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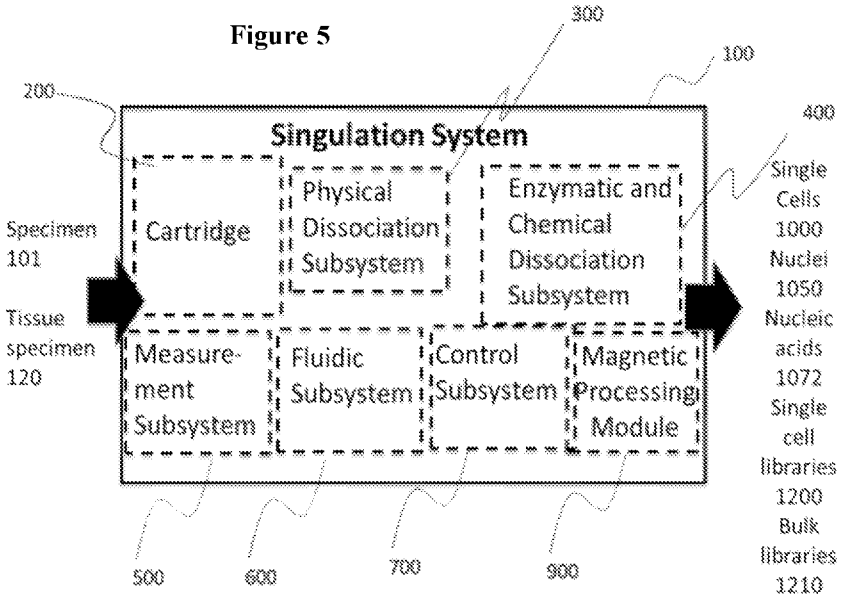
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(54) Title: METHOD AND APPARATUS FOR PROCESSING TISSUE SAMPLES

Figure 5



(57) Abstract: A system, methods, and apparatus are described to collect and prepare single cells, nuclei, subcellular components, and biomolecules from specimens including tissues. The system can perform enzymatic and/or physical disruption of the tissue to dissociate it into single-cells or nuclei in suspension or subcellular components including nucleic acids. In some embodiments, the titer of dissociated cells is monitored at intervals and the viability determined. In some embodiments, the processing is adjusted according to the measurements of the titer and viability. In some embodiments, the single-cells or nuclei in suspension are washed and resuspended in the buffer or media of choice. In some embodiments, the conditions are chosen to produce nuclei. In other embodiments, the single-cells or nuclei are purified by affinity paramagnetic bead processing. In some embodiments, matched bulk nucleic acid to the single-cells is produced. In other embodiments, single-cell libraries, or nuclei libraries, or matched bulk libraries, or bulk libraries are produced. The single cells or nuclei can then be further processed by FACS, DNA sequencing, mass spectrometry, fluorescence, or other methods. In other embodiments, the tissue processing is integrated with an analytical system to produce a sample-to-answer system such as a tissue-to-genomics system.

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[Continued on next page]

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[0001] Method and Apparatus for Processing Tissue Samples

(2) Cross Reference to related applications:

[0002] This application claims the benefit of the priority date of provisional patent application, 62/427,150, filed November 29, 2016, (Jovanovich et. al, "Method and Apparatus for Producing Single Cell Suspensions from Tissue and Other Samples), the contents of which are incorporated herein in their entirety and the benefit of the priority date of provisional patent application, 62/526,267, filed July 5, 2017, (Jovanovich et. al, "Method and Apparatus for Producing Single Cell Suspensions and Next Generation Sequencing Libraries for bulk DNA and Single-Cells from Tissue and Other Samples").

(3) Statement of federally sponsored research or development (if any):

[0003] None.

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[0004] None.

(5) Reference to a "Sequence Listing":

[0005] None.

(6) Background of the Invention

[0006] A) Field of Invention

[0007] This invention relates to the field of sample preparation from biological materials. More specifically, the invention relates to the processing of solid tissues into single cells, nuclei, biomolecules, and processed samples for bioanalysis.

[0008] B) Description of Related Art

[0009] Analysis of single cells and groups of cells is now beginning to provide information to dissect and understand how cells function individually and unprecedented insight into the range of individual responses aggregated in ensemble measurements. Single cell methods for electrophysiology, flow cytometry, imaging, mass spectrometry (Lanni, E.J., *et. al.* J Am Soc Mass Spectrom. 2014; 25(11):1897-907.), microarray (Wang L and KA Janes. Nat Protoc. 2013; 8(2):282-301.), and Next Generation Sequencing (NGS) (Saliba A.E., *et. al.* Nucleic Acids Res. 2014; 42(14):8845-60.) have been developed and are driving an increased understanding of fundamental cellular processes, functions, and interconnected networks. As the individual processes and functions are understood and differentiated from ensemble measurements, the individual information can in turn lead to discovery of how network processes among cells operate. The networks may be in tissues, organs, multicellular organisms, symbionts, biofilms, surfaces, environments, or anywhere cells interact.

[0010] Next Generation Sequencing (NGS) of single cells is rapidly changing the state of knowledge of cells and tissue, discovering new cell types, and increasing understanding of the diversity of how cells and tissue function. Single cell NGS RNA sequencing (Saliba A.E., *et. al.*, Nucleic Acids Res. 2014;42(14):8845-

60.) (Shapiro E. *et. al.*, Nat Rev Genet. 2013;14(9):618-30.) is unveiling the complexity of cellular expression, and the heterogenity from cell to cell, and from cell type to cell type (Buettner F. *et. al.*, Nat Biotechnol. 2015; 33(2):155-60.). *In situ* sequencing (Ke R *et. al.*, Nat Methods. 2013;10(9):857-60.), (Lee JH, *et. al.*, Nat Protoc. 2015;10(3):442-58.) (Lee JH, *et. al.*, Science. 2014, 21;343(6177):1360-3.) has shown the feasability of directly sequencing of fixed cells. However, for RNA, many fewer reads are generated with *in situ* sequencing, biasing against detection of low abundant transcripts. Photoactivatable tags have been used to capture mRNA from single cells (Lovatt, D., *et. al.*, Nat Methods. 2014;11(2):190-6.) from known location in tissue, albeit with low throughput capture and manual cell collection.

[0011] The NGS market has grown explosively over the last 10 years with costs reductions and throughput increases exceeding Moore's law. The applications have expanded from whole genome sequencing to RNA-Seq, ChIP-Seq, exome sequencing, to now single-cell sequencing, single nuclei sequencing, and many other exciting applications. The power and low cost of NGS is broadly changing life sciences and moving into translational medicine and the clinic as precision medicine begins. Until recent years essentially all of the NGS analysis was of 'bulk samples' where the nucleic acids of numerous cells had been pooled. There is a need for systems that integrate the sample preparation of single-cell suspensions, and single-cell libraries, and bulk libraries starting from original unprocessed specimens.

[0012] Single-cell sequencing is rapidly changing the state of knowledge of cells and tissue, discovering new cell types, and increasing the understanding of the diversity of how cells and tissue function. Single-cell RNA sequencing (Shapiro E. Biezuner T, Linnarsson S. Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet. 2013;14(9):618-30. PMID: 23897237) has highlighted the complexity of cellular expression, and the large heterogeneity from cell-to-cell, and from cell type-to-cell type (Buettner F. Natarajan KN, Casale FP, Proserpio V, Scialdone A, Theis FJ, Teichmann SA, Marioni JC, Stegle O. Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden subpopulations of cells. Nat Biotechnol. 2015; 33(2):155-60. PMID: 25599176). Single-cell sequencing (Wang., Y. and N.E. Navin. Advanced and Applications of single-cell sequencing technologies. Molecular Cell. 2015. 58:598-609. PMID 26000845.) is being applied to development, brain structure and function, tumor progression and resistance, immunogenetics, and more.

[0013] Single cell nucleic acid sequencing technology and methods using NGS and Next Next Generation Sequencing (NNGS), such as nanopores, are rapidly evolving. Common components are incorporation of a marker or barcode for each cell and molecule, reverse transcriptase for RNA sequencing, amplification, and pooling of sample for NGS and NNGS (collectively termed NGS) library preparation and analysis. Starting with isolated single cells in wells, barcodes for individual cells and molecules have been incorporated by reverse transcriptase template switching before pooling and polymerase chain reaction (PCR) amplification (Islam S. *et. al.* Genome Res. 2011; 21(7):1160-7.) (Ramsköld D. *et. al.* Nat Biotechnol. 2012; 30(8):777-82.) or on a barcoded poly-T primer with linear amplification (Hashimshony T. *et. al.* Cell Rep. 2012 Sep 27;2(3):666-73.) and unique molecular identifiers (Jaitin D.A. *et. al.* Science. 2014; 343(6172):776-9.).

[0014] Recent pioneering work has used the power of nanodroplets to perform highly parallel processing of mRNA from single cells with reverse transcription incorporating cell and molecular barcodes from freed primers (inDrop) (Klein A. M. *et. al.* Cell. 2015;161(5):1187-201.) or primers attached to paramagnetic beads (DropSeq) (Macosko E.Z. *et. al.* Cell. 2015;161(5):1202-14.) and using micronozzles

such as described by them or Geng T. et. al. Anal Chem. 2014;86(1):703-12 or others, and ; the lysis conditions and reverse transcriptase described by (Fekete R. A. and A. Nguyen. United States Patent 8,288,106. October 16, 2012) are incorporated by reference cited therein are incorporated by reference, including instrumentation, chemistry, workflows, reactions conditions, flowcell design, and other teachings. Both inDrop and DropSeq are scalable approaches have change the scale from 100s of cells previously analyzed to 1,000s and more.

[0015] Single-cell sequencing is now providing new information to biologists, genomic scientists, and clinical practitioners, and the single-cell market is growing explosively, perhaps the next great disruption in life sciences and medicine. Multiple companies are providing systems to take single-cell suspensions and create Single-cell RNA sequencing (scRNA-Seq) libraries that are analyzed by the robust NGS sequencing and analysis pipeline. No system integrates the upstream process to produce single-cell suspensions for NGS single-cell sequencing or has integrated from tissue to single-cell libraries.

[0016] The production of single-cells or nuclei or nucleic acids from solid and liquid tissue is usually performed manually with a number of devices used without process integration. A combination of gentle mechanical disruption with enzymatic dissociation has been shown to produce single-cells with the highest viability and least cellular stress response (Quatromoni JG, Singhal S, Bhojnagarwala P, Hancock WW, Albelda SM, Eruslanov E. An optimized disaggregation method for human lung tumors that preserves the phenotype and function of the immune cells. J Leukoc Biol. 2015 Jan;97(1):201-9. doi: 10.1189/jlb.5TA0814-373. Epub 2014 Oct 30.).

[0017] Many manual protocols for dissociating different tissues exist, for example, Jungblut M., Oeltze K., Zehnter I., Hasselmann D., Bosio A. (2009). Standardized Preparation of Single-Cell Suspensions from Mouse Lung Tissue using the gentleMACS Dissociator. JoVE. 29, doi: 10.3791/1266; Stagg AJ, Burke F, Hill S, Knight SC. Isolation of Mouse Spleen Dendritic Cells. Protocols, Methods in Molecular Medicine. 2001: 64: 9-22. Doi: 10.1385/1592591507.; Lancelin, W., Guerrero-Plata, A. Isolation of Mouse Lung Dendritic Cells. J. Vis. Exp. (57), e3563, 2011. DOI : 10.3791/3563; Smedsrod B, Pertoft H. Preparation of pure hepatocytes and reticuloendothelial cells in high yield from a single rat liver by means of Percoll centrifugation and selective adherence. J Leukocyte Biol. 1985: 38: 213-30.; Meyer J, Gonelle-Gispert C, Morel P, Bühler L Methods for Isolation and Purification of Murine Liver Sinusoidal Endothelial Cells: A Systematic Review. PLoS ONE 11(3) 2016: e0151945. doi:10.1371/journal.pone.0151945.; Kondo S. Scheef EA, Sheibani N, Sorenson CM. "PECAM-1 isoform-specific regulation of kidney endothelial cell migration and capillary morphogenesis", Am J Physiol Cell Physiol 292: C2070-C2083, (2007); doi: 10.1152/ajpcell.00489.2006.; Ehler, E., Moore-Morris, T., Lange, S. Isolation and Culture of Neonatal Mouse Cardiomyocytes. J. Vis. Exp. (79), e50154, doi:10.3791/50154 (2013).; Volovitz I Shapira N, Ezer H, Gafni A, Lustgarten M, Alter T, Ben-Horin I, Barzilai O, Shahar T, Kanner A, Fried I, Veshchev I, Grossman R, Ram, Z. A non-aggressive, highly efficient, enzymatic method for dissociation of human brain-tumors and brain-tissues to viable single cells. BMC Neuroscience (2016) 17:30 doi: 10.1186/s12868-016-0262-y; F. E Dwulet and M.E. Smith, "Enzyme composition for tissue dissociation," United States Patent 5,952,215, September 14, 1999.

[0018] For example, solid tissue of interest is usually dissected and then minced into 1-5 mm pieces by hand or a blender type of disruptor is used. Enzymes or a mixture of enzymes, such as collagenases, hydrauronadase, papain, proteases, DNase, *etc.*, are added and the specimen incubated, typically with

shaking or rotation to aid dissociation to prepare single cells or nuclei from tissue. In many procedures, the specimen is titurated multiple times or mechanically disrupted. The mechanical disruption may be through orifices, grinding, homogenization, forcing tissue through screens or filters, sonication, blending, bead-beating, rotors with features that dissociate tissue, and other methods to physically disrupt tissue to help produce single cells.

[0019] Following dissociation, in some embodiments the dissociated sample is passed through a filter, such as a 70 μm filter, to retain clumps of cells or debris. The filtrate which contains single cells or nuclei may be further processed to change the media or buffer; add, remove, or deactivate enzymes; concentrate cells or biomolecules, lyse red blood cells, or capture specific cell types. The processing typically involves multiple steps of centrifugation and resuspension, density gradients, or magnetic bead capture of specific cell types using antibodies or other affinity capture ligands, or fluorescent cell-activated sorting (FACS). The titer and viability of the single-cell suspension is usually determined using optical imaging with a microscope and haemocytometer, or an automated instrument. In many cases, the viability is determined using Trypan blue or fluorescent dyes. Quality control can include characterization of the nucleic acids by gel electrophoresis on an instrument such as a BioAnalyzer, or the determination of the expression of certain genes using reverse transcripatase and quantitative polymerase chain reaction (RT-qPCR), or other relevant methods.

[0020] The rapid production of nuclei can give a snapshot of gene expression (Habib N, Li Y, Heidenreich M, Swiech L, Avraham-Davidi I, Trombetta JJ, Hession C, Zhang F, Regev A. Div-Seq: Single-nucleus RNA-Seq reveals dynamics of rare adult newborn neurons. Science. 2016 Aug 26;353(6302):925-8. doi: 10.1126/science.aad7038. Epub 2016 Jul 28.; Grindberg RV, Yee-Greenbaum JL, McConnell MJ, Novotny M, O'Shaughnessy AL, Lambert GM, Araúzo-Bravo MJ, Lee J, Fishman M, Robbins GE, Lin X, Venepally P, Badger JH, Galbraith DW, Gage FH, Lasken RS. RNA-sequencing from single nuclei. Proc Natl Acad Sci U S A. 2013 Dec 3;110(49):19802-7. doi: 10.1073/pnas.1319700110. Epub 2013 Nov 18.).

[0021] The production of nuclei from tissue can be performed using a Dounce homogenizer in the presence of a buffer with a detergent that lyses cells but not nuclei. Nuclei can also be prepared starting from single cell suspensions (CG000124_SamplePrepDemonstratedProtocol_-_Nuclei_RevB, 10x Genomics, https://assets.contentful.com/an68im79xiti/6FhJX6yndYy0OwskGmMc8I/48c341c178feafa3ce21f5345ed3367b/CG000124_SamplePrepDemonstratedProtocol_-_Nuclei_RevB.pdf) by addition of a lysis buffer such as 10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl2 and 0.005% Nonidet P40 in nuclease-free water and incubation for 5 min on ice before centifugation to pellet the nuclei followed by resuspension in a resuspension buffer such as 1X PBS with 1.0% BSA and 0.2U/μl RNase Inhibitor. The nuclei may be repeatedly pelleted and resuspended to purify them or density gradients or other purification methods used. The titer and viability of the nuclei suspension is usually determined using optical imaging with a microscope and haemocytometer, or an automated instrument with viability determined using Trypan blue or fluorescent dyes.

[0022] The multi-process workflow to produce and characterize single-cells and nuclei from tissue is a usually performed manually using several devices without process integration, limiting the scalablity of single cell sequencing and the integration with downstream processes to create a sample-to-answer system. It is laborious and requires skilled technicians or scientists, and results in variability in the quality of the single-cells, and, therefore, in the downstream libraries, analysis, and data. The multiple steps and skill required

can lead to differing qualities of single cells or nuclei produced even from the same specimen. Today, the production of high quality single-cells can take months of optimization.

[0023] Standarization is necessary before routine single-cell preparation can be performed, particularly in clinical settings. In addition, the length of the process and the process of dissociation can lead to the tissue and cells changing physiology such as altering their expression of RNA and proteins in response to the stresses of the procedure, accentuated by potentially long processing times. A crucial recent insight is that cell processing methods can alter gene expression by placing cells under stress. For example, the use of protease to dissociate cells from tissue, confounding analysis of the true transcriptome (Lacar B, Linker SB, Jaeger BN, Krishnaswami S, Barron J, Kelder M, Parylak S, Paquola A, Venepally P, Novotny M, O'Connor C, Fitzpatrick C, Erwin J, Hsu JY, Husband D, McConnell MJ, Lasken R, Gage FH. Nuclear RNA-seq of single neurons reveals molecular signatures of activation. Nat Commun. 2016 Apr 19;7:11022. doi: 10.1038/ncomms11022. PMID: 27090946.).

[0024] Robust, automated sample preparation is required to simplify workflows before full integration can be achieved with downstream NGS analysis to produce true sample-to-answer systems in the future. Robust processes are required that will input a wide range of tissues from a wide range of organisms and tissues and produce high-quality single-cell or nuclei suspensions without intervention, at acceptable viability for suspensions, with minimal changes to gene expression patterns.

[0025] To achieve a standardized process will require a system that automates the sample preparation of cells or nuclei from tissue with a single-use disposable cartridge. In some cases, microvalves can be used in cartridges. Microvalves are comprised of mechanical (thermopneumatic, pneumatic, and shape memory alloy), non-mechanical (hydrogel, sol–gel, paraffin, and ice), and external (modular built-in, pneumatic, and non-pneumatic) microvalves (as described in: C. Zhang, D. Xing, and Y. Li., Micropumps, microvalves, and micromixers within PCR microfluidic chips: Advances and trends. Biotechnology Advances. Volume 25, Issue 5, September–October 2007, Pages 483–514; Díaz-González M., C. Fernández-Sánchez, and A. Baldi A. Multiple actuation microvalves in wax microfluidics. Lab Chip. 2016 Oct 5;16(20):3969-3976.; Kim J., Stockton AM, Jensen EC, Mathies RA.Pneumatically actuated microvalve circuits for programmable automation of chemical and biochemical analysis. Lab Chip. 2016 Mar 7;16(5):812-9. doi: 10.1039/c5lc01397f; Samad MF, Kouzani AZ. Design and analysis of a low actuation voltage electrowetting-on-dielectric microvalve for drug delivery applications. Conf Proc IEEE Eng Med Biol Soc. 2014;2014:4423-6. doi: 10.1109/EMBC.2014.6944605.; Samad MF, Kouzani AZ. Design and analysis of a low actuation voltage electrowetting-on-dielectric microvalve for drug delivery applications. Conf Proc IEEE Eng Med Biol Soc. 2014;2014:4423-6. doi: 10.1109/EMBC.2014.6944605.; Lee E, Lee H, Yoo SI, Yoon J. Photothermally triggered fast responding hydrogels incorporating a hydrophobic moiety for light-controlled microvalves. ACS Appl Mater Interfaces. 2014 Oct 8;6(19):16949-55. doi: 10.1021/am504502y. Epub 2014 Sep 25.; Liu X, Li S. An electromagnetic microvalve for pneumatic control of microfluidic systems. J Lab Autom. 2014 Oct;19(5):444-53. doi: 10.1177/2211068214531760. Epub 2014 Apr 17; Desai AV, Tice JD, Apblett CA, Kenis PJ.Design considerations for electrostatic microvalves with applications in poly(dimethylsiloxane)-based microfluidics. Lab Chip. 2012 Mar 21;12(6):1078-88. doi: 10.1039/c2lc21133e. Epub 2012 Feb 3.; Kim J, Kang M, Jensen EC, Mathies RA Lifting gate polydimethylsiloxane microvalves and pumps for microfluidic control. Anal Chem. 2012 Feb 21;84(4):2067-71. doi: 10.1021/ac202934x. Epub 2012 Feb 1; Lai

H, Folch A.Design and dynamic characterization of "single-stroke" peristaltic PDMS micropumps. Lab Chip. 2011 Jan 21;11(2):336-42. doi: 10.1039/c0lc00023j. Epub 2010 Oct 19).

[0026] Fluidic connections between cartridges and the instrument fluidics can be achieved by the use of spring-loaded connectors and modular microfluidic connectors as taught by Jovanovich, S. B. *et. al.* Capillary valve, connector, and router. February 20, 2001. U. S. Patent 6,190,616 and Jovanovich; S. B. *et. al.* Method of merging chemical reactants in capillary tubes, April 22, 2003, U.S. Patent 6,551,839; and Jovanovich, S., I. Blaga, and R. McIntosh. Integrated system with modular microfluidic components. US Patent 7,244,961. July 17, 2007. which are incorporated by reference and their teachings which describe the modular microfluidic connectors and details of modular microfluidic connectors, including their use as multiway valves, routers, and other functions including microfluidic circuits to perform flowthrough reactions and flow cells with internally reflecting surfaces.

[0027] The surface chemistries of the paramagnetic beads and conditions to bind cells or precipitate, wash, and elute nucleic acids and other biomolecules onto surfaces is well understood, (Boom, W. R. *et. al.* United States Patent 5,234,809. August 10, 1993.), (Reeve, M. and P. Robinson. United States Patent 5,665,554. September 9, 1997.), (Hawkins, T. United States Patent 5,898,071. April 27, 1999.), (McKernan, K. *et. al.* United States Patent, 6,534,262. March 18, 2003.), (Han, Z. United States Patent, 8,536,322. September 17, 2013.), (Dressman et al., "Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variation" Proc. Natl. Acad. Sci. 100(15):8817-8822 (2003)), (Ghadessy et al., "Directed evolution of polymerase function by compartmentalized self-replication", Proc. Natl. Acad. Sci. 98(8):4552-4557 (2000)), (Tawfik and Griffiths, "Man-made cell-like compartments for molecular evolution" Nat. Biotech. 16(7):652-656 (1998)), (Williams et al., "Amplification of complex gene libraries by emulsion PCR" Nat. Meth. 3(7):545-550 (2006)), and many chemistries are possible and within the scope of the instant disclosure.

(7) Brief Summary of the Invention

[0028] Disclosed herein is a Sample Processing System that processes original or processed samples for bioanalysis. The Sample Processing System processes are comprised of enzymatic and mechanical disruption mechanisms with integrated fluidic processes. This invention enables, among other things, the implementation of a Sample Processing System that inputs solid, liquid, or gaseous samples including tissue or other biological samples, and processes the samples for bioanalysis and other analyses.

[0029] In some embodiments, the sample or specimen is a tissue specimen. The tissue can be from any source such as a human, animal, or plant tissue. Examples of tissues include, without limitation, a biopsy sample, a cellular conglomerate, an organ fragment, whole blood, bone marrow, a biofilm, a fine needle aspirate, or any other solid, semi-solid, gelatinous, frozen or fixed three dimensional or two dimensional cellular matrix of biological . In another embodiment the released nucleic acid is bound to a membrane, chip surface, bead, surface, flow cell, or particle. The term specimen is used to mean samples and tissue specimens.

[0030] In one embodiment the Sample Processing System is used for tissue processing. A Tissue Processing System embodiment can be implemented as a flexible, extensible system that can process solid or liquid tissue and other samples into single cells, nuclei, organelles, and biomolecules with mechanical and enzymatic or chemical processes to produce single cells in suspension, nuclei, subcellular components, and

biomolecules such as macromolecules comprised of nucleic acids, comprised of DNA and RNA; proteins; carbohydrates; lipids; biomolecules with multiple types of macromolecules; metabolites; and other biological components, including natural products for bioanalysis. In some embodiments, the Tissue Processing System performs affinity or other purifications to enrich or deplete cell types, organelles such as nuclei, mitochondria, ribosomes, or other organelles, or extracellular fluids. In some embodiments the Tissue Processing System can perform NGS library preparation. In some embodiments, the Tissue Processing System processes tissue into single-cell libraries for sequencing including Sanger, NGS, NNGS and other nucleic acid sequencing technolgies, or protoeomics, or other analytical methods.

[0031] Disclosed herein are different embodiments of Sample Processing Systems that integrate two or more of the overall steps to take samples from specimens (*i.e.,* tissue, biofilms, other multi-dimensional matrices with cells or viruses, liquids) and prepare single cell or nuclei in suspensions or on surfaces, or further process the specimens into biomolecules including macromolecules comprised of nucleic acids, comprised of DNA and RNA; proteins; carbohydrates; lipids; biomolecules with multiple types of macromolecules; metabolites; and other biological components, including natural products). In some embodiments specimen can be processed into NGS sequencing libraries, or fully integrated with an analytical system to produce a sample-to-answer systems such as asample-to-answer genomic system.

[0032] In some embodiments the Sample Processing System can be integrated with downstream bioanalysis to create a sample-to-answer system. In a preferred embodiment of the Sample Processing System, a Tissue Processing System processing embodiment is integrated with a nucleic acid bioanalysis system to sequence nucleic acids from tissues. Integrated is used to mean the workflows directly interface or in other contexts that the physical system directly interfaces or is incorporated into a system, instrument, or device. In one embodiment, the Tissue Processing System is integrated with a nucleic acid sequencer to produce a sample-to-answer system.

[0033] The Sample Processing System can have multiple subsystems and modules that perform processing or analysis. In a preferred embodiment of the Sample Processing System, one or more cartridges performs one or more steps in the processing workflow. In some embodiments the cartridges have multiple processing sites such as processing chambers that can process more than one sample. In some embodiments a cap couples mechanical disruption on the cartridge from a Physical Dissociation Subsystem. In some embodiments reagents from an Enzymatic and Chemical Dissociation Subsystem are delivered to the cartridge by a Fluidic Subystem to regions that are used as Pre-Processing Chambers and Processing Chambers to disrupt or dissociate specimen and process the cells, subcellular components, and biomolecules for bioanalysis.

[0034] The addition of fluids can be controlled by a Fluidic Subsystem with the complete system controlled by software in a Control Subsystem which can include the user interface through a device comprised of monitor, embedded display, touch screen; or through audio commands through the system or an accessory devices such as a cell phone or microphone. In some instances the Control Subsytem can include interfaces to laboratory information management systems, other instruments, databases, analysis software, email, and other applications.

[0035] In some embodiments, the amount of dissociation is monitored at intervals during the dissociation and in some instances the viability determined during processing using a Measurement

Subsystem. The degree of dissociation and/or viability can be determined inside the main dissociation compartment and/or in a separate compartment or channel, and/or in the external instrument.

[0036] In some embodiments, cell imaging solutions, such as cell type specific antibodies, stains, or other reagents, can be added to the tissue or single cells or nuclei for additional processing or imaging. The imaging can capture cells, subcellular structures, or histological or other data. In some embodiments the images can be analyzed to direct the operation and workflow of the Sample Processing System through decisions trees, hash tables, machine learning, or artificial intelligence.

[0037] In some embodiments, single cells or nuclei in suspension or on surfaces are further processed using magnetic bead or particle technologies using a Magnetic Processing module to purify or deplete cell types, nuclei, nucleic acids, or other biomolecules.

[0038] The term singulated cells is used to mean single cells in suspension or on a surface or in a well including a microwell or nanowell such that they can be processed as single cells. The term singulated cells is also used at times to encompass single nuclei.

[0039] In one embodiment, the specimen is added to a cartridge which performs both physical and enzymatic dissociation of the tissue. In some embodiments the Singulator System performs tituration and other physical dissociation modalities as a step or steps in the process of singulating cells. The physical dissociation modalities include passing the specimen through screens, filters, orifices, grinding, blending, sonication, smearing,bead beating, and other methods known to one skilled in the art to physically disrupt tissue to help produce single cells or nuclei or nucleic acids or other biomolecules.

[0040] In one embodiment, the Sample Processing System is a Singulator System embodiment. The Singulator System described can input raw, unprocessed samples, or other primary or secondary samples, and output single cells or nuclei ready for single cell or nuclei analysis or for additional processing, *e.g.*, to purify specific cell types with antibodies or by cell sorting or growth, library preparation, or many other applications. A Singulator System embodiment dissociates single cells or nuclei from specimens such as tissue, blood, bodily fluid or other liquids or solids containining cells to produce single cells in suspensions or nuclei, or on surfaces, in matrices, or other output configurations. In a preferred Singulation System described embodiment, there is a cartridge that inputs tissue and/or other specimens and outputs single cells or nuclei, preferably of known titer in a buffer supplemented with media such as Hank's buffer with 2% fetal calf serum.

[0041] In some embodiments, the Sample Processing System, such as a Singulator System embodiment, uses enzymes to assist in the process of singulating cells including enzymes to preserve nucleic acids and prevent clumping. The enzymes are comprised of but not limited to collagenases (*e.g.*, collagenases type I, II, III, IV, and others), elastase, trypsin, papain, tyrpLE, hyaluronidase, chymotrypsin, neutral protease, , pronase, liberase, clostripain, caseinase, neutral protease (Dispase®), DNAse, protease XIV, RNase inhibitors, or other enzymes, biochemicals, or chemicals such as Triton X-100, Nonidet P40, detergents, surfactants, *etc*. In other embodiments, different reagents or mixtures of reagents are applied sequentially to dissociate the biological sample or specimen into single-cell suspensions.

[0042] In some embodiments the Singulator System produces cell suspensions of known titers and viability. In some embodiments the Singulator System monitors the viability and/or the amount of singulation of a sample and adjusts the treatment time and concentration of enzymes or other dissociation agents by

monitoring of the dissociation, for example by the production of single cells or nuclei. The monitoring can be in real time, in intervals, or endpoints or any combinations thereof.

[0043] The Singulator System can in some embodiments select from sets of reagents to dissociate tissue and adjust according to production of single cells or viability of cells as monitored by the system, in some instances in real time, at intervals, or as an endpoint. The single-cell suspensions produced by the Singulator System can be used to generate cells with therapeutic application, *e.g.*, re-grow new tissues and/or organs and/or organisms.

[0044] The Singulator System has advantages over existing technology and can produce single cells, nuclei, or biomolecules from tissue in an automated and standardized instrument that can in some embodiments process the specimens into NGS libraries or other preparations. The Singulator System will enable users, *e.g.*, researchers, clinicians, forensic scientists, and many disciplines to perform identical processing on biosamples, reducing user variability, and throughput constraints of manual processing.

[0045] Embodiments of the Singulation System can prepare single-cells or nuclei or nucleic acids for analysis by methods comprised of DNA sequencing, DNA microarrays, RNA sequencing, mass spectrometry, Raman spectroscopy, electrophysiology, flow cytometry, mass cytometry, and many other analytical methods well known to one skilled in the art including multidimensional analysis (*e.g.,* LC/MS, CE/MS, *etc.*). In addition, single-cell suspensions or on surfaces or matrices can be used to grow additional cells including genetically altered by methods such as CRISPR, engineered viral or nucleic acid sequences, in tissue culture, or to grow tissues or organs for research and therapeutic purposes.

[0046] The Singulator System embodiment described is compatible with commercially available downstream library preparation and analysis by both NGS and NNGS sequencers. The term NGS is used to connote either NGS or NNGS sequencers or sample preparation methods as appropriate. As contemplated herein, next generation sequencing or next-next generation sequencing refers to high-throughput sequencing, such as massivley parallel sequencing, (*e.g.,* simultaneously (or in rapid succession) sequencing any of at least 1,000, 100,000, 1 million, 10 million, 100 million, or 1 billion polynucleotide molecules). Sequencing methods may include, but are not limited to: high-throughput sequencing, pyrosequencing, sequencing-by-synthesis, single-molecule sequencing, nanopore sequencing, semiconductor sequencing, sequencing-by-ligation, sequencing-by-hybridization, RNA-Seq (Illumina), Digital Gene Expression (Helicos), next generation sequencing, Single Molecule Sequencing by Synthesis (SMSS) (Helicos), massively-parallel sequencing, Clonal Single Molecule Array (Solexa), shotgun sequencing, Maxam-Gilbert or Sanger sequencing, primer walking, sequencing using PacBio, SOLiD, Ion Torrent, Genius (GenapSys) or nanopore (*e.g.*, Oxford Nanopore, Roche) platforms and any other sequencing methods known in the art.

[0047] In another aspect provided herein is an apparatus, composition of matter, or article of manufacture, and any improvements, enhancements, and modifications thereto, as described in part or in full herein and as shown in any applicable Figures, including one or more features in one or more embodiment.

[0048] In another aspect provided herein is an apparatus, composition of matter, or article of manufacture, and any improvements, enhancements, and modifications thereto, as described in part of in full herein and as shown in any applicable Figures, including each and every feature.

[0049] In another aspect provided herein is a method or process of operation or production, and any improvements, enhancements, and modifications thereto, as described in part or in full herein and as shown in any applicable Figures, including one or more feature in one or more embodiment.

[0050] In another aspect provided herein is a method or process of operation or production, and any improvements, enhancements, and modifications thereto, as described in part or in full herein and as shown in any applicable Figures, including each and every feature.

[0051] In another aspect provided herein is a product, composition of matter, or article of manufacture, and any improvements, enhancements, and modifications thereto, produced or resulting from any processes described in full or in part herein and as shown in any applicable Figures.

[0052] In one embodiment the single-cell suspension is prepared for a bioanalysis module for downstream analysis including but not limited to sequencing, next generation sequencing, next next generation sequencing, proteomic, genomic, gene expression, gene mapping, carbohydrate characterization and profiling, lipid characterization and profiling, flow cytometry, imaging, DNA or RNA microarray analysis, metabolic profiling, functional, or mass spectrometry, or combinations thereof.

[0053] In another aspect provided herein is a data analysis system that correlates, analyzes, and visualizes the analytical information of a sample component such as its viability, degree of single cell or nuclei dissociation, with the processing step and measures the change over time, and/or amount of enyzmatic activity, and/or physical disruptions of the original biological specimen.

[0054] In another aspect provided herein is a data analysis system that correlates, analyzes, and visualizes the analytical information of a sample component such as its viability, degree of single cell or nuclei dissociation, with the processing step and measures the change over time, and/or amount of enyzmatic activity, and/or physical disruptions of the original biological specimen and adjusts the processing parameters from the analytical information.

[0055] The Singulator System is a novel platform that automates and standardizes the only portion of the single-cell NGS workflow that has not been automated. This will have broad impacts. Process standardization will be critical for comparison of data from lab to lab or research to researcher. The Human Cell Atlas project intends to freely share the multi-national results in an open database. However, with no standardization of the complete process, direct comparisons will greatly suffer from widely varying impacts of the first processing step of producing single-cells or nuclei from tissue. Additionally, when single-cell or nuclei sequencing becomes clinically relevant, the standardization and de-skilling of the production of single-cells or nuclei will be required to be performed by an automated instrument such as the Singulator System.

[0056] In another aspect, provided herein is a system comprising: (a) an instrument comprising: (i) one or more cartridge interfaces configured to engage a cartridge; (ii) a fluidics module comprising: (1) one or more containers containing one or more liquids and/or gasses; (2) one or more fluid lines connecting the containers with fluid ports in the cartridge interface; and (3) one or more pumps configured to move liquids and/or gasses into and/or out of the fluid port(s); (iii) a mechanical module comprising an actuator; (iv) optionally, a magnetic processing module comprising a source of magnetic force, wherein the magnetic force is positioned to form a magnetic field in the processing chamber; (v) optionally, a measurement module; (vi) optionally, a control module comprising a processor and memory, wherein the memory comprises code that, when executed by the processor, operates the system; and (b) one or more cartridges, each engaged with

one of the cartridge interfaces, wherein each cartridge comprises: (i) a sample inlet port; (ii) one or more cartridge ports communicating with the fluid ports in the cartridge interface; (iii) a preprocessing chamber communicating with the sample inlet port and with at least one cartridge port, and comprising a tissue disruptor configured for mechanical disruption of tissue, wherein the tissue disruptor engages with and is actuated by the actuator when the cartridge is engaged with the cartridge interface; (iv) a strain chamber communicating with the preprocessing chamber configured to separate cells and/or nuclei from disrupted tissue; (v) a processing chamber communicating with the strain chamber, optionally communicating with one or more cartridge ports and configured to perform one or more processing steps on separated cells and/or nuclei; and (vi) optionally, one or more waste chambers fluidically connected with the processing chamber. In one embodiment the tissue disruptor comprises a grinder, a pestle or a variable orifice. In another embodiment the system further comprises a barcode reader. In another embodiment the system comprises a measurement module (v) that performs optical imaging to measure titer, clumping, and/or viability of cells or nuclei or properties of biomolecules. In another embodiment the system comprises a measurement module (v) and a control system (vi), wherein the measurement module measures, and one or more time points, characteristics of a sample in the processing chamber, and control system comprises code that determines a state of the sample, *e.g.,* viability or degree of single cell or nuclei dissociation, and that adjusts processing parameters. In another embodiment the system further comprises (c) an analysis module, wherein an input port of the analysis module is in fluid communication with the processing chamber. In another embodiment the analysis module performs an analysis selected from one or more of: DNA sequencing, next generation DNA sequencing, next next generation DNA sequencing, proteomic analysis, genomic analysis, gene expression analysis, gene mapping, carbohydrate characterization and profiling, lipid characterization and profiling, flow cytometry, imaging, DNA or RNA microarray analysis, metabolic profiling, functional analysis, and mass spectrometry. In another embodiment the cartridge interface comprises a means of positioning the cartridge in the instrument that engages the fluidic module and the mechanical module and optionally is temperature controlled. In another embodiment the cartridge is disposable.

In another aspect provided herein is a method comprising: (a) providing a tissue sample to a preprocessing chamber; (b) automatically performing mechanical and enzymatic/chemical disruption of the tissue in the preprocessing chamber to produce disrupted tissue comprising released cells and/or nuclei and debris; (c) automatically moving the disrupted tissue into a strain chamber comprising a strainer and/or filter and separating the released cells and/or nuclei from the debris therein; and (d) automatically moving the released cells and/or nuclei into a processing chamber. In another embodiment (d) further comprises performing at least one processing step on the released cells and/or nuclei in the processing chamber. In another embodiment processing comprises one or more automatically performed processes selected from:(I) lysing cells; (II) capturing cells; (III) isolating nucleic acid; (IV) isolating protein; (V) converting RNA into cDNA; (VI) preparing one or more libraries of adapter tagged nucleic acids; (VII) performing PCR; (VIII) isolating individual cells or individual nuclei in nanodrops or nanoboluses; and (IX) outputting released cells and/or nuclei into output vessels such as 8 well strip tubes, microtiter plates, Eppendorf tubes, a chamber in the cartridge, or other vessels capable of receiving cell suspensions. In another embodiment the method further comprises: (e) automatically capturing the released cells and/or nuclei in the processing chamber by binding to magnetically attractable particles comprising moieties having affinity for the cells and/or nuclei and

applying a magnetic force to the processing chamber to immobilize the captured cells and/or nuclei. In another embodiment the method further comprises: (e) automatically monitoring cell and/or nuclei titer in the processing chamber and, when the titer reaches a desired level, exchanging a dissociation solution used to dissociate the tissue for a buffer.

In another aspect provided herein is a cartridge comprising: (i) a sample inlet port; (ii) one or more cartridge ports configured to communicate with fluid ports in a cartridge interface; (iii) a preprocessing chamber communicating with the sample inlet port and with at least one cartridge port, and comprising a tissue disruptor configured for mechanical disruption of tissue, wherein the tissue disruptor engages with and is actuated by the actuator when the cartridge is engaged with the cartridge interface; (iv) a strain chamber communicating with the preprocessing chamber configured to separate cells from disrupted tissue; (v) a processing chamber communicating with the strain chamber, optionally communicating with one or more cartridge ports and configured to perform one or more processing steps on separated cells; and (vi) optionally, one or more waste chambers fluidically connected with the processing chamber. In another embodiment the cartridge further comprises a cap that opens and closes the sample inlet port. In another embodiment the cap comprises a tissue disruptor element that moves about rotationally and back and forth along an axis. In another embodiment the cartridge further comprises a holder. In another embodiment the cartridge further comprises a top piece and a bottom piece connected by collapsible element which allow the top piece and/or the bottom piece to move relative to the holder. In another embodiment the holder comprises a mesh screen. In another embodiment the cartridge further comprises a grinding element for grinding tissue in the preprocessing chamber. In another embodiment the cartridge further comprises a barcode comprising information about the cartridge and/or its use. In another embodiment the cartridge further comprises a plunger configured to move slideably within the preprocessing chamber.

[0057] In another aspect provided herein is a variable orifice device for disrupting tissue comprising: (a) a first container and a second container fluidically connected through a flexible tube comprising a lumen; (b) an adjustable clamp positioned to clamp the flexible tube, wherein adjusting the clamp alters the cross-sectional area of the lumen; and (c) one or more pumps or devices operatively coupled with the first and/or second containers configured to push liquid in one container through the flexible tubing into the other container. In another embodiment the adjustable clamp comprises an eccentric cam operatively coupled to a motor, wherein rotating the cam closes or opens the clamp.

[0058] In another aspect provided herein is a method for disrupting tissue comprising: (a) providing a variable orifice device comprising first container and a second container fluidically connected through a flexible tube comprising a lumen; (b) moving a sample comprising tissue from one of the containers through the flexible tube to another one of the containers; (c) decreasing the cross-sectional area of the lumen and moving the sample from one of the containers through the flexible tube to another one of the containers; (d) repeating step (c) one or more times to disrupt the tissue.

[0059] In another aspect provided herein is a method of determining an amount of amplification of a nucleic acid molecule comprising amplifying the nucleic acid molecule with primers comprising random barcode (e.g., a barcode wherein each round of amplification adds in additional barcode to an amplified

nucleic acid molecule); and after amplification, counting incorporated barcodes, wherein the number of incorporated barcodes indicates the amount of amplification.

(8) Brief description of the drawings

[0060] The skilled artisan will understand that the drawings, described below, are for illustration purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0061] **Figure 1** shows a Sample Processing System that processes specimens or tissue specimens into biocomponents such as single cells or nuclei for bioanalysis.

[0062] **Figure 2** shows a Tissue Processing System that processes tissue specimens into biocomponents such as single cells or nuclei or other for bioanalysis.

[0063] **Figure 3** shows an example of the workflow to prepare and analyze single cells or nuclei for NGS analysis. Tissue samples are dissociated into singulated cells before processing into typically single-cell cDNA when samples can be pooled for library preparation, sequencing, and analysis. Different embodiments of the implementation can integrate different segments of the workflow.

[0064] **Figure 4** shows a high level overview of the workflow for a Singulator System to generate for example single cell or nuclei or biomolecules from a specimen or tissue specimen.

[0065] **Figure 5** shows an overview Singulator System and some exemplary modules. Tissue specimens or other specimens processed into single cells, nuclei, nucleic acids, single-cell libraries and other biologicals through the use of one or more cartridges and one or more of the Physical Dissociation Subsystem, the Enzymatic and Chemical Dissociation Subsystem, the Measurement Subsystem, the Fluidic Subsystem, the Control Subsystem, and a Magnetic Module.

[0066] **Figure 6A-K** shows an example of a process with a cartridge that processes specimens into single cells, nuclei, or other biocomponents.

[0067] **Figure 7A - G** shows a cartridge with an orifice.

[0068] **Figure 8** shows a device that can perform mechanical processing of specimens with fluidic and mechanical control.

[0069] **Figure 9** shows an automated device to create a variable orifice.

[0070] **Figure 10A - D** shows four types of automated mechanical dissociation methods and examples of tissue processing.

[0071] **Figure 11** is an example of part of a cartridge designed to physically disrupt a specimen with a rotating plunger.

[0072] **Figure 12A - C** illustrates a rotating disruptor with staggered grinding features on a rotor and stator.

