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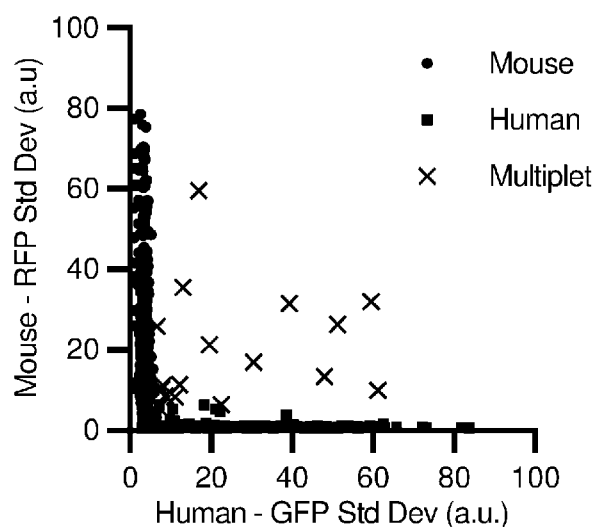


FIG 16A

(57) Abstract: Methods and systems for targeted cell lysis using high intensity focused ultrasound on a nanowell array covered with a membrane such as a gas permeable membrane are provided. The nanowells may have one or more shapes of one or more sizes in one or more patterns on the bottom of the nanowell. Changes in the shape, patterning, size, and spacing of the nanowells as well as the membrane thickness, and the ultrasound settings may be used to manipulate the percentage lysis, percentage crosstalk, and the number of wells cavitating.

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SYSTEMS AND METHODS FOR CELLULAR ACOUSTIC LYSIS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to and the benefit of the earlier filing of U.S. Provisional Application No. 63/597,316 filed on November 8, 2023, entitled “Systems and Methods for Single-Cell Acoustic Lysis”, which is incorporated by reference herein in its entirety.

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates generally to nanobubble-induced cavitation in nanowells.

BACKGROUND OF THE DISCLOSURE

[0003] Analysis of healthy and diseased tissues using single-cell omics offers high-resolution insight into biological processes. The challenge of single-cell lysis is to break the cellular membrane while preserving the molecular integrity and function of the released components. Current methods of lysis in a microfluidic format including mechanical, laser, acoustic, thermal, electrical, optical, and chemical methods have issues with protein degradation and cellular crosstalk.

[0004] Chemical lysis is the most common method for single cell lysis and uses various reagents to disrupt the cellular membrane and release intracellular contents. Commonly used reagents include detergents, chaotropic agents, enzymes, and organic solvents. While chemical methods are advantageous in terms of speed, scalability, and compatibility with a broad range of cell types and sample sizes, chemical lysis may have detrimental effects on the extracted cellular components including degradation, increased crosstalk, loss of function, and loss of structural integrity, leading to biased or inaccurate downstream analysis.

[0005] Mechanical lysis relies on the application of physical forces to break the cell membrane. For example, nanoscaled obstacles may be placed in microchannels creating shear forces to tear the cell structure. However, such methods may result in localized heating within a sample, leading to protein denaturation and aggregation.

[0006] Acoustic methods including bath sonication, probe sonication, or high-frequency ultrasound use sound waves to agitate and lyse cells creating stable or inertial cavitation. In stable cavitation, microbubbles oscillate due to acoustic pressure causing microstreams. Inertial cavitation occurs when gas bubbles grow to a critical size and undergo violent collapse, leading to the generation of high-speed jets, shock waves, and local heating. While ultrasound-mediated lysis is compatible with a broad range of cell types, it is inefficient and may result in heterogeneous

and contaminated results, especially when dealing with complex or heterogeneous cell populations. Miniaturized cavitation-induced lysis has the potential to allow for targeted analysis, however, it has proven difficult to implement due to problems such as increased pressure, inconsistent cavitation bubble size, issues with applying the cavitation homogeneously, and issues with controllability as cavitation is random and exponential by nature.

SUMMARY OF THE DISCLOSURE

[0007] The current disclosure provides systems and methods for miniaturized, controlled nanobubble induced cavitation for cell lysis. By miniaturizing and controlling the process, efficiencies are increased while maintaining the ability to lyse cells, mix their contents through a vessel, and maintain the integrity of nucleic acids, proteins, and enzymes.

[0008] A nanowell array (an array of wells with individual volumes of less than 100nL) made of a hydrophobic polymer with repeating geometric patterns on the bottoms of each nanowell is used to trap nanobubbles. While any method may be used to generate the nanobubbles, in some aspects, the nanobubbles are generated through solvent exchange. Nanobubbles may vary in size ranging from 10-500 nm. Using acoustical methods, such as high-intensity focused ultrasound (HIFU), cavitation may be induced using the nanobubbles as cavitation sites inside the nanowells. By altering the pattern on the bottom of each nanowell, the amount and size of the nanobubbles, and therefore the resulting cavitation, may be controlled. The size of each well, the spacing between each well within the array, the pattern on the bottom of each well, and the size of the pattern on the bottom of each well may be varied depending on the size of the cells and the amount and type of lysis desired. In some aspects, the nanowell array may be covered by a gas permeable membrane such that each well in the nanowell array is covered by at least part of the gas permeable membrane. The gas permeable membrane allows a measurable exchange of gas across the membrane over a time scale of minutes to hours. The thickness of the gas permeable membrane may be varied depending on the desired cellular activity. For example, varying the thickness of the gas permeable membrane may impact the amount of cavitation, the type of lysis, the amount of lysis, the range of the cavitation, and the amount of crosstalk between cells. In some aspects, the thickness of the membrane may be used to manipulate the number or type of cells being lysed. For example, membranes with a thickness of under 100 μm may be used for targeted lysis in which particular cells or sets of cells are lysed, while thicker membranes such as those at or over 150 μm may be used for high-throughput analysis in which all or most of the cells in the nanowell are lysed.

[0009] In some aspects, the type of acoustic signal, power, pulse repetition frequency, treatment time, and burst length provided by the acoustic system may be varied. In some aspects, the thickness of a gas permeable membrane used within the system such as high-intensity focused ultrasound (HIFU) may be varied. Such factors may be varied for a variety of reasons including cell size. In some aspects, such factors may be varied to reduce heat production. In some aspects, such factors may be varied to decrease cell crosstalk. In some aspects, such factors may be varied to increase the efficiency of cell lysis. In some aspects, such factors may be varied to increase the homogeneity of the resulting components. In some aspects, such factors may be varied to improve the consistency in the lysis from one nanowell to another. In some aspects, such factors may be varied to induce cavitation on a selection of nanowells in the nanowell array. In other aspects, cavitation may be induced in all wells in the nanowell array. In some aspects, such factors may be varied to lyse specific cells or sets of cells within a nanowell array.

[0010] The nanowell array may be used with a variety of omics and other single cell analysis including high-throughput transposase-accessible chromatin throughput sequencing (ATAC) and high-throughput mRNA sequencing among other types of sequencing.

[0011] In some aspects, the nanowell array may be covered with a gas permeable membrane and optionally placed in a tank containing a liquid or gel such as a water tank. In some aspects, an imaging device such as a microscope including an epifluorescence microscope may be positioned above the nanowell array and a HIFU transducer positioned below the nanowell array. The nanowell array and the HIFU transducer may be acoustically coupled, meaning that a media such as a liquid or gel connects the transducer with the nanowell array and is capable of transmitting acoustic waves through the media without significant attenuation of the waves. The impedance of the connecting media may be equivalent or similar to that of the nanowell array to minimize reflection of the acoustic waves at the array surface.

[0012] The use of the platform as described herein allows for cell lysis and enzymatic reactions to take place within single, individualized nanowells, and allows for temporal and spatial control over lysis. The design of the nanowell array may enhance acoustic cavitation resulting in high lysis, and low crosstalk.

[0013] These and other embodiments will be described in further detail in the detailed description, below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] FIG. 1. Process for single-cell HIFU lysis according to an embodiment.

[0015] FIGs. 2A-2D An embodiment of a custom nanowell tray without (FIGs. 2A and 2C) and with (FIGs. 2B and 2D) a gas-permeable hydrophobic membrane.

[0016] FIG. 3. A nanowell array-based assembly with a plurality of chambers according to an embodiment.

[0017] FIGs. 4A-4C. Characterization of polydimethylsiloxane (PDMS) gas permeable membranes according to embodiment showing the relationship between spin speed in revolutions per minute (RPM) and final membrane thickness (FIG. 4A); water-vapor permeability, as a proxy for gas permeability, of different thicknesses of membranes at room temperature with 1000 μm (410), 250 μm (406), 150 μm (408), 100 μm (404) and 0 μm (402) (ΔV =change in volume) (FIG. 4B); and the evaporation rate of membranes of different thicknesses (FIG. 4C).

[0018] FIGs. 5A, 5B. Illustrations of a High Intensity Focused Ultrasound (HIFU) apparatus according to an embodiment.

[0019] FIGs. 6A, 6B. Comparison of the performance of nanopatterns under the same high intensity focused ultrasound with a normalized number of cells cavitating for shapes of nanowell patterning * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by one-way ANOVA and Tukey's multiple comparisons test (FIG. 6A) and size of the entire nanopattern feature * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ by two-way ANOVA using Sidak's multiple comparison test (FIG. 6B).

[0020] FIGs. 7A-7H. Percent wells cavitating according to an embodiment as a function of shape of nanowells (hexagonal or circular) (FIG. 7A), without a nanopattern on the base of the well, membrane thickness in μm (FIG. 7B), well spacing (μm) (FIG. 7C), buffer (FIG. 7D), power (FIG. 7E), pulse repetition frequency (PRF) (FIG. 7F), treatment time (FIG. 7G), and burst length (FIG. 7H).

[0021] FIGs. 8A-8H. Percent cell lysis according to an embodiment as a function of shape of nanowells (hexagonal or circular) (FIG. 8A), without a nanopattern on the base of the well, membrane thickness in μm (FIG. 8B), well spacing (μm) (FIG. 8C), buffer (FIG. 8D), power (FIG. 8E), PRF (FIG. 8F), treatment time (FIG. 8G), and burst length (FIG. 8H).

[0022] FIGs. 9A-9H. Percent crosstalk in the focal zone according to an embodiment as a function of shape of nanowells (hexagonal or circular) (FIG. 9A), without a nanopattern on the base of the well, membrane thickness in μm (FIG. 9B), well spacing (μm) (FIG. 9C), buffer (9D), power (FIG. 9E), PRF (FIG. 9F), treatment time (FIG. 9G), and burst length (FIG. 9H).

[0023] FIGs. 10A-10H. Electron micrographs of nanopatterns tested including 2 μm squares (FIG. 10A), 5 μm squares (FIG. 10B), 10 μm squares with raised edges (FIG. 10C), flat wells (FIG.

10D), 2 μm lines (FIG. 10E), 2 μm nested concentric circles (FIG. 10F), 2 μm squares depressed into the polymer (inverse) (FIG. 10G), and 2 μm triangles (FIG. 10H).

[0024] FIG. 11. Results of orthogonal array testing to identify shape(s), membrane thickness (μm), well spacing (μm), buffer, power (W), PRF (Hz), treatment time (s), and burst length (μs) parameters to achieve >80% cell lysis and <20% crosstalk in one or more nanopatterned nanowells in a nanowell array.

[0025] FIGs. 12A, 12B. Quantification of the percent cell lysis (FIG. 12A) and percent crosstalk (FIG. 12B) of the optimized conditions (Nanowell array: Well size: 50 μm , Well spacing: 15 μm , Well shape: circle, Well nanopattern: 2x2x2 μm squares, Membrane thickness: 150 μm , Power: 60W, Treatment time: 1s, PRF: 50 Hz, Burst length: 10 μs , Frequency: 1100kHz).

[0026] FIGs. 13A, 13B. Examples of the effects of parameter variations on the percent cells lysed (y-axis) and percent crosstalk (x-axis), as a function of gas permeable membrane thickness (μm) (FIG. 13A) and power (FIG. 13B) in a nanowell array with 50 μm well diameter, 50 μm well depth, 15 μm spacing between wells, 2 μm x 2 μm x 2 μm square nanopatterning, 150 μm membrane thickness and HIFU parameters of: Power: 60W, Burst length: 10 μs , Frequency: 1100 MHz, Treatment time: 1s, PRF: 50 Hz.

[0027] FIGs. 14A, 14B. Examples of effects of parameters on the percent cells lysed (y-axis) and percent crosstalk (x-axis) and as a function of treatment time (seconds) (FIG. 14A) and pulse repetition frequency (FIG. 14B) in a nanowell array with 50 μm well diameter, 50 μm well depth, 15 μm spacing between wells, 2 μm x 2 μm x 2 μm square nanopatterning, 150 μm membrane thickness and HIFU parameters of: Power: 60W, Burst length: 10 μs , Frequency: 1100 MHz, Treatment time: 1s, PRF: 50 Hz.

[0028] FIG. 15 provides the effects of nanowell array, gas permeable membrane, and HIFU parameters on percent cell lysis and number of wells cavitating. Each point on the graph represents an independent combination of shape(s), membrane thickness (μm), well spacing (μm), buffer, power (W), PRF (Hz), treatment time (s), and burst length (μs).

[0029] FIGs. 16A-16D. Results of single-cell RNA sequencing (scRNA-seq) of individually lysed cells from a mixture of human HEK 293T and mouse NIH 3T3 cells using the protocol of Example 8 showing an imaging barnyard of primarily single cells with a low number of multiplets as determined by fluorescence (FIG. 16A) and the equivalent sequencing barnyard with similar proportions (FIG. 16B). The number of unique molecular identifiers (UMI) per cell (FIG. 16C), and number of UMI per gene for each cell (FIG. 16D) are also shown.

[0030] FIGs. 17A, 17B. Analysis of microscopy images of the nanowell array loaded with barcoded capture beads showing the mean intensity and standard deviation of individual nanowells with and without beads. Each point is an individual well within the nanowell array.

[0031] FIGs. 18A, 18B. Analysis of microscopy images of the nanowell array loaded with cells expressing a red fluorescent marker (RFP) showing the mean intensity and standard deviation of individual nanowells with and without RFP cells. Each point is an individual well within the nanowell array.

[0032] FIGs. 19A, 19B. Analysis of microscopy images of the nanowell array loaded with cells expressing a red fluorescent marker (RFP) showing the change (delta) in mean intensity and standard deviation of individual nanowells with RFP cells after treating the nanowell array with HIFU to identify lysed and non-lysed cells within each nanowell. Each point is an individual well within the nanowell array.

[0033] FIG.20. Results of single cell RNA sequencing of mouse brain cortex cells displayed as a U Map to identify the major cell types present in the mouse cortex. Each dot represents an individual cell.

[0034] FIGs. 21A, 22B. HIFU parameters and membrane thickness may be modified to achieve a smaller focal zone and targeted single cell lysis. FIG. 21B is the inset data in the square box from FIG.21A. At HIFU powers lower than 30W a thicker 150 μm membrane has no active cavitation and cell lysis. With a 64 μm membrane and a HIFU power of 5W, the focal zone of the HIFU can be reduced to a 14 well area.

[0035] FIGs. 22A – 22C. Quantification of cell lysis performance using targeted lysis. Individually selected cells were lysed and the number of cells/min (FIG. 22A), percent lysis of target cells (FIG. 22B), and percentage of off-target cell lysis (FIG. 22C) were measured.

[0036] FIGs. 23A, 23B. Digital droplet PCR results of selective lysis on mixtures of human and mouse cells showing the number of copies of prostaglandin E receptor 2 (PTGER2) cDNA converted mRNA released when targeting human cells in a background of mouse, targeting no cells as a negative and targeting all cells (FIG. 23A). Digital droplet PCR results when targeting different total number of human cells in a background of mouse cells are also shown (FIG. 23B).

[0037] FIGs. 24A-24D are images atomic force microscopy of bubble formation of the surface of: (FIG. 24A) a dry polystyrene nanowell array with a force of 2 nN, (FIG. 24B) after solvent exchange from ethanol to PBS with a force of 2 nN, (24C) the same area as (FIG. 24B) after solvent exchange with a force of 10 nN, and (FIG. 24D) the same area as (FIG. 24B) and (FIG. 24C) after solvent exchange returning to 2nN of force after 10 nN of force.

DETAILED DESCRIPTION

[0038] Various implementations of the present disclosure will be described in detail with reference to the drawings, wherein like reference numerals present like parts and assemblies throughout the several views. Additionally, any examples set forth in this specification are not intended to be limiting and merely set forth some of the many possible implementations.

[0039] Provided herein are arrays of nanowells that may be used with acoustic systems to perform cell lysis and enzymatic reactions within single, individualized nanowells in the nanowell array. The system provides temporal and spatial control over lysis resulting in high lysis and low crosstalk. In some aspects, the system provides targeted lysis of specific cells or sets of cells in a mixture of cells. Such systems may be used for a variety of purposes including tissue analysis using single-cell omics.

[0040] One or more aspects of the nanowell and the nanowell system may be varied depending on the desired outcome; guidelines for various combinations that result in intended outcome(s) are provided. For example, the size and shape of the nanowells may be varied. The type, size, repetition, and spacing of the pattern at the bottom of each nanowell may be varied. In some aspects, the type of acoustic signal, power, pulse repetition frequency (PRF), treatment time, and burst length provided by the acoustic system may be varied. In some aspects, the parameters of frequency, burst length, power, and PRF may impact the input pressure (pressure produced by the ultrasound transducer) and pressure inside the wells (pressure remaining after coupling effects and attenuation). In some aspects, the thickness of a gas permeable membrane used within the system may be varied. Such factors may be varied for a variety of reasons including cell size. In some aspects, such factors may be varied to reduce heat production. In some aspects, such factors may be varied to decrease cell crosstalk. In some aspects, such factors may be varied to increase efficiency of cell lysis. In some aspects, the various factors may be varied to alter the pressure inside the nanowells. In some aspects, such factors may be varied to increase the uniformity of the well contents relative to the contents of other wells. In some aspects, the factors may be varied to allow for targeted lysis of particular cells or sets of cells within a nanowell.

[0041] In some aspects, a plurality of factors may be adjusted to achieve the desired result. For example, with a smaller geometric pattern on the bottom of a nanowell, lower power can be used to have the same number of cells cavitate in comparison to a larger pattern. Thus, the pattern size influences the acoustic system used. In some embodiments, the aspects may be varied depending on the type of downstream analysis to be performed. For example, lower power high-intensity focused ultrasound (HIFU) such as less than 50W and/or pulse repetition frequency of less than 50Hz may be used to mix well contents without lysis. In other aspects, higher power

HIFU such as more than 70 W and/or pulse repetition frequency (PRF) of greater than 70Hz may be used to disrupt cell nuclei for DNA based sequencing outputs.

[0042] A nanowell array is a collection of nanowells in a single unit or plate. Nanowells may be circular, hexagonal, triangular, elliptical, or square among other shapes. As shown in FIGs. 8A and 9A, a hexagonal shape may increase the percent lysis in comparison to a circular shape. The nanowells may be a variety of sizes. For example, they may be 5 microns to 500 microns in diameter. In other aspects, they may be 40 microns to 60 microns in diameter. In some aspects they are 50 μm in diameter and 50 μm in depth. In some aspects, the size of the well may be dictated by the cells being analyzed. For example, the well may have a size such that only a single cell will fit in each well as shown for example, in FIG. 2A. In some aspects, the well may fit multiple cells as shown in FIG. 2C. The patterns at the bottoms of the nanowells may also have different shapes as shown in FIGs. 10A-10G in which protruding squares, parallel lines, nested concentric circles, triangles, and recessed squares (inverse) are depicted. The size of the patterns of the nanowells may also vary in size such that the width, depth, and height of the pattern varies between 1 μm to 20 μm including 2 μm x 2 μm x 2 μm , 5 μm x 5 μm x 5 μm , and 10 μm x 10 μm x 10 μm . In some aspects, at least one dimension of the pattern, that is one of the width, height, depth, or length is 0.2 μm to 11 μm , for example 2 μm , 5 μm , or 10 μm or any fraction or subset thereof.

[0043] In some aspects, the spacing between each nanowell in an array may be varied. For example, the nanowells may be spaced between 5 μm to 50 μm , for example, 1 μm , 2 μm , 5 μm , 10 μm , 15 μm , 20 μm , 25 μm , 30 μm , or 35 μm apart. Each nanowell in a nanowell array may be spaced the same or different distances apart, may have the same or different shapes and sizes, and may have the same or different patterns on the bottom, where each of the shape, size, spacing, and pattern are optionally selected independently.

[0044] In some aspects, the acoustic system used with the nanoarrays may be a high intensity focused ultrasound (HIFU) based system.

[0045] The size, shape, patterning, and spacing of the nanowells in the nanowell array may be used to achieve different effects depending on the type of cell, size of the cell, cell membrane thickness, desired uniformity of the resultant lysed cell samples as compared between wells, homogeneity of the cells, amount of throughput, desired amount of cavitation, and the importance of minimized crosstalk. As shown in FIG. 6B, the size of the nanopattern has a direct relationship with the uniformity of the lysis, the smaller the nanopattern, the more uniform and higher number of wells cavitating compared to larger nanopattern if all other conditions are kept constant. Further, smaller shapes with more corners showed a better response compared to other shapes

as shown in FIG. 6A with randomly surfaced nanowells (referred to herein as “flat”) reaching a maximum of 70% well cavitation while geometrically patterned nanowells reach a maximum of 98% well cavitation.

[0046] The nanowell array may be formed by any means generally known to those of ordinary skill in the art including soft lithography, laser ablation, modular molding, hot embossing, compression molding, and 3D or other printing methods and combinations thereof. In some aspects, the nanowell array may be formed using soft lithography. Soft lithography is a technique for replicating structures using an elastomeric stamping technique. In soft lithography, any type of elastomeric compound may be used to form the stamp, for example, polydimethylsiloxane (PDMS), tri-allyl-tri-azine:tri-thioltriacine 4:3 mixture, tri-allyl-tri-azine:tetra-thiolpentaerythritol 2:3 mixture, as well as other polymeric materials such as photocurable perfluoropolyethers, or cycloolefin copolymer. The mold may then be used to transfer a pattern onto a second material. In some aspects, the pattern may be transferred using hot embossing. In hot embossing, the stamp is heated above the glass transition temperature of the polymer substrate. Pressure is applied to the stamp and the concave topography is transferred to the softened polymer. The system is cooled, the mold is removed, and the polymer retains the relief structure of the stamp. Any polymer substrate that has low acoustic attenuation, hydrophobicity, and moldability can be used as the second material. In contrast to hydrophilic polymers, hydrophobic polymers allow for trapping of surface nanobubbles as described in further detail below. Exemplary hydrophobic polymers include polystyrene polymers, fluorocarbon polymers, cyclo olefin polymers, and poly(N-vinylpyrrolidone) (PVP) polymers or combinations thereof. In some aspects, the polymer substrate is polystyrene which has a glass transition temperature of $>100^{\circ}\text{C}$.

[0047] A nanowell array loaded with cells may be covered by a gas permeable membrane that acts as a pressure release system, allowing vapor to permeate through the membrane while maintaining the separation between cells and reagents in each well. In some aspects, the thickness of the membrane may be varied to manipulate the reflection and/or absorption of the ultrasound waves, impacting the forces generated in the nanowells and the resulting lysis of the cells in those nanowells.

[0048] The gas permeable membrane is arranged over the openings of the wells in the nanowell array. In some aspects, the gas permeable membrane may be covered with a layer of water to prevent it from drying out. The gas permeable membrane may be manufactured using any method generally used including spin coating, casting using a spacer, stencil printing, or a micromold. In some aspects, the membrane may be a PDMS membrane with a thickness between about 25 μm to 500 μm including 50 μm , 65 μm , 70 μm , 82 μm , 100 μm , 150 μm , 200 μm , 225 μm , 250 μm ,

325 μm , 390 μm , and 425 μm or any fraction thereof. As shown in FIG. 13A, variations in the thickness of the membrane impact the % lysis and the % crosstalk. Variations in the thickness of the membrane may be achieved, for example, by a change in the manufacturing method. For example, as shown in FIG. 4A, the membrane thickness is dependent on the speed of the spin coating. As water vapor permeability γ , gas permeability, and Young's modulus are inversely proportional to the thickness of an elastomeric membrane such as polydimethylsiloxane (PDMS) (see FIGs. 4B-4C), the thickness of the membrane may be varied to achieve the desired effect. In some aspects, the thickness of the membrane may be varied depending on the amount of cross-talk tolerated and/or the amount of cavitation required. For example, membranes of less than 50 μm are too thin to allow cavitation to occur. Further, membranes over 500 μm detach from the array by more than a threshold amount, increasing the amount of crosstalk. The thickness of the gas permeable membrane may additionally be varied depending on the intended downstream processing. For example, in high-throughput lysis, membranes of 100 μm to 200 μm may be used including 150 μm . For targeted cell lysis, membranes of 50 μm to 80 μm may be used, for example 70 μm .

[0049] As shown in FIG. 1, a cell suspension and/or reaction mixture may be loaded into a nanowell array 102. Reaction mixtures may include cells, cell nuclei, reagents, enzymes, antibodies, molecular biology reagents, beads, ligation reaction components, tagmentation reaction components, labeled cells, labeled cell nuclei, reverse transcription reaction components, and the like. After the cells are loaded, the array is capped with a membrane at 104 of the desired thickness, for example from 55 μm to 450 μm . In some aspects, it is 70 μm thick. In other aspects, it is 150 μm thick. In some aspects, the membrane is hydrophobic. In other aspects, the membrane is gas permeable. Such a membrane may act as a pressure release and control system. In some aspects, a liquid/gas interface between the membrane and the contents of the well may be dried to achieve membrane adherence. Once the array is capped, the mixture is imaged using, for example, epifluorescent microscopy at 106 and the cells targeted for lysis are identified. The cells are then lysed using HIFU cavitation-based lysis at 108. HIFU cavitation-based lysis may be performed on specific locations (focal areas) or throughout the array. In some aspects, the specific locations or focal areas may be identified using imaging. After the treatment, the membrane is lifted at 110 and the contents of the nanowell are extracted at 112 for further analysis.

[0050] While any density of cells may be loaded into the wells, in some examples, a cell suspension has a density such that only one cell or cell nucleus occupies at least 50% of the wells. In other aspects, the density of the cells may be greater or lower. In some aspects, the cell

density may be such that multiple cells including multiple types of cells are loaded into the wells. In some aspects, the cell density may be such that one or more wells are filled with one or more cells. In some aspects, some, but not all of the wells may be filled with at least one cell. In some aspects, the cell suspension may include only cells. In other aspects, the cell suspension may include only cell nuclei. In further aspects, the cell suspension may include cells and cell nuclei. The cells and/or cell nuclei may be labeled or unlabeled. For example, in some aspects, the cells or cell nuclei may be pre-barcoded cells or cell nuclei.

[0051] An exemplary compression molded nanowell array is shown in FIGs. 2A-3. As shown in FIGs. 2A and 2B, a nanowell array 202 separates each cell 204 of a solution into a respective well 206. A gas permeable membrane 208 is then placed over the array. As Shown in FIG. 2C, a nanowell array 202 separates one or more cells such as cell 204 and cell 210 into a plurality of wells in the nanowell array 202. The cells may be the same or different types of cells. As shown in FIG. 2C, in the first compartment, there are two cells 210 of a type of cell that is different than the cell 204. In subsequence nanowells, there may be a mixture of the type of cell 204 and the type of cell 210. The nanowell array 202 of FIG. 2D may also be covered by a gas permeable membrane 208.

