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(54) **METHODS AND SYSTEMS FOR USING ENCAPSULATED MICROBUBBLES TO PROCESS BIOLOGICAL SAMPLES**

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2015, now Pat. No. 9,982,290, filed as application No. PCT/US2013/063397 on Oct. 4, 2013.

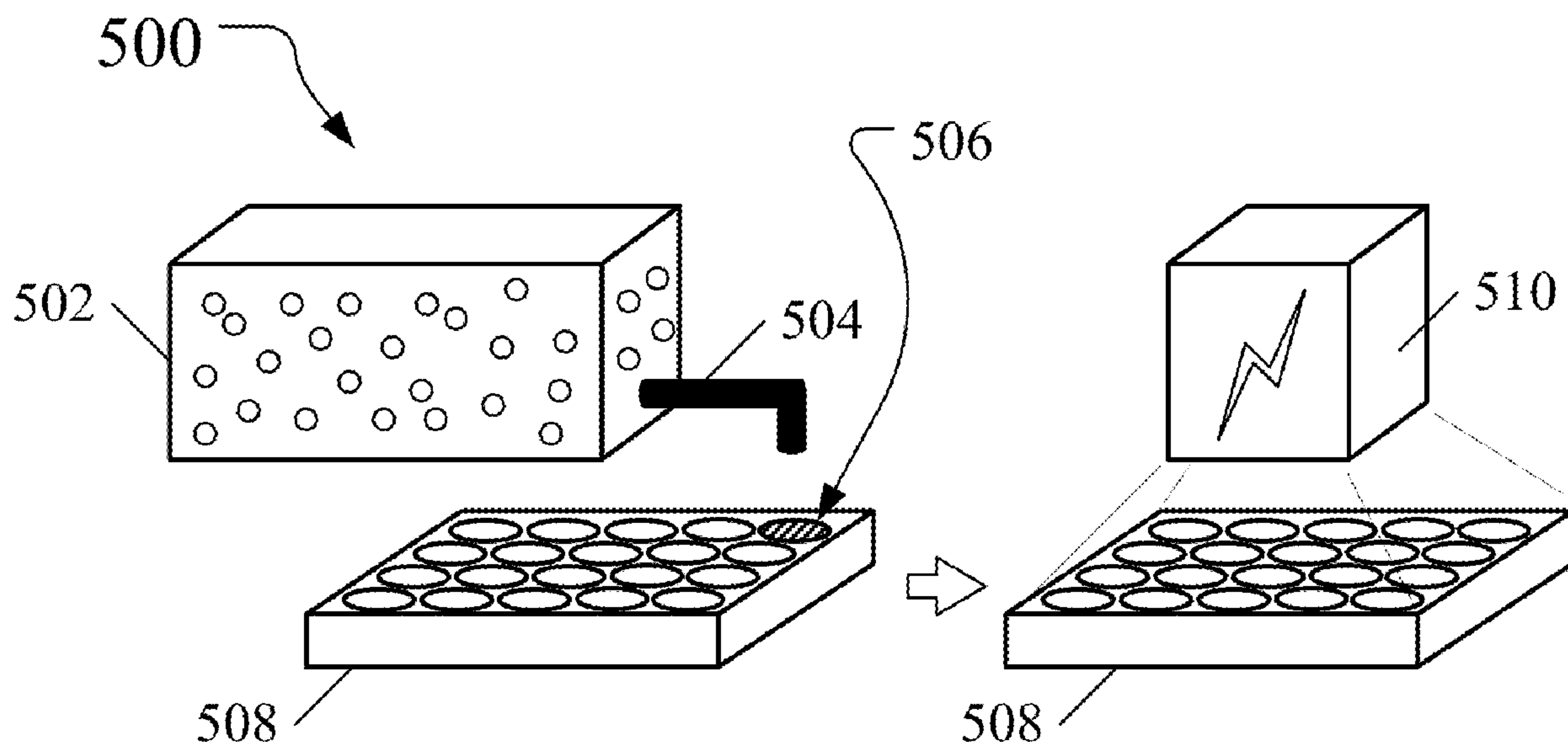
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(57) **ABSTRACT**

Methods and systems for using encapsulated microbubbles to process biological samples are disclosed. According to one aspect, a method for using encapsulated microbubbles to process a biological sample includes creating a mixture comprising encapsulated microbubbles mixed with a biological sample and adding activation energy to the mixture to cause at least some of the microbubbles to oscillate or burst and thereby process the sample, including effecting cell lysis, shearing DNA, and/or performing tissue dispersion.



