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(71) Applicants: **CELL MICROSYSTEMS, INC.** [US/US];
 P.O. Box 13169, 2 Davis Drive, Research Triangle Park,
 NC 27709 (US). **THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL OFFICE OF COMMER-**

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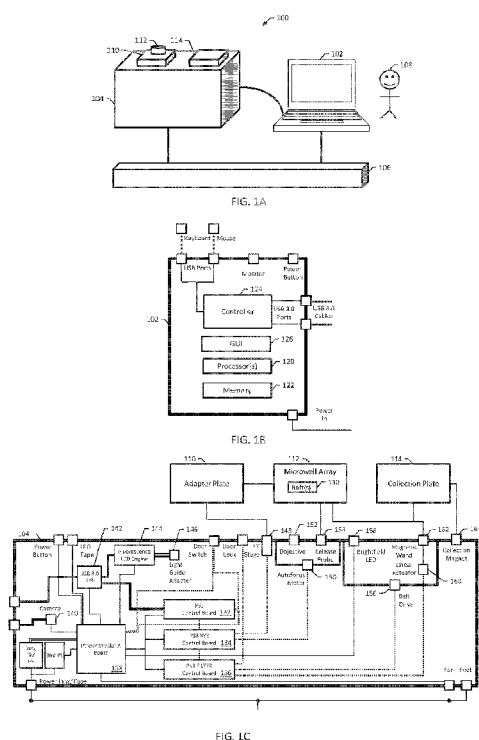
[—/US]; 109 Church St., Chapel Hill, NC 27516 (US).

(72) Inventors: **GEBHART, Steven, C.**; Cell Microsystems, Inc., 2 Davis Drive, Research Triangle Park, North Carolina, 27709 (US). **MCCLELLAN, Robert, W.**; Cell Microsystems, Inc., 2 Davis Drive, Research Triangle Park, NC 27709 (US). **TROTTA, Nicholas, C.**; Cell Microsystems, Inc., 2 Davis Drive, Research Triangle Park, NC 27709 (US). **ALLBRITTON, Nancy, L.**; Chapman Hall, Rm 241-cb#3216, The University of North Carolina, Chapel Hill, NC 27599-3216 (US). **ATTAYEK, Peter, Joseph**; 3312 Swansea St, Durham, NC 27713 (US). **ARMISTEAD, Paul, Michael**; 908 Chancellor's Ridge Drive, Durham, NC 27713 (US).

(74) Agent: **TAYLOR, Arles, A. Jr.**; Jenkins, Wilson, Taylor & Hunt, P.A., 3015 Carrington Hill Mill Boulevard, Suite 550, Morrisville, NC 27560 (US).

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(54) Title: AUTOMATED COLLECTION OF A SPECIFIED NUMBER OF CELLS



(57) Abstract: Embodiments of the disclosed subject matter provide an automated method and system to isolate and collect cells using computerized analysis of images of cells and their surroundings obtained from a digital imaging device or system. Embodiments of the disclosed subject matter make use of a "microwell array," which can comprise a formed, elastomeric grid of indentations or "wells." Many, most, or all of the wells in a microwell array can contain a releasable, microfabricated element, which can be referred to as a "raft." Embodiments of the disclosed subject matter provide a system and method for cell collection that includes computerized identification and collection of rafts with isolated single cells or a specific group or groups of cells, eliminating the need for continuous human identification and selection.

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AUTOMATED COLLECTION OF A SPECIFIED NUMBER OF CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/416,773 filed November 3, 2016, the disclosure of which is incorporated herein by reference in its entirety. This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/416,775 filed November 3, 2016, the disclosure of which is incorporated herein by reference in its entirety.
- 10 This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/430,094 filed December 5, 2016, the disclosure of which is incorporated herein by reference in its entirety. This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/526,177 filed June 28, 2017, the disclosure of which is incorporated herein by reference in its entirety.

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STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

 This invention was made with government support under grant number 2R42GM106421 awarded by the National Institutes of Health. The government has certain rights in the invention.

20

BACKGROUND

- The selection and isolation of single cells from a mixed population is a common procedure performed throughout biomedical research. For example, during
- 25 the development of cell lines that are genetically engineered, derived from stem cells, or grown from patient cell lines, single cells must be isolated and then cloned to form a homogeneous population. A variety of strategies exist to selectively identify and collect non-adherent cells from a mixed population, including fluorescence activated cell sorting (FACS), limiting dilution, panning, column chromatography and magnetic
- 30 sorting; furthermore, new techniques based on microfluidics and dielectrophoresis show promise in this area. To address the need to collect pure or enriched populations of adherent cells, investigators use these procedures by disaggregating or stripping the cells from their growth surface to create cell suspensions. Unfortunately, enzymatic or mechanical release imposes significant drawbacks including loss of cell

morphology, removal of cell surface markers, damage to cell membranes, alterations in cellular physiology and loss of viability.

To address this problem a cell isolation and recovery system was developed that uses polydimethylsiloxane (PDMS) microwell arrays comprising releasable, microfabricated elements, termed rafts, formed from biocompatible polystyrene or other materials. One version of such a system is called the CELLRAFT™ system. The rafts can be varied in size from tens to hundreds of microns to provide an adequate growth area for single cells or large colonies. The PDMS microwell array comprising the rafts can be visualized using, for example, an inverted microscope. Individual rafts containing the desired cells can be released from the array upon mechanical distortion of the microwell array, for example by the application of a gradual energy such as mechanical pushing or continuous vibration. In one example, individual rafts containing the desired cells can be visualized by a researcher or technician and then released from the array by mechanically pushing a probe attached to a microscope objective into the raft.

SUMMARY

Embodiments of the invention provide an automated method and system to isolate and collect cells using computerized analysis of images of cells and their surroundings obtained from a digital imaging device or system. Embodiments of the invention make use of a “microwell array,” which can comprise a formed, elastomeric grid of indentations or “wells.” Many, most, or all of the wells in a microwell array can contain a releasable, microfabricated element, which can be referred to as a “raft.” Embodiments of the invention provide a system and method for cell collection that includes computerized identification and collection of rafts with isolated single cells or a specific group or groups of cells, eliminating the need for continuous human identification and selection.

In some examples, a system is configured for releasing cell rafts from a microwell array. The system includes an imaging device configured for obtaining images of a microwell array, an actuator configured for releasing cell rafts from the microwell array, and a computer system comprising at least one processor and memory. The computer system is programmed for: obtaining one or more images of the microwell array using the imaging device; identifying, by analyzing the one or

more images of the microwell array, a selected cell raft; and controlling the actuator to release the selected cell raft from the microwell array.

The system can include one or more motors configured for moving the imaging device or the microwell array or both, and obtaining the one or more images
5 of the microwell array can include performing an array scan by: dividing the microwell array into a plurality of fields of view such that the fields of view, collectively, include each of the cell rafts; and for each field of view, controlling the one or more motors to orient the imaging device with the field of view of the microwell array and controlling the imaging device to obtain a respective image at the
10 field of view.

The system can include a microscope objective for the imaging system, and obtaining one or more images of the microwell array can include, for each field of view, autofocusing the microscope objective using one or more focus positions from at least one neighboring field of view. Autofocusing the microscope objective can
15 include sampling a plurality of sample images at a plurality of sample focus positions and interpolating a current focus position from focus scores of the sample images.

Identifying the selected cell raft can include counting, for each cell raft, a number of cells depicted in a sub-image of the cell raft and identifying at least one single-cell raft bearing a single isolated cell. Counting the number of cells depicted in
20 a sub-image comprises applying an intensity threshold to the sub-image to create a binary image, identifying unique objects in the binary image, and counting the number of identified unique objects. Identifying the single-cell raft can include determining a confidence score indicating a degree of confidence in the determination that the single-cell raft houses the single isolated cell.

Identifying the selected cell raft can include detecting, for each cell raft, a marker depicted in a sub-image of the cell raft, and gating the cell rafts based on detecting the marker. Identifying the selected cell raft can include assigning the selected cell raft to a mapped location of a collection plate. The system can include a mechanical cell raft collector, and the computer system can be configured for
25 controlling the mechanical cell raft collector to collect the selected cell raft after release from the microwell array and for controlling the mechanical cell raft collector to deposit the selected cell raft at the mapped location of the collection plate.
30

In some examples, a system is configured for monitoring a cellular process. For example, the system can be configured to perform a time-course analysis of a cell

or group of cells, e.g., for a drug or reagent challenge, or for changes in gene expression or cellular conditions such as cytotoxicity, cell growth, or metabolic state. The system includes an imaging device configured for obtaining images of a microwell array, the microwell array including cell rafts in microwells of the microwell array. The system includes a computer system comprising at least one processor and memory. The computer system is configured for at a first time, determining, using the imaging device, that at least one cell raft contains one or more cells undergoing a cellular process. The computer system is configured for, in response to determining that the at least one cell raft contains the one or more cells undergoing the cellular process, storing a location of the at least one cell raft on the microwell array. The computer system is configured for, at one or more times later than the first time, monitoring the cellular process by locating the at least one cell raft using the location and obtaining, using the imaging device, one or more images of the at least one cell raft.

Monitoring the cellular process can include storing the one or more images and associating each of the one or more images with a respective capture time of the image and the location of the at least one cell raft. Monitoring the cellular process can include presenting, in a graphical user interface, the one or more images and an initial image of the at least one cell raft.

Determining that the at least one cell raft contains one or more cells undergoing a cellular process can include: illuminating the at least one cell raft with light of a specified wavelength; in response to illuminating the at least one cell raft, obtaining, using the imaging device, an initial image of the at least one cell raft; and detecting a fluorescence signature of the cellular process in the initial image.

Monitoring the cellular process can include presenting, in a graphical user interface, a graph of fluorescence intensity in the one or more images over time.

Determining that the at least one cell raft contains one or more cells undergoing a cellular process can include determining that the at least one cell raft is a single-cell raft containing only a single cell and quantifying a baseline fluorescence level using the initial image. Monitoring the cellular process can include determining difference in measured fluorescence between the one or more images and the initial image.

In at least some embodiments, a computer-implemented method of collecting cells includes storing information about a microwell array containing a plurality of

rafts, and identifying, one or more cell rafts from among the plurality of rafts. A system like that described herein can be used to collect single-cell rafts. A single-cell raft is a raft with one isolated cell associated therewith, and often these are the cell rafts that would be selected. However, a system according to embodiments of the invention can also or instead be used to isolate rafts with some specified number of cells other than a single cell, or as another example, a raft with a single cell of one type and multiple cells of another type or a combination of specific numbers of cells of differing types. The system can then release a cell raft from the microwell array by controlling and using an actuator, and collect the cell raft using, as an example, a magnet. The system can optionally confirm a raft release prior to moving on to the next raft. Information about the microwell array can be stored in a memory device associated with or connected to a processor that executes the computer-implemented method.

In some embodiments, the storing of the information about the microwell array includes identifying and storing a size of the microwell array, identifying and storing an optimal focus position and an optimal exposure for the microwell array, and sectioning the microwell array and storing a translation required to match a set of microwells to a field of view. In some embodiments, the storing of the information about the microwell array includes calculating and storing an offset for the actuator.

In some embodiments, the identifying of the cell raft includes segmenting the plurality of rafts of the microwell array and counting the cell nuclei per raft for the plurality of rafts. In some embodiments the counting includes performing a watershed transform to define the cell nuclei relative to a fluorescence threshold. In some embodiments, the identifying of the cell rafts to be released further includes gating, or determining which of, the cell rafts are to be released by detection of a marker. As examples, a marker may include an intensity of one or more color channels (which may be indicative of fluorescence caused by a stain) and/or a size of the cell nuclei, the cell itself, or a structure other than the nucleus within the cell.

A computer system such as a workstation, personal computer, handheld computer, or embedded processing system can serve as part of the system and/or be connected to the imaging device, system and/or hardware, actuator and other equipment, and include or access non-transitory computer program code, which when executed by a processor, causes the system to execute all or a portion of the process

according to example embodiments of the invention. The computer program code can be stored in a storage device.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figures 1A-1C are diagrams of an example system for cell collection.

 Figures 2A-2B are isometric views of an example apparatus for cell collection.

 Figures 3A-3B illustrate an example method for cell collection.

 Figures 4A-4E are workflow diagrams showing interactions between user inputs, graphical user interface functions, hardware, and software for a system
10 according to example embodiments of the invention.

 Figure 5 is a flow diagram of an example method for determining an array position and type for a microwell array.

 Figure 6 is a flow diagram of an example method for navigating a microwell array.

15 Figures 7A-7C illustrate an example method for autofocusing a microscope lens on a field of view of a microwell array.

 Figure 8 is a flow diagram of an example method for identifying single-cell rafts in a microwell array.

 Figure 9 is a screen shot of an example screen of a graphical user interface for
20 selecting cells rafts and mapping a collection plate.

 Figure 10 is a screen shot of another example screen of the graphical user interface.

 Figures 11A-11I are a block diagram (Figure 11A) and a series of images (Figures 11B-11I) illustrating an example experiment.

25

DETAILED DESCRIPTION

Embodiments of the present invention now will be described more fully hereinafter with reference to the accompanying drawings, in which embodiments of the invention are shown. This invention may, however, be embodied in many
30 different forms and should not be construed as limited to the embodiments set forth herein. Terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the

terms “comprises” or “comprising,” when used in this specification, specify the presence of stated features, steps, operations, elements, or components, but do not preclude the presence or addition of one or more other features, steps, operations, elements, components, or groups thereof. Additionally, comparative, quantitative
5 terms such as “above”, “below”, “less”, “more”, are intended to encompass the concept of equality, thus, “less” can mean not only “less” in the strictest mathematical sense, but also, “less than or equal to.”

Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill
10 in the art to which this invention belongs. It will be further understood that terms used herein should be interpreted as having a meaning that is consistent with their meaning in the context of this specification and the relevant art and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein. It will also be understood that when an element is referred to as being “connected” or
15 “coupled” to another element, it can be directly connected or coupled to the other element or intervening elements may be present.

Single cell separation can be useful for a wide variety of scientific studies including for molecular analysis of individual cells (e.g., DNA, RNA, protein) and for obtaining colonies by clonal propagation from individual cells including cells after
20 transfection. A microwell array comprising a plurality of cell rafts in each well (a “microwell array”) has been shown to be useful in single cell isolation and collection. Separating single cells by isolation and collection from microwell arrays using manual methods with a standard microscope can take a considerable amount of time when seeking to obtain a large number of single cells for analysis or propagation.
25 Details on microwell arrays with cell rafts that can be used with a system and method of embodiments of the invention are described in U.S. Patent 9,068,155, which is incorporated herein by reference. An automated system that collects cells from microwell arrays that have been seeded with cells of interest can reduce the time and labor for conducting scientific and medical studies with single cells while also
30 including additional features such as imaging across multiple fluorescence channels.

A system for collecting cells, according to example embodiments of the invention includes an actuator, an imaging device, and a processor connected to the actuator and the imaging device, the processor being operable by use of stored executable computer program code to perform computer-implemented methods, e.g.,

as shown in Figures 3A-3B, 4A-4E, 5-6, 7A, and 8. The method and system of embodiments of the invention is presented in the figures by way of example as identifying single, isolated cells present on individual cell rafts in the microwell array, and collecting those cells by way of collecting single-cell rafts.

5 Figures 1A-1C are diagrams of an example system **100** for cell collection. The system **100** can be used to identify and collect multiple cells, cell colonies and rafts with various numbers of cells of different types. Figure 1A is an overview diagram of the system **100**. The system **100** includes a computer system **102**, an instrument assembly **104**, an experimental environment **106** (e.g., one or more pieces
10 of laboratory equipment such as power supplies and environmental control systems), and a user **108**. The instrument assembly **104** includes an optional adapter plate for receiving a microwell array **112** and a collection plate **114** for receiving cell rafts that have been selected and released from the microwell array **112**. The collection plate **114** can be organized into a standardized format, e.g., as an SBS collection plate.
15 Although a collection plate **114** is shown, the system **100** can alternatively use any appropriate collection structure, such as PCR strip tubes.

Typically, the user **108** would load or seed cells in media on the microwell array **112** and allow the cells to settle into individual cell rafts. The microwell array **112** is then placed into the adapter plate **110** of the system **100** for scanning and
20 image analysis. The system **100** can then release a cell raft from the microwell array **112** by controlling and using an actuator, and collect the cell raft with the isolated cell using, as an example, a magnet. For example, the actuator can be one or more motors configured to move a needle or similar device to release rafts. In some examples, the system **100** includes multiple actuators, including, possibly another
25 actuator to move a magnetic wand, and possibly actuators to move a stage, imaging optics, and other mechanical parts of the system.

Figure 1B is a block diagram of the computer system **102**. The computer system **102** includes at least one processor **120**, memory **122**, a controller **124** implemented as a computer program using the processor **120** and memory **122**, and a
30 graphical user interface (GUI) **126**. For example, the computer system **102** can be a desktop computer with a monitor and keyboard and mouse, or the computer system **102** can be a laptop or tablet computer or any other appropriate device. The computer system **102** is operatively coupled to the instrument assembly **104**, e.g., by universal serial bus (USB) cables.

The controller **124** is programmed for obtaining one or more images of the microwell array **112**; identifying, by analyzing the images, a selected cell raft of the microwell array **112**; and controlling the instrument assembly **104** to release the selected cell raft. The controller **124** can perform the example methods described
5 further with references to FIGS. 3A-3B, 4A-4E, 5-6, 7A, and 8. The GUI **126** is configured to present various control and results screens to the user **108** and to receive input from the user **108**.

Figure 1C is a block diagram of the instrument assembly **104**. The instrument assembly **104** can include various components for imaging individual cell rafts **130** on
10 the microwell array **130** and selectively releasing cell rafts **130** from the microwell array for placement into the collection plate **114**. For example, the instrument assembly **104** can include a power breakout board **138** and various control boards for controlling motors and actuators (e.g., PS3 control board **132**, PS3 XYZ control board **134**, and PS3 FILTER control board **136**). The motor control boards can contain TTL
15 and shutter functions that allow the controller **124** to control or address various components of the instrument assembly **104**.

The instrument assembly **104** can include a digital camera **140** or other appropriate imaging device, a communications hub (e.g., USB Hub **142**), a fluorescence light emitting diode (LED) engine **144**, and a light guide adapter **146**.
20 The fluorescence LED engine **144** can include multiple narrow-band LEDs configured to illuminate the microwell array **112** by way of the light guide adapter **146**.

The instrument assembly **104** includes a microscope system (e.g., an internal inverted digital microscope) including a motorized XY stage **148** and an autofocus
25 motor **150** configured for translating a microscope objective **152**. Typically, the camera **140** and the fluorescence LED engine **144** and microscope system are arranged in an epi-fluorescence configuration. In some examples, the microscope system includes a release probe **154** configured for individually releasing cell rafts **130** from the microwell array **112**. The release probe **154** can be actuated by the
30 autofocus motor **150**.

In some examples, the microscope system supports imaging of a region at least about 4 X 4 array features on a microwell array having rafts of 200 micron x 200 micron in dimension and approximately 8 X 8 array features if the rafts have a 100 micron x 100 micron array in a given field of view at a resolution of less than 2

microns per pixel. The microscope system may also support the capture of images using brightfield imaging (i.e. white light illumination and white light emission) and the capture of images in one or more fluorescent emission channels. In some examples, the instrument assembly **104** is capable of scanning an entire microwell array in under 20 minutes for all three fluorescent channels and brightfield assuming an exposure time 750 ms across all channels.

The release probe **154** typically comprises materials resistant to oxidation when exposed to saline or cell culture media. In some examples, the release probe **154** is a stainless steel 100 micron needle. The release probe **154** can have a possible travel distance of, e.g., at least 15 mm in the X and Y directions with respect to the center of the microwell array **112**.