[0052] As shown in FIG. 3, in some aspects, an optional gasket 304 is placed over a nanowell array 302. The polymer-based gasket 304 may be used for compartmentalizing a portion of the nanowell array. For example, the nanowell array may be compartmentalized in an area such as area 306 (referred to herein as a chamber) in which a group of nanowells are isolated from other nanowells in the nanowell array. In some aspects, a chamber may be used to isolate a region of the nanowells for smaller experiments. In some aspects, each nanowell within a chamber may contain the same or same types of cells, such that each chamber only contains one type of cells. In other aspects, various nanowells within a chamber may contain different types of cells, whether each well contains a single cell or multiple cells such that the nanowells within a chamber may contain different types of cells.

[0053] The systems and methods described herein use miniaturized, controlled nanobubble induced cavitation for cell lysis. The desired conditions and/or the desired types of cell lysis may be adjusted using the treatment frequency, burst length, treatment time, power, gas-permeable membrane thickness, spacing between the wells of the nanowell array, the size and amount of patterning on the bottom of a well of the nanowell array, the shape of a well of the nanowell array, the buffer and the like.

[0054] In some aspects, nanobubbles may be created on the surface of the nanowells prior to loading the nanowells with a cell suspension. Nanobubbles may be created using a variety of

methods. In some examples, nanobubbles may be formed through solvent exchange between two liquids with different gas solubilities, for example, alcohols and aqueous buffers. The process traps bubbles on the surface of the nanowells by using the nanowell patterns as bubble traps. Exemplary alcohols include ethanol, methanol, and isopropanol. Exemplary aqueous buffers include phosphate, PBS, bicarbonate, reverse transcriptase buffer, and DNA ligation buffer. Such nanobubbles may vary between 500 nm to 10 μ m in diameter, 10 nm to 10 μ m, 10 nm to 1 μ m or any fraction or subset thereof. In some aspects, the size of the nanobubbles may impact the amount of cavitation.

[0055] The amount and size of nanobubbles trapped by patterned nanowells may vary depending on the scale of the pattern. For example, when the size of the nanopattern was increased to 5 x 5 microns, bubbles were no longer seen in the nanowells. The ability of a pattern to trap air on the surface pockets was analyzed using rhodamine 6G, a dye that clusters specifically around the liquid-gas interface. When dye is present in ethanol, with no gas exchange, no apparent increased fluorescence was seen. However, after performing buffer exchange between ethanol and PBS, increased fluorescent intensity overlapping the nanopatterns was observed (not shown).

[0056] Micrographs of the nanowell surface taken with atomic force microscopy showed that the surfaces of unpatterned or “flat” nanowells have significant roughness and possess large, random artifacts (craters with $\sim$ 1 μ m diameter) due to the manufacturing process (FIG. 24A). Buffer exchange generates surface nanobubbles on the unpatterned surface (FIG. 24B). Atomic force microscopy cantilever force applied to the nanowell confirmed that the bubbles were not manufacturing artifacts. Upon increasing the peak force of the atomic force microscopy to 5 nN, there was a decrease in the number of nanobubbles present on the surface with nearly complete removal of the nanobubbles when the peak force was raised to 10nN (FIG. 24C), confirming the non-solid and transient nature of the surface nanobubbles. When the peak force was returned to the starting pressure of 2. nN, a small number of the surface nanobubbles reappeared, further confirming that the nanobubbles were not an artifact of the molding process (FIG. 24D).

[0057] HIFU may be used to induce cavitation on the surface nanobubbles inside the wells. In some aspects, the HIFU cavitation-based lysis is performed using an apparatus or platform such as the apparatus or platform of FIGs. 5A-5B in which a microwell chip 504 such as the nanowell array of FIGs. 2A-3, is placed on a motorized arm 502 between an epifluorescence microscope 506 and an HIFU transducer 508. In some aspects, the system may be in a water tank 510 as shown in FIG. 5B. The use of water above the sealed gas permeable membrane during biochemical reactions may assist in maintaining hydration levels in the nanowells. In some

aspects, the transducer may be 1 mm to 5 mm from the nanowell array or any fraction thereof. In some aspects, the transducer focal zone is within 10 mm of the bottom of the nanowell array.

[0058] In some aspects, the nanowell array and the HIFU transducer may be acoustically coupled, meaning that media such as a liquid or gel connects the transducer with the nanowell array and is capable of transmitting acoustic waves without significant attenuation of the waves. While a water tank is shown in FIG. 5B, the continuous media may be otherwise applied to the nanowell array such as by placing it directly on the nanowell, for example through the use of an ultrasound gel which is in contact with both the transducer and nanowell array. The impedance of the connecting media may be equivalent or similar to that of the nanowell array to minimize reflection of the acoustic waves at the array surface.

[0059] By altering the time, pulse repetition frequency, frequency, power, and temperature of the HIFU, the amount of crosstalk and efficiency of the lysis may be adjusted. For example, the HIFU may use a power of less than 150 W, a burst length shorter than 100 μ s, a frequency between 0.4 MHz and 15 MHz, a treatment time less than 60 s per treatment location, a pulse repetition frequency lower than 500 Hz. For example, the HIFU may use a power between 5 W and 150 W, a burst length between 1 μ s and 100 μ s, a frequency between 0.4 MHz and 15 MHz, a treatment time between 1 ms and 60 s per treatment location, a pulse repetition between 1 Hz and 500 Hz. In some aspects, the HIFU may run at 0.1 s -30 s per field of view. In some aspects, the HIFU may have a pulse repetition frequency of 10 Hz to 150 Hz. In some aspects, the HIFU may have a burst length of 10 μ s to 100 μ s. In some aspects, the HIFU may have a frequency of 0.5 mHz to 10 Mhz. In some aspects, the power used by the HIFU may be 1W to 200W. In some aspects, the temperature may be between 4°C and 70°C. As shown in FIGs. 14A and 14B, variations in treatment time and pulse repetition frequency may be used to alter both the amount of crosstalk and the percentage of cells that lyse in the focal zone. Different parameters may be used depending on the actions being performed. For example, if high throughput work is being performed, the HIFU may be used at 1 s per field of view or about 300 nanowells per second. The pulse repetition frequency may be 50 Hz, the burst length may be 10 μ s, the frequency may be 1.1. MHz, the power may be 60W, and the lysis may be performed at room temperature. In contrast, for targeted work, the HIFU may be used at 3 s per field of view, or about 10-15 nanowells per second, the frequency may be 3.4 MHz, the power may be 10W and the lysis may be performed at room temperature. In some aspects, the HIFU conditions may be altered depending on cell size or membrane thickness. For example, larger cells may require a lower power than smaller cells to achieve the desired effects. For example, cells of 20-25 μ m may be successfully lysed at 50W whereas cells with membranes of 10-20 μ m or cell nuclei may be

successfully lysed at 70W. Further, the HIFU conditions may be varied depending on the type of downstream analysis to be performed.

[0060] Cross talk and lysis may also be manipulated by varying the thickness of the gas permeable membrane. Further, such factors may be modified to achieve a smaller focal zone for targeted single cell lysis. For example, with a 70 μm membrane and a HIFU power of 10 W, the focal zone of the HIFU can be reduced to a small area, allowing for targeting and lysing of specific single cells visualized within the field of view of a microscope. For example, the cells of interest may be identified with microscopy or through other methods. The size of the focal zone may be reduced, for example, by reducing the power used during HIFU treatment and altering the thickness of the capping membrane.

[0061] By reducing the focal zone of the HIFU to a small number of wells, the system can be used to lyse a small number of cells, down to a single cell within the nanowell array as discussed in further detail with regard to Example 7. For example, focused lysis may be guided by imaging, such as microscopy, to select the specific wells, or groups of wells to be lysed. By altering the power, the diameter of the focal zone may be altered. In other aspects, the diameter of the focal zone may be altered by changing the thickness of the PDMS membrane, the frequency being used for the HIFU, the amount of pulse repetition, the burst length, and the treatment time. For example, in one example, HIFU was performed with a 50 μm membrane and the array was observed with a microscope to visualize the cavitating wells. With a power of 30 W, the focal zone (indicated by cavitating wells around the selected well) had a diameter of 1230 μm , which decreased to 890 μm at a power of 20 W, and 330 μm at 10 W. In another example, a range of PDMS membranes from 64 μm to 150 μm were tested with a HIFU transducer using a frequency of 3.410 MHz, a pulse repetition frequency of 30 Hz, a burst length of 10 μs , a treatment time of 1s and power ranging from 5W to 20 W was tested in FIG. 21 to measure the effect on the cavitation zone size within the nanowell array. In this example a power of 5W with a 64 μm thick membrane resulted in a focal zone of 14 nanowells. In another example, a PDMS membrane of 70 μm was tested with a HIFU transducer using a frequency of 3.410 MHz, a pulse repetition frequency of 30 Hz, a burst length of 10 μs , a treatment time of 1s and a power of 7W was applied to manually selected areas of the nanowell array containing fluorescently labelled cells which resulted in 16 lysed cells per minute, a lysis efficiency of 73% and off-target cell lysis of 10 cells per 100 target cells as shown in FIGs. 22A-22C.

[0062] The lysed cells may be used for downstream applications such as single cell mRNA sequencing and ATAC sequencing. For example, different types of cells labelled with different fluorescent markers may be loaded into the nanowell array along with DNA barcoded mRNA

capture beads. One of the cell types may be identified using microscopy imaging of the nanowell array, and selectively lysed using HIFU, or portions of the identified cell type may be lysed. The resulting mRNA captured on the barcoded beads can be analyzed to determine the amount of genetic material captured from the lysed cells relative to lysing all the cells or lysing none of the cells as shown in FIG. 23A and FIG. 23B indicating an enrichment of the target cell genetic material relative to treating all of the cells with HIFU and showing that increasing lysis of the target cells resulted in increasing amounts of genetic material from those cells without increasing genetic material from the non-targeted cells.

[0063] For some types of analysis, each cell in a nanowell may be labeled. Any labeling method known to one of ordinary skill in the art may be used, for example, barcodes such as molecular barcodes may be added to the bottom of the nanowell. In other aspects, barcoded beads may be added to the nanowell array. In other aspects, barcoded capture reagents may be bound to the nanowell surface. For example, barcoded capture beads, cells, and a reagent/enzyme mix may be added to the nanowells. Desirable reagents such as PBS allow for gas permeable membrane binding and adequate cavitation. Some conventional reagents such as polyethylene glycol or 1,3-propanediol inhibit gas permeable membrane binding and cavitation. Nanowell size may be selected based on the size of the bead. Generally, the ratio of the nanowell to the bead is above 1. Generally, the ratio of the bead to the nanowell is below 2. In some aspects, the ratio is between 1 and 2, inclusive. In some aspects, the ratio of the well size to the bead size is between 1.2 and 1.5, for example, 1.43.

[0064] Each nanowell in an array may be spaced apart from each other nanowell. The nanowells may be spaced between 5 μm and 20 μm including 5 μm , 10 μm , 15 μm , 20 μm , 25 μm , 30 μm apart (or any whole or partial measurement in between). As demonstrated herein, smaller well spacings (e.g., smaller than 2 μm) do not allow for sufficient contact between the top surface of the array and the gas permeable membrane for the membrane to adhere to the wells. Decreased adherence of the gas permeable membrane may lead to increased crosstalk between the wells during ultrasound treatment. Further, spacings that are too large may reduce loading efficiency as more cells are stranded on the top surface instead of settling into a microwell for treatment. Thus, in some aspects, the spacings may depend on the size of the cells.

[0065] Imaging using, for example, an optical microscope can be used to measure different aspects of the nanowell array such as barcoded bead loading, cell loading, cell lysis, cell phenotype, cell behavior, or cell interactions before and after HIFU application. In some examples, imaging may be used to select an area for HIFU application and lysis.

[0066] Various parameters of the contents of each nanowell may be analyzed before and after lysis. For example, intensity measurements may be extracted from images to determine the mean intensity and standard deviation in each nanowell. Brightfield intensity measurements, as shown in FIGs. 17A and 17B, may be used to identify nanowells with and without barcoded beads. Fluorescence levels from fluorescently labeled cells may be used to determine cell loading in each nanowell as shown in FIGs. 18A and 18B. Before lysis, the fluorescently labeled cells are visible within the wells, while the remaining contents of the wells do not fluoresce. After lysis, the fluorescent label is dispersed throughout the media in the well and the cell is no longer visible. Therefore, in some aspects, cell lysis may also be determined by measuring the mean fluorescence intensity and standard deviation of the fluorescence intensity of each nanowell before and after HIFU as shown in FIGs. 19A and 19B. In some aspects, computational analysis may be used to determine these measurements for each nanowell within the array and to select nanowells for HIFU lysis.

[0067] Cell lysed according to the systems and methods described herein may be used in a variety of downstream analytics. For example, they may be used in single cell high-throughput mRNA and protein sequencing or single cell high-throughput assay for transposase-accessible chromatin (ATAC) sequencing.

[0068] For example, single-cell RNA-seq may include solvent exchange from ethanol to phosphate buffered saline (PBS) to trap air on the inner surface of the nanowell. DNA barcoded RNA capture beads, single cells, and reverse transcription (RT) reagents may be loaded into the nanowell array. The array may then be capped using a polydimethylsiloxane (PDMS) membrane to isolate each nanowell. The nanowell array may then be visualized under the microscope. High-intensity focused ultrasound may then be applied to the nanowell array to lyse the cells and mix the contents of the nanowell causing RNA to be released from the cell and bind to the DNA capture beads. The nanowell array may then be incubated at 52°C to activate the RT enzyme. The contents of the nanowells including the barcoded beads and attached molecules may then be removed from the nanowells for downstream processing.

[0069] In other examples, single cell high-throughput mRNA and protein sequencing may include incubating the cells with barcoded antibodies for CITE-seq with poly-A regions, introducing the cells into the platform with buffer containing reverse transcription reaction components including DNA barcoded beads, centrifuging and washing the arrays, capping the wells with a gas-permeable membrane, performing HIFU to lyse the cells and release mRNA, performing RT inside the wells to attach the mRNA to the beads, uncapping the wells, removing the beads from the

wells and, performing random hexamer primer and PCR outside the wells to prepare the sequencing library, and sequencing.

[0070] In some aspects, the nanowell arrays may be used for targeted sequencing. Targeted sequencing may include the following steps. First, solvent exchange from ethanol to PBS traps air within the nanowell array. DNA barcoded mRNA capture beads may then be added to the nanowells. A cell suspension may then be added to the nanowell array and a PDMS membrane may be adhered to the array to seal the nanowells. Microscopy images of the nanowell array may then be taken and used to identify the cell population to target for lysis and downstream RNA sequencing. The cell population of interest may be identified without labels via morphology or cell behavior and/or with common methods for labelling cells for microscopy such as using fluorescent protein reporters or fluorescently labelled antibodies. HIFU may then applied to the identified areas of the nanowell array to lyse the targeted cells. The nanowell array contents are removed including but not limited to the beads and non-lysed cells and applied to a sucrose gradient. The gradient allows the beads to be separated from the unlysed cells and the unlysed cells are then removed. The beads may then be washed and reverse transcription reagents added to generate cDNA from the lysed cells. Targeted lysis is described in further detail with reference to Example 8.

[0071] The Exemplary Embodiments and Example(s) below are included to demonstrate particular embodiments of the disclosure. Those of ordinary skill in the art should recognize in light of the present disclosure that many changes can be made to the specific embodiments disclosed herein and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

First Set of Exemplary Embodiments.

[0072] 1. A nanowell array including a plurality of nanowells, wherein a bottom of each nanowell includes a geometric pattern.

[0073] 2. The nanowell array of embodiment 1, wherein the geometric pattern repeats.

[0074] 3. The nanowell array of embodiments 1 or 2, wherein the repeating geometric pattern is a plurality of squares or a plurality of parallel lines.

[0075] 4. The nanowell array of any of embodiments 1 to 3, wherein at least one dimension of the pattern is 0.2 μm to 11 μm .

[0076] 5. The nanowell array of any of embodiments 1 to 4, wherein a size of the pattern is 2 μm in at least one dimension.

- [0077]** 6. The nanowell array of any of embodiments 1 to 4, wherein a size of the pattern is 5 μm in at least one dimension.
- [0078]** 7. The nanowell array of any of embodiments 1 to 4, wherein a size of the pattern is 10 μm in at least one dimension.
- [0079]** 8. The nanowell array of any of embodiments 1 to 7, wherein each nanowell is 5 to 500 microns in diameter.
- [0080]** 9. The nanowell array of any of embodiments 1 to 7, wherein each nanowell is 40 to 60 microns in diameter.
- [0081]** 10. The nanowell array of any of embodiments 1 to 7, wherein each nanowell is 50 microns in diameter.
- [0082]** 11. The nanowell array of any of embodiments 1 to 10, wherein the nanowell array is a hydrophobic polymer.
- [0083]** 12. The nanowell array of embodiment 11, wherein the hydrophobic polymer includes polystyrene.
- [0084]** 13. The nanowell array of any of embodiments 1 to 12, wherein a spacing between each nanowell in the nanowell array is 1 μm to 500 μm .
- [0085]** 14. The nanowell array of any of embodiments 1 to 12, wherein a spacing between each nanowell in the nanowell array is 5 μm to 50 μm .
- [0086]** 15. The nanowell array of any of embodiments 1 to 12, wherein a spacing between each nanowell in the nanowell array is 15 μm .
- [0087]** 16. The nanowell array of any of embodiments 1 to 12, further including a gas permeable membrane structured to be attached to a top surface of the nanowell array.
- [0088]** 17. The nanowell array of embodiment 16, wherein the gas permeable membrane includes polydimethylsiloxane.
- [0089]** 18. The nanowell array of embodiment 16 or 17, wherein the gas permeable membrane is 20 μm to 250 μm thick.
- [0090]** 19. The nanowell array of any of embodiments 16 to 18, wherein the gas permeable membrane is about 150 μm thick.
- [0091]** 20. The nanowell array of any of embodiments 16 to 18, wherein the gas permeable membrane is about 70 μm thick.
- [0092]** 21. A method for cell lysis including: performing a solvent exchange in a nanowell array including a hydrophobic polymer including a plurality of nanowells; loading the nanowell array with a suspension; covering the nanowells with a gas permeable hydrophobic membrane; and subjecting the nanowell array to high intensity focused ultrasound (HIFU).

- [0093]** 22. The method of embodiment 21, wherein the suspension includes cells.
- [0094]** 23. The method of embodiment 21 or 22, wherein the loading results in only one cell in at least 50% of the occupied nanowells.
- [0095]** 24. The method of any of embodiments 21 to 23, wherein the suspension includes cell nuclei.
- [0096]** 25. The method of embodiment 24, wherein the loading results in only one cell nucleus in at least 50% of the occupied nanowells.
- [0097]** 26. The method of any of embodiments 21 to 25, wherein the suspension includes at barcodes, cells, and cell nuclei.
- [0098]** 27. The method of any of embodiments 21 to 26, wherein each nanowell of the plurality of nanowells has a geometric pattern on the bottom of the nanowell. 28. The method of embodiment 27, wherein the geometric pattern is a repeating pattern of a plurality of squares or a plurality of parallel lines.
- [0099]** 29. The method of embodiments 21 to 28, wherein at least one dimension of the pattern is 0.2 μm to 11 μm .
- [0100]** 30. The method of embodiments 21 to 29, wherein a size of the pattern is 2 μm in at least one dimension.
- [0101]** 31. The method of embodiments 21 to 29, wherein a size of the pattern is 5 μm in at least one dimension.
- [0102]** 32. The method of embodiments 21 to 29, wherein a size of the pattern is 10 μm in at least one dimension.
- [0103]** 33. The method of any of embodiments 21 to 32, wherein each nanowell is 5 to 500 microns in diameter.
- [0104]** 34. The method of any of embodiments 21 to 33, wherein each nanowell is 40 to 60 microns in diameter.
- [0105]** 35. The method of any of embodiments 21 to 34, wherein each nanowell is 50 microns in diameter.
- [0106]** 36. The method of any of embodiments 21 to 35, wherein the hydrophobic polymer includes polystyrene.
- [0107]** 37. The method of any of embodiments 21 to 35, wherein a spacing between each nanowell in the nanowell array is 1 μm to 500 μm .
- [0108]** 38. The method of any of embodiments 21 to 37, wherein a spacing between each nanowell in the nanowell array is 5 μm to 50 μm .

[0109] 39. The method of any of embodiments 21 to 38, wherein a spacing between each nanowell in the nanowell array is 15 μm .

[0110] 40. The method of any of embodiments 21 to 39, wherein the gas permeable membrane includes polydimethylsiloxane.

[0111] 41. The method of any of embodiments 21 to 40, wherein the gas permeable membrane is 20 μm to 250 μm thick.

[0112] 42. The method of any of embodiments 21 to 41, wherein the gas permeable membrane is 150 μm thick.

[0113] 43. The method of any of embodiments 21 to 42, wherein the gas permeable membrane is 70 μm thick.

[0114] 44. The method of any of embodiments 21 to 43, wherein the HIFU uses a power between 5 W and 150 W, a burst length between 1 to 100 μs , a frequency between 0.4 MHz and 15 MHz, a treatment time between 1 ms and 60 s per treatment location, a pulse repetition frequency from 1 Hz and 500 Hz.

[0115] 45. The method of any of embodiments 21 to 44, wherein the HIFU uses a power of 60W, a burst length of 10 μs , a frequency of 1100 MHz, a treatment time of 1s, and a pulse repetition frequency of 50 Hz.

[0116] 46. The method of any of embodiments 21 to 45, wherein the HIFU uses a power of 70W.

[0117] 47. The method of any of embodiments 21 to 46, wherein the HIFU is selectively applied to an area of the nanowell array.

[0118] 48. The method of embodiment 47, wherein the area for selective HIFU application is identified with microscopy imaging.

[0119] 49. The method of any of embodiments 21 to 48, further including imaging an area of the nanowell array prior to and after HIFU treatment.

[0120] 50. A method of high-throughput sequencing including: performing a solvent exchange in a hydrophobic polymer nanowell array, wherein the nanowell array includes a plurality of nanowells; loading the nanowell array with one or more reaction mixes; covering the nanowells with a gas permeable hydrophobic membrane; performing high intensity focused ultrasound (HIFU); incubating the one or more reaction mixes to form an analyte; and removing the gas permeable hydrophobic membrane.

[0121] 51. The method of embodiment 50, wherein the reaction mix includes cells.

[0122] 52. The method of embodiments 50 or 51, wherein the loading results in only one cell in at least 50% of the occupied nanowells.

[0123] 53. The method of embodiments 50, wherein the reaction mix includes cell nuclei.

- [0124]** 54. The method of embodiment 53, wherein the loading results in only one cell nucleus in at least 50% of the occupied nanowells.
- [0125]** 55. The method of embodiment 50, wherein the reaction mix comprises at least one of cell suspensions, cell nuclei suspensions, barcoded beads, and enzymes.
- [0126]** 56. The method of any of embodiments 50 to 55, wherein each nanowell of the plurality of nanowells has a with a geometric pattern on the bottom of each nanowell .
- [0127]** 57. The method of embodiment 56, wherein the geometric pattern is a repeating pattern of a plurality of squares or a plurality of parallel lines.
- [0128]** 58. The method of any of embodiments 50 to 57, wherein at least one dimension of the pattern is 0.2 μm to 11 μm .
- [0129]** 59. The method of any of embodiments 50 to 58, wherein a size of the pattern is 2 μm in at least one dimension.
- [0130]** 60. The method of any of embodiments 50 to 58, wherein a size of the pattern is 5 μm in at least one dimension.
- [0131]** 61. The method of any of embodiments 50 to 58, wherein a size of the pattern is 10 μm in at least one dimension.
- [0132]** 62. The method of any of embodiments 50 to 61, wherein each nanowell is 5 to 500 microns in diameter.
- [0133]** 63. The method of any of embodiments 50 to 62, wherein each nanowell is 40 to 60 microns in diameter.
- [0134]** 64. The method of any of embodiments 50 to 63, wherein each nanowell is 50 microns in diameter.
- [0135]** 65. The method of any of embodiments 50 to 64, wherein the hydrophobic polymer includes polystyrene.
- [0136]** 66. The method of any of embodiments 50 to 66, wherein a spacing between each nanowell in the nanowell array is 1 μm to 500 μm .
- [0137]** 67. The method of any of embodiments 50 to 66, wherein a spacing between each nanowell in the nanowell array is 5 μm to 50 μm .
- [0138]** 68. The method of any of embodiments 50 to 67, wherein a spacing between each nanowell in the nanowell array is 15 μm .
- [0139]** 69. The method of any of embodiments 50 to 68, wherein the gas permeable membrane includes polydimethylsiloxane.
- [0140]** 70. The method of any of embodiments 50 to 69, wherein the gas permeable membrane is 20 μm to 250 μm thick.

[0141] 71. The method of any of embodiments 50 to 70, wherein the gas permeable membrane is 150 μm thick.

[0142] 72. The method of any of embodiments 50 to 70, wherein the gas permeable membrane is 70 μm thick.

[0143] 73. The method of embodiment any of embodiments 50 to 72, wherein the reaction mix includes reverse transcription reaction components.

[0144] 74. The method of any of embodiments 50 to 72, wherein the HIFU is selectively applied to an area of the nanowell array.

[0145] 75. The method of embodiment 74, wherein the area for selective HIFU application is identified with microscopy imaging.

[0146] 76. The method of embodiment 75, further including imaging an area of the nanowell array prior to and after HIFU treatment.

[0147] 77. The method of any of embodiments 50 to 76, wherein the reaction mix includes ligation reaction components.

[0148] 78. The method of any of embodiments 50 to 77, wherein the reaction mix includes tagmentation reaction components.

[0149] 79. The method of any of embodiments 50 to 78, wherein the reaction mix includes cells pre-labeled with barcoded antibodies.

[0150] 80. The method of any of embodiments 50 to 80, wherein the reaction mix includes a hyperactive transposase loaded with DNA sequencing adapters.

[0151] 81. The method of any of embodiments 50 to 80, wherein the reaction mix includes DNA ligation reaction components.

[0152] 82. A high intensity focused ultrasound (HIFU) platform including: a nanowell array including a hydrophobic polymer including a plurality of nanowells with a geometric pattern on the bottom of each well; a gas permeable membrane adhered to a top surface of the nanowell array; an epifluorescence microscope positioned above the nanowell array; and a HIFU ultrasound transducer positioned below the nanowell array; wherein the nanowell array is acoustically coupled to the HIFU ultrasound transducer.

[0153] 83. The platform of embodiment 82, wherein the geometric pattern repeats.

[0154] 84. The platform of embodiment 82 or 83, wherein the repeating geometric pattern is a plurality of squares or a plurality of parallel lines.

[0155] 85. The platform of any of embodiments 82 to 84, wherein at least one dimension of the pattern is 0.2 μm to 11 μm .