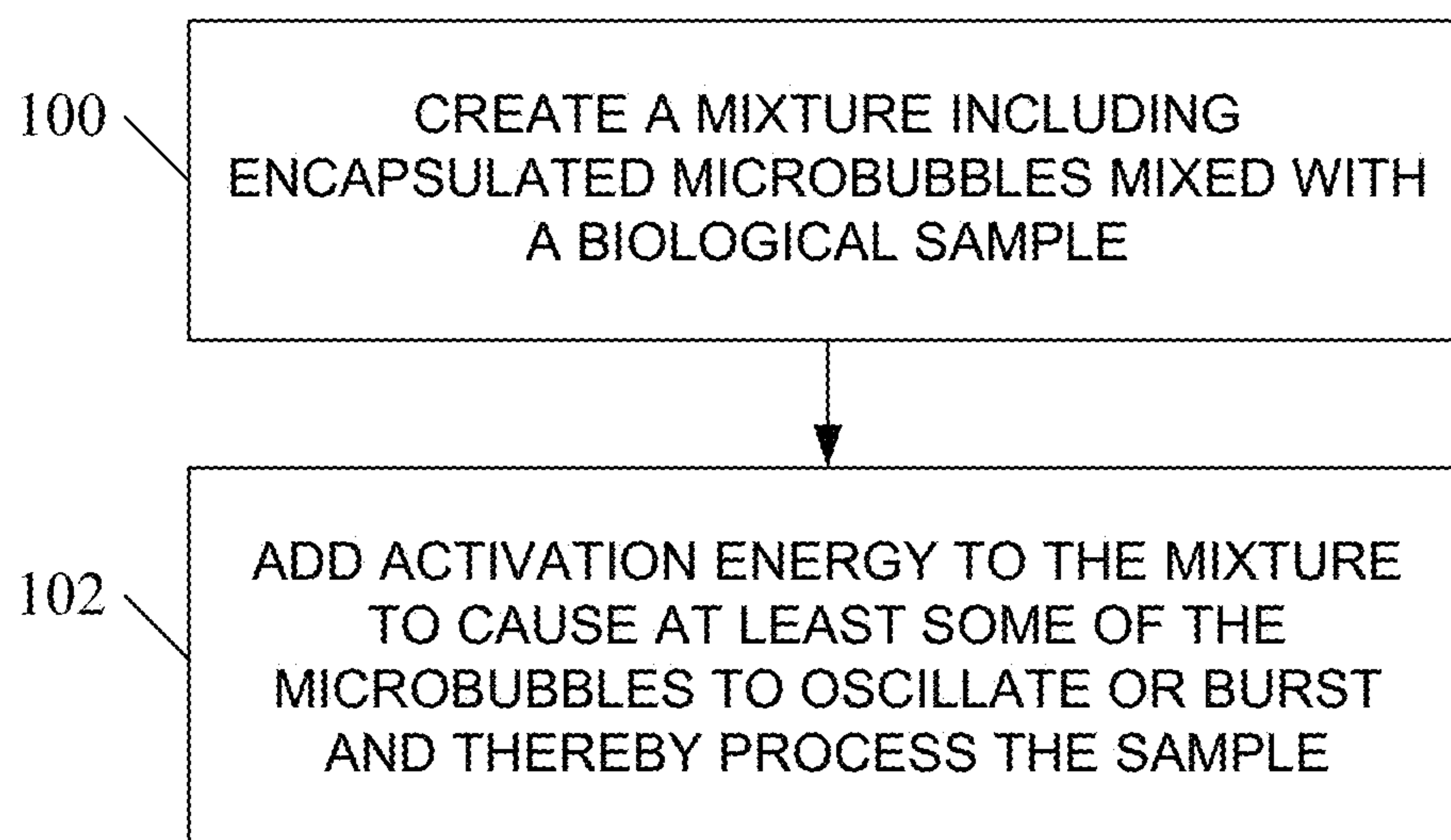


FIG. 1