[0073] **Figure 13** is a design of a magnetically coupled rotating disruptor with external spiral features to circulate fluid through a central circulation region as grinding features disrupt the tissue specimen.

[0074] **Figure 14** is a design of a magnetically coupled rotating disruptor that can exert adjustable force on the specimen. The disruptor has external spiral features to circulate fluid through a central circulation region as grinding features disrupt the specimen which is initially placed below the rotating disruptor.

[0075] **Figure 15A - D** show designs of rotating disruptors with different pitches of spiral external features.

[0076] **Figure 16A -E** shows an example of a rotating disruptor with external spiral features and an example ofa rotating disruptor with both internal and external spiral features.

[0077] **Figure 17A - E** illustrates a rotating disruptor assembly with a mesh top surface to grind the specimen and an internal spiral to circulate fluids.

[0078] **Figure 18** shows a three chamber cartridge with cap containing a plunger with two PreProcessing chambers and one Processing chamber for a variable orifice implementation.

[0079] **Figure 19** shows a cutaway of a cap design for a grinding implementation.

[0080] **Figure 20** shows seven paired grinder rotors and grinder stators.

[0081] **Figure 21** shows an AutoSingulator instrument with a cartridge.

[0082] **Figure 22A - B** shows the titer and viability of processing eight fresh mouse tissues into single cells using the AutoSingulator instrument with a cartridge.

[0083] **Figure 23** shows the titer of processing mouse tissue into nuclei using the AutoSingulator instrument with a cartridge.

[0084] **Figure 24A - B** shows the titer and viability of processing a range of sizes of mouse tissues into single cells using the AutoSingulator instrument with a cartridge.

[0085] **Figure 25** shows the titer and viability of processing a range of sizes of mouse tissues into nuclei

[0086] **Figure 26** illustrates using gene expression to monitor and develop processes on cartridges.

[0087] **Figure 27** shows the workflow for a Singulator System embodiment in the upper part of the figure, a single-sample design in the bottom left and a four-sample system design in the bottom right.

[0088] **Figure 28** shows the overall design concept of the Cell Singulation module for a prototype showing functional modules.

[0089] **Figure 29** shows an example of a Single-Sample Singulation System with mechanical disruption in the cartridge with a bank of enzymes and reagents on the instrument.

[0090] **Figure 30** shows a layout for a Magnetic Module.

[0091] **Figure 31** shows an example of a Single-Sample Singulation System with mechanical disruption in the cartridge with a bank of enzymes and reagents on the instrument with temperature control and a cartridge insertion mechanism.

[0092] **Figure 32** shows a detail of the cartridge insertion of a Single-Sample Singulation System.

[0093] **Figure 33** is an example of a user interface for a Singulation System.

[0094] **Figure 34** shows a cartridge and how the Fluidic Subsystem delivers reagents to the cartridge.

[0095] **Figure 35** shows electronics diagram and the hardware controlled by the electronics.

[0096] **Figure 36** illustrates an example of a cartridge cap and means of coupling to the instrument.

[0097] **Figure 37A - C** shows an example of a vertical cartridge that integrates processing of tissue.

[0098] **Figure 38** shows the modules in an Enhanced Singulator System.

[0099] **Figure 39** is an illustration of a flowcell and optical module.

[00100] **Figure 40** shows a five layer cartridge designed to integrate multiple functions into one cartridge.

[00101] **Figure 41** shows a Single Libarian embodiment with the workflow integrated and automated for multiple steps from tissue to single-cell libraries and other libraries, such as bulk nucleic acid libaries.

[00102] **Figure 42** is an example of a microfluidic nozzle used to create nanodroplets with beads and single-cells.

[00103] **Figure 43** is an exemplary workflow for the Single Librarian.

[00104] **Figure 44** shows an example of using transposons to produce a sequencing library from double stranded DNA from a specimen.

[00105] **Figure 45** shows an example workflow of library preparation from double stranded DNA.

[00106] **Figure 46** illustrates the processing of mRNA to cDNA on a bead.

[00107] **Figure 47** shows a sample-to-answer embodiment configured to process tissue into genetic information.

(9) Detailed Description of the Invention

[00108] NGS, mass spectrometry, FACS, and other modern high-throughput analysis systems have revolutionized life and medical sciences. The progression of information has been from the gross level of organism, to tissue, and now to single cell analysis. Single cell analysis of genomic, proteomic including protein expression, carbohydrate, lipid, and metabolism of individual cells is providing fundamental scientific knowledge and revolutionizing research and clinical capabilities.

[00109] All patents, patent applications, published applications, treatises and other publications referred to herein, both supra and infra, are incorporated by reference in their entirety. If a definition and/or description is set forth herein that is contrary to or otherwise inconsistent with any definition set forth in the patents, patent applications, published applications, and other publications that are herein incorporated by reference, the definition and/or description set forth herein prevails over the definition that is incorporated by reference.

[00110] As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having” or any other variation thereof, are intended to cover a non-exclusive inclusion. For example, a process, method, article, or apparatus that comprises a list of features is not necessarily limited only to those features but may include other features not expressly listed or inherent to such process, method, article, or apparatus. Further, unless expressly stated to the contrary, “or” refers to an inclusive-or and not to an exclusive-or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present). Both plural and singular means may be included.

[00111] Specimen: The term “specimen,” as used herein, refers to an *in vitro* cell, cell culture, virus, bacterial cell, fungal cell, plant cell, bodily sample, or tissue sample that contains genetic material. In certain embodiments, the genetic material of the specimen comprises RNA. In other embodiments, the genetic material of the specimen is DNA, or both RNA and DNA. In certain embodiments the genetic material is modified. In certain embodiments, a tissue specimen includes a cell isolated from a subject. A subject includes any organism from which a specimen can be isolated. Non-limiting examples of organisms include prokaryotes, eukaryotes or archaebacteria, including bacteria, fungi, animals, plants, or protists. The animal, for example, can be a mammal or a non-mammal. The mammal can be, for example, a rabbit, dog, pig, cow, horse, human, or a rodent such as a mouse or rat. In particular aspects, the tissue specimen is a human tissue sample. The tissue specimen can be liquid , for example, a blood sample, red blood cells, white blood cells, platelets, plasma, serum. The specimen, in other non-limiting embodiments, can be saliva, a cheek, throat, or nasal swab, a fine needle aspirate, a tissue print, cerebral spinal fluid, mucus, lymph, feces, urine, skin, spinal fluid, peritoneal fluid, lymphatic fluid, aqueous or vitreous humor, synovial fluid, tears, semen,

seminal fluid, vaginal fluids, pulmonary effusion, serosal fluid, organs, bronchio-alveolar lavage, tumors, frozen cells, or constituents or components of *in vitro* cell cultures. In other aspects, the tissue specimen is a solid tissue sample or a frozen tissue sample or a biopsy sample such as a fine needle aspirate or a core biopsy or a resection or other clinical or veternary specimen. In still further aspects, the specimen comprises a virus, bacteria, or fungus. The specimen can be an *ex vivo* tissue or sample or a specimen obtained by laser capture microdissection. The specimen can be a fixed specimen, including as set forth by U.S. Published Patent Application No. 2003/0170617 filed Jan. 28, 2003.

[00112] In some embodiments, the single cells can be analyzed further for biomolecules including one or more polynucleotides or polypeptides or other macromolecules. In some embodiments, the polynucleotides can include a single-stranded or double-stranded polynucleotide. In some embodiments, the polypeptide can include an enzyme, antigen, hormone or antibody. In some embodiments, the one or more biomolecules can include RNA, mRNA, cDNA, DNA, genomic DNA, microRNA, long noncoding RNA, ribosomal RNA, transfer RNA, chloroplast DNA, mitochondrial DNA, or other nucleic acids including modified nucleic acids and complexes of nucleic acids with proteins or other macromolecules.

[00113] It will be readily apparent to one of ordinary skill in the art that the embodiments and implementations are not necessarily inclusive or exclusive of each other and may be combined in any manner that is non-conflicting and otherwise possible, whether they be presented in association with a same, or a different, embodiment or implementation. The description of one embodiment or implementation is not intended to be limiting with respect to other embodiments and/or implementations. Also, any one or more function, step, operation, or technique described elsewhere in this specification may, in alternative implementations, be combined with any one or more function, step, operation, or technique described in the summary. Thus, the above embodiment and implementations are illustrative rather than limiting.

[00114] **Figure 1** shows a Sample Processing System **50** that can input specimen **101** and process them to produce biologicals such as single cells **1000** or nuclei **1050**, or other biocomponents comprised of subcellular components **1060**, and biomolecules **1070** such as macromolecules **1071** and nucleic acids **1072,** comprised of DNA **1073** and RNA **1074**; proteins **1075**; carbohydrates **1076**; lipids **1077**; biomolecules **1070** with multiple types of macromolecules **1071**; metabolites **1078**; and other biological components, including natural products **1079** for bioanalysis.

[00115] **Figure 2** shows a Tissue Processing System **80** that can input tissue specimens **120** and other specimens **101** and process them to produce biologicals such as single cells **1000** or nuclei **1050**, or other biocomponents comprised of subcellular components **1060**, and biomolecules **1070** such as macromolecules **1071** and nucleic acids **1072,** comprised of DNA **1073** and RNA **1074**; proteins **1075**; carbohydrates **1076**; lipids **1077**; biomolecules **1070** with multiple types of macromolecules **1071**; metabolites **1078**; and other biological components, including natural products **1079** for bioanalysis.

[00116] **Figure 3** shows the overall workflow for single-cell NGS sequencing from tissue through singulated cell preparation of single-cell suspensions to processing of the single-cells into cDNAs typically to library preparation, NGS sequencing, analysis, and applications. The first step is forming singulated cells in a suspension. In the workflow shown, this consists of mincing tissue, digesting extracellular matrix, filtering through a 70 μm filter, pelleting the cells, lysis of red blood cells with Ammonium-Chloride-Potassium (ACK) buffer, pelleting of the cells, and cryopreservation in fetal bovine serum with 10% DMSO. After the generation of droplets or capture of cells, the single-cell workflow shown in **Figure 3** is well integrated using

the installed ultrahigh throughput, low cost, NGS infrastructure. The downstream sequencing process, sequence readout, and analysis has great capacity at a low cost. However, the upstream production of single cells **1000** or nuclei **1050** suspensions is not integrated and relies on manual processing of specimens **101**.

[00117] Referring to **Figure 4**, the Singulation System **100** accepts one or more specimens 101 or tissue specimens **120** and processes them to produce biologicals such as single cells **1000** or nuclei **1050**, or other biocomponents comprised of subcellular components **1060**, and biomolecules **1070** such as macromolecules **1071** and nucleic acids **1072,** comprised of DNA **1073** and RNA **1074** and single cell libraries **1200** for bioanalysis.

[00118] Referring to **Figure 5**, in many embodiments, the Singulation System **100** processing is performed in cartridges **200** in the system. Tissue specimens **120** or other specimens **101** are converted to single cells **1000** or nuclei **150** through the use of cartridge **200** with one or more of the Physical Dissociation Subsystem **300**, the Enzymatic and Chemical Dissociation Subsystem **400**, the Measurement Subsystem **500**, the Fluidic Subsystem **600**, the Control Subsystem **700**, and the Magnetic Module **900**.

[00119] The Physical Dissociation Subsystem **300** can perform physical disruption by passing the specimen through orifices,grinding, rotating a rotor with features to dissociate tissue, forcing tissue through screens or mesh, sonication, blending, homogenization, bead beating, and other methods known to one skilled in the art to physically disrupt tissue to help produce single cells.

[00120] The Enzymatic and Chemical Dissociation Subsystem **400** can perform enzymatic disruption by adding formulations of a reagents or mixture of components comprised of but not limited to collagenases (*e.g.*, collagenases type I, II, III, IV, and others), elastase, trypsin, papain, hyaluronidase, chymotrypsin, neutral protease, clostripain, caseinase, neutral protease (Dispase®), DNAse, protease XIV, RNase inhibitors, or other enzymes, biochemicals, or chemicals such as EDTA, protease inhibitors, buffers, acids, or base.

[00121] Another aspect or the Enzymatic and Chemical Dissociation Subsystem **400** can perform chemical disruption or chemical and enzymatic disruption is by adding formulations of a chemicals that might disrupt tissue or cellular integrity such as Triton X-100, Tween, Nonident P40, other surfactants, or chemicals that can dissociate tissue into cells or produce nuclei or other organelles.

[00122] In other embodiments, different reagents or mixtures of reagents are applied sequentially to dissociate the biological sample or specimen into single cells. The physical and enzymatic/chemical dissociation systems can be separate from each other, or they can be co-located (*e.g.*, acting upon the sample simultaneously or sequentially).

[00123] In some embodiments, the amount of dissociation is monitored at intervals during the dissociation or at the endpoint, and in some instances the viability is determined during processing using a Measurement Subsystem **500**. The Measurement Subsystem **500** can be an optical imaging device to image cells using brightfield, phase contrast, fluorescence, chemiluminescence, near-field, or other optical readouts, or an electrical measurement, such as an impedance measurement of the change in conductivity, when a cell passes through a sensor, or other types of measurement.

[00124] The addition and movement of fluids can be performed by a Fluidic Subsystem **600**. The Fluidic Subsystem **600** can use syringe pumps, piezopumps, on-cartridge pumps and valves, pressure, pneumatics, or other components well known to one skilled in the art.

[00125] The Singulation System **100** can be controlled by software in a Control Subsystem **700** which can be comprised of a user interface **740** through a monitor, embedded display, or a touch screen **730**. In some instances the Control Subsytem **700** can include interfaces to laboratory information management systems, other instruments, analysis software, display software, databases, email, and other applications. The Control Subsystem **700** can include control software **725** and scripts that control the operation and in some embodiments the scripts can be revised, created, or edited by the operator.

[00126] In another aspect provided herein is a device for the dissociation of a biological sample, the device comprising: (i) a biological sample or specimen **101**;(ii) a cartridge **200** capable of dissociating tissue; (iii) an instrument to operate the cartridge **200** and provide fluids as needed (iv) a measurement module **500** such as an optical imaging to measure titer, clumping, and/or viability, (v) exchange of dissociation solution for buffer or growth media at the desired titer, and (vi) output vessels such as a chamber in the cartridge, 8 well strip tubes, microtiter plates, Eppendorf tubes or other vessels capable of receiving cell suspensions.

[00127] In another aspect provided herein is a device for the dissociation of a biological sample and the production of single-cell **1000** or nuclei **1050** suspensions or matched bulk nucleic acids **1010** or single cell libraries **1200** or matched bulk libraries **1210**, the device comprising: (i) a chamber or area to input a biological sample or specimen;(ii) a cartridge capable of dissociating tissue or specimen; (iii) an instrument to operate the cartridge and provide fluids as needed (iv) a measurement module such as an optical imaging to measure titer, clumping, and/or viability, (v) exchange of dissociation solution for buffer or growth media at the desired titer, (vi) the production of single-cell **1000** or nuclei **1050** suspensions or single cell libraries **1200**, and matched bulk nucleic acid libraries **1210**, in output vessels such as 8 well strip tubes, microtiter plates, Eppendorf tubes, a chamber in the cartridge, or other vessels capable of receiving cell suspensions.

[00128] Referring to **Figure 5,** a Magnetic Processing module **900** can use magnetic processing of magnetic and paramagnetic particles or beads, referred to as beads, to separate single cells **1000**, or cell types or nuclei **1050**, or other biocomponents comprised of subcellular components **1060**, and biomolecules **1070** such as macromolecules **1071** and nucleic acids **1072,** comprised of DNA **1073** and RNA **1074**; proteins **1075**; carbohydrates **1076**; lipids **1077**; biomolecules **1070** with multiple types of macromolecules **1071**; metabolites **1078**; and other biological components, including natural products **1079** for bioanalysis. In some embodiments the beads have a surface chemistry that facilitates the purification of the biologicals in conjunction with the chemical conditions. In other embodiments the bead have affinity molecules comprised of antibodies, aptamers, biomolecules, etc. that specifically purify certain biologicals such as cell types, nucleic acids, nuclei **1050**, or other components of tissue or samples.

[00129] In another aspect provided herein is a device for the dissociation and single-cell library preparation of a biological sample, the device comprising: (i) a chamber or area to input a biological sample or specimen;(ii) a cartridge **200** capable of dissociating tissue specimens **120** into single-cells **1000** and then produce single-cell libraries **1200**; (iii) an instrument to operate the cartridge **200** and provide fluids as needed (iv) a measurement subsystem **500** such as an optical imaging to measure titer, clumping, and/or viability, (v) exchange of dissociation solution for buffer at the desired titer, (vi) a magnetic processing or other processing chamber or tubing to perform magnetic separations, normalizations, purifications, and other magnetic processes, for example, to purify nucleic acids, couple enyzmatic reactions such as library preparation reactions, and other processes including producing single-cells or nuclei in isolation, such as

nanodrops, nanoboluses, or physical separation, (vii) output vessels such as 8 well strip tubes, microtiter plates, Eppendorf tubes, a chamber in the cartridge, or other vessels capable of receiving cell suspensions.

[00130] The basic elements of the Singulation System **100** can be configured in multiple ways depending on the specimen(s) **101** and analytes to be analyzed. In the following examples, a few of the numerous configurations are described in detail but in no way is the invention limited to these configurations as will be obvious to one skilled in the art. The Singulation System **100** can accommodate many different types of specimens **101**, comprised of fresh tissue; snap-frozen tissue; microtome slices (cryo, laser or vibrating) of tissue; fixed tissue; bulk material obtained by surgical excision, biopsies, fine needle aspirates; samples from surfaces, and other matrices.

[00131] There is a need to fill gap in the single-cell and nuclei NGS sample preparation pipeline by starting the workflow at processing raw solid tissues or liquids into single-cell **1000** and nuclei **1050** suspensions. The instant disclosure teaches how to produce a system that processes tissue specimens **120** and other samples into single-cells **1000** or nuclei **1050**, and other samples types then extend the processing all the way to libraries, such as single cell libraries **1200,** with little or no intervention by the operator once the process is started. This requires adapting to the widely varying starting types of tissue, with different requirements depending on the tissue, species, age, and state.

[00132] In the instant invention, many embodiments are possible. Systems with increasing capabilites can be developed as a series of embodiments, five are described: two embodiment as a Single Sample Singulator System **2000**, a Four Sample Singulator System **2400,** an Enhanced Singulator System **2500**, and the Single Librarian **3000** embodiments.

[00133] Two embodiments of a Single Sample Singulator System **2000** embodiment are described to process tissue into single-cell suspensions and purified single-cell subtypes or nuclei for many tissues. A Four Sample Singulator System **2400** is described to process four specimens **101** into single-cell **1000** or nuclei **1050** suspensions. An Enhanced Singulator System **2500** is described with additional capabilities to titer, adjust the buffer, and purify or deplete cell types, nuclei, organelles, or biomolecules. A Single Librarian **3000** embodiment is described that integration with single-cell library preparation, and bulk nucleic acid and library preparation and adds QC capabilities. It will be obvious to one skilled in the art that the systems can continue to be expanded with additional capabilities or be configured in many other embodiments.

[00134] This disclosure describes how to automate, integrate, and importantly standardize the complete process for single-cell **1000** or nuclei **1050** suspensions in a single Singulator System **100** system embodiment. The Singulator System **100** will greatly enable basic researchers, students, and translational researchers as well as clinicians and others with its ease of use and high performance. Designed for genomics and easily adaptable to other applications, the Singulator System **100** performs the most upstream sample preparation steps—from tissue specimens **120** or other sample types—and standardize the complete sample preparation process for single-cell **1000** and nuclei **1050** suspensions.

[00135] **Single-use cartridge designs.**

[00136] Cartridges **200** can be used to process tissue into single-cell **1000** suspensions or nuclei **1050** and are preferrablely single-use. The major workflow steps to produce single-cell suspensions **1000** are mechanical disruption of tissue, enzymatic dissociation, and straining to remove clumps, and optional cell type isolation by magnetic bead capture.

[00137] Ideally, the cartridge **200** will input specimen **101** and output viable singulated cells **1000** or nuclei **1050** in suspension and can be designed to incorporate magnetic and other downstream processing to also allow production of biomolecules **1070** such as nucleic acids **1072**. It is desirable that disposable cartridge **200** process multiple types of samples with mechanical disruption and enzymatic or chemical dissociation according to the tissue type and condition. The cartridge **200** can be designed to process tissue as quickly and as gently as possible, not expose the operator to the tissue being processed, and be manufacturable at low cost. Multiple mechanical methods may be needed to accommodate the wide range of tissues and their individual requirements: designs are shown that can be readily adapted to multiple different mechanical disruption methods comprising variable orifice **490**, grinding with rotating plungers **336**, pestles **361**, and straining and filtering using a plunger **362** as well as other mechanical methods without limitation.

[00138] Cartridges **200** can be designed for 3D printing, injection molding in plastics with single or double pulls and low labor assembly, or layered assembly of fluidic and other layers, combinations of methods, and other methods well known to one skilled in the art. Fluids can be delivered to cartridge **200** by a syringe pump **2130** or can be preloaded onto cartridge **200** or many combinations. In some embodiments, flexible tubing **493** can connect chambers and creates simple pinch valves **491** to direct flow. In other embodiments, channels are created in the cartridge **200** and valves can be incorporated such as pneumatic valves, or other valves.

[00139] **Example: Cartridge with Collapsable Features**

[00140] In a preferred embodiment, referring to **Figure 6**, cartridge **200** performs both a physical and enzymatic dissociation of the tissue. In this example, as shown in **Figure 6A** the cartridge has a cap **210** which can be opened and closed, and a holder **235** for the operator to handle. This embodiment has collapsable features **215** which allow top piece **241** and bottom piece **242** to move relative to the holder **235**. Other embodiments might use a plunger or have fixed walls. The holder **235** can have a mesh screen **225** and grinding features **220** which can be located on the cap **210** and the holder **235**. The grinding features **220** can be solid or allow fluids to pass through. A barcode **240** on holder **235** encodes tracking information of the cartridge **200** and usage. Fluidic connections **230** engage with the instrument to allow the Fluidic Subsystem **600** to add or remove liquids from the cartridge **200**. The Singulation System **100** can have pushbutton operation for either specialists or non-specialists to generate single-cell suspensions **1000** or other nuclei **1050** or other outputs from specimens **101** for medical, health, life science research, and other applications.

[00141] Referring to **Figure 6B**, cartridge **200** has a cap **210** which can be opened in a top piece **241** to allow the specimen **101** to be added into cartridge **200** while securing cartridge **200** by gripping holder **235**. Referring to **Figure 6C**, once the specimen **100** is placed in the cartridge, the cap **210** can be closed or placed on the cartridge to isolate the specimen **101** in cartridge **200** for processing.

[00142] In some embodiments the fluidics of the Singulation System **100** are incorporated onto cartridge(s) **200**. In some embodiments of the Singulation System **100,** the valves for the subsystems are microvalves, which in some embodiments are created in microchips with microchannels. Microvalves are well know to those skilled in the art. Microvalves can be actuated by, for example, mechanical force, pneumatic pressure, electrostatic force, piezoelectric force, thermal expansion force, *etc*. They may be have internal or external actuators. Pneumatic valves include, for example, diaphragm valves that employ a

flexible membrane of the pneumatic pressure or vacuum to close or open a fluid channel. Electrostatic valves may include, for example, a polysilicon membrane or a polyimide cantilever that is operable to cover a hole formed in a substrate. Piezoelectric valves may include external (or internal) piezoelectric disks that expand against a valve actuator. Thermal expansion valves may include a sealed pressure chamber bounded by a diaphragm. Heating the chamber causes the diaphragm to expand against a valve seat.

[00143] In some embodiments, cartridges **200** are used with functionality from a group of reagents, valves or microvalves, microchannels, syringe pumps, optical devices, integrated electronics for control of cartridge **200** functions, and lot tracking. In some embodiments microchips are used as parts of instrument or cartridges **200**. The cartridges **200** in some embodiments hold kits to perform the chemistries including all needed reagents, stains, library preparation chemistry, and other consumables. In other embodiments, the reagents or parts of the reagents are on board the instrument or added manually by the user. In some embodiments the reagents are stablized for long term room temperature storage by freeze drying or the addition of osmoprotectants.

[00144] Referring to **Figure 6D**, in a preferred embodiment, after specimen **101** has been added and the cap closed, cartridge **200** is placed into the Singulation System **100** which engages cartridge **200**. The Physical Dissociation Subsystem **300** engages a top actuator **310** and a bottom actuator **320** with the top piece **241** and bottom piece **242** respectively. The actuators can move up and down (Z direction) and in some embodiments either rotate or move horizonally in the X and Y directions. A thermal post **615** can engage the holder **235** to control the temperature of the cartridge or in other implementations the cartridge is held in a chamber with temperature control or in other embodiments the temperature is not controlled. In some embodiments, a thermal cycling temperature device may be incorporated. In others embodiments, a Peltier may control the temperature of one or more chambers including lowering the temperature. In some embodiments, a barcode reader **510** interrogates barcode **240** to track cartridge **200** used for specimen **101**.

[00145] The Fluidic Subsystem **600** engages with a modular microfluidic connector **620** located on the holder. The modular microfluidic connector **620** are true zero dead volume connectors and can join two or more capillaries to one, or join microchannels to microchips or cartridges, and be used as multiway microvalves. The modular microfluidic connectors **620** can have a linear array of microchannels, such as four, on both sides of the connection. The relative postion of one side of the connection can be moved to line up different sets of microchannels or close off a microchannel as taught in US Patent 7,244,961. In the embodiment taught in this disclosure, the modular microfluidic connectors are used to open and close fluidics to cartridge **200** to take aliquots for measurement, change media, and output the single cells.

[00146] Referring to **Figure 6E**, in one embodiment, the bottom actuator **320** is moved up towards the holder **235** and an enzymatic or chemical dissolution solution **410** can be added into the cartridge **200** through modular microfluidic connection **230** from Fluidic Subsystem **600**. The dissociation solution can be commercially available, such as Liberase™ DH Research Grade Roche or equivalent products, or be custom formulations to dissociate intercellular adhesion between cells and free the cells from the extracellular matrix. The dissociation solutions to dissociate the tissue and prevent clumping of single cells **1000** or nuclei **1050** can be comprised of collagenases (*e.g.*, collagenases type I, II, III, IV, and others), elastase, trypsin, papain, hyaluronidase, chymotrypsin, neutral protease, clostripain, caseinase, neutral protease (Dispase®), DNAse, protease XIV or other enzymes. The amount and concentration of the dissociation solution, time of contact, temperature, and other parameters can be optimized for particular tissue, organism, state of tissue, and age.

As the dissociation solution is added, air can be released through vent **211** which can be an air-permeable, liquid-impermeable membrane.

[00147] **Figure 6F, G, and H** show aspects of the cartridge **200** as it processes the specimen **101**. **Figure 6F** shows the bottom actuator **320** moving down at the same rate the top actuator **310** is moved down, forcing the dissolution solution through mesh **225** and into the bottom compartment of the cartridge **200**. Top actuator **310** can in some embodiments rotate (as illustrated) or move in the x and y axes (not shown) to grind the specimen **101** between grinding features **220**. **Figure 6G** shows the actuators moved up, and **Figure 6H** shows the actuators moved down again with specimen **101** being fragmented into smaller pieces or single cells **1000** and nuclei **1050** released. The timing of the movement of the actuators can be adjusted to the tissue type. The movement can titurate the specimen **101** through mesh **225**.

[00148] At appropriate intervals, aliquots can optionally be withdrawn through modular microfluidic connector **610** or other fluidic connectors or by pipetting, and the number of cells or the viability determined by Measurement Subsystem **500**. In some embodiments, a solution, such as a dye such as trypan blue, can be added to the aliquot to visualize live versus dead cells using a brightfield readout. The solutions can be imaging reagents, such as fluorescently directly conjugated antibodies or secondary antibodies, stains, fluorescent probes and dyes; imaging nanomaterials (including quantum dots and other nanoparticles), or other contrast or straining reagents. Many other compounds can be added if fluorescence or other imaging systems are used. In addition, in some embodiments, Measurement Subsystem **500** does not image the aliquot but measures light scattering or fluorescence of the aliquot to determine the number of cells, or the viability; in some instances, after an initial readout of the fluorescence assay of ATP or other intracellular components, all the cells in the aliquot can be lyzed to determine the total amount of ATP or other intracellular component to create a ratio of the percentage of viability. The Measurement Subsystem **500** can perform multiple readings as required and in some embodiments the amount of grinding, tituration, or enzymatic composition or concentrations can be adjusted based upon the measurements.

[00149] Referring to **Figure 6I**, after the appropriate amount of processing, which can be determined optionally by Measurement Subsystem **500**, both actuators are moved towards holder **235** to push the liquids through modular microfluidic connector and onto a filter **620** such as a 5 μm, or 10 μm, or 20 μm or other filter that retains the singulated cells. In some embodiments, tangential flow filtration is used.

[00150] Referring to **Figure 6J**, Fluidic Subsystem **600** can then deliver media **418** into the cartridge **200** through filter **620** to change the liquid from the dissolution solution **410** to the appropriate media **418** such as Hank's balance salt solution (HBSS) with 2% fetal calf serum or any other buffer, media, or solution. The amount of media **418** can be adjusted based upon the readout by Measurement Subsystem **500** to produce a desired titer such as 1, 10, 100, 1,000 or other numbers of cells/μL.

[00151] Referring to **Figure 6K**, the resuspended single cells can then be output through modular microfluidic connector **230** to strip tubes, Eppendorfs, microtiter plates, or other vessels, or into the next module for a multistage process, such as preparing sequencing libraries.

[00152] In some embodiment, labeling solutions such as antibodies or particles, such as paramagnetic beads, can be added to cartridge **200**. In other embodiments, the Fluidic Subsystem **600** is cleaned of debri, single cells, or tissue fragments by separate cleaning modules that operate in direct contact or non-contact mode utilizing various cleaning mechanisms including, but not limited to, mechanical brushes or chemical

agents comprised of ethanol, alcohols, detergents, non-ionic detergents, surfactants, water, buffers, acidic solutions, basic solutions, and other chemicals.

[00153] In other embodiments, solutions that dissolve the cellular membrane, such as 0.1% Triton X-100 or 0.005% Nonident P40 are added either alone or in combination with physical, chemical, acoustic, enzymatic, thermal, or other methods to produce cellular organelles such as nuclei, mitochondria, ribosomes, long non-coding RNA, or other nucleic acids.

[00154] **Example: Orifice Cartridge.**

[00155] In another example, **Figure 7A** shows a cartridge **200** designed to pass the specimen **101** through orifice **250** to physically disrupt tissue as well as relevant parts of the instrument. In a preferred embodiment, the cartridge **200** has four chambers with an orifice **250** located between chamber 1 **251** and chamber 2 **252** for tissue dissociation, a filter **625**, such as a 70 μm, or 50 μm, or 30 μm or other filter, between chamber 2 **252** and chamber 3 **253** for separation of dissociated cells from rest of the tissue, and filter **630**, such as a 5 μm, or 10 μm, or 20 μm or other filter, between chamber 3 **253** and chamber 4 **254** for removal of enzyme solution as well as debris.

[00156] In one embodiment, five actuators, **325, 326, 327, 328,** and **329** in the instrument engage with plungers **331, 332, 333, 334,** and **335** respectively on the cartridge in a manner to allow them to pull or push the plungers up or down. Piezoelectric pump **626** can access dissolution solution **410** from a reservoir and piezoelectric pump **627** can access media **418** from a reservoir. Strip heater **620** can control the temperature in the cartridge. Waste reservoir **430** collects waste from cartridge **200** as needed. The eye represents an optics imaging system **520** including illuminator and detector.

[00157] In some embodiments, the number of actuators can be one or more. In some embodiments, the actuators are syringe plungers, in others, on cartridge pumps, or off cartridge pressure or vacuum sources. In another embodiment one actuator such as a syringe plunger can move specimen **101** from chamber 1 **251** through orifice **250** into chamber 2 **252** which can have a plunger or be open to atmospheric or other pressure and serve as a reservoir for specimen **101** as it is processed through orifice **250.** In some embodiment a strainer or filter to disrupt tissue or filter single-cells for clumps can be incorporated.

[00158] **Figures 7B-G** illustrates one embodiment of the workflow. In the first step, **Figure 7B**, tissue specimen **120** is placed into chamber 1 **251**, either by removing plunger 1 **331** or through a cap (not shown). **Figure 7C** shows the lid/plunger **331** placed onto chamber 1 **251** and the cartridge **200** inserted into the instrument. Dissolution solution **410** is added to chamber 1 **251** using piezoelectric pump **626** and the solution is optionally heated using strip heater **620** to a constant temperature such as 37°C; Peltiers or other temperature control elements can also be utilized. The specimen **101** can be held at the desired temperature to incubate the specimen **101** for the time required.

[00159] As shown in **Figure 7C**, actuator 1 **325** can push down on plunger 1 **331** and actuator 2 **326** can pull up on plunger 2 **332** to force specimen **101** through orifice **250** which disrupts the tissue and into chamber 2 **252**. This can be reversed, with actuator 1 **325** pulling up and actuator 2 **326** pushing down to move the tissue back through orifice **250** into chamber 1 **251** causing dissociation. The cycle can be repeated as often as desired.

[00160] Referring to **Figure 7D**, both actuator 1 **325** and actuator 2 **326** can be pushed down to force dissociated tissue through filter **625** which separates clumps and non-dissociated tissue from singulated cells **1000** and move the solution into chamber 3 **253**. Strip heater **620** can be turned off. To further move the

[00161] Referring to **Figure 7E**, actuator 3 **327** pushes down on plunger 3 **333** and actuator 4 **328** pulls up plunger 4 **334** to force dissociating solution as well as debris smaller than the pore size of filter **630** into chamber 4 **254** while singulated cells **1000** remain on the chamber 3 **253** side of filter **630**.

[00162] Actuator 4 **328** can then push down on plunger 4 **334** while actuator 5 **329** can pull up on plunger 5 **335** to move the debris and enzyme solution to chamber 5 **255**. Actuator 5 **329** can then push down on plunger 5 **335** to move the debris and enzyme solution to waste **430**.

[00163] Optics **520** can interrogate the solution to determine the titer of single cells **1000** or nuclei **1050** and when desired the viability. Referring to **Figure 7F**, actuator 3 **327** pulls plunger 3 **333** up creating a temporary vacuum while piezoelectric pump **612** pumps media **418** into chamber 3 **253**. Media **418** can be a buffer, such as PBS, HBSS, etc, a solution to process the cell suspension such as red blood cell lysis solution or paramagnetic beads with antibodies, a growth media, an indicator solution or a combination.

[00164] To then change the media or wash the cells, actuator 3 **327** pushes plunger 3 **333** down while actuator 4 **328** pulls up plunger 4 **334** to move the added solution through filter **630** into chamber 3 **253** while singulated cells **1000** remain on the Chamber 3 **253** side of the filter **630**. The solutions can be moved back and forth between chambers 3 **253** and chamber 4 **254** as needed using actuators 3 **327** and 4 **328**.

[00165] These steps illustrated in **Figures 7D, 7E, and 7F** can be repeated to perform additional cycles of either media change, enzymology, or processing, with the cells washed and the filtrate moved into waste **430**.

[00166] Referring to **Figure 7G**, once the cells have been processed as desired, such as resuspension in the media of choice at the desired titer, cartridge **200** can be removed from the instrument and plunger 3 **333** removed and allowing the single cell suspension **1000** to be pipetted out with a device such as a syringe **370**. In other embodiments, the plunger 3 **333** can be pierced by a needle to remove the single cells **1000** to prevent aerosol formation or a cap **210** can provide access to the processed tissue specimen **120**.

[00167] **Example: Automated mechanical device.**

[00168] There are many ways to mechanically disrupt tissue. In one embodiment of a Singulator System **100**, a mechanical device, the AutoSingulator™ **2100,** automated and standardized multipled mechanical disruption methods. In one embodiment the device in **Figure 8** has a z-axis stepper **2110**, a rotary motor **2120**, and a syringe pump **2130** with a six-way valve **2140**, all controlled by control software **725**. **Figure 8** shows the AutoSingulator **2100** testing a-three-chamber cartridge **200** designed for a variable orifice mechanical disruption and mechanical processing with a cap **210** that has a mechanical device inside the cap **210**.

[00169] **Variable Orifice and Cartridge Example.**

[00170] A standard disruption method is trituration, passaging tissue through orifices which can be of successively smaller diameters of fixed orifices, *e.g.,* needles, Pasteur pipettes, *etc.* which works well for some tissues. However, the fundamental problems in developing a cartridge using orifices are incorporating the orifices in series, preventing clogging, and adapting the orifice sizes to the requirements of different tissue types. These problems are all solved by a variable orifice **490** that can be adjusted to create successively smaller orifices on demand or lumens of dfferent sizes.

[00171] As shown in **Figure 9**, an automated variable orifice device **2150** has been designed, built, and tested integrated with the AutoSingulator **2100**. A rotary motor **2155** with an optical sensor **2156** and a cam **2157** can compress a section of tubing **2158**, *e.g.*, 3 mm ID Tygon tubing, to change the internal cross-

section of the tubing to create a variable orifice **490** (obscured by the mechanism). As shown in **Figure 10A**, the automated variable orifice device **2150** has been tested with mouse lung with 0.1% Collagenase I and 0.05% DNAse and has produced viabilities up to 70% and titers of greater than 4 x 10^6 cells from tissue specimens **120** of approximately 100 mg.

[00172] Rotating physical dissociators

[00173] Rotation of one or more surfaces is a method to physically dissociate tissue and methods are herein disclosed. The rotating part can be directly driven by a mechanical coupling or use a magnetic coupling to achieve the rotation. The speed of rotation can be programmed by Control Subsystem **700** for example to 1 ,10, or 100 rotations per minute or other speeds and the direction reversed as desired. In some embodiments, the rotation can create fluid flow which can be exploited to move specimen **101** into grinding or crushing features. The rotating physical dissociators can be combined with features described for the cartridge with collapsable features or the orifice cartridge as will be obvious to one skilled in the art.

[00174] Referring to **Figure 11**, in a preferred embodiment, tissue specimen **120** is inserted into cartridge **200** with a chamber **340** where the physical disruption is performed. After the tissue specimen **120** is placed into chamber **340**, enzymatic or chemical dissolution solution **410** can be added and rotating plunger **336** is inserted, which can be incorporated into cap **210**, and cartridge **200** placed into the Singulator System instrument **100** where an actuator (not shown), that can rotate and move in the z direction, engages with the rotating plunger **336**. In another embodiment, enzymatic or chemical dissolution solution **410** is added through channel **343**. The rotating plunger **336** has grinding features **220** that can be molded or otherwise fabricated as a single piece or can be attached through adhesive **337**. The actuator can rotate rotating plunger **336** to grind tissue specimen **120** on filter **341** which can be comprised of a filter or mesh such as a stainless mesh with 70 μm, 150 μm, 220 μm, or other mesh sizes and is held in place by filter support **342**. The actuator can also push down or pull up on rotating plunger **336** to force tissue specimen **120** through filter **341** or a combination of rotation, and depressing and withdrawing rotating plunger **336** can be used to optimize performance for different tissue specimens **120**. An actuator assembly can incorporate force gauges to provide feedback for the pressure the plunger or actuator is exerting on tissue specimens **120** . In many instances, gentle pressure followed by withdrawal of the force will be appropriate with an incubation period to allow optional enzymatic activity to dissociate the loosened cells and aggregates. As singulated single-cell suspensions **1000** are produced, channel **343** can be used to move single cells **1000** or nuclei **1050** into other sections of the cartridge with functionality to wash the cells, titer, and determine viability.

[00175] **Figure 12** shows another preferred embodiment of rotating disruptor **344** with a grinder rotor **420 (Figure 12A)** and grinder stator **421 (Figure 12B)**. Referring to **Figure 12C**, grinder stator **421** is incorporated (or placed) into the bottom of chamber **340**. Tissue specimen **120** and enzymatic of chemical dissolution solution **410** are added followed by grinder rotor **420** which can be part of cap **200**. An optional stabilizer rod **252** can help maintain rotary alignment between grinder stator **421** and grinder rotor **420**. In the presence of an external rotating magnetic field, magnetic impellers **348** on grinder rotor **420** can propel the grinder rotor **420** to spin and staggered grinding teeth **355** on grinder rotor **420** and grinder stator **421** disrupt tissue specimen **120**. The force on the tissue specimen **120** can be adjusted by moving either the relative position of chamber **340** or magnetic stirrer **349**.

[00176] As shown in **Figure 10C**, a rotating disruptor **344** or grinding device has been attached to the AutoSingulator **2100** and used with Pre-Processing Chambers **440** after incubation for 30 min off device in 0.1% Collagenase I and 0.05% DNAse at 37 °C to disrupt fresh mouse lung to produce single-cells with 90% viabilities anda titer of $1.20x10^7$, sufficient for deep single-cell NGS.

[00177] **Figure 10B** shows pestle **361** attached to the AutoSingulator **2100** with dissociation in an Eppendorf tube**.** The picture on the right side of **Figure 10C** shows the dissociation of mouse liver using pestle **361** method to yield 33% viable single-cells in suspension at a titer of 3.8×10^6. The pestle method may be best suited for very hard tissues or producing nuclei or organelles.

[00178] **Figure 10D** shows plunger **362** attached to the AutoSingulator **2100** with dissociation of mouse lung through a 75 μm filter **341** in a simple cartridge **200**; the right panel shows dissociation of mouse spleen into single-cell suspensions with 70% viability with a 5.67×10^7 titer using this setup.

[00179] Referring to **Figure 13**, an example of a rotating disruptor **344** is shown. Rotating disruptor **341** is placed in chamber **340** and tissue specimen **120** is added. Enzymatic or chemical dissociation solution **410** can be added before or after tissue specimen **120**. The cartridge containing chamber **340** is engaged with the instrument which has a magnetic stirrer **349**. Magnetic stirrer **349** is used to rotate the rotating disrupter **344** using magnet impeller **348** attached to rotating disrupter **344**. As the rotating disrupter **344** spins, spiral features **347** pull tissue specimen **120** into the outside of rotating disrupter **344** where grinding features **346** can disrupt specimen **101** into smaller pieces. In some embodiments, grinding features **346** can have coarse and fine grinding features. In some embodiments, grinding features **346** can function as spiral features **347** to aid circulation of dissociation solution **410** and tissue specimen **120**. As rotating disruptor **344** spins, enzymatic or chemical dissociation solution **410** is pulled down and into central cirulation region **345** where the solution and any pieces of tissue specimen **120** flow up to the top of central cirulation region **345**. The rotation can be adjusted for speed and started and stopped as appropriate. Spiral features **347** can be designed to force the liquid either up and down depending on design intent.

[00180] **Figure 14** shows a rotating disruptor **344** driven by a magnetic stirrer **349** which is elevated with respect to the bottom of chamber **340**. In this example, tissue specimen **120** and enzymatic or chemical dissociation solution **410** are added to chamber **340** and rotating disruptor **341** is placed into the chamber. Magnetic impeller **348** will align to the height of magnetic stirrer **349**. If chamber **340** is moved up or down, magnetic impeller **348** magnets will pull the rotating disruptor **344** up or down to exert adjustable pressure by fine teeth **350** and rough teeth **351** on tissue specimen **120**. Liquid flow induced by rotory motion of rotating disruptor **344** forces smaller tissue fragments into and through the fine teeth **350**.

[00181] **Figure 15A-D** show two examples of rotating disruptors **344** with different pitches of spiral features **347** with large grinding features **346** on the bottom of the rotating disruptors **344**. In this example the gaps in spiral features **347** also can disrupt the specimen **101**.

[00182] **Figure 16A** and **B** show an embodiment where the rotating disruptor **344** is placed above specimen **101** and the liquid motion is induced by spiral features **347** on the outside of the rotating disruptor **344** with recirculation through central circulation region **345**. In addition to disruption as specimen **101** interacts with spiral features **347**, the bottom can be a grinding surface **357**. Magnet holes **356** are shown that hold magnets to create the magnetic impeller **348**. This example did not produce good yields or viabilities with mouse liver tissue. **Figure 16C, D,** and **E** show an embodiment where the rotating disruptor **344** is placed above specimen **101** and the liquid motion is induced by spiral features **347** on the outside of

rotating disruptor **344** and by internal spiral **357** on the inside of rotating disruptor **344** with recirculation through central circulation region **345**. In addition to disruption as specimen **101** interacts with spiral features **347** and internal spiral **357**, the bottom can be a grinding surface **357**. This embodiment can be 3D printed to create the internal spiral **357**. This design worked better than the one shown in **Figure 16A** with better circulation of the specimen **101** through the internal spiral **357** but also did not produce high yields and titers for mouse tissues.

