially electrically insulate the fluid for at least a short period of time. Those of ordinary skill in the art will be able to identify the conductivity of fluids. In one non-limiting embodiment, the fluid may be substantially hydrophilic, and the liquid surrounding the fluid may be substantially hydrophobic.

The electric field, in some embodiments, is generated from an electric field generator, i.e., a device or system able to create an electric field that can be applied to the fluid. The electric field generator may produce an AC field (i.e., one that varies periodically with respect to time, for example, sinusoidally, sawtooth, square, etc.), a DC field (i.e., one that is constant with respect to time), a pulsed field, etc. The electric field

generator may be constructed and arranged to create an electric field within a fluid contained within a channel or a microfluidic channel. The electric field generator may be integral to or separate from the fluidic system containing the channel or microfluidic channel, according to some embodiments. As used herein, "integral" means that portions of the components integral to each other are joined in such a way that the components cannot be manually separated from each other without cutting or breaking at least one of the components.

In another set of embodiments, droplets of fluid can be created from a fluid surrounded by a liquid within a channel by altering the channel dimensions in a manner that is able to induce the fluid to form individual droplets. The channel may, for example, be a channel that expands relative to the direction of flow, e.g., such that the fluid does not adhere to the channel walls and forms individual droplets instead, or a channel that narrows relative to the direction of flow, e.g., such that the fluid is forced to coalesce into individual droplets. In other embodiments, internal obstructions may also be used to cause droplet formation to occur. For instance, baffles, ridges, posts, or the like may be used to disrupt liquid flow in a manner that causes the fluid to coalesce into fluidic droplets.

In some cases, the channel dimensions may be altered with respect to time (for example, mechanically or electromechanically, pneumatically, etc.) in such a manner as to cause the formation of individual fluidic droplets to occur. For example, the channel may be mechanically contracted ("squeezed") to cause droplet formation, or a fluid stream may be mechanically disrupted to cause droplet formation, for example, through the use of moving baffles, rotating blades, or the like. Other examples of methods for creating droplets include those disclosed in Int. Pat. Apl. No. PCT/US2003/020542, filed June 30, 2003, entitled "Method and Apparatus for Fluid Dispersion," by Stone, *et al.*, published as WO 2004/002627 on January 8, 2004.

In some instances, the droplets may be created at relatively high rates. For instance, at least about 1 droplet per second may be created in some cases, and in other cases, at least about 10 droplets per second, at least about 20 droplets per second, at least about 30 droplets per second, at least about 100 droplets per second, at least about 200 droplets per second, at least about 300 droplets per second, at least about 500 droplets per second, at least about 750 droplets per second, at least about 1000 droplets per

second, at least about 1500 droplets per second, at least about 2000 droplets per second, at least about 3000 droplets per second, at least about 5000 droplets per second, at least about 7500 droplets per second, at least about 10,000 droplets per second, at least about 15,000 droplets per second, at least about 20,000 droplets per second, at least about 30,000 droplets per second, at least about 50,000 droplets per second, at least about 75,000 droplets per second, at least about 100,000 droplets per second, at least about 150,000 droplets per second, at least about 200,000 droplets per second, at least about 300,000 droplets per second, at least about 500,000 droplets per second, at least about 750,000 droplets per second, at least about 1,000,000 droplets per second, at least about 1,500,000 droplets per second, at least about 2,000,000 or more droplets per second, or at least about 3,000,000 or more droplets per second may be created.

Other examples of the production of droplets of fluid surrounded by a liquid are described in International Patent Application Serial No. PCT/US2004/010903, filed April 9, 2004 by Link, *et al.*, and International Patent Application Serial No. PCT/US03/20542, filed June 30, 2003 by Stone, *et al.*, published as WO 2004/002627 on January 8, 2004, each incorporated herein by reference.

In some embodiments, a species (for example, a cell) may be contained within the droplet, e.g., before or after formation. In some cases, more than one species may be present. Thus, for example, a precise quantity of a drug, pharmaceutical, or other agent can be contained within a droplet, e.g., in addition to a cell. For example, the species may be drug or other species that is suspected of being able to affect the interaction between an effector cell and a target cell within a droplet. Other species that can be contained within a droplet include, for example, biochemical species such as nucleic acids such as siRNA, mRNA, RNAi and DNA, proteins, peptides, or enzymes, or the like. Additional species that can be contained within a droplet include, but are not limited to, nanoparticles, quantum dots, proteins, indicators, dyes, fluorescent species, chemicals, amphiphilic compounds, detergents, drugs, or the like. Further examples of species that can be contained within a droplet include, but are not limited to, growth regulators, vitamins, hormones, or microbicides.

In certain instances, the invention provides for the production of droplets consisting essentially of a substantially uniform number of entities of a species therein (i.e., molecules, cells, particles, etc.). For example, about 90%, about 93%, about 95%,

about 97%, about 98%, or about 99%, or more of a plurality or series of droplets may each contain the same number of entities of a particular species. For instance, a substantial number of fluidic droplets produced, e.g., as described above, may each contain 1 entity, 2 entities, 3 entities, 4 entities, 5 entities, 7 entities, 10 entities, 15 entities, 20 entities, 25 entities, 30 entities, 40 entities, 50 entities, 60 entities, 70 entities, 80 entities, 90 entities, 100 entities, etc., where the entities are molecules or macromolecules, cells, particles, etc. In some cases, the droplets may each independently contain a range of entities, for example, less than 20 entities, less than 15 entities, less than 10 entities, less than 7 entities, less than 5 entities, or less than 3 entities in some cases.

As discussed, in some aspects, fluidic droplets may be screened and/or sorted, and in some cases, at relatively high rates. For example, a characteristic of a droplet may be sensed and/or determined in some fashion (e.g., as herein described), then the droplet may be directed towards a particular region of the device, for example, for sorting or screening purposes. For example, the fluidic droplets may be sorted into two or more than two channels, e.g., based on interaction of the cells within the droplets. In some embodiments, a characteristic of a fluidic droplet may be sensed and/or determined in some fashion, for example, as described herein (e.g., fluorescence of the fluidic droplet may be determined), and, in response, an electric field may be applied or removed from the fluidic droplet to direct the fluidic droplet to a particular region (e.g. a channel). Other techniques for sensing cells and/or for sorting cells that are known to those of ordinary skill in the art may also be used, in some embodiments of the invention.

In some cases, high sorting speeds may be achievable using certain systems and methods of the invention. For instance, at least about 1 droplet per second may be determined and/or sorted in some cases, and in other cases, at least about 10 droplets per second, at least about 20 droplets per second, at least about 30 droplets per second, at least about 100 droplets per second, at least about 200 droplets per second, at least about 300 droplets per second, at least about 500 droplets per second, at least about 750 droplets per second, at least about 1000 droplets per second, at least about 1500 droplets per second, at least about 2000 droplets per second, at least about 3000 droplets per second, at least about 5000 droplets per second, at least about 7500 droplets per second, at least about 10,000 droplets per second, at least about 15,000 droplets per second, at

least about 20,000 droplets per second, at least about 30,000 droplets per second, at least about 50,000 droplets per second, at least about 75,000 droplets per second, at least about 100,000 droplets per second, at least about 150,000 droplets per second, at least about 200,000 droplets per second, at least about 300,000 droplets per second, at least about 500,000 droplets per second, at least about 750,000 droplets per second, at least about 1,000,000 droplets per second, at least about 1,500,000 droplets per second, at least about 2,000,000 or more droplets per second, or at least about 3,000,000 or more droplets per second may be determined and/or sorted in such a fashion.

In one set of embodiments, a fluidic droplet may be directed by creating an electric charge (e.g., as previously described) on the droplet, and steering the droplet using an applied electric field, which may be an AC field, a DC field, etc. As an example, an electric field may be selectively applied and removed (or a different electric field may be applied, e.g., a reversed electric field) as needed to direct the fluidic droplet to a particular region. The electric field may be selectively applied and removed as needed, in some embodiments, without substantially altering the flow of the liquid containing the fluidic droplet. For example, a liquid may flow on a substantially steady-state basis (i.e., the average flowrate of the liquid containing the fluidic droplet deviates by less than 20% or less than 15% of the steady-state flow or the expected value of the flow of liquid with respect to time, and in some cases, the average flowrate may deviate less than 10% or less than 5%) or other predetermined basis through a fluidic system of the invention (e.g., through a channel or a microchannel), and fluidic droplets contained within the liquid may be directed to various regions, e.g., using an electric field, without substantially altering the flow of the liquid through the fluidic system.

In another set of embodiments, a fluidic droplet may be sorted or steered by inducing a dipole in the fluidic droplet (which may be initially charged or uncharged), and sorting or steering the droplet using an applied electric field. The electric field may be an AC field, a DC field, etc.

In other embodiments, however, the fluidic droplets may be screened or sorted within a fluidic system of the invention by altering the flow of the liquid containing the droplets. For instance, in one set of embodiments, a fluidic droplet may be steered or sorted by directing the liquid surrounding the fluidic droplet into a first channel, a second channel, etc.

In another set of embodiments, pressure within a fluidic system, for example, within different channels or within different portions of a channel, can be controlled to direct the flow of fluidic droplets. For example, a droplet can be directed toward a channel junction including multiple options for further direction of flow (e.g., directed toward a branch, or fork, in a channel defining optional downstream flow channels). Pressure within one or more of the optional downstream flow channels can be controlled to direct the droplet selectively into one of the channels, and changes in pressure can be effected on the order of the time required for successive droplets to reach the junction, such that the downstream flow path of each successive droplet can be independently controlled. In one arrangement, the expansion and/or contraction of liquid reservoirs may be used to steer or sort a fluidic droplet into a channel, e.g., by causing directed movement of the liquid containing the fluidic droplet. The liquid reservoirs may be positioned such that, when activated, the movement of liquid caused by the activated reservoirs causes the liquid to flow in a preferred direction, carrying the fluidic droplet in that preferred direction. For instance, the expansion of a liquid reservoir may cause a flow of liquid towards the reservoir, while the contraction of a liquid reservoir may cause a flow of liquid away from the reservoir. In some cases, the expansion and/or contraction of the liquid reservoir may be combined with other flow-controlling devices and methods, e.g., as described herein. Non-limiting examples of devices able to cause the expansion and/or contraction of a liquid reservoir include pistons and piezoelectric components. In some cases, piezoelectric components may be particularly useful due to their relatively rapid response times, e.g., in response to an electrical signal.

In certain aspects of the invention, sensors are provided that can sense and/or determine one or more characteristics of the fluidic droplets, and/or a characteristic of a portion of the fluidic system containing the fluidic droplet (e.g., the liquid surrounding the fluidic droplet) in such a manner as to allow the determination of one or more characteristics of the fluidic droplets. Characteristics determinable with respect to the droplet and usable in the invention can be identified by those of ordinary skill in the art. Non-limiting examples of such characteristics include fluorescence, spectroscopy (e.g., optical, infrared, ultraviolet, etc.), radioactivity, mass, volume, density, temperature, viscosity, pH, concentration of a substance, such as a biological substance (e.g., a protein, a nucleic acid, etc.), or the like.

In some cases, the sensor may be connected to a processor, which in turn, cause an operation to be performed on the fluidic droplet, for example, by sorting the droplet, adding or removing electric charge from the droplet, fusing the droplet with another droplet, etc. One or more sensors and/or processors may be positioned to be in sensing communication with the fluidic droplet. "Sensing communication," as used herein, means that the sensor may be positioned anywhere such that the fluidic droplet within the fluidic system (e.g., within a channel), and/or a portion of the fluidic system containing the fluidic droplet may be sensed and/or determined in some fashion. For example, the sensor may be in sensing communication with the fluidic droplet and/or the portion of the fluidic system containing the fluidic droplet fluidly, optically or visually, thermally, pneumatically, electronically, or the like. The sensor can be positioned proximate the fluidic system, for example, embedded within or integrally connected to a wall of a channel, or positioned separately from the fluidic system but with physical, electrical, and/or optical communication with the fluidic system so as to be able to sense and/or determine the fluidic droplet and/or a portion of the fluidic system containing the fluidic droplet (e.g., a channel or a microchannel, a liquid containing the fluidic droplet, etc.). For example, a sensor may be free of any physical connection with a channel containing a droplet, but may be positioned so as to detect electromagnetic radiation arising from the droplet or the fluidic system, such as infrared, ultraviolet, or visible light. The electromagnetic radiation may be produced by the droplet, and/or may arise from other portions of the fluidic system (or externally of the fluidic system) and interact with the fluidic droplet and/or the portion of the fluidic system containing the fluidic droplet in such as a manner as to indicate one or more characteristics of the fluidic droplet, for example, through absorption, reflection, diffraction, refraction, fluorescence, phosphorescence, changes in polarity, phase changes, changes with respect to time, etc. As an example, a laser may be directed towards the fluidic droplet and/or the liquid surrounding the fluidic droplet, and the fluorescence of the fluidic droplet and/or the surrounding liquid may be determined. "Sensing communication," as used herein may also be direct or indirect. As an example, light from the fluidic droplet may be directed to a sensor, or directed first through a fiber optic system, a waveguide, etc., before being directed to a sensor.

Non-limiting examples of sensors useful in the invention include optical or electromagnetically-based systems. For example, the sensor may be a fluorescence sensor (e.g., stimulated by a laser), a microscopy system (which may include a camera or other recording device), or the like. As another example, the sensor may be an electronic sensor, e.g., a sensor able to determine an electric field or other electrical characteristic. For example, the sensor may detect capacitance, inductance, etc., of a fluidic droplet and/or the portion of the fluidic system containing the fluidic droplet.

As used herein, a "processor" or a "microprocessor" is any component or device able to receive a signal from one or more sensors, store the signal, and/or direct one or more responses (e.g., as described above), for example, by using a mathematical formula or an electronic or computational circuit. The signal may be any suitable signal indicative of the environmental factor determined by the sensor, for example a pneumatic signal, an electronic signal, an optical signal, a mechanical signal, etc.

As a particular non-limiting example, a device of the invention may contain fluidic droplets containing one or more cells. The cells may be exposed to a signaling entity, such as a fluorescent signal marker that binds if a certain condition is present, for example, the marker may bind to a first cell type but not a second cell type, the marker may bind to an expressed protein, the marker may indicate viability of the cell (i.e., if the cell is alive or dead), the marker may be indicative of the state of development or differentiation of the cell, etc., and the cells may be directed through a fluidic system of the invention based on the presence/absence, and/or magnitude of the fluorescent signal marker. For instance, determination of the fluorescent signal marker may cause the cells to be directed to one region of the device (e.g., a collection chamber), while the absence of the fluorescent signal marker may cause the cells to be directed to another region of the device (e.g., a waste chamber). Thus, in this example, a population of cells may be screened and/or sorted on the basis of one or more determinable or targetable characteristics of the cells, for example, to select live cells, cells expressing a certain protein, a certain cell type, etc.

As mentioned, certain aspects of the invention are directed to the production of droplets using apparatuses and devices such as those described herein, for example, within microfluidic channels or other microfluidic systems. In some cases, e.g., with relatively large numbers of side channels, relatively large droplet production rates may

be achieved. For instance, in some cases, greater than about 1,000 droplets/s, greater than or equal to 5,000 droplets/s, greater than about 10,000 droplets/s, greater than about 50,000 droplets/s, greater than about 100,000 droplets/s, greater than about 300,000 droplets/s, greater than about 500,000 droplets/s, or greater than about 1,000,000 droplets/s, etc. may be produced.

In addition, in some cases, a plurality of droplets may be produced that are substantially monodisperse, in some embodiments. In some cases, the plurality of droplets may have a distribution of characteristic dimensions such that no more than about 20%, no more than about 18%, no more than about 16%, no more than about 15%, no more than about 14%, no more than about 13%, no more than about 12%, no more than about 11%, no more than about 10%, no more than about 5%, no more than about 4%, no more than about 3%, no more than about 2%, no more than about 1%, or less, of the droplets have a characteristic dimension greater than or less than about 20%, less than about 30%, less than about 50%, less than about 75%, less than about 80%, less than about 90%, less than about 95%, less than about 99%, or more, of the average characteristic dimension of all of the droplets. Those of ordinary skill in the art will be able to determine the average characteristic dimension of a population of droplets, for example, using laser light scattering, microscopic examination, or other known techniques. In one set of embodiments, the plurality of droplets may have a distribution of characteristic dimension such that no more than about 20%, no more than about 10%, or no more than about 5% of the droplets may have a characteristic dimension greater than about 120% or less than about 80%, greater than about 115% or less than about 85%, or greater than about 110% or less than about 90% of the average of the characteristic dimension of the plurality of droplets. The "characteristic dimension" of a droplet, as used herein, is the diameter of a perfect sphere having the same volume as the droplet. In addition, in some instances, the coefficient of variation of the characteristic dimension of the exiting droplets may be less than or equal to about 20%, less than or equal to about 15%, or less than or equal to about 10%.