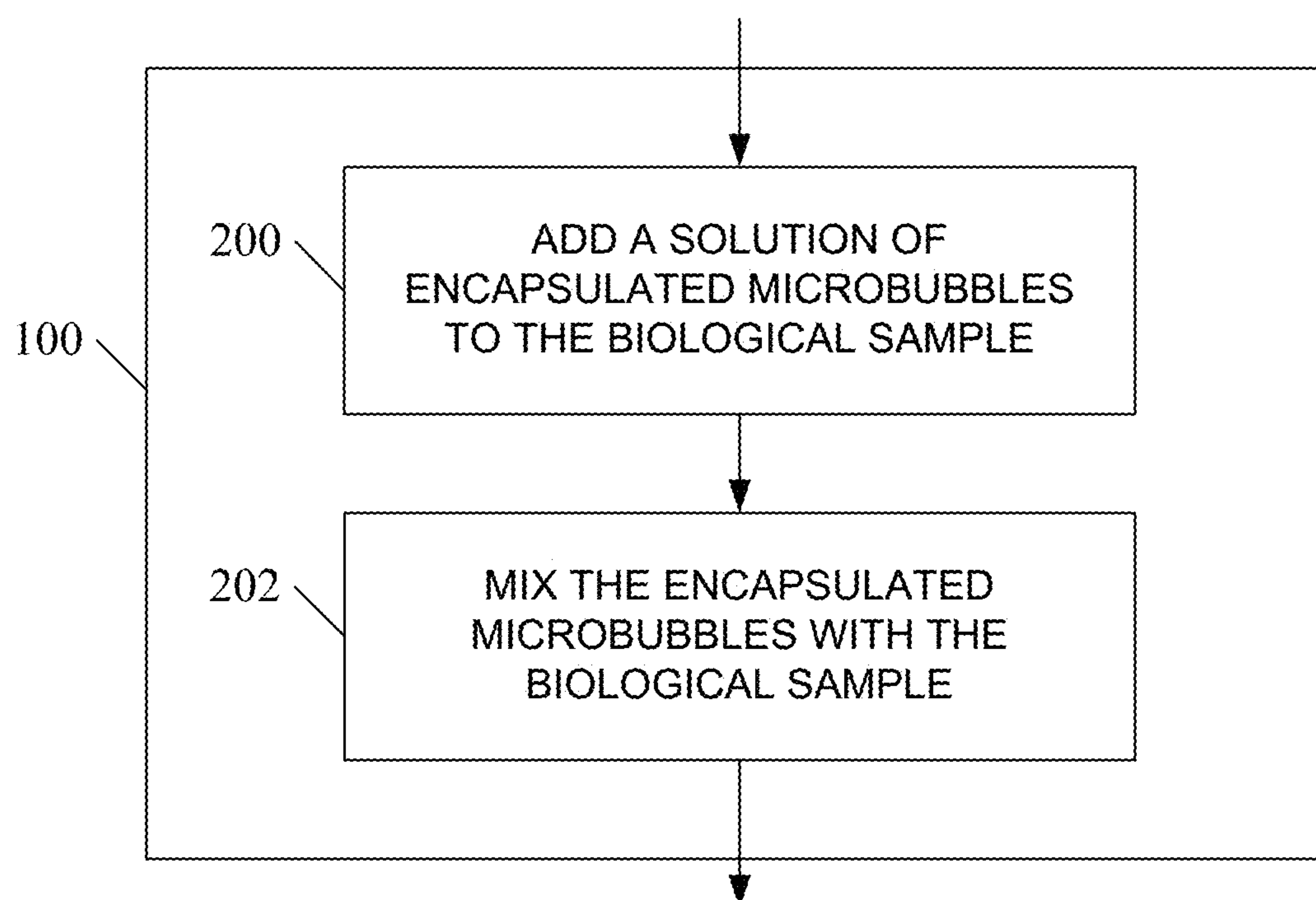


FIG. 2