The instrument assembly **104** includes a gantry assembly including a belt drive **156** for moving the gantry assembly, a brightfield LED **158** for illuminating the microwell array **112** during imaging, and a linear actuator **160** configured for moving a magnetic wand **162** to collect cell rafts after release. The gantry assembly can alternatively use a lead screw instead of a belt drive, or any other appropriate motor. The linear actuator **160** can be, e.g., a stepper motor configured to raise and lower the magnetic wand **162** into and out of the microwell array **112** and the collection plate **104**.

The instrument assembly **104** includes a collection magnet **164** positioned underneath the collection plate **114** to collect cell rafts into the collection plate **114** from the magnetic wand **162**. The collection magnet **164** can have a polarization opposite that of the magnetic wand **162** to repel the magnets within the magnetic wand **162** and pull the cell raft to the bottom of the collection plate **104**. The magnetic wand **162** typically comprises a material that is capable of being rendered sterile (e.g., rinsed with ethanol or isopropanol while removed from the instrument) so as not to contaminate the released cell raft or the media used in the collection plate **104**. The material for the magnetic wand **162** is also generally selected such that contact with the culture media does not cause any detectable decrement in cell viability or proliferation, or in the performance of molecular biology reagents, such as Taq polymerase, or reverse transcriptase.

Figures 2A-2B are isometric views of an example apparatus for cell collection. The apparatus is an example implementation of the instrument assembly **104** depicted in Figure 1C. As shown in Figure 2A, the adapter plate **110** and the collection plate

114 are positioned on top of the horizontal XY stage **148**. Some components, such as the electronic control boards **132**, **134**, and **136** are located below the XY stage **148**. The XY stage **148** is configured to move the microwell array **112** and the collection plate **104**. The XY stage **148** is electronically controllable for positioning cell rafts
5 for imaging (aligning cell rafts with the microscope objective **152**), releasing cell rafts (aligning cell rafts with the release probe **154**), and depositing cell rafts (aligning the magnetic wand **162** and selected locations of the collection plate **104** over the collection magnet **164**).

The gantry assembly, including the belt drive **156**, is positioned vertically over
10 the XY stage **148**. The gantry assembly is configured to move laterally to position the brightfield LED **158** for imaging and also to position the magnetic wand **162**. The gantry assembly positions the magnetic wand **162** over the microwell array **112** to collect rafts during release, and then the gantry assembly positions the magnetic wand **162** over the collection plate **114** to deposit cell rafts into selected locations of the
15 collection plate **114**.

The camera **140** and the autofocus motor **150** are located beneath the XY stage **148**, e.g., so that the autofocus motor **150** can move vertically with respect to the XY stage **148**. The fluorescence LED engine **144** and liquid light guide ports **146** are located below the XY stage **148** and coupled to a fluorescence filter cube **170**. The
20 fluorescence filter cube **170** is configured for fluorescence imaging, e.g., to allow light from the fluorescence LED engine **144** to reach the microwell array **112** and to block that light from reaching the camera **140**.

Figure 2B shows a cut-away view **172** of the microscope objective **152** and the release needle **154**. As shown, the release probe **154** is offset from the optical axis of
25 the microscope objective **152**, such that the release probe **154** does not intersect the field of view of the microscope objective **152**. With the release probe **154** not intersecting the field of view, a user may not be able to visualize release of a cell raft in real time, such that the system may have to move the microwell array **112** after release to confirm release by imaging. Nonetheless, it can be useful to position the
30 release probe **154** outside the field of view of the microscope objective **152** to improve imaging speed.

In some other examples, the release probe **154** is located within the field of view of the microscope objective **152**. Locating the release probe **154** in the field of view of the microscope objective **152** allows a user to visualize the release of a cell

raft in real time; however, such a location can require imaging through the acrylic window, which can reduce the transmission of the excitation and emission light and require longer integration times during scanning.

5 In either case, it can be useful to calibrate the offset between the center of the field of view of the microscope objective **152** and the puncture location of the release probe **154** on the microwell array **112**. Calibration can be performed, e.g., after every needle replacement, or at the start of every experiment, or one time during manufacturing. In some examples, the controller **124** of Figure 1B is programmed to perform automated calibration.

10 For example, the controller **124** can move the microwell array **112** to position the field of view of the microscope objective **152** with an array border, autofocus the microscope objective, and then puncture the array border with the release probe **154**. Then, the controller **124** moves the microwell array **112** to position the puncture location within the field of view of the microscope objective **152**, acquires an image
15 (e.g., using the brightfield LED **158**), and analyzes the image to locate the puncture position, e.g., by segmenting the image. The controller **124** can then calculate an offset. In some examples, the controller **124** repeats the process a specified number of times by moving to different locations and determines a calibration distance based on the offset positions, e.g., by averaging the offset positions.

20 Figures 3A-3B illustrate an example method for cell collection. Figure 3A is a flow diagram of the example method **300** for cell collection from a microwell array. The method **300** can be performed by the controller **124** of Figure 1, and the method **300** will be described with respect to the system **100** of Figure 1.

The method **300** includes initializing the system (**302**) and setting up an
25 experiment (**304**), which are described further below with reference to Figures 4A-4B. The method **300** includes determining whether to perform an array scan (**306**), e.g., by prompting the user **108** using the GUI **126**. If not performing an array scan, the method **300** includes navigating the microwell array **112** by user commands (**308**), e.g., received using the GUI **126**. Navigating by user commands can include
30 presenting images of the microwell **112** array in real-time as the user navigates the microwell array **112**. The method **300** then includes receiving a user selection of one or more cell rafts for cell collection (**310**), e.g., selected using the GUI **126**.

If performing the array scan, the method **300** includes performing the array scan by navigating the microwell array **112** without user input (**314**). The method **300**

then includes selecting one or more cell rafts for cell collection using image analysis (316). Figure 3B is a flow chart of an example method 350 for selecting cell rafts using image analysis. The method 350 includes detecting single cell rafts (352), gating the single cell rafts based on marker detection (354), and mapping the gated
5 single cell rafts to locations on a collection plate (356). Gating the single cell rafts generally includes selecting a subset of detected single cell rafts based on detection of fluorescence markers.

Array scanning, single-cell detection, and automated or semi-automated cell raft selection are described further below with reference to Figures 4C-4D. In some
10 examples, the array scan and identifying the cell rafts are performed in parallel. For example, the controller 124 can execute multiple threads in parallel, or use any appropriate parallel processing technique.

The method 300 includes releasing and transferring the selected cell rafts (312). Release and transfer are described further below with reference to Figure 4E.
15 The method 300 can optionally include exporting data describing an experiment, e.g., user input received, images captured, locations of detected single-cell rafts, records of released cell rafts, and any other appropriate data. Exporting data can include storing the data in a file on a local filesystem or transmitting the data to a remote system for storage.

As can be seen in Figures 3A-3B, a system according to example
20 embodiments can be used in a manual selection mode where the user identifies single cell rafts during real-time imaging (“real time imaging mode”) which may include storing such images in a memory device, but can also be used for a computer-implemented method of collecting cells, referred to as a “cytometric image analysis”
25 mode. In accordance with some embodiments, the user 108 selects through the GUI 126 to operate the system 100 in cytometric image analysis mode or real-time imaging mode. In real-time imaging mode, the user 108 is able to selectively navigate the imaging field of view within the microwell array 112 and select rafts for isolation based on visual inspection of real-time images. In cytometric image analysis mode, a
30 full scan of the microwell array is performed, and then quantitative image processing (e.g., of the fluorescence images) facilitates user selection of cells for isolation or automated selection of cells for collection.

In the cytometric image analysis mode, the system scans a microwell array and stores, in a memory device, information about a microwell array containing a

plurality of rafts, identifies, using a processor to perform image analysis and using the information, a cell raft having one or more cells from among the plurality of rafts, and releases, using the processor and an actuator, the cell raft from the microwell array. In some embodiments, the storing of the information about the microwell array
5 includes calculating and storing an offset for the actuator. In some embodiments, the system determines an address for each of a plurality of the microwells in the microwell array in order to expedite future operations by relying on known positions of specific rafts. The system can perform gating of the cell rafts to be released by the actuator based on detection of a marker, for example, based on intensity of a
10 fluorescent color or brightfield channel as determined from the imaging device, a size of a cell nucleus or other structure, or a combination of these markers. In some embodiments, the system collects cell rafts containing magnetic nanoparticles using a magnet and can optionally confirm that the cell raft has in fact been released.

Figures 4A-4E are workflow diagrams showing interactions between user
15 inputs, graphical user interface functions, hardware, and software for a system according to example embodiments of the invention. The workflow diagrams will be described with reference to the example system **100** of Figure 1. Each workflow diagram is horizontally divided into four regions. The top-most region shows actions performed by the user **108**. The region below the top-most region shows actions
20 carried out using the GUI **126**. The next region shows actions carried out using hardware (e.g., the instrument assembly **104**). The bottom-most region shows actions performed by the controller **124**.

Figure 4A illustrates the beginning of an example experiment. The user **108** retrieves cells, e.g., from an incubator and releases the cells from a plate. The user
25 **108** pre-treats a microwell array **112** and seeds cells on the microwell array **112**. The user **108** powers the instrument assembly **104** and the computer system **102** and launches the controller **124** and the GUI **126**. During initialization, the GUI **126** can display an initialization splash screen, while the instrument assembly **104** sets camera parameters and moves motors to home and then load positions. The controller **124**
30 initiates and verifies communication with the instrument assembly **104**, including the motor controllers, LED illuminator, and camera.

Figure 4B illustrates the next stage of the example experiment, experimental setup. The GUI **126** prompts the user **108** to place the microwell array **112** on the microscope stage. The user **108** places the microwell array **112** on the XY stage **148**

(e.g., onto the adapter plate **110**). The GUI **126** prompts the user **108** to select either a cytometric mode or a real-time analysis mode, receives a selection from the user **108**, and then displays a screen indicating the system is calibrating an array position for the microwell array **112**.

5 The controller **124** executes an array position and type routine, which includes controlling the instrument assembly **104** to obtain images of the microwell array **112** and identifying the orientation of the microwell array **112** and the type of microwell array **112**. The type of the microwell array **112** can characterize, e.g., the number and size of cell rafts on the microwell array **112**. The array position and type routine can
10 include performing an autofocus and navigation routine and identifying cell rafts, e.g., by performing image segmentation on brightfield images. An example array position and type routine is described further below with reference to Figure 5. In some examples, the system **100** provides the user **108** with user interface controls to control stage motors and visual feedback of brightfield images to position the corner of the
15 microwell array **112** within the field of view and click on it to identify the array position and type.

 If the user **108** selected the real-time imaging mode, then the controller **124** executes a real-time image acquisition routine. The user **108** sets imaging parameters and the field of view and reviews images in real-time, i.e., images as the instrument
20 assembly **104** navigates the microwell array **112**. The controller **124** controls the XY stage **148** and autofocus motor **150** in response to user input from the GUI **126** manipulating the field of view position. The controller **124** executes the autofocus and navigation routine to navigate the microwell array **112**, and at new fields of view, identifies cell rafts. The user **108**, after reviewing the images, selects one or more cell
25 rafts for release and transfer. If the user **108** selected the cytometric imaging mode, the GUI **126** optionally presents a screen for the user **108** to set imaging parameters for the array scan. The imaging parameters can alternatively be read from a file or receiving over a network connection.

 Figure 4C illustrates the next stage of the example experiment if the user **108**
30 selected the cytometric mode, array scanning and single-cell detection. The GUI **126** optionally presents a status screen during the array scan. The controller **124** divides the array extent into discrete fields of view, e.g., by maximizing the number of cell rafts within each field of view (i.e., minimizing the number of fields of view) while ensuring that no cell rafts are missed.

In general, the controller **124** uses an iterative state positioning and raft segmentation process to optimize the position of rafts within each field of view to maximize the number of cell rafts in each field of view and to calculate the translation required to image next set of cell rafts. The controller **124** can perform the iterative stage positioning and raft segmentation based on, e.g., the consistency of cell raft dimensions and spacing in arrays; a nominal margin between full cell rafts within a field of view and edges of the field of view; precision of state motor movements; and computational time to detect cell rafts within images. Alternatively, the controller **124** can translate a given distance between fields of view that ensures that at least one cell raft width of overlap and resolve duplicate cell raft images during processing.

The instrument assembly **104** moves the XY stage **148** to a first field of view. The controller **124** executes the autofocus and navigation routine, which can include identifying the cell rafts in the field of view, and then the controller **124** executes the image acquisition routine. The image acquisition routine can include lighting one or more specific light sources depending on the imaging parameters, e.g., whether a particular fluorescence channel is specified, and then controlling the camera **140** as specified, e.g., for a particular exposure time.

The controller **124** then controls the XY stage **148** to move to a next field of view and repeats the autofocus and navigation routine and image acquisition routine. The array scan continues until an end condition is reached, e.g., the entire microwell array **112** or a specified portion of the microwell array **112** has been imaged or a time limit is reached. The array scan can proceed in any appropriate directional manner, e.g., processing a row in one direction and then processing the next row in an opposite direction. During the array scan, the controller **124** assigns each detected cell raft an address. The addresses are useful, e.g., so that images of the cell raft and other data can be associated with the cell raft, and so that cell rafts can be located for release and transfer.

When the scan is complete, the GUI **126** presents an optional screen for receiving single-cell detection parameters from the user **108**. The user **108** can input, e.g., the color channel and a threshold for the color channel. The controller **124** performs an image analysis of the images obtained during the scan to identify single-cell rafts. For example, the controller **124** can segment cell nuclei in each cell raft using extracted sub-images of selected images. Typically, an image of a field of view of the microwell array **112** will include multiple cell rafts. Extracting a sub-image of

a particular cell raft can include segmenting the image to identify locations of the cell rafts and then isolating the portion of the image depicting the particular cell raft. Identifying single-cell rafts is discussed further below with reference to Figure 8. The controller **124** can then calculate and compile metrics for cell rafts and cell nuclei segmented in the selected images.

Figure 4D illustrates the next stage of the example experiment if the user **108** selected the cytometric mode, marker gating and plate mapping. During marker gating, the GUI **126** presents a screen for the user **108** to specify marker gating parameters. The user **108** defines fluorescence gates. For example, the user **108** can specify a threshold color intensity. The controller **124** calculates and compiles metrics for gated single-cell rafts, and the GUI **126** presents a screen displaying results.

A marker is a cellular feature, which may be represented by a nucleic acid, a protein, other type of organic or inorganic molecule, or cellular feature such as morphological characteristics and organellar size and structure, the detection of which within a given cell is used to identify or classify a given cell, or to distinguish it from other types of cells which may not contain the given marker or contain it to a different degree. The presence, relative amount or quantitative amount of a marker can be detected using a range of methods and materials. These include using dyes, including fluorescent dyes, which label cells, organelles or other cellular features such as cytoskeleton, nucleus, mitochondria and a range of other organellar compartments. Such dyes are well known in the art. They may also include using methods and materials for detecting native molecules with an optically detectable probe which may be represented by a nucleic acid with complementary sequence to the marker sequence, an antibody against a specific marker protein or transgenic approach where a gene expressing the marker is engineered to include a non-native moiety such as a fluorescent motif (green fluorescent protein, for example), antigen (HisX6 for example) or enzymatic activity (luciferase or alkaline phosphatase, for example).

In the context of this specification, a marker may also be a fluorescent dye, alone or in combination with one or more chemical or affinity moieties, that may be used to determine whether a cell is present, or whether a given cell is dead, alive, intact, not intact, or any other cellular condition or process. The term marker may also be used herein to describe the signal, such as a fluorescence or other optically detectable signal, that is detected by image analysis in the automated system and used

to identify or classify a given cell or to distinguish it from other types of cells or cells having different characteristics or features.

During plate mapping, the GUI **126** presents a screen for assigning cell rafts to wells in the collection plate **114**. For example, the user **108** can manually select cell rafts from a graphical display of cell rafts and assign cell rafts to a graphical display of wells in the collection plate **114**. In another example, the controller **124** can randomly assign cell rafts to available wells in the collection plate **114**. The controller **124** can select cell rafts based on user-specified criteria, e.g., by selecting each cell raft that is both determined to be a single-cell raft (e.g., with at least a specified level of confidence) and has a specified marker present (e.g., the detected marker is present at least at a threshold level). The controller **124** compiles a list of cell raft locations for collection plate wells.

In some examples, the GUI **126** displays, for all cell rafts within a currently selected marker gate, the approximate positions of the cell rafts on the microwell array **112** and a 2D histogram, or scatterplot, of fluorescence intensities within one or two of the imaging channels. Each cell raft within the gated population is represented on the scatterplot by a distinct visual indicator (e.g., a single yellow filled circle), whose (x,y) position is equal to the fluorescence intensity of the cell raft within the respective imaging channels corresponding to the two axes. Fluorescence intensities are integrated across the entire cell raft and are scaled relative to the maximum integrated cell raft intensity within the overall single-cell resident population for that imaging channel. Other indicators, e.g., colored flags, along each axis allow the user **108** to easily switch the imaging channel for a given axis.

In some examples, the GUI **126** can provide user interface controls that allow the user **108** to create marker gates on a fluorescence intensity scatterplot:

- **QUADRANT** – A single left-click on the scatterplot defines the position of a set of cross-hairs. A double-click within any of the four quadrants defined by the cross-hairs creates a new gate for that quadrant. Each of the quadrants can be used to create separate gates.
- **LINE** – Two left-clicks on the scatterplot define the trajectory of an infinite line across the scatterplot. A double-click on either side of the line creates a new gate. Both sides of the line can be used to create separate gates.

- ELLIPSE – A first left-click on the scatterplot defines the center of an ellipse. A second mouse-click defines one axis and radius of the ellipse relative to the center. A third mouse-click defines the orthogonal radius relative to the first axis. A double left-click inside the ellipse creates a new gate. Given the methodology for defining the ellipse, the ellipse may extend beyond the range of the scatterplot.
- POLYGON – Successive left-clicks on the scatterplot define the vertices of a polygon. To close the polygon, left-click on the original vertex. There is no limit on the number of vertices that can be defined, though the edges of the polygon cannot cross one another. A double left-click inside the polygon creates a new gate.

In some examples, the system **100** will allow the user **108** to define multiple ellipses and polygons on a single 2D scatterplot. If such gates overlap, when the user selects the overlapping region, the new gate is created from the original gate geometry. In some examples, when a previously created marker gate (parent gate) is selected as the current marker gate, the GUI **126** only displays the cell rafts within the selected gate on the scatterplot and array schematic. Consequently, when a new marker gate geometry is drawn on the scatterplot and used to create a new marker gate (child gate), only the cell rafts within the parent marker gate are screened for inclusion into the child gate, despite the fact that additional cell rafts within the overall single-cell resident population may fall within the child gate geometry.

Figure 4E illustrates the next stage of the example experiment, raft release and transfer. The selected cell rafts are released from the microwell array **112** and transferred to mapped locations on the collection plate **114**. The instrument assembly **104**, under control of the controller **124**, aligns the release probe **154** with the first calibrated cell raft position and aligns the magnetic wand **162** with the release probe **154**. Then, the instrument assembly **104** actuates the release probe **154** to release the cell raft, lowers the magnetic wand **162** into the microwell array **112**, and then raises the wand away from the microwell array **112**.

The controller **124** executes a confirm cell raft release and collection routine. For example, after releasing the cell raft, the controller **124** can move the XY stage **148** to image the cell raft that was released. The controller **124** can then perform image analysis on the obtained image to determine whether or not the cell raft is

depicted in the captured image. If the cell raft is not depicted, then the controller **124** can confirm release. If the cell raft is depicted, then the controller **124** can repeat the release routine with the release probe **154**, e.g., a specified number of times until stopping to report an error.