The average characteristic dimension or diameter of the plurality of droplets, in some embodiments, may be less than about 1 mm, less than about 500 micrometers, less than about 200 micrometers, less than about 100 micrometers, less than about 75 micrometers, less than about 50 micrometers, less than about 25 micrometers, less than

about 10 micrometers, or less than about 5 micrometers in some cases. The average characteristic dimension of a droplet (or plurality of droplets) may also be greater than or equal to about 1 micrometer, greater than or equal to about 2 micrometers, greater than or equal to about 3 micrometers, greater than or equal to about 5 micrometers, greater than or equal to about 10 micrometers, greater than or equal to about 15 micrometers, or greater than or equal to about 20 micrometers in certain cases.

In some embodiments, the fluidic droplets may each be substantially the same shape and/or size. The shape and/or size can be determined, for example, by measuring the average diameter or other characteristic dimension of the droplets. The term "determining," as used herein, generally refers to the analysis or measurement of a species, for example, quantitatively or qualitatively, and/or the detection of the presence or absence of the species. "Determining" may also refer to the analysis or measurement of an interaction between two or more species, for example, quantitatively or qualitatively, or by detecting the presence or absence of the interaction.

In some embodiments, a droplet may undergo additional processes. For example, as discussed, a droplet may be sorted and/or detected. For example, a species within a droplet may be determined, and the droplet may be sorted based on that determination. In general, a droplet may undergo any suitable process known to those of ordinary skill in the art. See, e.g., Int. Pat. Apl. No. PCT/US2004/010903, filed April 9, 2004, entitled "Formation and Control of Fluidic Species," by Link, *et al.*, published as WO 2004/091763 on October 28, 2004; Int. Pat. Apl. No. PCT/US2003/020542, filed June 30, 2003, entitled "Method and Apparatus for Fluid Dispersion," by Stone, *et al.*, published as WO 2004/002627 on January 8, 2004; Int. Pat. Apl. No. PCT/US2006/007772, filed March 3, 2006, entitled "Method and Apparatus for Forming Multiple Emulsions," by Weitz, *et al.*, published as WO 2006/096571 on September 14, 2006; Int. Pat. Apl. No. PCT/US2004/027912, filed August 27, 2004, entitled "Electronic Control of Fluidic Species," by Link, *et al.*, published as WO 2005/021151 on March 10, 2005, each of which is incorporated herein by reference in their entireties.

Certain aspects of the invention are generally directed to devices containing channels such as those described above. In some cases, some of the channels may be microfluidic channels, but in certain instances, not all of the channels are microfluidic. There can be any number of channels, including microfluidic channels, within the

device, and the channels may be arranged in any suitable configuration. The channels may be all interconnected, or there can be more than one network of channels present. The channels may independently be straight, curved, bent, etc. In some cases, there may be a relatively large number and/or a relatively large length of channels present in the device. For example, in some embodiments, the channels within a device, when added together, can have a total length of at least about 100 micrometers, at least about 300 micrometers, at least about 500 micrometers, at least about 1 mm, at least about 3 mm, at least about 5 mm, at least about 10 mm, at least about 30 mm, at least 50 mm, at least about 100 mm, at least about 300 mm, at least about 500 mm, at least about 1 m, at least about 2 m, or at least about 3 m in some cases. As another example, a device can have at least 1 channel, at least 3 channels, at least 5 channels, at least 10 channels, at least 20 channels, at least 30 channels, at least 40 channels, at least 50 channels, at least 70 channels, at least 100 channels, etc.

In some embodiments, at least some of the channels within the device are microfluidic channels. "Microfluidic," as used herein, refers to a device, article, or system including at least one fluid channel having a cross-sectional dimension of less than about 1 mm. The "cross-sectional dimension" of the channel is measured perpendicular to the direction of net fluid flow within the channel. Thus, for example, some or all of the fluid channels in a device can have a maximum cross-sectional dimension less than about 2 mm, and in certain cases, less than about 1 mm. In one set of embodiments, all fluid channels in a device are microfluidic and/or have a largest cross sectional dimension of no more than about 2 mm or about 1 mm. In certain embodiments, the fluid channels may be formed in part by a single component (e.g. an etched substrate or molded unit). Of course, larger channels, tubes, chambers, reservoirs, etc. can be used to store fluids and/or deliver fluids to various elements or systems in other embodiments of the invention, for example, as previously discussed. In one set of embodiments, the maximum cross-sectional dimension of the channels in a device is less than 500 micrometers, less than 200 micrometers, less than 100 micrometers, less than 50 micrometers, or less than 25 micrometers, less than about 10 micrometers, less than about 5 micrometers, or less than about 1 micrometer.

A "channel," as used herein, means a feature on or in a device or substrate that at least partially directs flow of a fluid. The channel can have any cross-sectional shape

(circular, oval, triangular, irregular, square or rectangular, or the like) and can be covered or uncovered. In embodiments where it is completely covered, at least one portion of the channel can have a cross-section that is completely enclosed, or the entire channel may be completely enclosed along its entire length with the exception of its inlets and/or outlets or openings. A channel may also have an aspect ratio (length to average cross sectional dimension) of at least 2:1, more typically at least about 3:1, at least about 4:1, at least about 5:1, at least about 6:1, at least about 8:1, at least about 10:1, at least about 15:1, at least about 20:1, at least about 30:1, at least about 40:1, at least about 50:1, at least about 60:1, at least about 70:1, at least about 80:1, at least about 90:1, at least about 100:1 or more. An open channel generally will include characteristics that facilitate control over fluid transport, e.g., structural characteristics (an elongated indentation) and/or physical or chemical characteristics (hydrophobicity vs. hydrophilicity) or other characteristics that can exert a force (e.g., a containing force) on a fluid. Non-limiting examples of force actuators that can produce suitable forces include piezo actuators, pressure valves, electrodes to apply AC electric fields, and the like. The fluid within the channel may partially or completely fill the channel. In some cases where an open channel is used, the fluid may be held within the channel, for example, using surface tension (i.e., a concave or convex meniscus).

The channel may be of any size, for example, having a largest dimension perpendicular to net fluid flow of less than about 5 mm or 2 mm, or less than about 1 mm, less than about 500 microns, less than about 200 microns, less than about 100 microns, less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about 30 microns, less than about 25 microns, less than about 10 microns, less than about 3 microns, less than about 1 micron, less than about 300 nm, less than about 100 nm, less than about 30 nm, or less than about 10 nm. In some cases, the dimensions of the channel are chosen such that fluid is able to freely flow through the device or substrate. The dimensions of the channel may also be chosen, for example, to allow a certain volumetric or linear flow rate of fluid in the channel. Of course, the number of channels and the shape of the channels can be varied by any method known to those of ordinary skill in the art. In some cases, more than one channel may be used. For example, two or more channels may be used, where they are positioned adjacent or proximate to each other, positioned to intersect with each other, etc.

In certain embodiments, one or more of the channels within the device may have an average cross-sectional dimension of less than about 10 cm. In certain instances, the average cross-sectional dimension of the channel is less than about 5 cm, less than about 3 cm, less than about 1 cm, less than about 5 mm, less than about 3 mm, less than about 1 mm, less than 500 micrometers, less than 200 micrometers, less than 100 micrometers, less than 50 micrometers, or less than 25 micrometers. The "average cross-sectional dimension" is measured in a plane perpendicular to net fluid flow within the channel. If the channel is non-circular, the average cross-sectional dimension may be taken as the diameter of a circle having the same area as the cross-sectional area of the channel. Thus, the channel may have any suitable cross-sectional shape, for example, circular, oval, triangular, irregular, square, rectangular, quadrilateral, or the like. In some embodiments, the channels are sized so as to allow laminar flow of one or more fluids contained within the channel to occur.

The channel may also have any suitable cross-sectional aspect ratio. The "cross-sectional aspect ratio" is, for the cross-sectional shape of a channel, the largest possible ratio (large to small) of two measurements made orthogonal to each other on the cross-sectional shape. For example, the channel may have a cross-sectional aspect ratio of less than about 2:1, less than about 1.5:1 , or in some cases about 1:1 (e.g., for a circular or a square cross-sectional shape). In other embodiments, the cross-sectional aspect ratio may be relatively large. For example, the cross-sectional aspect ratio may be at least about 2:1, at least about 3:1, at least about 4:1, at least about 5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 10:1, at least about 12:1, at least about 15:1, or at least about 20:1.

As mentioned, the channels can be arranged in any suitable configuration within the device. Different channel arrangements may be used, for example, to manipulate fluids, droplets, and/or other species within the channels. For example, channels within the device can be arranged to create droplets (e.g., discrete droplets, single emulsions, double emulsions or other multiple emulsions, etc.), to mix fluids and/or droplets or other species contained therein, to screen or sort fluids and/or droplets or other species contained therein, to split or divide fluids and/or droplets, to cause a reaction to occur (e.g., between two fluids, between a species carried by a first fluid and a second fluid, or between two species carried by two fluids to occur), or the like.

Non-limiting examples of systems for manipulating fluids, droplets, and/or other species are discussed below. Additional examples of suitable manipulation systems can also be seen in U.S. Patent Application Serial No. 11/246,911, filed October 7, 2005, entitled "Formation and Control of Fluidic Species," by Link, *et al.*, published as U.S. Patent Application Publication No. 2006/0163385 on July 27, 2006; U.S. Patent Application Serial No. 11/024,228, filed December 28, 2004, entitled "Method and Apparatus for Fluid Dispersion," by Stone, *et al.*, now U.S. Patent No. 7,708,949, issued May 4, 2010; U.S. Patent Application Serial No. 11/885,306, filed August 29, 2007, entitled "Method and Apparatus for Forming Multiple Emulsions," by Weitz, *et al.*, published as U.S. Patent Application Publication No. 2009/0131543 on May 21, 2009; and U.S. Patent Application Serial No. 11/360,845, filed February 23, 2006, entitled "Electronic Control of Fluidic Species," by Link, *et al.*, published as U.S. Patent Application Publication No. 2007/0003442 on January 4, 2007; each of which is incorporated herein by reference in its entirety.

Fluids may be delivered into channels within a device via one or more fluid sources. Any suitable source of fluid can be used, and in some cases, more than one source of fluid is used. For example, a pump, gravity, capillary action, surface tension, electroosmosis, centrifugal forces, etc. may be used to deliver a fluid from a fluid source into one or more channels in the device. A vacuum (e.g., from a vacuum pump or other suitable vacuum source) can also be used in some embodiments. Non-limiting examples of pumps include syringe pumps, peristaltic pumps, pressurized fluid sources, or the like. The device can have any number of fluid sources associated with it, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, etc., or more fluid sources. The fluid sources need not be used to deliver fluid into the same channel, e.g., a first fluid source can deliver a first fluid to a first channel while a second fluid source can deliver a second fluid to a second channel, etc. In some cases, two or more channels are arranged to intersect at one or more intersections. There may be any number of fluidic channel intersections within the device, for example, 2, 3, 4, 5, 6, etc., or more intersections.

A variety of materials and methods, according to certain aspects of the invention, can be used to form devices or components such as those described herein, e.g., channels such as microfluidic channels, chambers, etc. For example, various devices or components can be formed from solid materials, in which the channels can be formed via

micromachining, film deposition processes such as spin coating and chemical vapor deposition, physical vapor deposition, laser fabrication, photolithographic techniques, etching methods including wet chemical or plasma processes, electrodeposition, and the like. See, for example, *Scientific American*, 248:44-55, 1983 (Angell, *et al*).

In one set of embodiments, various structures or components of the devices described herein can be formed of a polymer, for example, an elastomeric polymer such as polydimethylsiloxane ("PDMS"), polytetrafluoroethylene ("PTFE" or Teflon®), or the like. For instance, according to one embodiment, a channel such as a microfluidic channel may be implemented by fabricating the fluidic system separately using PDMS or other soft lithography techniques (details of soft lithography techniques suitable for this embodiment are discussed in the references entitled "Soft Lithography," by Younan Xia and George M. Whitesides, published in the *Annual Review of Material Science*, 1998, Vol. 28, pages 153-184, and "Soft Lithography in Biology and Biochemistry," by George M. Whitesides, Emanuele Ostuni, Shuichi Takayama, Xingyu Jiang and Donald E. Ingber, published in the *Annual Review of Biomedical Engineering*, 2001, Vol. 3, pages 335-373; each of these references is incorporated herein by reference).

Other examples of potentially suitable polymers include, but are not limited to, polyethylene terephthalate (PET), polyacrylate, polymethacrylate, polycarbonate, polystyrene, polyethylene, polypropylene, polyvinylchloride, cyclic olefin copolymer (COC), polytetrafluoroethylene, a fluorinated polymer, a silicone such as polydimethylsiloxane, polyvinylidene chloride, bis-benzocyclobutene ("BCB"), a polyimide, a fluorinated derivative of a polyimide, or the like. Combinations, copolymers, or blends involving polymers including those described above are also envisioned. The device may also be formed from composite materials, for example, a composite of a polymer and a semiconductor material.

In some embodiments, various structures or components of the device are fabricated from polymeric and/or flexible and/or elastomeric materials, and can be conveniently formed of a hardenable fluid, facilitating fabrication via molding (e.g. replica molding, injection molding, cast molding, etc.). The hardenable fluid can be essentially any fluid that can be induced to solidify, or that spontaneously solidifies, into a solid capable of containing and/or transporting fluids contemplated for use in and with the fluidic network. In one embodiment, the hardenable fluid comprises a polymeric

liquid or a liquid polymeric precursor (i.e. a "prepolymer"). Suitable polymeric liquids can include, for example, thermoplastic polymers, thermoset polymers, waxes, metals, or mixtures or composites thereof heated above their melting point. As another example, a suitable polymeric liquid may include a solution of one or more polymers in a suitable solvent, which solution forms a solid polymeric material upon removal of the solvent, for example, by evaporation. Such polymeric materials, which can be solidified from, for example, a melt state or by solvent evaporation, are well known to those of ordinary skill in the art. A variety of polymeric materials, many of which are elastomeric, are suitable, and are also suitable for forming molds or mold masters, for embodiments where one or both of the mold masters is composed of an elastomeric material. A non-limiting list of examples of such polymers includes polymers of the general classes of silicone polymers, epoxy polymers, and acrylate polymers. Epoxy polymers are characterized by the presence of a three-membered cyclic ether group commonly referred to as an epoxy group, 1,2-epoxide, or oxirane. For example, diglycidyl ethers of bisphenol A can be used, in addition to compounds based on aromatic amine, triazine, and cycloaliphatic backbones. Another example includes the well-known Novolac polymers. Non-limiting examples of silicone elastomers suitable for use according to the invention include those formed from precursors including the chlorosilanes such as methylchlorosilanes, ethylchlorosilanes, phenylchlorosilanes, etc.

Silicone polymers are used in certain embodiments, for example, the silicone elastomer polydimethylsiloxane. Non-limiting examples of PDMS polymers include those sold under the trademark Sylgard by Dow Chemical Co., Midland, MI, and particularly Sylgard 182, Sylgard 184, and Sylgard 186. Silicone polymers including PDMS have several beneficial properties simplifying fabrication of various structures of the invention. For instance, such materials are inexpensive, readily available, and can be solidified from a prepolymeric liquid via curing with heat. For example, PDMSs are typically curable by exposure of the prepolymeric liquid to temperatures of about, for example, about 65 °C to about 75 °C for exposure times of, for example, at least about an hour. Also, silicone polymers, such as PDMS, can be elastomeric and thus may be useful for forming very small features with relatively high aspect ratios, necessary in certain embodiments of the invention. Flexible (e.g., elastomeric) molds or masters can be advantageous in this regard.

One advantage of forming structures such as microfluidic structures or channels from silicone polymers, such as PDMS, is the ability of such polymers to be oxidized, for example by exposure to an oxygen-containing plasma such as an air plasma, so that the oxidized structures contain, at their surface, chemical groups capable of cross-linking to other oxidized silicone polymer surfaces or to the oxidized surfaces of a variety of other polymeric and non-polymeric materials. Thus, structures can be fabricated and then oxidized and essentially irreversibly sealed to other silicone polymer surfaces, or to the surfaces of other substrates reactive with the oxidized silicone polymer surfaces, without the need for separate adhesives or other sealing means. In most cases, sealing can be completed simply by contacting an oxidized silicone surface to another surface without the need to apply auxiliary pressure to form the seal. That is, the pre-oxidized silicone surface acts as a contact adhesive against suitable mating surfaces. Specifically, in addition to being irreversibly sealable to itself, oxidized silicone such as oxidized PDMS can also be sealed irreversibly to a range of oxidized materials other than itself including, for example, glass, silicon, silicon oxide, quartz, silicon nitride, polyethylene, polystyrene, glassy carbon, and epoxy polymers, which have been oxidized in a similar fashion to the PDMS surface (for example, via exposure to an oxygen-containing plasma). Oxidation and sealing methods useful in the context of the present invention, as well as overall molding techniques, are described in the art, for example, in an article entitled "Rapid Prototyping of Microfluidic Systems and Polydimethylsiloxane," *Anal. Chem.*, 70:474-480, 1998 (Duffy *et al.*), incorporated herein by reference.