[00183] **Figure 17** is an example of a multiple piece assembly to form rotating disruptor **344**. **Figure 17B** shows external piece **360**. Mesh **358**, such as a stainless mesh with 70 μm, 150 μm, 220 μm, or other mesh sizes, or filters, is inserted into the top of external piece **360** to form the assembly shown in **Figure 17A**. **Figure 17C** shows internal piece **359** which is then inserted and press fit into the assembly to form the completed rotating disruptor **344** shown in **Figure 17D**. Internal piece **359** forms internal spiral **357**. Specimen **101** is added on top of rotating disruptor **344** and the rotation of the mesh **358** and fine teeth **350** disrupt specimen **101** with internal spiral **357** creating circulation to pull the specimen **101** down onto the mesh **358**. The speed of rotation can adjust the force of the pull onto mesh **358**. Larger sizes of mesh **358** can produce larger pieces of the specimen **101**. The fine teeth **350** can be supplemented with larger teeth as desired for different tissues.

[00184] **Cartridge Examples**

[00185] Cartridges **200** have been designed to accommodate multiple mechanical disruption methods—variable orifice, pestle, grinding, and straining—with mechanical transduction in many designs through the cartridge cap **210**. The cartridges can be designed for tissue samples of different sizes, such as ~3 mm^3 or larger and process the tissue in 0.3 to 1.0 mL of liquid, or for tissues < 3 mm^3 and process the tissue in volumes such as < 0.1 mL, or in 0.1 to 0.3 mL, or in greater than 0.3 to 1.0 mL or larger of liquid.

[00186] Referring to **Figure 18**, one embodiment of cartridge **200** has two basic functionalities: one or more Preprocessing Chambers **440**, to produce single-cells **1000** or nuclei **1050** and Processing Chamber **460** to perform the optional additional processing such as magnetic pulldown, red blood cell lysis, or library preparation including on or off-cartridge but on instrument nanodroplet/nanobolus single-cell cDNA processing.

[00187] The embodiment shown in **Figure 18** implements a variable orifice **490** with a three-chamber cartridge: two Preprocessing Chambers **440** pass specimen **101** back and forth through variable orifice **490** in tubing using a syringe plunger designed into cap **210** to create pressure or vacuum to move specimen **101**, and a Processing Chamber **460** that receives the single-cell **1000** or nuclei **1050** suspension which is strained through a 50 μm or other filter **341** as it enters Processing Chamber **460**. Pinch valve **471** is employed to open or close the path to Processing Chamber **460**. In a preferred embodiment, cap **210** can couple the mechanical motion from a Singulator System **100** such as the AutoSingulator **2100** to disrupt specimen **101** such as moving a plunger or spinning a rotating plunger **336**. In a preferred implementation, shown in **Figure 18**, the specimen **101** can be moved by a plunger that is coupled through the cap **210** and actuated by the Z-axis stepper **2130** on the instrument or rotated by the rotary motor **2120** as needed. In other implementations, syringe pump **2130** can deliver fluids to cartridge **200** to move the preprocessed single-cell suspension **1000** or nuclei suspensions **1200**, nucleic acids **1072**, biomolecules **1070**, subcellular components **1060**, or other products from pre-processing.

[00188] **Figure 19** shows a design with a cap **210** that couples a mechanical grinder rotor **420** in the cap **210** using cap coupler **211** to connect to rotary motor coupler **2125** on the instrument. The rotation motion of rotary motor **2120** is transmitted through rotary motor coupler **2125** to cap coupler **211** to rotate mechanical grinder rotor **420** in either direction while the vertical position of grinder rotor **420** is controlled by z axis steper **2110**. In some embodiments the grinder rotor **420** only travels downward to the bottom of the chamber while in others as described below it can be retracted by mechanisms such as magnetics, springs, or mechanical coupling.

[00189] **Figure 20** shows seven patterns of matching rotary grinders **420** and grinder stators **421** that have been designed and built. In add designs, the teeth on the rotary grinders **420** and grinder stators **421** are complementary and arranged in circular rows. **Figure 20A** has a set with three rows of equally sized teeth with sharp edges**. Figure 20B** has a set with five rows of successively smaller width of teeth as the center is approached on the rotary grinder **420** and the grinder stator **421**. **Figure 20C** has a set with three rows of equal sized sharp on the rotary grinder **420** and the grinder stator **421**. **Figure 20D** has a set with four rows of equal sized pointed 'shark's teeth' on the rotary grinder **420** and the grinder stator **421**. **Figure 20E** has a set with two rows of equal sized blunt 1.5 mm teeth on the rotary grinder **420** and the grinder stator **421**. **Figure 20F** has a set with two rows of equal sized blunt 1.0 mm teeth on the rotary grinder **420** and the grinder stator **421**. **Figure 20G** has a set with two rows of equal sized blunt 0.5 mm teeth on the rotary grinder **420** and the grinder stator **421**. The design in **Figure 20G** was used extensively and generated the highest titer and viability for the most types of tissues. In some embodiments, the spacing between the outside of the rotary grinder **420** and the grinder stator **421** is set to be 15 20, 25, 30, 35, 40, 45, or 50 μm or other spacing such that in some embodiments only single cells or nuclei can pass between the rotary grinder **420** and the grinder stator **421.**

[00190] **Figure 21** shows a cartridge **200** embodiment with a Pre-Processing chamber **440** with the grinder stator **421** and a mechanical grinder rotor **420** (with the **Figure 20G** design) connected through rotary motor coupler **2125** to rotary motor **2120** of the AutoSingulator **2100.** Rotary motor **2120** can control the rotation of the grinder rotor **420** against fixed stator **421** in the bottom of the Pre-Processing chamber **440** and the Z axis stepper **2110** controls the vertical position of grinder rotor **420**. The AutoSingulator **2100** delivers fluids, *e.g.,* enzymatic or chemical dissolution solution **410,** from syringe pump **2130** and six-way valve **2140** through Sweeney filter holder **347** holding a filter **341**to a reagent addition port **470** in Pre-Processing Chambers 440 through and can pull the pre-processed specimen **101** through Sweeney filter holder **347** holding a filter **341** such as a 70 μm filter. The setup in **Figure 21** has been tested for the singulation of cells and for the production of nuclei.

[00191] **Examples of tissue-specific workflows for cell singulation and nuclei production using the AutoSingulator**

[00192] **Figure 22** shows the results of producing single cells **1000** in suspension from five different mouse tissues using AutoSingulator **2100** setup as shown in **Figure 21.** A fresh tissue specimen **120** was placed in Pre-Processing Chamber **440** and positioned below the grinder rotor **420** attached to the AutoSingulator **2100**. Control software **725** then lowered the grinder rotor **420** into the Pre-Processing Chamber **440.** Dissolution enzymes added by syringe pump **2130** from 15 mL Falcon tubes to reagent addition port **470** on cartridge **200.**

[00193] Preferred embodiments for formulation of enzymes for each mouse tissue were used in HBBS without Ca or Mg. For lung 0.1% Collagenase II with 5u/mL Dispase and 0.03% DNase was used; for kidney 0.05% Collegenase I with 0.075% Papain and 0.03% DNase was used; for spleen, 0.05% Collagenase I with 0.03% DNase was used; for liver, 0.1% Collagenase IV with 0.05% Hyaluronidase and 0.03% DNase was used; for brain, 0.2% Papain with 0.03% DNase was used; and for gut, 0.1% Collagenase I with 0.025% Hyaluronidase and 0.03% DNase was used.

[00194] The operator then lowered grinder rotor **420** until it contacted the tissue. The rest of the operation was automated. The tissue specimen **120** was incubated for 30 min with the the grinder rotor **420** moved up and down every 5 min to mix. After a 30 min incubation at room termperature, the grinder rotor **420** was rotated seven times forward and seven times backward at 75 rpm against the tissue specimen **120** with fixed stator **421** in the bottom of the Pre-Processing Chamber **440**. The grinder rotor **420** was moved by the Z stepper **2110** about 200 μm down and the process repeated six to seven times until the grinder rotor **420** reached the bottom of the Pre-Processing Chamber **440** which had grinder stator **421**. When the grinder rotor **420** reached the bottom of Pre-Processing Chamber **440**, the dissociated sample was displaced through reagent addition port **470** through a 100 μm filter **341** held in a Sweeney filter holder **347** followed by a rinse with 3 mL of HBSS delivered backflushing through the 100 um filter **341** held in a Sweeney filter holder **347** into reagent addition port **470** and withdrawn back thorugh100 um filter **341** held in a Sweeney filter holder **347** and the output collected. The samples were centrifuged at 300 g for 5 min, the supernatant discarded, and the cells resuspended in RBC lysis buffer (G-Biosciences) for 3 min and then centrifuged at 300 g for 5 min and the pellet resuspended in 1 mL of HBSS. The viability and titer were determined on a Countess FL using Trypan blue. As shown in **Figure 22A**, the single cells had viabilities ranging 72 to 92%, and as shown in **Figure 22B** with yields of 2×10^6 to 8.3×10^6 cells for tissue specimens **120** from left to right of 125 mg of kidney, 65 mg of lung, 110 mg of lung, 70 mg of spleen, l70 mg of spleen, 180 mg of liver, and 160 mg of gut. The label of integrated on the X axis indicates that the complete process after lowering the grinder rotor **420** was performed by control software **725.**

[00195] **Figure 23** shows the production of nuclei **1050** from six mouse tissues, gut, kidney, lung, liver, brain, and spleen, using the AutoSingulator **2100** setup as shown in **Figure 21.** Tissue specimen **120** was added to the Pre-Processing Chamber **440** and then the Pre-Processing Chamber **440** was inserted under the grinder rotor **420** which was lowered by the software. The program then delivered Nuclei Homogenization Buffer (250 mM Sucrose, 25 mM KCl, 5mM $MgCl_2$, 10 mM Tris-HCl, and 0.1% Triton-X) reagent from a 15 mL Falcon tube using syringe pump **2130** through sweeney filter holder **347** to reagent addition port **470** on cartridge **200.** The AutoSingulator **2100** then immediately rotated grinder rotor **420** seven times forward and seven times backward at 75 rpm. The grinder rotor **420** was moved by the Z stepper **2110** 200 μm down and the process is repeated six to seven times until the grinder rotor **420** reached the bottom of Pre-Processing Chambers **440** which contained grinder stator **421**. When the grinder rotor **420** was at bottom of the chamber, the dissociated sample was displaced through reagent addition port **470** and through a 30 um filter **341** held in a Sweeney filter holder **347** followed by a rinse with 1 mL of Nuclei Homogenization Buffer delivered backflushing through the 30 um filter **341** held in a Sweeney filter holder **347** into reagent addition port **470** and withdrawn back thorugh 30 um filter **341** held in a Sweeney filter holder **347** and the output collected. The samples were then centrifuged at 500 g for 5 min at 4°C and the supernatant discarded. The nuclei **1050** pellet was resuspended in ice-cold Nuclei Storage Buffer (166.5

mM Sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl) and the titer determined on a Countess FL with Trypan blue staining. As shown in **Figure 23**, with tissue specimens **120** from left to right of gut, kidney, kidney, lung, lung, liver, and brain, the most tissues had yields of 1.7×10^5 to 4.4×10^5 nuclei per mg of tissue input while spleen yielded 1.6×10^6 nuclei per mg of tissue input (note the spleen sample is a 1:10 dilution to enable plotting on the same chart). Similar results were obtained when 0.1% Nonident P40 was used in place of the Triton-X.

[00196] Example: processing of small tissue samples.

[00197] Many tissue samples are only present in small amounts, such as core biopsies or fine needle aspirates, where 5 to 25 mg of tissue may be obtained. The AutoSingulator **2100** was shown to be able to process these small samples effectively using the setup as shown in **Figure 21** for the production of both single cells **1000** and nuclei **1050**.

[00198] **Figure 24** shows the results of processing a range of masses of four tissues to produce single cells **1000**. The processing was as described above for **Figure 21** except the filtration was performed manually using a 70 μm filter. Lung, kidney, and gut tissues from 100 mg to 20 mg had viabilities of 70-94% for most samples (**Figure 24A**) with yields over 10^5 viable cells per 5 mg of tissue (**Figure 24B**). The viability was similar until the mass was ~10 mg or less when the viability decreased. While the 5 mg specimens gave enough cell titer for downstream processing, the viabilities were low, indicating damage. Liver had generally lower viabilities and lower titers. The results demonstrate the cartridge **200** and AutoSingulator **2100** as shown in **Figure 21** can process tissue samples as small as 5 mg to produce single cells **1000** for single cell sequencing or cell biology or other applications.

[00199] **Figure 25** shows the results of processing a range of masses of four tissues to produce single nuclei **1050**. The processing was as described above for **Figure 23** except the filtration was performed manually using a 30 μm filter. The titers obtained were generally from1.5 to 4.5 $x10^5$ nuclei per mg of input tissue, yielding over 1,000,000 nuclei from a 5 mg tissue specimen **120** for most samples, ample for downstream single nuclei sequencing.

[00200] The results shown in **Figures 23** and **24** demonstrate the utility of the Singulator System **100** for processing tissue specimens **120** into single cells **1000** and nuclei **1050** for small samples such as core biopsy samples or fine needle aspirates. These results show the Singulator System **100** can be applied to prepare clinical samples for single cell NGS and single nuclei NGS applications.

[00201] Quality control metrics.

[00202] Speed, yield, viability, and cellular damage are key first QC metrics for high quality, reproducible workflows. As manual and automated disruption methods are refined, after initial screening, additional quality metrics of qPCR of IEG and other transcripts, RIN determination using capillary electrophoresis, and single-cell NGS can be used as more sophisticated metrics.

[00203] It is important to identify enzymatic methods to produce single cells **1000** or nuclei **1050** with minimal alterations of gene expression as a major improvement to the state-of-the-art. Combinations of less digestive enzymes into formulations with less cellular reactions can be tested. Additives can help freeze the state of the cell, such as transcription, membrane, or other inhibitors, to prevent clumping, and to preserve RNA.

[00204] **Figure 26** shows examples of where two different processing methods to extract RNA have either induced the IEG gene *fos* or not induced it. In this experiment, mouse lung was pre-processed using

rotating disruptor **344**, as shown in **Figure 21** after incubation for 30 min at 37°C in lung dissociation kit solution (Miltenyi 130-095-927). After the production of single cells **1000** in suspension, the cells were spun down at 300 g for 5 min and resuspended in HBSS. Aliquots of 10 μL were either lysed to release RNA by addition of 100 μL of the Extracta DNA kit (QuantaBio) with incubation at 95 °C for 30 min followed by addition of 100 μL of stabilization buffer or by addition of 200 uL CL buffer (10 mM Tris pH 8, 0.025 % Igepal CA-630 (Sigma 18896-50), 150 mM NaCl) (adapted from K. Shatzkes, B. Teferedegne, and H. Murata. A simple, inexpensive method for preparing cell lysates suitable for downstream reverse transcription quantitative PCR. Sci Rep. 2014; 4: 4659. PMCID: PMC3983595) with incubation for 5 min at room temperature. 1 μL of each sample was added to 5 μL of qScript XLT One-Step RT-PCR Tough Mix, ROX reagent (QuantaBio) with 4.5 μL of molecular biology grade water, and 0.5 μL of *actB* primer (Thermo Fisher, m01205647_g1) or 0.5 μL of *fos* primer (Thermo Fisher, Mm00487425_m1).

[00205] **Figure 26** shows *actB* was expressed in spleen for both treatments (E *actB* was the Extracta processed sample with *actB* primers and CL *actB* was the CL buffer processed sample with actB primers). For the IEG *fos* gene, the lysis using the CL buffer induced *fos* while the Extracta kit processing did not. Because the Extracta kit shows no induction of *fos*, the production of single cells in suspension **1000** by rotating disruptor **344** did not induce *fos*, demonstrating the method is gentle with this enzymatic processing.

[00206] **Singulator System embodiment**

[00207] In one embodiment of the Sample Processing System **50** as a Tissue Processing System **80**, as shown in **Figure 27,** the Singulator System **100** can perform powerful integrated tissue-to-genomics functionality for genomic scientists to simply and standardize the production of single-cell **1000** or nuclei **1050** suspensions, affinity purified single cells **1100,** affinity purified nuclei **1105** , nucleic acids **1072**, and bulk libraries **1210** from solid or liquid tissues. As will be obvious to one skilled in the art, the single cells **1000** and nuclei **1050** can also be used for cell biology, proteomics, metabolomics, and other analytical methods.

[00208] In this preferred embodiment a Cell Singulation module **800** and a Magnetic Processing module **900** are integrated into a Single-Sample Singulator System **2000** or into a Four-Sample Singulator System **2400**. Mechanical and enzymatic dissociation is performed in single-use cartridges **200** in the Pre-Processing chamber **440** to produce single-cell suspension **1000** or nuclei suspensions **1200**, nucleic acids **1072**, biomolecules **1070**, subcellular components **1060**, or other products from pre-processing. The samples can then be processed in the Processing chamber **460** by optional bead-based affinity purification of cell types by surface antigens to produce affinity purified single-cell suspensions **1100** or nuclear suspension by nuclear antigens **1105** or nucleic acids **1072**, biomolecules **1070**, subcellular components **1060** can be further processed into purified mRNA, NGS libraries, or other sample types.

[00209] To accomplish this, in a preferred embodiment, a Single-Sample Singulator System **2000** was designed with reagents **411** on-board the instrument and with cartridges **200** potentially with tissue-specific mechanical disruption modalities to accommodate the wide diversity of processing needs. The system can input raw, unprocessed tissue samples and output single-cells **1000** or nuclei **1050** in suspension, ready for processing into single cell NGS libraries off device or can process the single cells **1000** or nuclei **1050** into bulk libraries on the system.

[00210] **Example: A Single-Sample Singulation System.**

[00211] The Singulator System**100** can mechanically disrupt tissue and enzymatically dissociate the disrupted tissue in a cartridge **200** into single-cells **1000** or nuclei **1050** in suspension. As shown in the top of **Figure 27,** in one embodiment, a Cell Singulation module **800** combines the Physical Dissociation Subsystem **300** and the Enyzmatic and Chemical Dissociation Subsystem **400** to produce single-cell **1000** or nuclei **1050** suspensions. The instrument provides the mechanical motion and fluidics to the cartridge which in turn mechanically and enzymatically or chemically processes the tissue into single cells **1000** or nuclei **1050**. Multiple reagents **431** can be stored on the instrument with cooling as needed.

[00212] The Cell Singulation module **800** as shown in **Figure 28** combines the mechanical disruption of specimen **101** on cartridge **200**, adds enzymatic or chemical dissolution solution **410** and other fluids according to the protocols, and controls sample movement, pressures, and temperature. The Cell Singulation module **800** can move or rotate a syringe plunger, pestle, or grinder, using a z axis stepper **2110** with a rotary motor **2120** coupled through the cap **210**.

[00213] Referring to **Figure 27**, magnetic purification of cell types and nuclei using affinity capture reagents attached to paramagnetic beads using Magnetic Processing module **900** integrates the capabilities to produce affinity purified cell types **1100** and affinity purified nuclei **1105** or other organelles comprised mitochondria, transcription complexes, nucleosomes, ribosomes, and other subcellular structures starting from tissues or specimen **101**.

[00214] A 3D CAD representation of one embodiment of a Single-Sample Singulator System **2000** design packaged with a 'skin' is shown in **Figure 29**. The design is based upon the AutoSingulator **2100** and has two axis mechanical motion (Z axis stepper **2110** and rotary motor **2120**) integrated with fluidics based on a syringe pump with 1.6 μL resolution with a six-way valve (C2400MP, TriContinent) controlled by control software **725**. A small (~16 in^3) OEM computer **720** with Windows 10 and 85 Gbytes HD (Beelink, AP42) can run control software **725** to control the system with display on a 10" touchscreen **730** (eleduino, Raspberry Pi10). Chassis **1010** provides the framework to mount components and the exterior of the system.

[00215] This embodiment of the Single-Sample Singulator System **2000** has one syringe pump **2130** with a six-way valve **2140** to supply liquids, pressure, or vacuum to cartridge **200**. The cartridge **200** shown is similar to **Figure 18** with two Pre-Processing Chambers **440** and a single Processing Chamber **460**. Cartridge valves can be pinch valves **491**, which the instrument actuates, or other valves or have no valves on the cartridge **200** with all fluidic control from the instrument. A second six-way valve **2141** can access reagents (such as four digestive enzymes, magnetic beads, three buffers, two cleaning solution if two six-way valves **2140** are used) in small bottles through connecting tubing such as 1/16 ID tygon tubing or other tubing, capillaries, or fluidic lines. Actuators (not shown) can open and pinch close tubing in the cartridge **200**, and operate the variable orifice **490** using variable orifice device **2150** when desired. Two strip resistive heaters or Peltiers and controllers (not shown) can set the cartridge temperature in the Pre-Processing Chamber **440** and Processing Chamber **460**. A force gauge can be incorporated into the z-stage stepper **2110** to provide force-feedback control of the mechanical force on the specimen **101**; this can help develop very gentle mechanical processing steps. The interface with the cartridge **200** can be standardized by the development of a cartridge adapter that will be designed with each cartridge **200** to ensure simple insertion into the system. Reagents **411** are held in a ragent block **415** which can be cooled with a Peltier to minimize

degradation of reagents **411**. This embodiment of the single-sample Singulator System **2000** has a Magnetic Processing Module **900**.

[00216] Magnetic Processing module.

[00217] Referring to **Figure 30**, the Magnetic Processing module **900** can perform magnetic bead enrichment or depletion of cell types or organelles, capture nucleic acids to change buffers, and integrate downstream enzymatic workflows in Processing Chamber **460** or other locations such as in channels or tubing. Beads and wash solutions can be delivered to the Processing Chamber **460** from reagents **411** using six-way valve **2141** via a six-way valve **2140** in turn connected to syringe pump **2130**. While nanoparticles stay in solution, larger beads will settle out. This can be solved by resuspension of beads, *e.g.*, 1- 30 μm beads, using a rapid mechanical vibration of the bead container.

[00218] A single-sample Magnetic Processing module **900** can have a motor **930** moving a magnet **910** such as a neodymium magnet on an arm **920** to capture paramagnetic beads inside Processing Chamber **460** when the arm is in the horizontal position as shown or release the beads when it is in the raised vertical position (shown in a dashed outline). It will be obvious to one skilled in the art that many other configurations are possible, with in some embodiments magnet **910** moving in a linear fashion, or in a circle, or other geometries. Features can be incorporated into the cartridge **200** to improve magnetic processing performance such as having a region with a smaller distal distance to increase the magnetic field locally to improve bead capture. The field of the magnet **910** can also be directed by blocking certain regions with non-magnetic materials and enhanced in other areas of the field. Sensing devices such as optical sensors or magnetic sensors can be implemented for positional feedback. Motor **930** can be controlled using Control Subsystem **700**. Magnetic fields can also be produced using electromagnetic coils.

[00219] The Magnetic Processing module **900** can capture and purify cell types from eukaryotic, prokaryotics, or archea. Following creation of a single-cell suspensions, antibodies against cell surface components or other targets coupled to nanoparticles or paramagnetic beads, using standard coupling chemistries or commercially available beads with antibodies to cell surface proteins, can be added from reagents **411** by syringe pump **2130** and six-way valves **2140** and **2141** to Processing Chamber **460** containing the single-cell suspensions. Mixing can be by bubbling air through the Processing Chamber **460** or application of a 'stirring' magnetic field, or use of fluidics to agitate the single-cell suspensions and beads, or moving the sample with beads back and forth in tubing or channels, or between the two Pre-Processing Chambers **440** or other methods well known to one skilled in the art. The antibodies or other affinity agent will then bind target cells with the target antigen. The Magnetic Processing module **900** can move arm **920** to the horizontal position with magnet **910** positioned at the Processing Chamber **460**. The beads with captured cells, including from a media **418** such as containing enzymes or chemicals used to dissociated specimen **101,** are in turn captured by magnet **910**. The media **418** can be pumped out by syringe pump **2130** and as desired captured beads can be washed to remove cell debris, enzymes, buffer, and uncaptured cells with reagents **411** or to change buffers. The Magnetic Processing module **900** can then move arm **920** to the vertical position with magnet **910** positioned away from the Processing Chamber **460** to release the beads captured by the magnetic field of magnet **910**. The beads are then attached to single-cell suspensions that are now affinity purified for a desired subtype or depleted for a cell-subtype **1100**. Resuspending the beads in a different buffer or media **418** is an effective way to change buffer. It will be obvious to one skilled in the art that the beads can be used to deplete cell types, debris, or other material

from a cell suspension by holding the beads on the magnet and moving the now depleted fluid to a different chamber or output it to a tube or other device.

[00220] The Magnetic Processing module **900** can capture and purify nuclei **1050** or other subcellular components **1060** including organelles from eukaryotic organisms. Following creation of a suspension of nuclei **1050**, antibodies against nuclear surface components, such as biotinylated anti-nuclear protein *NeuN* antibodies or other nuclear targets, can coupled to nanoparticles or paramagnetic beads, using standard coupling chemistries such as streptavidin nanoparticles or commercially available beads with antibodies to nuclear proteins, can be added by syringe pump **2130** to Processing Chamber **460** containing the nuclei **1050** suspension. Mixing can be by bubbling air through the Processing Chamber **460** or application of a 'stirring' magnetic field, or use of fluidics to agitate the single-cell suspensions and beads, or moving the sample with beads back and forth in tubing or channels or other methods well known to one skilled in the art. The antibodies or other affinity agent will bind nuclei **1050** or other target organelles with the target antigen(s). The Magnetic Processing module **900** can move arm **920** to the horizontal position with magnet **910** positioned at the Processing Chamber **460**. The beads can then capture the nuclei **1050** bound to beads, including from a media such as containing chemicals or enzymes used to dissociated specimen **101** into nuclei **1050.** The captured nuclei, now bound to the beads, can be washed to remove cell debris, enzymes, buffer, and other unbound components or to change buffers. The Magnetic Processing module **900** can then move arm **920** to the vertical position with magnet **910** positioned away from the Processing Chamber **460**, which will release any beads captured in the magnetic field of magnet **910**. The beads are attached to affinity purified nuclei suspensions **1105**.

[00221] The Magnetic Processing module **900** can also be applied to process single cells **1000** or nuclei **1050** or tissue specimens **120** into nucleic acids **1072** and to further process the nucleic acids **1072** into bulk libraries. Tissue specimens can be lysed in Pre-Processing Chamber **440** by addition of chaotrophs, as described below, and the lysate can be strained through strainer **450**, and moved into Processing Chamber **460**. The lyzed tissue is then processed by magnetic beads to purify nucleic acids **1072**. In another embodiment, the single cells **1000** or nuclei **1050** in Processing Chamber **460** are lysed by addition of chaotrophs processed with magnetic beads to purify nucleic acids **1072**. In some embodiments, the purified nucleic acids **1072** are further processed into bulk libraries **1210** as described in **Figures 44**, **45**, and **46** or other librarian methods well known to one skilled in the art.

[00222] **Control Subsystem**

[00223] The systems are controlled by a Control Subsystem **700** that uses control software **725** to control electronics **710** that actuates modules and devices. Control software **725** runs on a computer **720** which can be a standalone computer **725** or a tablet **750**. The control software **725** is a rapid development software platform designed to accelerate development and commercialization. The software has support for IoT-based protocols, cloud-based protocols, Microsoft development tools and libraries, and machine learning technologies. The control software **725** Host provides a standardized scripting interface to develop, maintain, and run scripts, with a range of utilities to allow scripts to interact with the user and to interoperate with other software.

[00224] Control software **725** scripts are coded in any .Net languages and compiled to standardized DLL's; other languages are within the scope of the present invention. Once the scripting logic is developed,

the scripting host layer is replaced with a dedicated executable that references the same DLL and that executes the script, dramatically shortening the development cycle.

[00225] The control software **725** Library has precompiled DLL's that provide critical functionalities including scripting interface and base libraries, support for ZMQ and other IoT libraries for intercommunication, real-time scheduling engine for autonomously optimized non-deterministic scheduling of operations, databasing, image analysis, statistical analysis, data storage, and HDF5 numerical storage libraries. Supported hardware components include: a) pumps and valves using Cavro communication protocols, b) Tecan RSP/MSP robots, c) motor controllers and I/O devices (quadrature encoders, optical sensors, *etc.*), d) RS232, RS485, USB HID, and other generic interface devices, e) CAN devices using the KVASER™ Communication Library, f) LabSmith µProcess devices, including micro-pumps, valves, and pressure sensors, and g) Arduino based devices. Control software **725** can support hardware device added and integrate overall protocols through scripting. Other software can be substituted for the control software **725**.

[00226] **Example of another preferred embodiment of a single-sample Singulator System**

[00227] **Figure 31** shows the main functional elements of another preferred embodiment of single-sample Singulator System **2000,** the enclosure, fluidic, and electrical wiring are not shown on the figure for clarity. The fluidic circuits with a cartridge 200 are shown in **Figure 34** and the electronics in **Figure 35.**

[00228] Referring to **Figure 31**, the main functional parts are the fluidic subsystem **600** comprised of cartridge **200** inserted on cartridge slide **1450**, with temperature control by cartridge Peltier **1440**, the instrument fluidics comprised of syringe pump **2130** with six-way valve **2140**, two six-way valves **2141** and **2142,** to access reagents **411** held in reagent block **425** with temperature regulation by reagent peltier **1420**, and to connect to cartridge **200**, and mechanical motion by rotary motor **2120** and z axis stepper **2110**, with movement of magnet **910** by magnetic motor **930**.

[00229] **Example of processing a sample in a cartridge using a single-sample Singulator System**

[00230] **Figure 34** shows a design of a cartridge that incorporates a cap **210** that couples a mechanical grinder rotor **420.** The cap is inserted into a Pre-Processing Chamber **440** which has a grinder stator **421** at the bottom of the chamber. The process to utilize this embodiment of cartridge **200** is as follows.

[00231] **Figure 32** shows the detail of the cartridge interface **1500** with cartridge **200** on mechanical cartridge slide **1450** in cartridge detent **1455**. The operator inserts tissue specimen **120** into cartridge **200** and then cap **210** is placed on cartridge **200**. In some embodiments the cap **210** is keyed to only be placed in certain orientations and can lock onto cartridge **200** permanently, preventing inadvertant spillage of tissue specimen **120** or release of material from cartridge **200** during or after processing.

[00232] In a preferred embodiment, cartridge **200** and the cartridge interface **1500** have features for 'click-in docking' to the instrument and self-aligning connections to the instrument fluidic system. In the embodiment shown in **Figure 32**, cartridge **200** on cartridge slide **1450** is pushed into the instrument. Side brackets **1460** and slide **1450** align the cartridge **200**. When the slide **1450** is fully inserted, a mechanism such as a retractable ball socket **1462** engages with hole and locks cartridge **200** in the proper position against thermal transfer plate **1470** and docks cartridge ports **470**, **484**, **485**, and **486** with and spring -loaded fluidic connections **1410** such as 'pogo pins' **1415** or modular microfluidic connectors or other connectors **1417** with spring forces of, for example, 2 kg.

[00233] The operator can select a program to process tissue specimen **120** through user interface **740** on touchscreen **730** of tablet **750**. As shown in **Figure 33**, the operator can enter on user interface **740** a sample number and name, and select attributes such as the tissue type, organism (hidden behind dropdown menu), condition of tissue (*e.g.,* normal, cancerous, degraded, *etc.*), and to run a pre-programmed script. Other fields can be filled automatically by the software or a LIMS system such as date, cartridge ID (from a barcode), system ID, and software version.

[00234] **Figure 33** is an illustrative example of one of many embodiments of the user interface 740. In some embodiments, the operator can adjust the processing enzymatic or chemical solution for their tissue, or the processing time, temperature, or details of the mechanical disruption, or other attributes of the preprocessing and any processing steps such as magnetic bead purifications or enyzmatic reactions such as library preparation, PCR amplification, or other molecular biology, chemistry, or quality control steps. In some embodiments, Singulator System **100** can monitor the processing and adjust the processing according to rules set by the operator or preprogammed into control software **725**. Once the operator has selected the appropriate conditions and attributes for the tissue specimen **120**, the operator will direct the control software **725** to run and the instrument can run automatically.

[00235] Referring to **Figure 35**, control software **725** runs on computer **720** which in this embodiment is a tablet **750**. The figure shows power lines as heavy solid lines, control lines to electronics as light lines, and the hardware devices as dashed lines. This embodiment is supplied power (shown in heavy solid lines) from 24V DC power supply **2220** which drives 5V DC step down **2225** and 12 V DC stepdown **2230** as well as stepper driver board **2114**, heater relay board **2250**, and syringe pump and six-way valve controller **2131** with power daisy-chained to six-way valve controller **2210** and six-way valve controller **2212**. 5V DC step down **2225** powers computer **720** which in turn powers controller **2112** and controller **2122**. 12 V DC stepdown **2230** powers DC motor board relay board **2132**, DC motor board relay board **2134**, and heater relay board **2240**.

[00236] Computer **720** controls all devices. It has direct connections to syringe pump and six-way valve controller **2131** which in turn controls syringe pump **2130** and six-way valve **2140;** six-way valve controller **2210** which in turn controls six-way valve **2141**, and six-way valve controller **2212** which in turn controls six-way valve **2142**. Computer **720** connects to controller **2112** (control line not shown) which controls stepper driver board **2114** which in turn drives z-axis stepper **2110**. Computer **720** connects to controller **2114** (control line not shown) which controls DC motor relay board **2132** which drives magnetic motor **930**, DC motor relay board **2134** which drives rotary motor **2120**, heater relay board **2250** which drives reagent Peltier **1420**, and heater relay board **2240** which drives cartridge Peltier **1440**. Many other embodiments are within the scope of the invention and are obvious to one skilled in the art.

[00237] Referring to **Figure 34,** enzymatic or chemical dissolution solution **410** is delivered to Pre-Processing Chamber **440** from the appropriate reagent reservoir **2143** for example R1 through tubing **2163**, six-way valve **2140** using syringe pump **2130** through tubing **261** connected to six-way valve **2141** to line **495** to reagent addition port **470** on cartridge **200**. The temperature of cartridge **200** is controlled by cartridge Peltier **1440** by tablet **750**. The temperature of Pre-Processing Chambers 440 may be set to 37°C for processing tissue specimens **120** into cells and 4°C to process tissue specimens **120** into nuclei **1050** or other temperatures as the operator desires.

[00238] Using Z-axis stepper **2110,** rotary motor coupler **2125** is lowered until it engages with cap 2**10.** In some embodiments the engagement is using a locking mechanism such as a crown shaped coupler **2126** shown in **Figure 36**. In a preferred embodiment two magnets **2128** are located on rotary motor coupler **2126** and two magnets or a bar of ferromagnetic material **2129** on cap **210** is attracted; this embodiments rotationally aligns cap **210** and rotary motor coupler **2126**. In another embodiment rotary motor coupler **2126** has a single embedded permanent magnet **2128** which attracts a magnet or ferromagnetic material **2129** including iron, nickel, cobalt, or other materials. In some embodiments this attraction is used to raise grinder rotor **420** after it has been depressed. In other embodiments, cap **210** has a spring inside the cap to raise grinder rotor **420** when desired when Z-axis stepper **2110** is raised. Other mechanism such as using a hex shaped coupler with a matching hexagon receptor on cap **210** with a spring load, or a gripping chuck, or many other configurations are possible without limitation.

[00239] When the cap **210** has been engaged by the rotary motor coupler **2125** and the appropriate enzymatic or chemical dissolution solution **410** has been delivered to Pre-Processing Chamber **440**, Z-axis stepper **2110** can move rotary grinder **420** down. In some embodiments a force sensor monitors the force used to move rotary grinder **420** down to ascertain when tissue specimen **120** is encountered. In some embodiments rotary motor **2120** is rotating as Z-axis stepper **2110** is lowered and the current draw is monitored to to ascertain when tissue specimen **120** is encountered. In other embodiment, rotary grinder **2120** is moved to a position without any force feedback. Rotary motor **2120** can be actuated and the tissue disrupted according to the desired program for the tissue.

[00240] The program may include many variations of moving in one direction, then reversing direction. In some versions, the grinder may move downward during the grinding and then move upwards to relieve pressure on the tissue. In some embodiments rotary grinder **420** can be rotated at slow speeds such as 25, 50, or 75 rpm or slower; in other embodiments at speeds such as 100, 200, 500, or 1,000 rpm or more. The speed of the rotation can be changed or the direction reversed and the position controlled by Z-axis stepper **2110**. In other embodiments, cartridge **200** has a plunger, variable orifice, pestle or other mechanical disruption device.

[00241] The Pre-Processing chamber **440** can be temperature controlled by thermal transfer plate **1470** which is controlled by cartridge Peltier **1440**. In many cell singulation protocols, the tissue is incubated at 37°C for 30 min and for many nuclei **1050** protocols, the tissue is incubated at 4°C. The system can accommodate a wide range of temperatures, incubation times, and mechanical disruption protocols.

[00242] Referring to **Figure 34**, after mechanical and enzymatic disruption, the rotary grinder **420** can be moved to the bottom of Pre-Processing Chamber **440** which displaces the now dissociated tissue specimen **120** to the level of outlet port **471**. Vacuum can be applied to waste chamber **431** using syringe pump **2130** through six-way valve **2140** and line **2161** through six-way valve **2141** and line **498** to upper port **486** of waste chamber **431** of cartridge **200**; the vacuum in waste chamber **431** pulls through line **477** connected to port **478** and port **479** to Processing Chamber **460** and line **475** connected to port **476** and port **474** on strainer **450**, through line **472** connected to outlet port **471** and port **473** to pull the now dissociated tissue specimen **120** through line **472** into strain chamber **450** and through a filter **341,** such as a 70 μm or a 50 μm or 30 μm or other filter, to remove clumps of cells **1000**, nuclei **1050**, or debris. The filtered sample is then pulled by the vacuum through port **474** and line **475** and port **476** into Processing Chamber **460**. The amount of vacuum can be minimized to lessen shearing by filter **341** if desired. For the production of single

cells **1000** and nuclei **1050**, the user can remove Processing Chamber cap **465** and pipette out the processed sample.

[00243] Waste chambers **431** and **432** are designed to be connected to the top and the bottom of Processing Chamber **460** respectively. This allows waste chamber **432** to withdraw liquid from the bottom of Processing Chamber **460** when vacuum is applied while waste chamber **431** will not withdraw liquid from Processing Chamber **460** in most circumstances. Waste chambers **431** and **432** can optionally contain a liquid absorbent or solid absorbent.

[00244] In some instances, the operator will have selected a program that further processes the dissocated tissue specimen **120** in Processing Chamber **460**. For example, the sample can be further processed by magnetic processing with the Magnetic Processing Module **900**. For cells, antibodies to capture specific cell types coupled to magnetic beads or particles can be added to Processing Chamber **460** by syringe pump **2130** from a reagent reservoir such as magnetic beads in **2145 R8** through line **2166** and six-way valve **2142** through line **2162** and six-way valve **2140** to line **2161** to six-way valve **2141** and line **497** to reagent addition port **485** on Processing Chamber **460**. The beads and cells can be mixed by moving them back and forth in line **475** into the bottom of strain chamber **450** by applying vacuum or pressure through lines **472** and **495** and **2161** using syringe pump **2130** and six-way valves **2141** and **2140**. After mixing and incubation for the desired time, the specific cells for the antibodies attached to the magnetic beads can be collected by using Magnetic Processing module **900** to move magnet **910** close to Processing Chamber **460**, such as within 1 mm, or 5 mm, or 1 cm, and waiting for 1 min, or 2 min, or 5 min or other times. The magnetic beads are captured on the side of Processing Chamber **460** or in a line such as **475**. The uncaptured cells in the enzymatic or chemical dissolution solution **410** can be then removed by applying vacuum to the lower port **483** on processing chamber **460** through line **482** to port **481** on waste chamber **432** by using port **484** and line **496** through six-way valve **2141** and line **2161** and six-way valve **2140** with syringe pump **2130**. The desired buffer can then be added from a reagent reservoir such as **2145 R7** through port **485** and line **497** and the magnet **910** moved away from Processing Chamber **460**. The cells can be resuspended by again mixing in line **475** as described. Additional cycles of wash can be applied when desired. The purified cells attached to the magnetic beads through the antibodies or other affinity reagents can then be removed through Processing Chamber cap **465**.

[00245] It will be obvious to one skilled in the art that many variations of magnetic bead processing can be used including depletion of types of cells, removal of cellular debris or tissue debris, capture of nuclei **1050** or subcellular components **1060**, processing of nucleic acids **1072**, or other biomolecules **1070**. Other processes can also be performed in Processing Chamber **460** such as library preparation or other reactions as described herein.

[00246] **Example using a vertical cartridge in the Singulator system**

[00247] Another preferred embodiment of cartridge **200** is shown in **Figures 37 A-C.** This vertical cartridge **200** is designed to be injection molded and then sealed with a material such as a heat sealable plastic or laser welded, ultrasonically welded or other means to seal cartridge **200**. It has two chambers for processing samples which facilitates improved mixing during processing steps.

[00248] Referring to **Figures 37 A-C**, a typical Process Flow is as follows. The operator inserts tissue specimen **120** into the Pre-Processing Chambers **440** through sample inlet port **425** and places cap **210** (not shown) onto cartridge **200** and inserts cartridge **200** into the Singulator System **100**, Tissue Processing

System **80**, or Sample Processing System **50** as described above. After selection of the appropriate program, the instrument makes the mechanical connection to cap **210** through rotary motor coupler **2125** and fluid/gas connections to the Fluid/Gas Inlets/Outlets **480**. The instrument also contacts the Pre-Processing Chambers **440** and the two Processing Chambers **441** and **442** from the back of cartridge **200** with elements such as cartridge Peltier **1440** which can heat or cool said fluid chambers.

[00249] Enyzmatic or chemical dissolution solution **410** is injected into the Pre-Processing Chamber **440** through the fluid channel **441**. The solution may be heated or cooled by the action of the temperature regulation elements engaged with Pre-Processing Chamber **440**. The enyzmatic or chemical dissolution solution **410** can contain enzymes or chemicals to help dissociate the tissue specimen **120** or convert cells to nuclei 1050. The grinder rotor **420** is then mechanically rotated and brought up/down by the Singulator System **100** whereby tissue specimen **120** is separated into smaller and smaller pieces by the action of the grinding features on the grinder rotor **420** and grinder stator **421**Single cell **1000** or nuclei **1050** production is achieved by the combined action of the grinding elements and incubation/exposure of the tissue specimen **120** to reagents **411**, *e.g.,* enzymes, or chemicals , or combinations of enzymes and chemicals as described herein. After the tissue disruption is sufficiently advanced, the grinder rotor **420** is brought completely down until it touches the grinder stator **421** whereby the singulated cells **1000** or nuclei **1050** in the enyzmatic or chemical dissolution solution **410** are pushed around and above the grinder rotor **420**.

[00250] All the Fluid/Gas Inlets/Outlets **480** are then sealed and the singulated cells **1000** or nuclei **1050** suspension, or nucleic acids **1072** are pulled from the Pre-Processing Chambers **440** through channel **442** to Strain Chamber **450** and then through channel **443** into the Processing Chamber **461** by applying negative pressure through channels **446** or **444**. A filter **431** in Strain Chamber **450** prevents undissociated tissue, cell aggregates, and debris from entering the Processing Chamber **461**. Waste Chamber **431** can containing a liquid absorbent or solid absorbent to prevent any liquid from exiting through the Fluid/Gas Inlets/Outlets **480** and into the Singulator System **100**.

[00251] If desired, the single cell **1000** or nuclei **1050** suspension or other prepared tissue specimen **120** can then be mixed through Channel **448** by applying alternative negative (and or positive) pressure to channels **444** and **445** to move the sample back and forth from Processing Chamber **461** to Processing Chamber **462**. If no further processing is desired, the operator can pull out the single cell **1000** or nuclei **1050** suspension or other processed sample through an opening or processing chamber cap **465** (not shown) in the top wall of Processing Chamber **461** or Processing Chamber **462**.