- [0156]** 86. The platform of any of embodiments 82 to 85, wherein a size of the pattern is 2 μm in at least one dimension.
- [0157]** 87. The platform of any of embodiments 82 to 86, wherein a size of the pattern is 5 μm in at least one dimension.
- [0158]** 88. The platform of any of embodiments 82 to 87, wherein a size of the pattern is 10 μm in at least one dimension.
- [0159]** 89. The platform any of embodiments 82 to 88, wherein each nanowell is 5 to 50 microns in diameter.
- [0160]** 90. The platform of any of embodiments 82 to 89, wherein each nanowell is 40 to 60 microns in diameter.
- [0161]** 91. The platform of any of embodiments 82 to 90, wherein each nanowell is 50 microns in diameter.
- [0162]** 92. The platform of any of embodiments 82 to 91, wherein the hydrophobic polymer includes polystyrene.
- [0163]** 93. The platform of any of embodiments 82 to 92, wherein a spacing between each nanowell in the nanowell array is 1 μm to 500 μm .
- [0164]** 94. The platform of any of embodiments 82 to 93, wherein a spacing between each nanowell in the nanowell array is 5 μm to 50 μm .
- [0165]** 95. The platform of any of embodiments 82 to 94, wherein a spacing between each nanowell in the nanowell array is 15 μm .
- [0166]** 96. The platform of any of embodiments 82 to 95, wherein the gas permeable membrane includes polydimethylsiloxane.
- [0167]** 97. The platform of any of embodiments 82 to 96, wherein the gas permeable membrane is 20 μm to 250 μm thick.
- [0168]** 98. The platform of any of embodiments 82 to 97, wherein the gas permeable membrane is 150 μm thick.
- [0169]** 99. The platform of any of embodiments 82 to 97, wherein the gas permeable membrane is 70 μm thick.
- [0170]** 100. The HIFU platform of any of embodiments 82 to 99, wherein a transducer focal zone is within 10 mm of the bottom of the nanowell array.
- [0171]** 101. A method of using the HIFU platform of any of embodiments 82 to 100.

Second Set of Exemplary Embodiments.

[0172] 1. A method including: loading wells of a polymer nanowell array including a plurality of nanowells with one or more reaction mixes; covering the loaded wells with a membrane; treating the nanowell array with high intensity focused ultrasound (HIFU); incubating the reaction mixes in the nanowell array to form an analyte; and removing the membrane.

[0173] 2. The method of embodiment 1, wherein the membrane is 20 μm to 250 μm thick.

[0174] 3. The method of embodiment 1, wherein the membrane is 150 μm thick.

[0175] 4. The method of embodiment 1, wherein the membrane is 70 μm thick.

[0176] 5. The method of any of embodiments 1 to 4, wherein the membrane is hydrophobic.

[0177] 6. The method of any of embodiments 1 to 5, wherein the membrane is a gas permeable membrane.

[0178] 7. The method of any embodiment 6, wherein the gas permeable membrane includes polydimethylsiloxane.

[0179] 8. The method of any of embodiments 1 to 7, wherein the method produces a library of analytes.

[0180] 9. The method of any of embodiments 1 to 8, wherein the reaction mix includes cells.

[0181] 10. The method of embodiment 9, wherein the loading of the polymer nanowell array results in a plurality of nanowells including one or more cells of the reaction mix.

[0182] 11. The method of any of embodiments 1 to 10, wherein the reaction mix includes cell nuclei.

[0183] 12. The method of any of embodiments 1 to 11, wherein the reaction mix includes at least one of cell suspensions, cell nuclei suspensions, barcoded beads, and enzymes.

[0184] 13. The method of any of embodiments 1 to 12, wherein the polymer of the polymer nanowell array is hydrophobic.

[0185] 14. The method of any of embodiments 1 to 13, wherein the polymer of the polymer nanowell array includes polystyrene.

[0186] 15. The method of any of embodiments 1 to 14, wherein each nanowell of the plurality of nanowells has a geometric pattern on the bottom of each nanowell.

[0187] 16. The method of embodiment 15, wherein the geometric pattern includes a repeating pattern of a plurality of squares or a plurality of parallel lines.

[0188] 17. The method of embodiment 15 or embodiment 16, wherein at least one dimension of the pattern is 0.2 μm to 11 μm .

[0189] 18. The method of any of embodiments 15 to 17, wherein a size of the pattern is 2 μm in at least one dimension.

- [0190]** 19. The method of any of embodiments 15 to 17, wherein a size of the pattern is 5 μm in at least one dimension.
- [0191]** 20. The method of any of embodiments 15 to 17, wherein a size of the pattern is 10 μm in at least one dimension.
- [0192]** 21. The method of any of embodiments 1 to 21, wherein each nanowell of the nanowell array is 5 to 500 microns in diameter.
- [0193]** 22. The method of embodiment 21, wherein each nanowell of the nanowell array is 40 to 60 microns in diameter.
- [0194]** 23. The method of embodiment 22, wherein each nanowell of the nanowell array is 50 microns in diameter.
- [0195]** 24. The method of any of embodiments 1 to 23, wherein the HIFU is selectively applied to an area of the nanowell array.
- [0196]** 25. The method of embodiment 24, wherein the area for selective HIFU application is identified with microscopy imaging.
- [0197]** 26. The method of embodiment 25, wherein the microscopy imaging is epifluorescent microscopy.
- [0198]** 27. The method of any of embodiments 1 to 26, wherein treating the nanowell array with HIFU includes applying HIFU using a power of between 0.01 W and 250 W with a burst length between 1 to 100 μs , a frequency between 0.2 MHz and 50 MHz, a treatment time between 1 ms and 60 s per treatment location, and a pulse repetition frequency from 1 Hz and 500 Hz.
- [0199]** 28. The method of any of embodiments 1 to 26, treating the nanowell array with HIFU includes applying HIFU using a power between 1 W and 100 W, a burst length between 1 to 20 μs , a frequency between 0.4 MHz and 15 MHz, a treatment time between 100 ms and 10 s per treatment location, a pulse repetition frequency from 10 Hz and 100 Hz.
- [0200]** 29. The method of any of embodiments 1 to 26, wherein treating the nanowell array with HIFU includes applying HIFU using a power of 60W, a burst length of 10 μs , a frequency of 1100 MHz, a treatment time of 1s, and a pulse repetition frequency of 50 Hz.
- [0201]** 30. The method of any of embodiments 1 to 26, wherein treating the nanowell array with HIFU includes applying HIFU using a power of 8W, a burst length of 10 μs , a frequency of 3410 MHz, a treatment time of 1s, and a pulse repetition frequency of 30 Hz.
- [0202]** 31. The method of any of embodiments 1 to 26, wherein treating the nanowell array with HIFU includes applying HIFU using a power of 70W.
- [0203]** 32. A high intensity focused ultrasound (HIFU) platform including: a polymer nanowell array including a plurality of nanowells; a membrane adhered to a top surface of the nanowell

array; a microscope positioned above the nanowell array; and a HIFU ultrasound transducer positioned below the nanowell array; wherein the nanowell array is acoustically coupled to the HIFU ultrasound transducer.

[0204] 33. The HIFU platform of embodiment 32, wherein the polymer nanowell is hydrophobic.

[0205] 34. The HIFU platform of embodiment 32, wherein the hydrophobic polymer includes polystyrene.

[0206] 35. The HIFU platform of any of embodiments 32 to 34, wherein the microscope is an epifluorescent microscope.

[0207] 36. The HIFU platform of any of embodiments 32 to 35, wherein the membrane is 20 μm to 250 μm thick.

[0208] 37. The HIFU platform of any of embodiments 32 to 36, wherein the membrane is 150 μm thick.

[0209] 38. The HIFU platform of any of embodiments 32 to 37, wherein the membrane is 70 μm thick.

[0210] 39. The HIFU platform of any of embodiments 32 to 38, wherein the membrane is hydrophobic.

[0211] 40. The HIFU platform of any of embodiments 32 to 39, wherein the membrane is a gas permeable membrane.

[0212] 41. The HIFU platform of embodiment 40, wherein the gas permeable membrane includes polydimethylsiloxane.

[0213] 42. The HIFU platform of any of embodiments 32 to 41, further including a geometric pattern on the bottom of each nanowell of the nanowell array.

[0214] 43. The HIFU platform of any of embodiments 32 to 42, wherein the geometric pattern includes a repeating pattern of a plurality of squares or a plurality of parallel lines.

[0215] 44. The HIFU platform of any of embodiments 32 to 43, wherein at least one dimension of the pattern is 0.2 μm to 11 μm .

[0216] 45. The HIFU platform of any of embodiments 32 to 44, wherein a size of the pattern is 2 μm in at least one dimension.

[0217] 46. The HIFU platform of any of embodiments 32 to 44, wherein a size of the pattern is 5 μm in at least one dimension.

[0218] 47. The HIFU platform of any of embodiments 32 to 44, wherein a size of the pattern is 10 μm in at least one dimension.

[0219] 48. The HIFU platform of any of embodiments 32 to 47, wherein each nanowell of the nanowell array is 5 to 50 microns in diameter.

[0220] 49. The HIFU platform of any of embodiments 32 to 47, wherein each nanowell of the nanowell array is 40 to 60 microns in diameter.

[0221] 50. The HIFU platform of any of embodiments 32 to 47, wherein each nanowell of the nanowell array is 82 microns in diameter.

[0222] 51. The HIFU platform of any of embodiments 32 to 50, wherein a spacing between adjacent nanowells in the nanowell array is 1 μm to 500 μm .

[0223] 52. The HIFU platform of embodiment 51, wherein a spacing between adjacent nanowells in the nanowell array is 5 μm to 50 μm .

[0224] 53. The HIFU platform of embodiment 52, wherein a spacing between adjacent nanowells in the nanowell array is 15 μm .

[0225] 54. The HIFU platform of any of embodiments 32 to 54, wherein a transducer focal zone is within 10 mm of the bottom of the nanowell array.

[0226] 55. A method of using the HIFU platform of any of embodiments 32 to 54.

Example 1. Effect of nanopatterned surfaces on high-intensity focused ultrasound response.

[0227] Nanowell arrays made of polystyrene, and created with thermal compression molding were created. Each nanowell array contained nanowells of 50 μm diameter, 50 μm depth, and 15 μm spacing. The bottom of each nanowell and/or nanowell array was patterned with geometric designs including 2 μm triangles, concentric lines, inverse squares, and 2 μm , 5 μm and 10 μm lines, and squares. One nanowell array was flat with no nanopattern on the bottom of the nanowells. Each nanowell array was placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with N_2 and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. The 7mm x 7mm chambers, similar to chambers 306 of FIG. 3, allow for experiments to be conducted within portions of a nanowell array.

[0228] 100 μL of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μL of ethanol was then removed from the gasket chamber and 50 μL of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μL) was removed and replaced with 100 μL PBS. The 100 μL PBS was then removed and replaced

with an additional 100 μ L PBS. 100 μ L of liquid was then removed and MCF7 cells, labeled with the cell nuclei stain SYBRGreen were loaded by adding 100 μ L of the prepared solution into the chamber (~3,000 cells) and allowing them to settle for ten minutes. A gas permeable 150 μ m PDMS membrane was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. The nanowell array was placed in the HIFU-microscope apparatus (FIGs. 5A and 5B). HIFU was applied to the nanowell array with the following parameters: Power: 60W, Pulse repetition frequency: 50 Hz, Burst length: 10 μ s, Frequency: 1.1 MHz, Treatment time: 3 s. Images from the microscope were taken of the cell nuclei before, during and after HIFU treatment and the number of cell cavitating were measured. This data is shown in FIG. 6A and FIG. 6B. FIG. 6A compares all the different nanopatterns that were 2 μ m in size with the 2 μ m squares having the highest amount of cavitation. FIG. 6B compares nanopatterns of different sizes for the line and square nanopatterns demonstrating that the 2 μ m square nanopattern has the highest cavitation.

Example 2. Orthogonal array testing to identify optimal parameters for individualized cell lysis

[0229] Nanowell arrays with varying geometries including well shape being circular or hexagonal, and well spacings from 5 to 20 μ m were tested using membrane thicknesses between 50 to 200 μ m, different buffers such as PBS and a general reverse transcription buffer, and varied HIFU parameters for power, pulse repetition frequency, treatment time, burst length to determine well cavitation, percent cell lysis, and percent crosstalk.

[0230] The nanowell arrays were placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with nitrogen and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. 100 μ L of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μ L of ethanol was then removed from the gasket chamber and 50 μ L of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μ L) was removed and replaced with 100 μ L PBS. The 100 μ L PBS was then removed and replaced with an additional 100 μ L PBS. 100 μ L of liquid was then removed and the cells were loaded by adding 100 μ L of the prepared solution into the chamber (3,000 cells) and allowing them to settle for ten

minutes. Gas permeable PDMS membranes of varying thicknesses from 50 to 200 μm was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. After dehydration, the nanowell arrays were treated with the above HIFU parameters in triplicate. The data were interpreted as number of wells cavitating (FIGs. 7A-7H), percent cell lysis (FIGs. 8A-8H) and percent cell crosstalk (FIGs. 9A-9H). The well geometry chosen was circular with 15 μm spacing. FIG. 11 displays all data points for all conditions where the optimal conditions had less than 20% crosstalk and more than 80% lysis. The optimal HIFU conditions as a result of this testing were 1.1 MHz frequency, 60 W for power (FIG. 13B), 50 Hz pulse repetition frequency (FIG. 14B), 10 μs burst length, 3s treatment time (FIG. 14A), with a membrane thickness of 150 μm (FIG. 13A).

Example 3. High throughput cell lysis in a nanowell array using optimal conditions

[0231] Nanowell arrays made of polystyrene, and created with thermal compression molding were created containing nanowells of 50 μm diameter, 50 μm depth, and 15 μm spacing between nanowells. The bottom of the nanowells were patterned with geometric designs consisting of 2 μm squares. Each nanowell array was placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with N₂ and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. 100 μL of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μL of ethanol was then removed from the gasket chamber and 50 μL of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μL) was removed and replaced with 100 μL PBS. The 100 μL PBS was then removed and replaced with an additional 100 μL PBS. 100 μL of liquid was then removed and MCF7 cells expressing GFP were loaded by adding 100 μL of the prepared solution into the chamber (~3,000 cells) and allowing them to settle for ten minutes. A gas permeable 150 μm PDMS membrane was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. This was done in triplicate in three different chambers. Each nanowell array was placed in the HIFU-microscope apparatus (FIG. 5A). HIFU was applied to the nanowell array with the following parameters: Power: 60W, Pulse repetition frequency: 50 Hz, Burst length: 10 μs , Frequency: 1.1 MHz, Treatment time: 1 s and

this was rastered across 12,000 nanowells containing the ~3,000 cells. Images from the microscope were taken of the cells before and after HIFU treatment. These images were analyzed to count the percentage of cells lysed and percentage of cells that lysed and spilled into adjacent wells (crosstalk). Percent cell lysis is shown in FIG. 12A and percent crosstalk in FIG. 12B.

Example 4. Single cell mRNA sequencing of a cell mixture

[0232] Nanowell arrays made of polystyrene, and created with thermal compression molding were created containing nanowells of 50 μm diameter, 50 μm depth, and 15 μm spacing. The bottom of the nanowells were patterned with geometric patterns consisting of 2 μm squares.

[0233] Each nanowell array was placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with N_2 and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. 100 μL of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μL of ethanol was then removed from the gasket chamber and 50 μL of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μL) was removed and replaced with 100 μL PBS. The 100 μL PBS was then removed and replaced with an additional 100 μL PBS. Barcoded capture beads were prepared and diluted by adding 7.5 μL of beads to 82.5 μL of PBS with RNase inhibitor. The beads were then loaded into the nanowells by adding 100 μL of the diluted beads to the chamber and allowing them to settle for two minutes. 100 μL of liquid was then removed and A mixture of HEK-293T-zsGreen cells expressing a green fluorescent protein and NIH 3T3-tdTomato cells expressing a red fluorescent protein were loaded by adding 100 μL of the prepared solution into the chamber (~500 of each cell type) and the cells were centrifuged into the wells. The RT master mix was prepared by combining 42.5 μL H_2O , 30 μL dNTPs, 20 μL 5X Maxima mix, 2.5 μL RNase inhibitor, and 5 μL Maxima enzyme, totaling 100 μL . 100 μL of liquid was removed from the chamber, and 98 μL of the RT master mix was added. The chamber was incubated at room temperature for 5 minutes. 50 μL of the liquid was then removed from the chamber. A gas permeable 150 μm PDMS membrane was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. PCR tape was adhered to the top of the gasket to prevent evaporation. The nanowell array was placed in the HIFU-microscope apparatus

(FIGs. 5A-5B). HIFU was applied to the nanowell array with the following parameters: Power: 60W, Pulse repetition frequency: 50 Hz, Burst length: 10 μ s, Frequency: 1.1 MHz, Treatment time: 1 s and this was rastered across 12,000 nanowells containing the ~1,000 total cells. Images from the microscope were taken of the cells before and after HIFU treatment. After that 100 μ L of PBS FBS was added on top of the membrane. The nanowell array was placed onto a thermocycler with the lid at 52°C and incubated at 25°C for 15 minutes and 52°C for 30 minutes. The PCR tape was removed, and the PDMS membrane was removed with tweezers. The solution in the chamber was pipetted up and down to dislodge the beads from the wells and transferred to a 0.2 mL PCR tube. After reverse transcription, an exonuclease treatment was performed with the following reaction mixture: 10 μ L NEB 3.1 10X buffer, 85 μ L H₂O, and 5 μ L ExoI (exonuclease I). The mixture was incubated at 37°C for 50 minutes in the rotator. This was followed by washes. After exonuclease treatment, a second-strand synthesis mix was prepared, consisting of 20 μ L Maxima 5X RT buffer, 40 μ L PEG 8000, 10 μ L 10 mM dNTPs, 1 μ L 1 mM dN-SMRT, 2.5 μ L Klenow enzyme, and 26.5 μ L H₂O. The beads were resuspended in the second-strand synthesis reaction mixture and incubated at 37°C for 1 hour in the rotator and followed by washes. Then whole transcriptome amplification was performed with: 1 μ L Primer IS-PCR (100 μ M), 50 μ L Kapa HiFi 2X, and 49 μ L H₂O. PCR was performed on the beads with the following cycles: 95°C for 3 minutes, 4 cycles of 98°C for 20 seconds, 65°C for 45 seconds, and 72°C for 3 minutes, followed by 4 cycles of 98°C for 20 seconds, 67°C for 20 seconds, and 72°C for 3 minutes, with a final extension at 72°C for 5 minutes and a hold at 4°C. The DNA was stored at 4°C. This was purified using Magnetic Ampure beads at a 0.6X ratio. The DNA was eluted into pure water. The DNA was prepared for Illumina sequencing using standard Illumina library preparation.

[0234] FIG. 16A contains data from the microscopy images of the nanowell array before HIFU showing the standard deviation of the fluorescence intensity of the Human GFP cells and mouse RFP cells in the nanowell array. FIG. 16B contains the complementary sequencing data showing the number of transcripts for the cells sequenced in the array. FIGs. 16C and 16D show the number of UMIs and genes respectively for the human and mouse cells from the sequencing data. FIG. 17, FIG. 18, and FIG. 19 show image analysis of the nanowell array from this example to determine the bead loading, cell loading, and cell lysis.

Example 5. Single cell mRNA sequencing of mouse brain cortex

[0235] Nanowell arrays made of polystyrene, and created with thermal compression molding were created containing nanowells of 50 μ m diameter, 50 μ m depth, and 15 μ m spacing. The bottom of the nanowells were patterned with geometric patterns consisting of 2 μ m squares. Each

nanowell array was placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with N₂ and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. 100 µL of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 µL of ethanol was then removed from the gasket chamber and 50 µL of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 µL) was removed and replaced with 100 µL PBS. The 100 µL PBS was then removed and replaced with an additional 100 µL PBS. Barcoded capture beads were prepared and diluted by adding 7.5 µL of beads to 82.5 µL of PBS with RNase inhibitor. The beads were then loaded into the nanowells by adding 100 µL of the diluted beads to the chamber and allowing them to settle for two minutes. A single cell suspension of mouse brain cortex cells was created by mechanical and enzymatic digestion of mouse brain cortex tissue. 100 µL of liquid was then removed from the nanowell array chamber and a single cell suspension of mouse brain cortex cells was loaded by adding 100 µL of the prepared solution into the chamber (~3000 cells) and the cells were centrifuged into the wells. The RT master mix was prepared by combining 42.5 µL H₂O, 30 µL dNTPs, 20 µL 5X Maxima mix, 2.5 µL RNase inhibitor, and 5 µL Maxima enzyme, totaling 100 µL. 100 µL of liquid was removed from the chamber, and 98 µL of the RT master mix was added. The chamber was incubated at room temperature for 5 minutes. 50 µL of the liquid was then removed from the chamber. A gas permeable 150 µm PDMS membrane was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. PCR tape was adhered to the top of the gasket to prevent evaporation. The nanowell array was placed in the HIFU-microscope apparatus (FIGs. 5A and 5B). HIFU was applied to the nanowell array with the following parameters: Power: 60W, Pulse repetition frequency: 50 Hz, Burst length: 10 µs, Frequency: 1.1 MHz, Treatment time: 1 s and this was rastered across 12,000 nanowells containing the ~3,000 total cells. Images from the microscope were taken of the cells before and after HIFU treatment. After that 100 µL of PBS FBS was added on top of the membrane. The nanowell array was placed onto a thermocycler with the lid at 52°C and incubated at 25°C for 15 minutes and 52°C for 30 minutes. The PCR tape was removed, and the PDMS membrane was removed with tweezers. The solution in the chamber was pipetted up and down to dislodge the beads from the wells and transferred to a

0.2 mL PCR tube. After reverse transcription, an exonuclease treatment was performed with the following reaction mixture: 10 μ L NEB 3.1 10X buffer, 85 μ L H₂O, and 5 μ L ExoI. The mixture was incubated at 37°C for 50 minutes in the rotator. This was followed by washes. After exonuclease treatment, a second-strand synthesis mix was prepared, consisting of 20 μ L Maxima 5X RT buffer, 40 μ L PEG 8000, 10 μ L 10 mM dNTPs, 1 μ L 1 mM dN-SMRT, 2.5 μ L Klenow enzyme, and 26.5 μ L H₂O. The beads were resuspended in the second-strand synthesis reaction mixture and incubated at 37°C for 1 hour in the rotator and followed by washes. Then whole transcriptome amplification was performed with: 1 μ L Primer IS-PCR (100 μ M), 50 μ L Kapa HiFi 2X, and 49 μ L H₂O. PCR was performed on the beads with the following cycles: 95°C for 3 minutes, 4 cycles of 98°C for 20 seconds, 65°C for 45 seconds, and 72°C for 3 minutes, followed by 4 cycles of 98°C for 20 seconds, 67°C for 20 seconds, and 72°C for 3 minutes, with a final extension at 72°C for 5 minutes and a hold at 4°C. The DNA was stored at 4°C. This was purified using Magnetic Ampure beads at a 0.6X ratio. The DNA was eluted into pure water. The DNA was prepared for Illumina sequencing using standard Illumina library preparation.

[0236] FIG. 20 shows the resulting UMAP of 2,315 total cells depicting 14 different cell clusters consisting of neuronal restricted progenitor cells (NRPs), oligodendrocyte precursor cells (OPCs), Astrocytes, oligodendrocytes, mature neurons, GABA neurons, Choroid plexus cells (CPCs), Ependymocytes, macrophages, microglia, endothelial cells, pericytes, and vascular smooth muscle cells (Vascular SMCs). Analysis of differentially expressed genes from each of the cell clusters identified key marker genes corresponding to each cell type from known mouse brain databases. There was expression of the key marker genes for each cell type: astrocytes (*Gja1*), OPCs (*Pdgfra*), oligodendrocytes (*Cldn11*), Mature neurons (*Snhg11*, *Snap25*, *Syt1*), GABA-2 (*Npy*, *Gad1*, *Gad2*), GABA-1 (*Penk*, *Gpr88*, *Pcp4*, *Gad1*, *Gad2*), NRPs (*Ccnd2*, *Sox11*), ependymocytes (*Ccdc153*), choroid plexus cells (*Ttr*, *Prr32*), macrophages (*Lyz2*, *Pf4*), microglia (*Cx3cr1*, *Tmem119*), endothelial cell (*Ly6c*, *Cldn5*), pericytes (*Vtn*, *Kcnj8*), and vascular smooth muscle cells (*Acta2*).

Example 6. Single cell mRNA sequencing of human peripheral blood mononuclear cells

[0237] Nanowell arrays made of polystyrene, and created with thermal compression molding were created containing nanowells of 50 μ m diameter, 50 μ m depth, and 15 μ m spacing. The bottom of the nanowells were patterned with geometric patterns consisting of 2 μ m squares. Each nanowell array was placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with N₂ and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any

remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. 100 μ L of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μ L of ethanol was then removed from the gasket chamber and 50 μ L of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μ L) was removed and replaced with 100 μ L PBS. The 100 μ L PBS was then removed and replaced with an additional 100 μ L PBS. Barcoded capture beads were prepared and diluted by adding 7.5 μ L of beads to 82.5 μ L of PBS with RNase inhibitor. The beads were then loaded into the nanowells by adding 100 μ L of the diluted beads to the chamber and allowing them to settle for two minutes. Human peripheral blood mononuclear cells (PBMCs) were labeled with Alexa Fluor 647 labelled anti CD14 antibodies to label CD14 monocytes and SYBR Green to label all cell nuclei. 100 μ L of liquid was then removed from the nanowell array chamber and a single cell suspension of mouse brain cortex cells was loaded by adding 100 μ L of the prepared solution into the chamber (~3000 cells) and the cells were centrifuged into the wells. The RT master mix was prepared by combining 42.5 μ L H₂O, 30 μ L dNTPs, 20 μ L 5X Maxima mix, 2.5 μ L RNase inhibitor, and 5 μ L Maxima enzyme, totaling 100 μ L. 100 μ L of liquid was removed from the chamber, and 98 μ L of the RT master mix was added. The chamber was incubated at room temperature for 5 minutes. 50 μ L of the liquid was then removed from the chamber. A gas permeable 150 μ m PDMS membrane was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. PCR tape was adhered to the top of the gasket to prevent evaporation. The nanowell array was placed in the HIFU-microscope apparatus (FIGs. 5A and 5B). HIFU was applied to the nanowell array with the following parameters: Power: 60W, Pulse repetition frequency: 50 Hz, burst length: 10 μ s, Frequency: 1.1 MHz, Treatment time: 1 s and this was rastered across 12,000 nanowells containing the ~3,000 total cells. Images from the microscope were taken of the cells before and after HIFU treatment. After that 100 μ L of PBS FBS was added on top of the membrane. The nanowell array was placed onto a thermocycler with the lid at 52°C and incubated at 25°C for 15 minutes and 52°C for 30 minutes. The PCR tape was removed, and the PDMS membrane was removed with tweezers. The solution in the chamber was pipetted up and down to dislodge the beads from the wells and transferred to a 0.2 mL PCR tube. After reverse transcription, an exonuclease treatment was performed with the following reaction mixture: 10 μ L NEB 3.1 10X buffer, 85 μ L H₂O, and 5 μ L ExoI. The mixture was incubated at 37°C for 50 minutes in the rotator.