Another advantage to forming channels or other structures (or interior, fluid-contacting surfaces) from oxidized silicone polymers is that these surfaces can be much more hydrophilic than the surfaces of typical elastomeric polymers (where a hydrophilic interior surface is desired). Such hydrophilic channel surfaces can thus be more easily filled and wetted with aqueous solutions than can structures comprised of typical, unoxidized elastomeric polymers or other hydrophobic materials.

In some aspects, such devices may be produced using more than one layer or substrate, e.g., more than one layer of PDMS. For instance, devices having channels with multiple heights and/or devices having interfaces positioned such as described herein may be produced using more than one layer or substrate, which may then be assembled or bonded together, e.g., e.g., using plasma bonding, to produce the final

device. As a specific example, a device as discussed herein may be molded from masters comprising two or more layers of photoresists, e.g., where two PDMS molds are then bonded together by activating the PDMS surfaces using O_2 plasma or other suitable techniques. For example, in some cases, the masters from which the PDMS device is cast may contain one or multiple layers of photoresist, e.g., to form a 3D device. In some embodiments, one or more of the layers may have one or more mating protrusions and/or indentations which are aligned to properly align the layers, e.g., in a lock-and-key fashion. For example, a first layer may have a protrusion (having any suitable shape) and a second layer may have a corresponding indentation which can receive the protrusion, thereby causing the two layers to become properly aligned with respect to each other.

In some aspects, one or more walls or portions of a channel may be coated, e.g., with a coating material, including photoactive coating materials. For example, in some embodiments, each of the microfluidic channels at the common junction may have substantially the same hydrophobicity, although in other embodiments, various channels may have different hydrophobicities. For example a first channel (or set of channels) at a common junction may exhibit a first hydrophobicity, while the other channels may exhibit a second hydrophobicity different from the first hydrophobicity, e.g., exhibiting a hydrophobicity that is greater or less than the first hydrophobicity. Non-limiting examples of systems and methods for coating microfluidic channels, for example, with sol-gel coatings, may be seen in International Patent Application No. PCT/US2009/000850, filed February 11, 2009, entitled "Surfaces, Including Microfluidic Channels, With Controlled Wetting Properties," by Abate, *et al.*, published as WO 2009/120254 on October 1, 2009, and International Patent Application No. PCT/US2008/009477, filed August 7, 2008, entitled "Metal Oxide Coating on Surfaces," by Weitz, *et al.*, published as WO 2009/020633 on February 12, 2009, each incorporated herein by reference in its entirety. Other examples of coatings include polymers, metals, or ceramic coatings, e.g., using techniques known to those of ordinary skill in the art.

As mentioned, in some cases, some or all of the channels may be coated, or otherwise treated such that some or all of the channels, including the inlet and daughter channels, each have substantially the same hydrophilicity. The coating materials can be used in certain instances to control and/or alter the hydrophobicity of the wall of a

channel. In some embodiments, a sol-gel is provided that can be formed as a coating on a substrate such as the wall of a channel such as a microfluidic channel. One or more portions of the sol-gel can be reacted to alter its hydrophobicity, in some cases. For example, a portion of the sol-gel may be exposed to light, such as ultraviolet light, which can be used to induce a chemical reaction in the sol-gel that alters its hydrophobicity. The sol-gel may include a photoinitiator which, upon exposure to light, produces radicals. Optionally, the photoinitiator is conjugated to a silane or other material within the sol-gel. The radicals so produced may be used to cause a condensation or polymerization reaction to occur on the surface of the sol-gel, thus altering the hydrophobicity of the surface. In some cases, various portions may be reacted or left unreacted, e.g., by controlling exposure to light (for instance, using a mask).

In addition, as mentioned, certain aspects of the invention relate to antibodies that bind selectively to proteins or other targets. An antibody is a protein that typically includes at least two heavy (H) chains and two light (L) chains inter- by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_H1, C_H2 and C_H3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or V_L) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system.

The term "antigen-binding fragment" of an antibody as used herein, refers to one or more portions of an antibody that retain the ability to specifically bind to an antigen (e.g., a cytokine). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments

encompassed within the term "antigen-binding fragment" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_H1 domains; (ii) a F(ab')$_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and CH1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment which consists of a V_H domain or the variable domain of a heavy-chain antibody, such as a camelid heavy-chain antibody (e.g. V_{HH}); (vi) an isolated complementarity determining region (CDR); and (vii) polypeptide constructs comprising the antigen-binding fragments of (i) – (vi). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional procedures, such as proteolytic fragmentation procedures, expression of recombinant nucleic acids, or the like. The fragments are screened for utility in the same manner as are intact antibodies.

Isolated antibodies of the invention encompass various antibody isotypes, such as IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD, IgE. As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by heavy chain constant region genes. Antibodies of the invention can be full length or can include only an antigen-binding fragment such as the antibody constant and/or variable domain of IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD or IgE or could consist of a Fab fragment, a F(ab')$_2$ fragment, and a Fv fragment.

Antibodies of the present invention can be polyclonal, monoclonal, or a mixture of polyclonal and monoclonal antibodies. Antibodies of the invention can be produced by methods disclosed herein or by a variety of techniques known in the art, and many such antibodies can readily be obtained commercially.

Aspects of the invention encompass both polyclonal and monoclonal antibodies, including antibodies prepared using techniques that are known in the art. A monoclonal antibody typically refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody may display a single binding specificity and

affinity for a particular epitope. A monoclonal antibody may display a single binding specificity and affinity for a particular epitope. The polyclonal antibody typically refers to a preparation of antibody molecules that comprises a mixture of antibodies active that specifically bind a specific antigen.

A process of monoclonal antibody production may include obtaining immune somatic cells with the potential for producing antibody, in particular B lymphocytes, which have been previously immunized with the antigen of interest either *in vivo* or *in vitro* and that are suitable for fusion with a B-cell myeloma line. Mammalian lymphocytes typically are immunized by *in vivo* immunization of the animal (e.g., a mouse) with the desired protein or polypeptide, e.g., a cytokine. Such immunizations are repeated as necessary at intervals of up to several weeks to obtain a sufficient titer of antibodies. Once immunized, animals can be used as a source of antibody-producing lymphocytes which can be cloned and recombinantly expressed, as discussed further below. Following the last antigen boost, the animals are sacrificed and spleen cells removed. Mouse lymphocytes give a higher percentage of stable fusions with the mouse myeloma lines described herein. Of these, the BALB/c mouse is preferred. However, other mouse strains, rat, rabbit, hamster, sheep, goats, camels, llamas, frogs, etc. may also be used as hosts for preparing antibody-producing cells. Mouse strains that have human immunoglobulin genes inserted in the genome (and which cannot produce mouse immunoglobulins) can also be used. Examples include the HuMAb mouse strains produced by Medarex/GenPharm International, and the XenoMouse strains produced by Abgenix. Such mice produce fully human immunoglobulin molecules in response to immunization.

Those antibody-producing cells that are in the dividing plasmablast stage fuse preferentially. Somatic cells may be obtained from the lymph nodes, spleens and peripheral blood of antigen-primed animals, and the lymphatic cells of choice depend to a large extent on their empirical usefulness in the particular fusion system. The antibody-secreting lymphocytes are then fused with (mouse) B cell myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The resulting fused cells, or hybridomas, are cultured, and the resulting colonies screened for the production of the

desired monoclonal antibodies. Colonies producing such antibodies are cloned, and grown either *in vivo* or *in vitro* to produce large quantities of antibody.

Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of the desired hybridomas. Examples of such myeloma cell lines that may be used for the production of fused cell lines include, but are not limited to Ag8, P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4.1, Sp2/0-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7, S194/5XX0 Bul, all derived from mice; R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210 derived from rats and U-266, GM1500-GRG2, LICR-LON-HMy2, UC729-6, all derived from humans. Those of ordinary skill in the art will be aware of numerous routine methods to produce monoclonal antibodies.

Fusion with mammalian myeloma cells or other fusion partners capable of replicating indefinitely in cell culture is effected by standard and well-known techniques, for example, by using polyethylene glycol ("PEG") or other fusing agents.

Methods of raising polyclonal antibodies are well known to those of ordinary skill in the art. As a non-limiting example, polyclonal antibodies may be raised by administering a polypeptide subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The polypeptide can be inoculated with (e.g., injected at) a total volume of 100 microliters per site at six different sites, typically with one or more adjuvants. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times every six weeks. A sample of serum is collected 10 days after each boost. Polyclonal antibodies are recovered from the serum, preferably by affinity chromatography using acetylated cytochrome to capture the antibody. Those of ordinary skill in the art will be aware of numerous routine methods to produce polyclonal antibodies.

In other embodiments, antibodies may be recombinant antibodies. Recombinant antibodies generally include antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from an animal (e.g., a mouse) that is transgenic for another species' immunoglobulin genes, genetically engineered antibodies, antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial antibody library, or

antibodies prepared, expressed, created or isolated by any other means that involves splicing of immunoglobulin gene sequences to other DNA sequences.

Antibodies or antigen-binding fragments of the invention are, preferably, isolated. In some cases, the isolated antibody is not present in an organism that endogenously produces the antibody, i.e., the antibody has been isolated from the organism. In some cases, isolated antibodies and antigen-binding fragments thereof refer to an antibody (or antigen-binding fragment thereof) that is substantially free of other antibodies (or antigen-binding fragments) having different antigenic specificities. An isolated antibody that specifically binds to an epitope, isoform or variant of a polypeptide (e.g., a cytokine) may, however, have cross-reactivity to other related antigens, e.g., a mutant form of the cytokine, or a polypeptide from other species (e.g., homologs in other species). Moreover, an isolated antibody (or antigen-binding fragment thereof) may be substantially free of other cellular material and/or chemicals.

Antibodies of the invention include, but are not limited to antibodies that specifically bind to a cytokine. As used herein, "selective binding" or "specific binding" refers to antibody binding to a predetermined antigen with a preference that enables the antibody to be used to distinguish the antigen from others. In some embodiments, the antibody binds to a single protein and is able to distinguish that protein from all other proteins. In other embodiments, the antibody binds to several different related proteins and is able to distinguish those proteins from all other proteins.

In some embodiments, multiple antibodies, such as multiple different monoclonal antibodies, are incorporated into the polymer system at any given time, resulting in an extracorporeal circuit that is capable of removing more than one cytokine at a time. In so doing, the highly specific nature of the system is preserved and yet expanded to remove groups of proteins in a fashion never before described.

In some embodiments, an antibody or antigen-binding fragment thereof, of the invention, can specifically bind to an antigen with sub-nanomolar affinity. The dissociation constants can be about 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9} M or less, preferably about 1×10^{-10} M or less, more preferably 1×10^{-11}M or less. In a particular embodiment the binding affinity is less than about 5×10^{-10}M.

Examples of specific binding include, in the case of a receptor/ligand binding pair, the ligand would specifically and/or preferentially select its receptor from a

complex mixture of molecules, or vice versa. An enzyme would specifically bind to its substrate, a nucleic acid would specifically bind to its complement, an antibody would specifically bind to its antigen. Other examples include, nucleic acids that specifically bind (hybridize) to their complement, antibodies specifically bind to their antigen, and the like. The binding may be by one or more of a variety of mechanisms including, but not limited to ionic interactions, and/or covalent interactions, and/or hydrophobic interactions, and/or van der Waals interactions, etc.

In some aspects, an antibody or antigen-binding fragment thereof binds to a conformational epitope of a polypeptide such as a cytokine. To determine if the selected antibodies bind to conformational epitopes, each antibody can be tested in assays using native protein (e.g., non-denaturing immunoprecipitation, flow cytometric analysis of cell surface binding) and denatured protein (e.g., Western blot, immunoprecipitation of denatured proteins). A comparison of the results will indicate whether the antibodies bind conformational epitopes. Antibodies that bind to native protein but not denatured protein are those antibodies that bind conformational epitopes, and are preferred antibodies.

In some embodiments, a ligand, rather than an antibody, is used for selective or specific binding to a substance such as a protein.

An antibody or antigen-binding fragment thereof of the invention can be linked to a detectable label. A detectable label of the invention may be attached to antibodies or antigen-binding fragments thereof of the invention by standard protocols known in the art. In some embodiments, the detectable labels may be covalently attached to an antibody or antigen-binding fragment thereof of the invention. The covalent binding can be achieved either by direct condensation of existing side chains or by the incorporation of external bridging moieties. Many bivalent or polyvalent agents are useful in coupling protein molecules to other proteins, polypeptides or amine functions, etc. For example, the literature is replete with coupling agents such as carbodiimides, diisocyanates, glutaraldehyde, and diazobenzenes. This list is not intended to be exhaustive of the various coupling agents known in the art but, rather, is exemplary of the more common coupling agents.

A variety of definitions are now provided which will aid in understanding various aspects of the invention. Following, and interspersed with these definitions, is further disclosure that will more fully describe the invention.

A "droplet," as used herein, is an isolated portion of a first fluid that is completely surrounded by a second fluid. In some cases, the first fluid and the second fluid are substantially immiscible. It is to be noted that a droplet is not necessarily spherical, but may assume other shapes as well, for example, depending on the external environment. The diameter of a droplet, in a non-spherical droplet, is the diameter of a perfect mathematical sphere having the same volume as the non-spherical droplet. The droplets may be created using any suitable technique, as previously discussed.

As used herein, a "fluid" is given its ordinary meaning, i.e., a liquid or a gas. A fluid cannot maintain a defined shape and will flow during an observable time frame to fill the container in which it is put. Thus, the fluid may have any suitable viscosity that permits flow. If two or more fluids are present, each fluid may be independently selected among essentially any fluids (liquids, gases, and the like) by those of ordinary skill in the art.

Certain embodiments of the present invention provide a plurality of droplets. In some embodiments, the plurality of droplets is formed from a first fluid, and may be substantially surrounded by a second fluid. As used herein, a droplet is "surrounded" by a fluid if a closed loop can be drawn around the droplet through only the fluid. A droplet is "completely surrounded" if closed loops going through only the fluid can be drawn around the droplet regardless of direction. A droplet is "substantially surrounded" if the loops going through only the fluid can be drawn around the droplet depending on the direction (e.g., in some cases, a loop around the droplet will comprise mostly of the fluid by may also comprise a second fluid, or a second droplet, etc.).

In most, but not all embodiments, the droplets and the fluid containing the droplets are substantially immiscible. In some cases, however, they may be miscible. In some cases, a hydrophilic liquid may be suspended in a hydrophobic liquid, a hydrophobic liquid may be suspended in a hydrophilic liquid, a gas bubble may be suspended in a liquid, etc. Typically, a hydrophobic liquid and a hydrophilic liquid are substantially immiscible with respect to each other, where the hydrophilic liquid has a greater affinity to water than does the hydrophobic liquid. Examples of hydrophilic

liquids include, but are not limited to, water and other aqueous solutions comprising water, such as cell or biological media, ethanol, salt solutions, etc. Examples of hydrophobic liquids include, but are not limited to, oils such as hydrocarbons, silicon oils, fluorocarbon oils, organic solvents etc. In some cases, two fluids can be selected to be substantially immiscible within the time frame of formation of a stream of fluids. Those of ordinary skill in the art can select suitable substantially miscible or substantially immiscible fluids, using contact angle measurements or the like, to carry out the techniques of the invention.

The following documents are incorporated herein by reference in their entireties: International Patent Application No. PCT/US04/10903, filed April 9, 2004, entitled "Formation and Control of Fluidic Species," by Link, *et al.*, published as WO 2004/091763 on October 28, 2004; International Patent Application No. PCT/US03/20542, filed June 30, 2003, entitled "Method and Apparatus for Fluid Dispersion," by Stone, *et al.*, published as WO 2004/002627 on January 8, 2004; International Patent Application No. PCT/US04/27912, filed August 27, 2004, entitled "Electronic Control of Fluidic Species," by Link, *et al.*, published as WO 2005/021151 on March 10, 2005; and U.S. Pat. No. 8,337,778. Also incorporated herein by reference in their entireties are International Patent Application No. PCT/US2008/008563, filed July 11, 2008, entitled "Droplet-Based Selection," by Weitz, *et al.*, published as WO 2009/011808 on January 22, 2009; and International Patent Application No. PCT/US2009/004037, filed July 10, 2009, entitled "Systems and Methods of Droplet-Based Selection," by Weitz, *et al.*, published as WO 2010/005593 on July 10, 2009.

In addition, U.S. Provisional Patent Application Serial No. 62/008,341, filed June 5, 2014, entitled "Protein Analysis Assay System," by Weitz, *et al.*, is incorporated herein by reference in its entirety.

The following examples are intended to illustrate certain embodiments of the present invention, but do not exemplify the full scope of the invention.

EXAMPLE 1

Quantitative protein analysis of single cells is rarely achieved due to technical difficulties of detecting minute amounts of proteins present in one cell. This example develops a mix-and-read assay for drop-based label-free protein analysis of single cells.