5 After confirming release, the controller **124** transfers the released cell raft to the assigned well for that cell raft in the collection plate **114**. The GUI **126** can update a status screen depicting, e.g., the cell rafts that have been released and the cell rafts remaining for release. The controller **124** then selects another cell raft for release, aligns the release probe **154**, actuates the release probe **154**, and then controls
10 the magnetic wand **162** to collect the released cell raft.

The controller **124** repeats the process for each cell raft that has been selected for release. When each of the cell rafts selected for release has been transferred, the GUI **126** presents a screen indicating completion. The user **108** retrieves the microwell array **112** and the collection plate from the system.

15 The user **108** can use the GUI **126** to save a summary file, e.g., to a local hard drive. The summary file may be compatible with any suitable database structure and typically includes data on: 1) imaging channel used for cell identification; 2) imaging channels used for cytometric analysis; 3) description of the selected marker's fluorescence signal gates used for cytometric channels (e.g., center point of circle and
20 radius) 4) exposure time used for each imaging channel; 5) total scan time (if performed); 6) position of the single-cell raft in the microwell array **112** that is assigned to each well of the collection plate **114**; 7) mean fluorescence signal intensity (in relative fluorescence units – RFUs) in each imaging channel for each collected cell raft; and 8) date and time of day run was performed when the user **108**
25 acknowledged placing the microwell array **112** on the system. The controller **124** can export images from all active imaging channels selected by the user **108** for each retrieved and collected single-cell rafts in a standard image file format compatible with the image.

Figure 5 is a flow chart of an example method **500** for determining an array
30 position and type for a microwell array. The method **500** can be performed by the controller **124** of Figure 1 and will be described with respect to the system **100** of Figure 1. In general, the method **500** includes searching for a specified position (e.g., a corner) of the microwell array **112** in an automated manner using, e.g., known array geometry, manufacturing tolerances, and cell rafts detected in images.

The method **500** includes moving the XY stage **148** to an initial position (**502**), e.g., an estimated position for imaging one of the corners of the microwell array **112**. The method **500** includes autofocusing the microscope system, executing an image acquisition routine, and segmenting the obtained image to determine locations of cell
5 rafts within the obtained image (**504**). The method **500** can include calculating an average cell raft size, e.g., for a square cell raft, the length of the sides (L).

The method **500** includes determining whether to move the XY stage **148** to locate a corner of the microwell array **112** (**506**), e.g., determining whether a corner is depicted in the obtained image. For example, if one or more cell rafts are detected
10 within a threshold distance (e.g., a distance $2*L$) of the top of the image, the method **500** can include determining to translate towards the top (e.g., by a distance L); and if one or more cell rafts are detected within a threshold distance of the left side of the image, the method **500** can include determining to translate towards the left (e.g., by a distance L).

15 If moving the XY stage **148**, the method includes moving the XY stage **148** to move closer to a corner of the microwell array **112** (**508**) and repeating the search for a corner of the microwell array **112**. When the corner is found, the method **500** includes **510** optionally moving the XY stage **148** into a final position based on the location of the corner for determining the array type and orientation and autofocusing
20 and obtaining an image at the final position.

The method **500** includes determining the array type and orientation based on the image at the final position (**512**). For example, the array type can be coded into the microwell array **112** by coding a pattern into a specified corner (e.g., top-left corner) of the microwell array **112**. Cell rafts can provide digital information by
25 being present or absent (i.e., so that microwells are filled or unfilled). The method **500** includes determining whether to image additional areas of the microwell **112** to determine the type and orientation (**514**). For example, the method **500** can repeat for each of four corners of the microwell array **112**.

Figure 6 is a flow chart of an example method **600** for an autofocus and
30 navigation routine. The method **600** can be performed by the controller **124** of Figure 1 and will be described with respect to the system **100** of Figure 1.

The method **600** includes, at a particular field of view of the microwell array **112**, autofocusing and obtaining an initial image (**602**). The method **600** includes detecting cell rafts within the initial image (**604**). The method **600** includes moving

the XY stage **148** to maximize the number of cell rafts in the field of view (**606**). For example, if the cell rafts are askew in the initial image, moving the XY stage **148** can include moving the XY stage **148** to center the cell rafts with the boundaries of the field of view. The method **600** includes obtaining a new image after the movement
5 (**608**). The method **600** includes detecting cell rafts in the new image (**610**). The method **600** includes indexing the cell rafts in the new image (**612**), e.g., by assigning addresses to each cell raft detected in the new image. The addresses can be, e.g., row and column addresses.

For example, the following process can be performed with each increment in
10 the field of view while imaging the microwell array **112**:

1. The autofocus routine is executed and the brightfield image with the best autofocus score within the stack of images is selected for segmentation.
2. Using an OpenCV routine, the Otsu method is used to calculate the two pixel-intensity thresholds that maximize the inter-class variance across the
15 three classes of pixels within the image. The upper threshold only is applied to convert the brightfield grayscale image to a binary image.
3. Using OpenCV routines, bounding boxes for each unique detected object, i.e. set of contiguous bright pixels, are calculated.
4. The bounding boxes are subsequently bandpass filtered to eliminate any
20 object whose area deviates by more than 15% from the nominal cell raft area; and since cell rafts are nominally square, objects are also eliminated whose X and Y dimensions deviate by more than 10% from one another.
5. The cell raft row containing the most cell raft objects is selected to determine the skew of the array within the field of view. A first-order
25 polynomial is calculated using a linear regression to the centers of the cell raft bounding boxes and the slope dictates the skew direction.
6. The four corner cell raft objects are selected, and based on the skew direction, their bounding boxes are used to calculate the margin between the cell rafts and the edges of the field of view.
7. The XY stage is moved to center the nominal cell raft sub-array within the
30 field of view and the Z focus drive is adjusted to the point of best focus interpolated during the autofocus routine.
8. A new brightfield image is acquired and steps 2-4 are repeated.

9. The segmented cell rafts are sorted by row and column and appropriately indexed based on the indices assigned to the previous field of view and the translation direction of the XY stage.

Figure 7A is a flow chart of an example method **700** for autofocusing a microscope objective. The method **700** can be performed by the controller **124** of Figure 1 and will be described with respect to the system **100** of Figure 1.

In some examples, the system **100** performs a rough autofocus function at the beginning of an experiment and then a fine autofocus function with every shift in the field of view of the imaging microscope. The first autofocus procedure for a given experimental run is performed after the microwell array **112** is loaded and the system begins initialization. A system configuration file can specify the X and Y positions for the XY stage **148** that align the microscope objective and system field of view with the upper-left quadrant of a single-reservoir microwell array or the upper-left reservoir of a quad-reservoir microwell array. The system navigates to that position and performs a rough autofocus procedure as follows:

1. The focus drive is moved to the minimum position in the focus search range as defined in the configuration file.
2. A brightfield image is acquired using, e.g., 2x binning and a dynamically determined exposure time.
3. In parallel:
 - a. The image data is transferred from the camera and a focus score is calculated that quantifies the image contrast based on the variance in pixel intensities across the image.
 - b. The focus drive is moved up, e.g., 100 μm .
4. If the focus drive has not reached the top of the search range as defined in the configuration file, repeat steps 2 and 3. If it has, the software extracts the focus position with maximum focus score.

The system **100** can then autofocus the microscope objective at a particular field of view using any appropriate technique. For example, the controller **124** can execute a Z-stack routine to search for an autofocused position on the Z axis. The Z-stack routine selects bottom and top search limits along the Z axis and a fixed step size between sample focus positions. In some examples, during an array scan, the search limits can apply for every field of view within the microwell array **112**. The Z-stack routine then acquires an image and calculates a focus score at each sample focus

position and then either extracts the position with the best focus score or interpolates a best focus position based on the sample focus positions. Other examples of search routines include the golden search method and Brent's method.

5 The method 700 illustrated in Figure 7 can be considered a modification to a Z-stack routine to leverage information gained from neighboring fields of view. The method 700 can be useful, e.g., during an array scan, so that search limits can potentially be narrowed to improve the speed or the accuracy or both of autofocusing.

10 The method 700 includes moving the XY stage 148 to a new field of view and moving the microscope objective, along the Z axis, to an end point (e.g., bottom or top) of a possible focus range (702). In some examples, the method 700 includes determining, if available, the possible focus range (or otherwise determining a number of sample focus positions) based on focus positions from at least one neighboring field of view of the microwell array 112.

15 For instance, focus positions from neighboring fields of view are not typically available at the beginning of an array scan, but typically would be available as the scan progresses. In some examples, the method 700 includes selecting top and bottom search limits along the Z axis and step size between sample focus positions based on an average of any focus positions already determined within eight neighboring fields of view. Figure 7B illustrates a range of sample focus positions
20 along the Z axis.

The method 700 includes acquiring, using the camera 140, an image at a sample focus position (704). The method 700 includes moving the microscope objective, along the Z axis, the microscope objective to a next sample focus position (706), e.g., by moving the microscope objective up in a fixed step size such as 28
25 microns. In method 700 includes, transferring the image data to the computer system 102 (708) and calculating a focus score for the image (710). Any appropriate type of quantitative focus metric can be used for the focus score, e.g., the focus score can be determined based on a gray-level variance.

30 The method 700 can optionally include calculating the focus score in parallel with moving the microscope objective to the next sample focus position. The method 700 includes waiting (712), if needed, for both operations to complete. The method 700 includes determining whether there are more sampling focus positions to sample (714), e.g., whether the microscope objective is at the top (or bottom) of the possible

focus range. If there are more sampling focus positions to sample, the method **700** repeats imaging and calculating a focus score for the next positions.

The method **700** includes interpolating the Z axis position of best focus using the Z locations of the sample focus points and the focus scores at the sample focus points (716). Figure 7C illustrates an example plot of sample focus positions (on the horizontal axis) and focus scores (on the vertical axis). For example, the method **700** can include extracting the Z axis position of the sample focus point with the best focus score (Z_1 , FS_1 in Figure 7C) and the two sample focus points on either side (along the Z axis) of that sample focus point (Z_2 , FS_2 and Z_3 , FS_3 in Figure 7C). Then the interpolated Z axis position (Z_{focus}) can be calculated as follows:

$$Z_{focus} = Z_1 + SIGN(Z_2 - Z_3) \left(\frac{FS_2 - FS_3}{FS_1 - FS_3} \right) \left(\frac{Z_{step}}{2} \right)$$

The SIGN operator yields a positive or negative sign depending on the difference between the Z axis positions of Z_2 and Z_3 , and Z_{step} is the step size between sample focus positions. The interpolated focus position can then be used for any appropriate imaging for the system **100**.

Although the system **100** is described as autofocusing at each field of view, the system **100** can, in general, use any appropriate technique for focusing the microscope objective. For example, the system can perform an interpolation of a focus plane at any array position within the array by fitting a surface to the subset of focal planes measured versus (x,y) positions within the array. Surface fitting can be based on, e.g., a model derived from physical properties of the array (dimensions, elasticity, etc.) and the weight of the fluid within the reservoir. For example, the system **100** can use a thin-plate spline, which: calculates a minimally bended (i.e. minimal second derivatives) smooth surface that represents a least-squares minimization between the surface and the control points; uses a radial basis function $U(r) = r^2 \ln(r)$ between the control points; and uses an L-U decomposition to calculate the second-order polynomial weights and first-order coefficients for the surface as a function of (x,y).

Figure 8 is a flow chart of an example method **800** for identifying single-cell rafts in a microwell array. The method **800** can be performed by the controller **124** of Figure 1 and will be described with respect to the system **100** of Figure 1.

The method **800** includes, for a selected cell raft, extracting a sub-image of the cell raft (**802**). The method **800** includes removing an outside border of the image

(804) (e.g., by masking) and applying an intensity threshold (e.g., a user-specified threshold) to the image to create a binary image (806).

The method 800 includes identifying unique objects within the binary image, e.g., using any appropriate object detection algorithm (808). The method 800
5 includes filtering the identified unique objects based on size (810), e.g., by discarding identified unique objects having fewer than a threshold number of pixels (e.g., 10 pixels). The method 800 includes counting the remaining objects after filtering (812) and identifying the selected cell raft if only a single cell is depicted.

The method 800 optionally includes determining, if the selected cell raft is
10 identified as a single-cell raft, a confidence score indicating a degree of confidence in the determination that the selected cell raft is a single-cell raft (814). For example, the selected cell raft can be further processed using its fluorescence morphology to assign a confidence level to the single-cell classification. For each candidate single-cell cell raft:

- 15 • Local maxima within the grayscale pattern of the detected fluorescence object are identified using a combination smoothing and dilation process:
 - A 9x9 flat structuring element (SE) is defined with a zero at its origin.
 - Using SE , a grayscale dilation is applied to the masked image (I):
20 $GD = I \boxplus SE$
 - The zero at the origin of the structuring element has the effect that the grayscale intensity of GD equals the intensity of I at positions of maximum intensity within I over a 9x9 local region.
 - The masked image (I) is subtracted from the dilated image (GD):
 $SI(x, y) = GD(x, y) - I(x, y)$
 - 25 ○ Elements within $SI(x, y)$ equal to zero represent local maxima within the original masked image $I(x, y)$.
 - The local maxima are analyzed to determine a confidence score that the object corresponds to a single nucleus. The cell raft starts with a rating of HIGH confidence. For every combination of two detected local maxima
30 within the grayscale image of the fluorescent object:
 - The pixel intensities along a line between the two local maxima are compared against a theoretical linear gradient between them. If the

5 actual intensity dips below 75% of the linear gradient at any point along the line, the cell raft is demerited by one level (from HIGH to MEDIUM, MEDIUM to LOW, or LOW to removal from the candidate list). If the intensity dips below 50% of the linear gradient, the cell raft is demerited by two levels. (NOTE: the demerits are cumulative over all combinations of identified local maxima.)

- 10 ○ As a measure of circularity of the object, the distance between the two maxima (D) is compared to their respective distances (DT_1 , DT_2) from the edge of the detected object (as measured by the distance transform value applied to the complement of the thresholded image).
- If $2 * D > (DT_1 + DT_2)^2$, the cell raft is removed from the single-cell candidate list.

15 In some examples, any cell raft candidates remaining after the culling process are classified according to their confidence level and available for filtering based on confidence rating, size, or maximum intensity before inclusion in the preliminary single-cell population for marker gating.

In general, the controller **124** can use any appropriate image analysis techniques for identifying single-cell rafts. In some examples, the controller **124** can, for a sub-image of a cell raft, threshold the sub-image; if the number of discrete objections is one after thresholding, calculate a distance transform of a complement of the thresholded image; create a negative of the distance transform; set background pixels to $-\text{Inf}$ (or other appropriate placeholder value); identify and mark local minima to differentiate a single cell from a cluster of cells; and perform a watershed transform to draw boundaries around cells.

Figure 9 is a screen shot of an example screen of a graphical user interface for selecting cells rafts and mapping a collection plate, e.g., for a real-time imaging mode as described above with reference to Figure 3A. Figure 9 illustrates an example “Imaging” screen GUI (e.g., from the GUI **126** of Figure 1) that combines multiple elements of system control and sample capture. The user interface further provides user interface controls that allow the user to move the field of view in each direction or move to any position with a single mouse click, as well as to allow the user to adjust the focus of the imaging optics or initiate autofocus. The user interface further

allows the user to turn on or off the brightfield and red, green, and blue fluorescent channels, set the exposure time and gain for each fluorescent channel, the binning for each fluorescent channel, and allow the user to adjust the exposure time independently for each active imaging channel.

5 As shown in Figure 9, the user can click on or select a raft of interest within any of the images, inspect a zoomed image of the raft, and select a particular raft for isolation. The user interface indicates to the user the position of the currently displayed field of view on the microwell array. The GUI screen also displays a map of the collection plate (96-well format shown as an example) and permits the user to
10 assign the single-cell raft selected for isolation to a specific position within the collection plate specified at the beginning of the experiment.

 Using display elements from Figure 9, the GUI can also produce a screen that can be used to monitor the release and transfer process. The GUI screen can display the list of rafts selected for isolation, allow the user to initiate the isolation process via
15 raft release from the microwell array, to transfer rafts to the collection plate via the magnetic wand, and to track the progress of the isolation process by displaying time to completion and each deposit in the collection plate map.

 When using the system **100** in cytometric image analysis mode, an “Imaging” tab GUI containing elements similar to Figure 9 can be displayed, allowing the user to
20 navigate the system field of view within the microwell array and visualize real-time images and pixel histograms for the purposes of setting the imaging parameters for the three fluorescence imaging channels to be used during the full automated scan of the microwell array. An “array scanning” tab GUI within the cytometric image analysis mode can be displayed that allows the user to initiate a full scan of the
25 microwell array, track the progress of the scan, and pause/resume the full automated scan of the microwell array. The GUI displays the position of the current field of view within the array as well as the images acquired from that field of view.

 Figure 10 is a screen shot of another example screen of the graphical user interface. Figure 10 illustrates a “Cell Gating” or cell identification tab GUI within
30 the cytometric image analysis mode. The Cell Gating tab GUI allows the user to set the parameters by which fluorescent objects within the primary imaging channel are segmented to determine the number of cells on each raft within the array. The controls allow the user to set the intensity threshold for the object segmentation, review the effects of the setting change on the segmentation, and apply various filters

within the population of single-cell rafts to further screen candidate rafts from the population. These settings can be applied to the method **800** for single cell detection so that the user can optimize the system's identification of single-cell rafts with greater than 95% efficiency. The user interface displays a histogram of signals for the

5 "Cell Identification" channel with the X direction representing pixel intensity and the Y axis representing the number of pixels within the full array scan at a given signal intensity. A user can use a mouse to set an indicator of the current single cell gating threshold on the histogram.

The user interface allows the user to change the single cell gating threshold to

10 a value representing a normalized percentile calculated as a function of both minimum (0%) and maximum pixel intensity (100%) detected during the full array scan, as well as a confidence interval for the single cell count on the array. The GUI screen will also indicate to the user the total number of rafts identified as containing cells during the full array scan of the microwell array, and the number of single cell-resident rafts

15 which meet the user-specified threshold parameters for subsequent isolation.

The user interface can display a "Marker Gating" tab within the cytometric image analysis mode. After completing the cell gating processes, an additional screen in the user interface will display an image of the full microwell array with the positions highlighted where single cell-resident rafts are located and the total number

20 of single cell-resident rafts found on the array. The user interface screen can also display a 2D histogram, providing a single point representing each single-cell raft, plotted along the X and Y axis for each of two cytometric marker channels specified by the user. The interface can further provide user interface controls that allow the user to apply cytometric marker gates on the histogram in any appropriate format,

25 e.g., as described above with reference to Figure 4D.

The user interface also allows the user to use a mouse to click the positions of single cell-resident rafts within the full image of the microwell array or on the 2D scatter plot, as well as to view images of the selected single-cell raft in all active fluorescent channels and brightfield. The interface further allows (e.g., in the real-

30 time imaging mode) the user to randomly select single-cell rafts in all active fluorescent channels and brightfield. Using a plate mapping display similar to the plate mapping display shown in Figure 9, the user can assign the selected single-cell raft to a specific position within the collection plate specified at the beginning of the experiment.

The systems and methods described in this specification are applicable to many problems in cell biology where a cellular process is monitored through a time varying fluorescence signature. The effect of specific transgenes, siRNA or small molecules on cellular proliferation or survival could readily be monitored in model cell systems, and cells displaying unusual properties such as extreme gene expression, cell proliferation or extended survival could be isolated for expansion and more detailed study. In the field of cancer biology, the system could also be used in primary human tumors, which contain diverse cell populations, to isolate and study individual cells with unique temporal characteristics.

For example, a time-course analysis can be accomplished using the functionality inherent within the cytometric image analysis mode. In the case of an application involving a drug or reagent challenge, a full array scan can be performed at $t = t_1$ – before the addition of a drug or reagent – for the purposes of 1) identifying single-cell rafts within the array and 2) quantifying a baseline fluorescence level within one of the reporter fluorescence imaging channels. After the addition of the drug or reagent and sufficient incubation time, a second full array scan can be performed at $t = t_2$. The system can subsequently enable single-cell raft selection for isolation by calculating and displaying the difference in measured fluorescence between t_1 and t_2 and by allowing the user to create gates based on that difference.