This was followed by washes. After exonuclease treatment, a second-strand synthesis mix was prepared, consisting of 20 μ L Maxima 5X RT buffer, 40 μ L PEG 8000, 10 μ L 10 mM dNTPs, 1 μ L 1 mM dN-SMRT, 2.5 μ L Klenow enzyme, and 26.5 μ L H₂O. The beads were resuspended in the second-strand synthesis reaction mixture and incubated at 37°C for 1 hour in the rotator and followed by washes. Then whole transcriptome amplification was performed with: 1 μ L Primer IS-PCR (100 μ M), 50 μ L Kapa HiFi 2X, and 49 μ L H₂O. PCR was performed on the beads with the following cycles: 95°C for 3 minutes, 4 cycles of 98°C for 20 seconds, 65°C for 45 seconds, and 72°C for 3 minutes, followed by 4 cycles of 98°C for 20 seconds, 67°C for 20 seconds, and 72°C for 3 minutes, with a final extension at 72°C for 5 minutes and a hold at 4°C. The DNA was stored at 4°C. This was purified using Magnetic Ampure beads at a 0.6X ratio. The DNA was eluted into pure water. The DNA was prepared for Illumina sequencing using standard Illumina library preparation.

[0238] Microscopy images of the nanowell array showed nuclei in cyan and CD14 in magenta, enabling us to quantify the proportion of CD14+ cells at 34.1%. The resulting UMAP of 3,382 total cells identifies the major PBMC cell types such as B cells, plasma cells, dendritic cells (DC), FCGR3+ monocytes, CD14+ monocytes, naive CD4 T cells, memory CD4 T cells, CD8 t cells, natural killer cells (NK cells) and platelets. The proportion of CD14+ monocytes in the sequencing data (34.5%) was similar to the imaging data (34.1%).

Example 7. Effect of modifying high intensity focused ultrasound and membrane thickness on cavitation focal area

[0239] Nanowell arrays made of polystyrene, and created with thermal compression molding were created containing nanowells of 50 μ m diameter, 50 μ m depth, and 15 μ m spacing. The bottom of the nanowells were patterned with geometric patterns consisting of 2 μ m squares. The nanowell arrays were placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with nitrogen and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers as shown in FIG. 3 was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array.

[0240] 100 μ L of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μ L of ethanol was then removed from the gasket chamber and 50 μ L of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with

PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μ L) was removed and replaced with 100 μ L PBS. The 100 μ L PBS was then removed and replaced with an additional 100 μ L PBS. Gas permeable PDMS membranes of varying thicknesses from 64 to 150 μ m were floated on top of the remaining solutions. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. After dehydration, the nanowell arrays were treated with HIFU parameters in triplicate. In this example, the frequency was 3.41 MHz, burst length was 10 μ s, the pulse repetition frequency was 30 Hz, and the treatment time was 1 second. The power was varied from 0 to 20W as shown in FIGs. 21A-21B, with an optimal power response of 5 to 10 W and membrane thickness of 64 μ m.

Example 8. Targeted lysis of individual user selected cells using HIFU within the nanowell array

[0241] Nanowell arrays made of polystyrene, and created with thermal compression molding were created containing nanowells of 50 μ m diameter, 50 μ m depth, and 15 μ m spacing. The bottom of the nanowells were patterned with geometric patterns consisting of 2 μ m squares. The nanowell arrays were placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with nitrogen and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. 100 μ L of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μ L of ethanol was then removed from the gasket chamber and 50 μ L of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μ L) was removed and replaced with 100 μ L PBS. The 100 μ L PBS was then removed and replaced with an additional 100 μ L PBS. 100 μ L of liquid was then removed and the cells were loaded by adding 100 μ L of the prepared solution into the chamber (1,000 cells) and allowing them to settle for ten minutes. A 64 μ m gas permeable PDMS membrane was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. After dehydration, the nanowell arrays were treated with HIFU parameters in triplicate. The frequency was 3.41 MHz, burst length 10 μ s, pulse repetition frequency 30 Hz, power 7W, and the treatment time was 1 second. The rate of cellular lysis was

measured as 16 cells per minute (FIG. 22A), with a lysis efficiency of 70% (FIG. 22B), and 10% off-target cells lysed (FIG. 22C). Custom automation software was used to move the HIFU focal zone to the location of specific cells to derive the cells targeted per unit time metric.

Example 9. Enrichment of cellular nucleic acids using targeted lysis within the nanowell array using microscopy imaging

[0242] Nanowell arrays made of polystyrene, and created with thermal compression molding were created containing nanowells of 50 μm diameter, 50 μm depth, and 15 μm spacing. The bottom of the nanowells were patterned with geometric patterns consisting of 2 μm squares. Each nanowell array was placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with N_2 and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. 100 μL of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μL of ethanol was then removed from the gasket chamber and 50 μL of PBS was added. The PBS and ethanol were mixed by pipetting up and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μL) was removed and replaced with 100 μL PBS. The 100 μL PBS was then removed and replaced with an additional 100 μL PBS. Barcoded capture beads were prepared and diluted by adding 7.5 μL of beads to 82.5 μL of PBS with RNase inhibitor. The beads were then loaded into the nanowells by adding 100 μL of the diluted beads to the chamber and allowing them to settle for two minutes. 100 μL of liquid was then removed and a mixture of HEK-293T-zsGreen cells expressing a green fluorescent protein and NIH 3T3-tdTomato cells expressing a red fluorescent protein were loaded by adding 100 μL of the prepared solution into the chamber (~100 human cells and 1,000 mouse cells in 1% FBS in PBS) and the cells were centrifuged into the wells. 50 μL of the liquid was then removed from the chamber. A gas permeable 70 μm PDMS membrane was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. This procedure was repeated for 2 more chambers with the only difference being that the other two chambers were sealed with 150 μm PDMS membranes. PCR tape was adhered to the top of the gasket to prevent evaporation. The nanowell array with the 70 μm membrane was placed in the HIFU-microscope apparatus (FIG. 5). HIFU was applied to the nanowell array with the following parameters: Power: 7W, Pulse repetition frequency: 30

Hz, Burst length: 10 μ s, Frequency: 3.140 MHz, Treatment time: 1 s and this was applied only to the nanowells containing the human cells by imaging the nanowell array and only selecting the nanowells with human cells. One of the chambers with the 150 μ m membrane was not subjected to HIFU and the other chamber with the 150 μ m membrane received the following HIFU treatment: Power: 60W, Pulse repetition frequency: 50 Hz, Burst length: 10 μ s, Frequency: 1.1 MHz, Treatment time: 1 s and this was rastered across full chamber. Images from the microscope were taken of the cells before and after HIFU treatment. After that 100 μ L of PBS FBS was added on top of the membrane. The arrays were held at 25°C for 15 minutes. The PCR tape was then removed and the PDMS membrane was removed with tweezers. The solution in the chamber was pipetted up and down to dislodge the beads from the wells. 100 μ L of the solution was removed and transferred to a 0.2 mL PCR tube. A 1.2 mL sucrose gradient was prepared with 200 μ L each of 60%-10% sucrose in PBS at a 10% step size. After 7 minutes, the supernatant was removed, and the beads were recovered and transferred to a 0.2 mL PCR tube followed by subsequent washes. Then the reverse transcription master mix was added consisting of 22.5 μ L H₂O, 20 μ L 5X RT buffer, 40 μ L 30% PEG, 10 μ L dNTPs, 2.5 μ L RNase inhibitor, and 5 μ L Maxima RT enzyme. The mixture was incubated in an end-over-end rotator at 52°C for 1 hour and washed with TE-tween. After reverse transcription, a pre-amplification PCR was performed for the PTGER2 gene using gene specific primers, followed by AMPure cleanup of the PCR product and digital droplet PCR to quantify the number of PTGER2 copies from mouse and human cells. This data is shown in FIG. 23A.

Example 10. Controlling release of cellular nucleic acids using targeted lysis within the nanowell array using microscopy imaging

[0243] Nanowell arrays made of polystyrene, and created with thermal compression molding were created containing nanowells of 50 μ m diameter, 50 μ m depth, and 15 μ m spacing. The bottom of the nanowells were patterned with geometric patterns consisting of 2 μ m squares. Each nanowell array was placed in a glass petri dish, covered with isopropyl alcohol, and sonicated for 15 minutes. The nanowell array was then dried with N₂ and transferred to a clean petri dish. The petri dish with the nanowell array was then incubated at 63°C for 15 minutes to evaporate any remaining isopropyl alcohol. A silicone gasket creating 7mm x 7mm chambers was then adhered to the nanowell array to ensure each chamber was sealed to the nanowell array. 100 μ L of 100% ethanol was then added to each chamber by pipetting the ethanol into one corner of the gasket chamber and allowing it to disperse over 5 minutes. 50 μ L of ethanol was then removed from the gasket chamber and 50 μ L of PBS was added. The PBS and ethanol were mixed by pipetting up

and down and the process was repeated for a total of 3 dilutions with PBS. The procedure was repeated for a total of 3 dilutions with PBS. All of the solution (~100 μ L) was removed and replaced with 100 μ L PBS. The 100 μ L PBS was then removed and replaced with an additional 100 μ L PBS. Barcoded capture beads were prepared and diluted by adding 7.5 μ L of beads to 82.5 μ L of PBS with RNase inhibitor. The beads were then loaded into the nanowells by adding 100 μ L of the diluted beads to the chamber and allowing them to settle for two minutes. 100 μ L of liquid was then removed and a mixture of HEK-293T-zsGreen cells expressing a green fluorescent protein and mouse NIH 3T3-tdTomato cells expressing red fluorescent proteins were loaded by adding 100 μ L of the prepared solution into the chamber (~200 human cells and 200 mouse cells in 1% FBS in PBS) and the cells were centrifuged into the wells. 50 μ L of the liquid was then removed from the chamber. A gas permeable 70 μ m PDMS membrane was floated on top of the remaining solution. The remaining fluid was then removed via pipette and the array was incubated at 37°C for 10 minutes to seal the microwells. This procedure was repeated for 3 more chambers with 3 of the chambers sealed with the same 70 μ m membrane and one chamber sealed with a 150 μ m PDMS membrane. PCR tape was adhered to the top of the gasket to prevent evaporation. The nanowell arrays with the 70 μ m membrane were placed in the HIFU-microscope apparatus (FIGs. 5A and 5B). HIFU was applied to the nanowell array with the following parameters: Power: 7W, Pulse repetition frequency: 30 Hz, Burst length: 10 μ s, Frequency: 3.140 MHz, Treatment time: 1 s. In one chamber with a 70 μ m membrane no cells were treated with HIFU, in the next chamber with a 70 μ m membrane 25 human cells were targeted and lysed, in the next chamber with a 70 μ m membrane 50 human cells were targeted and lysed. The location of the nanowells containing the human cells was guided by microscopy imaging of the cells within the nanowell array. The last chamber with the 150 μ m membrane received the following HIFU treatment: Power: 60W, Pulse repetition frequency: 50 Hz, Burst length: 10 μ s, Frequency: 1.1 MHz, Treatment time: 1 s and this was rastered across full chamber. Images from the microscope were taken of the cells before and after HIFU treatment. After that 100 μ L of PBS FBS was added on top of the membrane. The arrays were held at 25°C for 15 minutes. The PCR tape was then removed and the PDMS membrane was removed with tweezers. The solution in the chamber was pipetted up and down to dislodge the beads from the wells. 100 μ L of the solution was removed and transferred to a 0.2 mL PCR tube. A 1.2 mL sucrose gradient was prepared with 200 μ L each of 60%-10% sucrose in PBS at a 10% step size. After 7 minutes, the supernatant was removed, and the beads were recovered and transferred to a 0.2 mL PCR tube followed by subsequent washes. Then the reverse transcription master mix was added consisting of 22.5 μ L H₂O, 20 μ L 5X RT buffer, 40 μ L 30% PEG, 10 μ L dNTPs, 2.5 μ L RNase inhibitor, and 5 μ L Maxima RT

enzyme. The mixture was incubated in an end-over-end rotator at 52°C for 1 hour, and washed with TE-tween. After reverse transcription, a pre-amplification PCR was performed for the PTGER2 gene using gene specific primers, followed by AMPure clean up of the PCR product and digital droplet PCR to quantify the number of PTGER2 copies from human and mouse cells. This data is shown in FIG. 23B. An increasing amount of PTGER2 copies from human cells was seen as more human cells were lysed without an increase in mouse PTGER2.

Example 11. Pre-tagmented nuclei assay for transposase-accessible chromatin (ATAC)

[0244] Starting with tagmented nuclei (that is, fragmented and tagged) with barcoded Illumina p7 transposase adapters, up to $\lambda=10$ /well (based on 96 tn5 barcodes) MCF7 nuclei were loaded on the array (used 50,000 nuclei, barcoded using S3-tn5 adapters (Mulqueen et al., 2021 doi.org/10.1038/s41587-021-00962-z)). The array was then spun down at 20-100rcf for 2 min. Buffer exchange was performed using 100 μ l of a reaction mix containing: 72 μ l dH₂O; 1 μ l Tris-HCl pH=7.5; 5 μ l 100 mM MgCl₂; 1 μ l 100 mM KOAc; 2.5 μ l 100 mM DTT; 0.5 μ l 100 mM ATP; 5 μ l 9°N Ligase (NEB); 5 μ l Tte-UvdD helicase; 5 μ l ET-SSB (Extremely thermostable single stranded DNA binding protein) at 20 ng/ μ l; 1 μ l T4 Polynucleotide Kinase; 2 μ l 10 μ M Splint then cap and dry at 37°C for 10 min. HiFu was run with nuclei parameters (Nanowell array: 50 μ m well diameter, 50 μ m well depth, 15 μ m spacing between wells, 2 μ m x 2 μ m x 2 μ m square nanopatterning, 150 μ m membrane thickness; HIFU parameters: Power: 70W, Burst length: 10 μ s, Frequency: 1100 MHz, Treatment time: 1s, PRF: 50 Hz) and then the array was incubated at 45°C for 30 min. The beads were then removed from the wells and washed 2xPBS and eluted into amplification mix containing 25 μ l 2x Watchmaker U DNA polymerase master mix; 1.5 μ l TrueSeq i5 indexing primer; 1.5 μ l TrueSeq i7 indexing primer; 0.5 μ l 100X SYBR Green; 21.5 μ l dH₂O and amplified for 17 cycles.

Example 12. One-pot assay for transposase-accessible chromatin (ATAC)

[0245] Beads were loaded into nanowells and then MCF7 cells in PBS were added. The array was spun down 20-100rcf 2:00. A buffer exchange using 100 μ l from a master mix containing: 72 μ l dH₂O; 1 μ l Tris-HCl pH=7.5; 5 μ l 100 mM MgCl₂; 1 μ l 100 mM KOAc; 2.5 μ l 100 mM DTT; 0.5 μ l 100 mM ATP; 5 μ l 9°N Ligase (NEB); 5 μ l Tte-UvdD helicase; 5 μ l ET-SSB (Extremely thermostable single stranded DNA binding protein) at 20 ng/ μ l; 1 μ T4 Polynucleotide Kinase; 2 μ l 10 μ M Splint; 5 μ l tn5 transposase loaded with Nextera p7 only; and 5 μ l USER enzyme (OMIT) was performed. The gas permeable membrane was then placed on the array, excess liquid was removed, and the array was incubated at 37°C 10:00. HIFU was run with cell parameters (Nanowell array: 50 μ m well diameter, 50 μ m well depth, 15 μ m spacing between wells, 2 μ m x

2 μm x 2 μm square nanopatterning, 150 μm membrane thickness; HIFU parameters: Power: 60W, Burst length: 10 μs , Frequency: 1100 MHz, Treatment time: 1s, PRF: 50 Hz) and the array was then incubated at 45°C for 30:00. The beads were then removed from the wells, washed 2xPBS and eluted into amplification mix containing 25 μl 2x Watchmaker U DNA polymerase master mix; 1.5 μl TrueSeq i5 indexing primer; 1.5 μl Nextera i7 indexing primer; 0.5 μl 100X SYBR™ Green; and 5 μl dH₂O. The mix was then amplified according to manufacture protocol for 17 cycles.

Closing Paragraphs

[00246] As will be understood by one of ordinary skill in the art, each embodiment disclosed herein can comprise, consist essentially of or consist of its particular stated element, step, ingredient or component. Thus, the terms “include” or “including” should be interpreted to recite: “comprise, consist of, or consist essentially of.” The transition term “comprise” or “comprises” means has, but is not limited to, and allows for the inclusion of unspecified elements, steps, ingredients, or components, even in major amounts. The transitional phrase “consisting of” excludes any element, step, ingredient or component not specified. The transition phrase “consisting essentially of” limits the scope of the embodiment to the specified elements, steps, ingredients or components and to those that do not materially affect the embodiment. A material effect would cause a statistically significant difference in one or more of the amount of cavitation, bubble size, cell lysis, and cross-talk. A material effect would cause a statistically significant difference in the homogeneity of the lysed cell components.

[00247] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. When further clarity is required, the term “about” has the meaning reasonably ascribed to it by a person skilled in the art when used in conjunction with a stated numerical value or range, i.e. denoting somewhat more or somewhat less than the stated value or range, to within a range of $\pm 20\%$ of the stated value; $\pm 19\%$ of the stated value; $\pm 18\%$ of the stated value; $\pm 17\%$ of the stated value; $\pm 16\%$ of the stated value; $\pm 15\%$ of the stated value; $\pm 14\%$ of the stated value;

$\pm 13\%$ of the stated value; $\pm 12\%$ of the stated value; $\pm 11\%$ of the stated value; $\pm 10\%$ of the stated value; $\pm 9\%$ of the stated value; $\pm 8\%$ of the stated value; $\pm 7\%$ of the stated value; $\pm 6\%$ of the stated value; $\pm 5\%$ of the stated value; $\pm 4\%$ of the stated value; $\pm 3\%$ of the stated value; $\pm 2\%$ of the stated value; or $\pm 1\%$ of the stated value.

[00248] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[00249] The terms “a,” “an,” “the” and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[00250] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[00251] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited

in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0252] Furthermore, numerous references have been made to patents, printed publications, journal articles, other written text, and website content throughout this specification (referenced materials herein). Each of the referenced materials is individually incorporated herein by reference in their entirety for their referenced teaching(s), as of the filing date. For instance, with regard to chemical compounds, nucleic acid, and amino acid sequences referenced herein that are available in a public database, the information in the database entry is incorporated herein by reference as of the date of the filing date.

[0253] It is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.

[0254] The particulars shown herein are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of various embodiments of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for the fundamental understanding of the invention, the description taken with the drawings and/or examples making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

[0255] Definitions and explanations used in the present disclosure are meant and intended to be controlling in any future construction unless clearly and unambiguously modified in the example(s) or when application of the meaning renders any construction meaningless or essentially meaningless. In cases where the construction of the term would render it meaningless or essentially meaningless, the definition should be taken from Webster's Dictionary, 11th Edition or a dictionary known to those of ordinary skill in the art, such as the Oxford Dictionary of Biochemistry and Molecular Biology, 2nd Edition (Ed. Anthony Smith, Oxford University Press, Oxford, 2006), and/or A Dictionary of Chemistry, 8th Edition (Ed. J. Law & R. Rennie, Oxford University Press, 2020).

LISTING OF CLAIMS

What is claimed is:

1. A method comprising:
loading wells of a polymer nanowell array comprising a plurality of nanowells with one or more reaction mixes;
covering the loaded wells with a membrane;
treating the nanowell array with high intensity focused ultrasound (HIFU);
incubating the reaction mixes in the nanowell array to form an analyte; and
removing the membrane.
2. The method of claim 1, wherein the membrane is 20 μm to 250 μm thick.
3. The method of claim 1, wherein the membrane is 150 μm thick.
4. The method of claim 1, wherein the membrane is 70 μm thick.
5. The method of claim 1, wherein the membrane is hydrophobic.
6. The method of claim 1, wherein the membrane is a gas permeable membrane.
7. The method of claim 6, wherein the gas permeable membrane comprises polydimethylsiloxane.
8. The method of claim 1, wherein the method produces a library of analytes.
9. The method of claim 1, wherein the reaction mix comprises cells.
10. The method of claim 9, wherein the loading of the polymer nanowell array results in a plurality of nanowells comprising one or more cells of the reaction mix.
11. The method of claim 1, wherein the reaction mix comprises cell nuclei.
12. The method of claim 1, wherein the reaction mix comprises at least one of cell suspensions, cell nuclei suspensions, barcoded beads, and enzymes.

13. The method of claim 1, wherein the polymer of the polymer nanowell array is hydrophobic.
14. The method of claim 13, wherein the polymer of the polymer nanowell array comprises polystyrene.
15. The method of claim 1, wherein each nanowell of the plurality of nanowells has a geometric pattern on a bottom of each nanowell.
16. The method of claim 15, wherein the geometric pattern comprises a repeating pattern of a plurality of squares or a plurality of parallel lines.
17. The method of claim 15, wherein at least one dimension of the pattern is 0.2 μm to 11 μm .
18. The method of claim 15, wherein a size of the pattern is 2 μm in at least one dimension.
19. The method of claim 15, wherein a size of the pattern is 5 μm in at least one dimension.
20. The method of claim 15, wherein a size of the pattern is 10 μm in at least one dimension.
21. The method of claim 1, wherein each nanowell of the nanowell array is 5 to 500 microns in diameter.
22. The method of claim 21, wherein each nanowell of the nanowell array is 40 to 60 microns in diameter.
23. The method of claim 22, wherein each nanowell of the nanowell array is 50 microns in diameter.
24. The method of claim 1, wherein the HIFU is selectively applied to an area of the nanowell array.
25. The method of claim 24, wherein the area for selective HIFU application is identified with microscopy imaging.

26. The method of claim 25, wherein the microscopy imaging is epifluorescent microscopy.
27. The method of claim 1, wherein treating the nanowell array with HIFU comprises applying HIFU using a power of between 0.01 W and 250 W with a burst length between 1 to 100 μ s, a frequency between 0.2 MHz and 50 MHz, a treatment time between 1 ms and 60 s per treatment location, and a pulse repetition frequency from 1 Hz and 500 Hz.
28. The method of claim 1, treating the nanowell array with HIFU comprises applying HIFU using a power between 1 W and 100 W, a burst length between 1 to 20 μ s, a frequency between 0.4 MHz and 15 MHz, a treatment time between 100 ms and 10 s per treatment location, a pulse repetition frequency from 10 Hz and 100 Hz.
29. The method of claim 1, wherein treating the nanowell array with HIFU comprises applying HIFU using a power of 60W, a burst length of 10 μ s, a frequency of 1100 MHz, a treatment time of 1s, and a pulse repetition frequency of 50 Hz.
30. The method of claim 1, wherein treating the nanowell array with HIFU comprises applying HIFU using a power of 8W, a burst length of 10 μ s, a frequency of 3410 MHz, a treatment time of 1s, and a pulse repetition frequency of 30 Hz.
31. The method of claim 1, wherein treating the nanowell array with HIFU comprises applying HIFU using a power of 70W.
32. A high intensity focused ultrasound (HIFU) platform comprising:
a polymer nanowell array comprising a plurality of nanowells;
a membrane adhered to a top surface of the nanowell array;
a microscope positioned above the nanowell array; and
a HIFU ultrasound transducer positioned below the nanowell array;
wherein the nanowell array is acoustically coupled to the HIFU ultrasound transducer.
33. The HIFU platform of claim 32, wherein the polymer nanowell is hydrophobic.

34. The HIFU platform of claim 32, wherein the polymer is a hydrophobic polymer, which hydrophobic polymer optionally comprises polystyrene.
35. The HIFU platform of claim 32, wherein the microscope is an epifluorescent microscope.
36. The HIFU platform of claim 32, wherein the membrane is 20 μm to 250 μm thick.
37. The HIFU platform of claim 32, wherein the membrane is 150 μm thick.
38. The HIFU platform of claim 32, wherein the membrane is 70 μm thick.
39. The HIFU platform of claim 32, wherein the membrane is hydrophobic.
40. The HIFU platform of claim 32, wherein the membrane is a gas permeable membrane.
41. The HIFU platform of claim 40, wherein the gas permeable membrane comprises polydimethylsiloxane.
42. The HIFU platform of claim 32, further comprising a geometric pattern on a bottom of each nanowell of the nanowell array.
43. The HIFU platform of claim 42, wherein the geometric pattern comprises a repeating pattern of a plurality of squares or a plurality of parallel lines.
44. The HIFU platform of claim 42, wherein at least one dimension of the pattern is 0.2 μm to 11 μm .
45. The HIFU platform of claim 42, wherein a size of the pattern is 2 μm in at least one dimension.
46. The HIFU platform of claim 42, wherein a size of the pattern is 5 μm in at least one dimension.

47. The HIFU platform of claim 42, wherein a size of the pattern is 10 μm in at least one dimension.
48. The HIFU platform of claim 32, wherein each nanowell of the nanowell array is 5 to 50 microns in diameter.
49. The HIFU platform of claim 32, wherein each nanowell of the nanowell array is 40 to 60 microns in diameter.
50. The HIFU platform of claim 32, wherein each nanowell of the nanowell array is 82 microns in diameter.
51. The HIFU platform of claim 32, wherein a spacing between adjacent nanowells in the nanowell array is 1 μm to 500 μm .
52. The HIFU platform of claim 51, wherein a spacing between adjacent nanowells in the nanowell array is 5 μm to 50 μm .
53. The HIFU platform of claim 52, wherein a spacing between adjacent nanowells in the nanowell array is 15 μm .
54. The HIFU platform of claim 32, wherein a transducer focal zone is within 10 mm of a bottom of the nanowell array.
55. A method of using the HIFU platform of claim 32.