This high-throughput example method quantifies absolute, rather than relative, amounts of proteins and does not involve antibody labeling or mass spectrometry.

The average abundance of proteins in a single mammalian cell is estimated to be 100,000 copies, corresponding to 16 femtomolar if the cell is lysed in a typical 10 microliter microwell assay. Conventional quantitative immunoassays have picomolar detection limits at best, and therefore, lack the sensitivity to quantify proteins at the level of single cells. However, drop-based microfluidics enable single-cell encapsulation in picoliter water-in-oil drops. Accordingly, the in-drop concentration of a mammalian protein with average abundance in a single cell exceeds 1 nanomolar, which is well within the detection range of conventional quantitative immunoassays.

This example illustrates a Protein Assay via Induced Gene Expression (PAIGE), which is a one-pot mix-and-read assay that allows quantitative protein measurements of single cells encapsulated in drops. To demonstrate PAIGE, this example illustrates the detection of a target antibody specific to an *in vitro* expressed epitope (Fig. 3A). The target antibody (Ab) forms a ternary complex bridging two *in vitro* expressed fusion proteins. The first fusion protein (AD-Ag) contains the activation domain (AD) fused to the antibody epitope (Ag). The second fusion protein (A/G-DB) contains the hybrid protein A/G fused to the DNA binding domain (DB). The resulting antibody ternary complex binds to the promoter region of the reporter DNA and activates expression of the fluorescent GFP reporter (Fig. 3A). This example uses monoclonal anti-Myc, which specifically binds the c-myc epitope, as a model system. In microwell assays, PAIGE expressing c-myc epitope in the fusion protein AD-myc activates the GFP expression only in the presence of anti-Myc, but not different antibodies, such as anti-MBP (Fig. 5). In the absence of anti-Myc, the two expressed fusion proteins (AD-myc and A/G-DB) do not interact and consequently only a basal level of GFP is expressed (Fig. 5). Furthermore, the fluorescence of PAIGE in microwell assays is linearly correlated to the concentration of anti-Myc (Fig.3C, squares, bulk). These data establish that PAIGE expressing a given epitope can be used to detect and quantify an antibody that is specific to the epitope.

This example applies PAIGE in drop-based microfluidics, whose workflow includes encapsulation, off-chip incubation, fluorescence detection and drop sorting (Fig. 3B). PAIGE is first encapsulated with different concentrations of pure anti-Myc in drops

and measure their fluorescence after incubation. A linear correlation of drop fluorescence was observed with anti-Myc concentrations ranging from 2 nM to 200 nM, corresponding to 30,000 to 2,000,000 anti-Myc molecules per drop (Fig. 3C, circles, drop). This linear correlation was in good agreement with that of bulk measurements in microwells, in spite of ~500,000 fold reduction in volume. In both drop and bulk experiments, large standard deviations at ~2 nM of anti-Myc (or 30,000 molecules per drop) were observed, suggesting that the sensitivity of PAIGE for anti-Myc is at the low nM range. However, by performing in drops, PAIGE has the sensitivity of detecting proteins from single cells. Additionally, the linear concentration range in drops is larger than that for microwells (2 nM to 48 nM), indicating that drop-based PAIGE increased the dynamic range of detection. In both cases, a saturation or slight decrease in the fluorescence was observed at very high concentrations of anti-Myc (e.g., >2,000,000 molecules per drop), which may be attributed to a large number of anti-Myc binding to both fusion proteins that prevents further formation of antibody ternary complexes (Fig. 3A). However, it is estimated that most mammalian proteins have less than 2,000,000 copies per cell.

To demonstrate single-cell antibody quantification, 9E10 hybridoma cells that produce anti-Myc were co-encapsulated with PAIGE and a cell-lysis agent in drops (Fig. 3B). These cells were diluted to achieve a Poisson loading such that 10% of drops contain single cells, 90% are empty, and a negligible number of drops contain multiple cells. After incubation, the heatmap of the fluorescence from 10,000 drops exhibited a bimodal distribution in which ~10% of drops were bright (Fig. 3D). Individual bright drops were sorted and verified to determine that they contained 9E10 cells by RT-PCR amplification of the beta-2-microglobulin gene, which is expressed constitutively in all hybridoma cells, followed by Sanger sequencing (Fig. 6). Based on the linear correlation of drop fluorescence with anti-Myc concentrations, it was determined that bright drops contained on average 880,000 anti-Myc molecules in a single hybridoma cell (Fig. 3C, dashed line). The contents from lysed cells could affect the PAIGE signal in drops, and the accuracy of the estimate that is based on the linear correlation of pure anti-Myc. However, the cell lysate was diluted ~ 8-fold in drops in the PAIGE assay. Moreover, it is believed that the mammalian factors were unlikely to significantly affect the bacterial transcription and translation machineries in PAIGE. This result agrees with an ensemble

estimate of the amount of anti-Myc secreted from a bulk 9E10 culture, which is on average 1,000,000 anti-Myc per cell. This may be the first drop-based single-cell quantification of an antibody, and the first high-throughput single-cell quantification of a protein that is not a fluorescent protein or an enzyme whose product is a fluorescent molecule. By further optimizing PAIGE, such as increasing the binding affinity towards the target molecule, its sensitivity may be improved at the low nM range, e.g., corresponding to ~30,000 molecules per cell. Since the average abundance of proteins in a single mammalian cell is estimated to be 100,000 copies, drop-based PAIGE may be used to measure medium to low abundance proteins from single cells.

In principle, drop-based PAIGE can be customized to detect and quantify any molecule of interest from single cells, provided that appropriate binders can be created (an example scheme is shown in Fig. 7). To correlate the target molecule concentration with drop fluorescence, one of the binding interactions for the activation of the fluorescent reporter may be rate-limiting. These binding interactions can be tuned by using a variety of methods (e.g., evolutionary methods) to engineer protein binders with appropriate affinities.

Fig. 3 shows detection and quantification of antibody in single hybridoma cells using drop-based PAIGE. Fig. 3A is a scheme of PAIGE for detecting an antigen (Ag)-specific antibody (Ab). Two fusion proteins, AD-Ag and A/G-DB, were constitutively expressed under T7 promoter (T7) from input DNA templates by T7 RNA polymerase (step 1). The antibody binds to both Ag and A/G, forming a ternary complex that binds to the upstream-activation sequence (UAS) on the reporter DNA and recruits AD near the promoter-bound RNA polymerase (RNAP) (step 2). AD activates RNAP (step 3) to express the reporter gene, which produces GFP (step 4).

Fig. 3B shows the workflow of drop-based PAIGE. Single cells are encapsulated with PAIGE and a lysis buffer in drops. After off-chip incubation, drops were re-injected into a fluorescence-activated drop-sorting (FADS) device to measure their fluorescence and sort them based on a fluorescence threshold. Fig. 3C shows titration curves of pure anti-myc in microwell (squares) and drop-based (circles) PAIGE. The dashed line shows the average number of anti-Myc molecules in a single cell. The fluorescence of anti-Myc in microwells or drops was normalized by that of microwells or drops without anti-Myc. The normalized activated fluorescence, F, was obtained by

subtracting one. Fig. 3D shows a heat map showing the distribution of drops in terms of their normalized activated fluorescence, *F*, and width recorded as the PMT's pulse duration (left). The corresponding fluorescence histogram is shown on the right. The inset fluorescence microscopy image shows that after incubation a small fraction of the drops is bright.

Fig. 5 shows detection of anti-Myc in a microwell plate using PAIGE. The GFP fluorescence of PAIGE reactions was monitored during incubation at 37 °C in a microplate reader. Reactions expressing the c-myc epitope were performed in the presence or absence of 240 nM anti-Myc. As additional negative controls, monitor the fluorescence of reactions expressing c-myc were monitored in the presence of 240 nM anti-MBP, and YEEI peptide in the presence of 240 nM anti-Myc.

Fig. 6 shows agarose gel image of amplification products from RT-PCR of a 350 bp region of mRNA encoding beta-2-macroglobulin from single cells. M: 100 bp DNA marker. 3 bright drops were sorted into 10 microliter water drops, and distributed into 8 wells for RT-PCR (lanes 1 to 8). The products were successfully amplified from two bright drops and their sequences verified by Sanger sequencing.

Fig. 7 shows a scheme of universal PAIGE for detecting and quantifying any target molecule of interest in solution. Appropriate binders (X and Y) in the fusion proteins were expressed from DNA templates (step 1) and bound to different regions of a target. Such interactions resulted in a ternary complex that binds to the upstream-activation sequence (UAS) on the reporter DNA and recruited AD near the promoter-bound RNA polymerase (RNAP) (step 2). AD activated RNAP (step 3) to express the reporter gene, which produced GFP (step 4). If one of the binding interactions (X/target or Y/target) is rate-limiting for the reactions, the concentration of the target should be linearly correlated to the GFP concentration.

EXAMPLE 2

In addition to quantifying proteins, drop-based PAIGE can be designed to detect enzymatic activity from single cells. To demonstrate this versatility, this example customizes drop-based PAIGE to detect Abl tyrosine kinase activity present in single chronic myelogenous leukemia cells (K562). Here, the interaction between the two fusion proteins is mediated through phosphorylation of a substrate peptide by Abl tyrosine kinase (Fig. 8A). In microwell PAIGE, GFP expression may be activated by

Abl kinase, as shown in Fig. 8. Then, drop-based PAIGE is verified to show that the drop fluorescence increases with the kinase activity by performing titration experiments with increasing amounts of pure Abl kinase (Fig. 8C). Next, single K562 cells are co-encapsulated with PAIGE and the drop fluorescence after incubation is measured.

The fluorescence (F) had a trimodal distribution, which included bright drops, a small middle population of low-fluorescence drops, 1.5>F>0.8 and dark drops, as shown in Fig. 4A. Bright drops contained cells with high kinase activity, whereas dark drops did not contain cells due to the Poisson loading. Interestingly, the detection of the low-fluorescence drops suggests a small subpopulation, 5%, of K562 cells had low kinase activity. Using a threshold of 1.5>F>0.8 (see below), ~6% of K562 cells were determined to exhibit low kinase activity. To support this notion, K562 cells were treated with imatinib, an Abl tyrosine kinase inhibitor, and the kinase activity was monitored in single cells for 12, 24, 36, and 48 hours (Figs. 4B-4E).

The same threshold of 1.5>F>0.8 was used to quantify the number of drops in the trimodal distributions at different time points in spite of overlapping peaks (Figs. 4B-4E). The number of fluorescent drops (bright drops plus low-fluorescence drops) approximated the number of total encapsulated single K562 cells since dark drops generally did not contain cells. As shown in Figs. 4C-4F, unlike the DMSO control, where the fraction of cells with low kinase activity remained ~5%, the corresponding fraction of imatinib-treated cells increased to ~25% after 24 hours, remained at 25% after 36 hours, and fell to ~6% after 48 hours (see below). These imatinib-sensitive cells underwent drug-induced apoptosis followed by necrosis, as supported by single-cell staining experiments (Fig. 9). At 24-hr imatinib treatment, the similar fractions of cells (25% vs 28%, Fig. 4F and Fig. 9) exhibiting low fluorescence activity and undergoing apoptosis suggests that the kinase inhibition by imatinib initiates apoptosis in the same population of cells. At 36-hr imatinib treatment, the fraction of cells with low kinase activity remains at 25% (Fig. 4F), whereas the fraction of apoptotic cells increases to almost 50% (Fig. 9). It is believed that at this time point, half of these apoptotic cells have completely lost their kinase activity and could not be detected by the drop-PAGE assay. At 48-hr imatinib treatment, the fractions of both low-kinase-activity cells and apoptotic cells fell to around ~6% (Fig. 4F and Fig. 9). It is believed that at this late stage of drug treatment, the majority of imatinib-sensitive cells have undergone

extensive necrosis. These cells were lysed before or during centrifugation, and were subsequently removed from intact cells before the single-cell drop and microscopic analyses (Fig. 10). After 48 hours, this fraction fell to 7% (Fig. 4F).

Most of the imatinib-sensitive cells at this stage underwent drug-induced apoptosis followed by necrosis, as supported by single-cell staining experiments (Fig. 9). It is hypothesized that these necrotic cells were either removed by centrifugation before drop analysis, or if encapsulated in drops, exhibited little kinase activity resulting in dark drops. After 48 hours, the remaining drug-treated cells still exhibited high kinase activity indicating resistance to imatinib (Fig. 4E). These data illustrate that drop-based PAIGE may be used for assessing the responses of heterogeneous cancer cells to drug treatment with single-cell resolution. Furthermore, the drop sorting capabilities allow single-cell genetic analysis of both drug-sensitive and drug-resistant cells.

In summary, these examples demonstrated a high-throughput mix-and-read label-free method that allows quantification of absolute amounts of proteins in single cells. Current single-cell protein analysis methods measured relative amounts by using antibodies to label/capture target proteins. Other drop-based single-cell protein analysis methods require co-encapsulating cells with capturing beads in single drops. Compared to flow cytometry, drop-based PAIGE has the additional advantage of not requiring pretreatment of cells. In contrast to single-cell mass spectrometry methods, these drop-based methods preserve cell contents for further genetic analyses, which provides a link between genotype and phenotype for studies of cell heterogeneity.

Fig. 4 shows monitoring kinase activity of drug-treated single leukemia cells using drop-based PAIGE. Figs. 4A-4E are heat maps showing the distribution of drops containing K562 cells treated with imatinib for 0, 12, 24, 36, and 48 hours (left). The corresponding fluorescence histograms are shown on the right. The dashed lines separate three groups of drops: bright and low-fluorescence drops containing cells that express high and low kinase activities, respectively, and dark drops without cells. Fig. 4F shows fractions of cells with low kinase activity as determined by drop-based PAIGE during the treatment of imatinib (circles) or DMSO (squares) as control.

Fig. 8A shows a scheme of PAIGE for detecting kinase activity. AD was fused to a kinase substrate peptide and SH2 was fused to DB. Both fusion proteins, AD-peptide and SH2-DB, were constitutively expressed under T7 promoters from input

DNA templates (step 1). Phosphorylation of the substrate peptide by a kinase (step 2) results in the peptide's binding to SH2-DB which was bound to the upstream-activation sequence (UAS) on the reporter DNA (step 3). AD activated the promoter-bound RNA polymerase (RNAP) (step 4) to express the reporter gene, which produced GFP (step 5). Fig. 8B shows detection of Abl kinase activity in a microwell plate using PAIGE. The GFP fluorescence was monitored during incubation at 37 °C in a microplate reader. Fig. 8C shows a titration curve of pure Abl kinase using drop-based PAIGE. The fluorescence of drops with Abl kinase vas normalized by that of drops without Abl kinase. The normalized activated fluorescence, F, is obtained by subtracting one.

Fig. 9 shows the fractions of leukemia K562 cells undergoing apoptosis and necrosis during drug treatments as determined by cell staining. The cells were treated with 10 micromolar imatinib and DMSO was the control. The fraction of apoptotic cells increased steadily during first 36 hours due to kinase inhibition of imatinib. After 36 hours, the apoptotic fraction dropped dramatically because in this time interval the imatinib-sensitive cells became necrotic and were removed by centrifugation during the sample preparation process.

Fig.1 shows work flow of the experimental steps for monitoring the kinase activity, apoptosis and necrosis of single K562 cells during imatinib treatment, according to one embodiment.

EXAMPLE 3

Following are materials and methods used in the above examples. These are by way of example only and should not be construed as being limiting.

General PAIGE reagents. In these experiments, PAIGE was derived from the *in vitro* two-hybrid system (IVT2H) and contained 144 nM purified *E.coli* RNA polymerase core enzyme (New England Biolabs), 1.2 micromolar purified recombinant *E. coli* IHF, 0.8 units/microliters murine RNAase inhibitor (New England Biolabs), 0.2 ng/microliter (45 pM) plasmid DNA expressing *E. coli* sigma factor 54, 0.2 ng/microliter (~40-60 pM) DNA constructs expressing hybrid fusion proteins, 4.4 nM linear reporter DNA expressing the reporter GFP, and the reconstituted protein synthesis system containing T7 RNA polymerase and purified *E. coli* translational components (PURExpress, New England Biolabs).

PAIGE for antibody detection. The genes for c-myc epitope peptide (EQKLISEEDL) and a chimeric IgG-binding Fc receptor protein A/G were commercially synthesized (Integrated DNA Technologies) and cloned into the C-terminus of AD and N-terminus of DB, generating the hybrid fusion proteins, AD-myc and A/G-DB, respectively. As a control, *E. coli* maltose-binding protein (MBP) was also cloned into the C-terminus of AD to give AD-MBP. For antibody detection, DNA constructs expressing AD-myc or AD-MBP and A/G-DB were mixed with PAIGE reagents and different concentrations (2.4-240 nM) of purified monoclonal anti-Myc antibody (Sigma Aldrich) or 240 nM anti-MBP (NEB). For detection in microwell plates (384-well microplate, Corning, Lowell, MA), 10 microliters of reaction mixtures were incubated at 37 ^{o}C for 4 hours and GFP fluorescence (excitation 513 nm, emission 532 nm) was measured using a Spectramax M5 microplate reader (Molecular Devices).