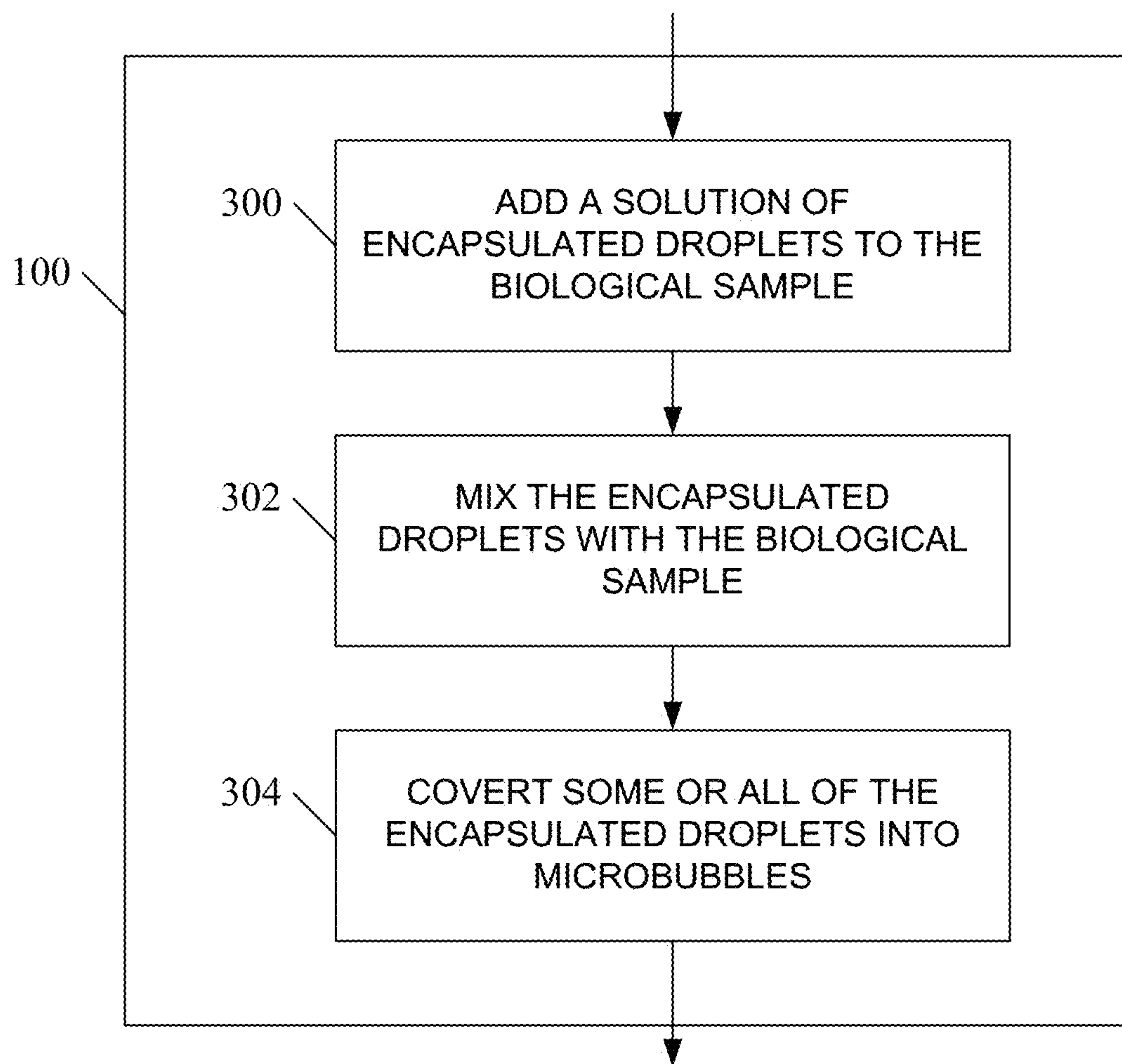


FIG. 3