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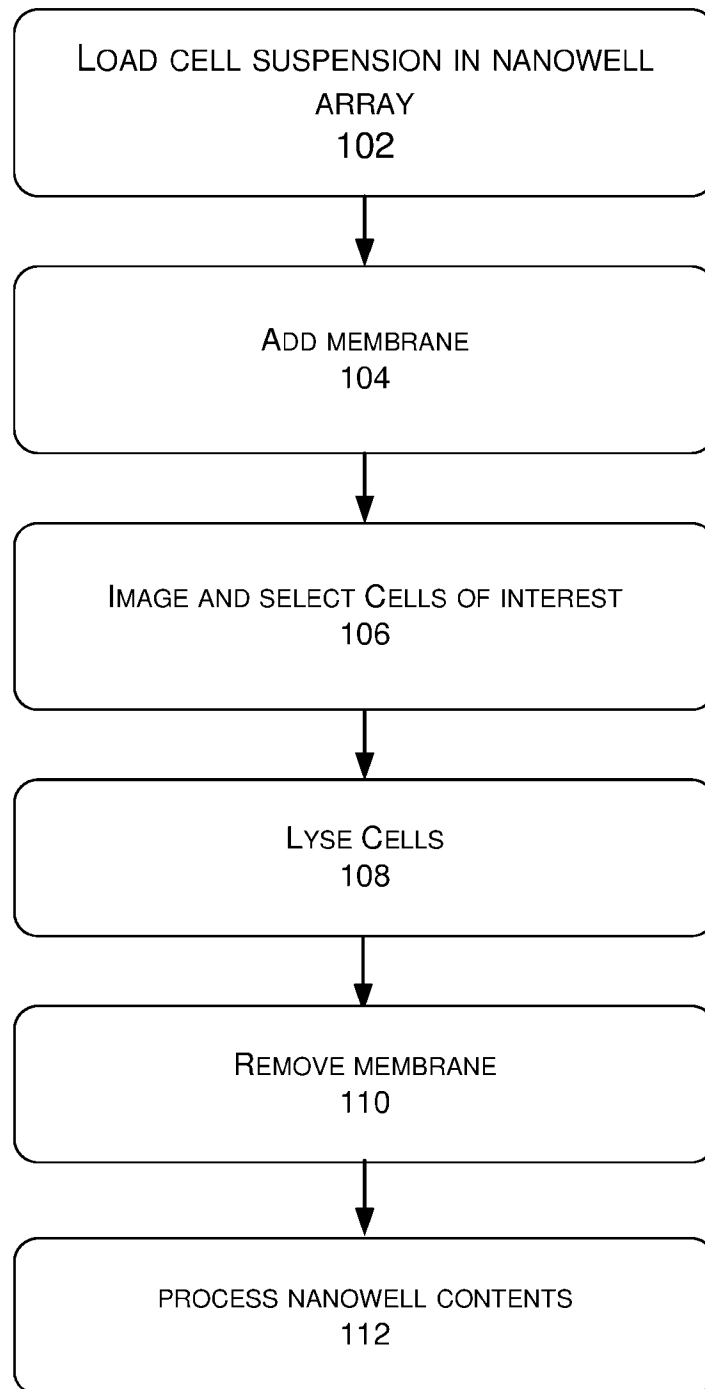
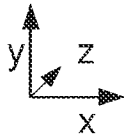
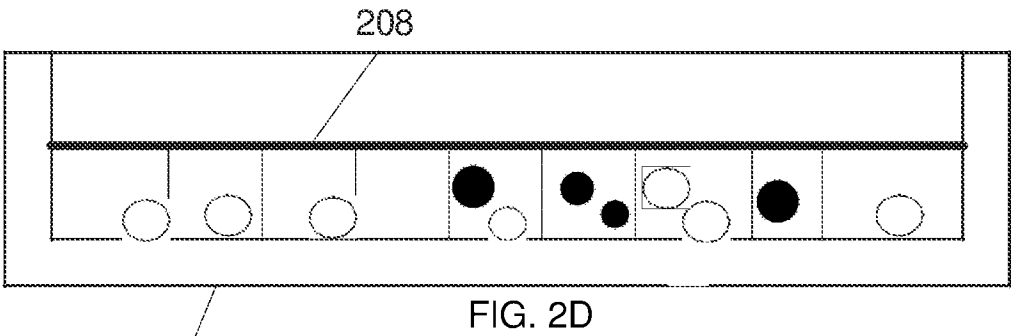
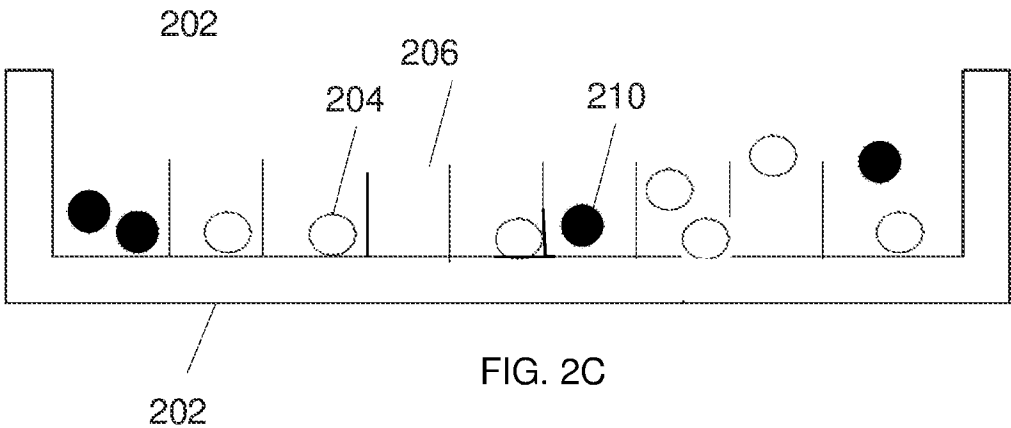
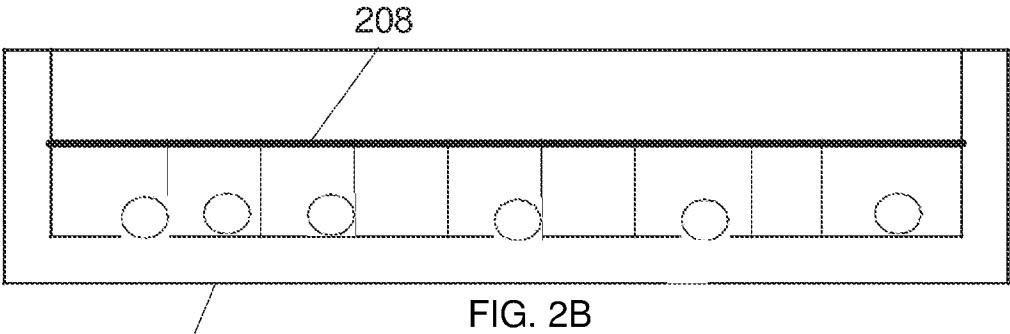
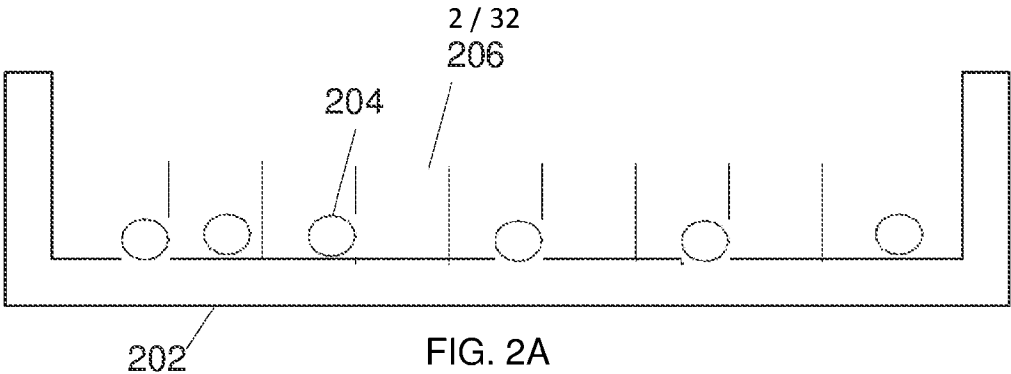


FIG. 1



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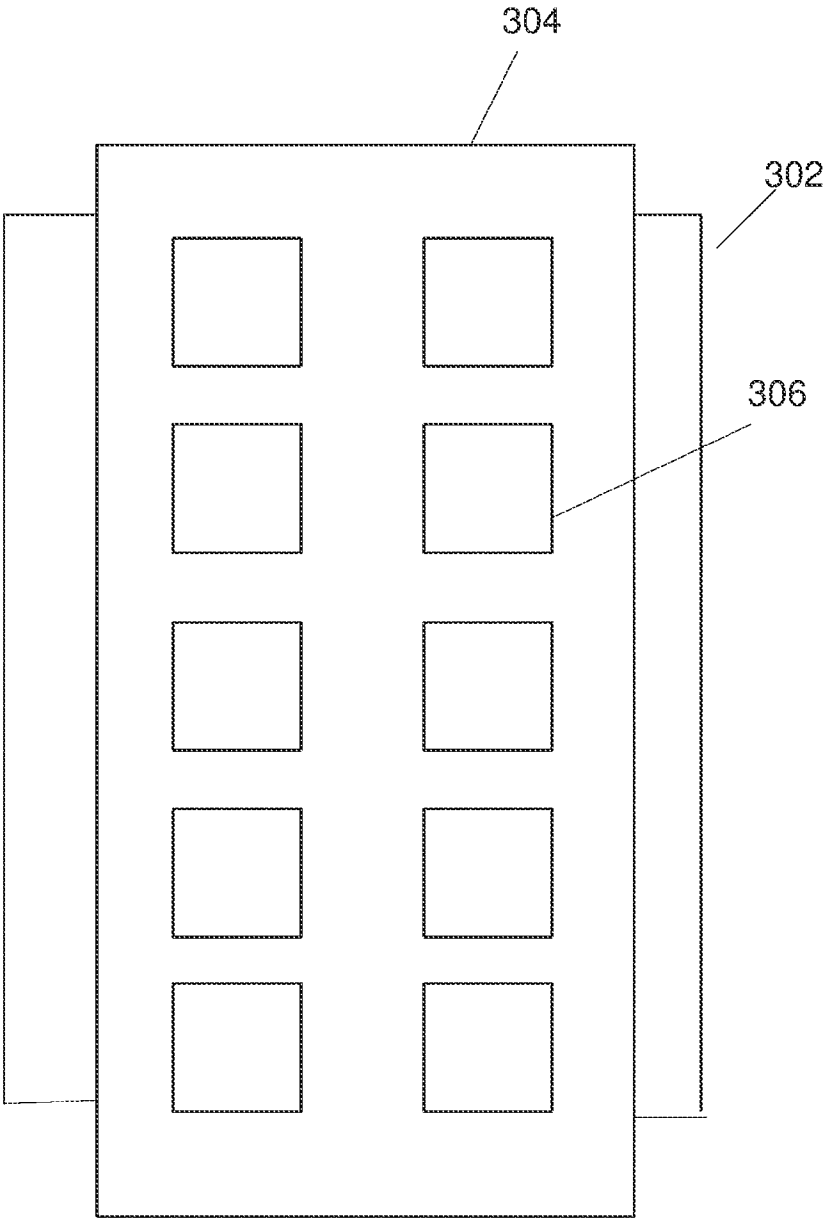


FIG. 3

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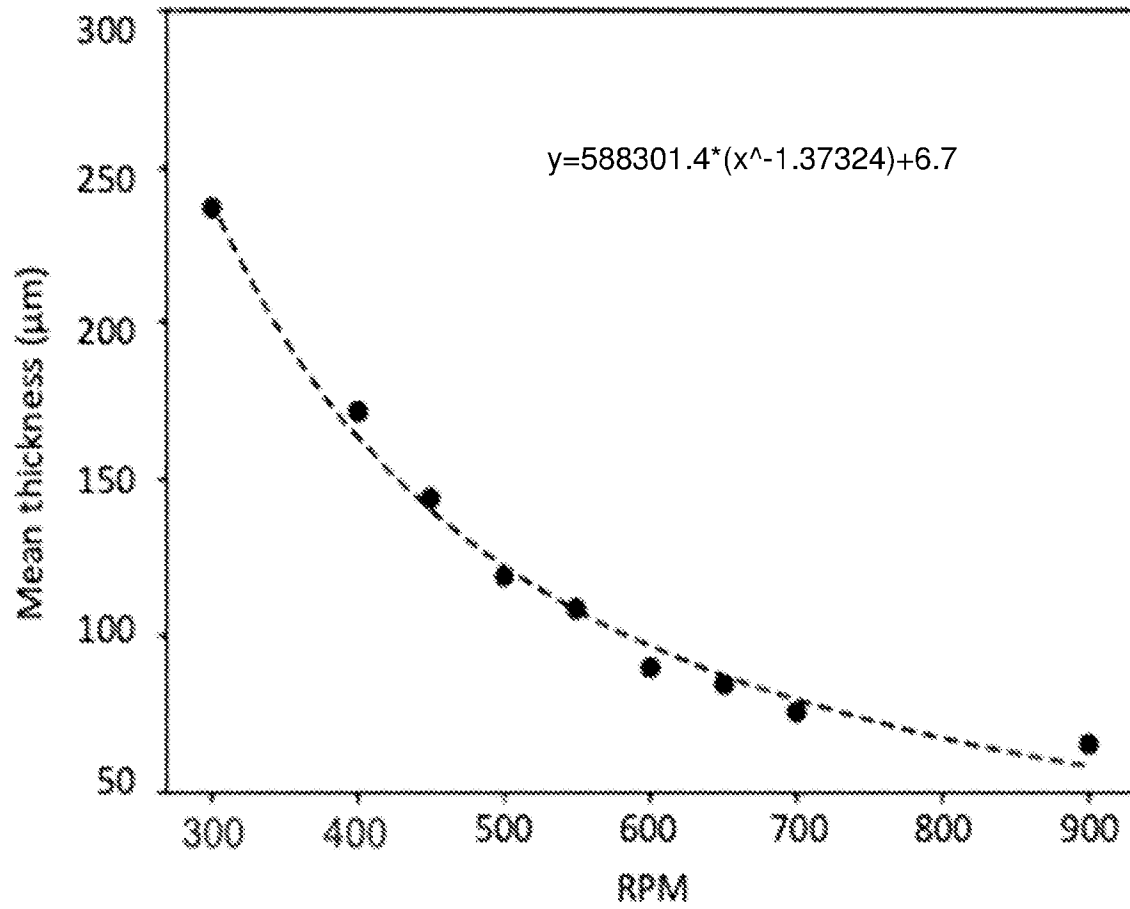


FIG. 4A

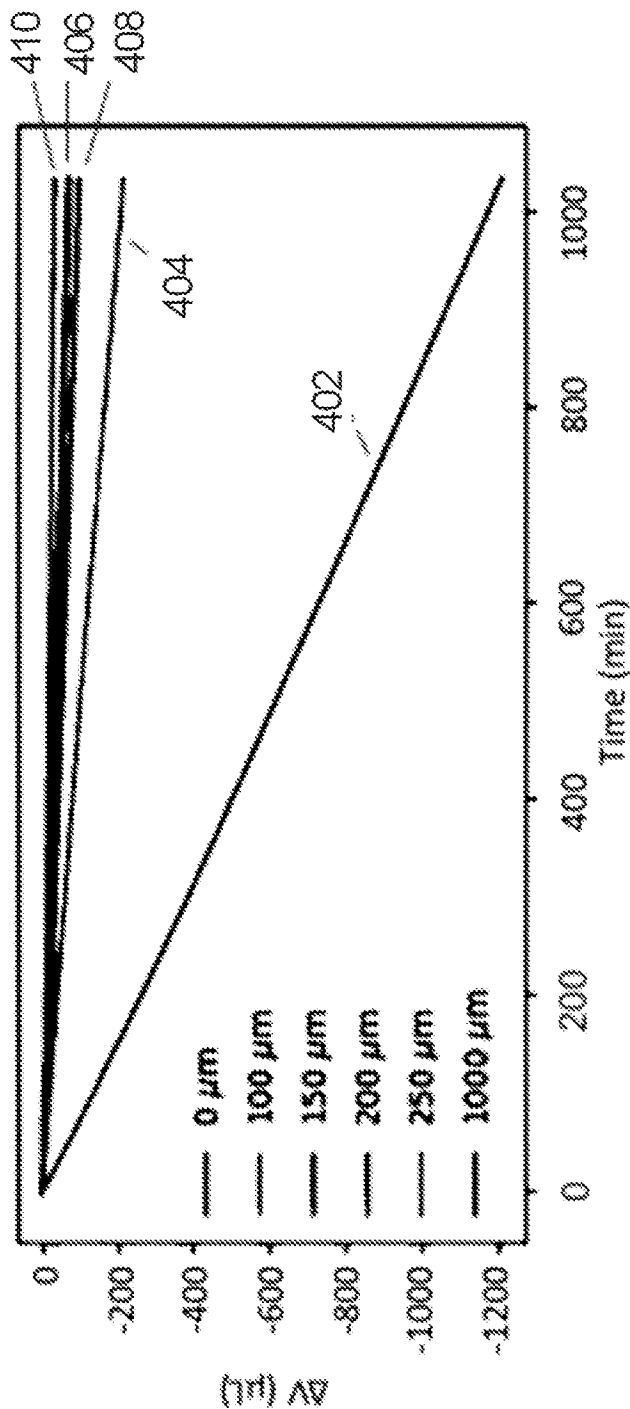


FIG. 4B

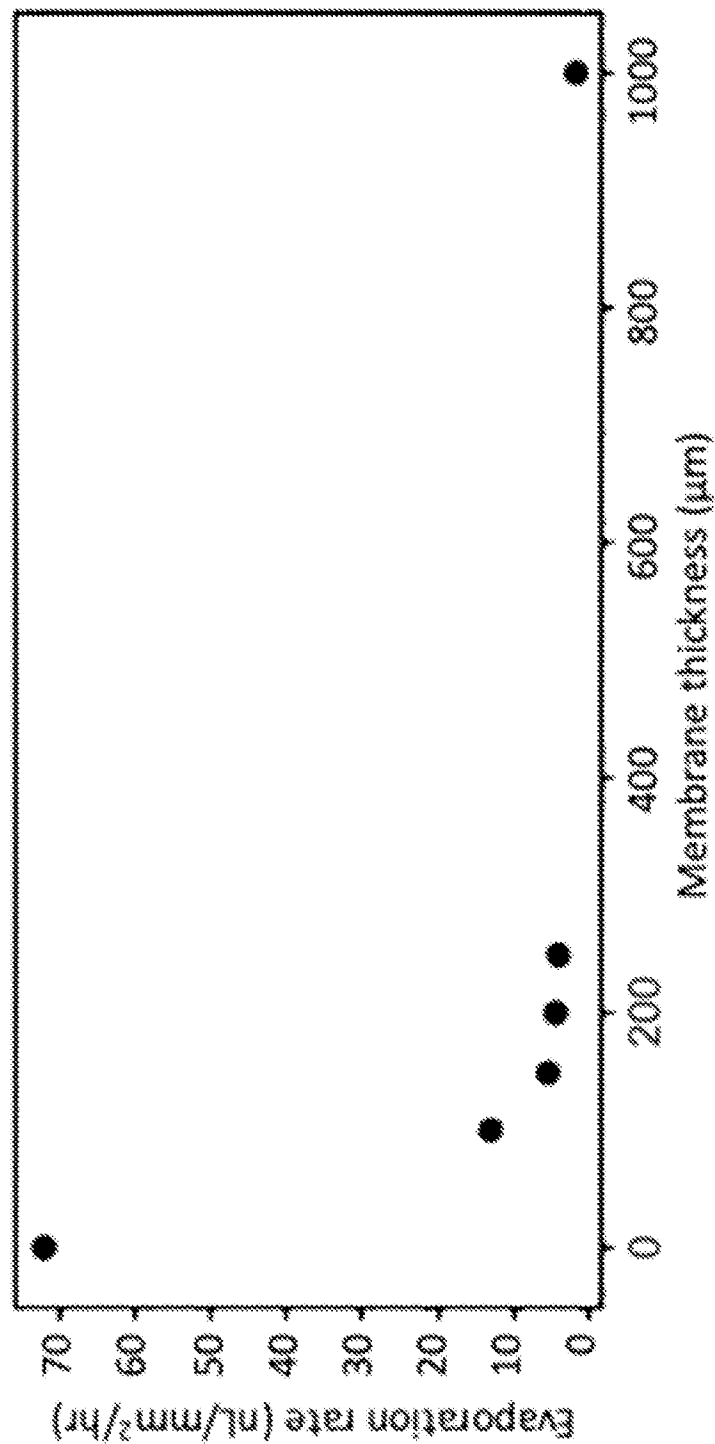


FIG. 4C

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FIG. 5A

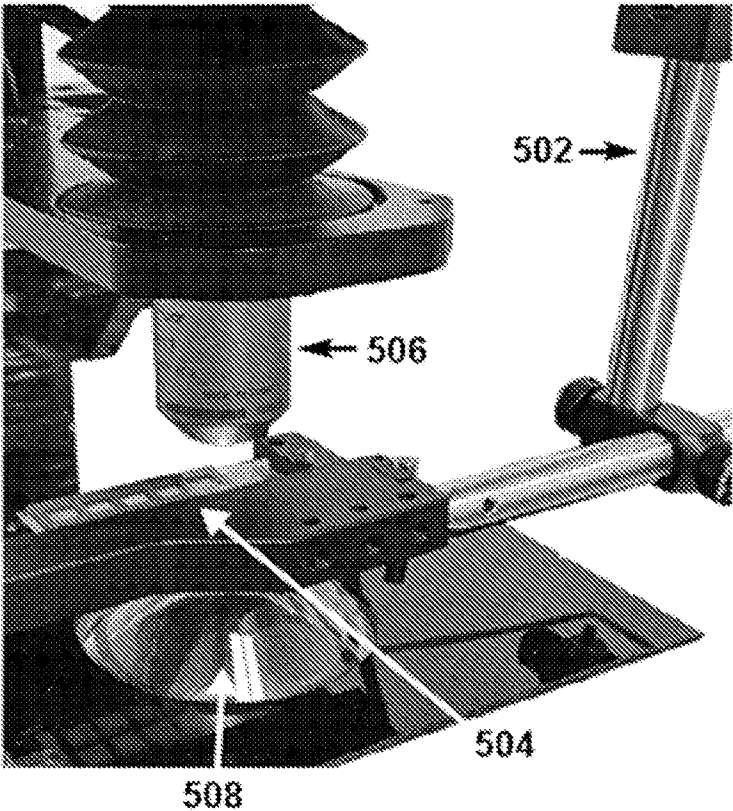
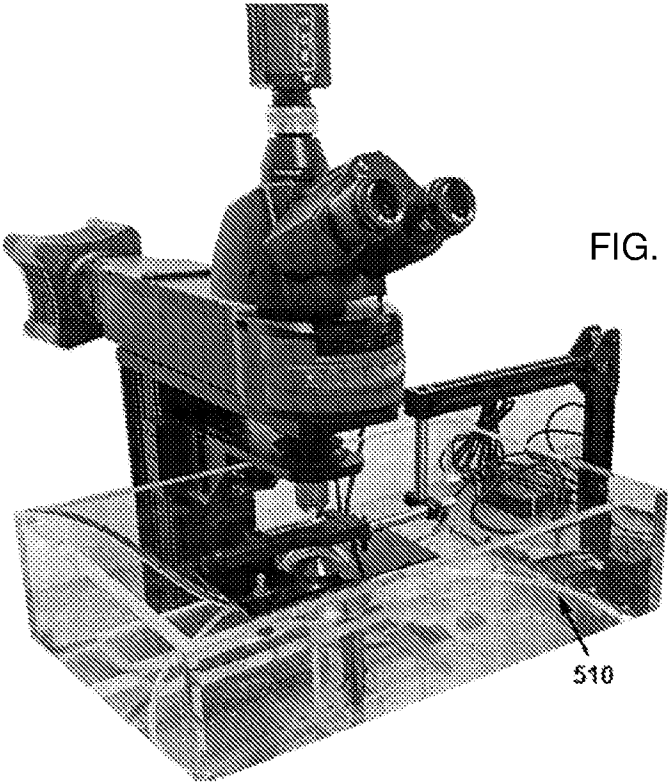


FIG. 5B



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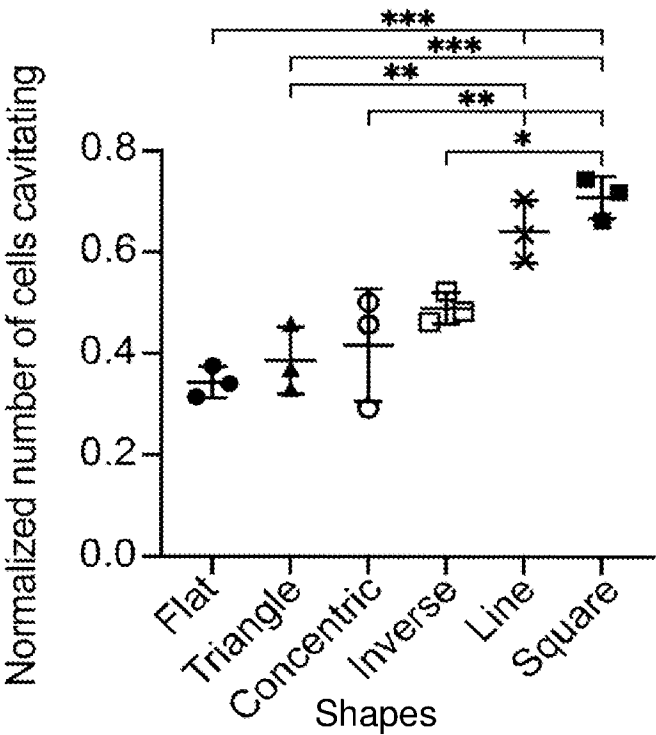


FIG. 6A

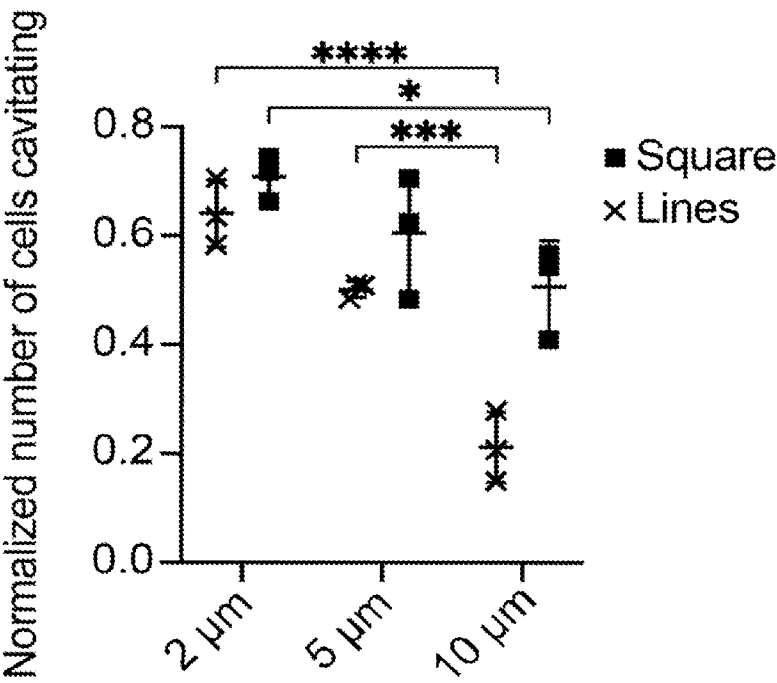


FIG. 6B

FIG. 7A

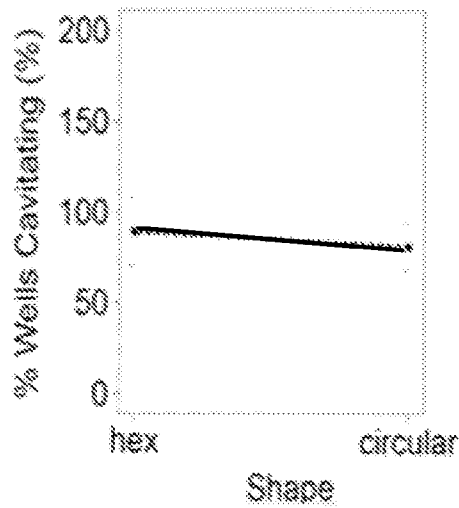


FIG. 7B

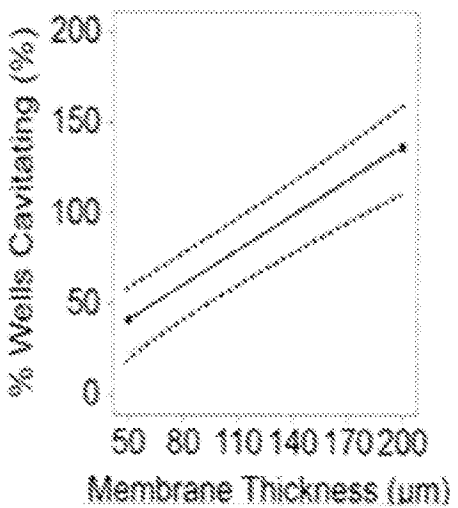


FIG. 7C

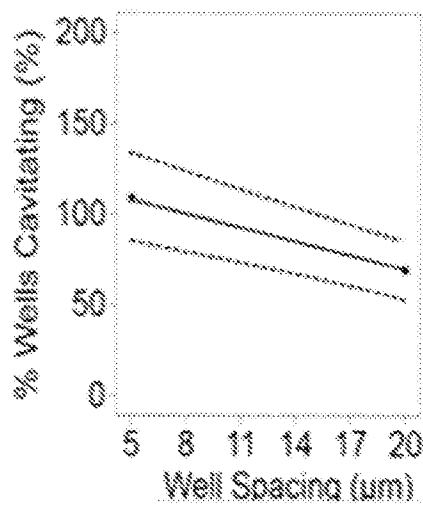
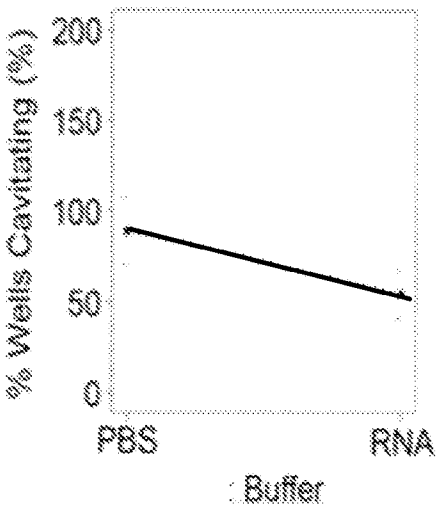