[00252] For the positive selection or depletion of specific cell types, or nuclei **1050**, or subcellular componets **1060**, or biomolecules **1070**, or for washing the cells and/or for exchanging the buffer, the single cell **1000** or nuclei **1050** suspensions can be further processed by using cell-specific, or nuclei-specific, or other affinity reagents coupled to magnetic beads or using paramagnetic bead purification of nucleic acids **1072** or other methods. For example, cell-type specific or nuclei-specific, or other affinity magnetic beads and reaction solutions are injected through Channel **444** into Processing Chamber **461**. The beads are incubated with the single cell **1000** or nuclei **1050** suspension by mixing though channel **448** as described above, whereby the magnetic beads bind to their target cells. Then, magnet(s) **910** is/are applied to the backside of Processing Chambers **461** and/or **462** depending where the sample is moved to, whereby the magnetic beads (and attached cells or nuclei or other biocomponents) are attracted to and held at the Processing Chamber **461** or **462** wall(s). The single cell **1000** or nuclei **1050** solution now depleted of

specific targets is pulled into Processing Chamber **461** by applying negative pressure to channel **444** (and/or positive pressure to channels **445** and **446** and then sequentially into the Waste Chamber **432** containing a liquid or solid absorbent substance by applying a negative pressure through channels **447** and **449**.

[00253] Simultaneously or subsequently, washing solution can be injected through channel **444** and the beads attached to magnet **910** can be washed with a wash buffer by combinations of mixing, magnetic release/application and pulling liquid to the Waste Chamber **432**. This process can be repeated one or more times. Similar processing can also be used to resuspend the single cells **1000** or nuclei **1050** in a specific buffer or growth solution.

[00254] After the single cells **1000** or nuclei **1050** are in the desired output buffer, the magnet **910** is released, the cells homogeneously resuspended by mixing in channel **448**, and then the single cell **1000** or nuclei **1050** suspension is pulled either into Processing Chamber **461** or **462**. The operator can then pull out the single cell **1000** or nuclei **1050** suspension through an opening in the top wall of Processing Chamber **461** or **462** covered by a foil-seal, or septum, or processing chamber cap **465** or other mechanism (not shown). Other processing/reaction/fluidic elements can be added to the cartridge as desired to enable additional processing modes in including without limitation tangential flow filtration, optical interrogation, library preparation, and nucleic acid purification.

[00255] **Four-Sample Singulator System**

[00256] A Four-Sample Singulator System **2400** is shown in **Figure 27 bottom right**. Tissue specimens **120** are loaded into any of four cartridges **200**, the appropriate program selected on the touchscreen **730** graphical user interface **740**, and the system executes the appropriate workflow for the tissue and genomic application. The core of the Four-Sample Singulator System **2400** can be the Single-Sample Singulator System **2000**, expanded to accept four cartridges. The expansion can be comprised of the addition of cartridge interface assemblies with cartridge Peltier **1440**, fluidic connections, a Z-axis stepper **2110**, rotary motor **2120**, a six-way valve **2142**, control boards, and other components for each additional cartridge added. In one embodiment one or up to four cartridges **200** can be run at the same time with the same or different programs.

[00257] **Enhanced Singulator System**

[00258] The workflow of the Singulator System **100** can be extended downstream in an Enhanced Singulator System **2500** as shown in **Figure 38** and additional processing capabilities added to integrate and simplify the workflows for genomics and other applications for operators. An optical module **2600** can be added to determine titer and viability. A tangential flow filtration module **2700** can be added to replace the enzymatic mixture with a buffer of choice at a volume to produce the proper titer and buffer for downstream processes. Downstream processing in the Processing Chamber **460** can be extended to lyse red blood cells and perform chemistries such bulk library preparation. Many other modules and applications can be added.

[00259] **Optical module.**

[00260] The determination of the viability and number of cells is critical to produce titered cell suspensions **1300** automatically for downstream processing without further intervention. Currently, after cells are prepared from tissue, separate instruments are used to count the number of cells and viability, *e.g.,* FACS, a cell counter, or a microscope, and centrifugation to wash and concentrate the cells, or FACS to select certain cell types and remove debris.

[00261] An optical module **2600** can be incorporated into an Enhanced Singulator System **2500** to interrogate samples for titer, viability, and process control to potentially produce less stressed cells. The viability determination can be performed using bright-field illumination with an added stain, *e.g.*, Trypan Blue, or with fluorescence live/dead stains such as SYTOX Green or others. Viability staining can be detrimental to the viability of the cells, interfere with downstream labeling, or require optical quality cartridges.

[00262] Referring to **Figure 39**, a preferred embodiment is to take a small aliquot, such as 10 to 50 μL, from a port in the cartridge **200**, such as the Processing chamber cap **465**, with a pipettor **2660** on a two-axis stage **2665**, move the pipettor **2660** to flowcell **2620**, and dispense the sample into reservoir **2621** and then the pipettor **2660** picks up and adds the appropriate dye, *e.g.,* Trypan Blue, or other, and optionally uses pipettor **2660** to mix the aliquot with the dye in reservoir **2621** by pipetting up and down. In some embodiments no dye is used.

[00263] The mixed aliquot and dye is then moved into the flowcell **2620** by applying vacuum on line **2627** through waste container **2626** and line **2625** and connector **2622** to pull the mixed aliquot and dye into flowcell **2620**. Alternatively, the dimensions of the flowcell **2620** may sufficiently small for the mixed aliquot and dye to be pulled in by capillary action with the aliquot and dye can be premixed in the pipettor **2660** or other place. In another embodiment, pipettor **2660** can seal at the end of reservoir **2621** to push the mixed aliquot and dye into flowcell **2620**. Many other methods of moving the aliquot are envisioned and within the scope of the present invention including pneumatic pumps, peristalic pumps, electrokinetic pumps, mechanical pumps, and other pumps located on or off the device, as well as many other ways to move the aliquot. The mixed aliquot and dye in flowcell **2620** can then be interrogated by an optical imaging device **2675** to measure brightfield or fluorescence or other images of the cells or nuclei in flowcell **2620.**

[00264] In one embodiment, as shown in **Figure 39,** a 'staring' epifluorescent imager **2675** interrogates flowcell **2620**. A light source **2610**, comprised of LED, lasers, laser diodes, halogen lamps, or other light sources, outputs beam **2615** which is reflected by beam-splitter **2630** and focused by lens **2617** to illuminate the sample in flowcell **2620**. Emitted fluorescent signal **2631** passes through lens **2617** and hits beamsplitter **2630** which will allow the longer wavelength fluorescence to pass through creating fluorescent signal **2632**, which in turn can pass through laser-line blocker **2635** and optical filter **2640** before reaching detector **2650**. Detector **2650** can have many different embodiments comprised of CCD cameras, preferrably with greater than 10 megapixels, photomultiplier tubes, avalanche photodiodes, photodiodes, CMOS detectors, or other sensors. In a preferred embodiment, detector **2650** has an array of detection sensor elements to form pixels of image. In some embodiments, imager **2675** can stare without moving, and in other embodiments part of the imager **2675** may physically move to scan the flowcell **2620** such as a flying head imager, or a galvoscanner, or other implementations well known to one skilled in the art. The optical path can be adapted to the light source **2610** and in some embodiments will use a diffuse beam without a beamsplitter for brightfield illumination. In some embodiments, confocal imaging can be used to improve signal-to-noise. In some embodiments, multiple detectors, beamsplitters, and filter sets are used to separate different wavelengths of light or otherwise process the light.

[00265] In a preferred embodiment, the flowcell **2620** geometry is made from optical glass with a 100 μm channel. In other embodiments, arrays of fluidic channels are used in flowcell **2620** to allow multiple aliquots to be detected. In some embodiments, one or more glass capillaries with burned windows are used as

flowcell **2620**. In some embodiment, pipettor **2660** and two axis robot **2665** are replaced with fluidic plumbing to deliver the aliquot to reservoir **2621**.

[00266] In some embodiments, the imager **2675** and detector **2650** can be autofocused with flowcell **2620**. In one embodiment, the autofocusing moves imager **2675** and detector **2650** using motor **2680** to move in small increments, e.g., less than 1 μm, less than 2 μm, less than 5 μm, less than 10 μm, less than 20 μm, less than 25 μm, less than 50 μm, or less than 100 μm to focus on features on flowcell **2620** or the Raman line of water in flowcell **2620** or other features. The features may be the top or bottom surface of the flowcell **2620** or may be features designed into flowcell **2620** to simplify focusing such as a grid of lines or 3-D features. Software interprets the images to determine the focal plane for best resolution. In another embodiment, the detector **2650** and the flowcell **2620** are rigidly fixed optically to place flowcell **2620** always in the plane of focus.

[00267] After the mixed aliquot with dye has been interrogated by imager **2675,** in one embodiment, the mixed aliquot and dye is then moved into waste container **2626** by applying vacuum on line **2627** through waste container **2626**, line **2625,** and connector **2622** to pull the mixed aliquot and dye from flowcell **2620** into waste container **2626**. The flowcell **2620** is then cleaned for reuse, such as by having pipettor **2660** pipetting cleaning solutions, such as 100 mM NaOH followed by 10 mM Tris HCl, pH 7 followed by deionized water into reservoir **2621** and after a suitable incubation time, pulling the cleaning solution into waste **2626** as described. Many other cleaning protocols are within the scope of the invention.

[00268] Camera control and image acquisition can be based on Point Grey/FLIR Spinnaker SDK optimized for machine vision applications or other image processing software such as Image J freeware, Cell Profiler, or other software. The output of the imaging device **2675** can be processed in software to quantify total number of viable cells and non-viable cells or to detect subcellular components **1060** and nuclei **1050** or quantify biomolecules **1070** such as nucleic acids **1072**. In some embodiments, chemicals or biologicals can be added to the aliquot to allow measurement of their impact on freshly produced cells **1000** or nuclei **1050** or other cellular components. In some embodiments, with two or more fluidic channels, chemicals or biologicals can be added to one or more of two or more identical aliquots but not to another aliquot which can serve as the control. In some embodiments, the single cells **1000** can be imaged and genetically modified such as with CRISPR and the cells collected for subsequent usage.

[00269] Monitoring of cell titer and viability at intervals will enable the Singulator System **100** to adjust the mechanical or enzymatic regime to gentler or harsher enzymatic and mechanical conditions as needed for a tissue that dissociates easier than expected or harder. For example, cancerous tissues have different properties than normal tissues and may need individual adjustment and optimization of disruption conditions for best results. Singulator System **100** can process images from imager **2675** with Control Subsystem **700** control software **725** to monitor the tissue dissociation rate by the number of cells or cellular components produced per time interval. When applicable, the operator or control software **725** can increase or decrease the mechanical disruption or the enzymatic or chemical formulation changed to stronger or weaker solutions.

[00270] **Tangential flow filtration module.**

[00271] The production of titered single cells for direct processing by single cell DNA sequencing or scRNA-Seq can simplify the tasks for the genomic scientist. The optical module **2600** can measure the number and viability of the single cells and the single cell **1000** or nuclei **1050** suspensions can be adjusted for titer, typically by dilution. In the lab, the workflow involves centrifugation, washing, and resuspension to

replace the buffer and remove debris, or by FACS sorting. In a fluidic device accomplishing this can be done using magnetic bead processing or filtration; however, 'dead-end' filtration is prone to clogging, can shear cells, and recovery of filtered cells can be problematic. These problems have been solved in the biopharmaceutical industry by using tangential flow filtration (TFF).

[00272] In one embodiment, referring to **Figure 40**, a five-layer cartridge **2705** with full process integration was designed. This cartridge is laser cut from acrylic, and assembled with pressure-sensitive adhesive (ARcare 90445) with embedded tangential flow filters **2710** and strainer filter **2711**. The layered cartridge, and similar ones designed only to test TFF, can be assembled a variety of tangential flow filters **2710** *(e.g.*, 0.8 μm Durapore, 5 μm Durapore, Isopore, Fluoropore). The five-layer cartridge **2705** also has optical areas **2720** to interrogate the viability and titer of the single-cell **1000** or nuclei **1050** suspension in the cartridge **2705** at different parts of the workflow. The cells are gently moved tangential to the filters by a syringe plunger or other method connected to the AutoSingulator **2100** while buffers are circulated on the opposing side of the tangential flow filters **2710** by syringe pumps **2130**.

[00273] TFF can be incorporated in many emobodiments of cartridges **200** in the Processing Chamber **460** to add the ability to concentrate cell suspensions, remove debris, and change buffers. The implementation is an interplay between cartridge design and the on-instrument process development. Cartridge **200** can be designed to incorporate parallel filters into the molding process, routinely done for syringe filters. The Enhanced Singulator System **2500** can seal the cartridge **200** and provide the circulation of buffer driven by pumps comprised of peristaltic pumps, micropumps (*e.g.,* TCS Micropumps), or others directed by control software **725**. The TFF module can be incorporated in many cartridge **200** designs and with many embodiments of the Sample Processing System **50**, Tissue Processing System **80**, or the Singulator System **100**.

[00274] **Downstream processing: red blood cell lysis.**

[00275] The Enhanced Singulator System **2500** has the capability to perform additional biochemistry after single cells **1000**, nuclei **1050**, or biomolecules **1070** have been produced or purified. Syringe pump **2130** can deliver reagents to the Preprocessing Chamber **440** or Processing Chamber **460** of the cartridge **200** which enables multiple process options.

[00276] In many procedures, red blood cells (RBC) are present in high titer in the starting tissue and need to be removed by perfusion or later by lysis. Red blood cell lysis can be added as an option to the workflow after production of single-cell suspensions **1000** or purified single-cell suspensions **1100** or other outputs as follows. RBC lysis solution (*e.g.*, 0.5% ammonium chloride or commercially available solutions) is moved by syringe pump **2130** into Processing Chamber **460** and mixed with the single-cell **1000** in suspensions by methods such as bubbling, fluid flow, magnetic stirring, or other methods, and the lysis solution and the single-cell **1000** suspensions incubated for five minutes or less at room temperature or other temperatures. The time course and temperature can be optimized to adjust parameters to conditions that favor high viability for the tissue specimen **120** with the requirements of the RBC lysis. After lysis, the RBC lysis solution can be removed or diluted to protect the other cell types either by TFF processing or rapid dilution with buffer.

[00277] **Single Librarian embodiment**

[00278] The Enhanced Singulator System **2500** embodiment can be extended to create a Single Librarian **3000** embodiment with integrated optical analysis to determine viability and titer, tangential flow

filtration to wash cells or nuclei to replace the buffer and adjust the titer, magnetic processing to capture nucleic acids and integrate pooled library enzymatic steps, and integration of single-cell/nuclei nanodroplet or nanobolus processing and library preparation. Real-time titer and viability data enables adapting tissue processing reagents and mechanical disruption in almost real-time using machine learning or other analytical methods: the system could potentially autotune sample preparation of single-cells **1000**, nuclei **1050** or other cellular components using singulation and viability metrics or production metrics such as the concentration of cellular components.

[00279] The Single Librarian **3000** embodiment as shown in **Figure 41** and an exemplary workflow shown in **Figure 43** adds capabilities to produce 1) titered single-cell suspensions **1300** that have been imaged and the titer adjusted in the buffer for downstream processing, 2) matched bulk nucleic acid preparations **1010**, 3) single-cell libraries **1200**, 4) single nuclei libraries **1250**, and 5) matched bulk libraries **1210**. Bulk is used to refer to preparations where more than one and frequently numerous single cells have been pooled or processed together and the individual signals such as DNA or RNA sequence are no longer distinquishable except as a composite signal. Matched is used to mean the nucleic acid preparation or library can be a bulk preparation or library that is from part of a batch of single cells **1000** or nuclei **1050** that are used to create a single cell library **1200** or single nuclei library **1250** and can serve as the bulk aggregated control for the single cells **1000** or nuclei **1050**.

[00280] In a preferred embodiment, the Single Librarian **3000** can be configured to process any number of tissue samples automatically with tissue-specific disposable cartridges and enzymatic formulations to produce single-cell **1000** and nuclei **1050** suspensions and libraries, such a single sample, or four, or eight, or 12, or 96, or 384, or more samples. The processing time for single-cell **1000** or nuclei **1050** suspensions can be less than 2 min, or less than ten min, or less than 30 min, or less than two hours or less than four hours or other times. The processing can use optimized enzyme formulations for the production of single-cells **1000** or nuclei **1050**. Magnetic bead processing can purify cell types, or nuclei, or organelles, or nucleic acids, or link biochemical reactions for library preparation.

[00281] Nanodroplets without single-cells **2810** or with single-cells **2820** can be produced with microfluidic nozzles **2800,** as shown in **Figure 42**, or nanoboluses can be produced by using modular microfluidic connectors **620**, three line connectors with a “T”, or other configurations with the boluses being created as the acqueous single cells **1000** or nuclei **1050** or other sample produced by the Enhanced Singulator System **2500** go through the noozle with an emulsion oil introduced through the side channels. While **Figure 42** shows the sample in agarose, in many implementations the single cells **1000** can be in a buffer without agarose. Emulsion oils are comprised of immiscible fluids (such as FluorInert®; mineral oil; silicone-based oil; fluorinated oils; Droplet Generation Oil (Biorad, #1863005); emulsion oil (Life Technologies, Part No. 4469000); 73% Tegosoft DEC (Diethylhexyl Carbonate), 20% mineral oil, and 7% ABIL WE; 0.12% Span (v/v), 0.00325% Tween 80 (v/v), 0.0000125% Triton X-100 (v/v) in mineral oil; and other formulations.

[00282] An optical module **2600** can determine titer, viability, and QC samples during preparation, and the information can used for real-time process optimization by adjusting parameters comprising temperature, enzyme concentration and formulation including purified enzymes and use of acetate counterion salts in buffers and osmoprotectants such as glycine betaine, proline, glutamate, threhalose, *etc.* or chemicals and conditions.

[00283] The Single Librarian **3000** embodiment can integrate the workflows and processing of solid tissues from raw specimens to genomic samples to de-skill the workflow for single-cell sequencing and standardize production of single-cell suspensions and libraries. This will help researchers at laboratories in educational and research institutions, biopharmaceuticals, and applied markets (*e.g.,* food testing, agriculture, animal sciences, *etc.*), and ultimately the clinical community to access single-cell sequencing and to NGS sample preparation for tissue.

[00284] The Single Librarian **3000** embodiment can function as follows in one embodiment, as shown in the workflow outlined in **Figure 43** for single cells. Tissue specimen **120** is added to cartridge **200** in the Cell Singulator module **800** where mechanical and enzymatic or chemical dissociation occurs as described above. The resultant single-cell **1000** or nuclei **1050** suspension or other products is strained and can then be optionally affinity purified in Magnet module **900**. To produce matched bulk nucleic acid, an aliquot of the single-cell **1000** or nuclei **1050** suspension can be moved from Pre-Processing Chamber **440** to Processing Chamber **460**. The aliquot of single-cells can have 7 M guanidine, sodium isocyanate, or other chaotrophs added along with paramagnetic beads such as SPRI beads (Beckman Coulter), Dynabeads (Thermo Fisher), or many other beads with COOH or other surface coatings or no coatings. The tissue specimen **120** can also be lysed directly without producing single cells **1000** or nuclei **1050** as an intermediary step. Alternatively, a lysis agent can be added and then the freed nucleic acid precipitated upon the beads using salt and polyethylene glycol or other chemistries. The amount of beads can be chosen to be limit the amount of nucleic acids **1072** that can bind, thereby normalizing the nucleic acid concentration. The chaotroph will lyse the cells, releasing the nucleic acid **1072** which is then forced onto the bead surface in a standard bead-based nucleic acid purification. The nucleic acid **1072** can be washed, such as with 70% ethanol, one or more times. If desired, the nucleic acid can be eluted into a buffer such as 10 mM Tris HCl or water to form matched bulk nucleic acid **1010** which can be output for off instrument processes or used as the starting material for a matched bulk nucleic acid library **1210**. To process matched bulk nucleic acid **1010** into matched bulk nucleic acid library **1210**, the matched bulk nucleic acid **1010** is processed differently for DNA and RNA libraries.

[00285] **DNA Library Production using Tagmentation**

[00286] Libraries for NGS can be prepared using tagmentation with transposons including the Nextera Tagmentation (http://www.epibio.com/docs/default-source/protocols/nextera-dna-sample-prep-kit-(illumina--compatible).pdf?sfvrsn=4). In this embodiment, referring to **Figure 44**, double stranded DNA **820** is produced in Processing Chamber **460** and transposons, *e.g.*, Nextera enzyme, reaction mix, and water are added by syringe pump **2130**. The reaction is incubated for example at 55°C for 5 min. A bead purification is performed using Magnetic module **900** to remove reactants and purify the double stranded product with transposon inserted into the DNA on the beads which are still in the Processing Chamber **460**. Syringe pump **2130** is used to add Nuclease-Free Water, Nextera Adaptor 2 (or other barcoded adapters), Nextera PCR Enzyme, PCR Buffer, and Nextera Primer Cocktail. If the Processing Chamber **460** also has a thermal cycling capability in the instrument with cartridge Peltier **1440**, nine cycles of PCR can be performed. A bead purification is performed to remove reactants and purify the double stranded DNA product before elution into buffer or water. The double stranded bulk DNA library **1010** is now ready to QC and bridge amplification on the flow cell of the sequencer. Many variations of the method described here are within the instant disclosure and are obvious to one skilled in the art.

[00287] **DNA Library Production using polishing, end repair, and ligation.**

[00288] Another embodiment of the workflow to produce libraries is illustrated in **Figure 45**. Double-stranded DNA attached to a bead, such as double stranded cDNA attached to bead **684,** or bulk nucleic acid absorbed onto beads, can if needed be fragmented enzymatically, e.g. Fragmentase® (New England Biolabs, M0348), with restriction enzymes, nucleases, or other enzymes, or chemically. Enyzmes or chemicals can be added to Processing Chamber **460** by syringe pump **2130** and incubated. Following fragmentation, the now fragmented nucleic acid can be forced onto paramagnetic beads, the magnetic beads captured, and the nucleic acid **1072** purified by washes.

[00289] The fragmented nucleic acid can be end-polished in Processing Chamber **460** by addition of reaction mix and enzymes, for example, the NEBNext® End Repair Module (NEB E 6050S) reagents, from syringe pump **2130** to generate end-polished DNA product **810**, an end-polished, blunt-ended double-stranded DNA having 5´-phosphates and 3´-hydroxyls; other kits such as Agilent PCR polishing kit 200409 and other enzymology can perform the same function. Following end polishing, a magnetic separation is performed in Processing Chamber **460** to remove reactants and enzymes from end-polished DNA product **810**.

[00290] Following polishing, A-tailing is used to generate fragments ready to ligate with a primer with a complementary T overhang and to prevent concatamer formation during ligation. A-tailing can be performed using commercially available kits such as the NEBNext® dA-Tailing Module (NEB E6053S) with enzyme and master mix added from the syringe pump **2130** to Processing Chamber **460** containing end-polished DNA product **810** and incubating the reaction to produce blunt-ended double-stranded DNA having 5´-phosphates with an A residue overhang on the 3' end, A-tailing DNA product **815**. Following A tailing, a magnetic separation is performed in Processing Chamber **460** to remove reactants and enzymes from A-tailing DNA product **815**.

[00291] A double stranded second primer **611** with a complementary T overhang can be ligated by DNA ligase onto the 3' end of A-tailing DNA product **815**. DNA ligase, DNA ligase reaction mix, and second primer **611** (such as NEB Next Adapter) are added by syringe pump **2130** to Processing Chamber **460** and incubating the reaction. DNA ligation can be performed using commercially available kits or reactions, e.g. NEBNext® Quick Ligation Module, NEB E6056S. Following DNA ligation, a magnetic separation is performed in Processing Chamber **460** to remove reactants and enzymes. The product is now a double stranded DNA product **820** that has incorporated second sequencing primer **611** or can have two adapters attached depending on the workflow. The product of the ligation can be a matched bulk nucleic acid library **1210**. The fragment sizes for the downstream NGS analysis can be selected by a two step 'heart cut' precipitation onto beads, with one cut selecting for fragments longer than a lower cutoff, *e.g.*, 400 bases, and the second cut selecting for fragments shorter than a high cutoff, *e.g.*, 600 bases.

[00292] For bulk RNA, after nucleic acid purification to produce bulk matched nucleic acid **1010**, the RNA can optionally be fragmented by addition of metal cations from syringe pump **2130** to Processing Chamber **460** followed by magnetic bead purification to produce purified fragmented nucleic acid. The polyadenylated RNA can then be converted to cDNA using a poly T primer and reverse transcriptase in Processing Chamber **460**. The cDNA can now be treated as described above for DNA Library Production using polishing, end repair, and ligation or with Tagmentation to produce bulk matched RNA libraries.

[00293] Production of single-cell libraries from polyadenylated mRNA in single-cell or nuclei suspensions.

[00294] In one embodiment of the single-cell **1000** or nuclei **1050** library workflow, after production of single-cell suspensions **1000** in the Preprocessing Chamber **440** in the Cell Singulation module **800**, the cells are moved through the strainer into the Processing Chamber **460**. Referring to **Figure 46**, the single-cell **1000** or nuclei **1050** suspensions can be optionally purified for specific cell type using affinity purification in Magnetic module **900**. The single-cell suspension is then counted in the Optical module **2600**, and the titer and buffer adjusted as needed in the TFF module **2700**.

[00295] The single-cell suspension in appropriate buffer is then mixed with beads which can have poly T containing primers embedded for mRNA, and moved to a microfluidic nozzle **2800**, e.g., as shown in **Figure 42** either connected through fluidics or using the 2 axis robot **2665** and pipettor **2660** to deliver the sample to the microfluidic nozzle **2800.** The single-cell suspension and beads are passed through microfluidic nozzle **2800** to produce nanodroplets **2810**, some of which will contain a bead **2820** and some nanodroplets containing both a bead and a single-cell.

[00296] The processing of the single-cells can be as described in International Patent Publication WO 2017/075,293 (Jovanovich and Wagner, "Method and apparatus for encoding cellular spatial position information"), the contents of which are incorporated herein in their entirety. The same methods can be utilized in the Single Librarian without the use of spatial barcodes.

[00297] A preferred embodiment for mRNA is described in more detail. In one embodiment, single channel fluidics are used. Referring to **Figure 46**, barcoded oligonucleotide-functionalized beads **680** with a poly T sequence as the capture region on oligonucleotide **601** in lysis/reverse transcriptase mix is added to the single-cell suspension. Monodisperse nanodroplets from single cells with beads with lysis/RT mix, are produced using a nozzle **2800.** The output of the microfluidic nozzle **2800** is moved back to the Processing Chamber **460** if needed. Cells are lysed by lysis buffer, heating, or other methods and polyadenylated mRNA **681** captured onto the oligonucleotide to form captured mRNA structure **682**. The oligonucleotides **601** can be barcoded for spatial barcode information **607** if desired as well as cellular barcodes **608** and molecule barcodes **609**. Amplification primer **604** and sequencing primer **605** may be included on the oligonucleotide, or may be added in downstream library preparation methods as needed. The amplification primers **604** can be for T7 polymerase for amplified RNA production, PCR, rolling circle transcription-based amplification, rapid amplification of cDNA ends, continuous flow amplification, and other amplification methods.

[00298] After lysis and capture of the mRNA onto the poly T**,** a reverse transcriptase reaction is performed in Processing Chamber **460** to produce cDNA attached to bead **683**, formed from the mRNA, and now containing the cellular and molecular barcodes as well as the optional spatial barcode in addition to any sequencing and amplification primers attached to the bead **680** through an optional cleavable linker. Cleavage of the linker can release the cDNA from the bead when desired. A photocleavable or chemical cleavable linker and fluorescent tag(s) to aid in quality control and process development is included in the instant disclosure. As required, fragmentation of the RNA or cDNA can be performed using methods comprised of chemical, biochemical, and physical methods. Alternative preferred embodiments include performing an RNA ligase reaction to covalently join the mRNA to one strand of the double stranded oligonucleotide after lysis and capture of the mRNA onto oligonucleotide-functionalized beads **680** with a poly T sequence as the capture region **610**, or ligating RNA to a single stranded RNA or DNA attached to the

bead. The produced cDNA can then be used in the library preparation as described above for bulk nucleic acid library preparation. In an alternative embodiment, the cDNA still attached to the bead can be ligated with a second primer or adapter to produce a library. In some embodiments the cDNA can be directly readout on a nanopore or other sequencer.

[00299] Determination of amplification of nucleic acid and normalization thereof

[00300] In many applications in genomics, an amplification step is required to produce enough material for the downstream analysis instrument. For example, in NGS after library creation, a PCR step may be required before loading the DNA sequencer. While PCR amplification is straightforward, many targets may amplify unevenly, leading to uncertainty about the actual amount of the target in the unamplified library. This prevents determination of the absolute amount of the target.

[00301] scRNA-Seq is a novel method to sequence mRNA from single-cells. After capture of the mRNA typically onto a bead with a poly T sequence, the mRNA is processed with reverse transcriptase to produce cDNA using primers that may have barcodes for the cell and the molecules that are then made into a library.

[00302] The amount of amplification for each molecule can be measured and used to normalize the resulting sequencing data to minimize amplification or readout biases. To do this, the PCR amplification primer can incorporate a set of enumeration barcodes such as a three base long barcode that is random. Once the fragment is amplified and sequenced, the number of bases that appear in the enumeration barcodes can be counted to determine the degree of amplification. A three base enumeration barcode would be useful for up to a 64-fold amplification: enumeration barcodes with more bases could extend the range as high as desired. For the example where three bases are used in the enumeration barcode, the representation of each of the 64 possible sequences is determined and that number is used to normalize the representation of that molecule in the final NGS data, such that if 32 combinations were found in a first sequence and 16 in a second sequence, the depth of the first sequence would be adjusted by a factor of two with respect to the second sequence to normalize for the amplification and readout biases.

[00303] Example: Sample-to-answer system

[00304] In other embodiments a Sample Processing System **50** is combined with an analyzer **4000** to create a sample to answer system. In a preferred embodiment, referring to **Figure 47**, the Single Librarian **3000** is physically integrated with a genetic analyzer such as a NGS system. In this embodiment, the Single Librarian **3000** processes a specimen **101**, preferrably a tissue specimen **120**, into single cell libraries **1200**, or nuclei libraries **1250** or bulk libraries **1210** or other materials which then can be analyzed on an genetic analyzer **4000** comprised of a nucleic acid sequencer, more preferably an NGS or NNGS sequencer, to produce genetic analysis information **4500** such as DNA sequence, or RNA sequence, or single nucleotide variations, or nucleic acid modifications. The prepared library is fluidically moved into the analyzer **4000** and delivered to its flowcell or other input for analysis or further processing such as bridge amplification. This may replace the on-board library preparation found on some NGS sequencers or directly couple to a nanopore or other analytical device.

[00305] This produces a tissue-to-answer genomic system **5000** capable of performing bulk sequencing of tissue, or single cell sequencing of tissue, or single nuclei sequencing of tissue, or mitochondria sequencing of tissue, or other sample-to-answer genetic analysis for nucleic acids **1072**, DNA **1073**, RNA **1074** comprised of microRNAs, long non-coding RNA, ribosomal RNA, message RNAs, etc.

[00306] As used herein, the following meanings apply unless otherwise specified. The word “may” is used in a permissive sense (i.e., meaning having the potential to), rather than the mandatory sense (i.e., meaning must). The words “include”, “including”, and “includes” and the like mean including, but not limited to. The singular forms “a,” “an,” and “the” include plural referents. Thus, for example, reference to “an element” includes a combination of two or more elements, notwithstanding use of other terms and phrases for one or more elements, such as “one or more.” The term “or” is, unless indicated otherwise, non-exclusive, i.e., encompassing both “and” and “or.”

[00307] It should be understood that the description and the drawings are not intended to limit the invention to the particular form disclosed, but to the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the present invention as defined by the appended claims. Further modifications and alternative embodiments of various aspects of the invention will be apparent to those skilled in the art in view of this description. Accordingly, this description and the drawings are to be construed as illustrative only and are for the purpose of teaching those skilled in the art the general manner of carrying out the invention. It is to be understood that the forms of the invention shown and described herein are to be taken as examples of embodiments. Elements and materials may be substituted for those illustrated and described herein, parts and processes may be reversed or omitted, and certain features of the invention may be utilized independently, all as would be apparent to one skilled in the art after having the benefit of this description of the invention. Changes may be made in the elements described herein without departing from the spirit and scope of the invention as described in the following claims. Headings used herein are for organizational purposes only and are not meant to be used to limit the scope of the description.

(10) Claims

WHAT IS CLAIMED IS:

1. A system comprising:

(a) an instrument comprising:

(i) one or more cartridge interfaces configured to engage a cartridge;

(ii) a fluidics module comprising:

(1) one or more containers containing one or more liquids and/or gasses;

(2) one or more fluid lines connecting the containers with fluid ports in the cartridge interface; and

(3) one or more pumps configured to move liquids and/or gasses into and/or out of the fluid port(s);

(iii) a mechanical module comprising an actuator;

(iv) optionally, a magnetic processing module comprising a source of magnetic force, wherein the magnetic force is positioned to form a magnetic field in the processing chamber;

(v) optionally, a measurement module;

(vi) optionally, a control module comprising a processor and memory, wherein the memory comprises code that, when executed by the processor, operates the system; and

(b) one or more cartridges, each engaged with one of the cartridge interfaces, wherein each cartridge comprises:

(i) a sample inlet port;

(ii) one or more cartridge ports communicating with the fluid ports in the cartridge interface;

(iii) a preprocessing chamber communicating with the sample inlet port and with at least one cartridge port, and comprising a tissue disruptor configured for mechanical disruption of tissue, wherein the tissue disruptor engages with and is actuated by the actuator when the cartridge is engaged with the cartridge interface;

(iv) a strain chamber communicating with the preprocessing chamber configured to separate cells and/or nuclei from disrupted tissue;

(v) a processing chamber communicating with the strain chamber, optionally communicating with one or more cartridge ports and configured to perform one or more processing steps on separated cells and/or nuclei; and

(vi) optionally, one or more waste chambers fluidically connected with the processing chamber.

2. The system of claim **1**, wherein the tissue disruptor comprises a grinder, a pestle or a variable orifice.

3. The system of claim **1**, further comprising a barcode reader.

4. The system of claim **1**, comprising a measurement module (v) that performs optical imaging to measure titer, clumping, and/or viability of cells or nuclei.

5. The system of claim **1**, comprising a measurement module (v) and a control system (vi), wherein the measurement module measures, and one or more time points, characteristics of a sample in the processing chamber, and control system comprises code that determines a state of the sample, e.g., viability or degree of single cell or nuclei dissociation, and that adjusts processing parameters.

6. The system of claim **1**, further comprising:

(c) an analysis module, wherein an input port of the analysis module is in fluid communication with the processing chamber.

7. The system of claim 6, wherein the analysis module performs an analysis selected from one or more of: DNA sequencing, next generation DNA sequencing, next next generation DNA sequencing, proteomic analysis, genomic analysis, gene expression analysis, gene mapping, carbohydrate characterization and profiling, lipid characterization and profiling, flow cytometry, imaging, DNA or RNA microarray analysis, metabolic profiling, functional analysis, and mass spectrometry.

8. The system of claim **1**, wherein the cartridge interface comprises a means of positioning the cartridge in the instrument that engages the fluidic module and the mechanical module and optionally is temperature controlled.

9. The system of claim **1**, wherein the cartridge is disposable.

10. A method comprising:

(a) providing a tissue sample to a preprocessing chamber;

(b) automatically performing mechanical and enzymatic/chemical disruption of the tissue in the preprocessing chamber to produce disrupted tissue comprising released cells and/or nuclei and debris;

(c) automatically moving the disrupted tissue into a strain chamber comprising a strainer and/or filter and separating the released cells and/or nuclei from the debris therein; and

(d) automatically moving the released cells and/or nuclei into a processing chamber.

11. The method of claim **9**, wherein (d) further comprises performing at least one processing step on the released cells and/or nuclei in the processing chamber.

12. The method of claim **11**, wherein processing comprises one or more automatically performed processes selected from:

(I) lysing cells;

(II) capturing cells;

(III) isolating nucleic acid;

(IV) isolating protein;

(V) converting RNA into cDNA;

(VI) preparing one or more libraries of adapter tagged nucleic acids;

(VII) performing PCR;

(VIII) isolating individual cells or individual nuclei in nanodrops or nanoboluses; and

(IX) outputting released cells and/or nuclei into output vessels such as 8 well strip tubes, microtiter plates, Eppendorf tubes, a chamber in the cartridge, or other vessels capable of receiving cell suspensions.

13. The method of claim **9**, further comprising:

(e) automatically capturing the released cells and/or nuclei in the processing chamber by binding to magnetically attractable particles comprising moieties having affinity for the cells and/or nuclei and applying a magnetic force to the processing chamber to immobilize the captured cells and/or nuclei.

14. The method of claim **9**, further comprising:

(e) automatically monitoring cell and/or nuclei titer in the processing chamber and, when the titer reaches a desired level, exchanging a dissociation solution used to dissociate the tissue for a buffer.

15. A cartridge comprising:

(i) a sample inlet port;

(ii) one or more cartridge ports configured to communicate with fluid ports in a cartridge interface;

(iii) a preprocessing chamber communicating with the sample inlet port and with at least one cartridge port, and comprising a tissue disruptor configured for mechanical disruption of tissue, wherein the tissue disruptor engages with and is actuated by the actuator when the cartridge is engaged with the cartridge interface;

(iv) a strain chamber communicating with the preprocessing chamber configured to separate cells from disrupted tissue;

(v) a processing chamber communicating with the strain chamber, optionally communicating with one or more cartridge ports and configured to perform one or more processing steps on separated cells; and

(vi) optionally, one or more waste chambers fluidically connected with the processing chamber.

16. The cartridge of claim **15**, further comprising a cap that opens and closes the sample inlet port.

17. The cartridge of claim 16, wherein the cap comprises a tissue disruptor element that moves about rotationally and back and forth along an axis.

18. The cartridge of claim **15**, further comprising a holder.

19. The cartridge of claim **18**, further comprising a top piece and a bottom piece connected by collapsible element which allow the top piece and/or the bottom piece to move relative to the holder.

20. The cartridge of claim **18**, wherein the holder comprises a mesh screen.

21. The cartridge of claim **18**, further comprising a grinding element for grinding tissue in the preprocessing chamber.

22. The cartridge of claim **18**, further comprising a barcode comprising information about the cartridge and/or its use.

23. The cartridge of claim **15**, further comprising a plunger configured to move slideably within the preprocessing chamber.

24. A variable orifice device for disrupting tissue comprising:

(a) a first container and a second container fluidically connected through a flexible tube comprising a lumen;

(b) an adjustable clamp positioned to clamp the flexible tube, wherein adjusting the clamp alters the cross-sectional area of the lumen; and

(c) one or more pumps or devices operatively coupled with the first and/or second containers configured to push liquid in one container through the flexible tubing into the other container.

25. The device of claim **24**, wherein the adjustable clamp comprises an eccentric cam operatively coupled to a motor, wherein rotating the cam closes or opens the clamp.

26. A method for disrupting tissue comprising:

(a) providing a variable orifice device comprising first container and a second container fluidically connected through a flexible tube comprising a lumen;

(b) moving a sample comprising tissue from one of the containers through the flexible tube to another one of the containers;

(c) decreasing the cross-sectional area of the lumen and moving the sample from one of the containers through the flexible tube to another one of the containers;

(d) repeating step (c) one or more times to disrupt the tissue.

27. A method of determining an amount of amplification of a nucleic acid molecule comprising amplifying the nucleic acid molecule with primers comprising a barcode wherein each round of amplification adds in additional barcode to an amplified nucleic acid molecule; and after amplification, counting incorporated barcodes, wherein the number of incorporated barcodes indicates the amount of amplification.