Various permutations are possible for this workflow where a fluorescence value is compared between two different time points. If the two time points are minutes or hours apart, the cells may be left inside the instrument. If the two time points are many hours or days apart, then the microwell array containing the cells can be removed from the instrument between imaging runs and placed in a standard cell culture incubator.

An additional application for two-point time-course analysis is the evaluation of clonal colony propagation during a transfection experiment with a fluorescent reporter gene. The preliminary scan would identify single-cell rafts which are required for a clonal colony. The secondary scan would allow evaluation of total colony fluorescence, indicative of overall colony size and efficacy of the transfection within the colony.

To take the time-course capability further, the system can perform any number of additional array scans at times $t = t_3, t_4, \dots, t_N$, quantify the fluorescence intensity within the reporter imaging channel, and display plots of measured intensity versus

time. In addition to creating single-cell raft population gates based on single-time-point fluorescence intensities or intensity changes between two time points as previously described, the software can further enable the user to create gates based on the shapes of the fluorescence time curves. Curve shape based gating capability can be facilitated using dimensionality reduction, clustering, and classification techniques such as principal component analysis, Fourier transforms, wavelet transforms, hierarchical cluster analysis, or linear discriminant analysis.

While fluorescent intensity is an excellent measurement of the abundance of an analyte within a single cell, there is also potential to use this capability to examine the localization of an analyte within a given cell. For example, many membrane proteins respond to a given ligand or other stimulus by internalization, typically through endocytosis, and translocating to other cellular compartments or organelles such as the nucleus. Analysis of proteins displaying this behavior may require not only monitoring fluorescent intensity, but also monitoring changes in the localization pattern of its fluorescent signal. In the case described above for example, comparing a protein's plasma membrane-linked signal to its nucleus-linked signal may provide an indication of overall signal transduction through a given pathway. Many types of analytes could take advantage of this method, including proteins, nucleic acids and organelles themselves such as endoplasmic reticulum, endosomes or mitochondria. Image analysis algorithms for these types of time course experiments would likely employ a segmentation-based method, identifying objects and imputing their geometry to predict the nature of the cellular compartment to which they are localized, or allowing the user to ascribe a specific fluorescent signal to a given cellular compartment.

Figures 11A-11I illustrate an example experiment where an automated, computer- controlled microwell release and collection system comprising an actuator, an imaging device, and a processor, including image processing and analysis algorithms is used with a microwell array to examine and isolate transfected K562 cells (a human myelogenous leukemia cell line). The cells were transfected using standard techniques with a single-stranded oligodeoxynucleotide repair template containing the leukemia-associated S34F mutation along with a plasmid containing genes encoding the Cas9 nuclease, an enhanced green fluorescent protein (EGFP) reporter gene, and one of two sgRNAs designed to bind to specific sequences in the U2AF1 gene. Cells were incubated at 37°C, 5% CO₂ in 2 mL of pre-equilibrated

culture medium for 24 hours following transfection. The transfected cells were then stained with 1 μ M calcein AM or CellTracker Deep Red for 30 min, washed and imaged following the respective staining protocol and seeded onto microwell arrays. The cell rafts were immediately imaged to identify the position of cell rafts with a single cell. Cell rafts with greater than one cell were excluded from subsequent analyses. The arrays were then imaged every 12 h until 72 h post-transfection.

The components of the automated system were generally similar to the components of the system **100** of Figure 1. The imaging device in the automated system comprised a MVX10 MacroView upright microscope (Olympus, Center Valley, PA) equipped with an ORCA-Flash4.0 CMOS camera (Hamamatsu, Bridgewater, NJ) that was used to acquire bright field and fluorescence images. A plan apochromat objective lens (1X with numerical aperture of 0.25) paired with a magnification zoom enabled a wide range of effective magnifications (0.63X – 6.3X) during imaging. The sample and objective movement was automated using a PS3H122 Motorized Focus Drive and a H138A motorized XY translational stage (Prior Scientific Inc., Rockland, MA). A Lambda 10-3 optical filter changer positioned an emission filter wheel (LB10-NWE), an excitation filter wheel with SmartShutter (LB10-NWIQ) and a stand-alone SmartShutter shutter (IQ25-SA) (Sutter Instrument, Novato, CA). A filter set (89000 – ET – Sedat Quad; Chroma Technology Corp, Bellows Falls, VT) with 5 excitation bandpasses (350 $\pm$ 50 nm, 402 $\pm$ 15 nm, 490 $\pm$ 20 nm, 555 $\pm$ 25 nm, 645 $\pm$ 30 nm) and 4 emission bandpasses (455 $\pm$ 50 nm, 525 $\pm$ 36 nm, 605 $\pm$ 52 nm, 705 $\pm$ 72 nm) permitted fluorescence measurement in the blue, green, red and far red wavelengths. An arc lamp (Lumen 200, Prior Scientific Inc., Rockland, MA) was used for illumination. All microscopy equipment was controlled by custom software written in MATLAB (MathWorks, Natick, MA) and used a Micro-Manager (Open Imaging, San Francisco, CA) core.

At varying times, brightfield and fluorescence images of the microwell array were acquired. An overlap of at least 300 μ m (spacing between cell rafts + cell raft width) between imaged fields of view was used in all experiments to ensure full image coverage. For experiments to identify EGFP- expressing K562 cells, the cells were first stained with CellTracker Deep Red. Bright field and fluorescence images of the arrays were acquired 24 h to 72 h post-transfection at 12 h intervals.

A custom MATLAB program was used to control the microscope in the system for automated acquisition of bright field and fluorescence array images at

designated time points and to process and analyze images. A graphical user interface (e.g., similar to the GUI 126 of Figure 1) enabled user input, including fluorescence channel selection, camera exposure time, and microwell array geometry. The user interface was also used to direct the user to manually locate and focus on the 4 corners and center of the microwell array. The 5 identified points from each array were fit to a thin-plate spline in order to predict the position and focal plane for each field-of-view for the array based on interpolated planes from the fit based on the array dimensions.

At all magnifications, the cell rafts possessed high contrast borders under bright field illumination. The cell rafts were segmented and assigned array locations using the bright field images. Flat-field correction was performed on each bright field image to correct for uneven illumination intensity. Each bright field image was thresholded using Otsu's method and the pixels assigned a 1 or 0 based on their value above or below the threshold value. To remove debris on the arrays from consideration, binary images were further processed to fill the interior of each cell raft border and objects larger than $1.5\times$ or smaller than $0.5\times$ the known cell raft size were eliminated from analyses. Using this strategy, the positions of all cell rafts were identified at each time point and prior to cell raft isolation. Background noise was removed from fluorescence images by applying a top-hat filter. Otsu's method was then used to threshold each image and convert the image to binary. A watershed algorithm was applied to the binary image to separate cells in contact enabling the counting of fluorescent cells. The spatial resolution required for image acquisition was optimized by considering the pixel size, array image time, cell raft segmentation accuracy and cell identification success.

The release probe in the automated system consisted of two Delrin components: a motor housing for a small stepper linear actuator (20DAM10D2U- K; 15 mm travel; Portescap, West Chester, PA) and a needle mount. The needle mount possessed a clear polycarbonate window with a small hole through which a needle was secured. The clear, polycarbonate window permitted bright field microscopy with the needle in place. The linear actuator was controlled by a custom MATLAB program interfaced to an Arduino Uno (SparkFun Electronics, Boulder, CO) equipped with a motor shield (Adafruit Industries, New York, NY). An automated mechanical system with customized software written in MATLAB was developed to release individual cell rafts when supplied with a target cell raft list by the imaging analysis software (e.g., similar to the system 100 of Figure 1). A stepper linear actuator

controlled the Z position of the release probe used to puncture the microwell array and dislodge the cell raft in an individual microwell. The release probe and motor were mounted beneath the microscope stage aligned with the optical path of the microscope objective. The user interface enabled the user to select the Z-travel distance during
5 actuation for cell raft release. Cell rafts targeted for automated release were successfully dislodged with between 94 and 100 percent efficiency, depending on the number of times the release needle pierced the microwell array at the target location.

A motorized magnetic wand in the automated system was designed to capture, transfer, and deposit the superparamagnetic cell rafts into a collection vesicle. The
10 retrieval wand was fabricated by placing a cylindrical NdFeB magnet (3.175 mm diameter, 25.4 mm length) within a hollow polycarbonate cylinder (4.76 mm outer diameter, 3.18 mm inner diameter, 63.5 mm length). The cylinder was blocked at both ends leaving the magnet able to move freely along the central axis of the cylinder. The retrieval wand was mounted to the microscope objective using Delrin components and
15 its vertical movement controlled by a linear actuator (L12-30-50-06-R; Firgelli Technologies Inc., Victoria, BC, Canada, travel distance of 30 mm). The linear actuator was controlled by a custom MATLAB program interfacing with an Arduino Uno equipped with a motor shield. To capture cell rafts, the magnetic collection wand was placed in the medium above a microwell array within 2 mm of a released
20 cell raft. Once removed from the array, the cell raft was held on the wand tip by a cylindrical magnet within the wand as well as the surface tension of the fluid droplet on the wand tip. Cell rafts were deposited into a 96-well plate with an efficiency of 100%.

Prior to recovering cell rafts from the array, the release needle location in the
25 field of view was recorded. The X-Y-Z location of the collection wand relative to the array was also calibrated as well as its position relative to that of the wells in the 96-well plate. For cell raft release a selected cell raft was expelled from the microwell array by piercing the PDMS substrate with the needle using the cell raft release system. The magnetic collection wand was immersed into the array medium attracting
30 the released magnetic cell raft. The collection wand then moved to and was immersed within a well of a nearby 96-well plate containing culture medium. A NdFeB block magnet (101.6×76.2×6.35 mm) below the 96-well plate attracted the cell raft into the well. The block magnet was also positioned such that the cylindrical magnet in the collection wand was repelled by the block magnet.

A customized MATLAB program and user interface was used to integrate the actions of the various components of the system, allowing coordinated movement of the cell raft release probe during imaging, cell raft collection upon raft release and microscope stage movement to place collected cell rafts into a 96-well plate. Using the user interface, the user initiated a cell raft release, adjusting the travel distance until the needle just breaks through the PDMS. The user identifies the needle location, and the software stores the needle location in relation to the XY stage position. Next the GUI allows the user to manipulate the XY stage and cell raft collection system to place the wand tip into the 4 corners of the cell raft array and 4 corner wells of the 96-well plate. This information is then used to interpolate collection and deposition positions for each cell raft.

The automated system was used identify the position of cell rafts with a single cell and to track the duration and intensity of EGFP fluorescence of every cell. EGFP-positive cells were examined every 12 hours over a 72 hour period. Cell rafts that contained an EGFP-expressing cell at any time point were identified at the completion of the imaging time course by the image analysis software. A total of 220 cell rafts starting with a single cell contained at least one fluorescent cell during the imaging time, corresponding to a transfection efficiency of 1.9% . Cell rafts containing fluorescent cells were identified with 100% sensitivity, and the automated cell raft system was able to release cell rafts with >98% efficiency and collect cell rafts with 100% efficiency. The single-cell rafts were targeted for release and collection into 96-well plates. Cells that expanded into colonies were genetically analyzed to determine the presence of successful gene editing. Two K562 colonies were generated containing the S34F mutation in U2AF1, demonstrating the ability to sort cells based on the temporal evolution of fluorescent protein expression and providing selected cells and colonies that were successfully gene-edited.

The automated system also was used to analyze the temporal evolution of EGFP expression in transfected cells on the microwell array, and whether there were identifiable trends in EGFP expression that predicted whether a cell would proliferate after cell raft isolation. The mean fluorescence per cell obtained by the imaging system over 72 hours was used to categorize cells on individual cell rafts as low fluorescence for the entire duration, high fluorescence for the entire duration, low expression followed by high expression, and high expression followed by low expression. Cells that proliferated post-isolation were distributed stochastically

throughout the four groups. There was no discernable difference in fluorescence expression at any single time point between the post-isolation proliferative and non-proliferative groups, and there was no correlation between proliferation of cells on the microwell array and proliferation after isolation of the cell raft. Analysis of the temporal evolution of EGFP expression also demonstrated that transfected K562 cells could take up to 72 hours to express EGFP following transfection. Furthermore, the automated system allows the user to correlate expression data taken over time on single-cell rafts in a microwell array with downstream molecular analysis, in this case a determination of successful gene editing in isolated clones derived from single cells assayed on a microwell array.

Figure 11A shows a schematic of image processing and analysis used to identify and isolate cell rafts containing transfected K562 cells. Figure 11B shows a raw brightfield image of a microwell array. Figure 11C shows a bright field image of the same microwell array after a flat-field correction was applied. Figure 11D illustrates that thresholding of the corrected image yielded a binary image marking the cell raft borders. Figure 11E illustrates morphological filtering that was applied to fill in the cell rafts and remove any cell rafts touching the image border. Figure 11F shows a fluorescence image of two touching cells loaded with calcein AM. Figure 11G illustrates a top-hat filter that was applied to the fluorescence image to remove background noise. Figure 11H shows that thresholding of the top-hat filtered image yielded a binary image with the two cells connected. Figure 11I shows the results of applying a watershed algorithm to separate the touching cells.

In one embodiment of the invention, an automated, computer-controlled microwell release and collection system comprising an actuator, an imaging device, and a processor including image processing and analysis algorithms is used with a microwell array to examine the simultaneous measurement of T cell function with recovery of individual T cells.

An automated system (similar to the system **100** of Figure 1) was used to measure, in a functional assay on the array, the ability of individual T cells to kill a population of target cells and to measure the time dependence of T cell mediated killing, or cytotoxicity, “on-array”. The system was further used to viably sort specific cells into a 96-well plate for clonal populations that can be used as probes for monitoring antigen presentation under different situations and in different cell types.

A human T cell culture was generated against the influenza M1p antigen. Isolated peripheral blood mononuclear cells were used to obtain CD8⁺ cells using standard methods. The cytotoxic T lymphocyte (CTL) culture was initiated by incubating the CD8⁺ T cells with M1p pulsed DCs in CTS AIM V media with 10% human AB serum (complete media, CM) supplemented with IL-21. After a 3 d incubation, the cells were supplemented with CM plus IL-7 and IL-15 every 2 d. CTLs were restimulated with M1p-pulsed DCs 11 d after culture initiation in CM containing IL-21, IL-7 and IL-15. IL-2 was added 19 d after initiation of the culture. The CTLs were restimulated 21 and 34 d after culture initiation and were cryopreserved in aliquots 41 d after initiation of the culture. Cryopreserved CTLs were thawed and restimulated with M1p pulsed DCs. After 3 d, the CTLs were isolated using the CD8⁺ T cell isolation kit and maintained in CM supplemented with IL-7, IL-15 and IL-2. CD8⁺ T cells were plated on microwell arrays 2 to 3 d later. M1p/HLA-A*02:01 tetramer enumeration showed that 48.4% of CD8⁺ T cells were specific for M1p. The bulk culture displayed antigen specific cytotoxicity against M1p pulsed autologous DCs compared to autologous DCs pulsed with the leukemia associated antigen PR1.

Dendritic cells (DCs) were differentiated from CD34⁺ cells isolated from cryopreserved leukapheresis products obtained from the Hematopoietic Progenitor Cell Laboratory at UNC Hospitals using the CD34 Microbead Kit UltraPure (Miltenyi Biotec). CD34⁺ cells were incubated for 12 d in CTS AIM V media with 10% human AB serum (complete media, CM), supplemented with GM-CSF, Flt3-ligand, SCF and IL-4 as described in the supplemental methods to yield immature DCs that were cryopreserved for future use. Immature DCs were differentiated into mature DCs by incubation with GM-CSF, IL-4 and TNF- α for 2 d and GM-CSF, IL-4, TNF- α , IFN- α and IL-6 for 2 d. Matured DCs were co-incubated with peptides (M1p or PR1) for at least 18 h.

Microwell arrays were coated in 0.1 wt% bovine gelatin in PBS and incubated at 37°C for ≥ 2 h after which the gelatin solution was aspirated and the array was washed $\times 3$ with PBS before plating cells.

The microwell arrays used for the analysis were comprised of a regular pattern (70 $\times$ 70) of cell rafts on which cells are cultured and assayed over time by microscopy. The cell rafts fabricated from a magnetic polystyrene possess a concave surface of 120 μ m depth enhancing retention of cells on their surfaces during assay

setup and performance. The microwell arrays were seeded with autologous DCs (“target” cells) that had been pulsed with either M1p or PR1 peptides and labeled with Hoechst dye. Target cells were applied to the array in phenol-red free RPMI 1640 supplemented with human AB serum, penicillin, streptomycin and HEPES at a ratio of 30 cells per cell raft (147,000 cells per array). CD8⁺ T cells were labeled with CellTracker Deep Red and placed onto the array at a cell:cell raft ratio of 1:1 in order to maximize the number of cell rafts that contained a single T cell. Cells settled onto the array in a stochastic manner so that the number in each well across the array followed the Poisson distribution, meaning that roughly 1/3 of the wells (36.8% or 1803 wells) are predicted to possess a single CD8⁺ T cell, 26.4% (1294) are predicted to contain >1 T cell and 36.8% (1803) are expected to have 0 T cells. The media overlaying the array contained Sytox Green, a DNA binding dye that is membrane impermeable.

The assay was designed to identify cell rafts that contained a single T cell and showed a high rate of cytotoxicity against target cells as evidenced by increasing green fluorescence over a 6 h time course. Because both the target cells and T cells were applied to the media over the microwell array, individual cell rafts can have different numbers and ratios of target and T cells. Image acquisition, processing, and analysis was accomplished in a similar manner to that described above with reference to the system **100** of Figure 1. Fluorescence images of the arrays were obtained every 30 min for 6 h while in the incubator housing the microscope and the development of green fluorescence over time was measured for each cell raft.

Fluorescence images were processed in a similar manner to the processing described above with reference to Figures 4A-4E. The fluorescence images were analyzed to determine the intensity, area, position and number of cells displaying each fluorophore (Hoechst, Sytox Green and CellTracker Deep Red). Top hat filtering and Otsu’s thresholding was applied to each image to produce a binary mask of the cells on the microwell array. Within the mask created by each fluorophore, the intensity, location and number of pixels was recorded for each cell raft. A watershed algorithm was applied to each image in the far red channel (corresponding to cells stained with CellTracker Deep Red) to count the number of individual cells on each cell raft.

In parallel with the image acquisition, the MATLAB program processed and analyzed the acquired images. The MATLAB GUI was used to select the desired combination of bright field and fluorescence channels for imaging. The processing

time of each set of images was faster than the microscope stage movement and image acquisition, so no additional time was added to the total scan time. The bright field images were used to identify individual cell raft locations. Due to the elastomeric nature of PDMS, image analysis was needed to accurately locate the exact positions of cell rafts on the array. The cell raft segmentation method included background estimation, flat-field correction, thresholding and morphological filtering. The vast majority of cell rafts ($99.8 \pm 0.8\%$) were correctly identified without false positives ($n = 100$ images, 100-121 cell rafts per image).