FIG. 7D



Factor Coding: Actual
Response: % Wells Cavitating (%)
----- 95% Confidence Interval Bands

FIG. 7E

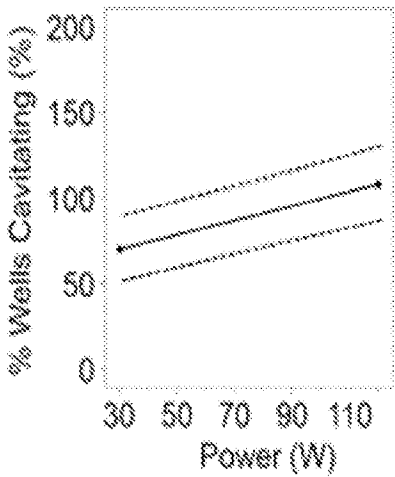
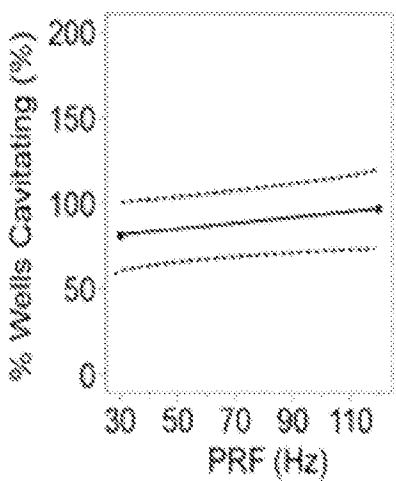


FIG. 7F



Factor Coding: Actual
Response: % Wells Cavitating (%)
—— 95% Confidence Interval Bands

FIG. 7G

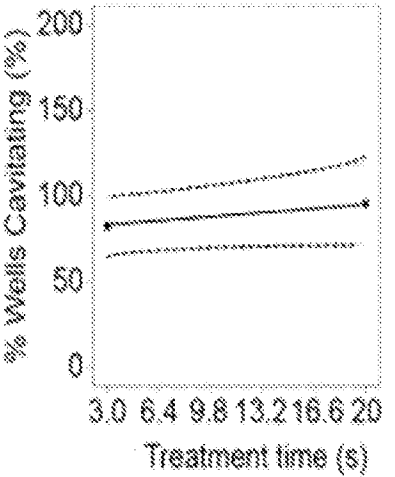
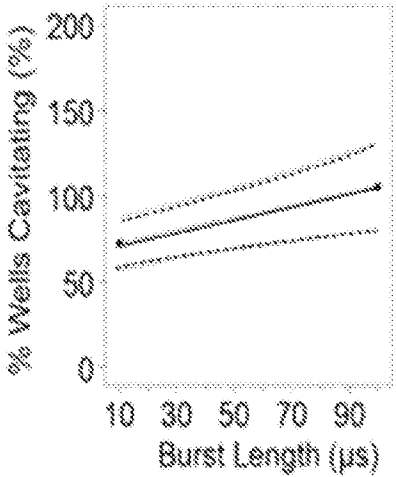
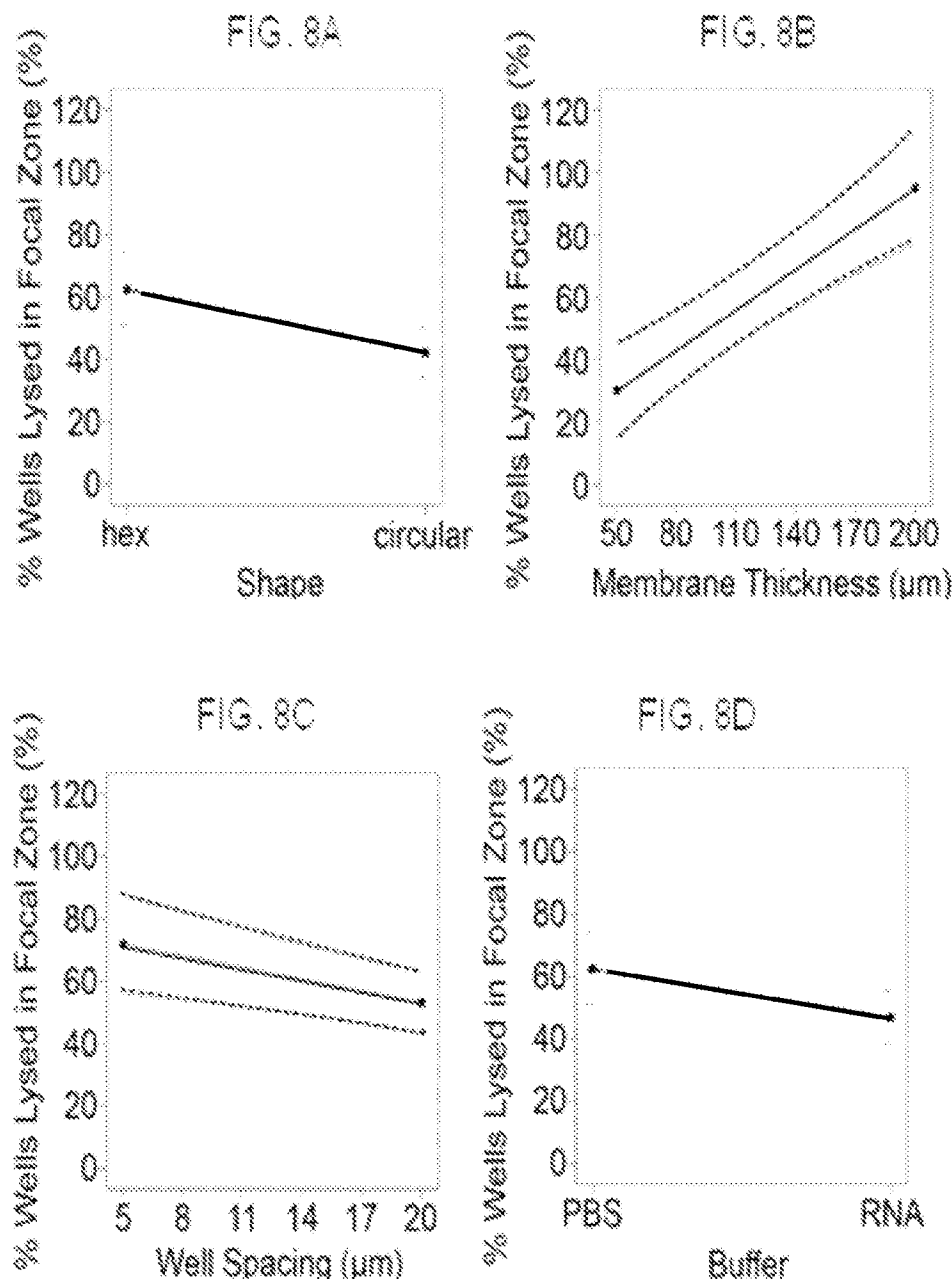
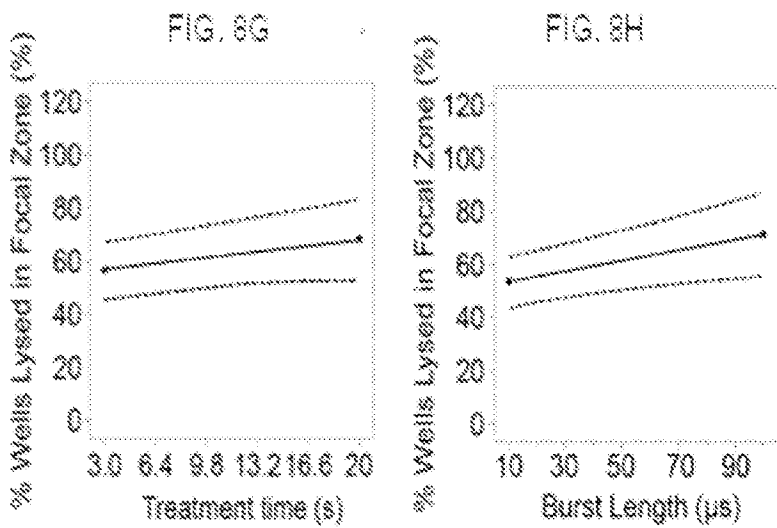
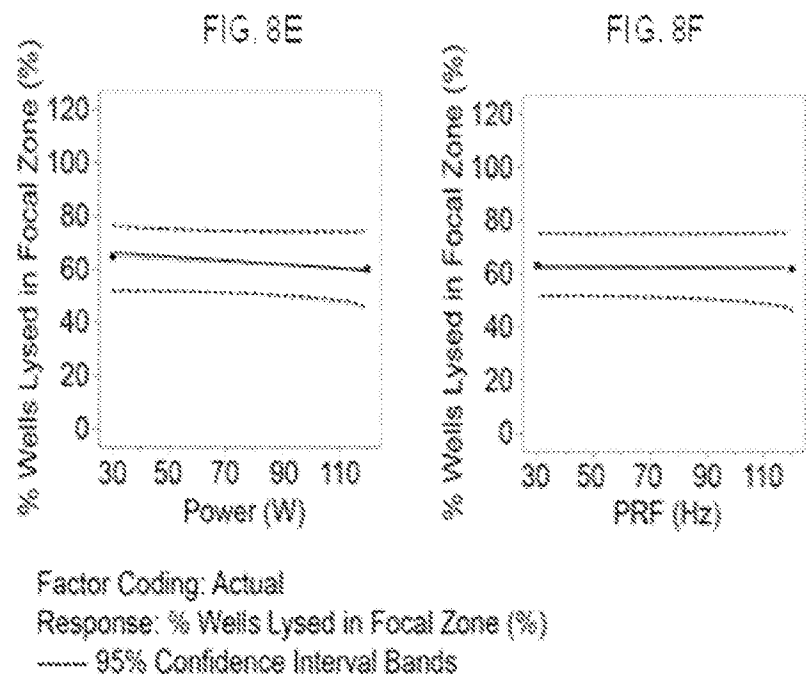


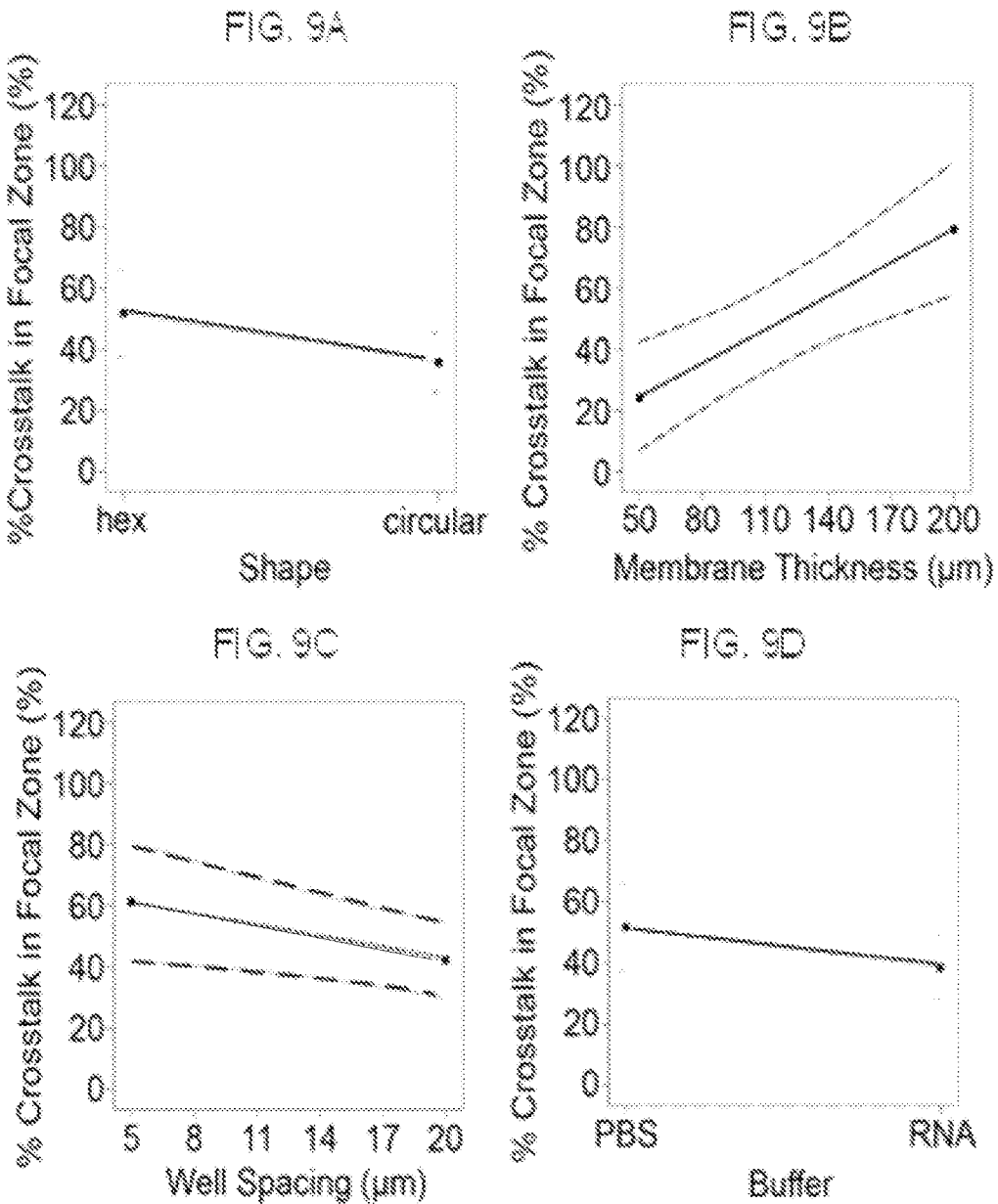
FIG. 7H



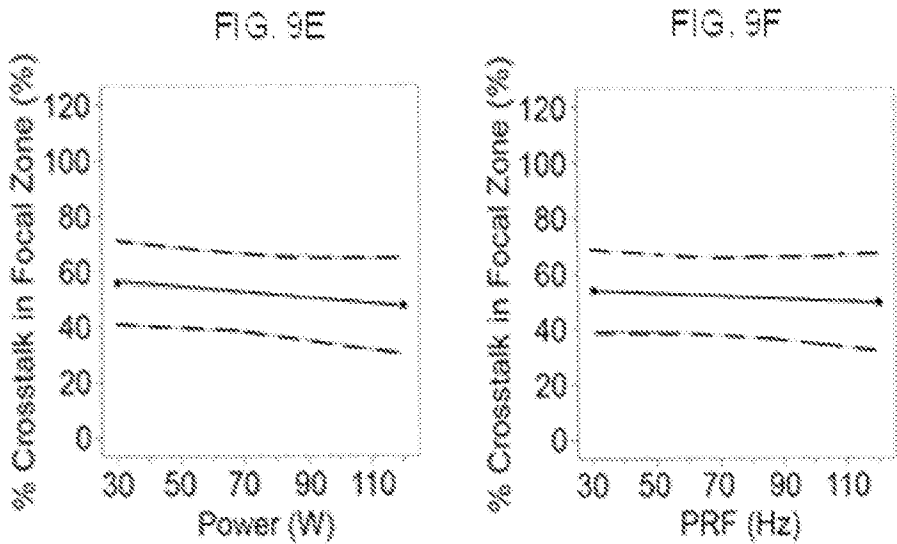


Factor Coding: Actual
Response: % Wells Lysed in Focal Zone (%)
—— 95% Confidence Interval Bands

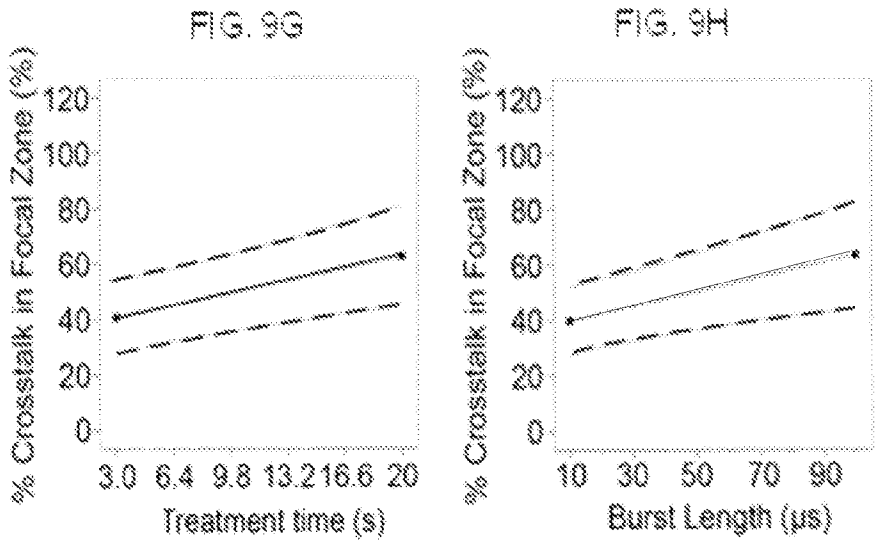




Factor Coding: Actual
Response: % Crosstalk in Focal Zone (%)
----- 95% Confidence Interval Bands



Factor Coding: Actual
Response: % Crosstalk in Focal Zone (%)
----- 95% Confidence Interval Bands



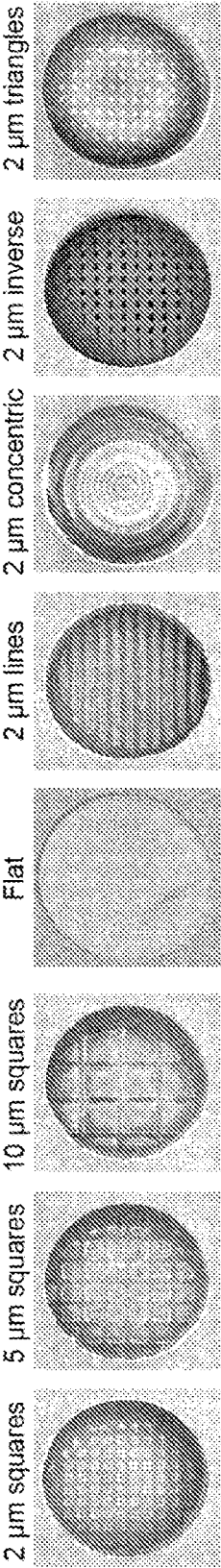


FIG. 10A FIG. 10B FIG. 10C FIG. 10D FIG. 10E FIG. 10F FIG. 10G FIG. 10H

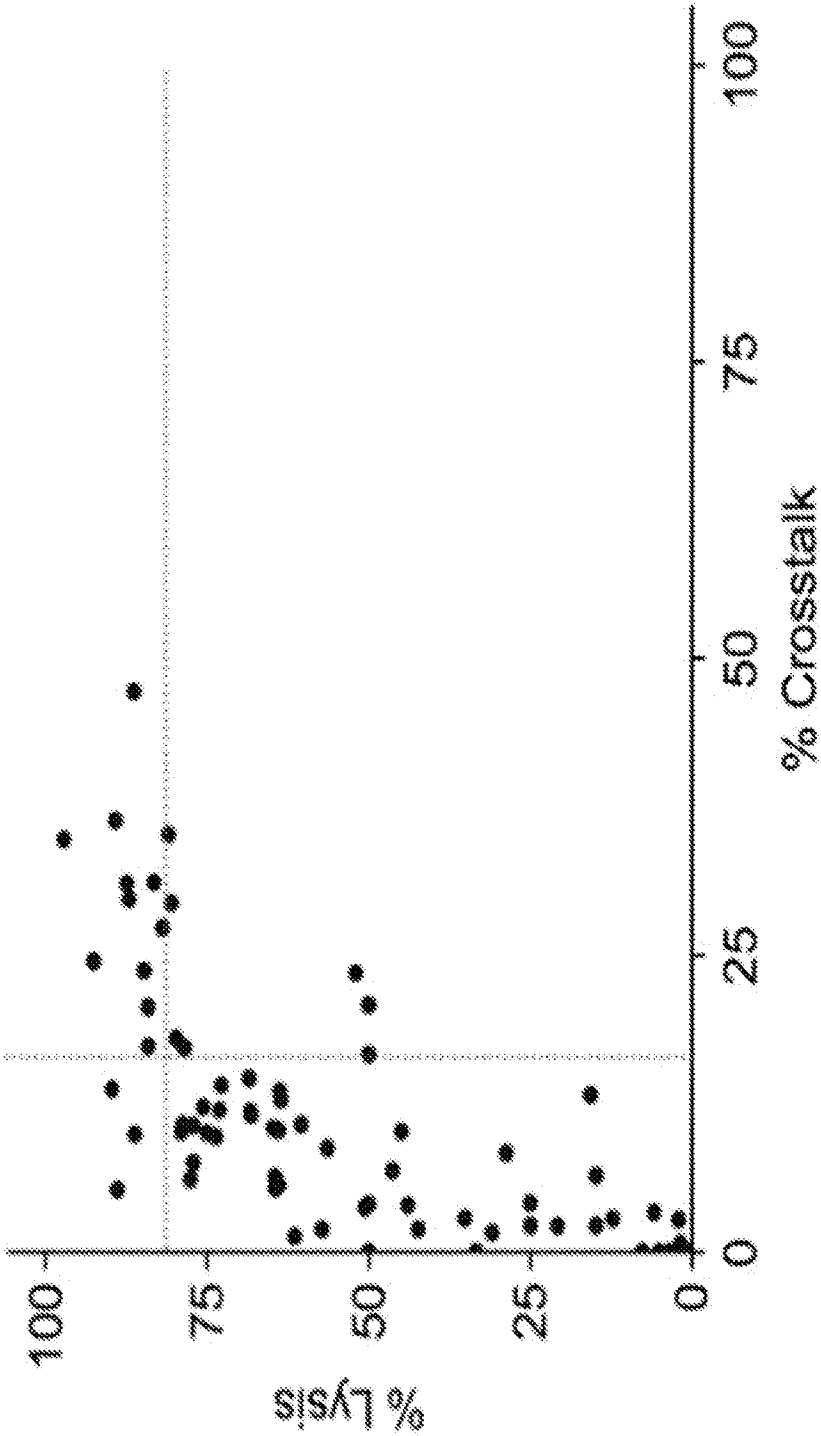


FIG. 11

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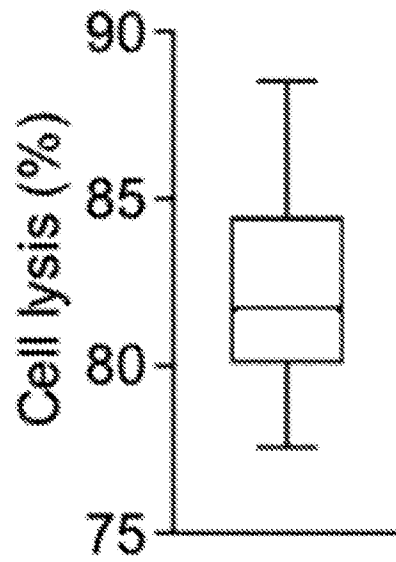