Figure 1

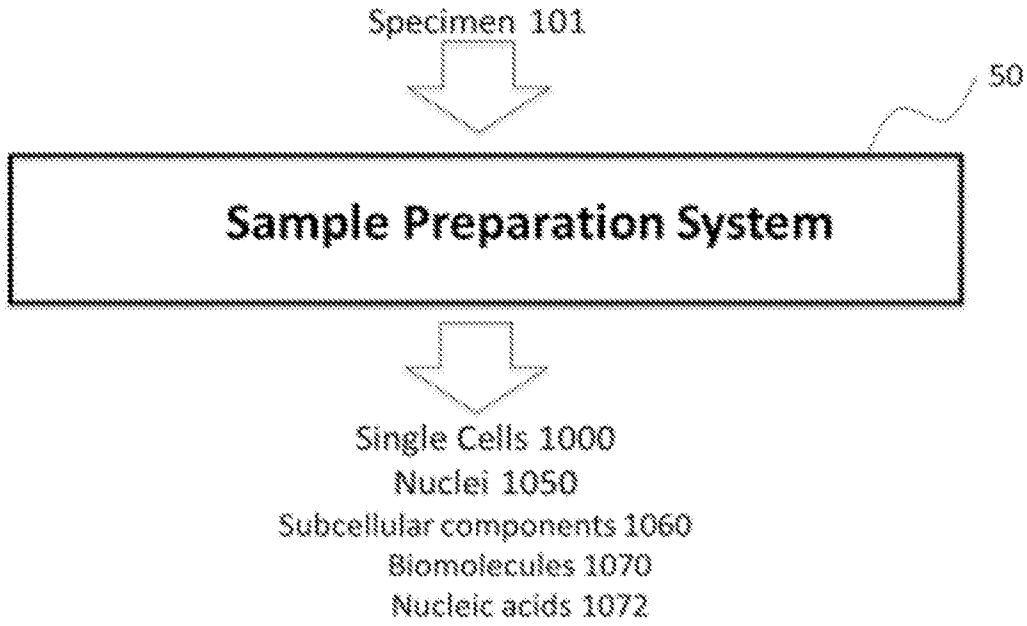


Figure 2

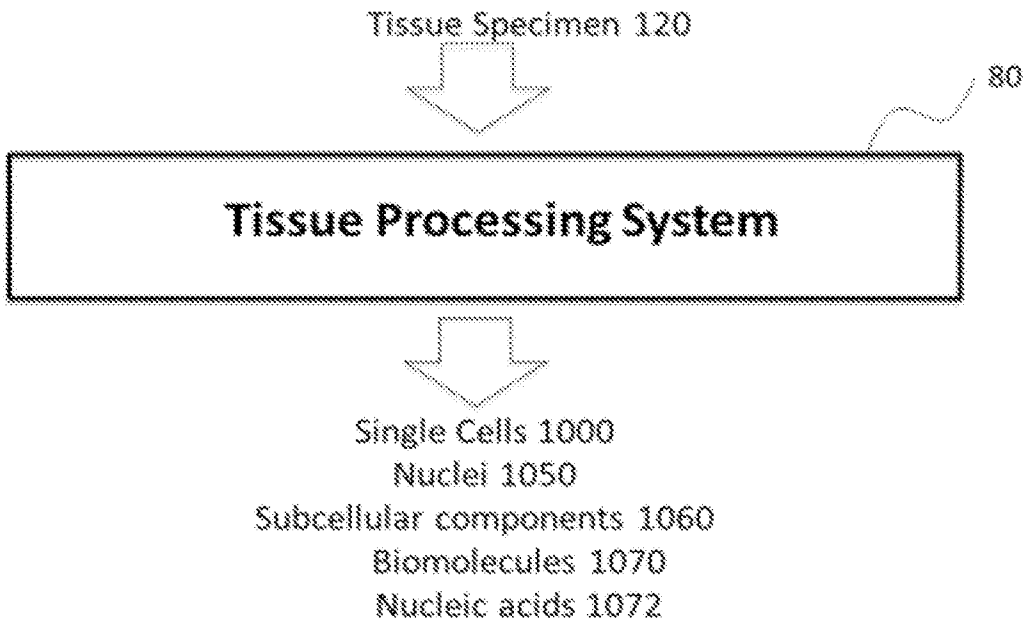


Figure 3

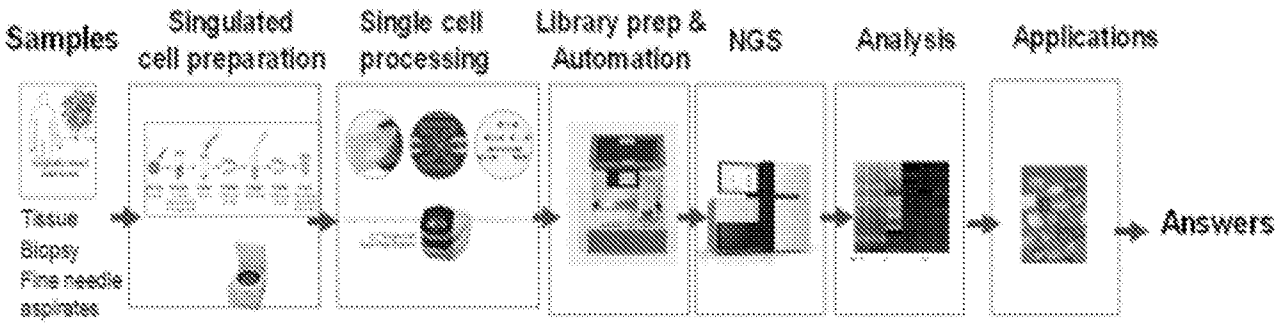


Figure 4

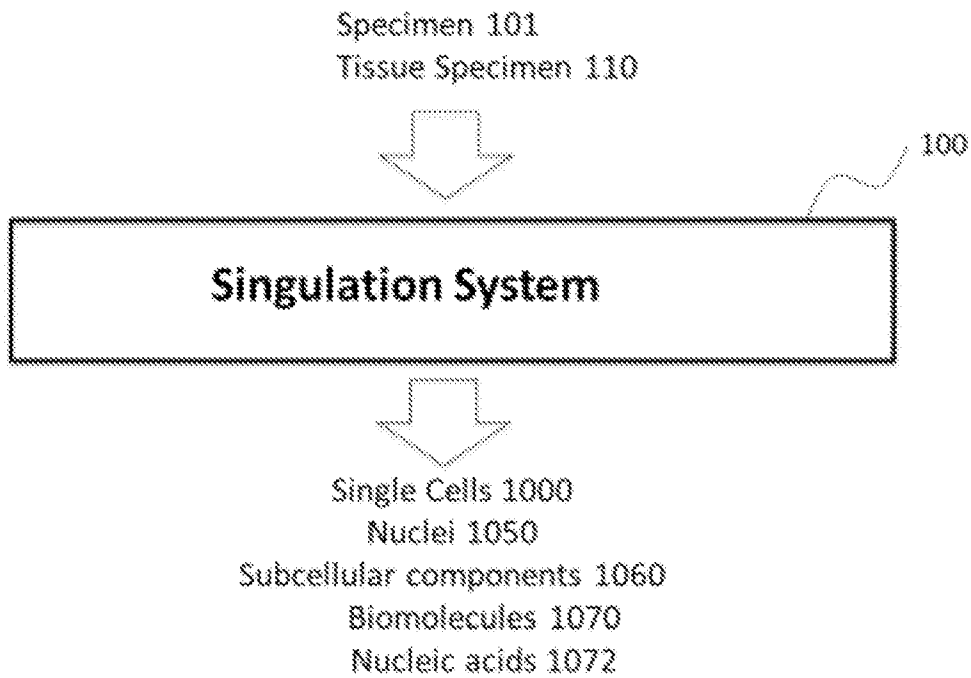


Figure 5

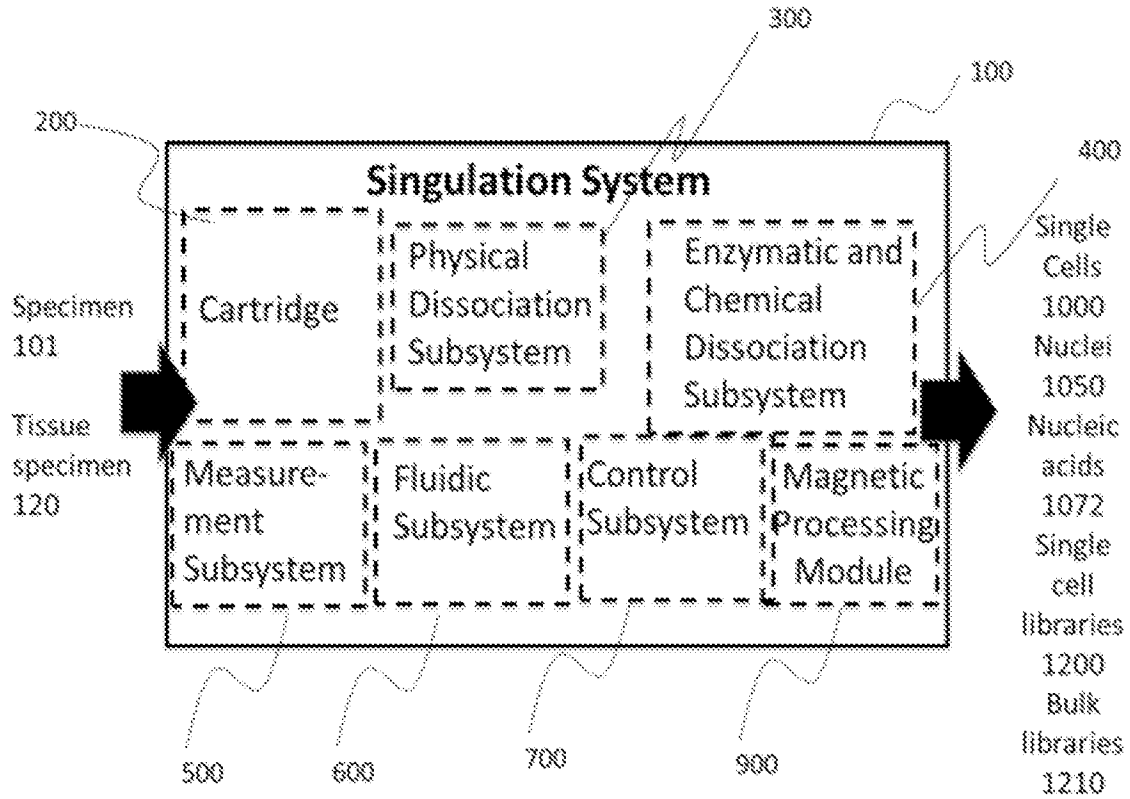


Figure 6A.

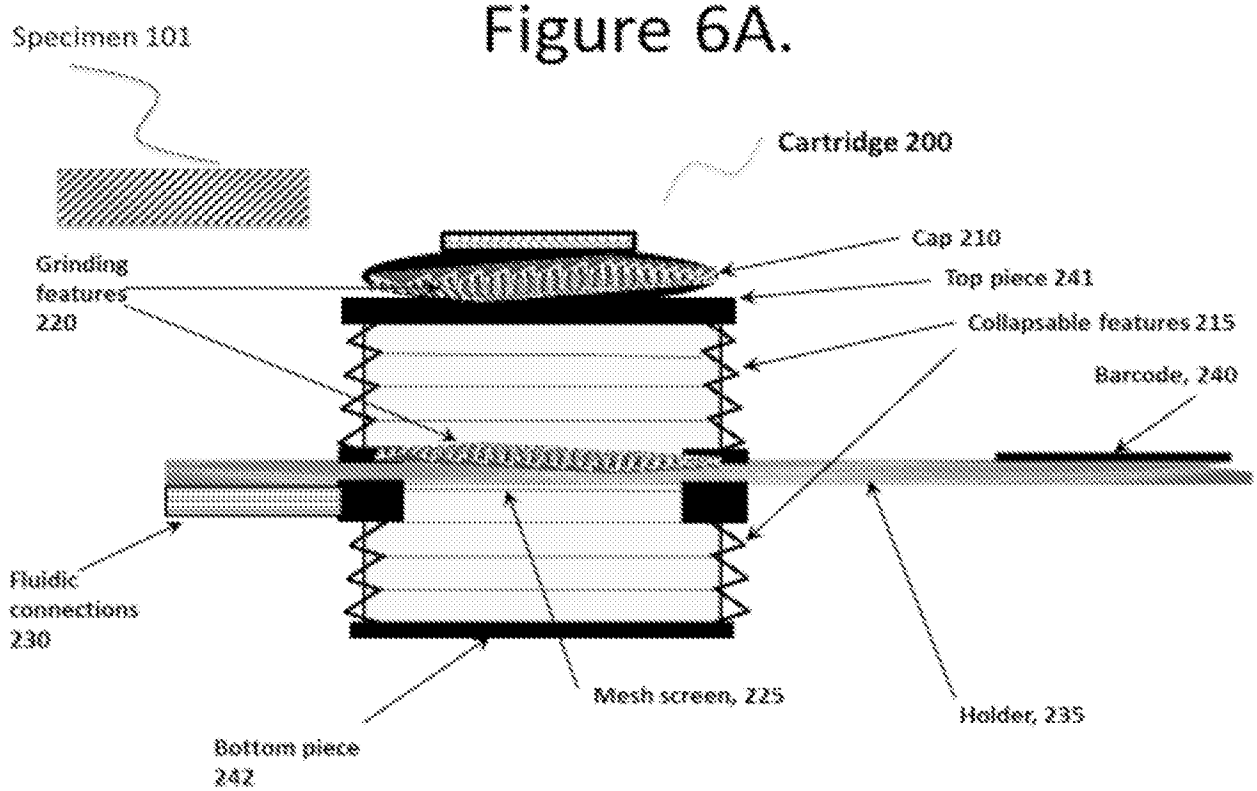


Figure 6B.

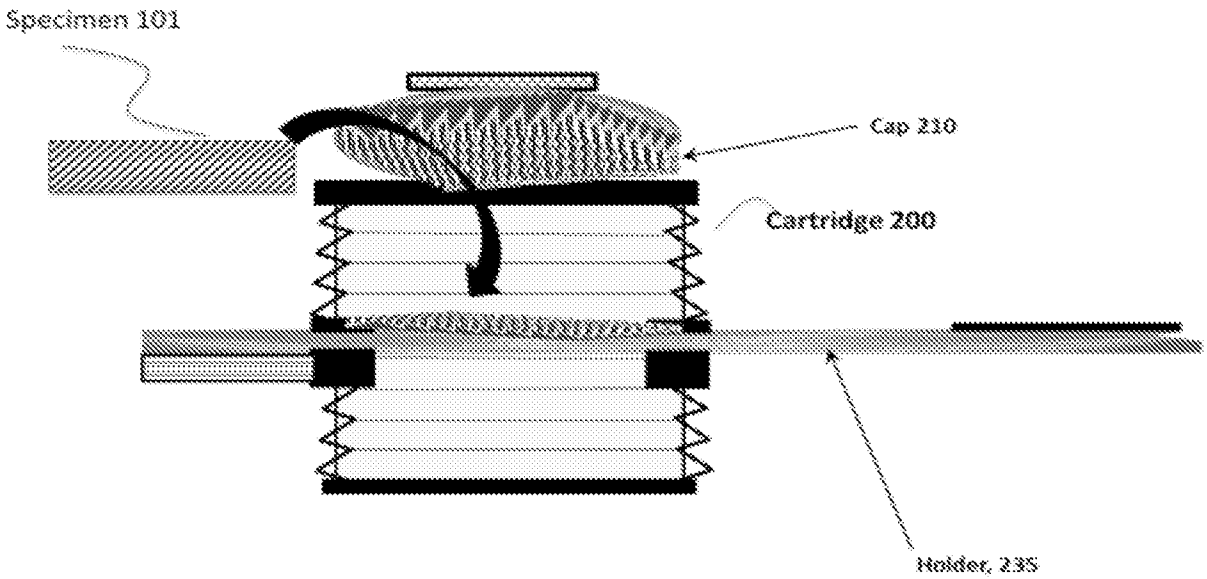


Figure 6C.

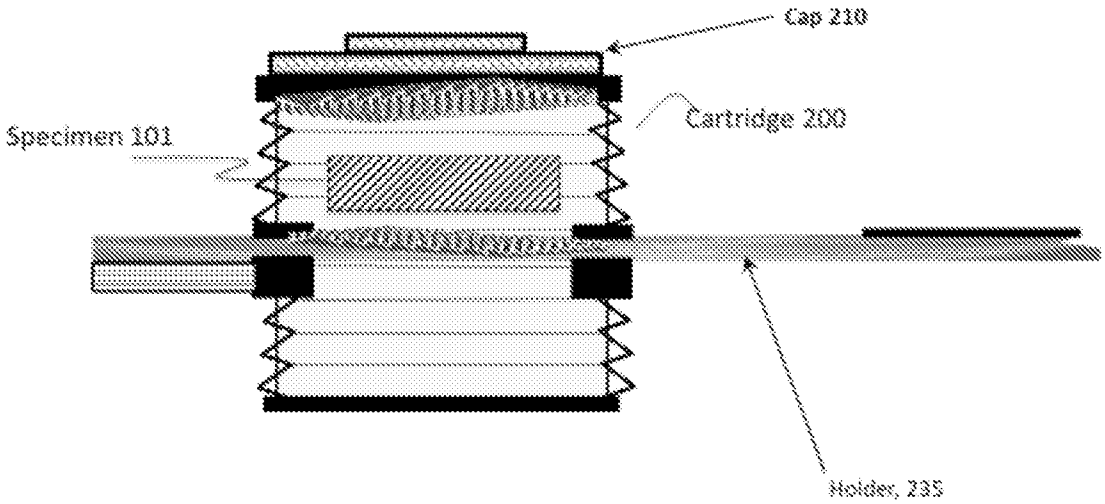


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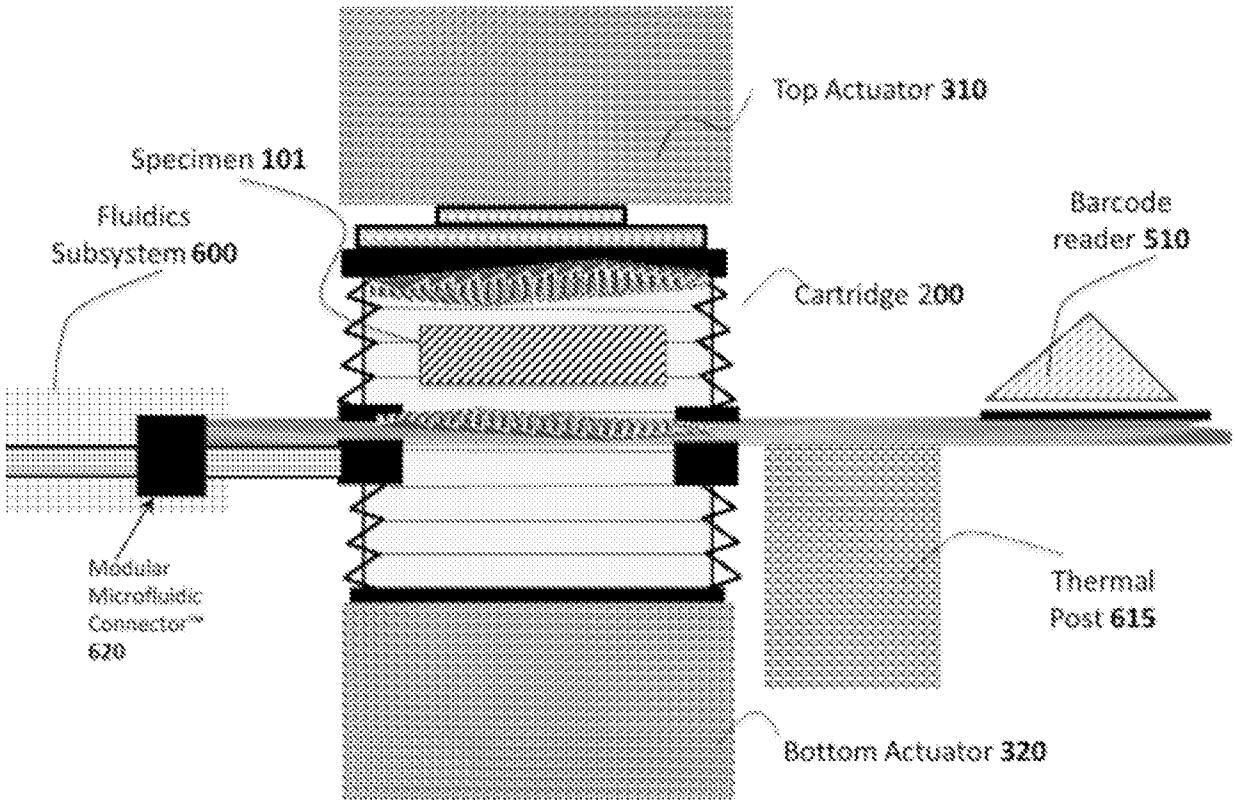


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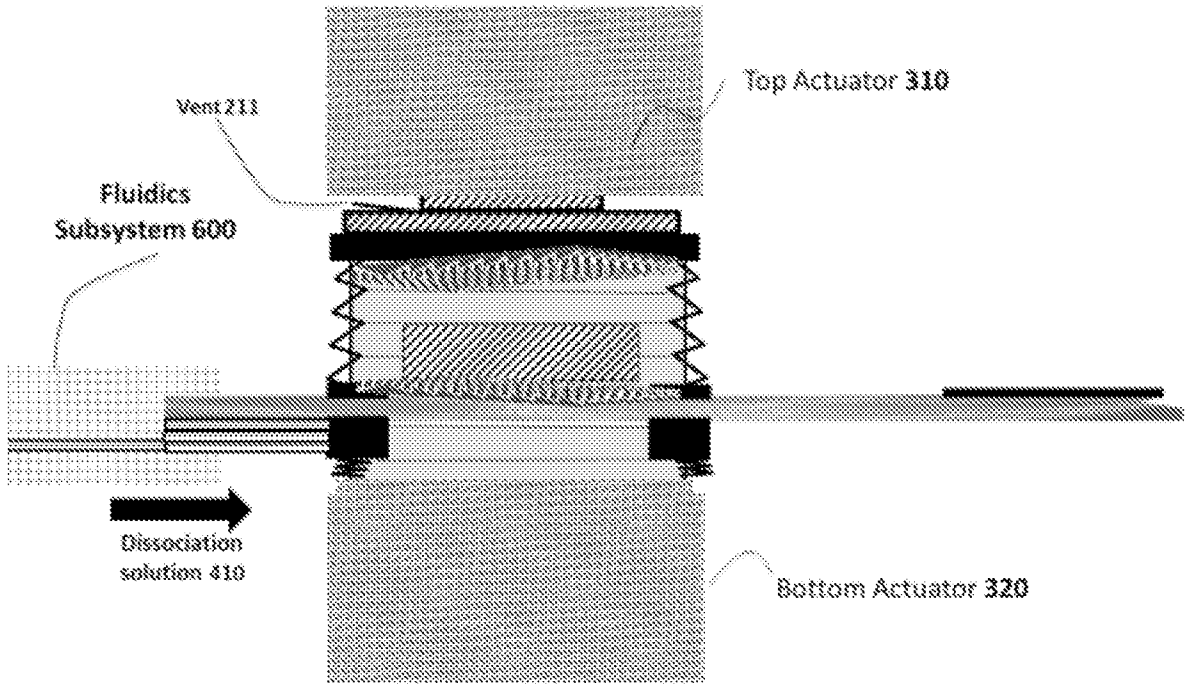


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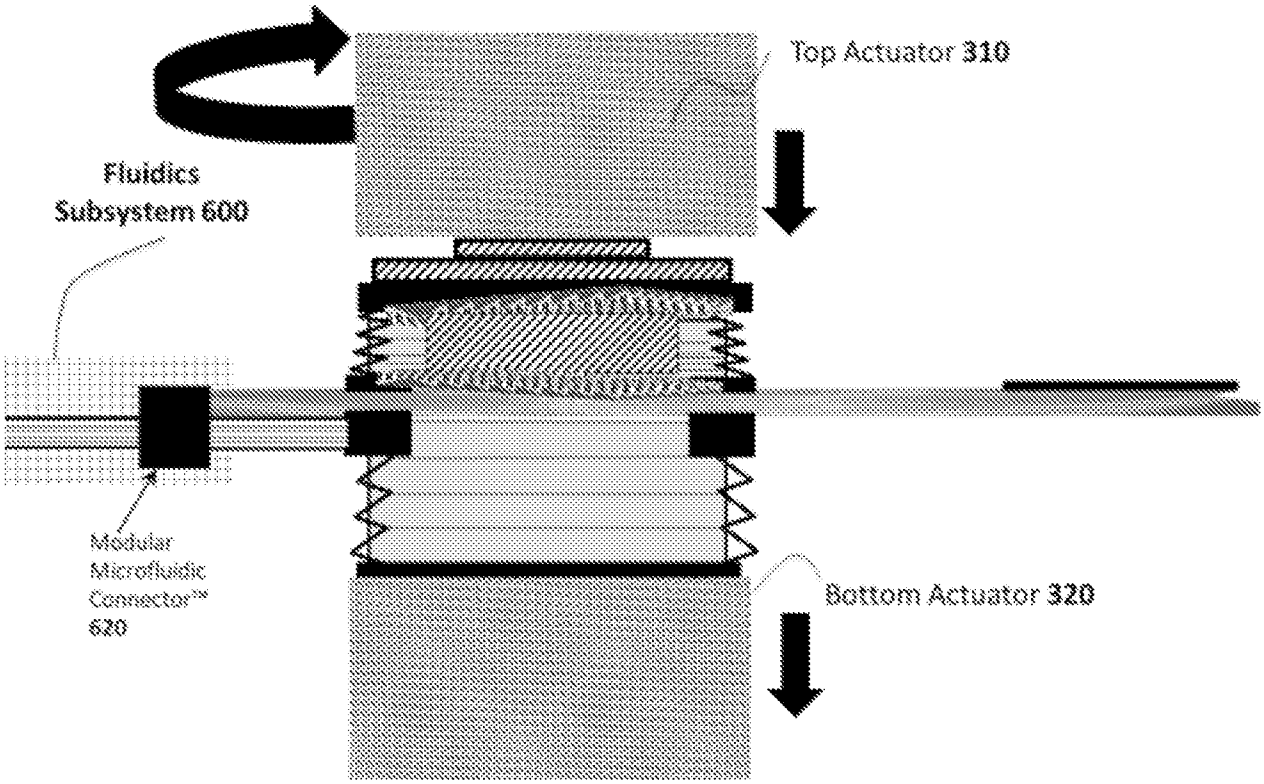


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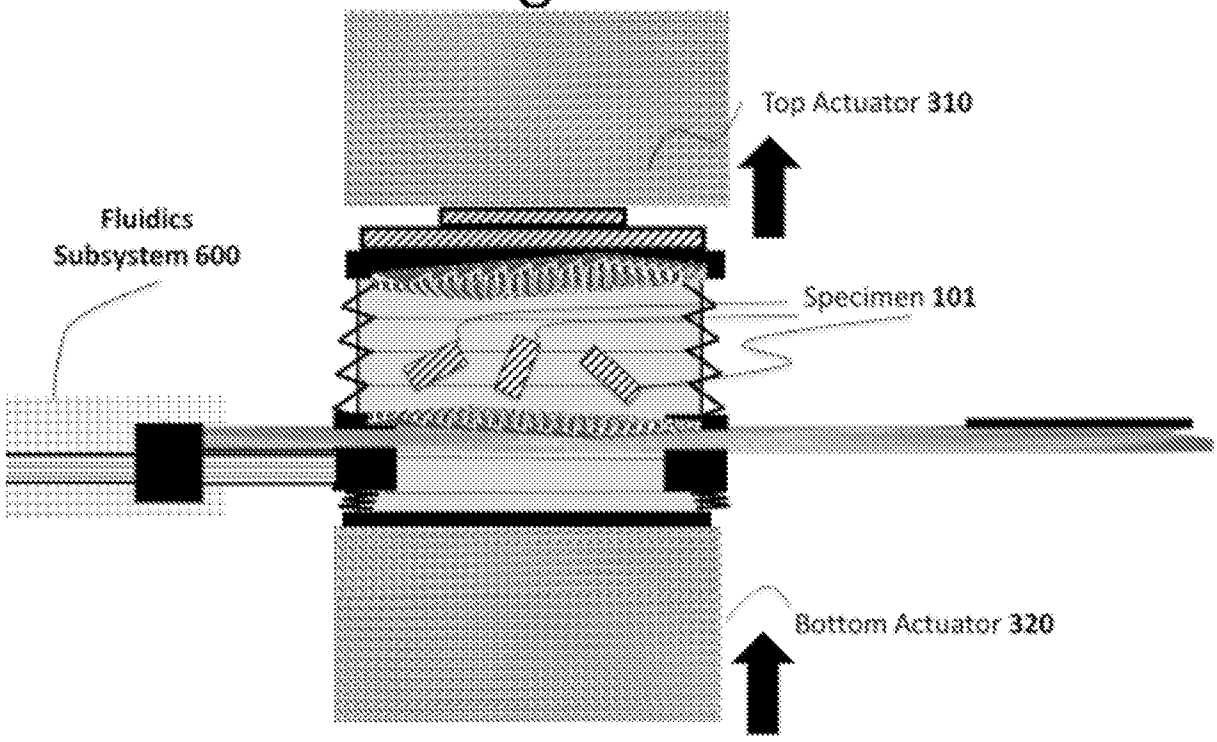


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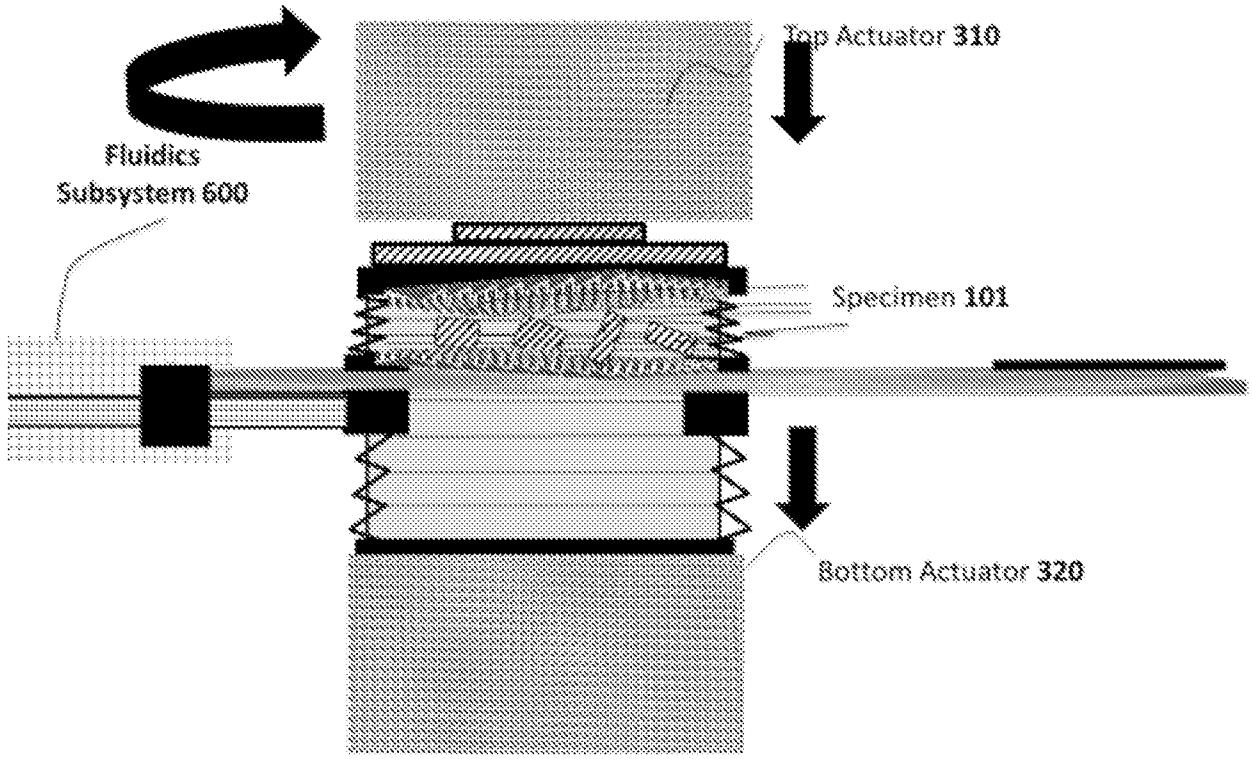


Figure 6I

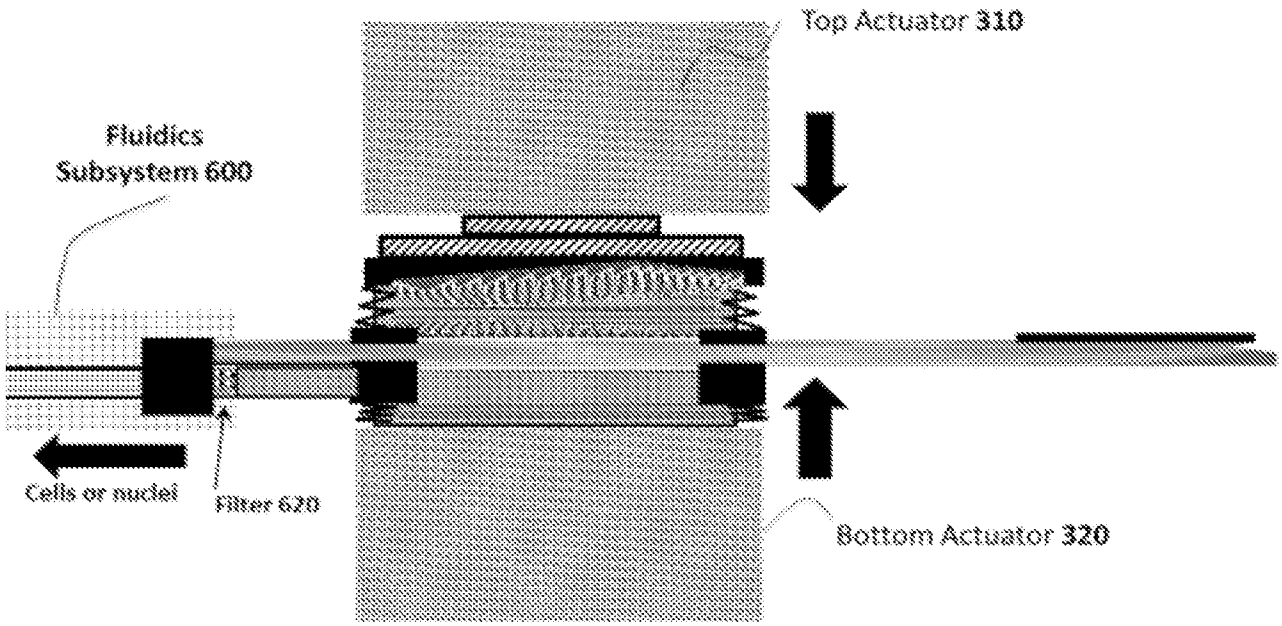


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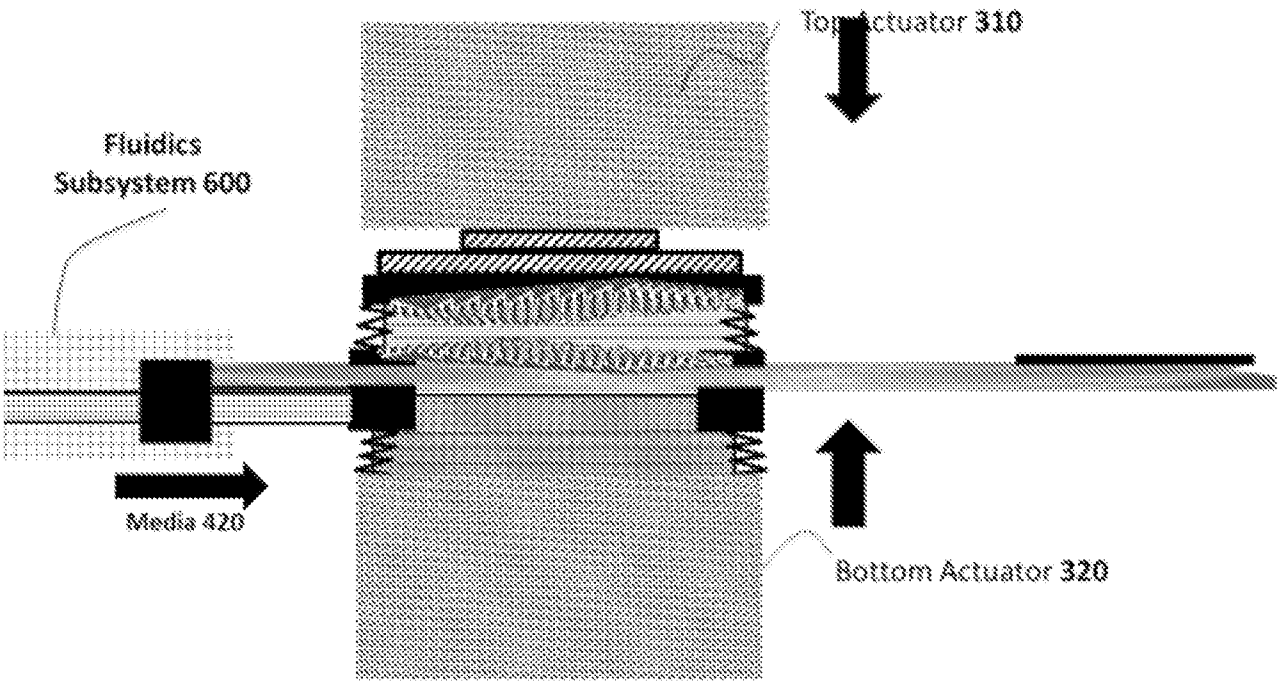


Figure 6K

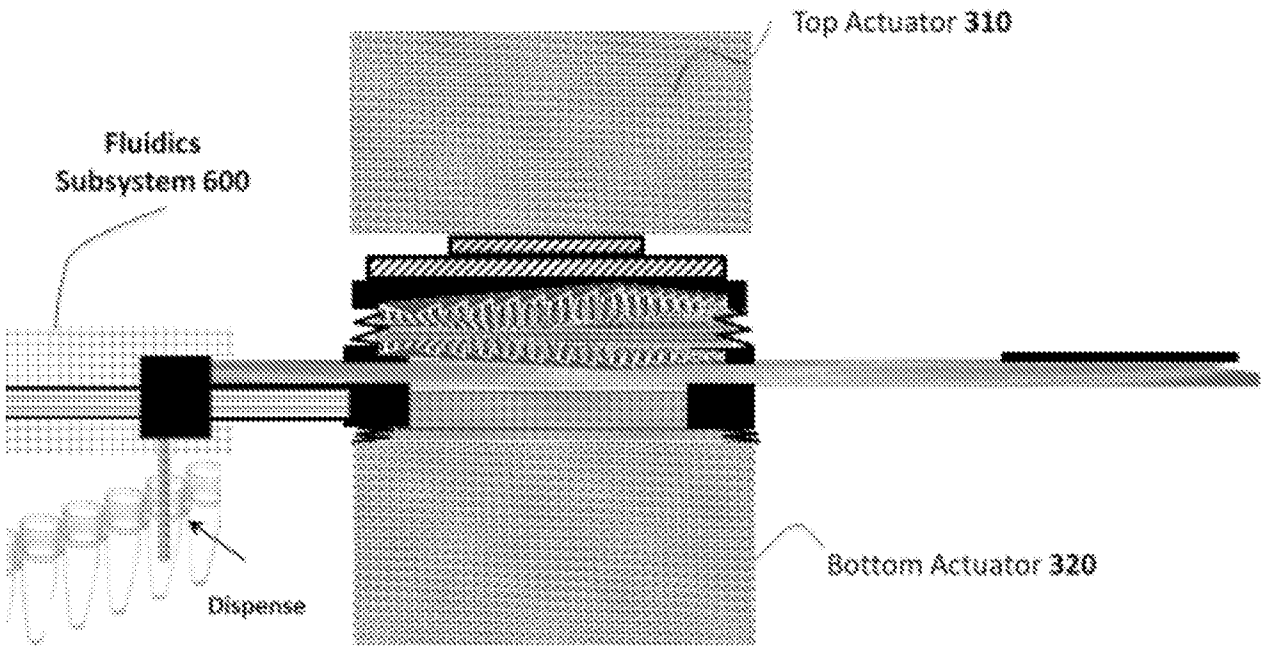


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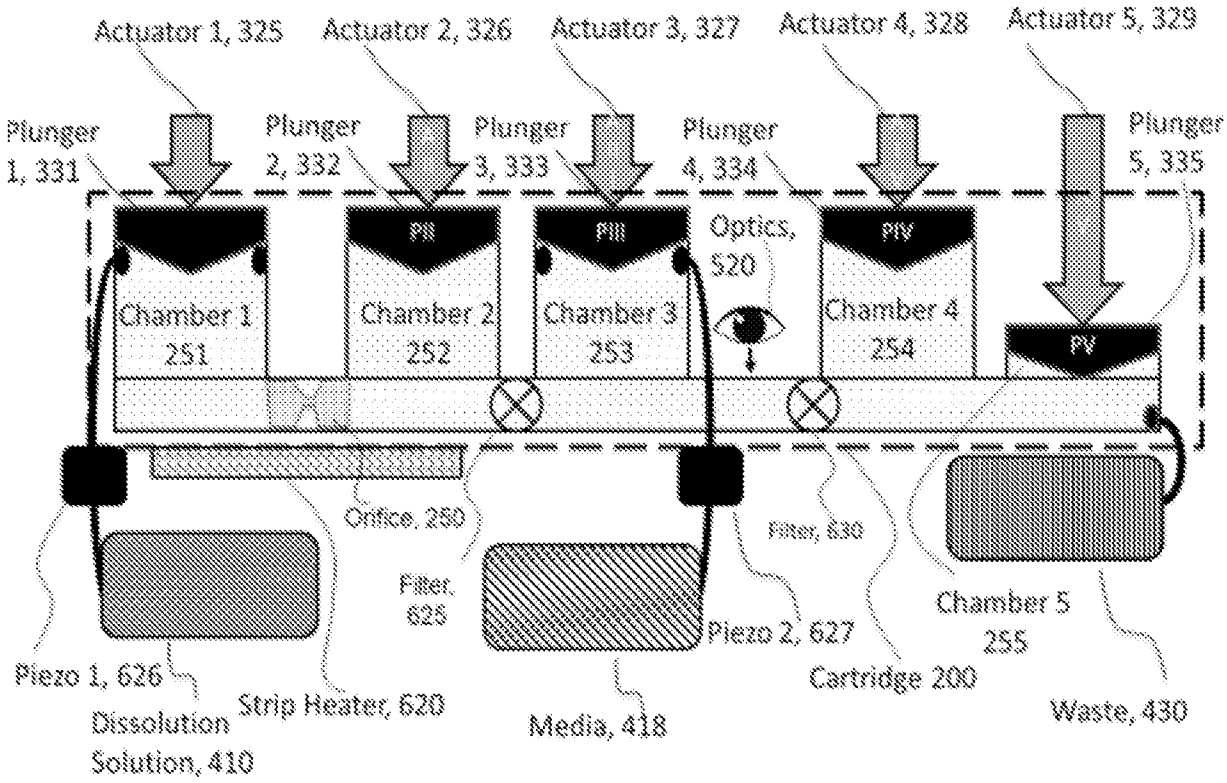


Figure 7B

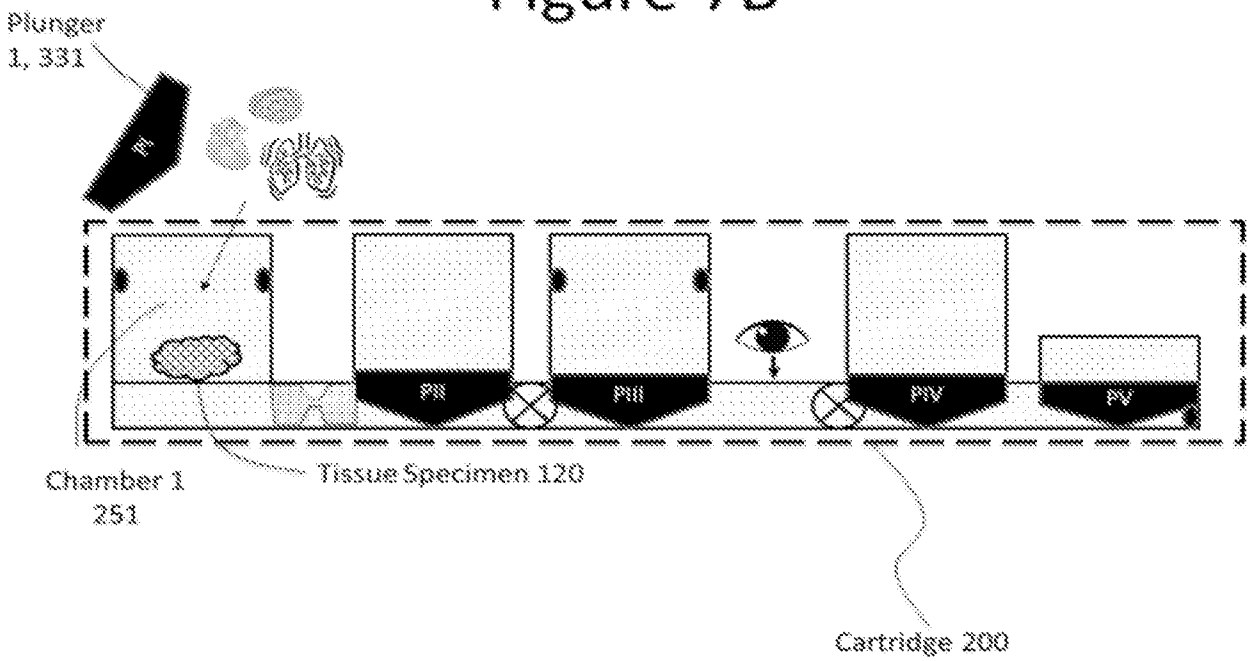


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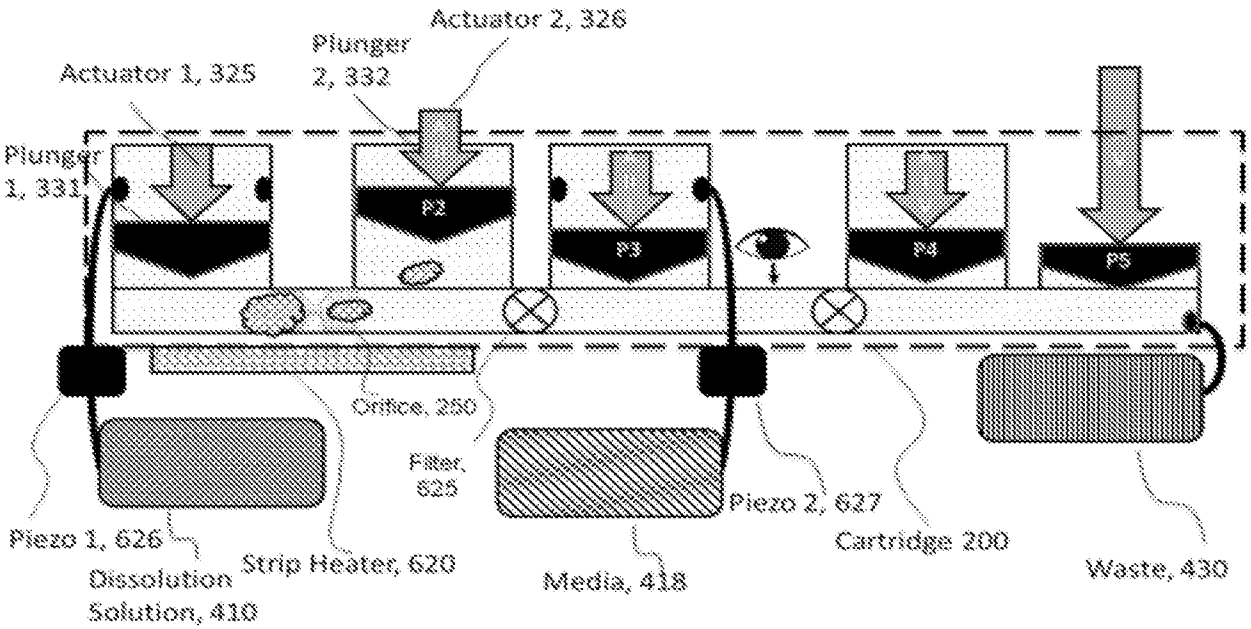