PAIGE for kinase detection. *In vitro* detection of the protein interaction in response to the phosphorylation of a kinase substrate peptide YEEI by Src kinase has been described previously. In these examples, DNA constructs expressing AD-YEEI and SH2-DB were mixed with PAIGE reagents with or without 360 nM purified recombinant Abl kinase (Sigma-Aldrich). To detect the fluorescence, 10 microliters of the reaction mixtures were incubated in 384 microwell plates (Corning) at 37 ^{o}C for 4 hours and measured using a Spectramax M5 microplate reader (excitation 513 nm, emission 532 nm, Molecular Devices).

Preparation of cells for single-cell analysis and drug treatments. The hybridoma cell line MYC 1-9E10.2 (9E10) and the leukemia cell line K562 (ATCC) were maintained in Dulbecco's modified Eagle's medium DMEM (Cellgro/Mediatech) supplemented with 10% low-endotoxin fetal calf serum (HyClone), 100 U penicillin/ml, and 100 microgram/ml streptomycin. Cells were harvested and counted before they were co-flowed into the microfluidic device for single-cell analysis. To inhibit the kinase of K562 cells, they were incubated in the presence of 10 micromolar of imatinib. As a control, K562 cells were cultured in the presence of DMSO. Cells were collected at 12, 24, 36, and 48 hours by centrifugation. This centrifugation step separated intact cells, which were collected at the bottom of the centrifuge tube, from lysed cells which remained in the supernatant. An Annexin V-FITC Apoptosis Detection Kit (Sigma) was used to detect apoptosis. This kit also provided propidium iodide to detect necrosis cells

as red fluorescence. Cells undergoing apoptosis or necrosis are counted under fluorescence microscopy.

Fabrication of microfluidic devices. Polydimethylsiloxane (PDMS) microfluidic devices were fabricated using standard soft lithographic methods. The microfluidic channel walls were rendered hydrophobic by treating them with Aquapel (PPG). The electrodes of the PDMS sorting device were made by heating the device to 80 °C and filling the channels with Indalloy 19 (51In, 32.5 Bi, 16.5 Sn; 0.020 inch diameter, 1 inch = 2.54 cm), a low melting point metal alloy (Indium Corporation).

Formation of monodisperse aqueous drops and drop-based PAIGE reactions. A microfluidic chip was used that contained a co-flow drop maker with a cross section of 25 micrometer^2 to generate 35 micrometer monodisperse aqueous drops in fluorinated oil, HFE-7500 (3M, Saint Paul, MN, U.S.A), containing 1% (w/w) Krytox-PEG diblock co-polymer surfactant (RAN Biotech). The PAIGE reagents and different concentrations (Figs. 3C and 8C) of pure anti-Myc, kinase or cells were encapsulated in drops via co-flow in different channels. To achieve optimal concentrations for PAIGE reactions, the resistance of the channel for PAIGE reagents was adjusted to obtain a 4:1 (PAIGE reagents to protein or cells) co-flow ratio. For both pure anti-Myc and cell lysis, 25% NP40 was added to the PAIGE reagents to achieve a 1% working concentration. Off-chip incubation was conducted at 37 °C for 6 hours.

Fluorescence-activated drop sorting (FADS) analysis. A microfluidic fluorescence-activated drop sorter was used to isolate bright drops. After incubation, drops were re-injected into the sorter at a flow rate of 30 microliters/h and evenly spaced by HFE-7500 oil with surfactant flowing at a rate of 180 microliters/h through a 40 micrometer x 40 micrometer channel. When a drop passes through the laser's focal point, its fluorescence was collected by a microscope objective and focused onto a photomultiplier tube (PMT) (Hammamatsu). The pulse height was used as the measure of drop fluorescence. The pulse width, which was the duration of time for a drop to pass through the laser detector, was used as the measure of drop size. The PMT was connected to a custom computer LabView program running on a real-time field-programmable gate array card (National Instruments). All drops were gated based on pulse width to exclude merged drops. Pulse width is routinely used to plot the data in drop-based microfluidics. Pulse width is proportional to drop size, reflecting the

monodispersity of drops. Encapsulation of cells or other materials can affect the local hydrodynamics of the pinch-off process, resulting in changes of the drop size, and thus the observed pulse width. To protect the sorted drops from evaporation and facilitate their handling, they were collected into a tip loaded with 20 microliters of drops containing pure water. Single sorted drops were isolated by distributing the emulsion into microwells whose number is determined by Poisson loading. For example, 10 bright drops were distributed into 30 microwells.

For K562 cells after drop-based PAIGE reactions, the droplets exhibit a trimodal distribution in fluorescence (Fig. 4A). To quantify the number of bright drops, low-fluorescence drops and dark drops, the distribution was used without drug treatment to define the threshold for each population of drops, since it had relatively well-defined and separate peaks (Fig. 4A). The same threshold was then applied to the distributions at 12, 36 and 48 hours. Specifically, the fluorescence threshold was set at the first minimum (F=0.8) of the trimodal distribution to distinguish between low-fluorescence drops and dark drops, and at the second minimum (F=1.5) of the trimodal distribution to distinguish between low-fluorescence drops and bright drops.

RT-PCR amplification of mRNA from single sorted hybridoma 9E10 cells and Sanger sequencing. The emulsion in the microwells was broken with 10 microliters of 20% of 1H,1H,2H,2H-perfluorooctanol (Alfa Aesar) followed by vortexing and centrifugation. Five microliters of ddH_2O was added to each well. The aqueous phase containing cellular RNA for RT-PCR was pipetted out. The 25 microliter RT-PCR cocktail was composed of 1 microliter of OneStep RT-PCR Enzyme Mix with 1x buffer (Qiagen), 400 micromolar dNTPs, 0.25 micromolar beta-2-macroglobulin forward and reverse primers, and 5 microliters of the cellular RNA. The RT-PCR was done in one step by combining RT and PCR reactions. Thermocycling conditions were: 50 °C for 30 min, 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s, followed by 72 °C for 5 min. The PCR products were purified with GenElute™ Gel Extraction Kit (Sigma) and sent out for Sanger sequencing.

While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or

one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other

than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

When the word "about" is used herein in reference to a number, it should be understood that still another embodiment of the invention includes that number not modified by the presence of the word "about."

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

CLAIMS

What is claimed is:

1. A method of determining a target antibody, comprising:

producing a first fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising a binding domain;

producing a second fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising an activation domain;

binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target antibody within a compartment having a volume of less than about 1 microliter;

binding an RNA polymerase to the activation domain;

binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein; and

determining expression of the protein, thereby determining the target antibody.

2. The method of claim 1, wherein the nucleic acid comprises a promoter that the RNA polymerase binds.

3. The method of any one of claims 1 or 2, wherein the nucleic acid comprises an upstream activating sequence that the binding domain binds.

4. The method of any one of claims 1-3, wherein the nucleic acid encodes a fluorescent protein.

5. The method of claim 4, comprising determining expression of the protein by determining fluorescence of the protein.

6. The method of any one of claims 1-5, wherein the nucleic acid encodes green fluorescent protein.

7. The method of any one of claims 1-6, wherein the first portion of the first fusion protein comprises an antigen that the target antibody is able to bind.

8. The method of any one of claims 1-7, wherein the target antibody comprises an Fab portion that is able to bind to the first portion of the first fusion protein.

9. The method of any one of claims 1-8, wherein the first portion of the second fusion protein comprises an Fc binding domain that the target antibody is able to bind.

10. The method of any one of claims 1-9, wherein the first portion of the second fusion protein comprises Protein A and/or Protein G.

11. The method of any one of claims 1-10, wherein the target antibody comprises an Fc portion that is able to bind to the first portion of the first fusion protein.

12. The method of any one of claims 1-11, wherein the target antibody is produced by a cell.

13. The method of claim 12, wherein the target antibody is secreted by the cell.

14. The method of any one of claims 12 or 13, further comprising lysing the cell.

15. The method of any one of claims 1-14, wherein the acts are performed in the order recited.

16. The method of any one of claims 1-15, wherein the compartment is a microfluidic droplet.

17. The method of claim 16, wherein the first fusion protein, the second fusion protein, the target antibody, the RNA polymerase, and the nucleic acid are contained within the microfluidic droplet.

18. The method of claim 17, wherein determining expression of the protein comprises determining fluorescence of the microfluidic droplet to determine expression of the protein.

19. The method of any one of claims 17 or 18, further comprising sorting the microfluidic droplet based on the expression of the protein.

20. The method of any one of claims 17-19, wherein the target antibody is produced by the cell.

21. The method of claim 20, wherein the cell is contained within the microfluidic droplet.

22. The method of any one of claims 20 or 21, wherein the target antibody is secreted by the cell.

23. The method of any one of claims 20-22, further comprising lysing the cell within the microfluidic droplet.

24. The method of claim 17-23, wherein the microfluidic droplet is one of a plurality of droplets.

25. The method of claim 24, wherein the droplets have a distribution in diameters such that no more than 5% of the droplets have a diameter greater than about 110% and/or less than about 90% of the overall average cross-sectional dimension of the droplets.

26. The method of any one of claims 24 or 25, wherein the droplets have an average population of cells of less than about 1 cell per droplet.

27. The method of any one of claims 24-26, wherein the droplets have an average population of cells of less than about 1 cell per 10 droplets.

28. The method of any one of claims 24-27, wherein the droplets have a characteristic dimension of no more than about 1 micrometer.

29. A method of determining a target antibody, comprising:

producing a first fusion protein within a microfluidic droplet, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising a binding domain;

producing a second fusion protein within the microfluidic droplet, wherein the first fusion protein comprises a first portion able to bind to the target antibody and a second portion comprising an activation domain;

binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target antibody;

binding an RNA polymerase to the activation domain;

binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein;

determining expression of the protein, thereby determining the target antibody; and

sorting the microfluidic droplet based on the expression of the protein.

30. The method of claim 29, wherein the nucleic acid comprises a promoter that the RNA polymerase binds.

31. The method of any one of claims 29 or 30, wherein the nucleic acid comprises an upstream activating sequence that the binding domain binds.

32. The method of any one of claims 29-31, wherein the nucleic acid encodes a fluorescent protein.

33. The method of claim 32, comprising determining expression of the protein by determining fluorescence of the protein.

34. The method of any one of claims 29-33, wherein the nucleic acid encodes green fluorescent protein.

35. The method of any one of claims 29-34, wherein the first portion of the first fusion protein comprises an antigen that the target antibody is able to bind.

36. The method of any one of claims 29-35, wherein the target antibody comprises an Fab portion that is able to bind to the first portion of the first fusion protein.

37. The method of any one of claims 29-36, wherein the first portion of the second fusion protein comprises an Fc binding domain that the target antibody is able to bind.

38. The method of any one of claims 29-37, wherein the first portion of the second fusion protein comprises Protein A and/or Protein G.

39. The method of any one of claims 29-38, wherein the target antibody comprises an Fc portion that is able to bind to the first portion of the first fusion protein.

40. The method of any one of claims 29-39, wherein the target antibody is produced by a cell.

41. The method of claim 40, wherein the target antibody is secreted by the cell.

42. The method of any one of claims 40 or 41, further comprising lysing the cell.

43. The method of any one of claims 29-42, wherein the acts are performed in the order recited.

44. The method of any one of claims 29-43, wherein the first fusion protein, the second fusion protein, the target antibody, the RNA polymerase, and the nucleic acid are contained within the microfluidic droplet.

45. The method of any one of claims 29-44, wherein determining expression of the protein comprises determining fluorescence of the microfluidic droplet to determine expression of the protein.

46. The method of any one of claims 29-45, wherein the target antibody is produced by the cell.

47. The method of claim 46, wherein the cell is contained within the microfluidic droplet.

48. The method of any one of claims 46 or 47, wherein the target antibody is secreted by the cell.

49. The method of any one of claims 46-48, further comprising lysing the cell within the microfluidic droplet.

50. The method of any one of claims 29-49, wherein the microfluidic droplet is one of a plurality of droplets.

51. The method of claim 50, wherein the droplets have a distribution in diameters such that no more than 5% of the droplets have a diameter greater than about 110% and/or less than about 90% of the overall average cross-sectional dimension of the droplets.

52. The method of any one of claims 50 or 51, wherein the droplets have an average population of cells of less than about 1 cell per droplet.

53. The method of any one of claims 50-52, wherein the droplets have an average population of cells of less than about 1 cell per 10 droplets.

54. The method of any one of claims 50-53, wherein the droplets have a characteristic dimension of no more than about 1 micrometer.

55. A method of determining a target, comprising:

producing a first fusion protein *in situ*;

producing a second fusion protein *in situ*;

binding the first fusion protein and the second fusion protein to the target and to an RNA polymerase to form a complex within a compartment having a volume of less than about 1 microliter;

binding the complex to a nucleic acid to express a protein encoded by the nucleic acid; and

determining expression of the protein, thereby determining the target.

56. The method of claim 55, wherein the first fusion protein comprises an antibody or a fragment thereof that can bind the target.

57. The method of any one of claims 55 or 56, wherein the second fusion protein comprises an antibody or a fragment thereof that can bind the target.

58. The method of any one of claims 55-57, wherein the compartment is a microfluidic droplet.

59. The method of claim 58, wherein the first fusion protein, the second fusion protein, the target, the RNA polymerase, and the nucleic acid are contained within the microfluidic droplet.

60. The method of any one of claims 58 or 59, further comprising sorting the microfluidic droplet based on the expression of the protein.

61. The method of any one of claims 55-60, wherein the nucleic acid comprises a promoter that the RNA polymerase binds.

62. The method of any one of claims 55-61, wherein the nucleic acid comprises an upstream activating sequence that the binding domain binds.

63. The method of any one of claims 55-62, wherein the nucleic acid encodes a fluorescent protein.

64. The method of claim 63, comprising determining expression of the protein by determining fluorescence of the protein.

65. The method of any one of claims 55-64, wherein the nucleic acid encodes green fluorescent protein.

66. The method of any one of claims 55-65, wherein the target is produced by a cell.

67. The method of claim 66, wherein the target is secreted by the cell.

68. A method of determining a target antibody, comprising:

producing a first fusion protein *in situ*;

producing a second fusion protein *in situ*;

binding the first fusion protein and the second fusion protein to a target antibody and to an RNA polymerase to form a complex within a compartment having a volume of less than about 1 microliter; and

binding the complex to a nucleic acid to express a protein encoded by the nucleic acid; and

determining expression of the protein, thereby determining the target antibody.

69. The method of claim 68, wherein the compartment is a microfluidic droplet.

70. The method of claim 69, wherein the first fusion protein, the second fusion protein, the target antibody, the RNA polymerase, and the nucleic acid are contained within the microfluidic droplet.

71. The method of claim 70, further comprising sorting the microfluidic droplet based on the expression of the protein.

72. The method of any one of claims 68-71, wherein the nucleic acid comprises a promoter that the RNA polymerase binds.

73. The method of any one of claims 68-72, wherein the nucleic acid comprises an upstream activating sequence that the binding domain binds.

74. The method of any one of claims 68-73, wherein the nucleic acid encodes a fluorescent protein.

75. The method of claim 74, comprising determining expression of the protein by determining fluorescence of the protein.

76. The method of any one of claims 68-75, wherein the nucleic acid encodes green fluorescent protein.

77. The method of any one of claims 68-76, wherein the target antibody is produced by a cell.

78. The method of claim 77, wherein the target antibody is secreted by the cell.

79. A method of determining a target, comprising:

producing a first fusion protein *in situ*, wherein the first fusion protein

comprises a first portion able to bind to the target and a second portion comprising a binding domain;

producing a second fusion protein *in situ*, wherein the first fusion protein comprises a first portion able to bind to the target and a second portion comprising an activation domain;

binding the first portion of the first fusion protein and the first portion of the second fusion protein to the target within a compartment having a volume of less than about 1 microliter;

binding an RNA polymerase to the activation domain;

binding the binding domain to a nucleic acid, wherein the RNA polymerase is able to express at least a portion of the nucleic acid to produce a protein; and

determining expression of the protein, thereby determining the target.

80. The method of claim 79, wherein the first portion of the first fusion protein comprises an antibody or a fragment thereof that can bind the target.

81. The method of any one of claims 79 or 80, wherein the first portion of the second fusion protein comprises an antibody or a fragment thereof that can bind the target.

82. The method of any one of claims 79-81, wherein the compartment is a microfluidic droplet.

83. The method of claim 82, wherein the first fusion protein, the second fusion protein, the target, the RNA polymerase, and the nucleic acid are contained within the microfluidic droplet.

84. The method of claim 83, further comprising sorting the microfluidic droplet based on the expression of the protein.

85. The method of any one of claims 79-84, wherein the nucleic acid comprises a promoter that the RNA polymerase binds.

86. The method of any one of claims 79-85, wherein the nucleic acid comprises an upstream activating sequence that the binding domain binds.