The analyses performed on the microwell arrays compared development of green fluorescence over time in cell rafts that contained either 0 or 1 CD8⁺ T cell. Cell rafts with 0 T cells were considered the control and reflected the rate of spontaneous target cell death over the time of the investigation. A total magnification of 4 $\times$ was used for scanning cell rafts, resulting in a pixel size of 1.62 $\mu\text{m}/\text{pixel}$. This magnification was chosen because it allowed for easy identification of single cells while maintaining a large field of view to minimize image acquisition time. Each 70 $\times$ 70 microwell array required 49 images per channel at this magnification with a 5% overlap of images. Image acquisition of a single array using bright field (100 ms camera exposure) plus 3 fluorescence channels (200 ms camera exposure each) required 216 ± 4 s to complete ($n = 10$). An autofocus algorithm was used to maintain focus for each image and required 99 ± 2 s to complete. All of the automatically acquired focal planes were accurate to within the microscope objective's depth of field at 4 $\times$ magnification ($\pm 21.8 \mu\text{m}$) compared to the manually selected focal planes ($n = 50$). The resulting total microwell array scan time (autofocus and image acquisition) was 315 ± 5 s.

The locations of individual cell rafts were determined using the image processing and analysis software. Cell rafts containing a single CD8⁺ cell were identified using CellTracker Deep Red fluorescence. These cell rafts were sorted based on the increase in Sytox Green fluorescence intensity. Automatically identified cell rafts were re-screened after gelatin encapsulation to ensure that a single CellTracker Deep Red-positive cell remained on the cell raft.

The automated system and processes enabled the measurement of the rates of T cell mediated killing for single cells, measured by fluorescence microscopy, was quantified in each cell raft with each cell raft designed to contain a population of fluorescently labeled antigen-presenting target cells and 1 CD8⁺ T cell. This allowed

for the identification of highly cytotoxic CD8⁺ T cells in as little as 2 h. Cytotoxicity or cell killing was measured by summing the Sytox Green pixel intensity within the Hoechst fluorescence area on each cell raft. Using the Hoechst positive regions on each cell raft as a mask for the Sytox Green fluorescence greatly reduced spurious measurements due to debris and disintegrating cells and their fragments. The effector cell count and cytotoxicity information was recorded for each cell raft at each time point to generate temporal traces of cytotoxicity corresponding to specific numbers of effector cells present. The rates of target cell death among the individual CD8⁺ T cells varied greatly; however, individual T cells maintained their rates of cytotoxicity throughout the time course of the experiment enabling rapid identification of highly cytotoxic CD8⁺ T cells.

Upon completion of the cytotoxicity assay, the arrays were overlaid with a thin layer of gelatin as described previously. The incubator surrounding the microscope was cooled to 24°C just prior to gelatin overlay of the array. The culture media above the array was replaced with 5 wt% bovine gelatin in PBS and the array was centrifuged. The array was then incubated for 10 min at 37°C, excess gelatin was aspirated and then incubated at 4°C for 5 min to solidify the gelatin within the cell rafts. Cold (4°C) culture media was overlaid onto the arrays. The cell raft with gel-encapsulated cell(s) is then readily captured by an overlaying magnetic wand dipped into the media above the array. The wand with the captured cell raft is then placed into the well of a 96-well plate facilitated by a magnet under the plate to pull the cell raft down to the bottom of the well.

Cell rafts that contained a CD8⁺ T cell and showed a high rate of target cell death were individually released from the array using the needle-release device. Released rafts were then captured by a magnetic wand mounted on a computer controlled 3-axis motor and deposited into a 96-microwell plate in a similar manner to that described above with reference to Figure 1. Cell rafts with highly active CD8⁺ T cells were individually transferred to wells of a 96-well plate containing feeder cells, using a needle-release device coupled to the microscope. Three sorted T cells were clonally expanded. All three clones expressed high-avidity T cell receptors for M1p/HLA*02:01 tetramers.

This method can be used to determine the sequence of the T cell receptors (TCRs) in clones known to be cytotoxic to selected target cells and the data could be used for the development of immunotherapeutics. TCRs exist as heterodimeric protein

complexes consisting of a TCR α chain paired with a TCR β chain, which are expressed on the T cell's surface. The paired chains bind with high affinity to the target peptide/MHC, which were in this instance the M1p/HLA-A*02:01 complex. The portions of the TCR α and TCR β chains that impart the specificity of the interaction with the target peptide/MHC are known as the CDR3 regions. If the CDR3 regions and flanking V and J segments of the TCR α and TCR β chains are known, full-length transgenic TCR constructs can be produced and transfected into primary human T cells to change their specificity towards the target peptide/MHC. This approach could have a broad application in cancer immunotherapy where a cancer patient's T cells could be transduced with a transgenic TCR, containing TCR α and TCR β chain sequences, which targets a cancer-specific peptide/MHC complex. The ability to determine the activity of small numbers of effector T cells in very short time scales could also be used for immune monitoring as T cell therapies are developed for clinical use. Such assays could be used to screen T cells cultures for antigen specificity against multiple antigens to reduce the risk of off-target cytotoxicity that has been observed in early T cell therapy studies. In addition, modifications of the array geometry can be easily modified to identify rare cytotoxic T cells, which could enable the identification and subsequent cloning of rare tumor antigen-specific CD8⁺ T cells. Assays of this kind can alternatively be used to interrogate the effects of multiple cellular populations interacting at the same time such as studies investigating target cell resistance to T cell mediated cytotoxicity or the screening of drug candidates that modulate the activity of T cells.

A processor together with the actuator, camera, motors, magnet, and other components, as well as executable code can form the various means for carrying out an embodiment of the invention. In some embodiments, a general-purpose processor such as a DSP, microcontroller or microprocessor is used and non-transitory firmware, software, or microcode can be stored in a tangible storage device that is associated with the system. A self-contained computer such as a personal computer, mobile computer, or an embedded controller based system can be connected to the other hardware. Any such functionalities may be referred to herein as a "processor" or a "microprocessor." A storage device may be a memory integrated into the processor, or may be a memory chip that is addressed by the controller or processor to perform control functions. Such firmware, software or microcode is executable by the processor and when executed, causes the processor to perform its control functions.

Such firmware or software could also be stored in or on a device such as an optical disk or traditional removable or fixed magnetic medium such as a disk drive connected to or within the cell isolation system.

5 It should be noted that any software as well as any data and information necessary to support the execution of instructions for any embodiment of the invention can be placed in a removable storage medium for development purposes or for maintenance and update purposes. Such a storage device may be accessed either directly or over a network, including the Internet.

10 Although specific embodiments have been illustrated and described herein, those of ordinary skill in the art appreciate that any arrangement which is calculated to achieve the same purpose may be substituted for the specific embodiments shown and that the invention has other applications in other environments. This application is intended to cover any adaptations or variations of the present invention. The following claims are in no way intended to limit the scope of the invention to the
15 specific embodiments described herein.

CLAIMS

1. A system for releasing cell rafts from a microwell array, the system comprising:

- an imaging device configured for obtaining images of a microwell
5 array, the microwell array comprising a plurality of cell rafts;
an actuator configured for releasing cell rafts from the microwell array;
and
a computer system comprising at least one processor and memory, the
computer system configured for:
10 obtaining one or more images of the microwell array using the
imaging device;
identifying, by analyzing the one or more images of the
microwell array, a selected cell raft from among the plurality of cell rafts; and
controlling the actuator to release the selected cell raft from the
15 microwell array.

2. The system of claim 1, comprising one or more motors configured for moving the imaging device or the microwell array or both, wherein obtaining the one or more images of the microwell array comprises performing an array scan by:

- 20 dividing the microwell array into a plurality of fields of view such that
the fields of view, collectively, include each of the cell rafts; and
for each field of view, controlling the one or more motors to orient the
imaging device with the field of view of the microwell array and controlling the
imaging device to obtain a respective image at the field of view.

25 3. The system of claim 2, comprising a microscope objective for the imaging system, and wherein obtaining one or more images of the microwell array comprises, for each field of view, autofocusing the microscope objective using one or more focus positions from at least one neighboring field of view.

30 4. The system of claim 3, wherein autofocusing the microscope objective comprises sampling a plurality of sample images at a plurality of sample focus positions and interpolating a current focus position from focus scores of the sample images.

5 5. The system of any preceding claim, wherein identifying the selected cell raft comprises counting, for each cell raft, a number of cells depicted in a sub-image of the cell raft and identifying at least one single-cell raft housing a single isolated cell.

10 6. The system of claim 5, wherein counting the number of cells depicted in a sub-image comprises applying an intensity threshold to the sub-image to create a binary image, identifying unique objects in the binary image, and counting the number of identified unique objects.

15 7. The system of claim 5 or claim 6, wherein identifying the single-cell raft comprises determining a confidence score indicating a degree of confidence in the determination that the single-cell raft houses the single isolated cell.

20 8. The system of any preceding claim, wherein identifying the selected cell raft comprises detecting, for each cell raft, a marker depicted in a sub-image of the cell raft, and gating the cell rafts based on detecting the marker.

25 9. The system of any preceding claim, wherein identifying the selected cell raft comprises assigning the selected cell raft to a mapped location of a collection plate.

30 10. The system of claim 9, comprising a mechanical cell raft collector, wherein the computer system is configured for controlling the mechanical cell raft collector to collect the selected cell raft after release from the microwell array and for controlling the mechanical cell raft collector to deposit the selected cell raft at the mapped location of the collection plate.

35 11. A computer-implemented method of collecting cells comprising:
 storing, in a memory device, information about a microwell array containing a plurality of rafts;
 identifying, using a processor to perform image analysis and using the information, a cell raft from among the plurality of rafts; and

releasing, using the processor and an actuator, the cell raft from the microwell array.

12. The computer-implemented method of claim 1 wherein the storing of the
5 information about the microwell array further comprises:

identifying and storing a size of the microwell array;

identifying and storing an optimal focus position and an optimal exposure for the microwell array;

10 sectioning the microwell array using the processor and storing a translation required to match a set of microwells to a field of view.

13. The computer-implemented method of claim 2 further comprising determining an address for each of a plurality of microwells in the microwell array.

15 14. The computer-implemented method of claim 2 or claim 3 further comprising calculating and storing an offset for the actuator.

15. The computer-implemented method according to any preceding claim wherein the identifying of the cell raft further comprises:

20 segmenting, using the processor, the plurality of rafts of the microwell array;

counting, using the processor, cell nuclei per raft for the plurality of rafts.

25 16. The computer-implemented method of claim 5 wherein the counting of the cell nuclei further comprises performing, using the processor, a watershed transform to define the cell nuclei relative to a fluorescence threshold.

30 17. The computer-implemented method of claim 5 or claim 6 further comprising gating, using the processor, the cell rafts to be released by the actuator based on detection of a marker.

18. The computer-implemented method of claim 7 wherein the detection of the marker is based on intensity of a color channel, a size, or both.

19. The computer-implemented method according to any preceding claim further comprising collecting, using the processor and a magnet, the cell raft with the isolated cell.

5

20. The computer-implemented method any preceding claim further comprising confirming by the processor, after releasing the cell raft, that the cell raft has in fact been released.

10

21. The computer-implemented method according to any preceding claim wherein the cell raft is a single-cell raft, a single-cell raft being a raft with one isolated cell associated therewith.

15

22. The computer-implemented method according to any of the preceding claims comprising generating a plurality of different graphical user interface screens for controlling operations associated with collecting the cells.

20

23. The computer-implemented method according to any of the preceding claims, comprising monitoring a cellular process through a time-varying fluorescence signature.

25

24. The computer-implemented method according to claim 23, comprising monitoring, using fluorescent intensity, an abundance of an analyte within a given cell, a localization of an analyte within a given cell, or both.

30

25. A tangible storage medium including non-transitory computer program code for performing the computer-implemented method according to any of claims 11 through 24.

26. A system for collecting cells, the system comprising:

an actuator;

an imaging device; and

a processor connected to the actuator and the imaging device, the processor being operable by use of stored executable computer program code

to perform the computer-implemented method according to any of claims 11 through 24.

27. Apparatus for collecting cells, the apparatus comprising:

- 5 means for obtaining images of cells;
 means for releasing a cell raft; and
 means for performing the computer-implemented method according to any of claims 11 through 24.

10 28. A system for monitoring a cellular process, the system comprising:

 an imaging device configured for obtaining images of a microwell array, the microwell array comprising a plurality of cell rafts;

 a computer system comprising at least one processor and memory, the computer system configured for:

15 at a first time, determining, using the imaging device, that at least one cell raft contains one or more cells undergoing a cellular process;

 in response to determining that the at least one cell raft contains the one or more cells undergoing the cellular process, storing a location of the at least one cell raft on the microwell array; and

20 at one or more times later than the first time, monitoring the cellular process by locating the at least one cell raft using the location and obtaining, using the imaging device, one or more images of the at least one cell raft.

25 29. The system of claim 28, wherein monitoring the cellular process comprises storing the one or more images and associating each of the one or more images with a respective capture time of the image and the location of the at least one cell raft.

30 30. The system according to any of claims 28-29, wherein monitoring the cellular process comprises presenting, in a graphical user interface, the one or more images and an initial image of the at least one cell raft.

31. The system according to any of claims 28-30, wherein determining that the at least one cell raft contains the one or more cells undergoing a cellular process comprises:

illuminating the at least one cell raft with light of a specified wavelength;

in response to illuminating the at least one cell raft, obtaining, using the imaging device, an initial image of the at least one cell raft; and

detecting a fluorescence signature of the cellular process in the initial image.

32. The system of claim 31, wherein monitoring the cellular process comprises presenting, in a graphical user interface, a graph of fluorescence intensity in the one or more images over time.

33. The system according to any of claims 31-32, wherein determining that the at least one cell raft contains the one or more cells undergoing a cellular process comprises determining that the at least one cell raft is a single-cell raft containing only a single cell and quantifying a baseline fluorescence level using the initial image.

34. The system according to any of claims 31-33, wherein monitoring the cellular process comprises determining difference in measured fluorescence between the one or more images and the initial image.

35. A method for monitoring a cellular process, the method comprising:
at a first time, determining, by at least one processor using an initial image of at least one cell raft of a microwell array, that the at least one cell raft contains one or more cells undergoing a cellular process;

in response to determining that the at least one cell raft contains the one or more cells undergoing the cellular process, storing in a memory device a location of the at least one cell raft on the microwell array; and

at one or more times later than the first time, monitoring, by the at least one processor, the cellular process by locating the at least one cell raft using

the location and obtaining, using the imaging device, one or more images of the at least one cell raft.

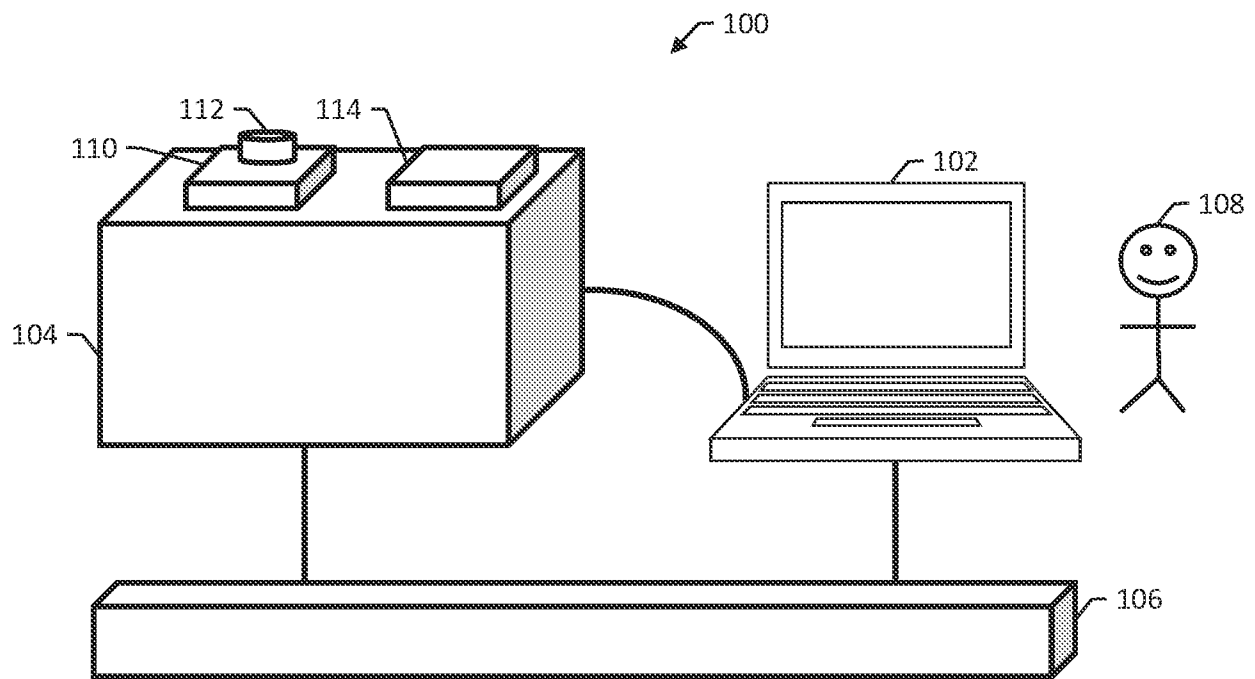


FIG. 1A

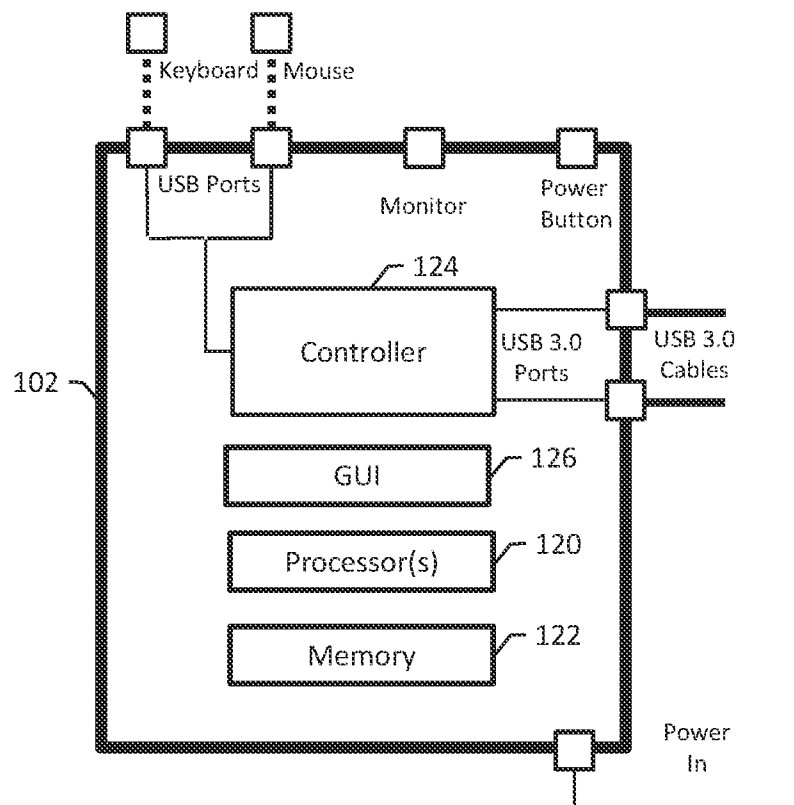
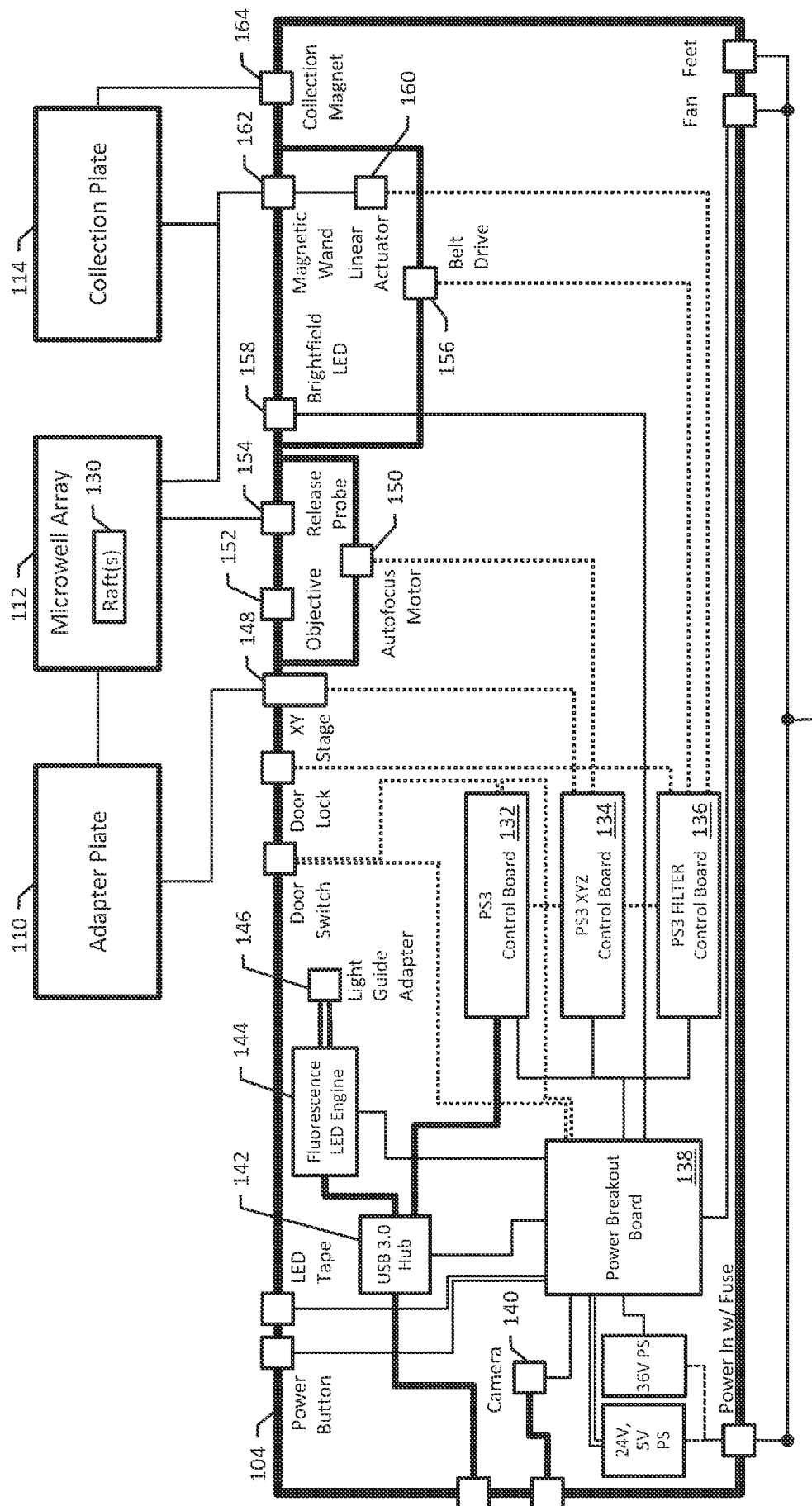