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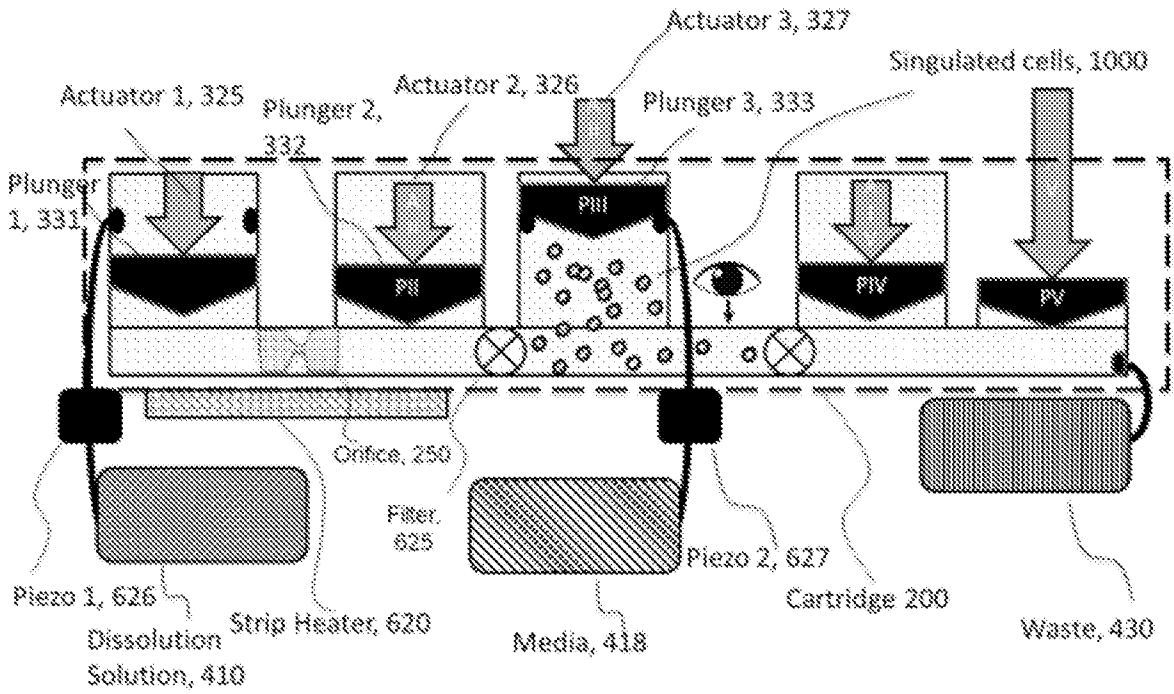


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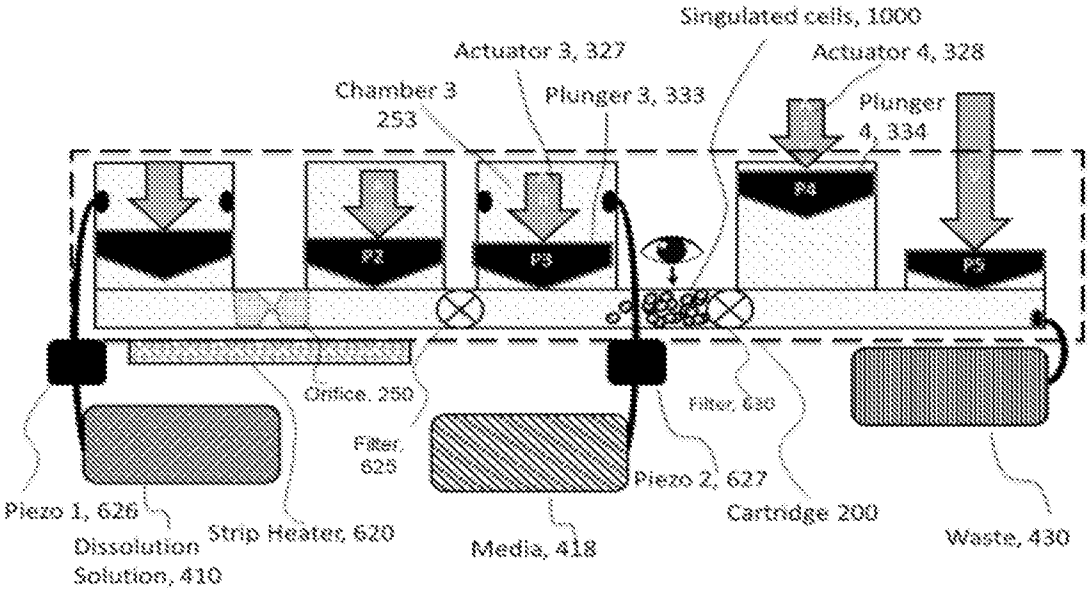


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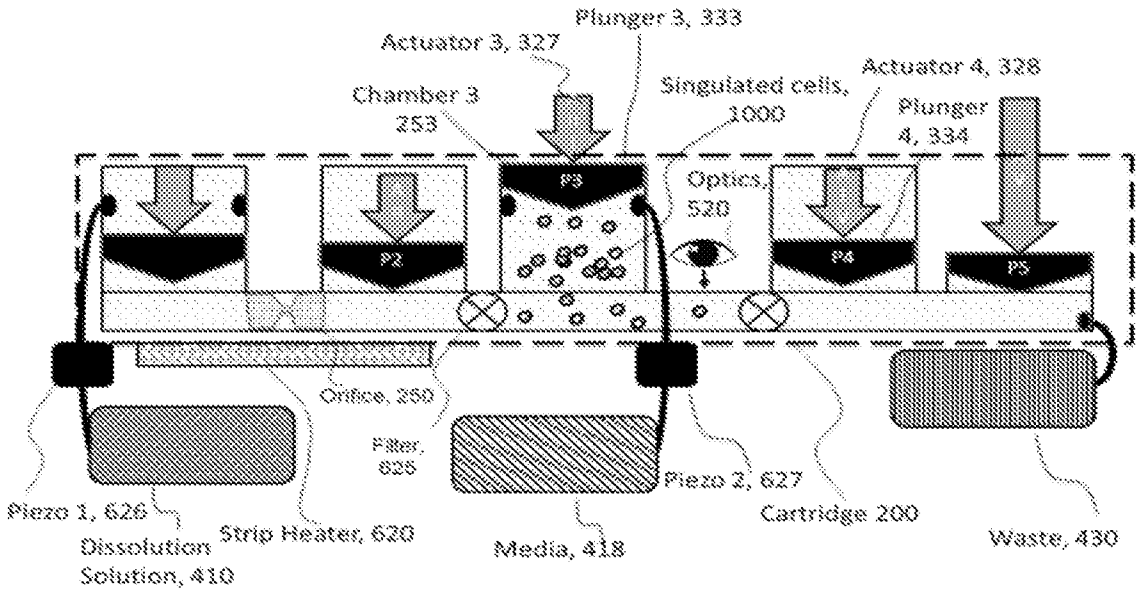


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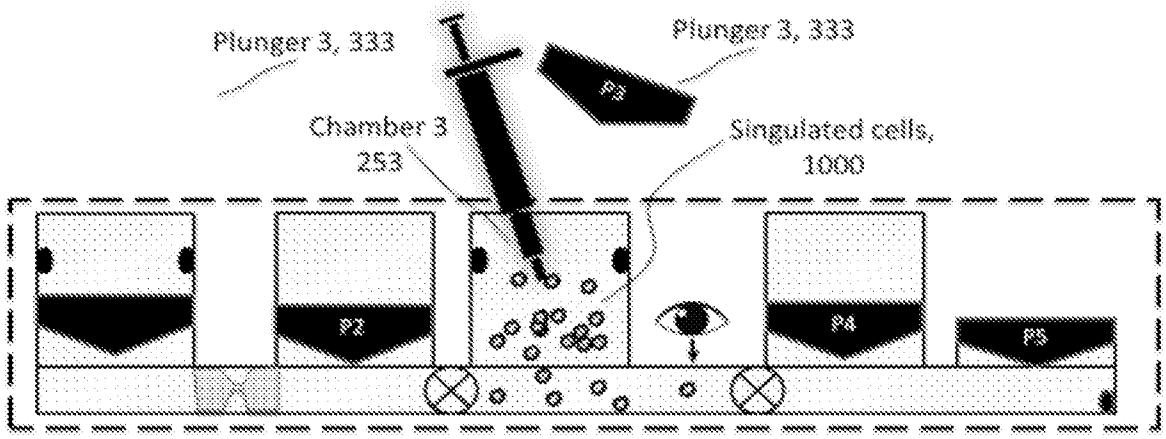


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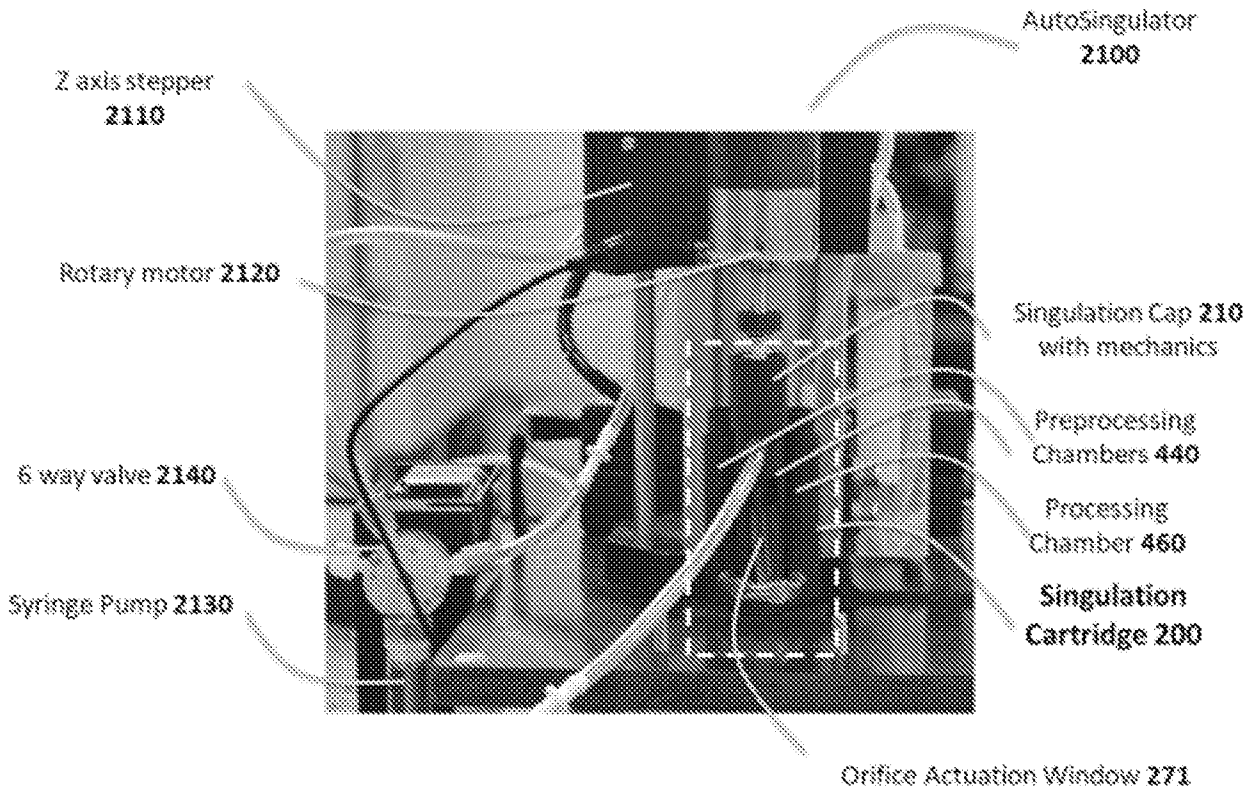


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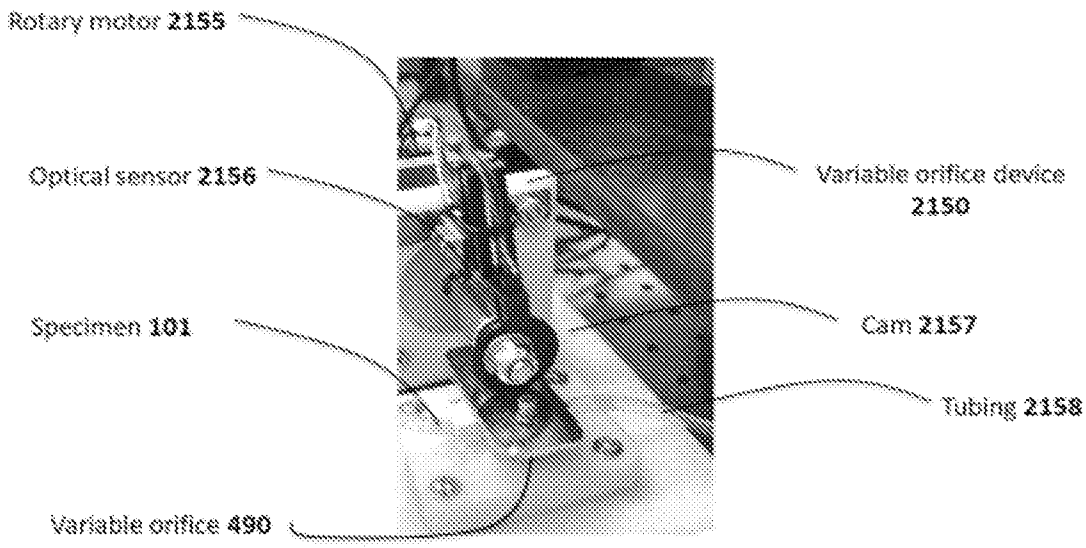


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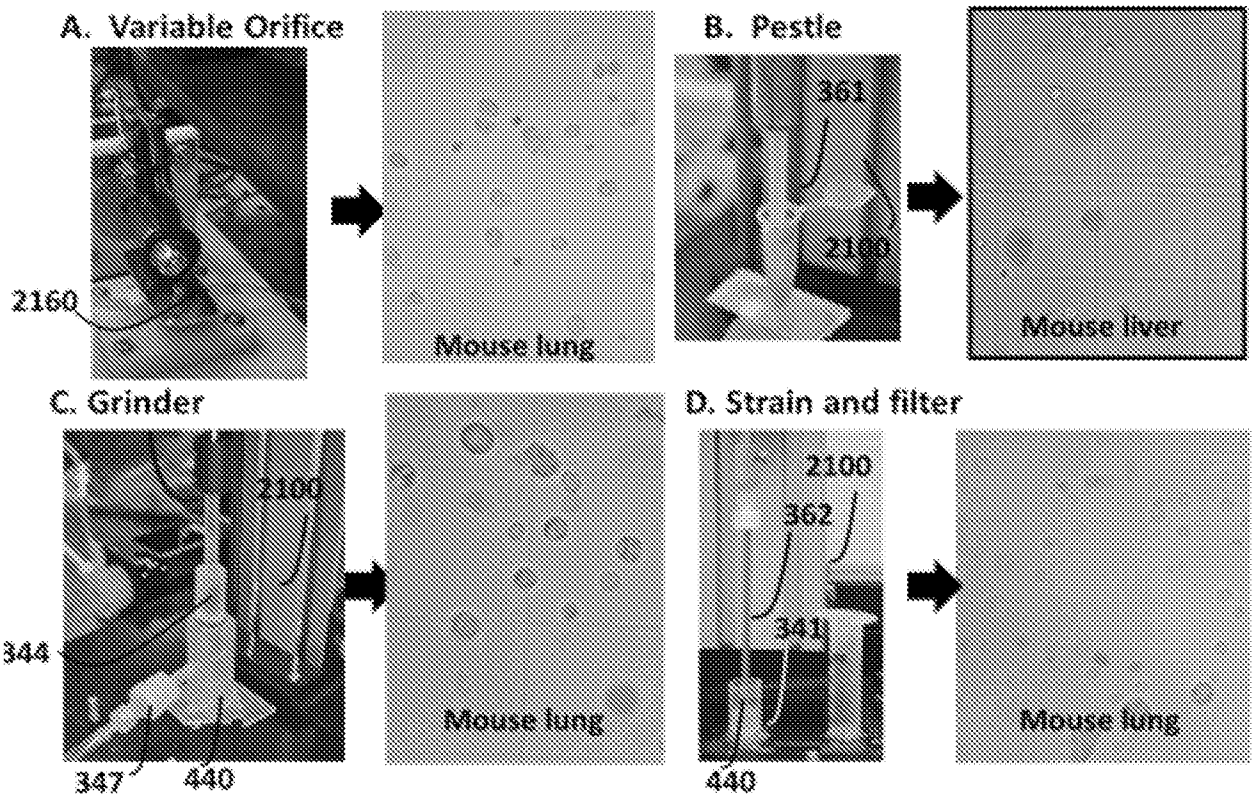


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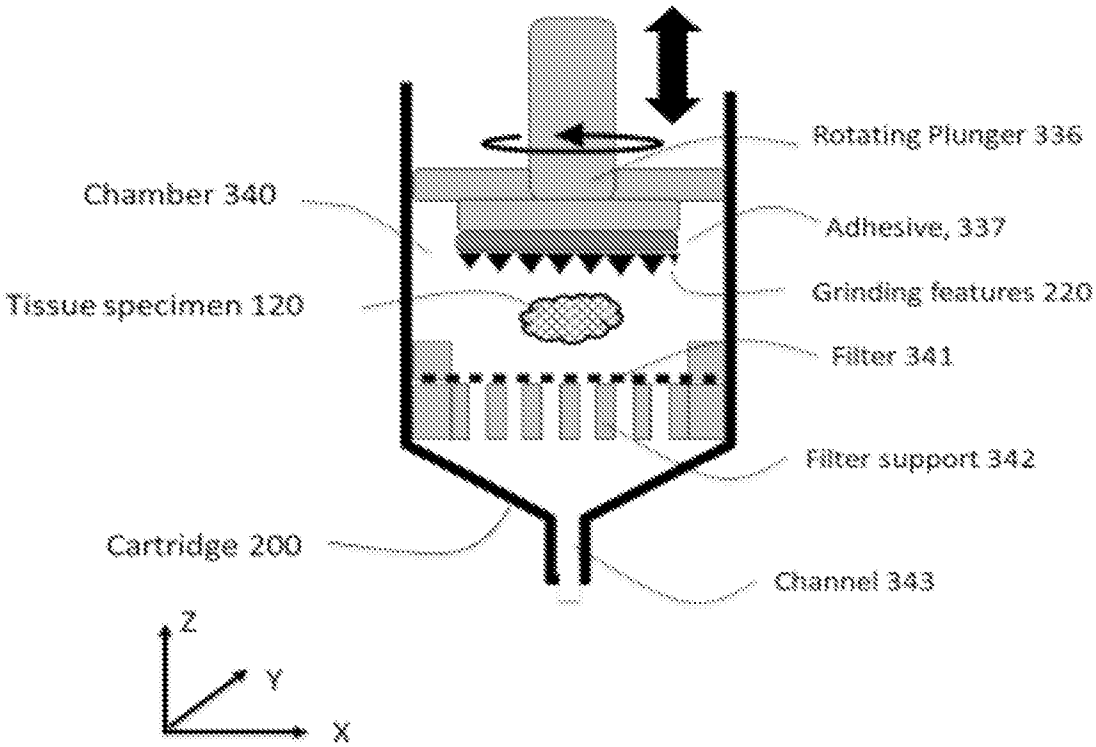


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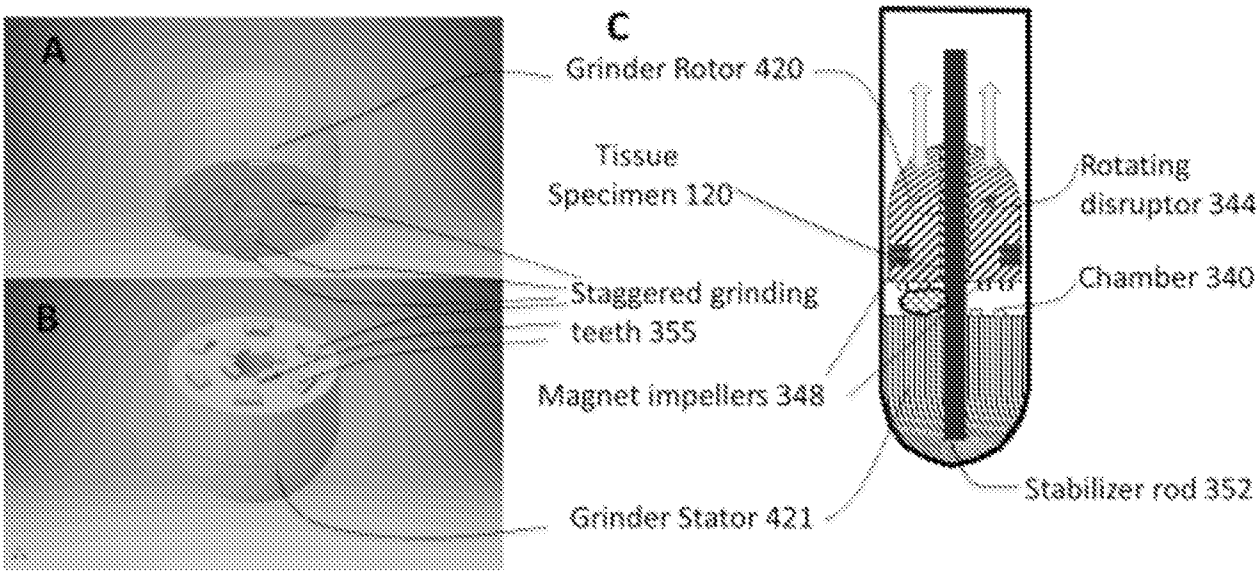


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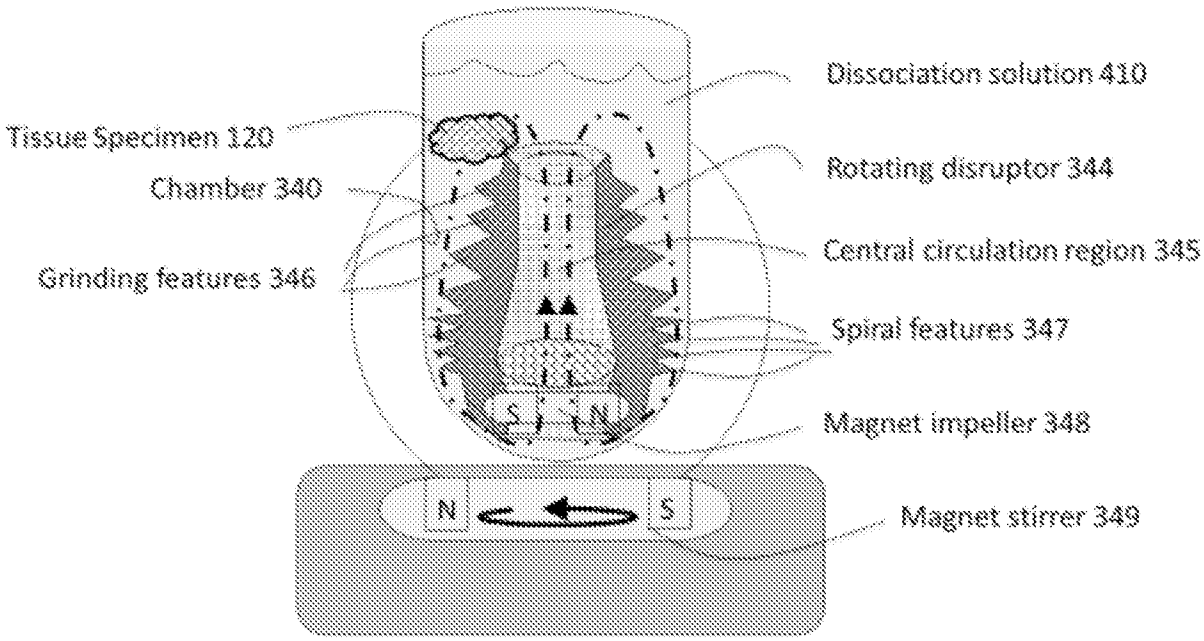


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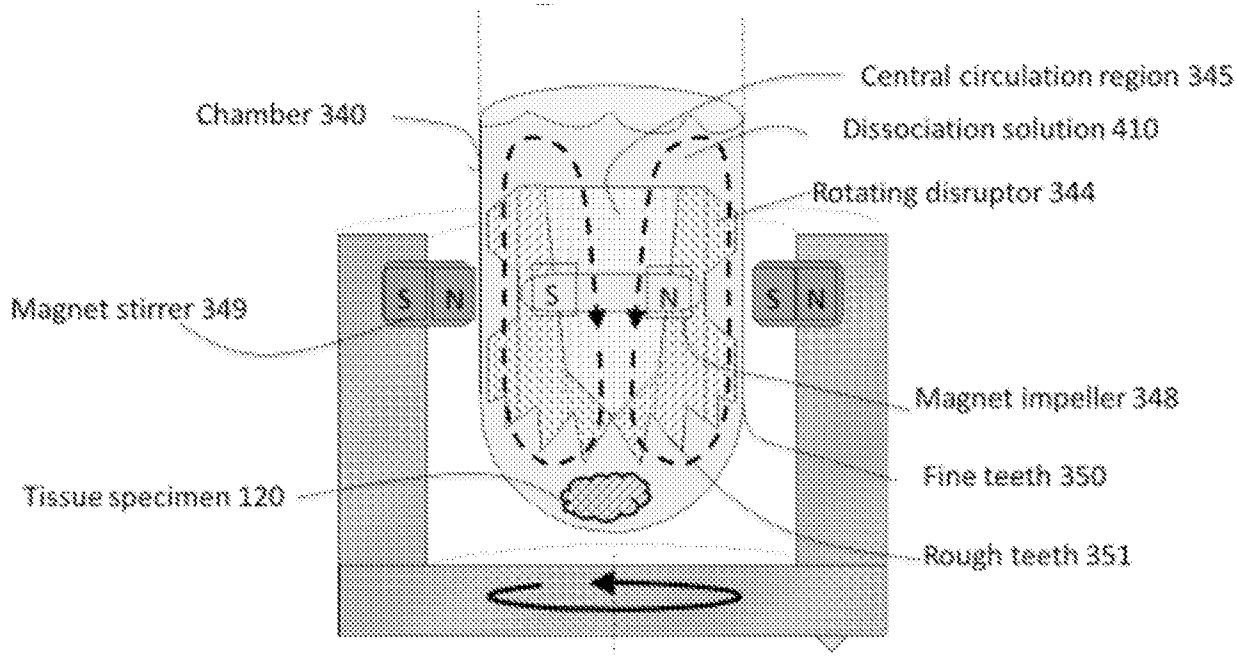


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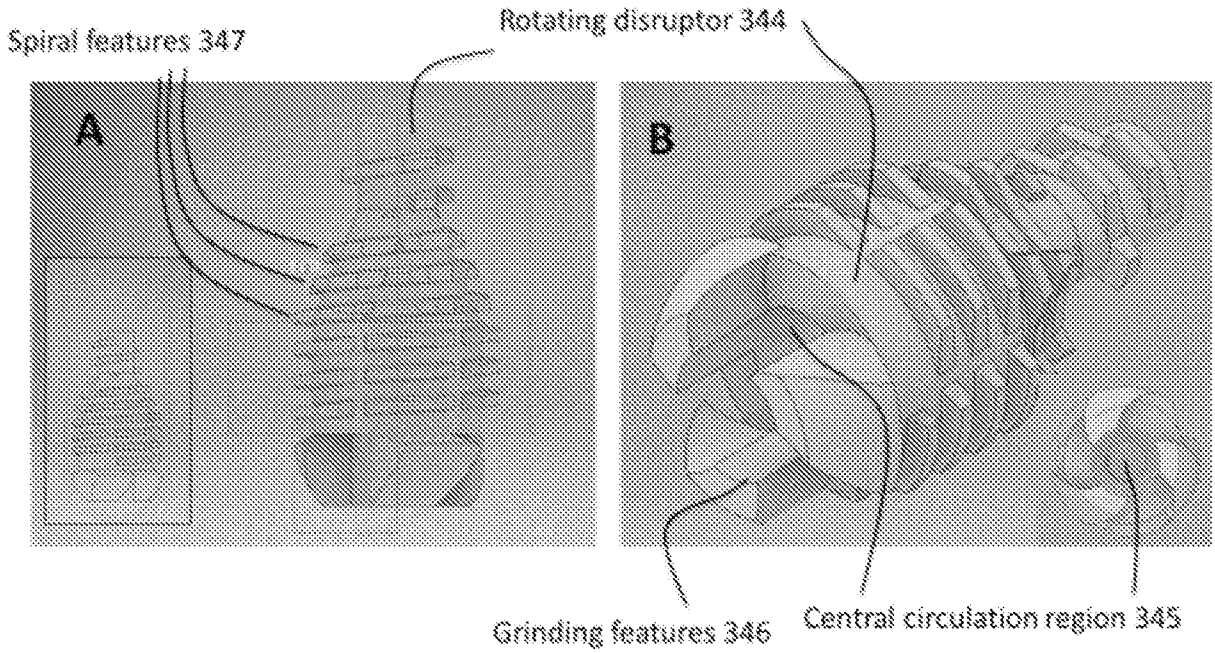

Spiral features 347
Rotating disruptor 344
A
B
Grinding features 346
Central circulation region 345
Spiral features 347
Rotating disruptor 344
C
D
Central circulation region 345
Grinding features 346

Figure 16

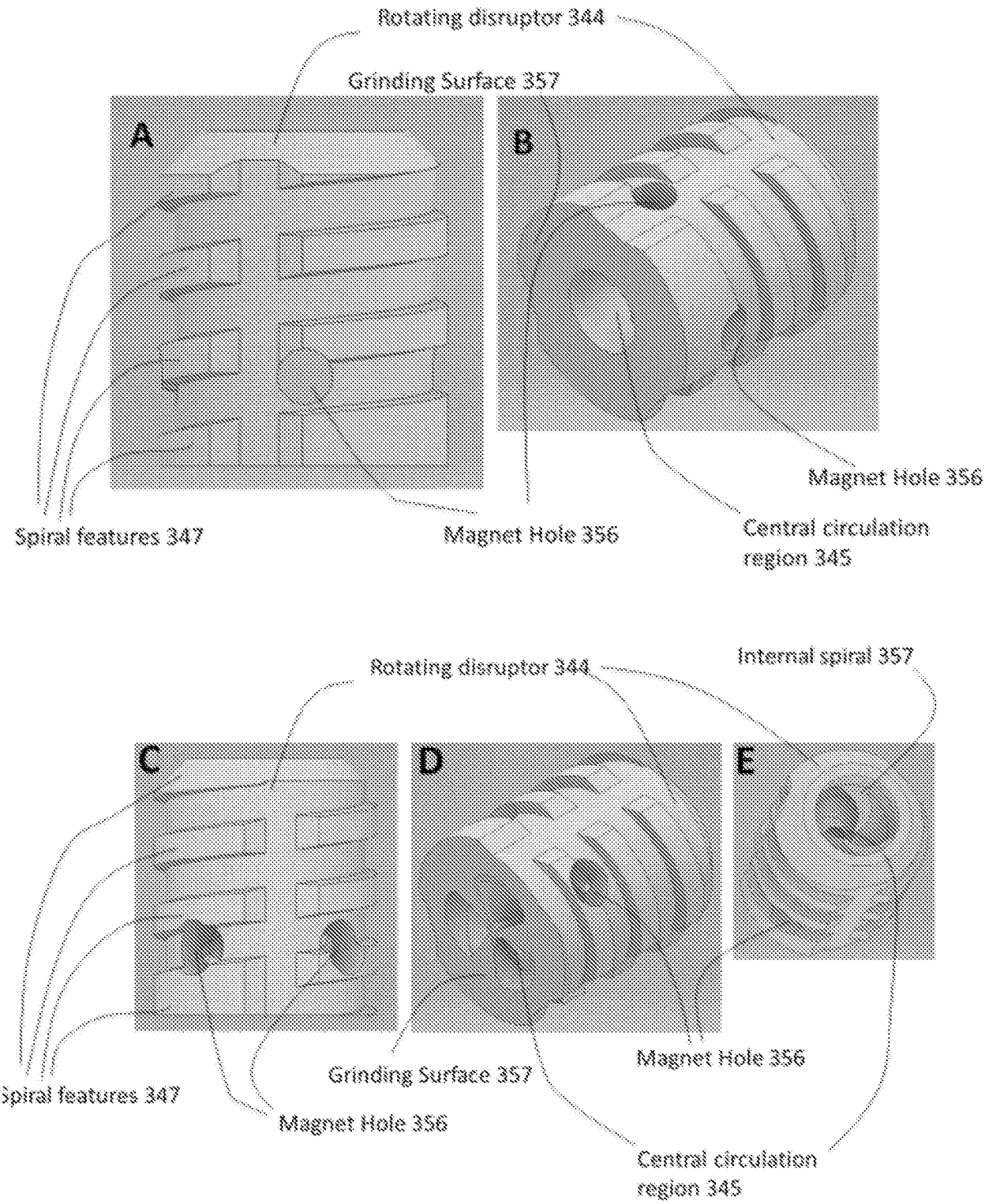

Rotating disruptor 344
Grinding Surface 357
A
B
Magnet Hole 356
Spiral features 347
Magnet Hole 356
Central circulation region 345
Internal spiral 357
Rotating disruptor 344
C
D
E
Magnet Hole 356
Grinding Surface 357
Spiral features 347
Magnet Hole 356
Central circulation region 345

Figure 17

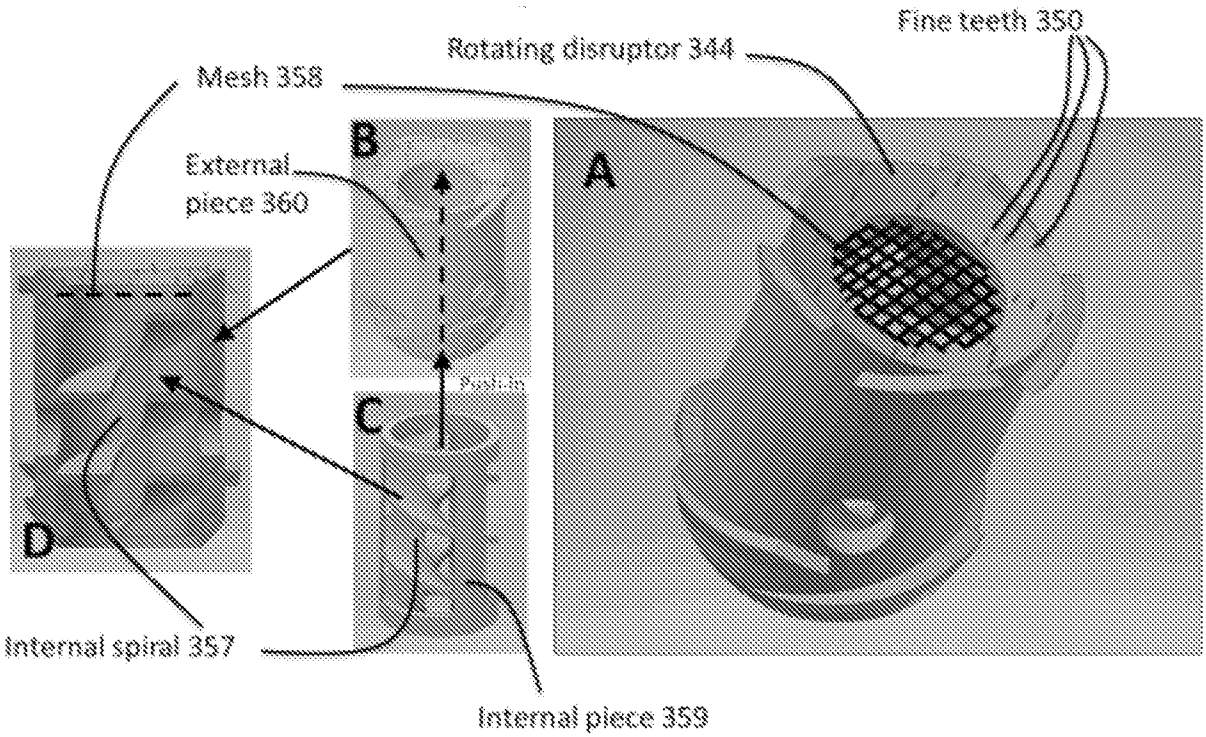


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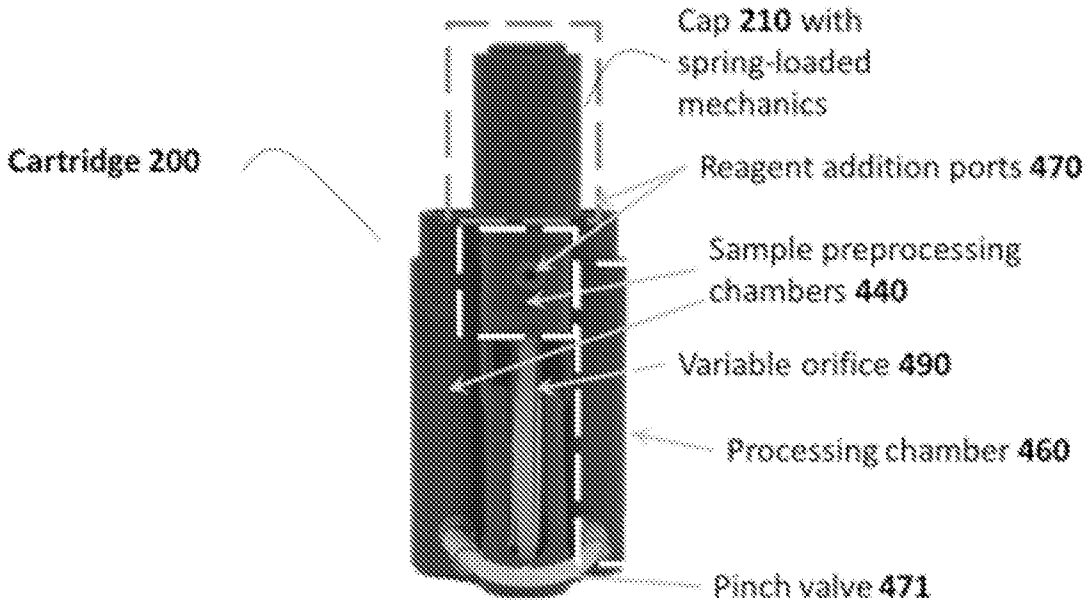


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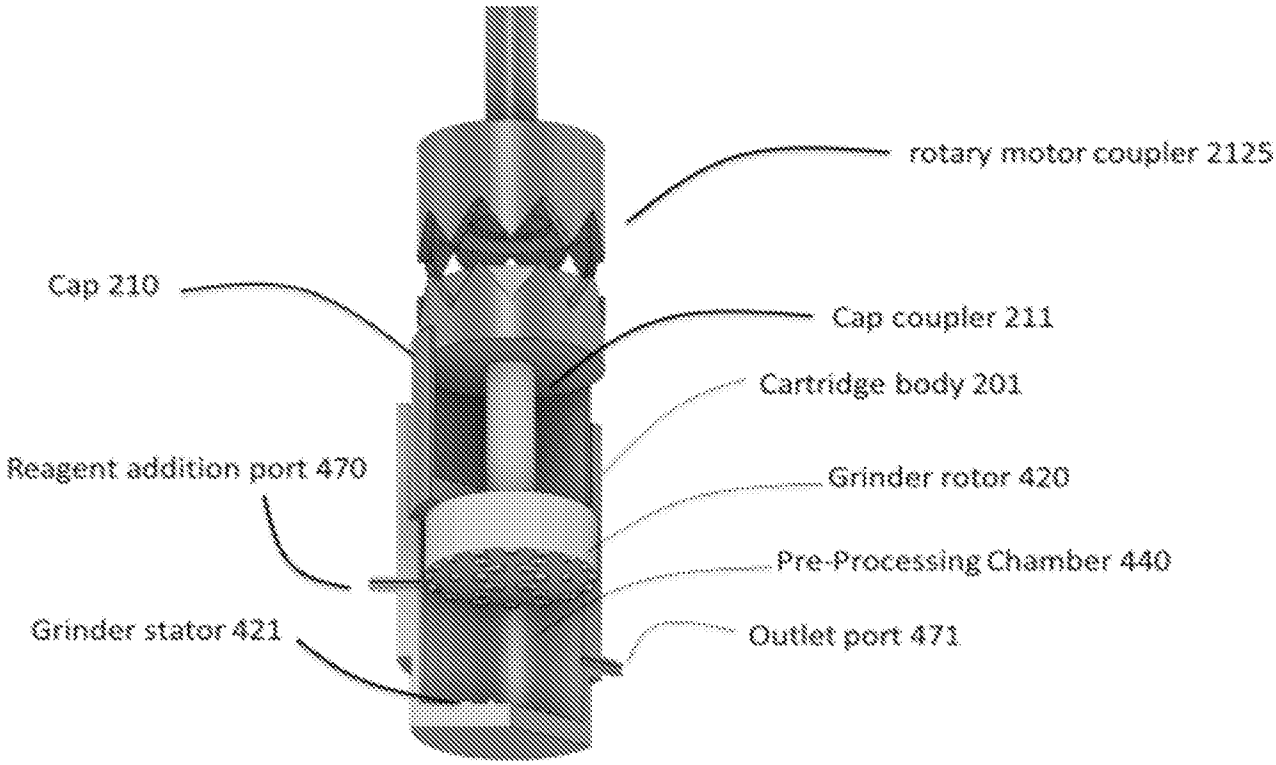


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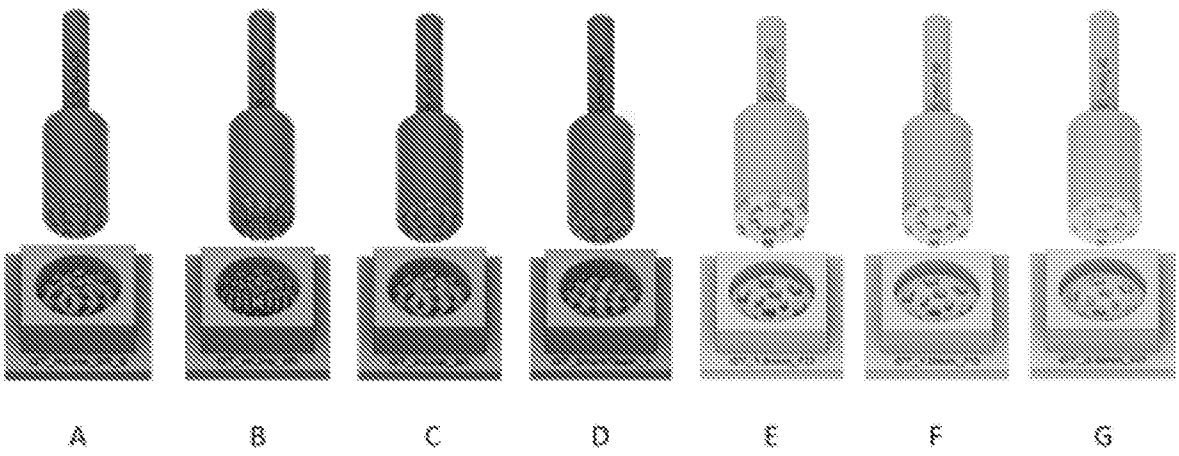


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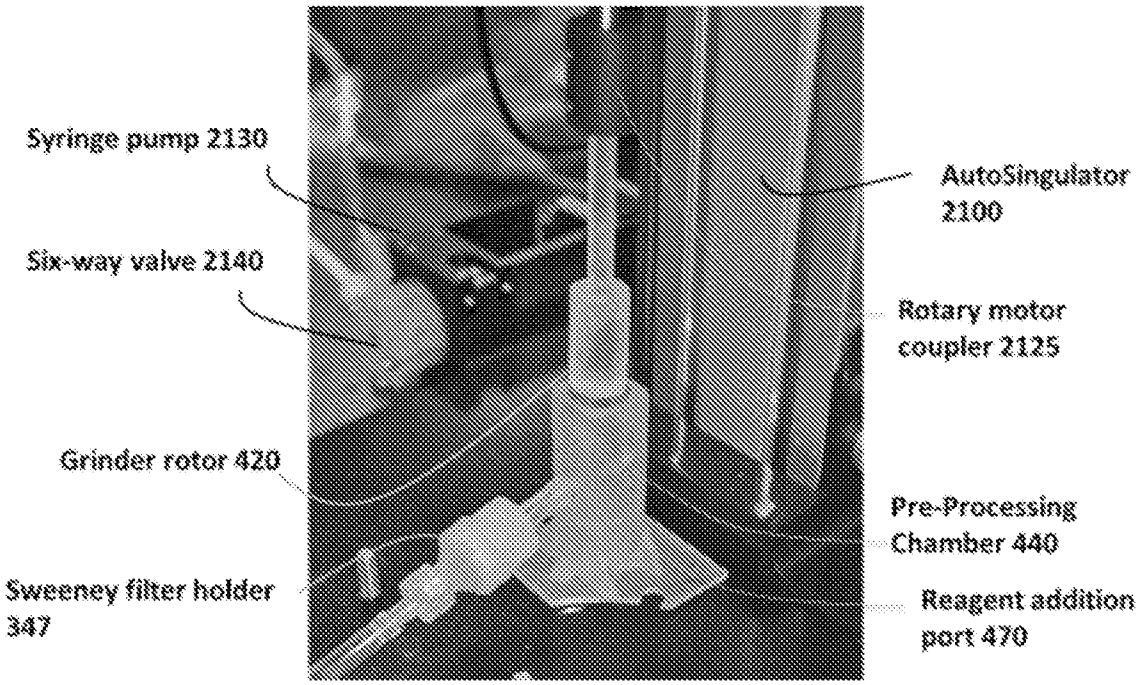