87. The method of any one of claims 79-86, wherein the nucleic acid encodes a fluorescent protein.

88. The method of claim 87, comprising determining expression of the protein by determining fluorescence of the protein.

89. The method of any one of claims 79-88, wherein the nucleic acid encodes green fluorescent protein.

90. The method of any one of claims 79-89, wherein the target is produced by a cell.

91. The method of claim 90, wherein the target is secreted by the cell.

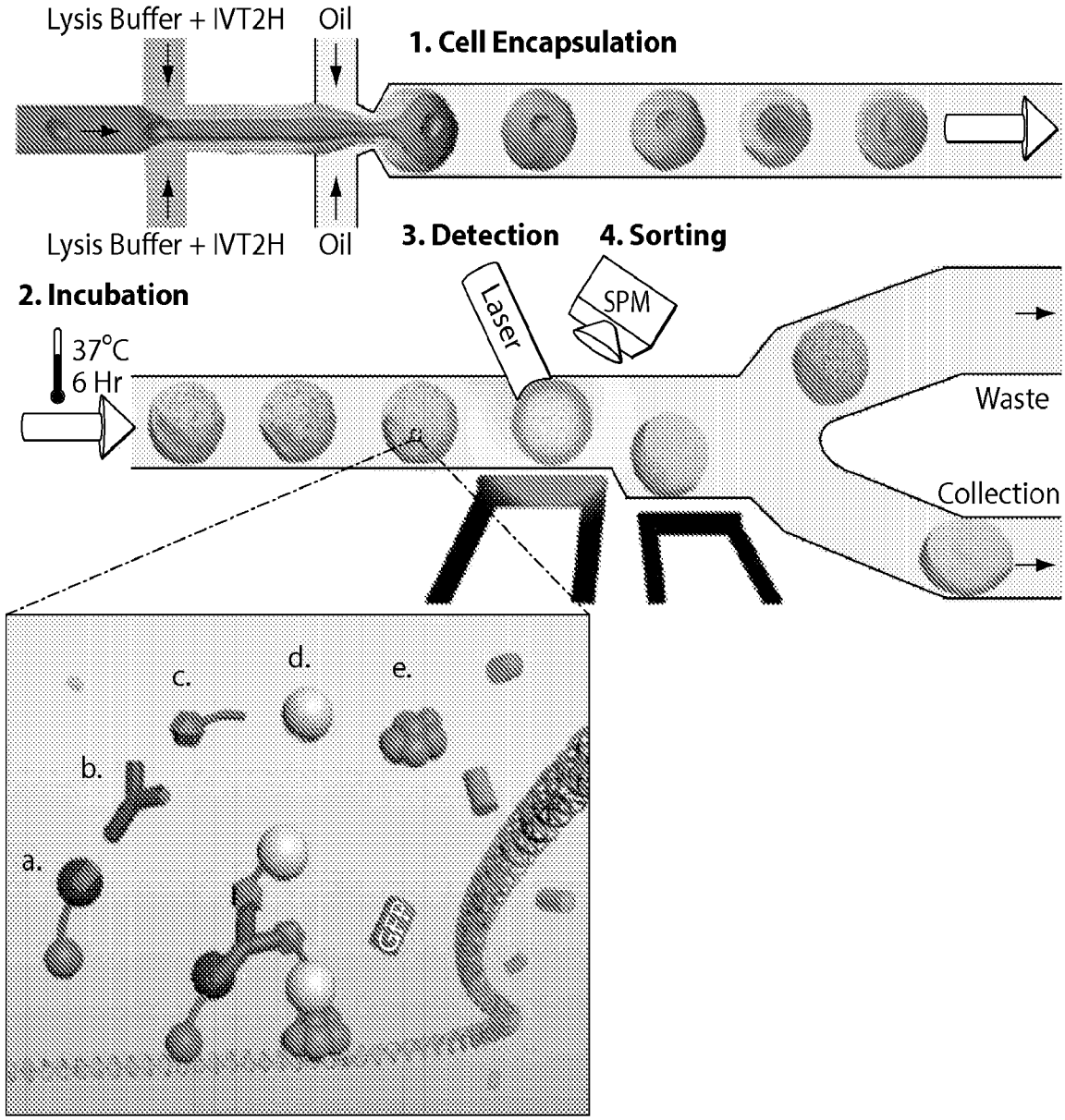
Lysis Buffer + IVT2H
Oil
1. Cell Encapsulation
Lysis Buffer + IVT2H
Oil
3. Detection
4. Sorting
2. Incubation
37°C
6 Hr
Laser
SPM
Waste
Collection
a.
b.
c.
d.
e.

Fig. 1

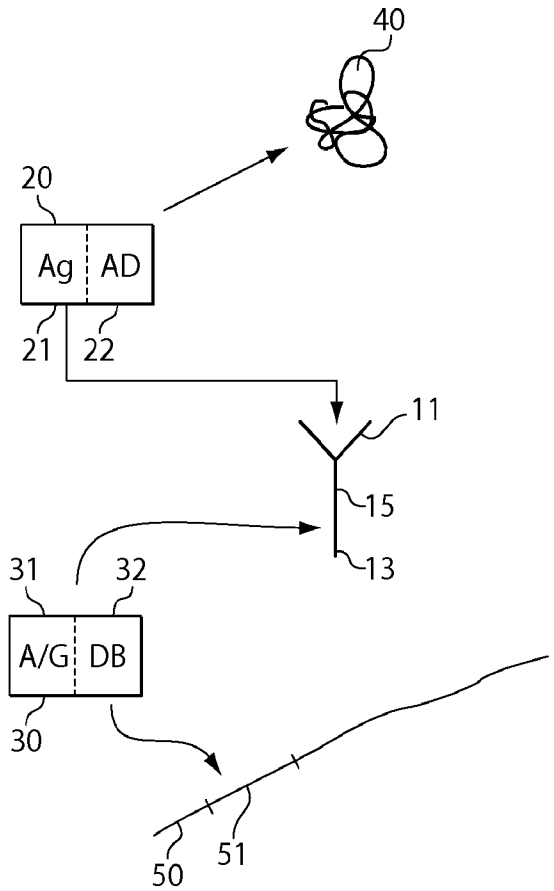
40
20
Ag
AD
21
22
11
15
13
31
32
A/G
DB
30
50
51

Fig. 2A

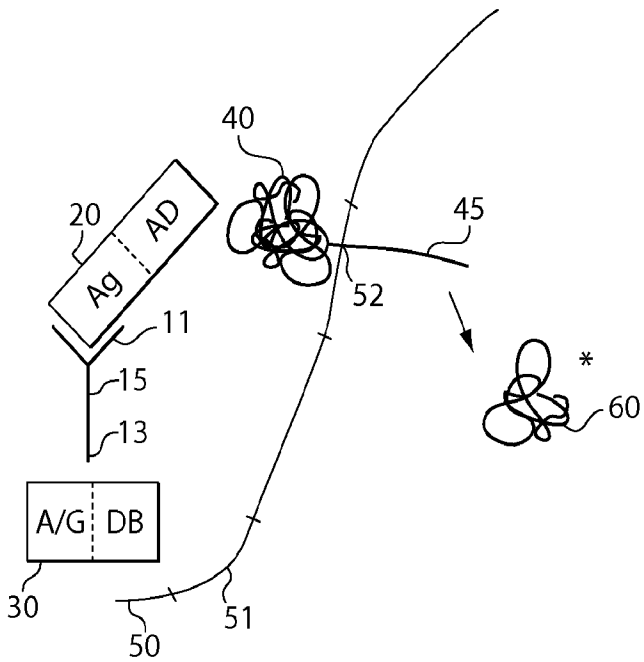
40
20
Ag
AD
45
52
11
15
13
*
60
A/G
DB
30
50
51

Fig. 2B

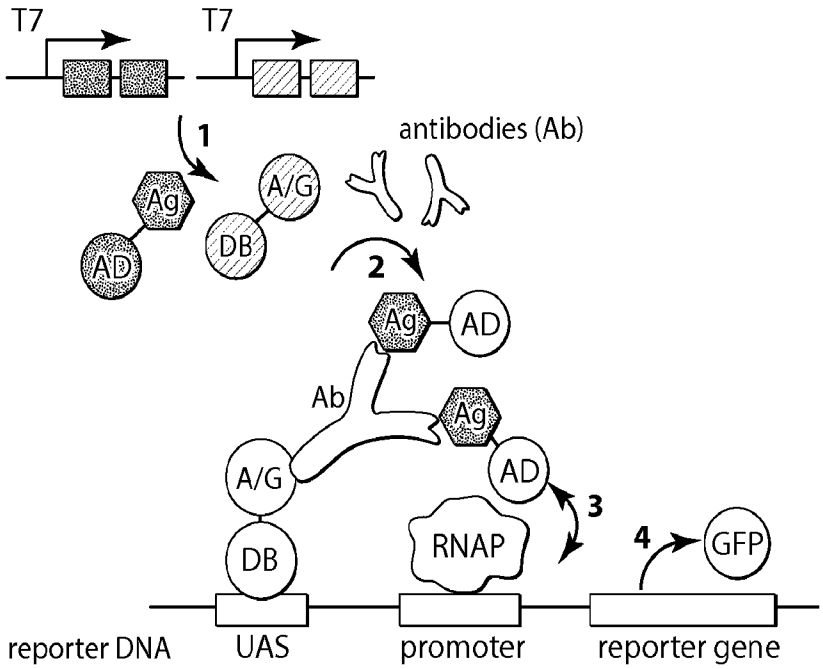
T7
T7
1
antibodies (Ab)
Ag
A/G
AD
DB
2
Ag
AD
Ab
Ag
A/G
AD
3
DB
RNAP
4
GFP
reporter DNA
UAS
promoter
reporter gene

Fig. 3A

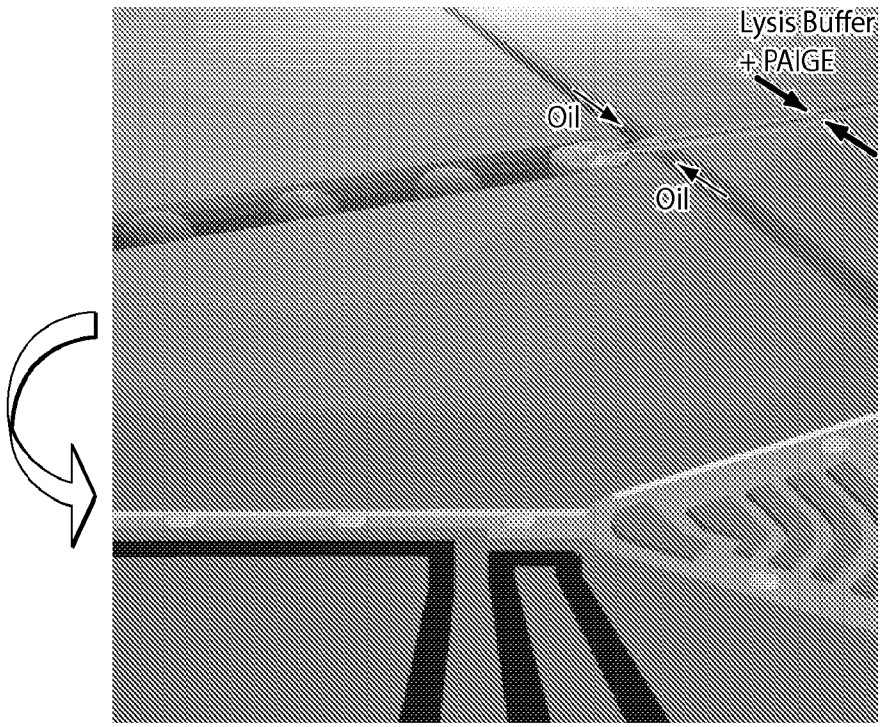
Lysis Buffer
+ PAIGE
Oil
Oil

Fig. 3B

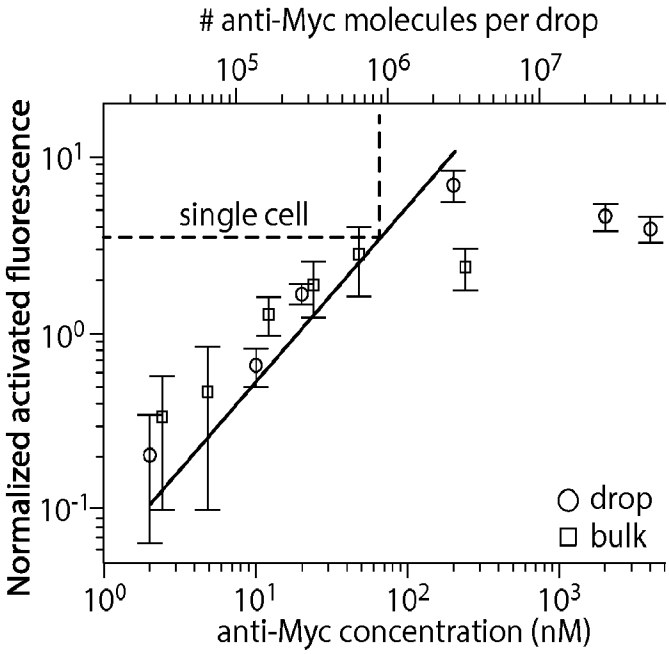
anti-Myc molecules per drop
10^5
10^6
10^7
Normalized activated fluorescence
10^1
10^0
10^{-1}
single cell
drop
bulk
10^0
10^1
10^2
10^3
anti-Myc concentration (nM)

Fig. 3C

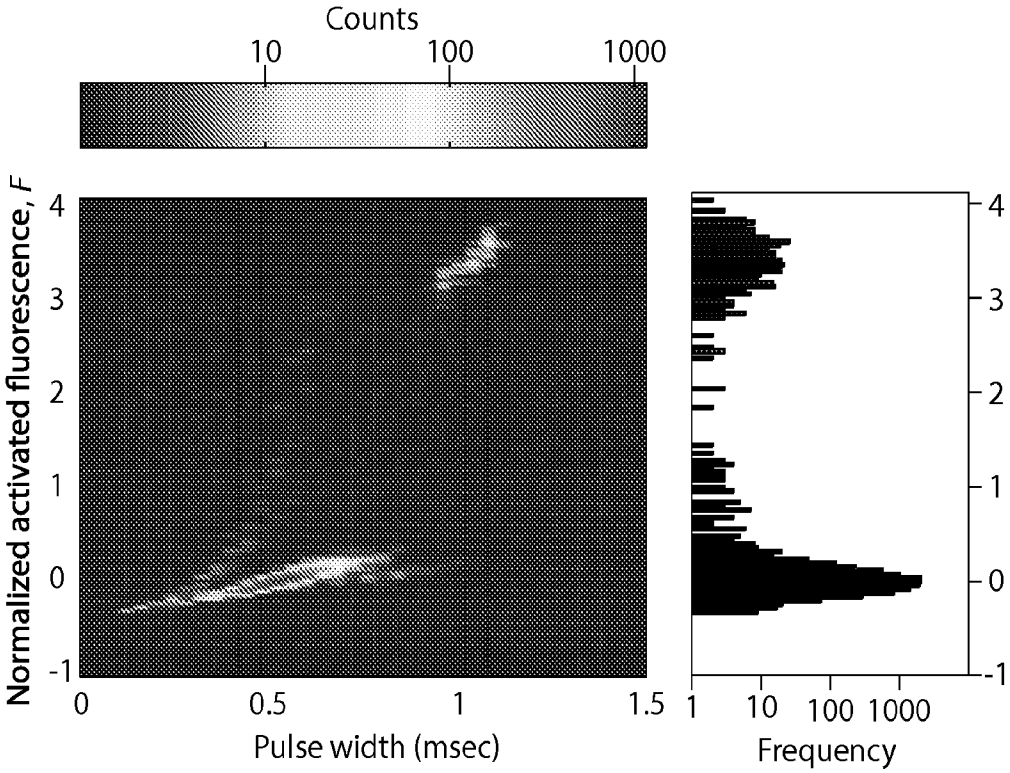
Counts
10
100
1000
Normalized activated fluorescence, F
4
3
2
1
0
-1
0
0.5
1
1.5
Pulse width (msec)
1
10
100
1000
Frequency