FIG. 12A

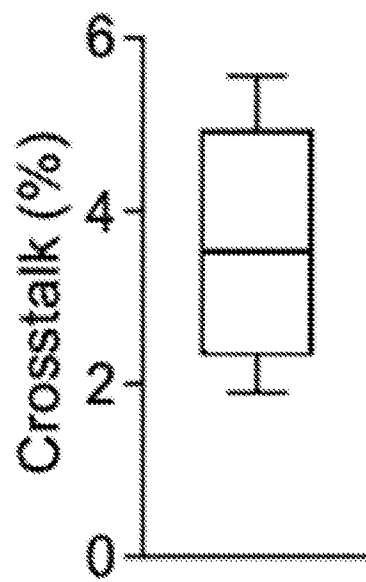


FIG. 12B

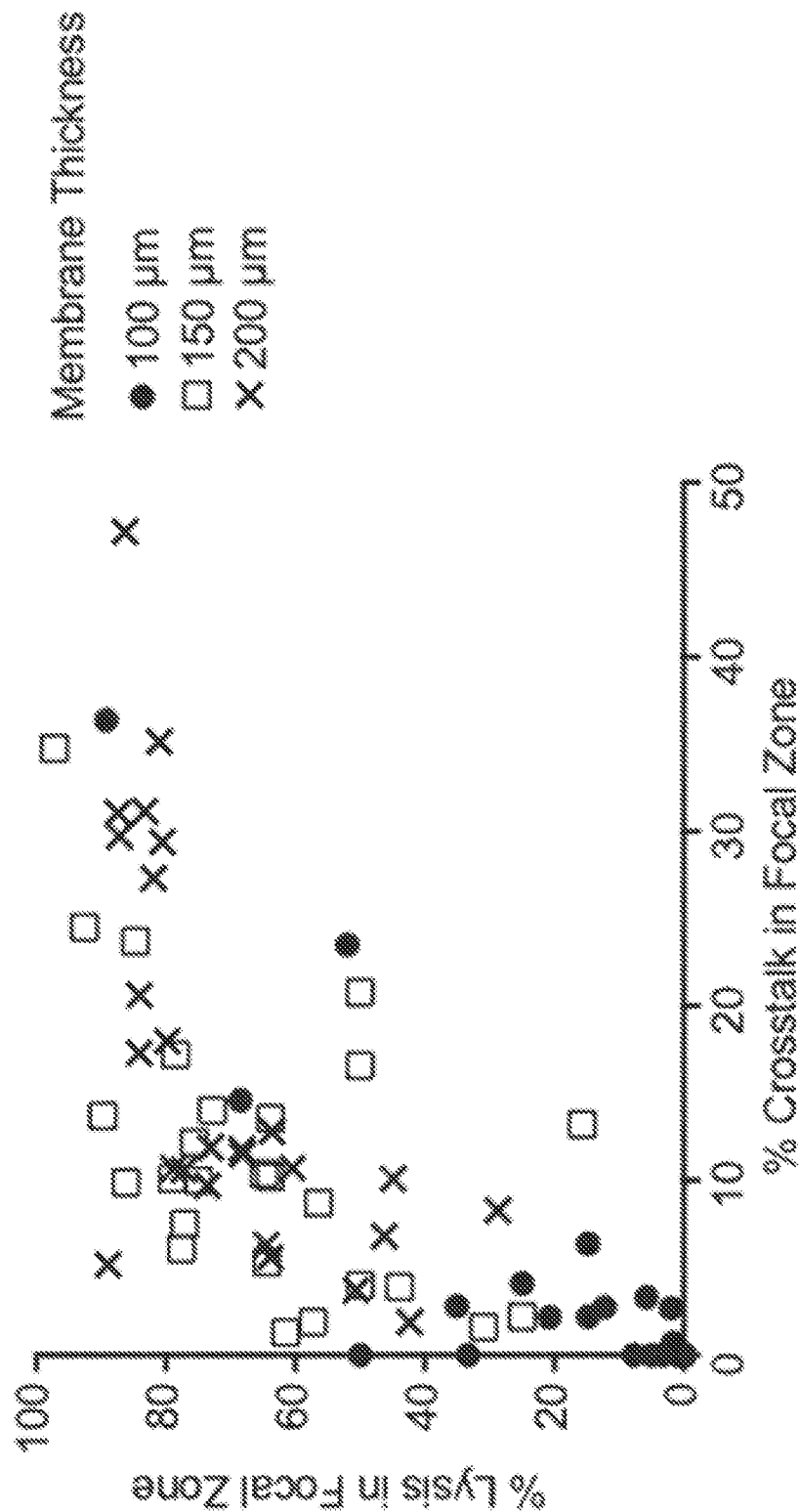


FIG. 13A

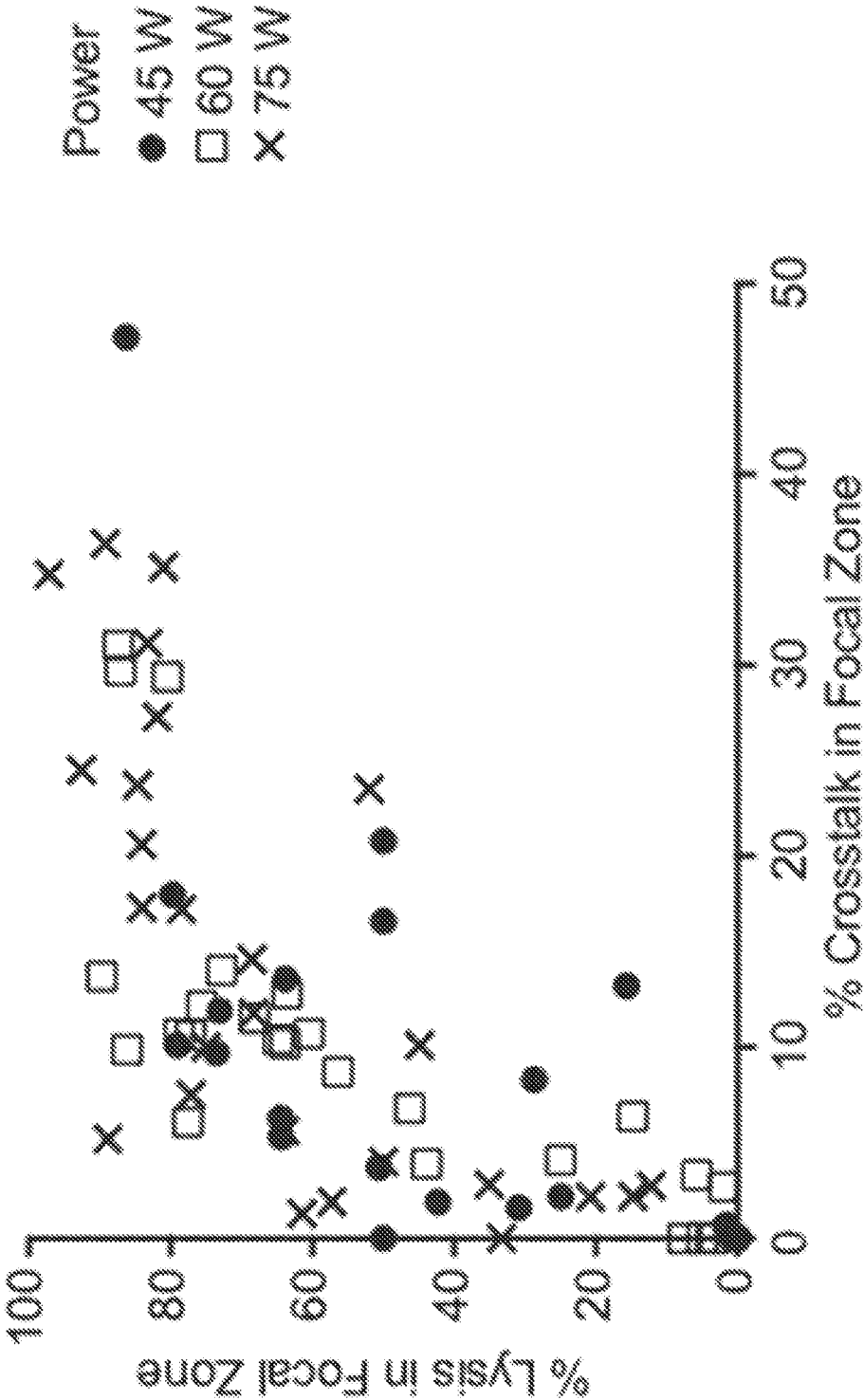
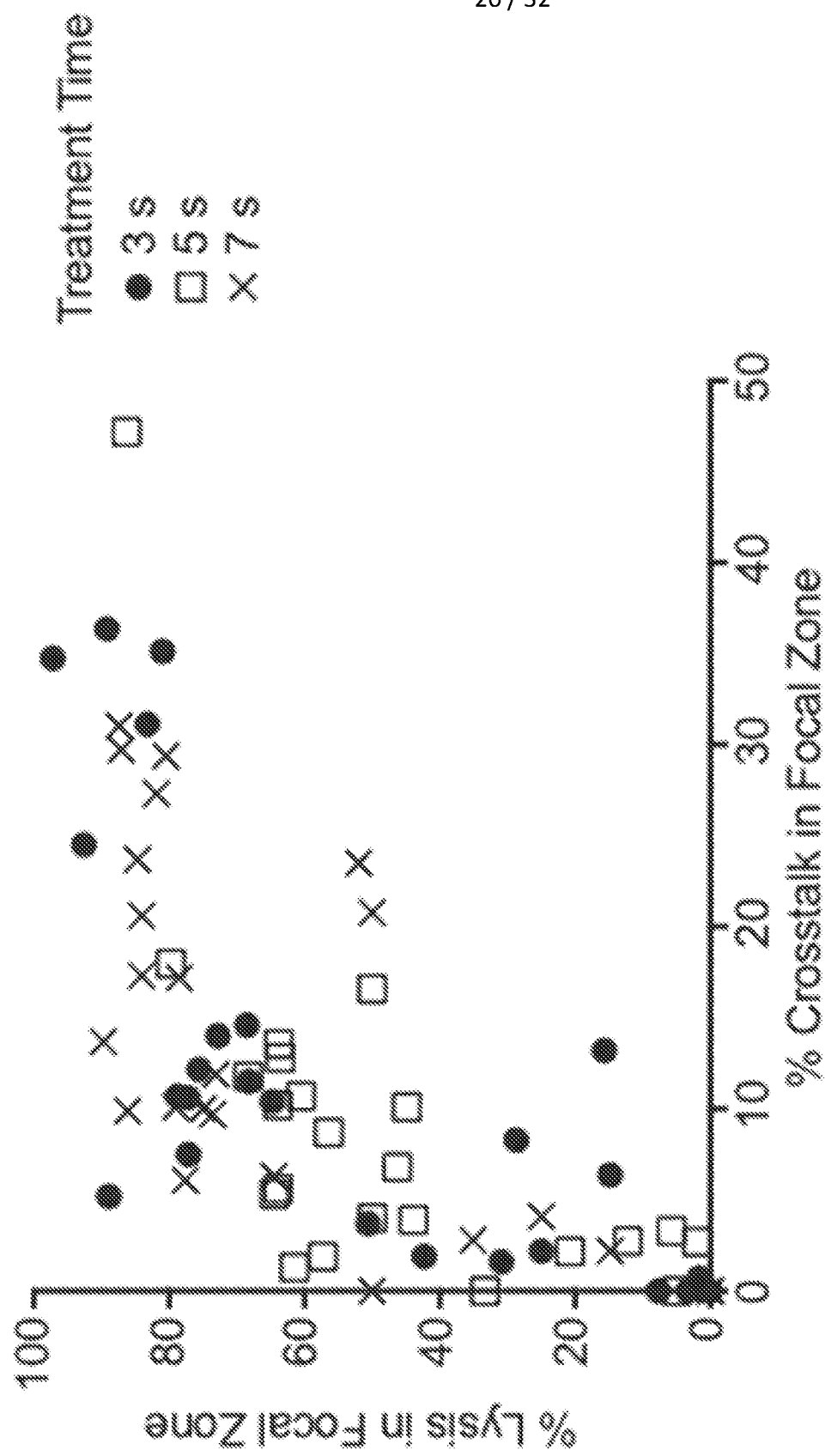


FIG. 13B



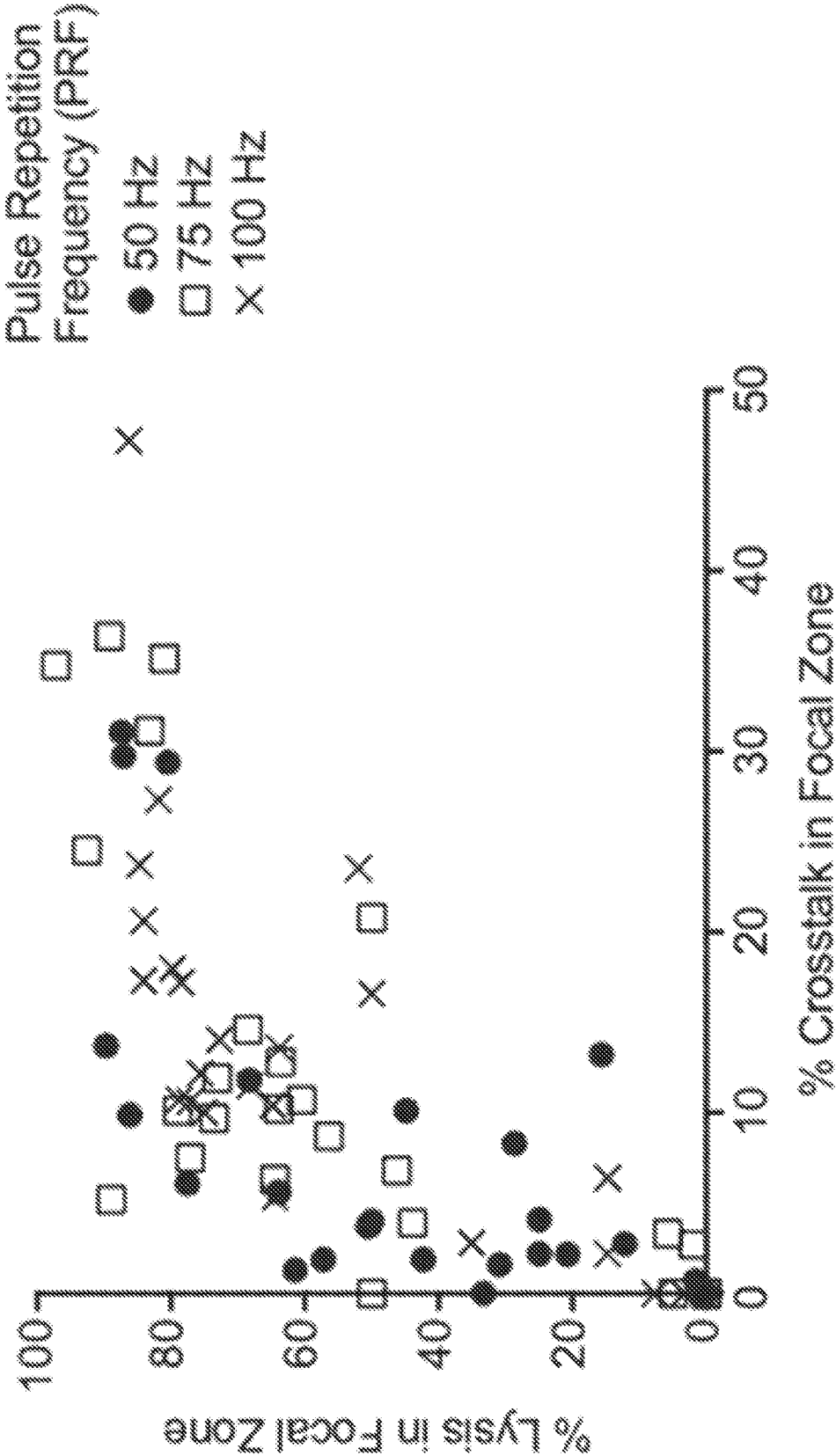


FIG. 14B

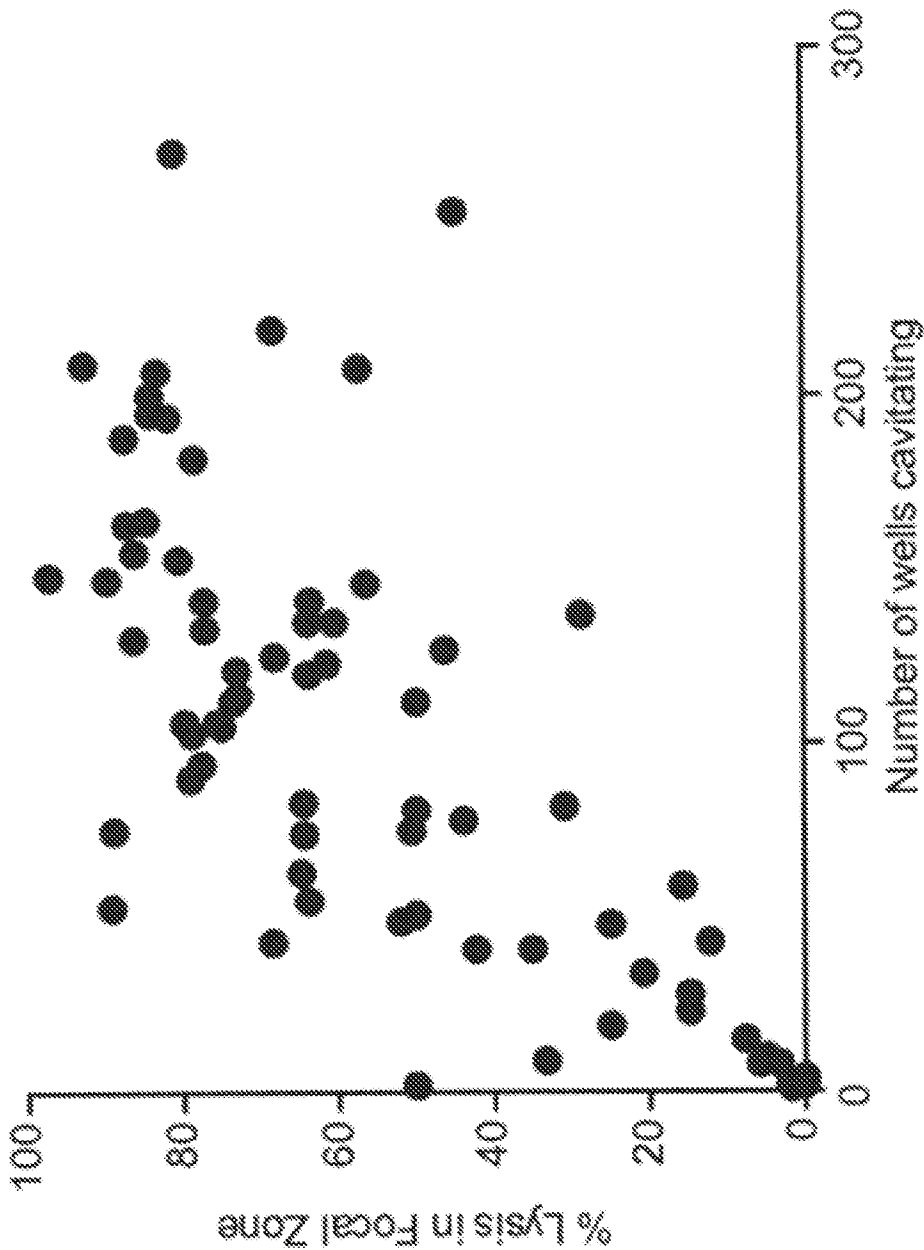


FIG. 15

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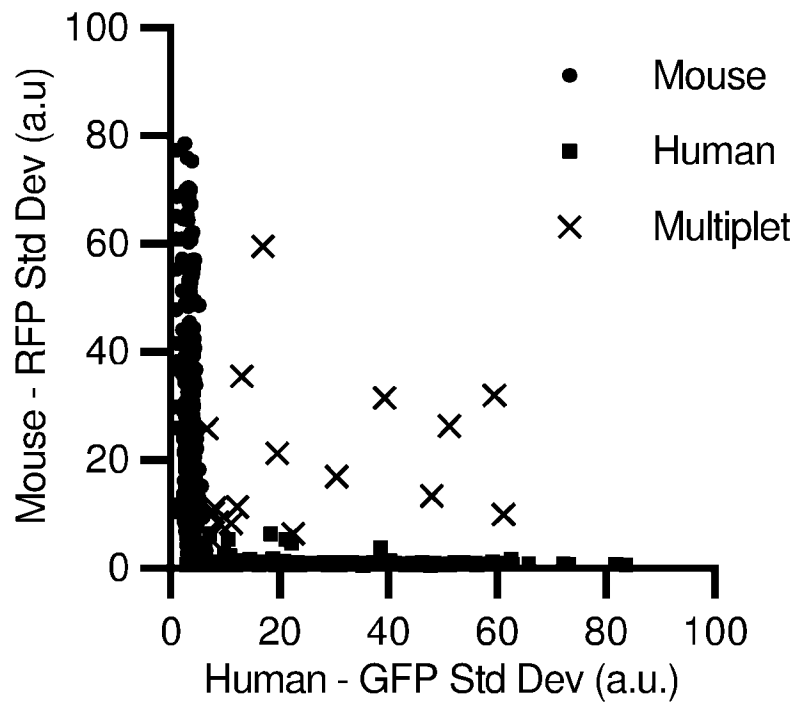