FIG. 1B



U
H
G
E

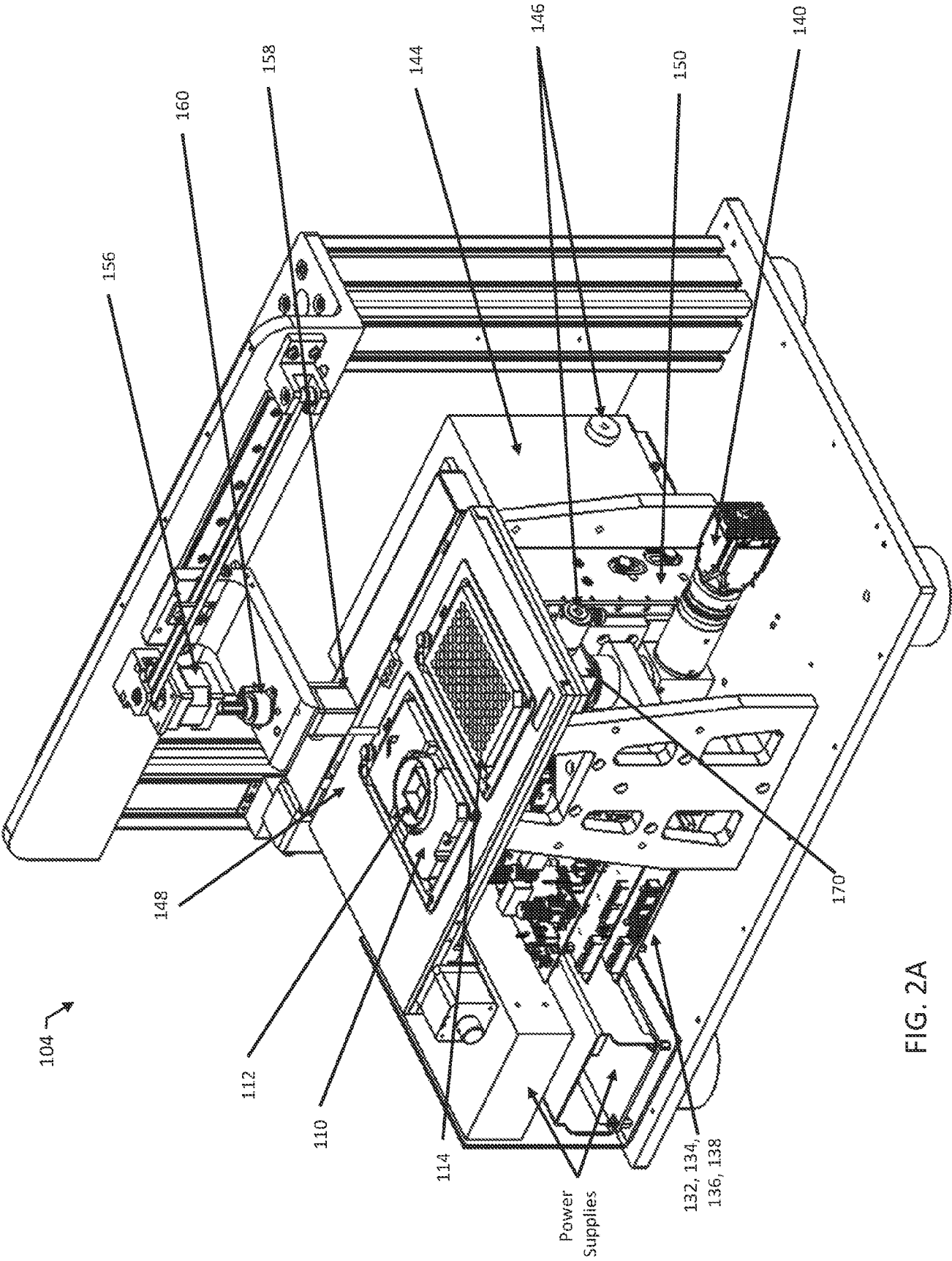


FIG. 2A

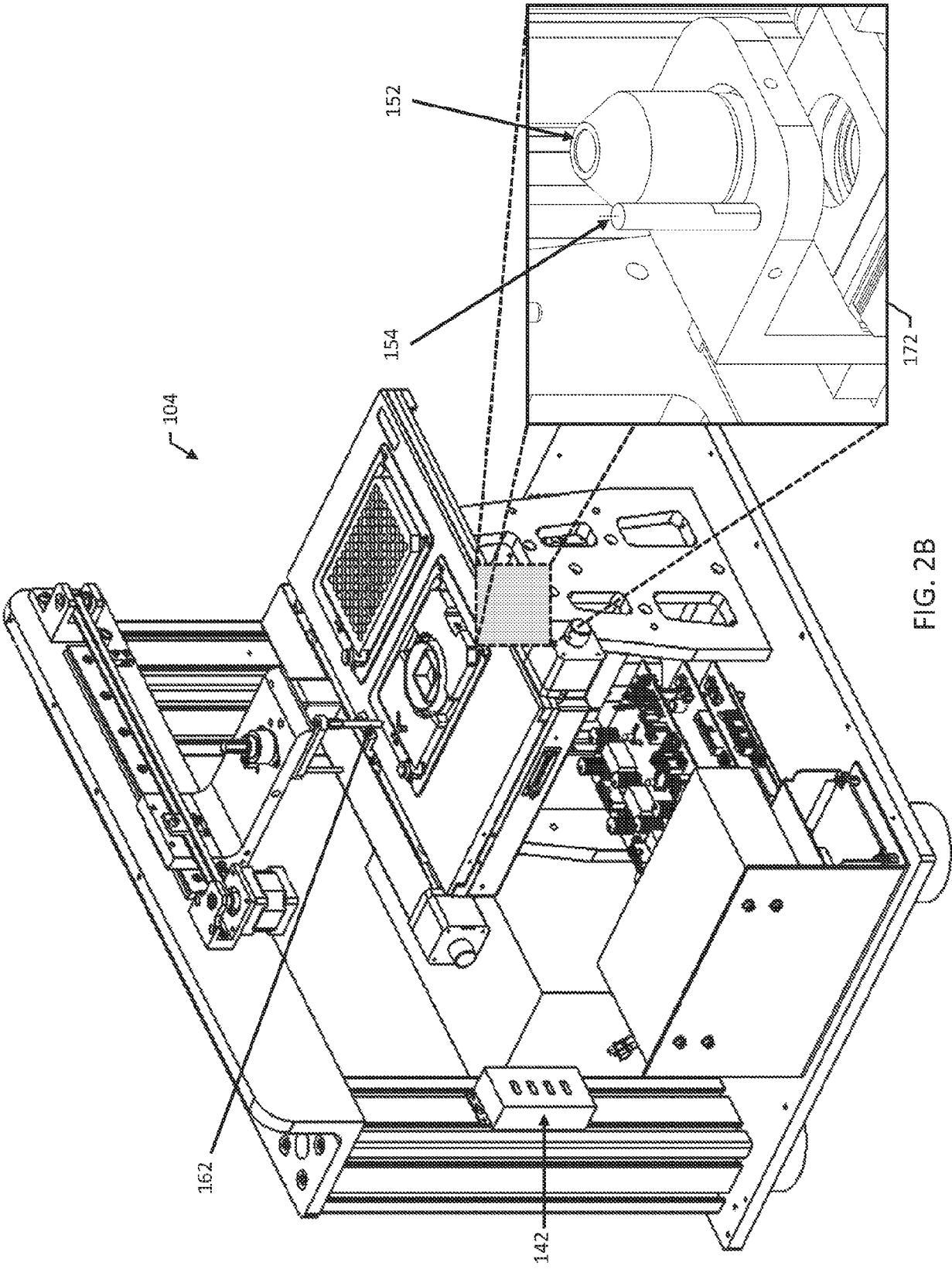


FIG. 2B

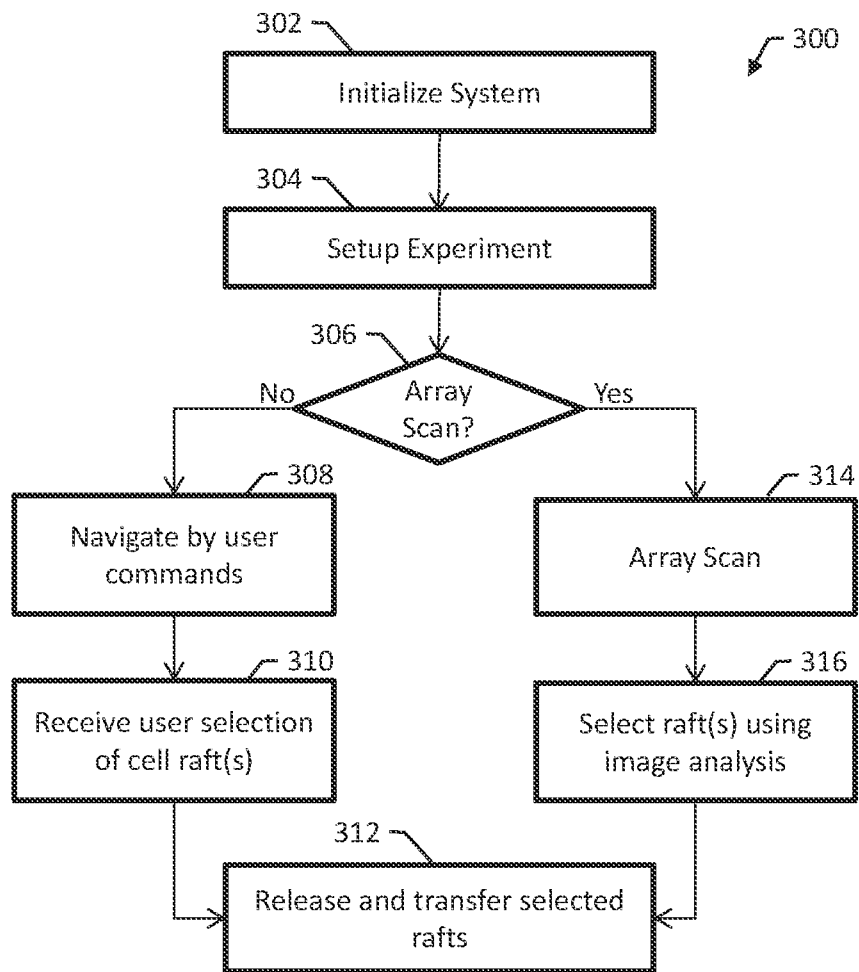


FIG. 3A

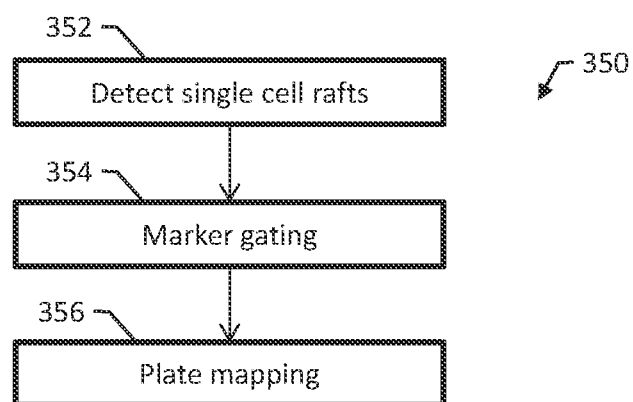


FIG. 3B