Fig. 3D

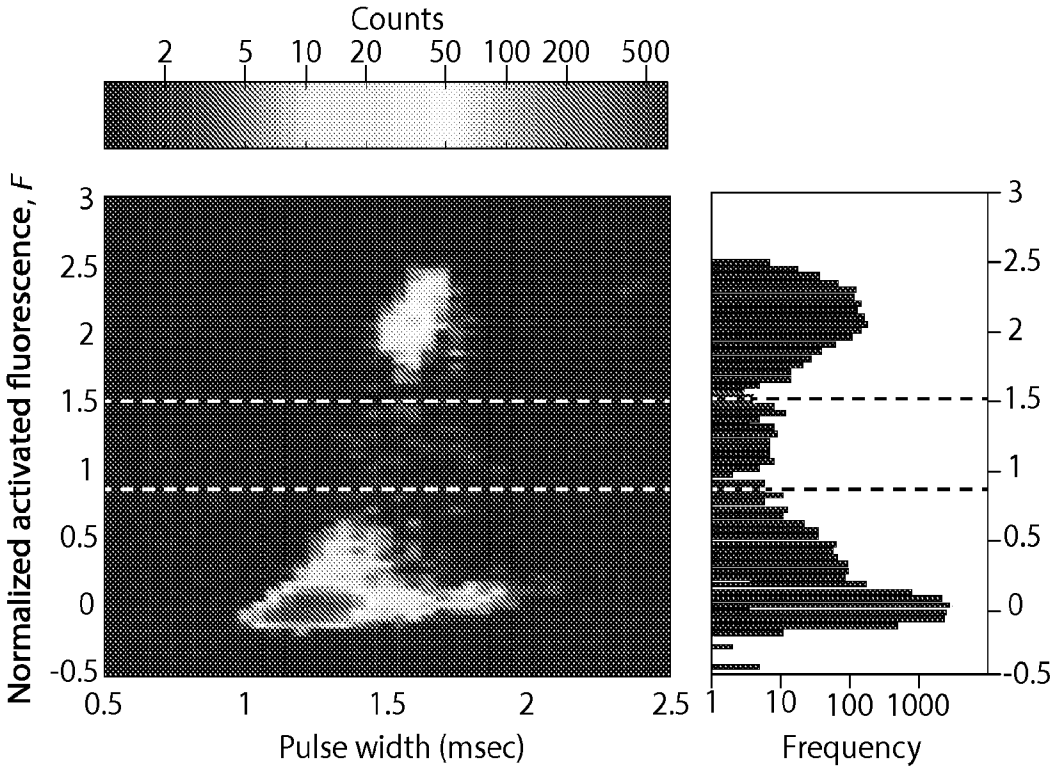
Counts
2 5 10 20 50 100 200 500
Normalized activated fluorescence, F
3
2.5
2
1.5
1
0.5
0
-0.5
0.5 1 1.5 2 2.5
Pulse width (msec)
1 10 100 1000
Frequency

Fig. 4A

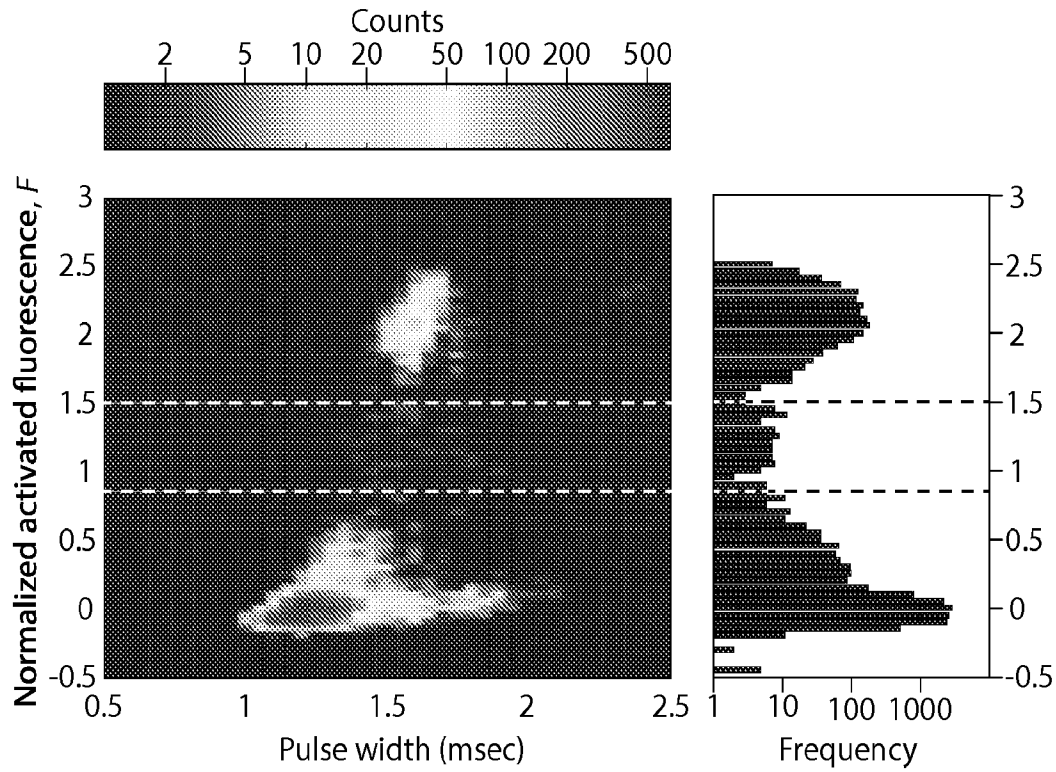
Counts
2 5 10 20 50 100 200 500
Normalized activated fluorescence, F
3
2.5
2
1.5
1
0.5
0
-0.5
0.5 1 1.5 2 2.5
Pulse width (msec)
1 10 100 1000
Frequency

Fig. 4B

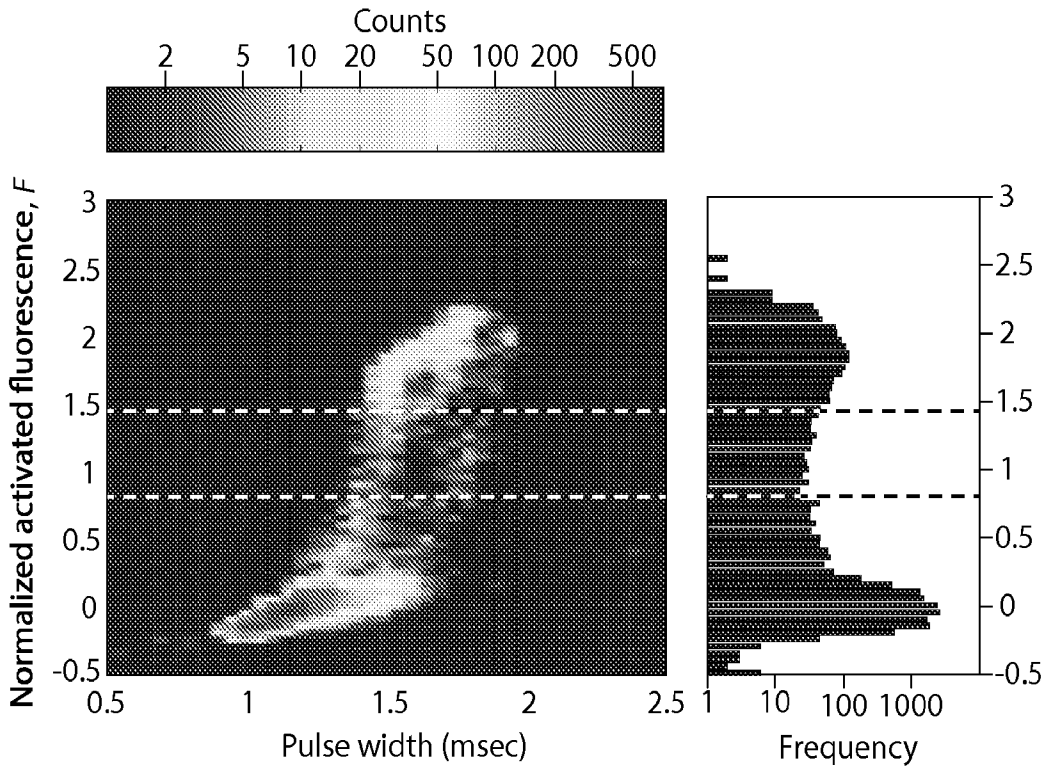
Counts
2 5 10 20 50 100 200 500
Normalized activated fluorescence, F
3
2.5
2
1.5
1
0.5
0
-0.5
0.5 1 1.5 2 2.5
Pulse width (msec)
1 10 100 1000
Frequency

Fig. 4C

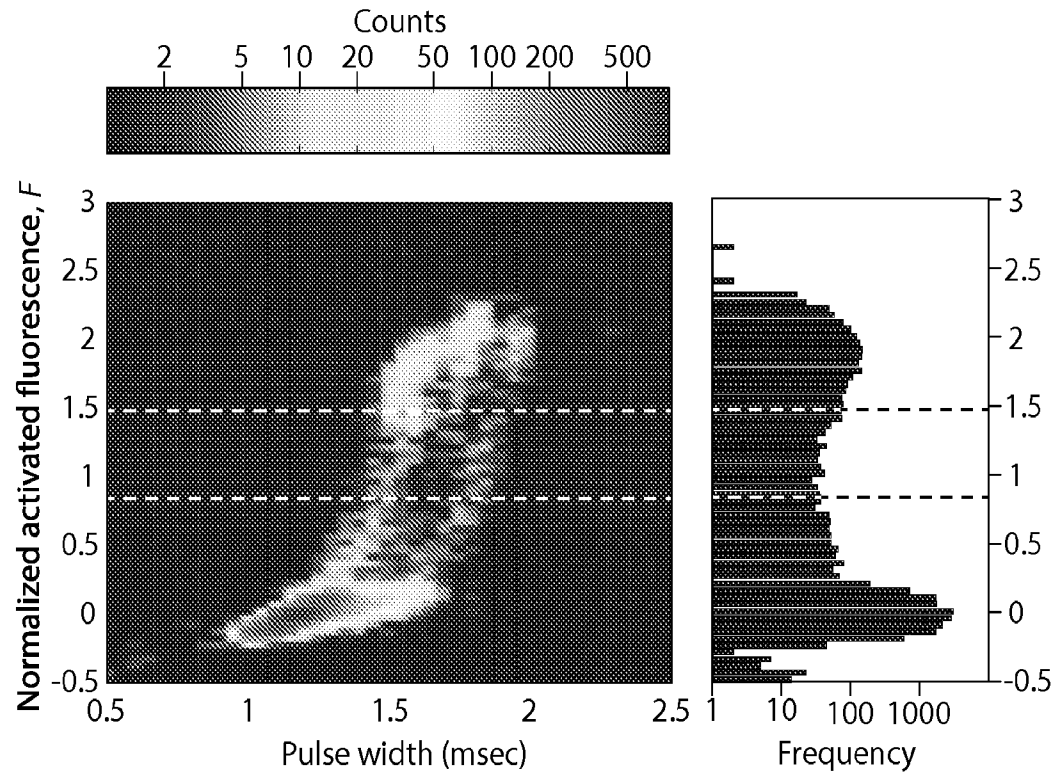
Counts
2 5 10 20 50 100 200 500
Normalized activated fluorescence, F
3
2.5
2
1.5
1
0.5
0
-0.5
0.5 1 1.5 2 2.5
Pulse width (msec)
1 10 100 1000
Frequency

Fig. 4D

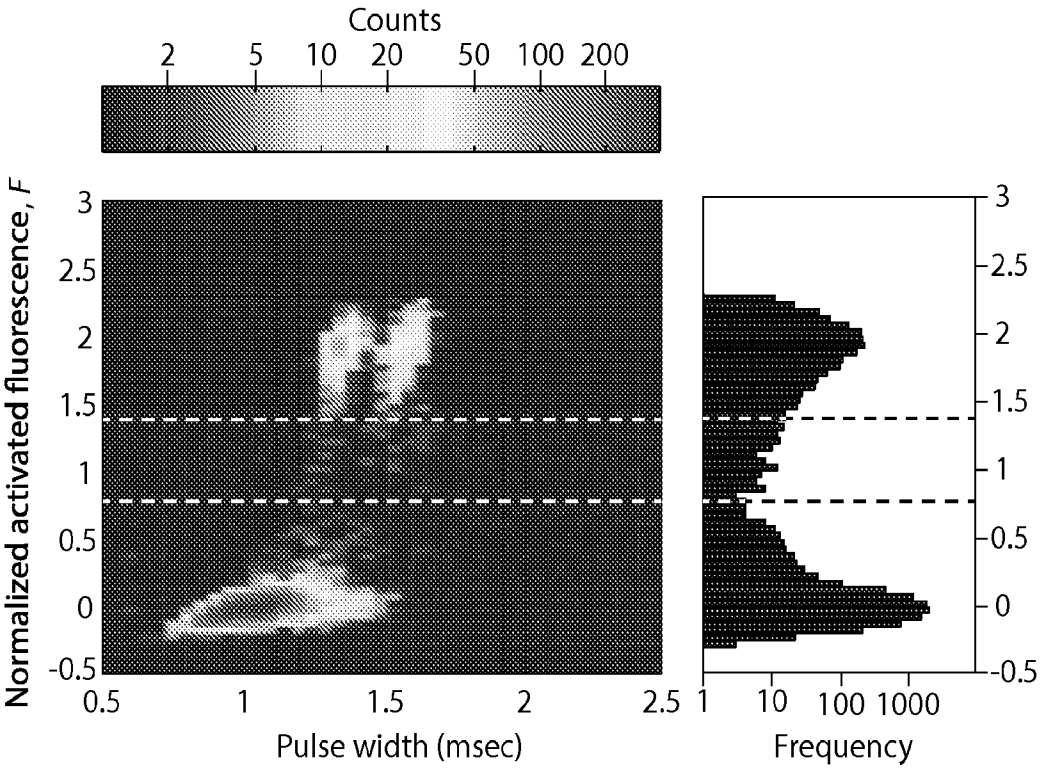
Counts
2 5 10 20 50 100 200
Normalized activated fluorescence, F
3
2.5
2
1.5
1
0.5
0
-0.5
0.5 1 1.5 2 2.5
Pulse width (msec)
1 10 100 1000
Frequency

Fig. 4E

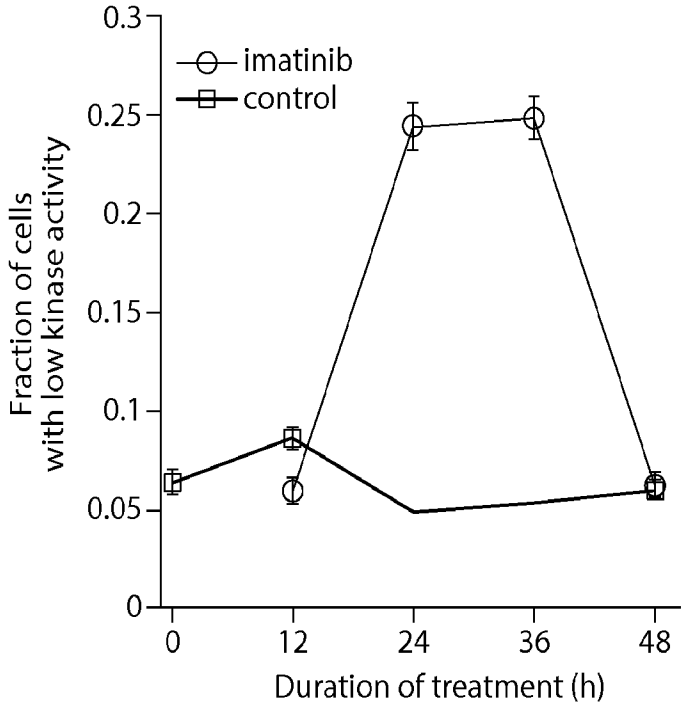
imatinib
control
Fraction of cells with low kinase activity
0.3
0.25
0.2
0.15
0.1
0.05
0
0 12 24 36 48
Duration of treatment (h)

Fig. 4F

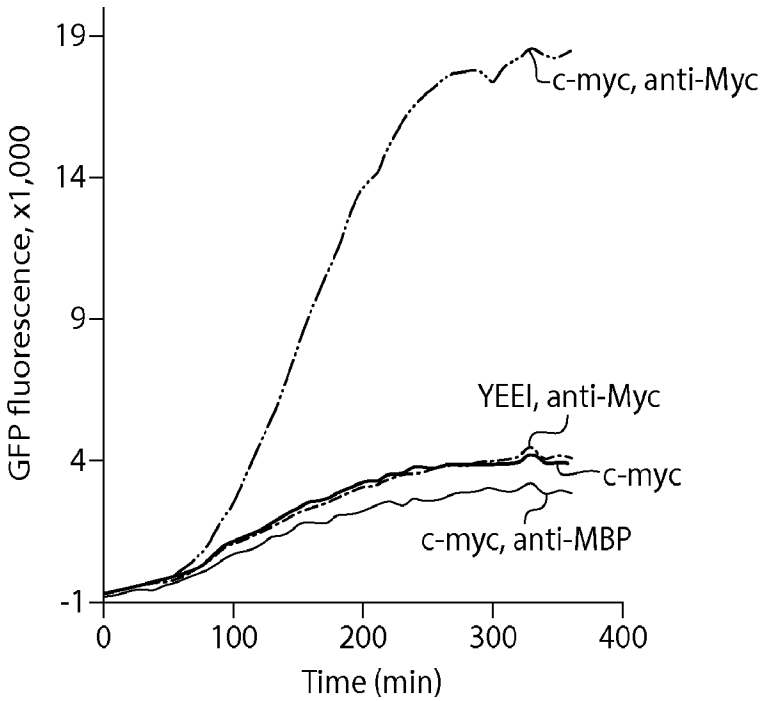
19
14
9
4
-1
GFP fluorescence, x1,000
0
100
200
300
400
Time (min)
c-myc, anti-Myc
YEEI, anti-Myc
c-myc
c-myc, anti-MBP