Syringe pump 2130
Six-way valve 2140
Grinder rotor 420
Sweeney filter holder 347
AutoSingulator 2100
Rotary motor coupler 2125
Pre-Processing Chamber 440
Reagent addition port 470

Figure 22A

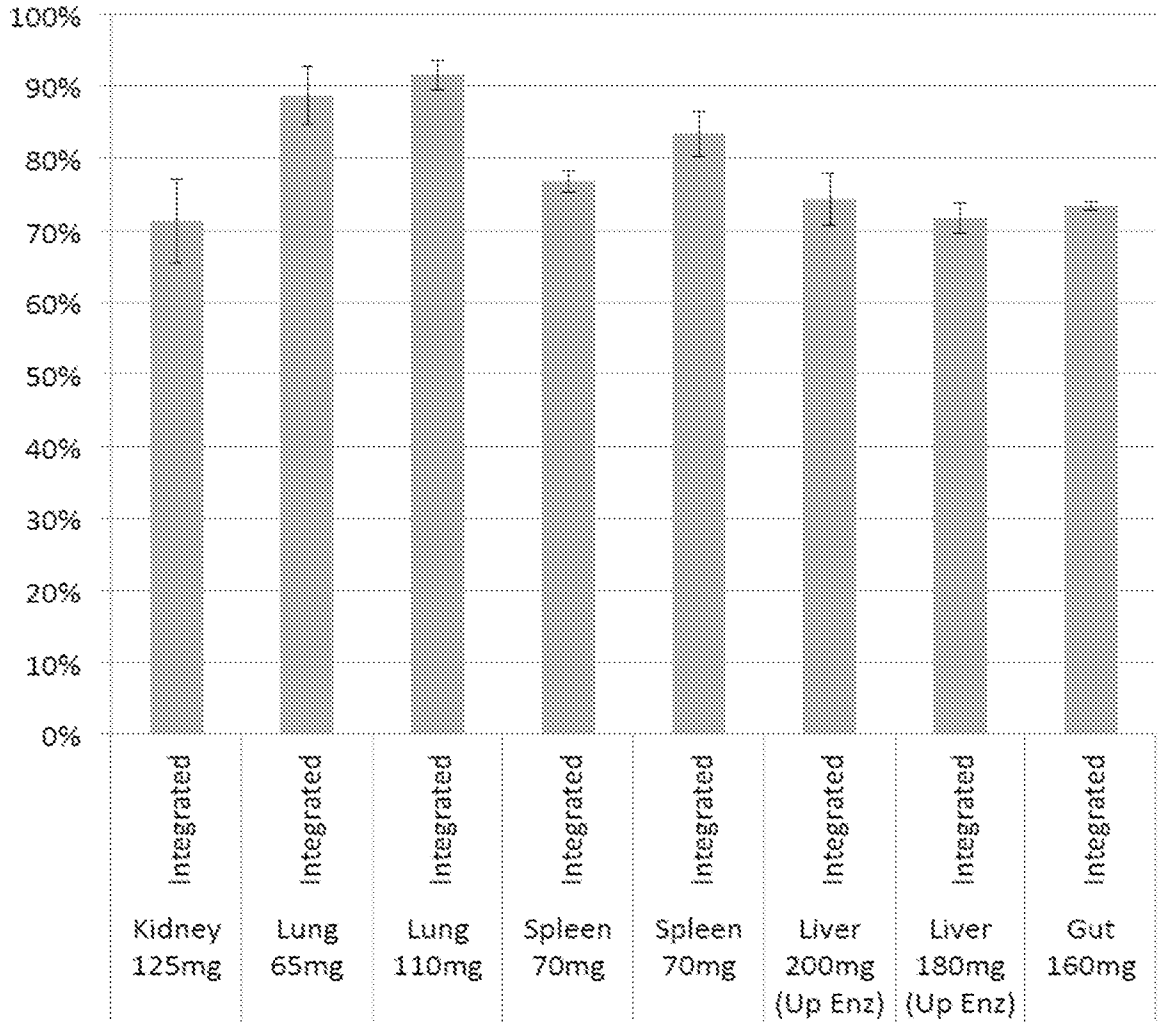


Figure 22B

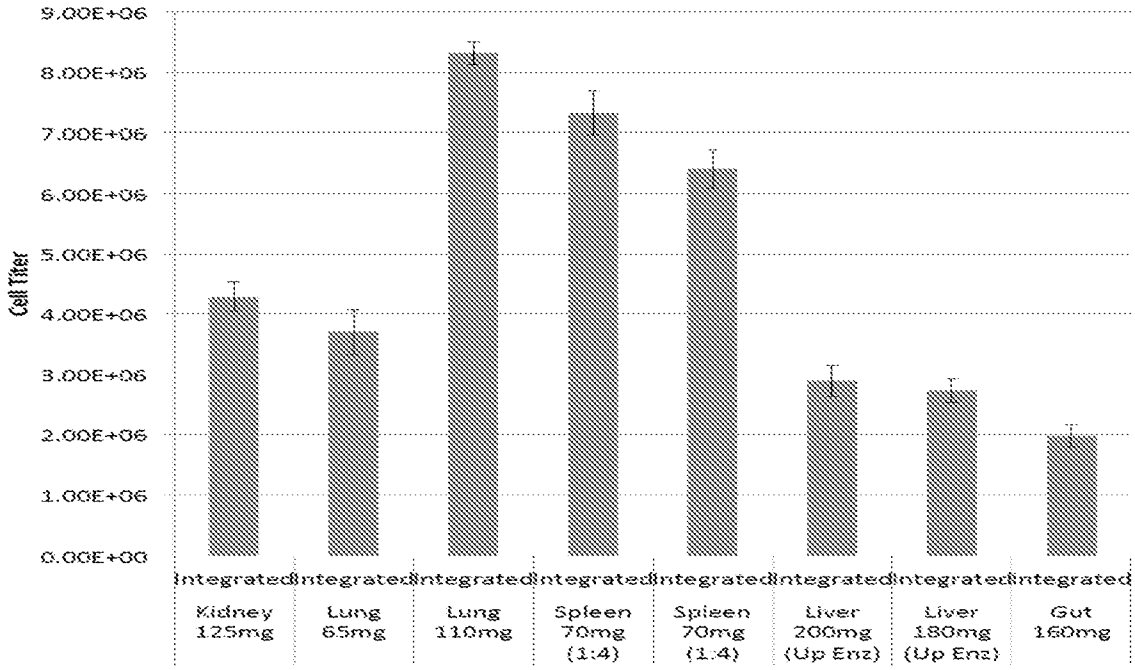


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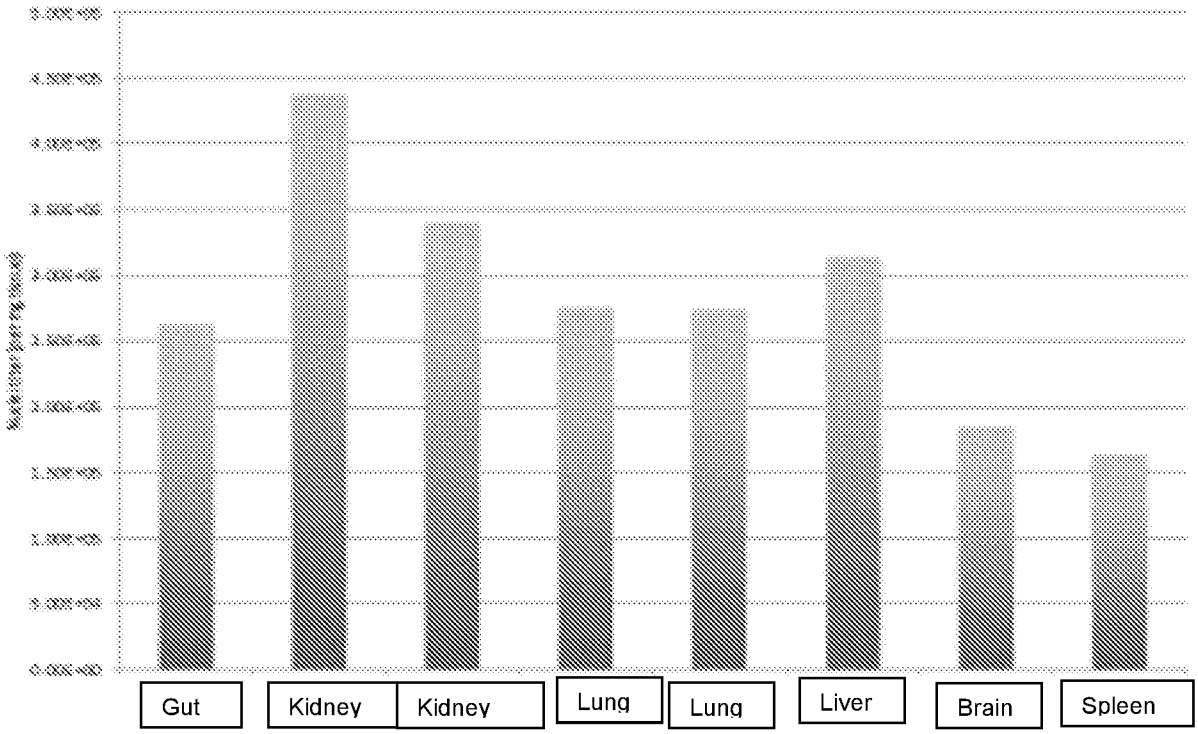


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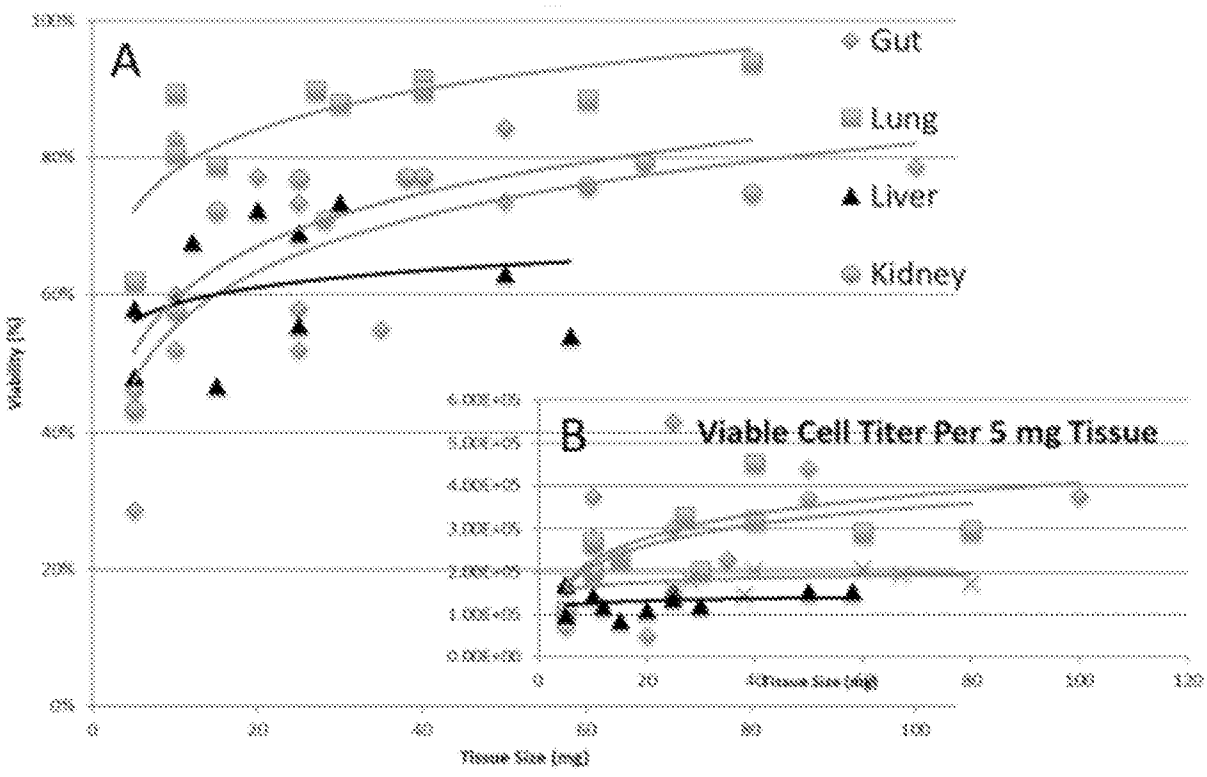


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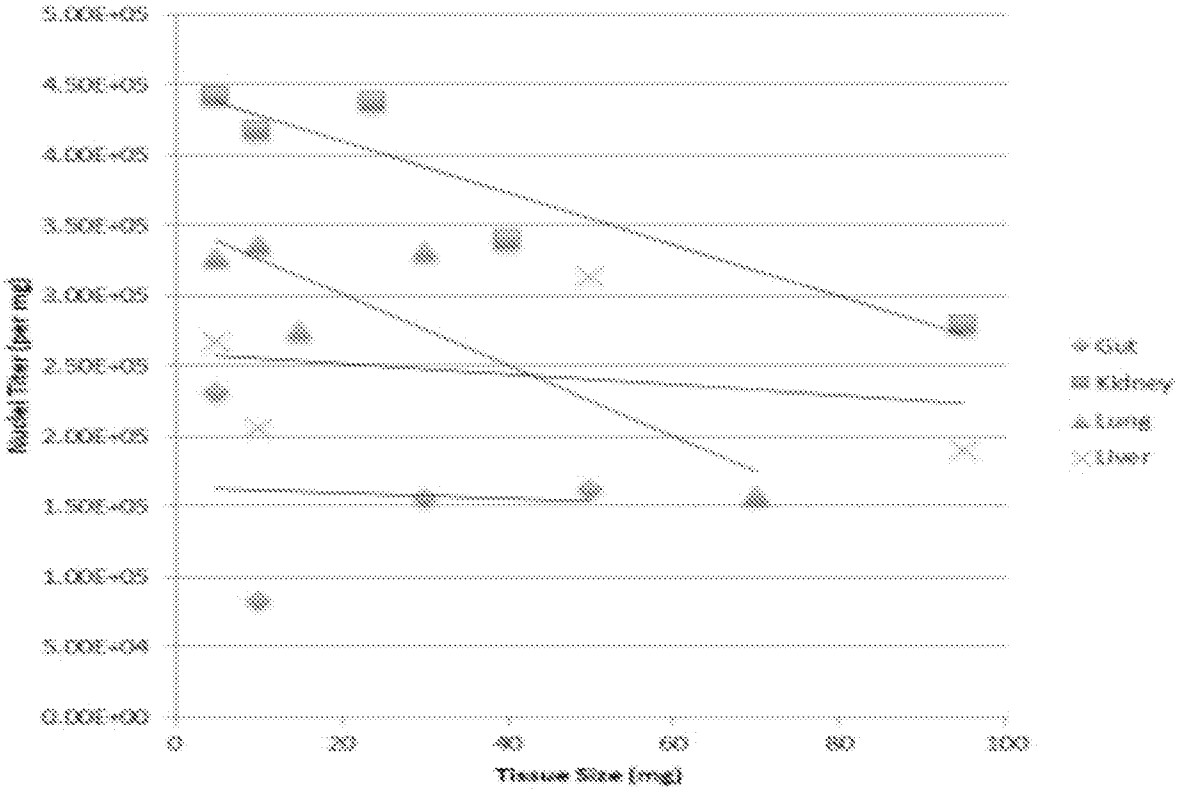


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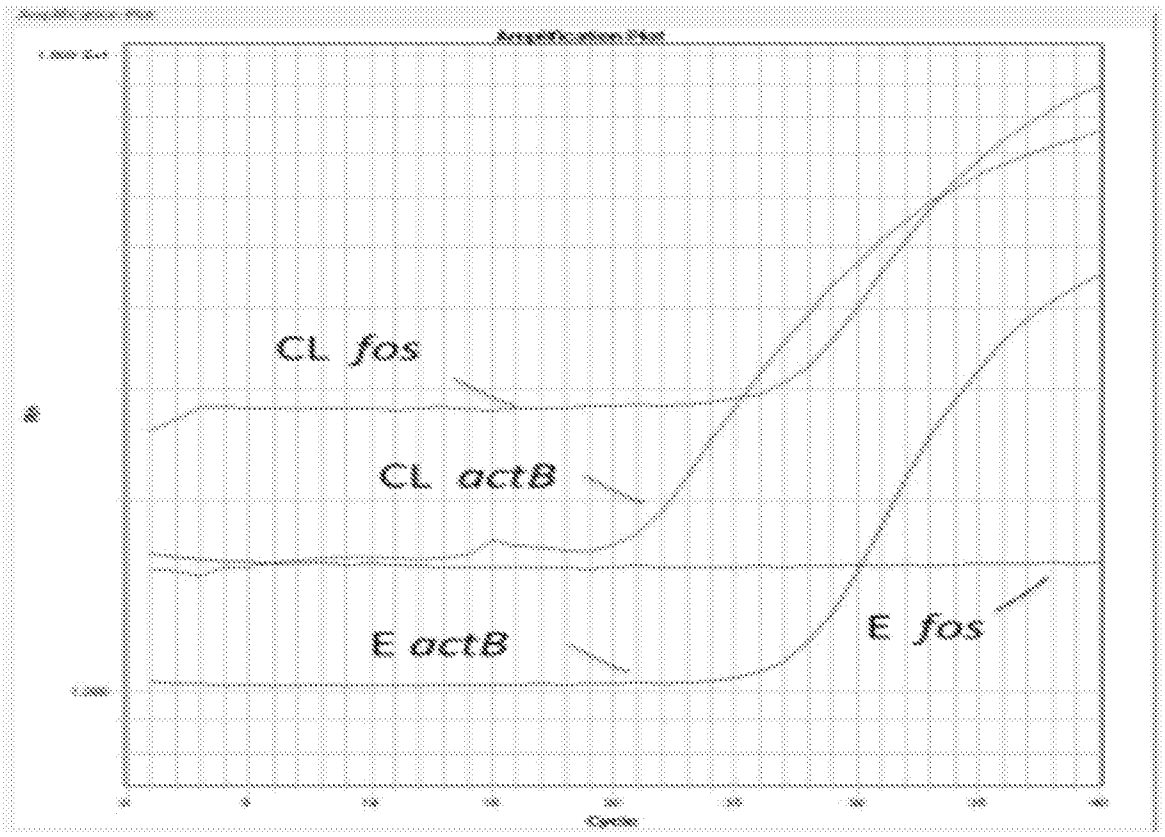


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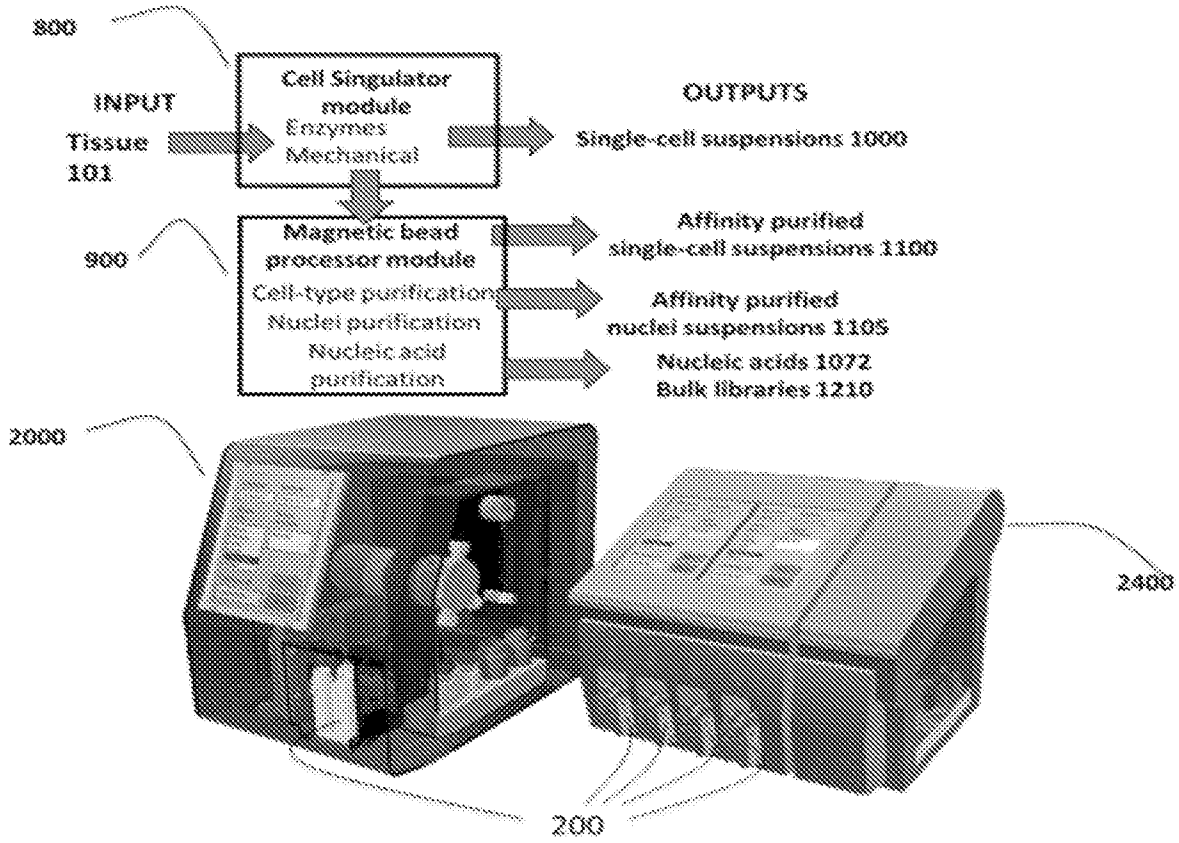


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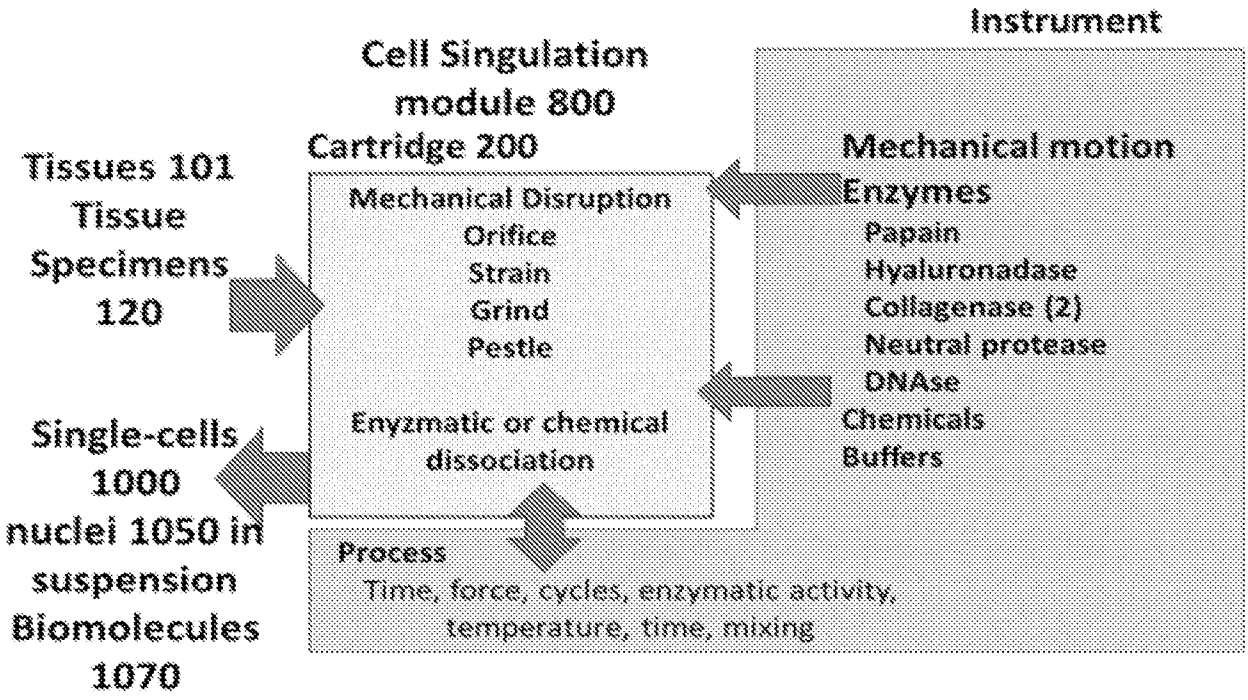


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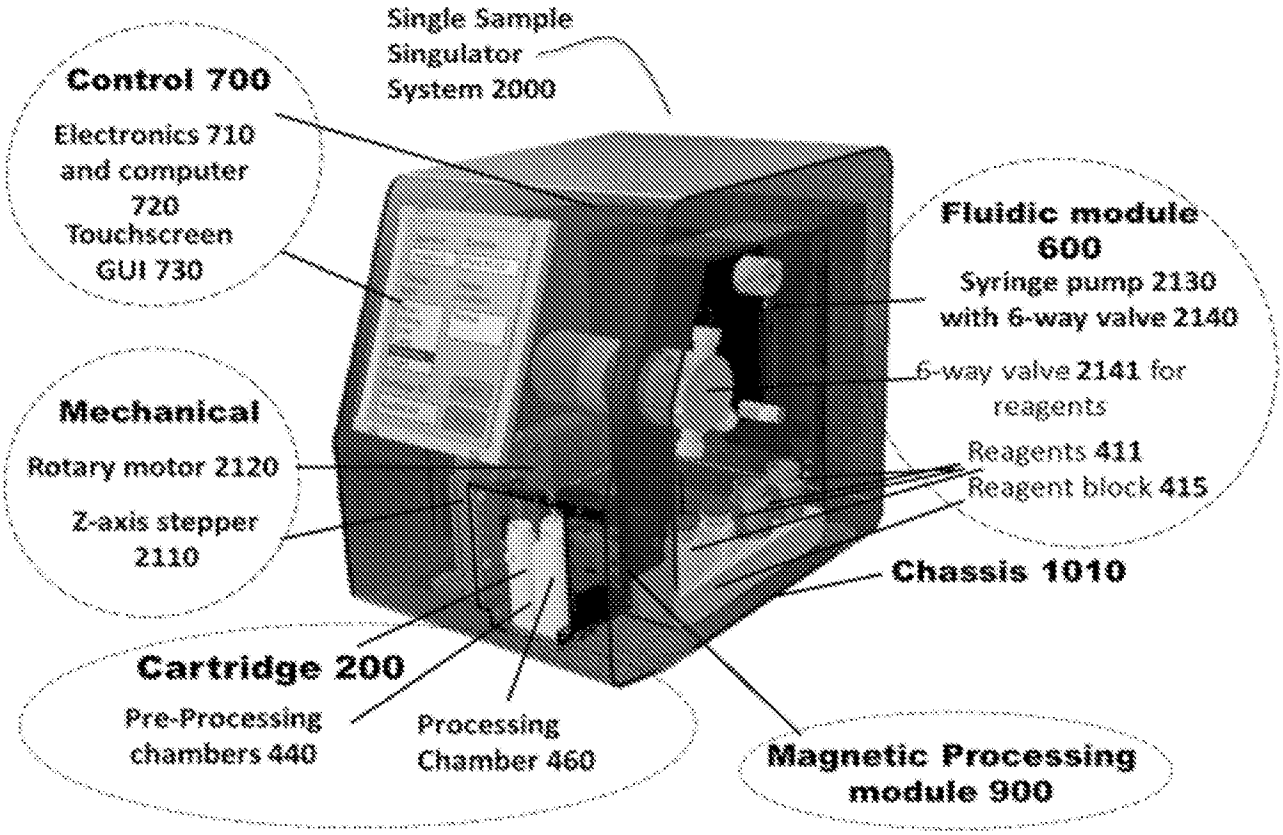


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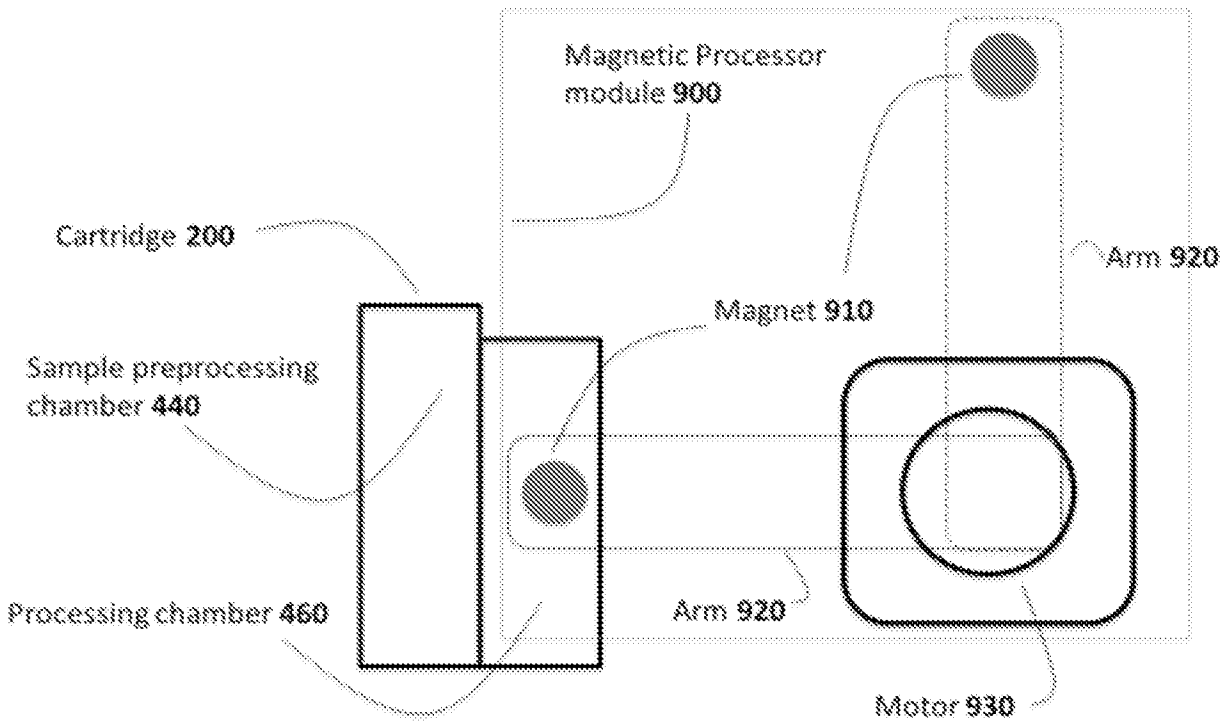


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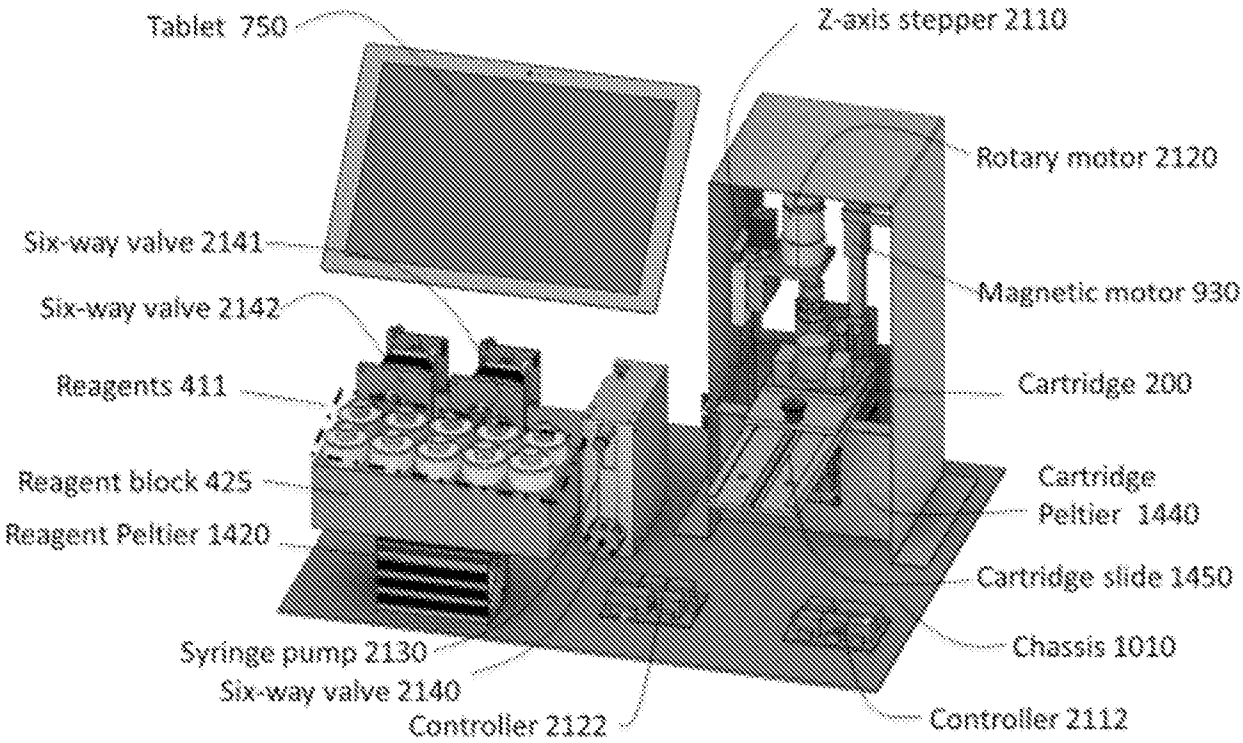


Figure 32

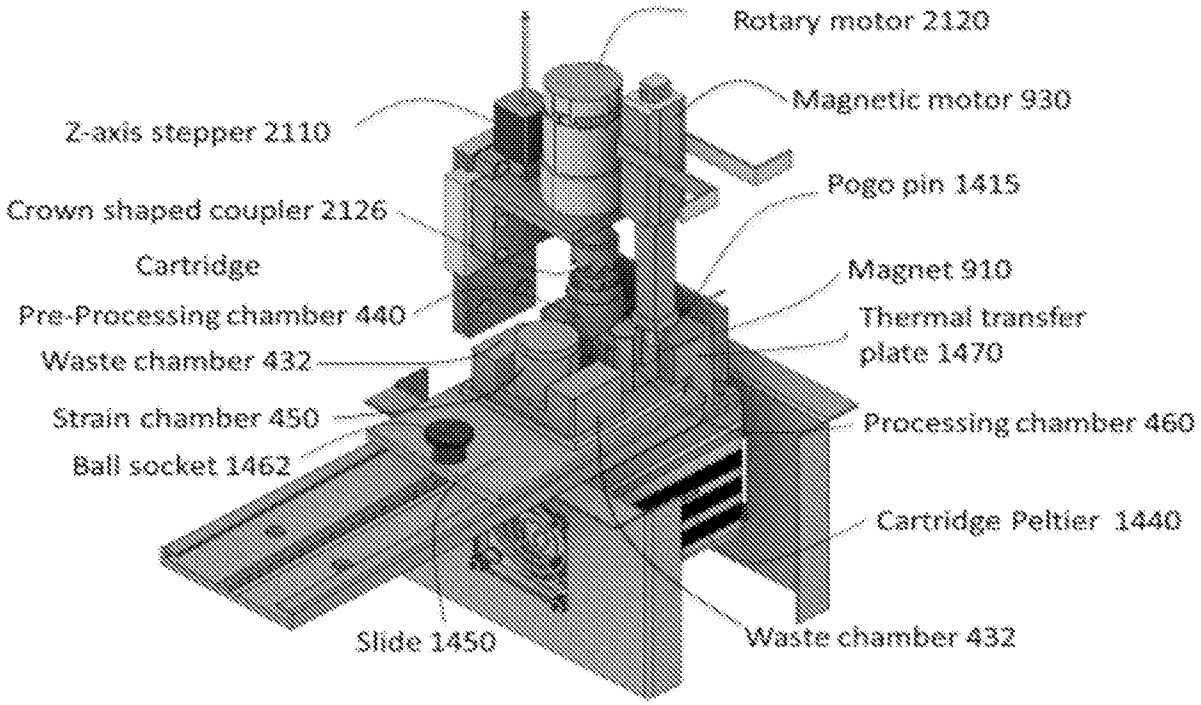


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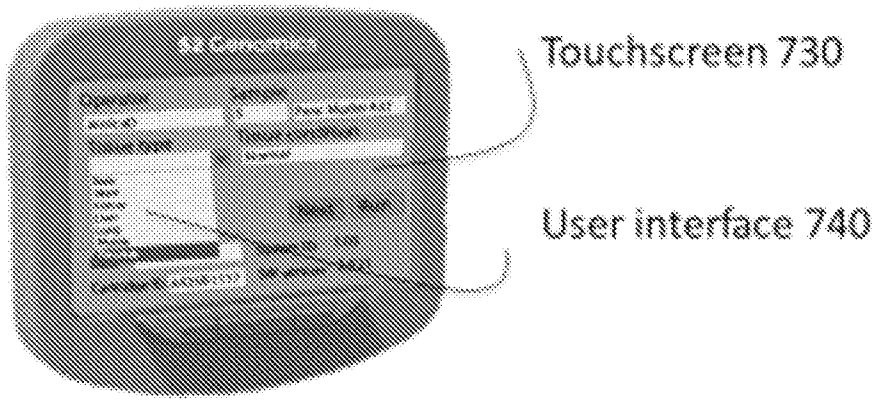


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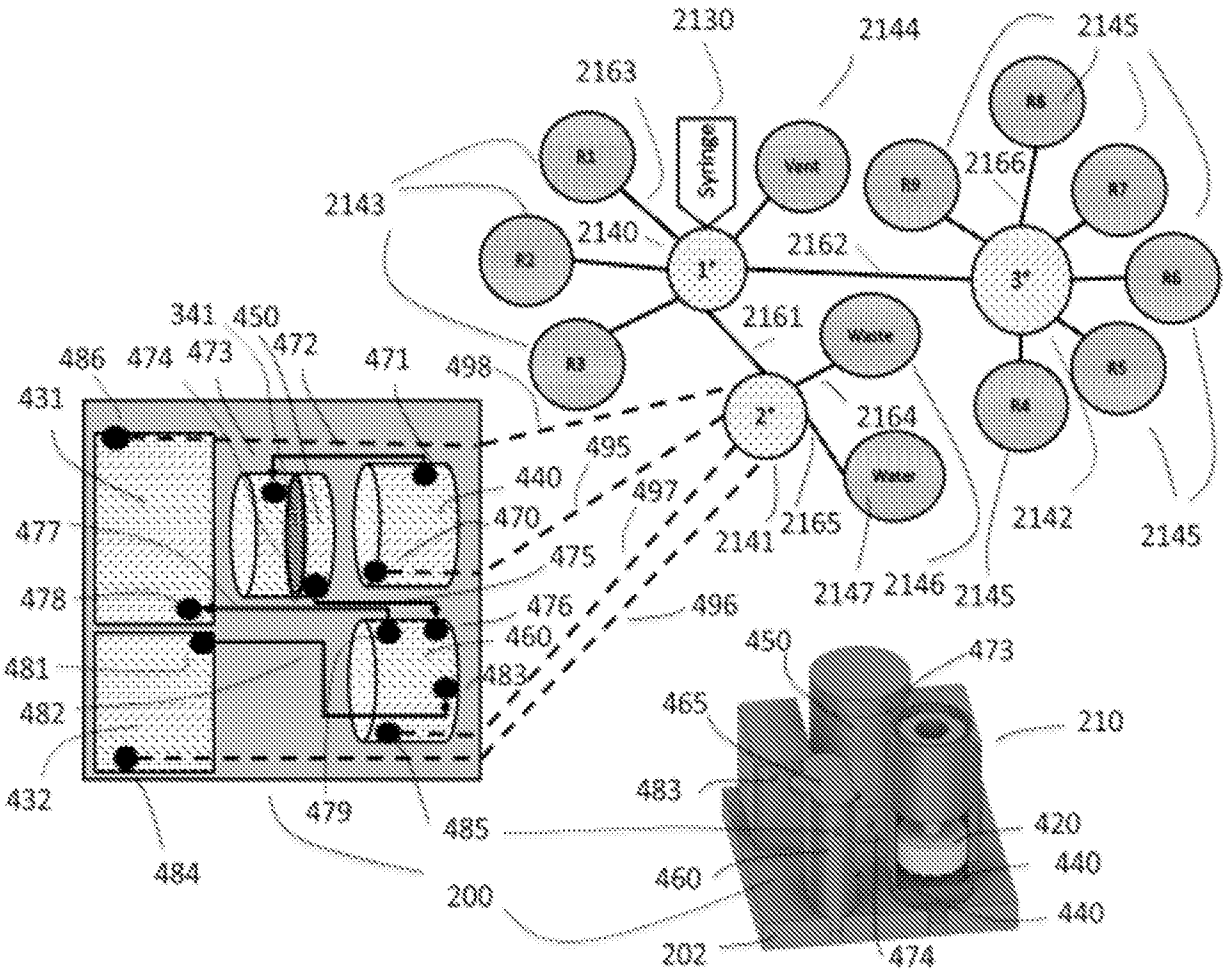


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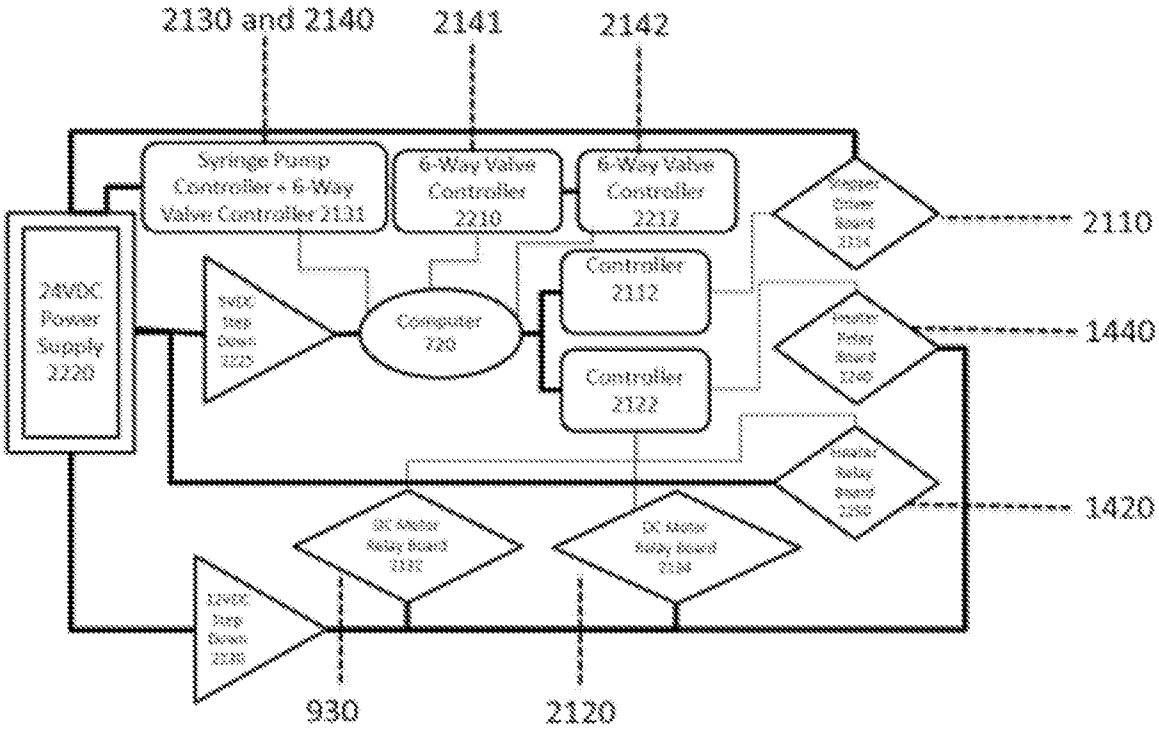