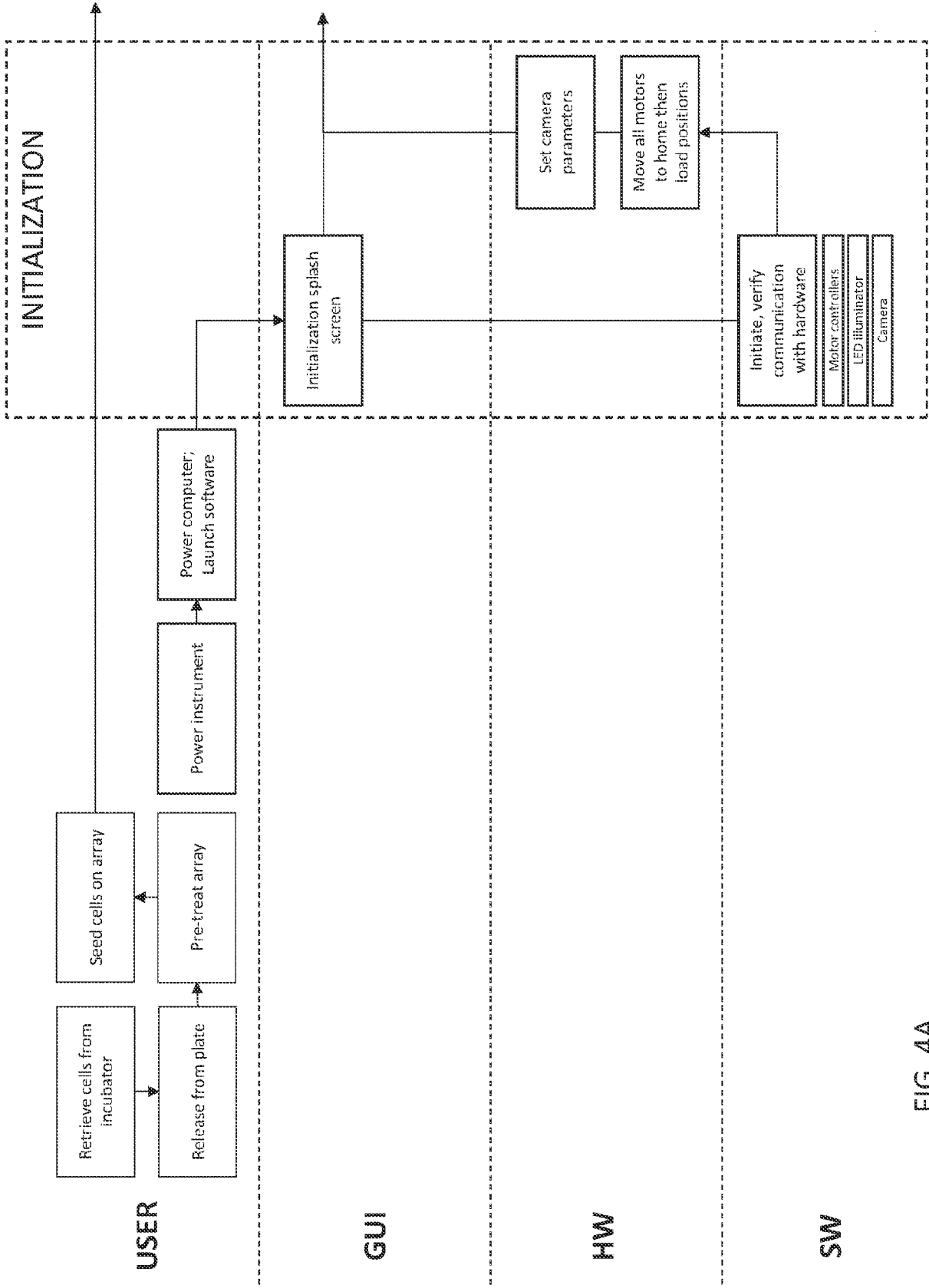


FIG. 4A

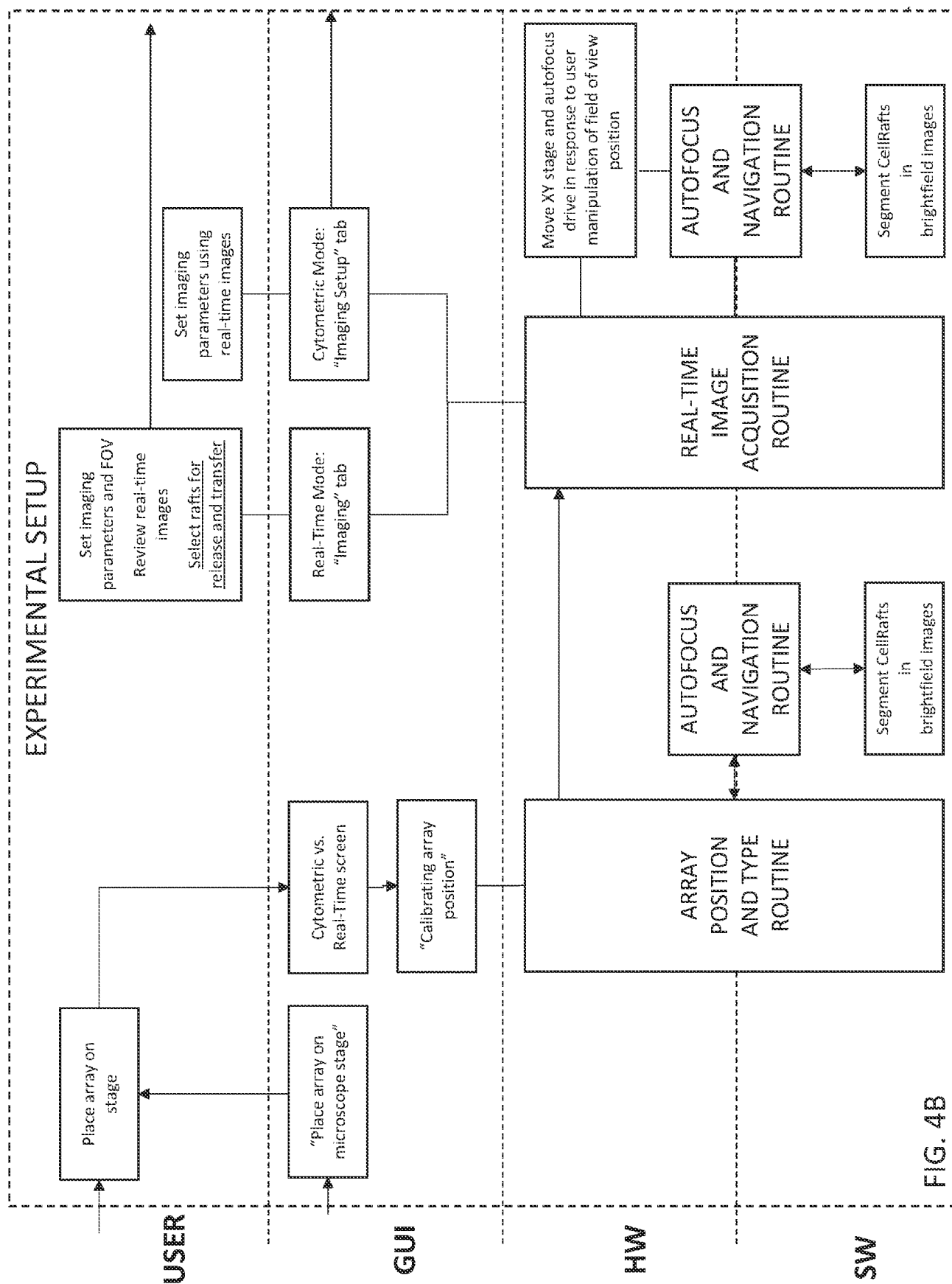


FIG. 4B

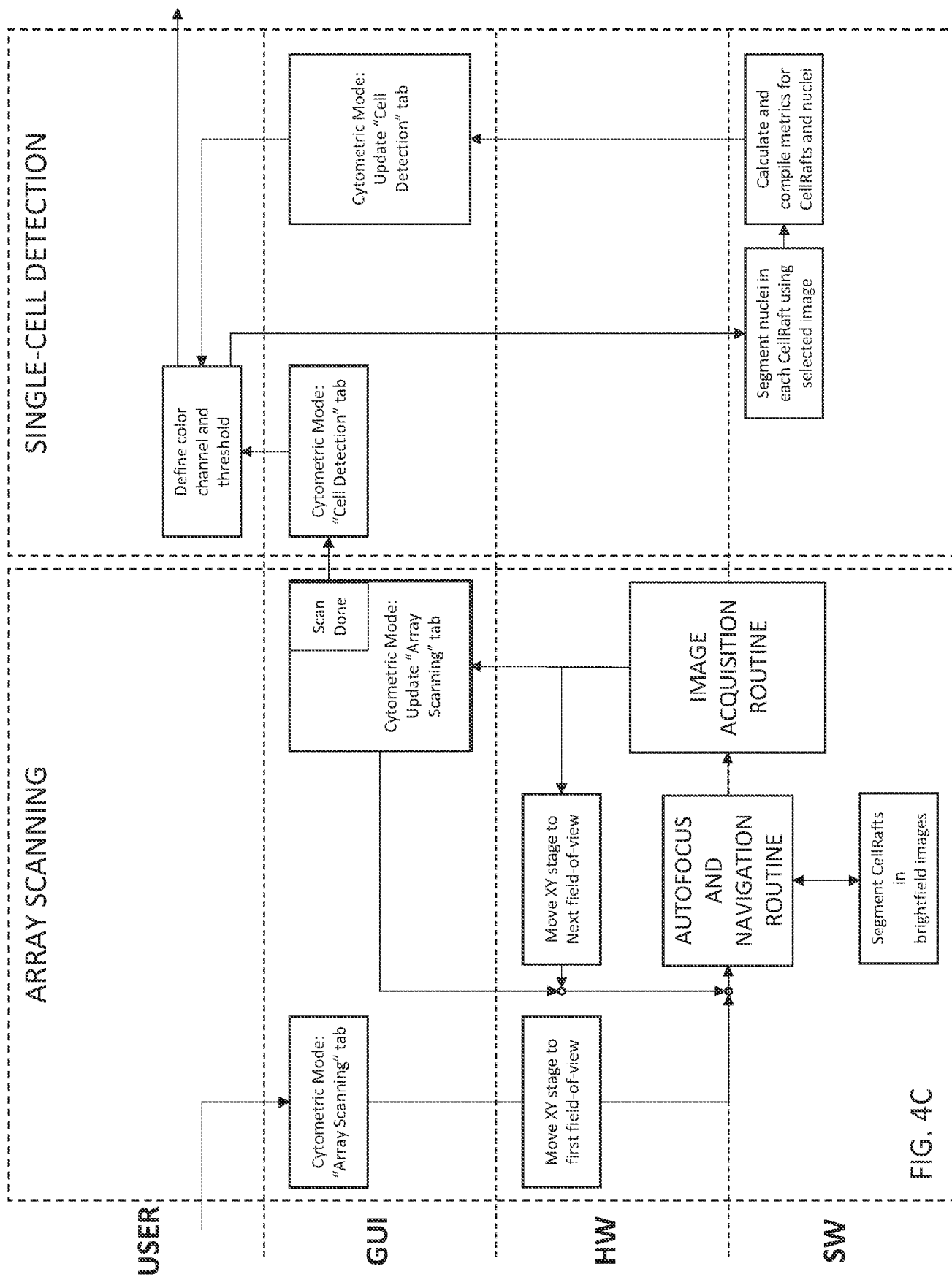


FIG. 4C

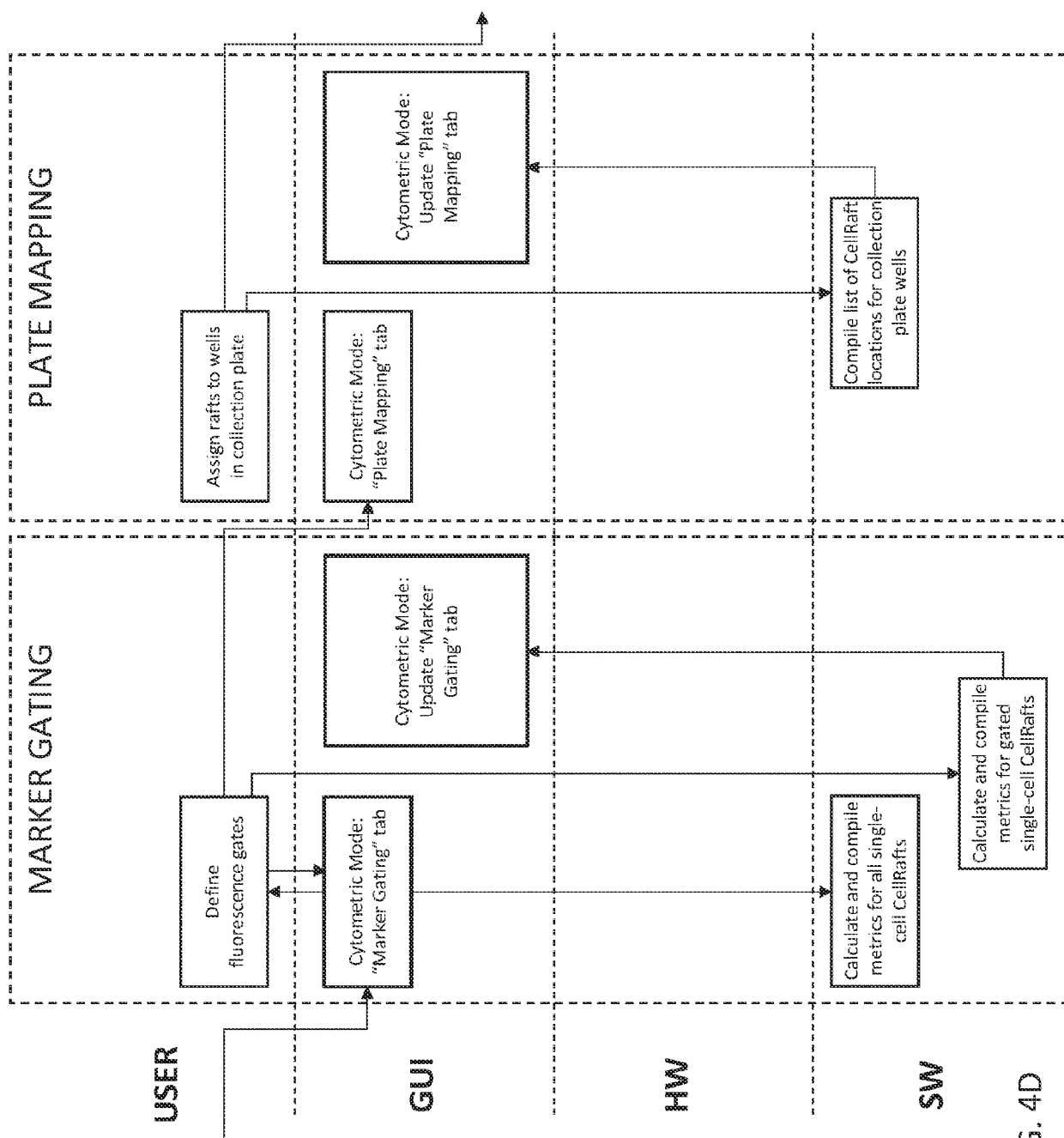
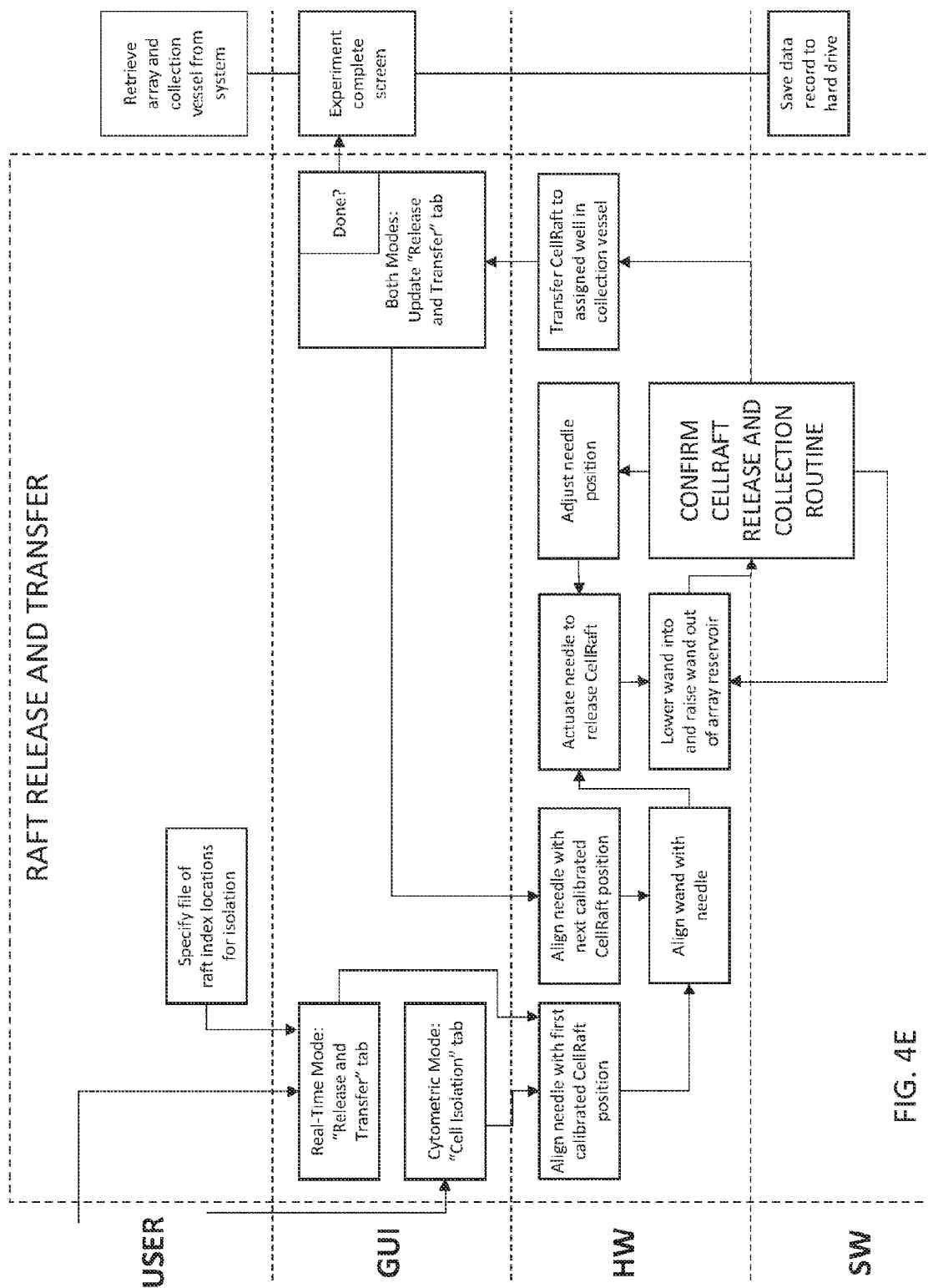