Fig. 5

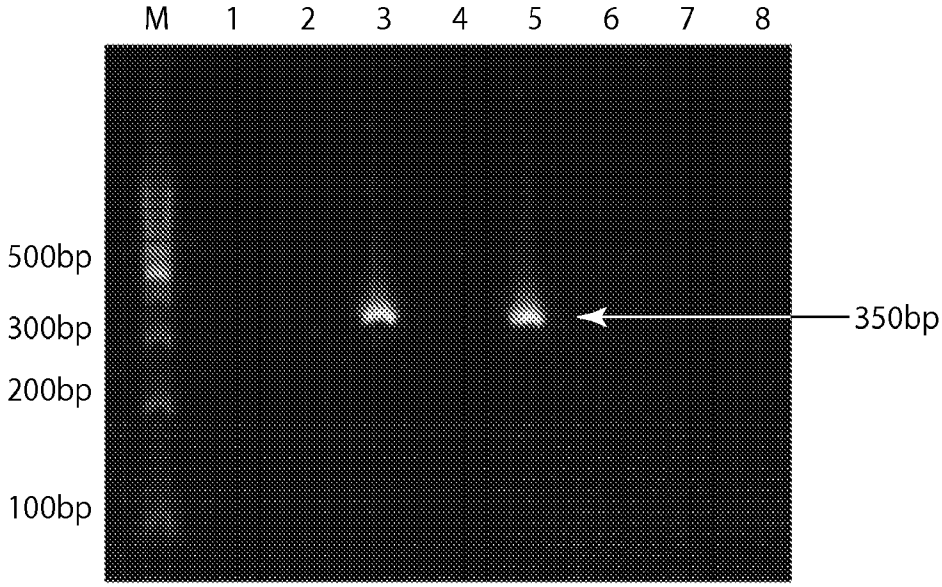
M
1
2
3
4
5
6
7
8
500bp
300bp
200bp
100bp
350bp

Fig. 6

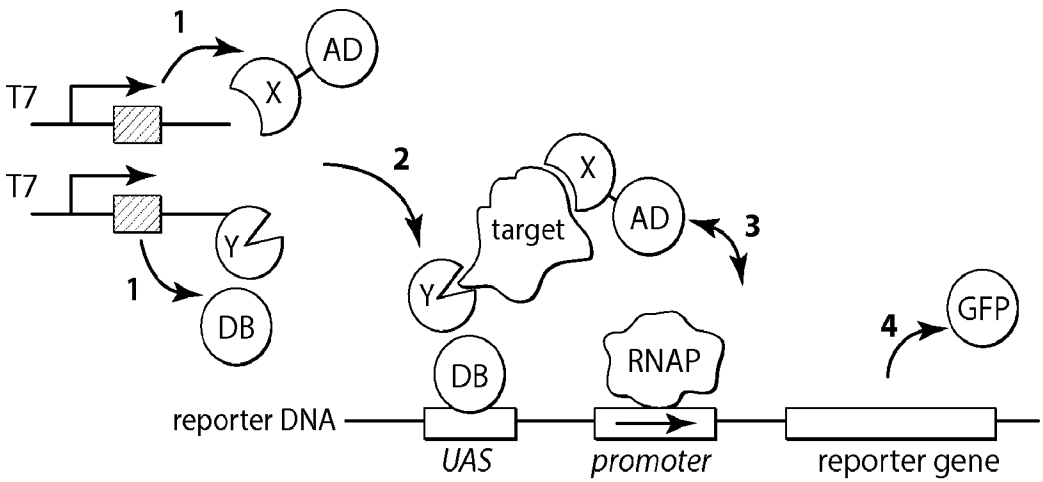
1
AD
X
T7
T7
Y
1
DB
2
X
target
AD
3
Y
DB
RNAP
4
GFP
reporter DNA
UAS
promoter
reporter gene

Fig. 7

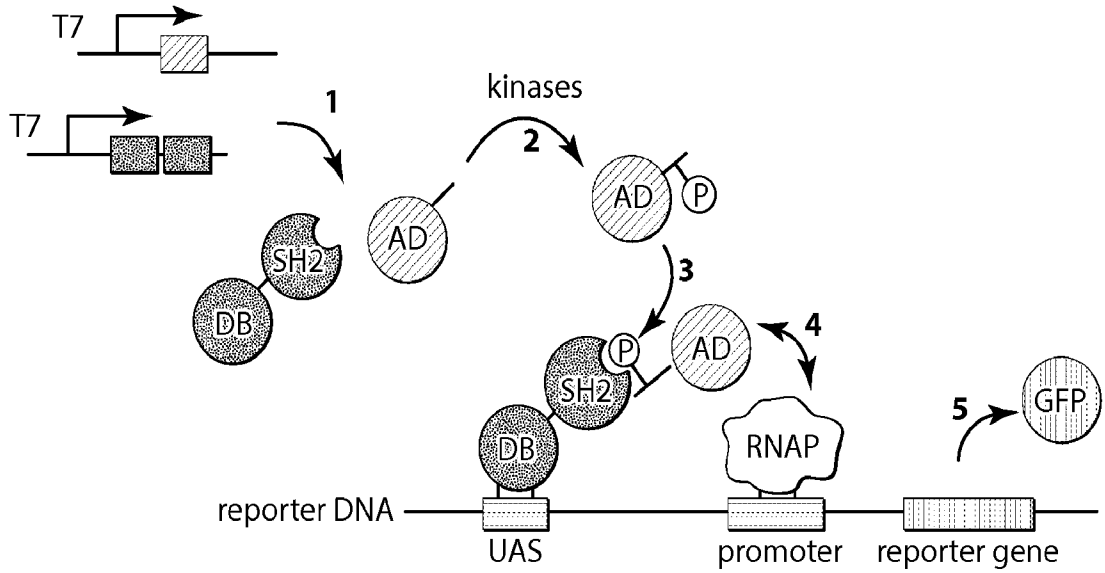
T7
T7
1
kinases
2
AD
P
SH2
DB
AD
3
P
AD
4
SH2
DB
RNAP
5
GFP
reporter DNA
UAS
promoter
reporter gene

Fig. 8A

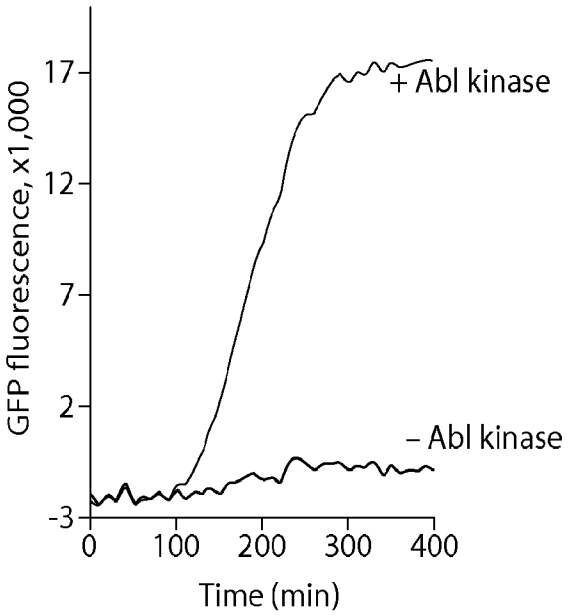
GFP fluorescence, x1,000
17
12
7
2
-3
0
100
200
300
400
Time (min)
+ Abl kinase
– Abl kinase

Fig. 8B

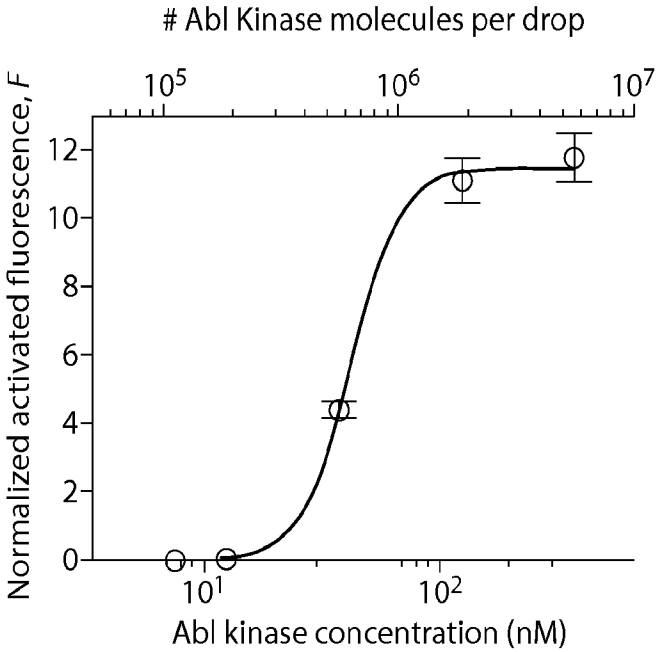
Abl Kinase molecules per drop
10^5
10^6
10^7
Normalized activated fluorescence, F
12
10
8
6
4
2
0
10^1
10^2
Abl kinase concentration (nM)

Fig. 8C

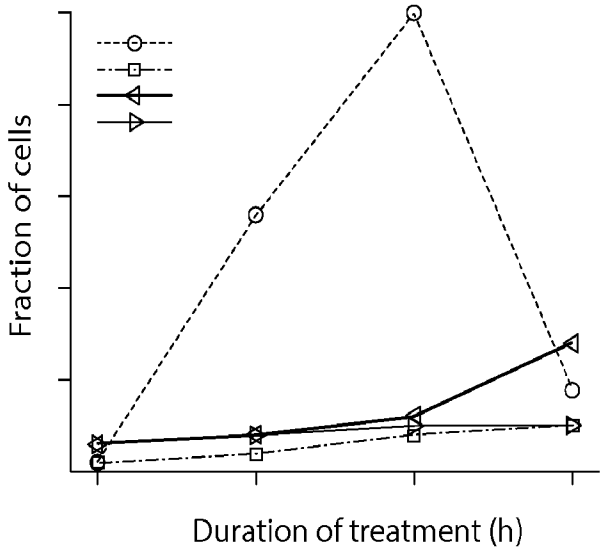

Fig. 9

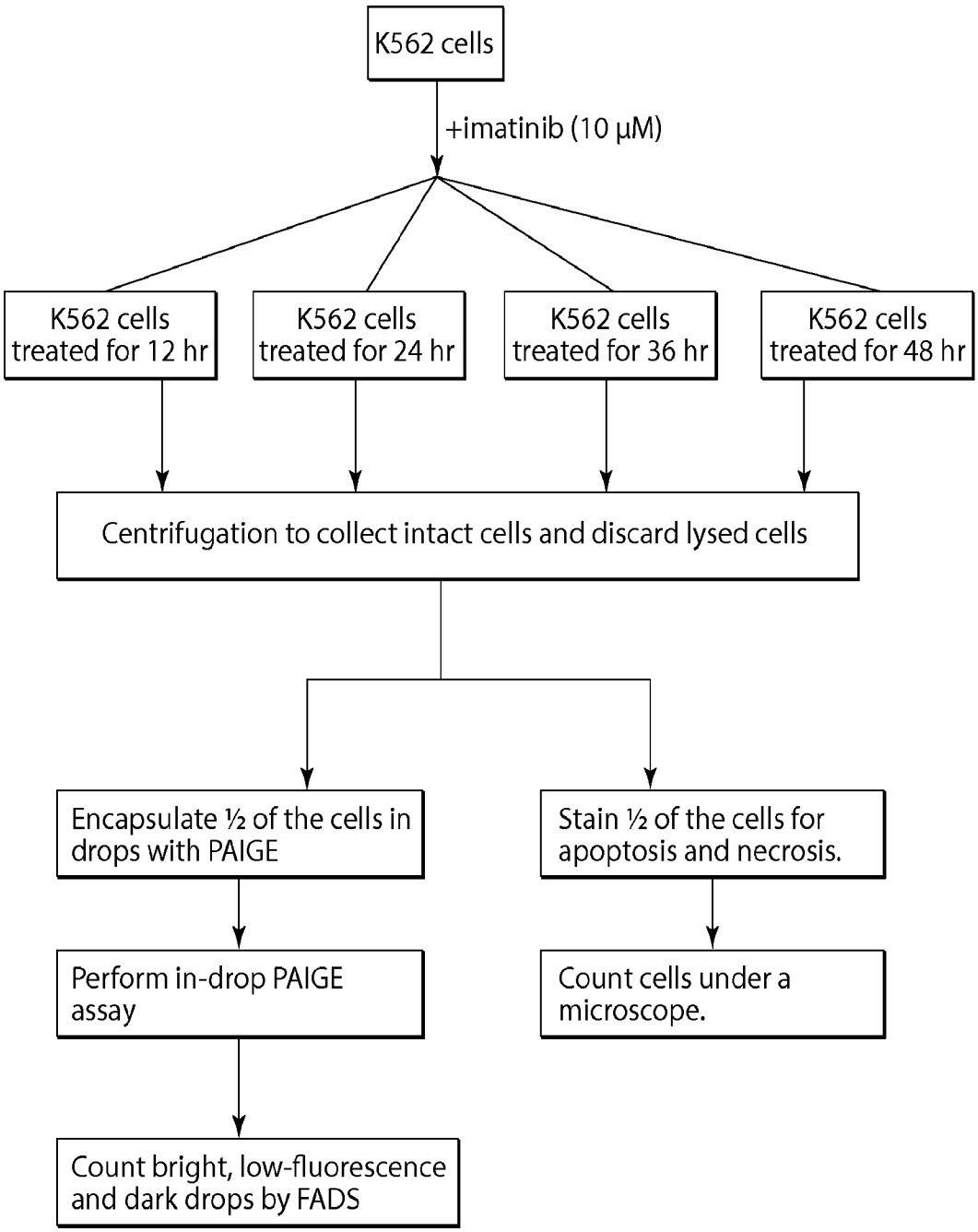
K562 cells
+imatinib (10 μM)
K562 cells treated for 12 hr
K562 cells treated for 24 hr
K562 cells treated for 36 hr
K562 cells treated for 48 hr
Centrifugation to collect intact cells and discard lysed cells
Encapsulate ½ of the cells in drops with PAIGE
Stain ½ of the cells for apoptosis and necrosis.
Perform in-drop PAIGE assay
Count cells under a microscope.
Count bright, low-fluorescence and dark drops by FADS

Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/33924

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/53; C12P 21/04; C40B 30/04, 40/10, 50/08 (2015.01)
CPC - C07K 2319/00, 2319/705, 2319/715; B01J 2219/00725; B01J 2219/0059
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)
CPC: C07K 2319/00, 2319/705, 2319/715; B01J 2219/00725; B01J 2219/0059

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C07K 2319/00, 2319/705, 2319/715; B01J 2219/00725; B01J 2219/0059 (text search)
USPC: 435.7.1, 69.1; 506/9,18, 27 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Scholar; Google Patents Search terms: Microfluidic, droplet, encapsulation, in vitro 2 hybrid system (IVT2H), protein interaction, fusion protein antigen, antibody, RNA polymerase, enhancer, transcription, GFP, reporter, antibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009/0170069 A1 (GHOSH et al.) 02 July 2009 (02.07.2009). Especially para [0086], [0124], sheet 8 fig 8.	1-3, 29-31, 55-57, 68-72, 79-81
A	MAZUITS et al. Single-cell analysis and sorting using droplet-based microfluidics. Nat Protoc May 2013 Vol 8 No 5 Pages 870-891. Especially abstract, pg 874 fig 4.	1-3, 29-31, 55-57, 68-72, 79-81
X,T	Abbaspourrad et al. Label-free single-cell protein quantification using a drop-based mix-and-read system. Sci Rep 3 August 2015 Vol 5 No 12756 Pages 1-7. Especially pg 3 fig 1A.	1-3, 29-31, 55-57, 68-72, 79-81
A,P	ZHOU et al. Engineering bacterial transcription regulation to create a synthetic in vitro two-hybrid system for protein interaction assays. J Am Chem Soc ePub 26 September 2014 Vol 136 No 40 Pages 14031-14038. Especially abstract, pg 14032 fig 1.	1-3, 29-31, 55-57, 68-72, 79-81
A	Chokkalingam et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. Lab Chip. 21 December 2013 Vol 13 No 24 Pages 4740-4747. Especially pg 4741 fig 1.	1-3, 29-31, 55-57, 68-72, 79-81

☐ Further documents are listed in the continuation of Box C. ☐

* 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 application or patent 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
13 August 2015 (13.08.2015)	03 SEP 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/33924

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.: 4-28, 32-54, 58-67, 73-78, 82-91
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.