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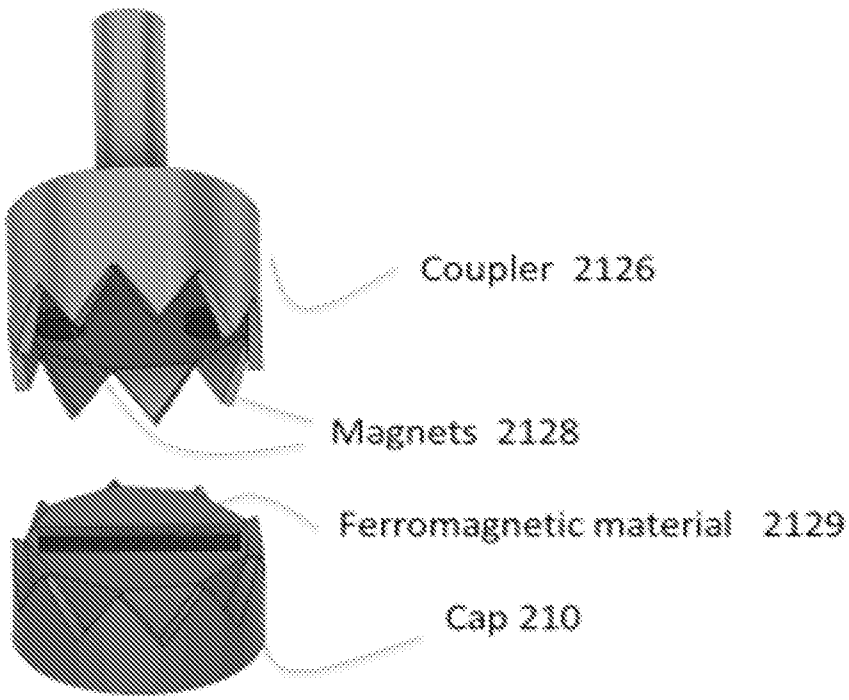


Figure 37A

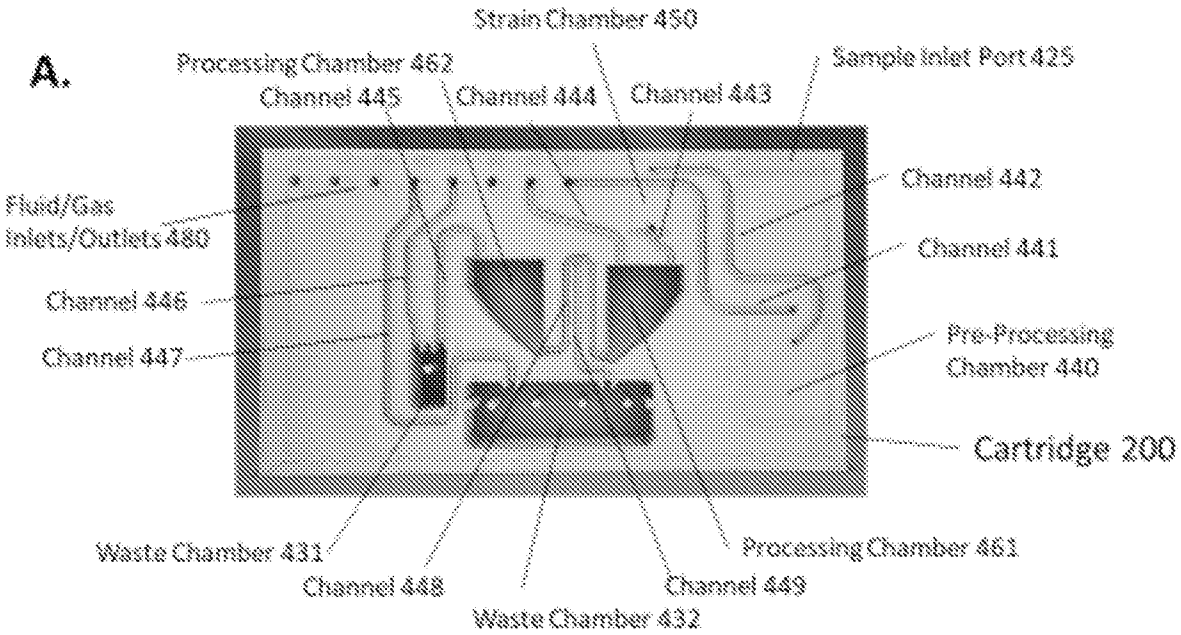


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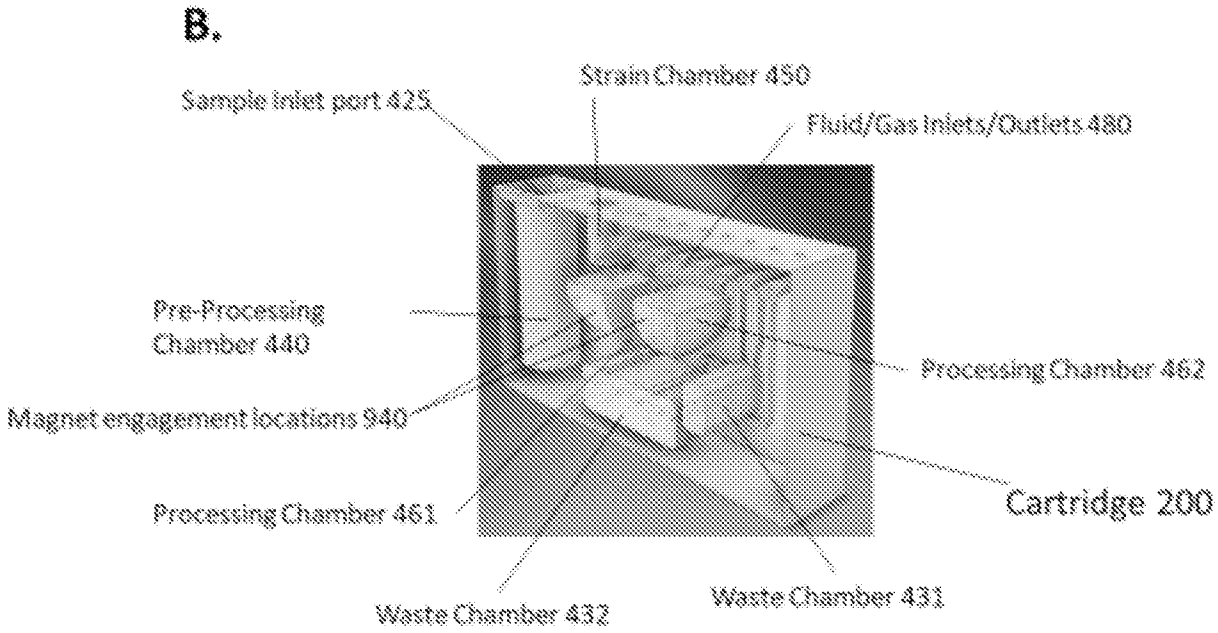


Figure 37C

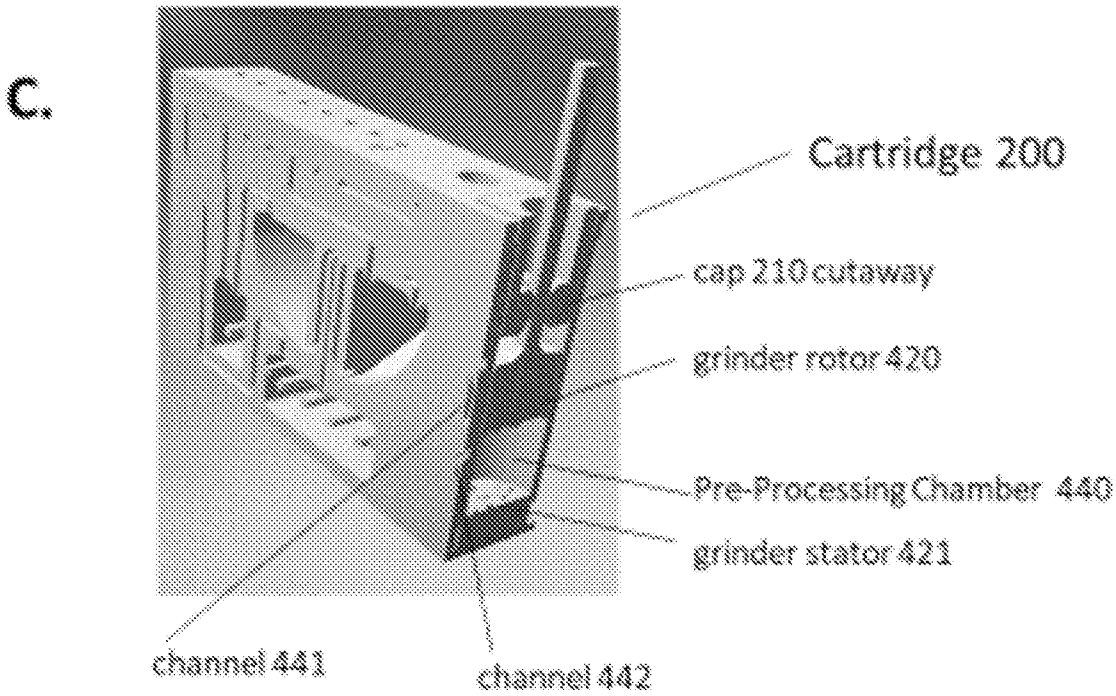


Figure 38

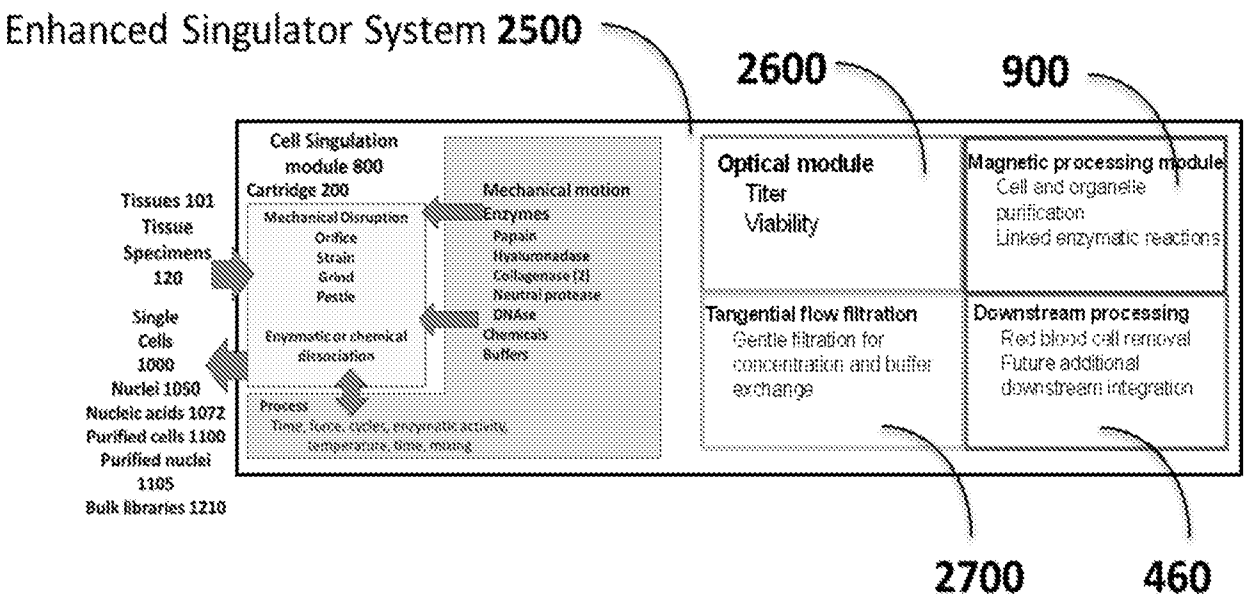


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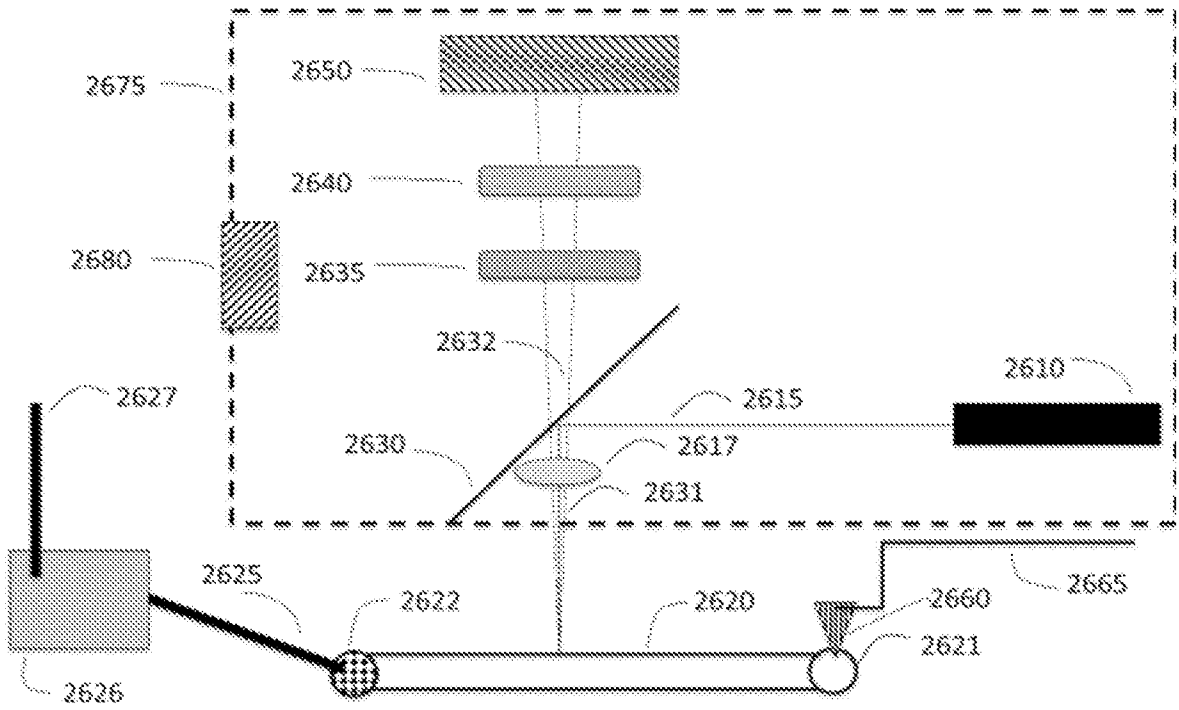


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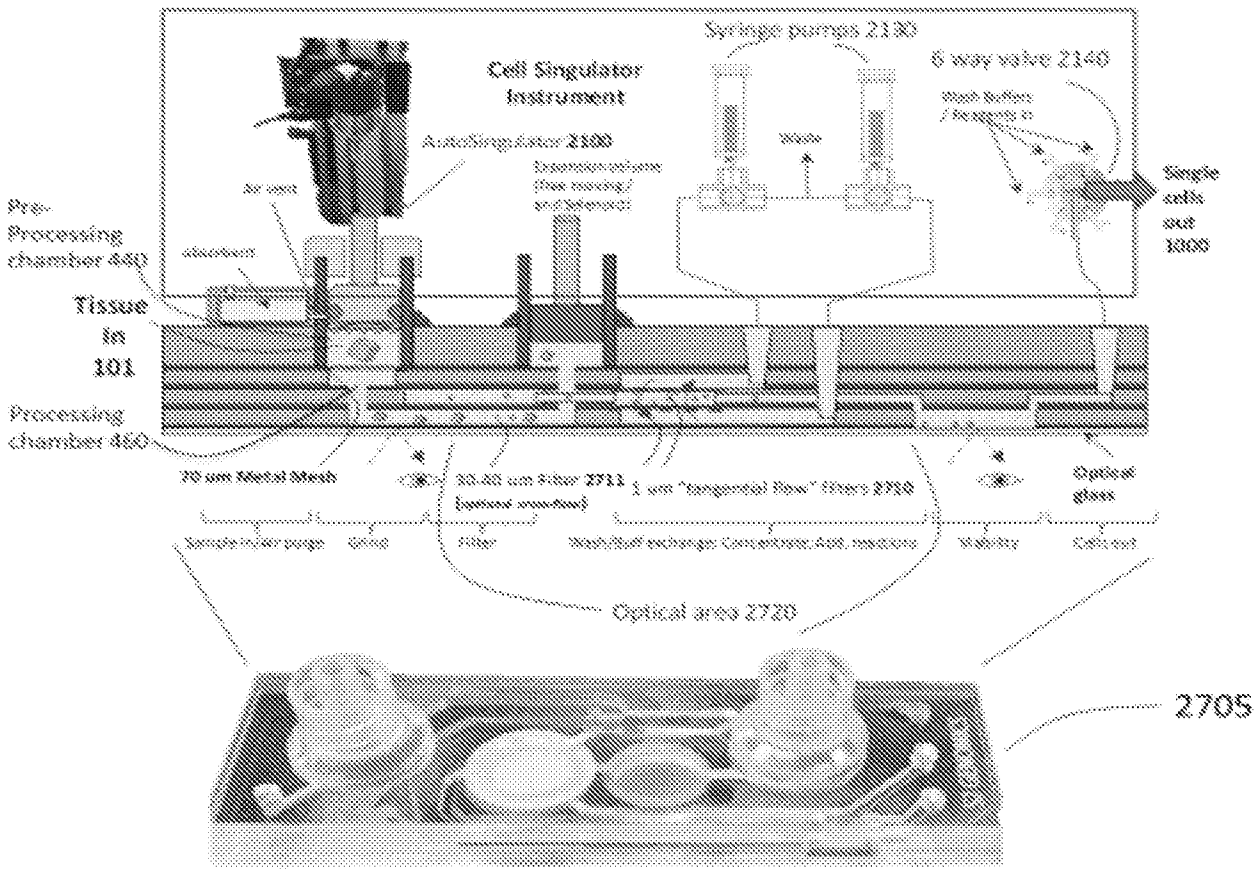


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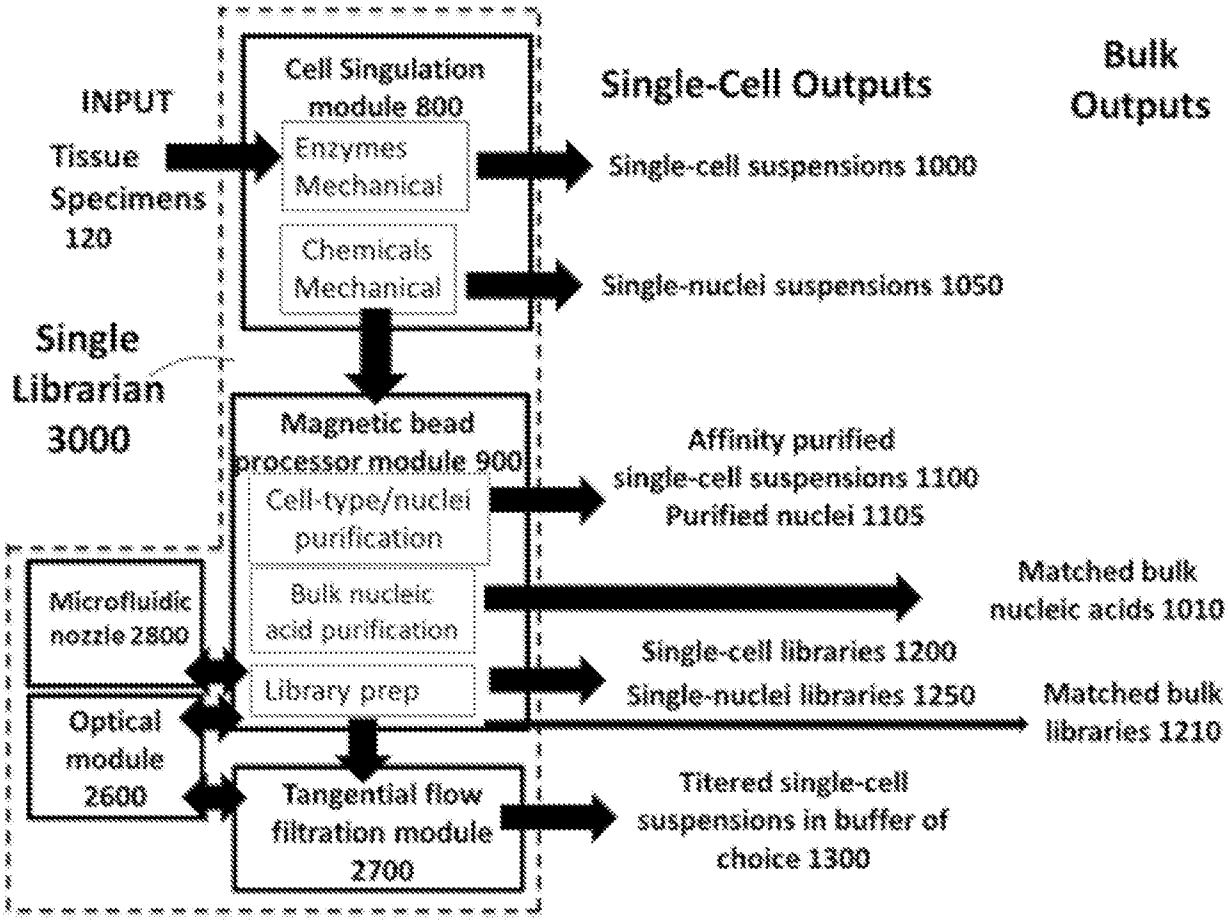


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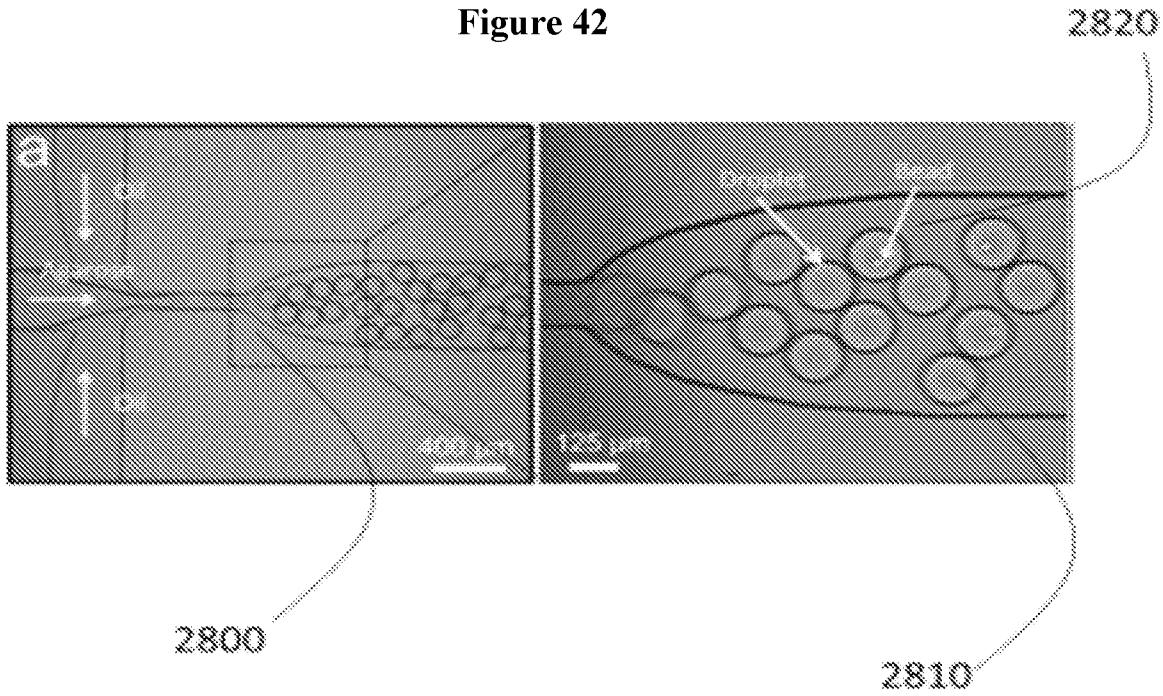


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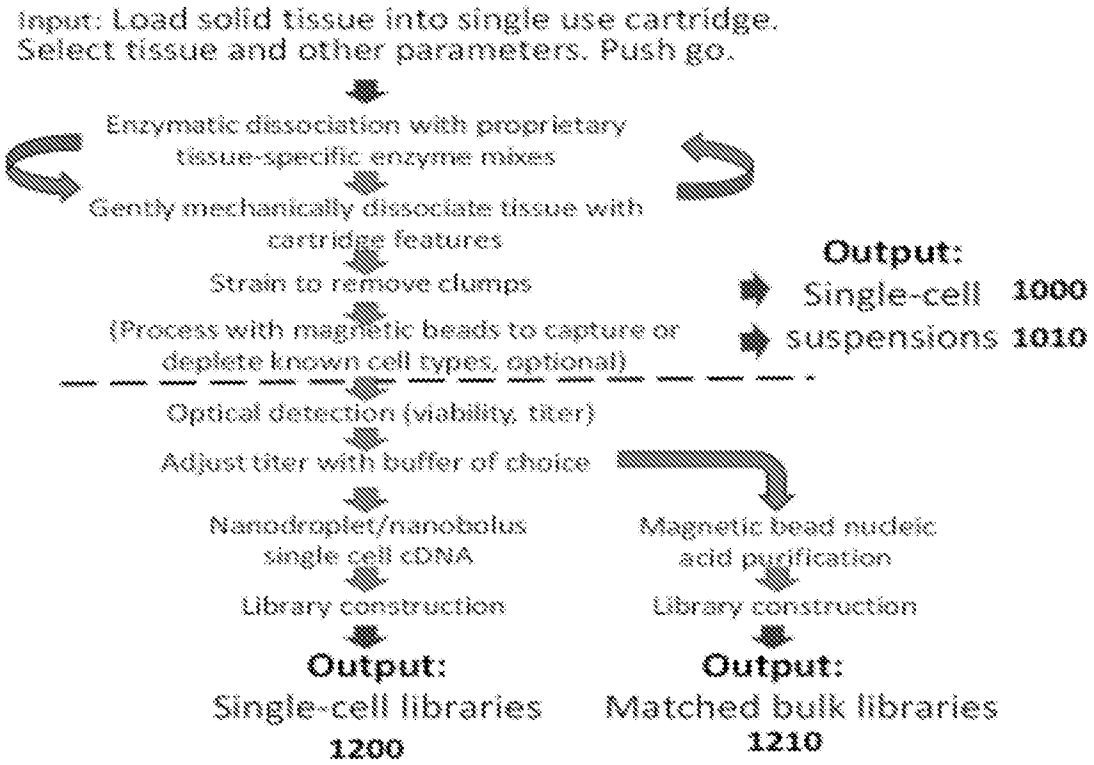


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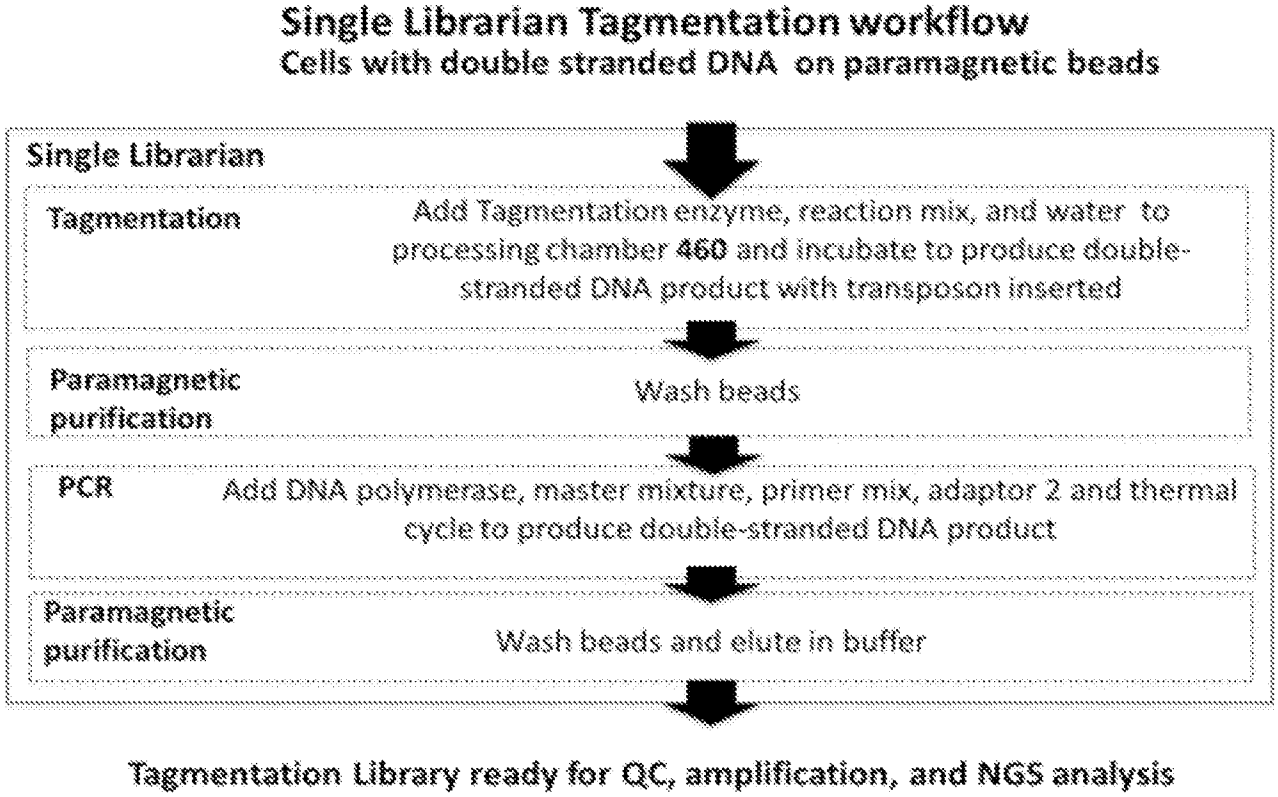


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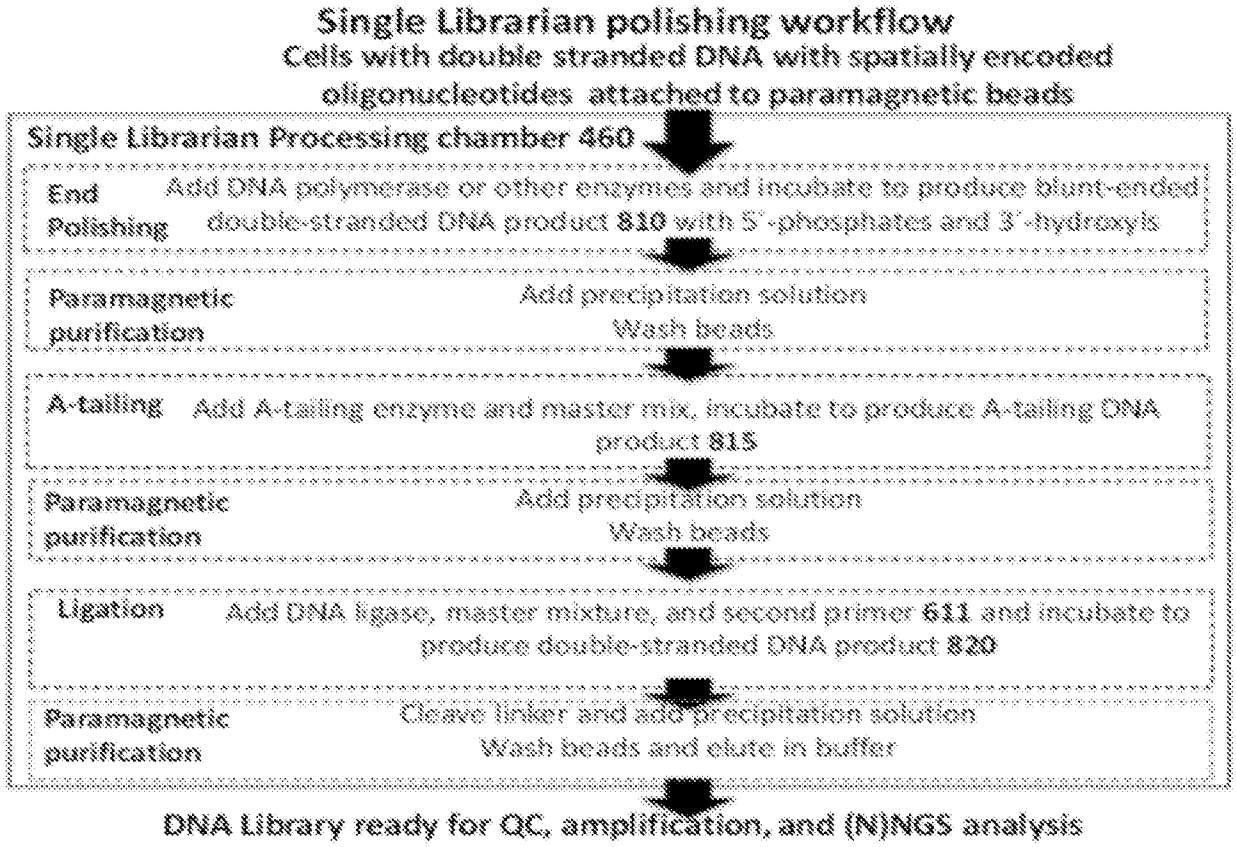


Figure 46

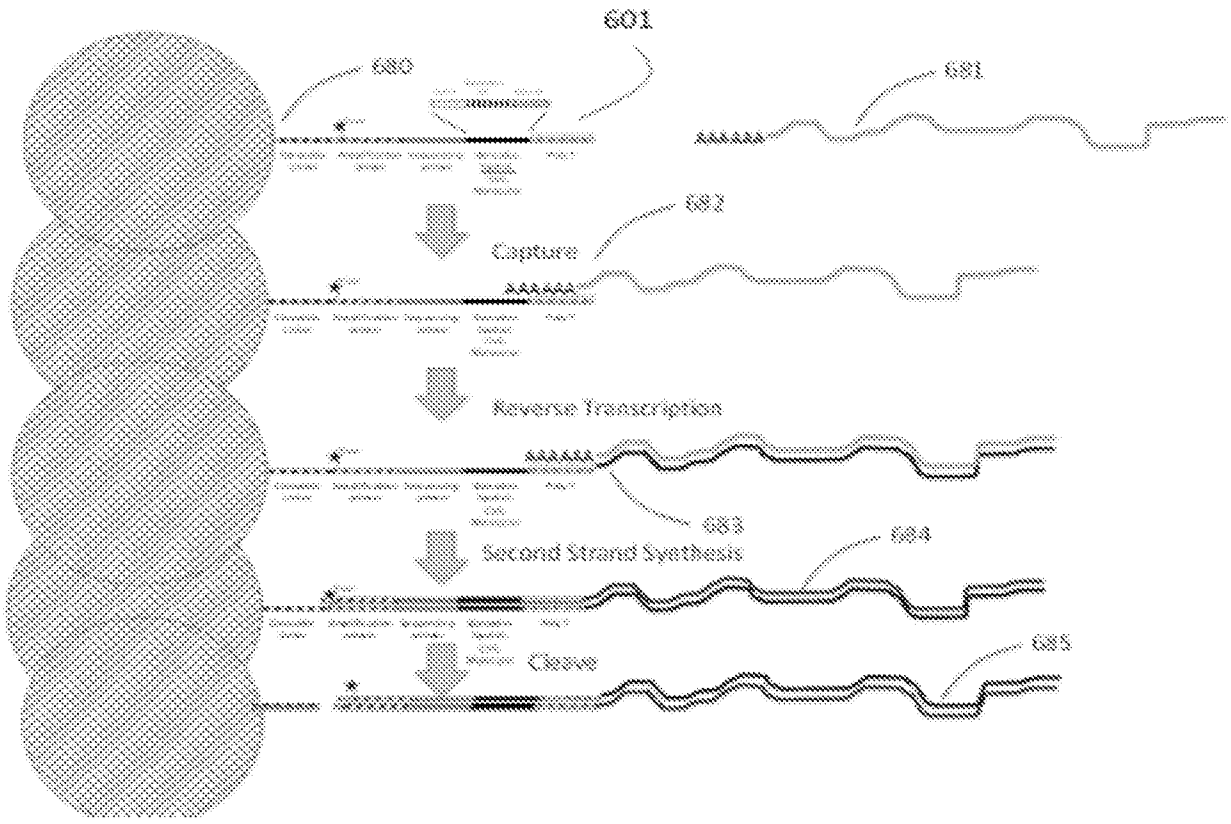
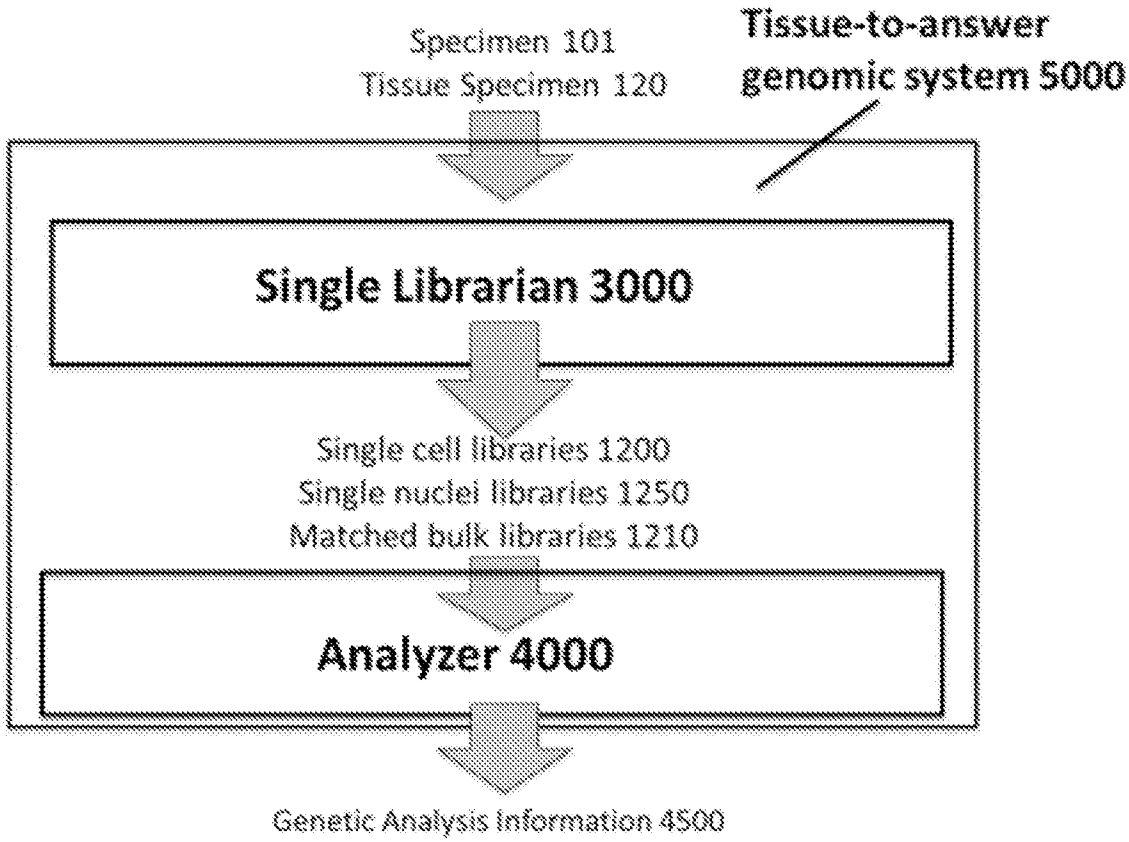


Figure 47


Specimen 101
Tissue Specimen 120
Tissue-to-answer genomic system 5000
Single Librarian 3000
Single cell libraries 1200
Single nuclei libraries 1250
Matched bulk libraries 1210
Analyzer 4000
Genetic Analysis Information 4500

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/63811

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12M 3/00, 3/06, 3/08; C12N 1/08, 15/09, 15/10 (2017.01)

CPC - C12M 3/00, 3/06, 3/08; C12N 1/08, 15/09, 15/10, 15/1003, 15/1017

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.
A	US 5,114,858 A (WILLIAMS, JG et al.) May 19, 1992; abstract; figures 1-6; columns 4, 9-16; claim 3	1-9, 15-23
A	US 2014/0377880 A1 (7685297 CANADA INC.) December 25, 2014; figures 1-2; paragraphs [0046], [0048]	1-9, 15-23
A	US 2008/0307904 A1 (PRESSMAN, NJ et al.) December 18, 2008; paragraphs [0009], [0011]-[0012]	1-9, 15-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 January 2018 (25.01.2018)	29 MAR 2018

Name and mailing address of the ISA/	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/63811

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

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

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

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Within the Next Supplemental Box-***-

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.:
1-9, 15-23

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
Information on patent family members

International application No.
PCT/US17/63811

Continuation of Box No. III - Observations where unity of invention is lacking:

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. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-9 and 15-23 are directed towards a system for separating cells and/or nuclei from disrupted tissue.

Group II: Claims 10-14 are directed towards a method of producing disrupted tissue.

Group III: Claims 24-26 are directed towards a variable orifice device for disrupting tissue.

Group IV: Claim 27 is directed towards a method of determining an amount of amplification of a nucleic acid molecule.

The inventions listed as Groups I-IV 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:

Group I includes (a) an instrument comprising: (i) one or more cartridge interfaces configured to engage a cartridge; (ii) a fluidics module comprising: (1) one or more containers containing one or more liquids and/or gasses; (2) one or more fluid lines connecting the containers with fluid ports in the cartridge interface; and (3) one or more pumps configured to move liquids and/or gasses into and/or out of the fluid port(s); (iii) a mechanical module comprising an actuator; (iv) optionally, a magnetic processing module comprising a source of magnetic force, wherein the magnetic force is positioned to form a magnetic field in the processing chamber; (v) optionally, a measurement module; (vi) optionally, a control module comprising a processor and memory, wherein the memory comprises code that, when executed by the processor, operates the system; and (b) one or more cartridges, each engaged with one of the cartridge interfaces, wherein each cartridge comprises: (i) a sample inlet port; (ii) one or more cartridge ports communicating with the fluid ports in the cartridge interface; (iii) a preprocessing chamber communicating with the sample inlet port and with at least one cartridge port, and comprising a tissue disruptor configured for mechanical disruption of tissue, wherein the tissue disruptor engages with and is actuated by the actuator when the cartridge is engaged with the cartridge interface; (iv) a strain chamber communicating with the preprocessing chamber configured to separate cells and/or nuclei from disrupted tissue; (v) a processing chamber communicating with the strain chamber, optionally communicating with one or more cartridge ports and configured to perform one or more processing steps on separated cells and/or nuclei; and (vi) optionally, one or more waste chambers fluidically connected with the processing chamber, which are not present in Groups II-IV.

Group II includes automatically performing mechanical and enzymatic/chemical disruption of the tissue to produce disrupted tissue comprising released cells and/or nuclei and debris; automatically moving the disrupted tissue into a strain chamber; and automatically moving the released cells and/or nuclei into a processing chamber, which are not present in Groups I and III-IV.

Group III includes a variable orifice device for disrupting tissue comprising: (a) a first container and a second container fluidically connected through a flexible tube comprising a lumen; (b) an adjustable clamp positioned to clamp the flexible tube, wherein adjusting the clamp alters the cross-sectional area of the lumen; and (c) one or more pumps or devices operatively coupled with the first and/or second containers configured to push liquid in one container through the flexible tubing into the other container, which are not present in Groups I-II and IV.

Group IV includes a method of determining an amount of amplification of a nucleic acid molecule comprising amplifying the nucleic acid molecule with primers comprising a barcode wherein each round of amplification adds in additional barcode to an amplified nucleic acid molecule; and after amplification, counting incorporated barcodes, wherein the number of incorporated barcodes indicates the amount of amplification, which are not present in Groups I-III.

The common technical features of Groups I-IV are providing a tissue sample to a preprocessing chamber; performing mechanical disruption of the tissue in the preprocessing chamber to produce disrupted tissue comprising released cells and/or nuclei; moving the disrupted tissue into a strain chamber and separating the released cells and/or nuclei; and moving the released cells and/or nuclei into a processing chamber.

These common technical features are disclosed by US 2003/0215935 A1 (COON). Coon discloses providing a tissue sample to a preprocessing chamber (placing tissue sample 75 in perfusion tank 12 (preprocessing chamber); figures 1-3; paragraph [0035] -[0036]); performing mechanical disruption of the tissue in the preprocessing chamber to produce disrupted tissue comprising released cells and/or nuclei (circulating a digesting buffer and slicing the tissue sample (mechanical disruption) in the perfusion tank 12 to produce digested tissue (released cells); figures 1-3; paragraphs [0035]-[0036]); moving the disrupted tissue into a strain chamber and separating the released cells and/or nuclei (the collection buffer containing the tissue sample contents (disrupted tissue) is drained through drain 24 to filters 67, 68, 69 and 70 (strain chamber) to separate the released cells; figures 1-3; paragraphs [0035]-[0036]); and moving the released cells and/or nuclei into a processing chamber (the isolated cells are moved to filter cartridge 55 (processing chamber) used for further processing (e.g. in a centrifuge); figures 1-3; paragraphs [0035]-[0036]).

Since the common technical features are previously disclosed by the Coon reference, the common features are not special and so Groups I-IV lack unity.