FIG. 4D



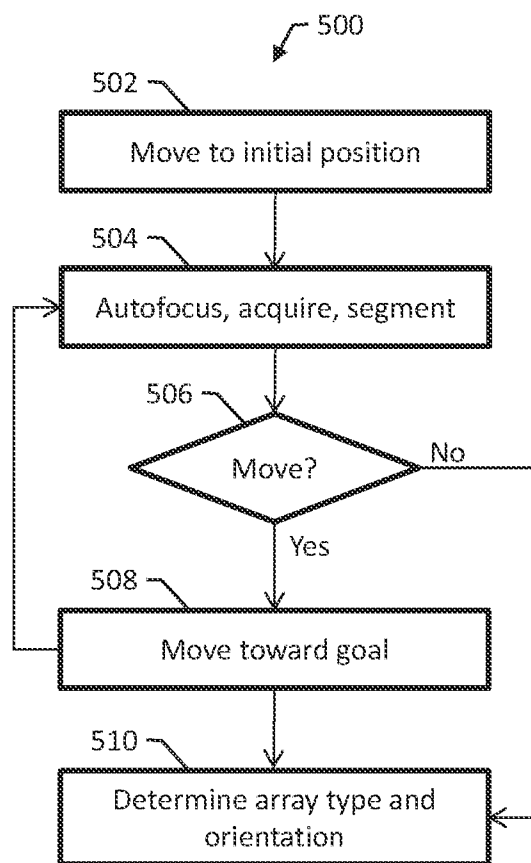


FIG. 5

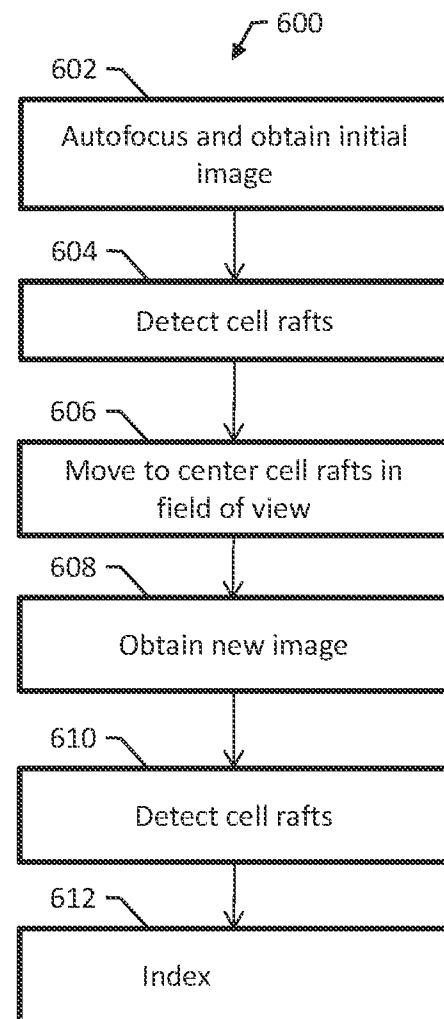


FIG. 6

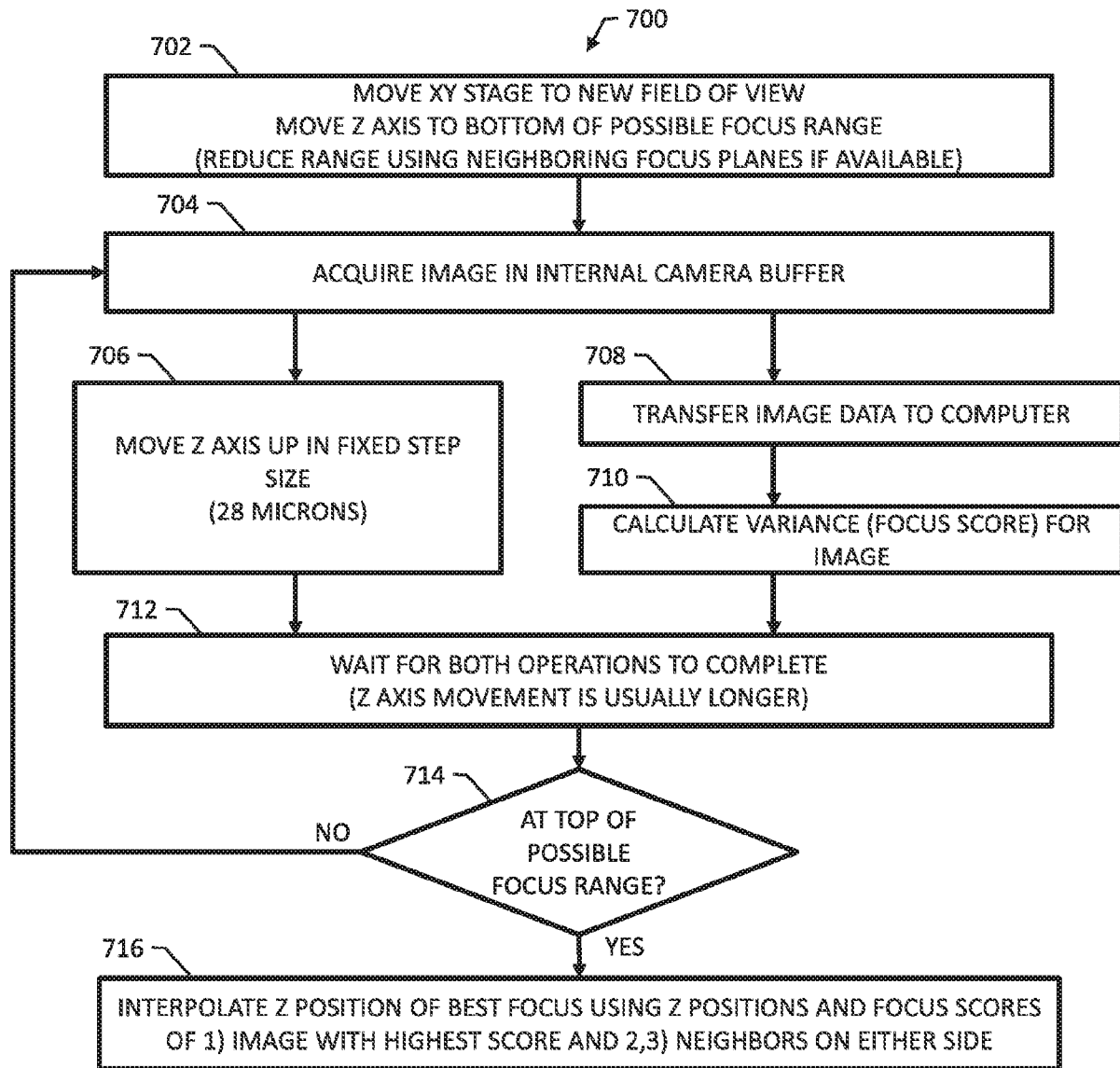


FIG. 7A

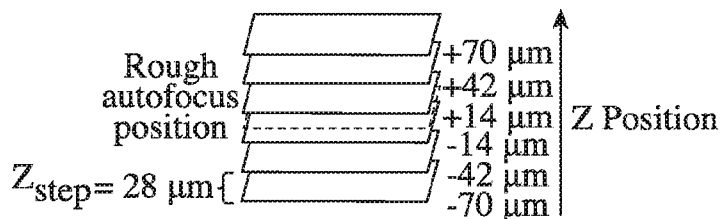


FIG. 7B

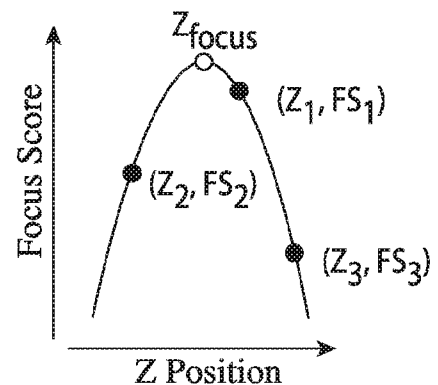


FIG. 7C

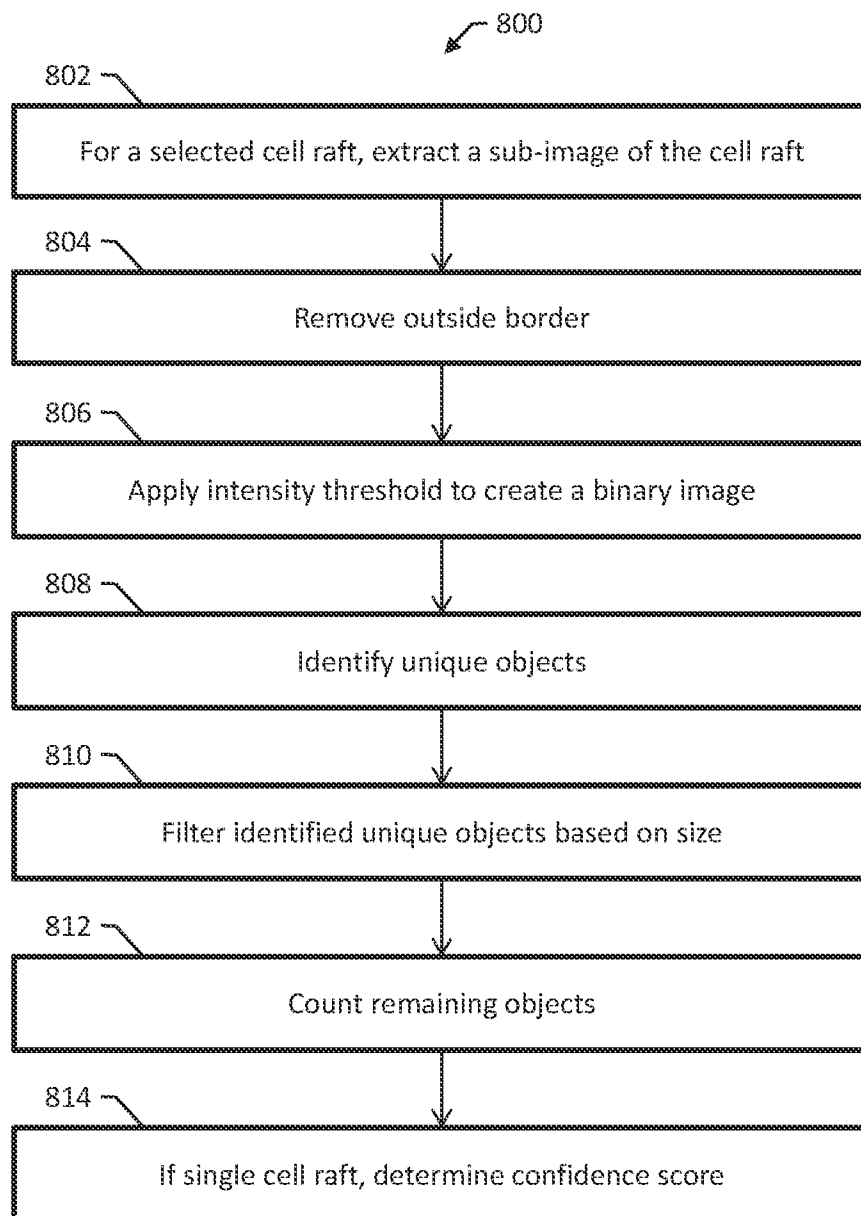


FIG. 8

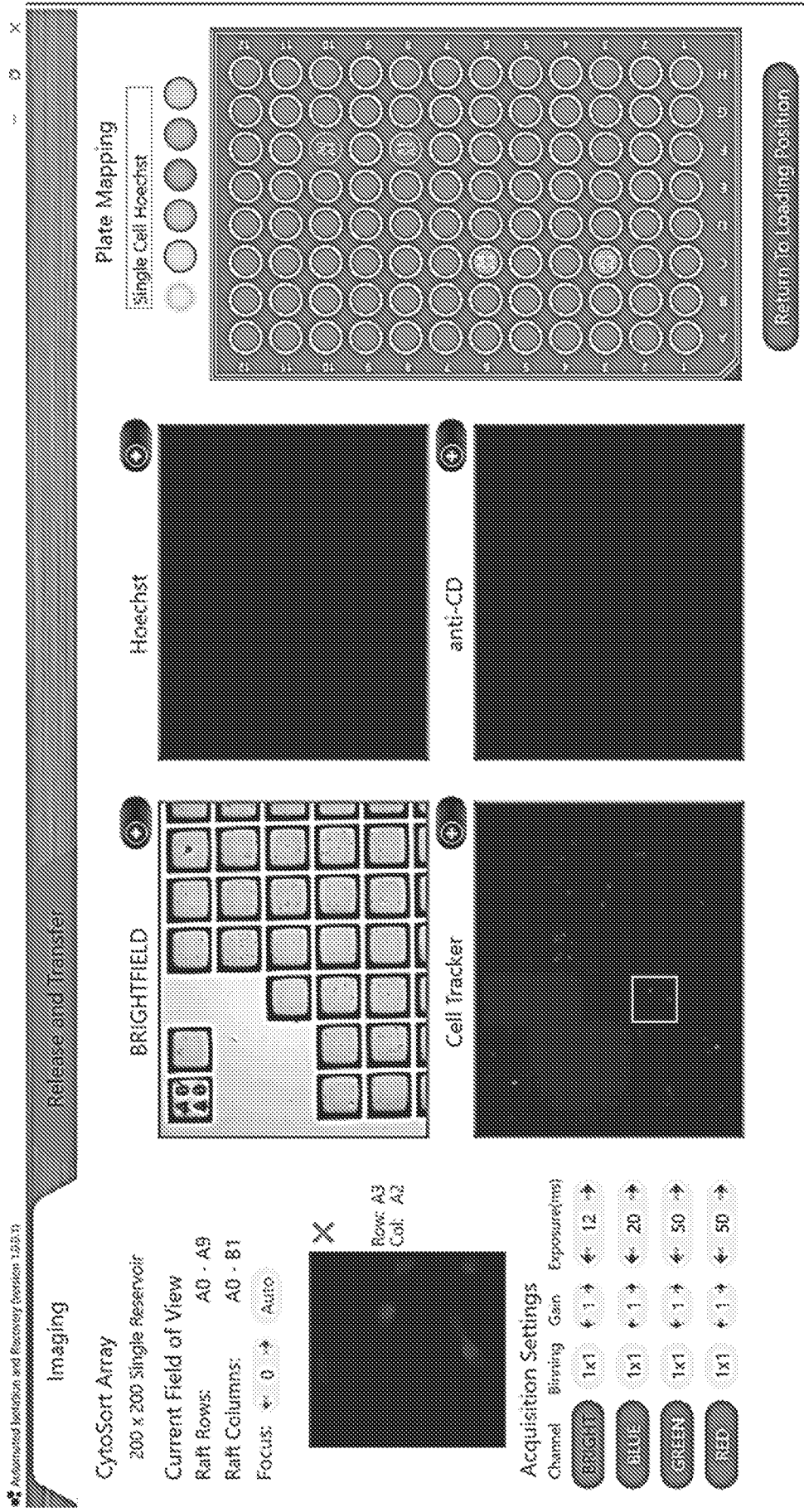
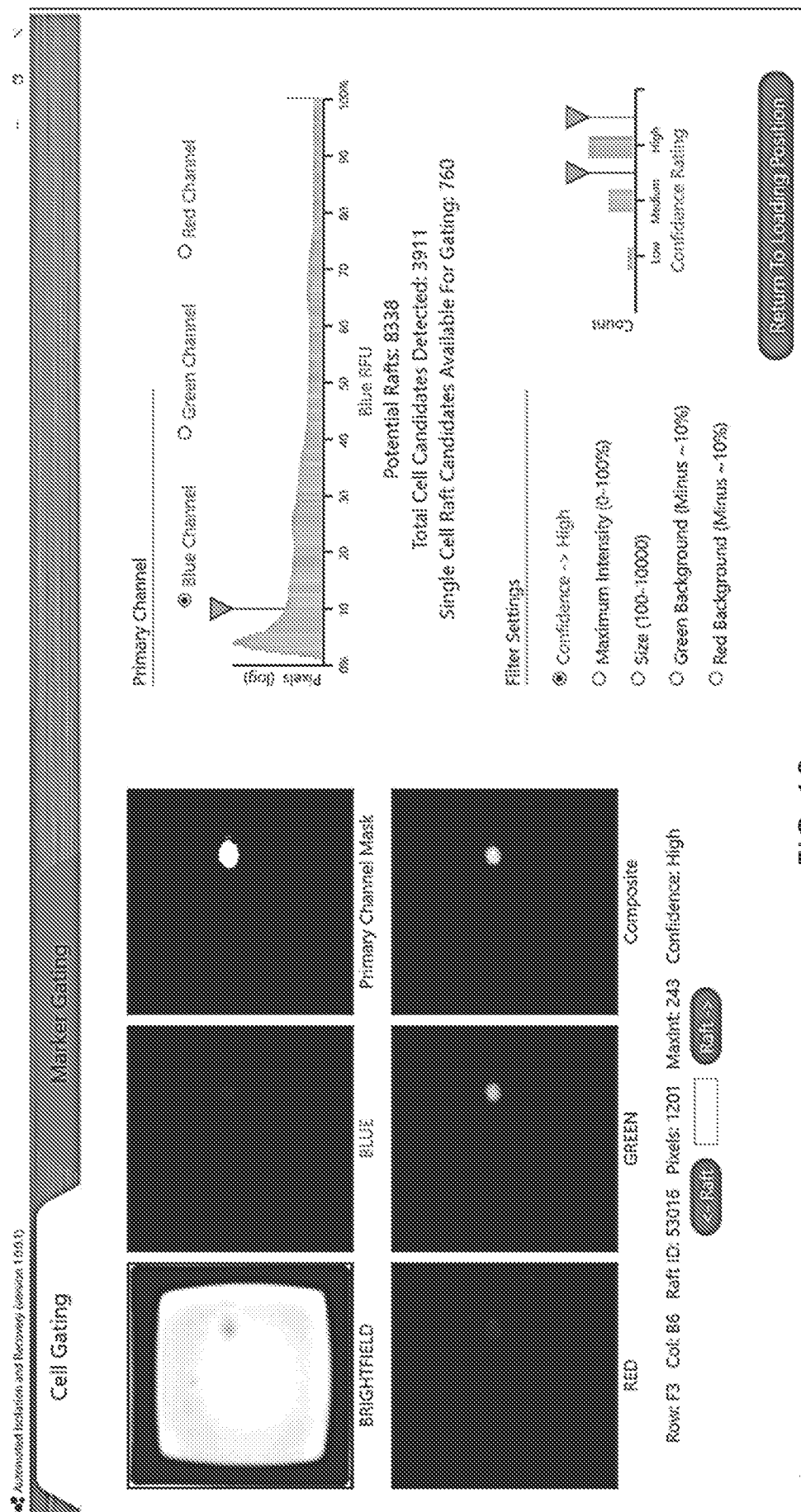
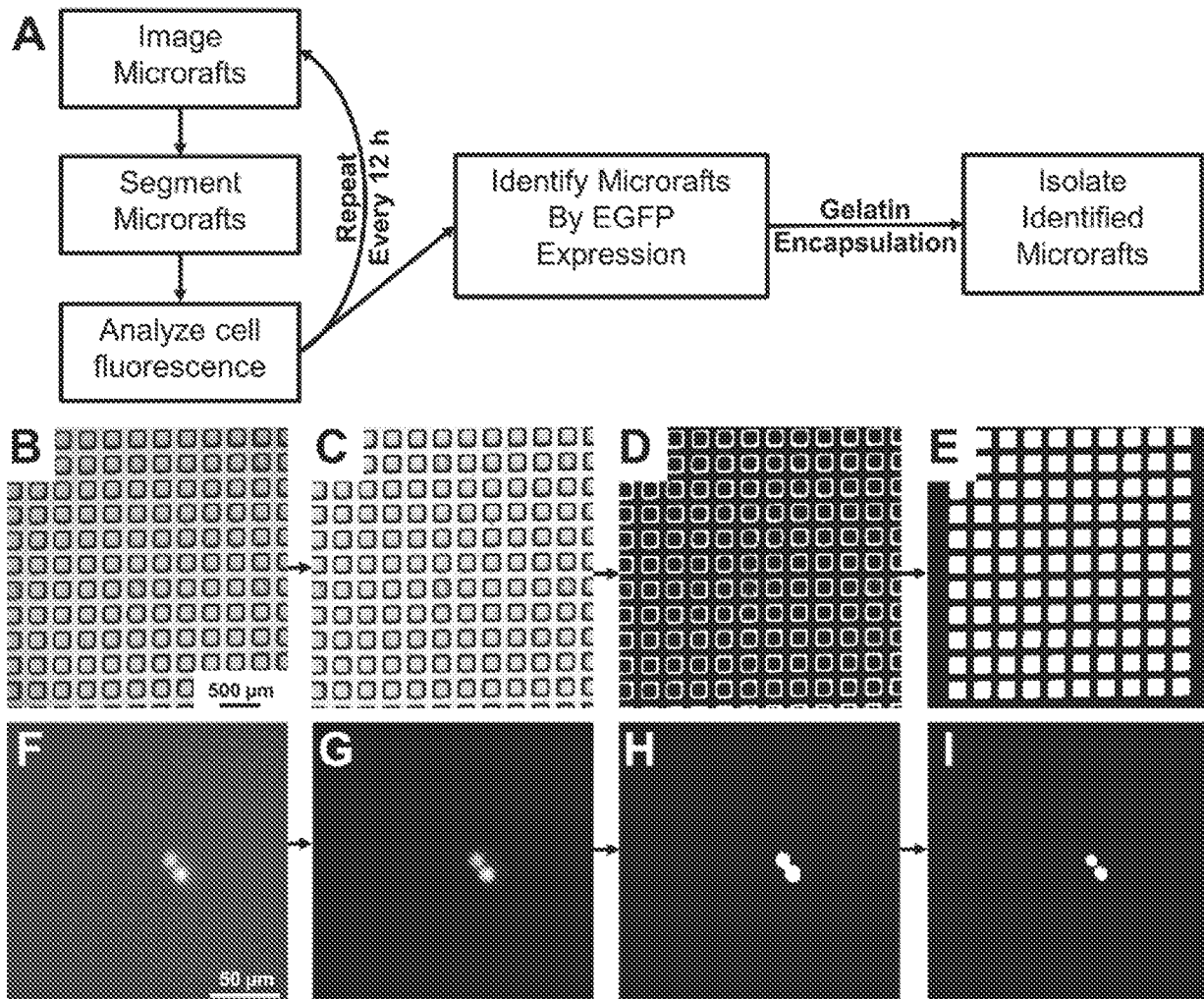


FIG. 9





INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/59979

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C40B 30/06, 50/06, 60/12, 60/14; G01N 21/76, 21/25, 21/64, 33/53, 35/02; C12M 1/32 (2018.01)
 CPC - A61B 34/32; C40B 30/06, 50/06, 60/12, 60/14; G01N 21/76, 21/25, 21/64, 33/53, 35/02; C12M 1/32, 1/12, 1/00, 1/34, 3/00; C12N 1/14, 1/20, 5/16; C12Q 1/02, 1/08; H01J 37/26; G06F 19/10

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)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	(GRACZ, AD et al.) A high throughput platform for stem cell-niche co-cultures and downstream gene expression analysis. Nature Cell Biology. March 2015, Epub 9 February 2015, Vol. 17, No. 3; pages 340-349; page 2, 3rd paragraph; page 3, 1st paragraph; page 3, 4th paragraph; page 6, 2nd paragraph; page 9, 3rd paragraph; page 10, 1st paragraph; page 12, 3rd paragraph; page 12, 5th paragraph; Supplemental pages 6-7; DOI: 10.1038/ncb3104	1-4, 5/1-4, 6/5/1-4, 11-13, 14/12-13, 28-29, 30/28-29, 35
X	US 9,068,155 B2 (ALLBRITTON, N et al.) 30 June 2015; entire document	1-4, 5/1-4, 6/5/1-4, 11-13, 14/12-13, 28-29, 30/28-29, 35
Y	(MOLECULAR DEVICES CORPORATION) Meta Imaging Series MetaMorph Basic Commands. Version 7.0.15 Product Manual [online]. 2006 [availability before priority date verified by web.archive.org; retrieved on 10 April 2018] Retrieved from the Internet: <URL: http://www.well.ox.ac.uk/_asset/file/metamorph-basic-commands.pdf>; page 228, 7th paragraph; page 238, 6th paragraph	3-4, 5/1-4, 6/5/1-4, 12-13, 14/12-13
A	(CARPENTER, AE et al.) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biology. 31 October 2008, Vol. 7, No. 10; pages 1-11; DOI: 10.1186/gb-2006-7-10-r100	1-4, 5/1-4, 6/5/1-4, 11-13, 14/12-13, 28-29, 30/28-29, 35
A	(WELCH, JD et al.) Selective single cell isolation for genomics using microraft arrays. Nucleic Acids Research. 30 September 2016, Epub 16 August 2016, Vol. 44, No. 17; pages 8292-8301; DOI: 10.1093/nar/gkw700	1-4, 5/1-4, 6/5/1-4, 11-13, 14/12-13, 28-29, 30/28-29, 35



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier 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

12 April 2018 (12.04.2018)

Date of mailing of the international search report

25 APR 2018

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/59979

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.: 7-10, 15-27, 31-34
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/59979

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	(DONG, X et al.) Accurate identification of single nucleotide variants in whole genome amplified single cells. <i>Nature Methods</i> . May 2017, Epub 20 March 2017, Vol. 14, No. 5, pages 491-493; DOI: 10.1038/nmeth.4227	1-4, 5/1-4, 6/5/1-4, 11-13, 14/12-13, 28-29, 30/28-29, 35
P, X	WO 2017/151582 A1 (THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL) 08 September 2017; entire document	1-4, 5/1-4, 6/5/1-4, 11-13, 14/12-13, 28-29, 30/28-29, 35
E, X	WO 2018/017892 A1 (TAKARA BIO USA, INC.) 25 January 2015; entire document	1-4, 5/1-4, 6/5/1-4, 11-13, 14/12-13, 28-29, 30/28-29, 35