FIG 16A

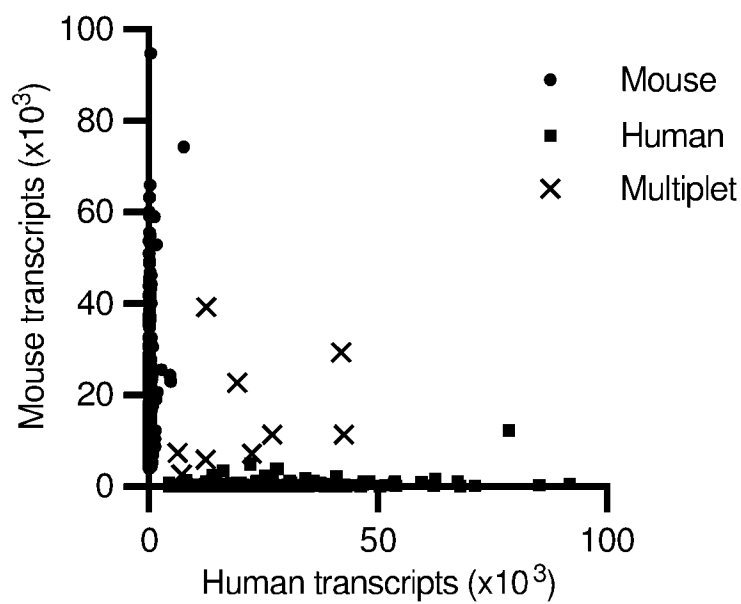


FIG 16B

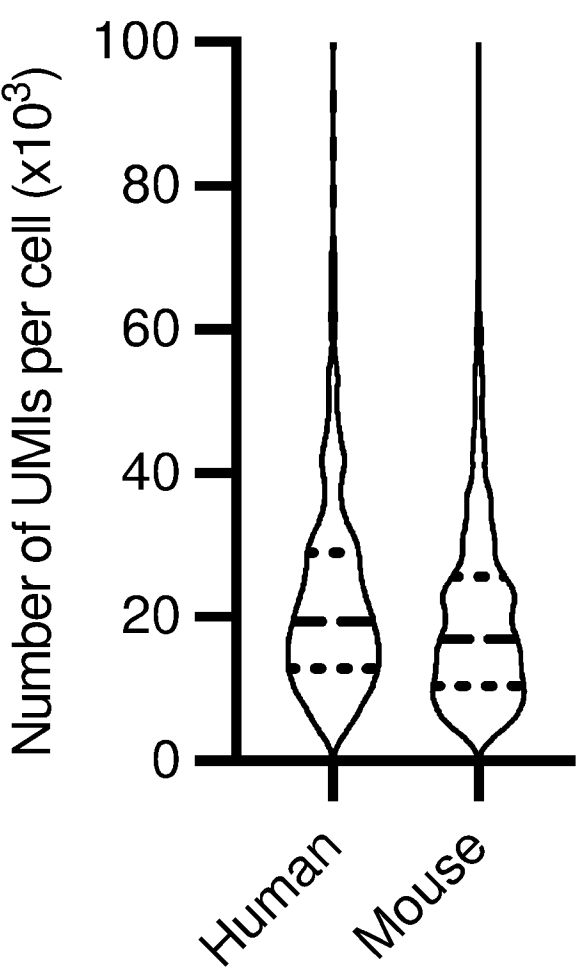


FIG. 16C

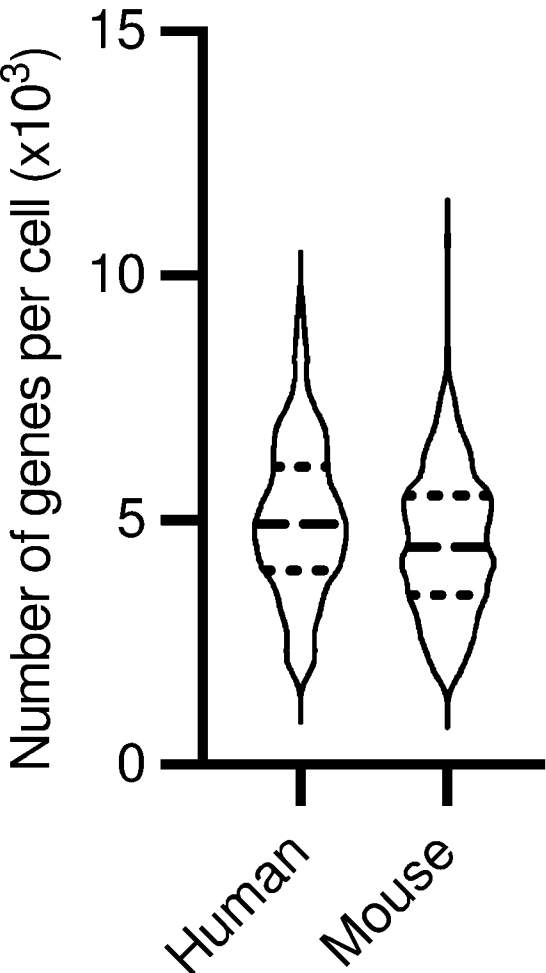


FIG. 16D

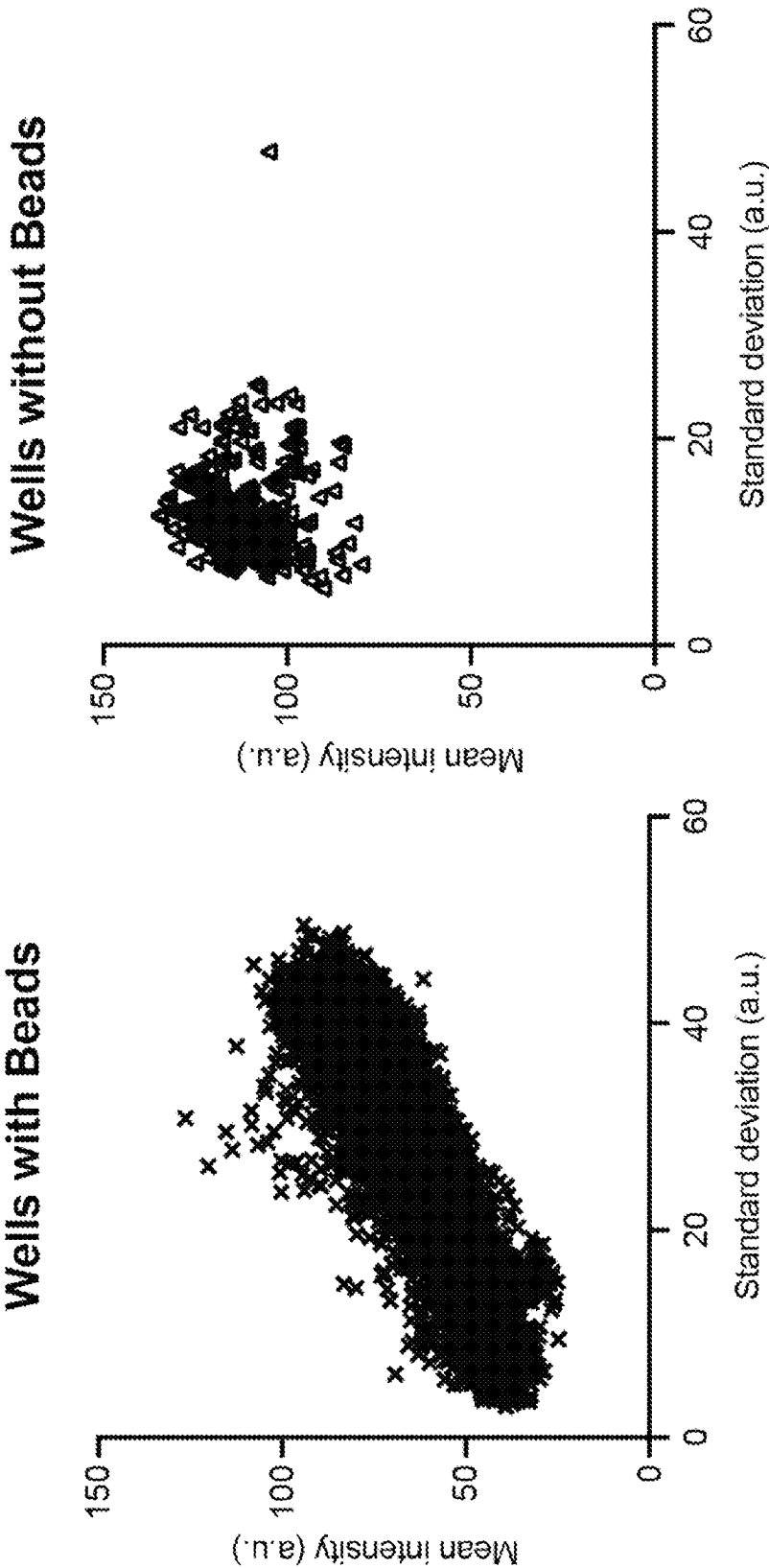


FIG. 17A

FIG. 17B

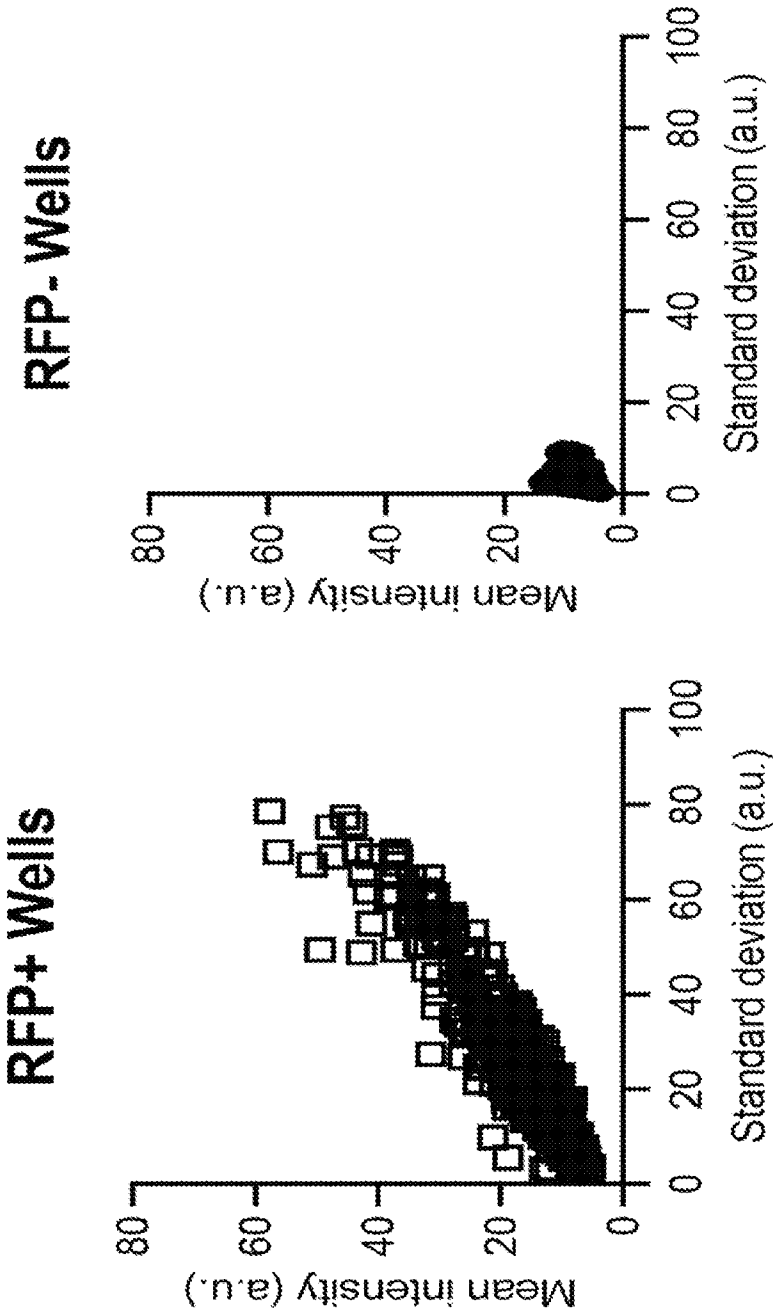


FIG. 18A

FIG. 18B

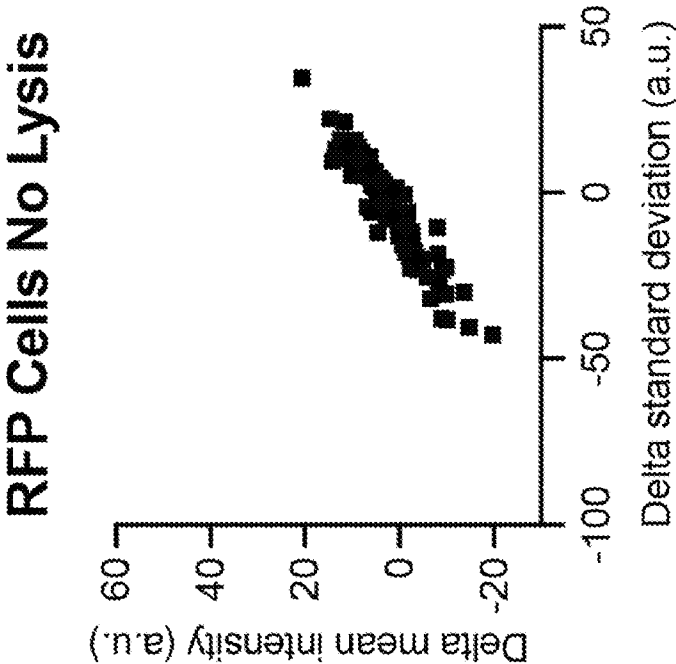


FIG. 19B

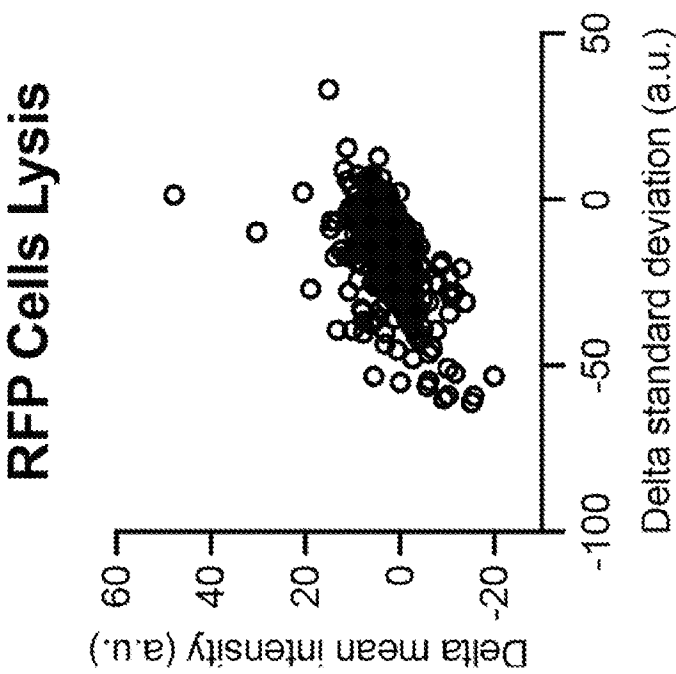


FIG. 19A

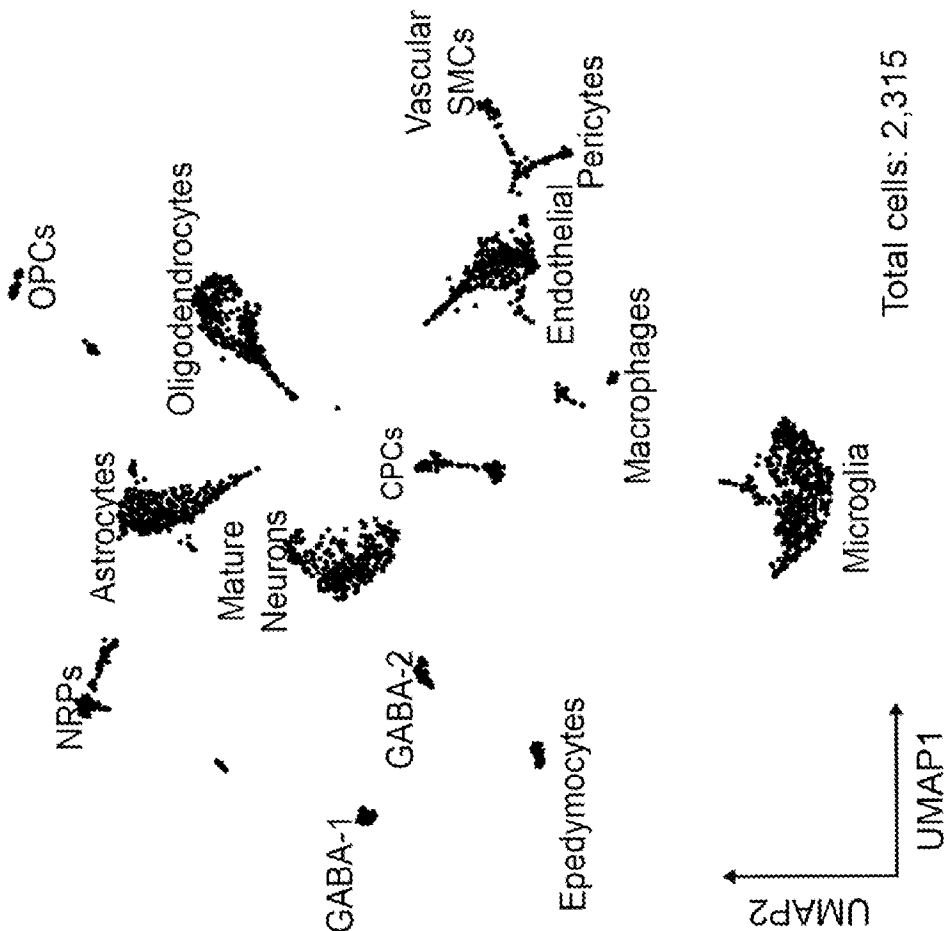


FIG. 20

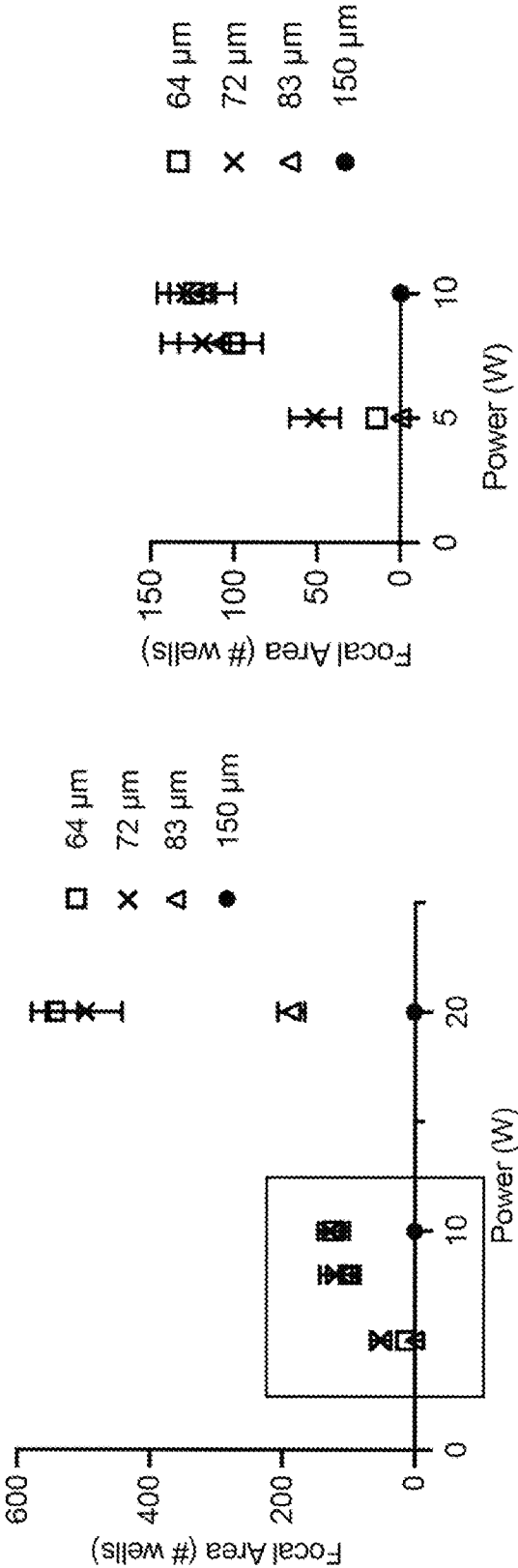


FIG. 21B

FIG. 21A

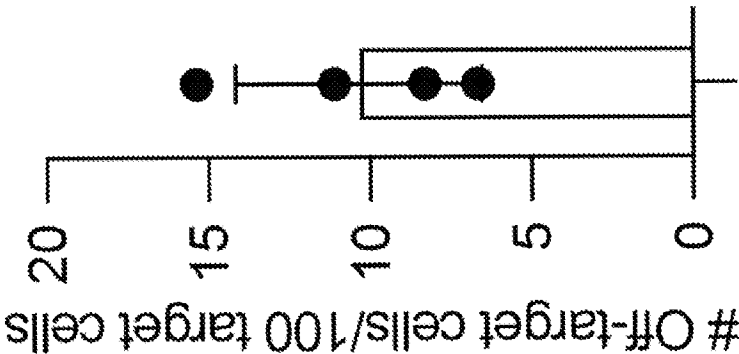


FIG. 22C

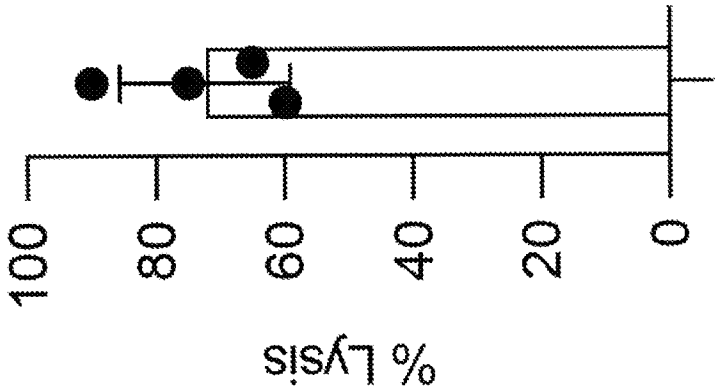


FIG. 22B

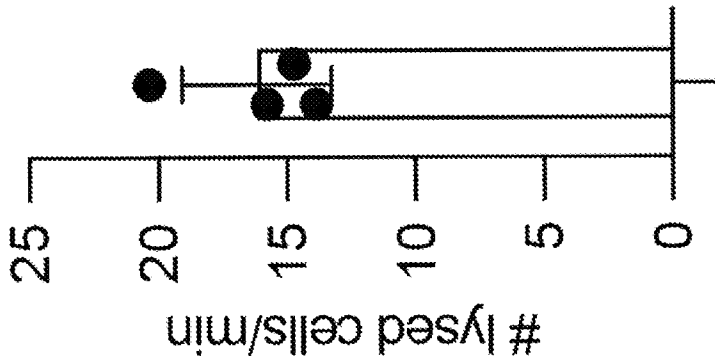


FIG. 22A

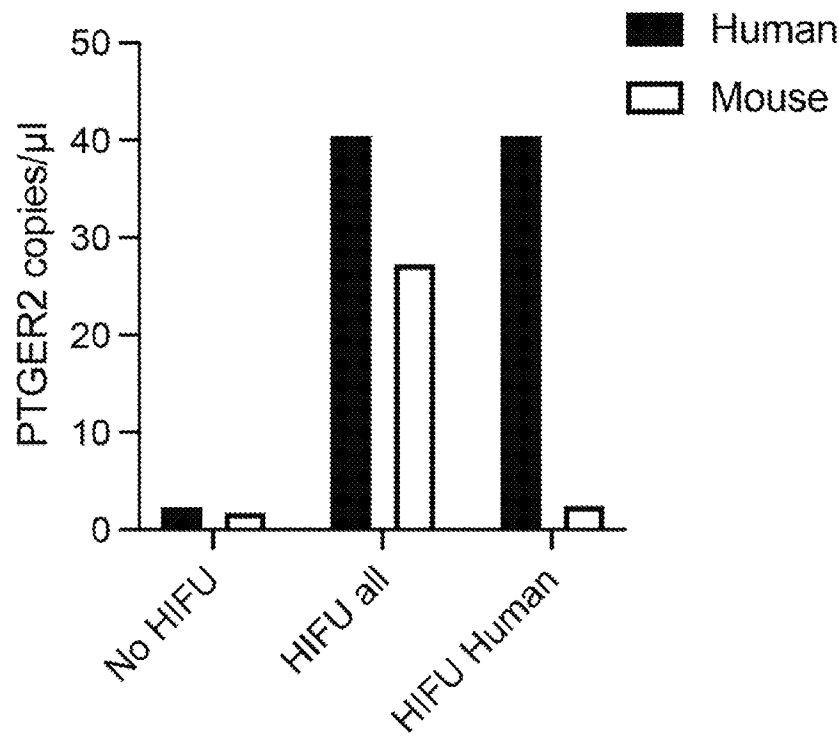


FIG. 23A

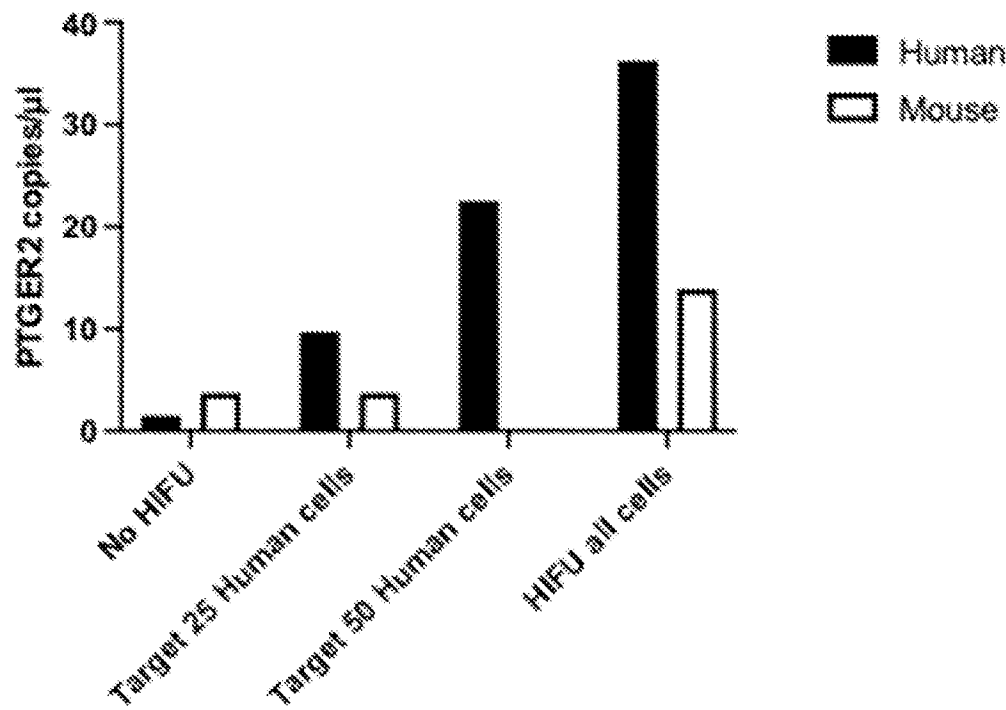


FIG. 23B

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FIG. 24A

Dry, 2 nN

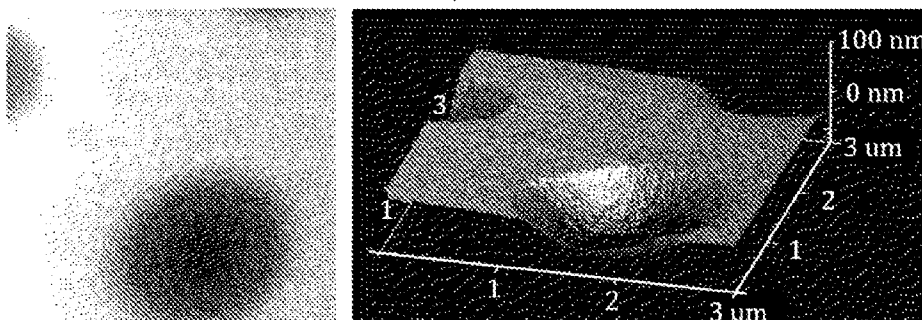


FIG. 24B

After solvent exchange, 2 nN

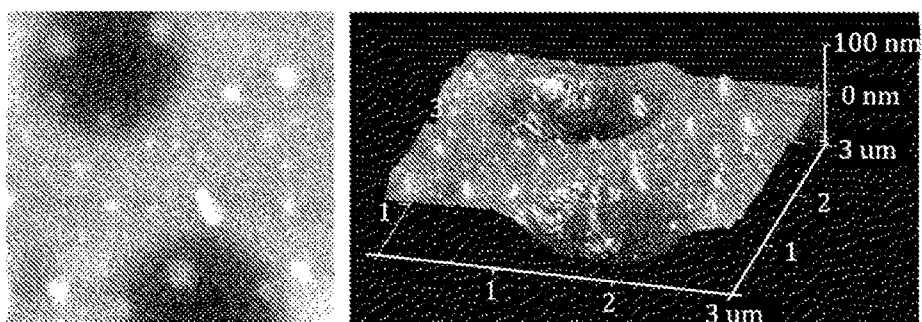


FIG. 24C

After solvent exchange, 10 nN

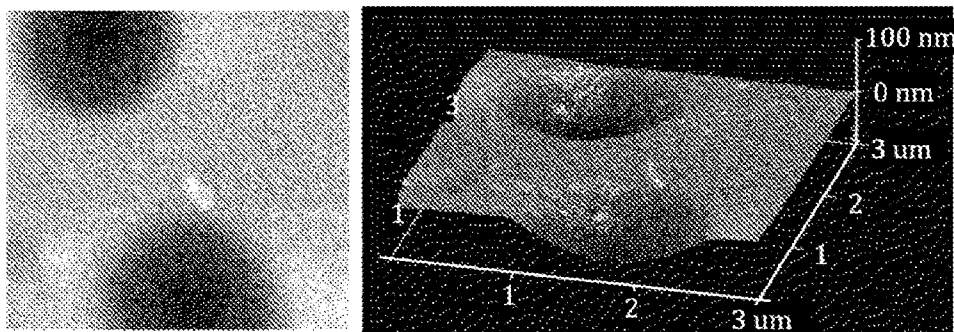
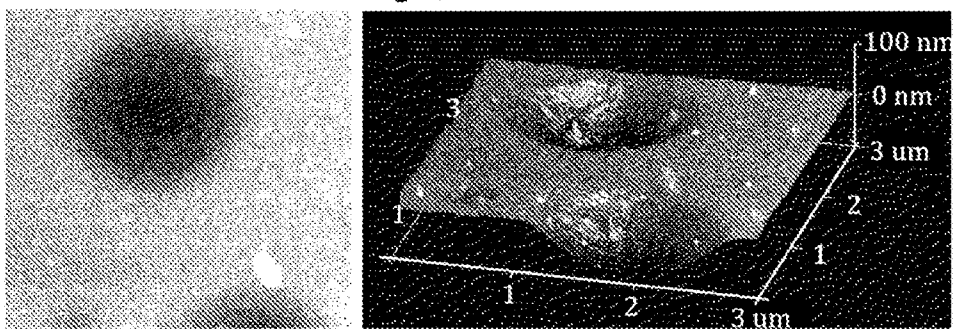


FIG. 24D

After solvent exchange, Return to 2 nN after 10 nN



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/055275

A. CLASSIFICATION OF SUBJECT MATTERIPC: **C12N 1/06** (2025.01); **C12M 1/42** (2025.01); **C12Q 1/6806** (2025.01); **C12N 13/00** (2025.01); **C12M 1/33** (2025.01); **C12M 3/08** (2025.01); **B01L 3/00** (2025.01)CPC: **C12N 1/06**; **C12N 1/066**; **C12M 1/42**; **C12M 45/02**; **C12Q 1/6806**; **C12N 13/00**; **C12M 1/33**; **C12M 3/08**; **B01L 3/50855**; **B01L 2300/0829**

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.
Y	US 2015/0005203 A1 (CRACKERBIO, INC.) 01 January 2015 (01.01.2015) entire document, especially para [0031], [0058], [0067]	1-24, 31
A	entire document, especially para [0031], [0058], [0067]	25-30
Y	US 2014/0228255 A1 (10X TECHNOLOGIES, INC.) 14 August 2014 (14.08.2014) entire document, especially para [0128-0129], [0158]	1-24, 31
A	entire document, especially para [0128-0129], [0158]	25-30
Y	WO 2022/144754 A1 (MOLECULAR DEVICES (AUSTRIA) GMBH) 07 July 2022 (07.07.2022) entire document, especially para [0003], [007]	6, 7, 14
Y	US 2018/0119139 A1 (ILLUMINA, INC.) 03 May 2018 (03.05.2018) entire document, especially para [0242]	8



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

“D” document cited by the applicant in the international application

“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

25 February 2025 (25.02.2025)

Date of mailing of the international search report

06 March 2025 (06.03.2025)

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Telephone No. PCT Help Desk: 571-272-4300

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:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: Claims 1-31 drawn to a method of incubating reaction mixtures.

Group II: Claims 32-55, drawn to a high intensity focused ultrasound (HIFU) platform.

The inventions listed in the above-mentioned groups do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I includes the special technical feature of incubating the reaction mixes, not included in the other group.

Group II includes the special technical feature of a microscope positioned above the nanowell array, not included in the other group.

Common Technical Features:

The only technical feature shared by Groups I and II that would otherwise unify the groups, is a polymer nanowell array comprising a plurality of nanowells, covering the wells with a membrane, and the nanowell array is acoustically coupled to a source of high intensity focused ultrasound. However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is disclosed by WO 2023/196253 A1 to ACOUSTIC BIO, INC. (hereinafter Acoustic Bio) (12 October 2023).

Acoustic Bio discloses a polymer nanowell array comprising a plurality of nanowells (para [0003]: a solution that presents precise alignment of an acoustic beam with respect to a feature (e.g., a nanowell) in a microfluidic device), covering the wells with a membrane (para [0004]: a microfluidic device comprising an array of wells, wherein a well of said array of wells has a volume of less than about one microliter, wherein said array of wells is in fluidic communication with a channel, and wherein said microfluidic device further comprises a cover, wherein said cover encloses said channel and said array of wells), and the nanowell array is acoustically coupled to an ultrasound transducer (para [0004]: an acoustic transducer configured to apply one or more acoustic beams to a feature of said microfluidic device...operate at a frequency of about 1 megahertz (MHz) to about 50 MHz...acoustic transducer is configured to apply a focused acoustic beam on a spot having a size of about 25 micrometers (pm) (the specified parameters provide a high intensity focused ultrasound)).

As the common technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Accordingly, the inventions listed as the above Groups lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature providing a contribution over prior art.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/055275

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

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.: **1-31**

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/US2024/055275

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2023/196253 A1 (ACOUSTIC BIO) 12 October 2023 (12.10.2023) entire document, especially para [0004]	1-31