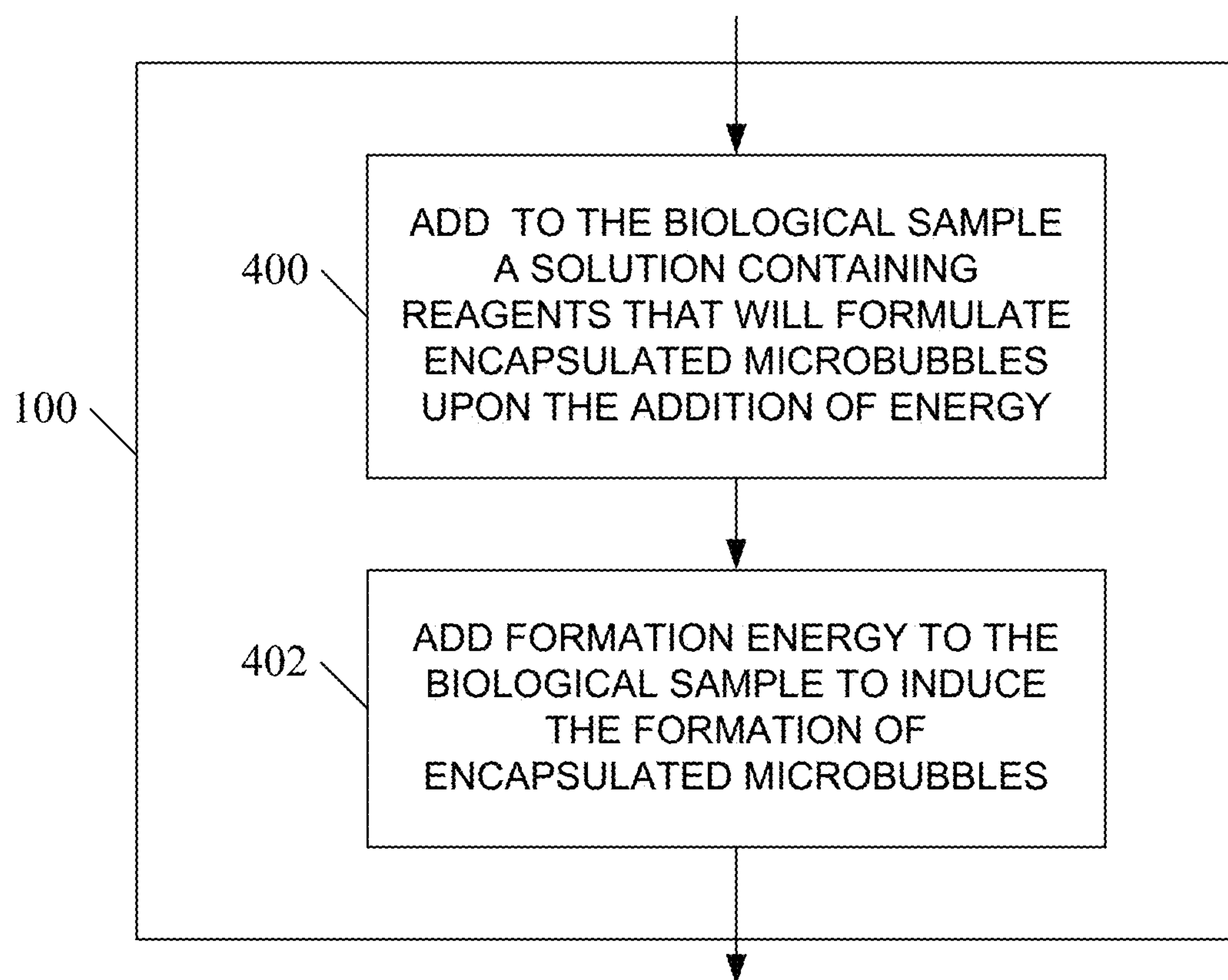


FIG. 4

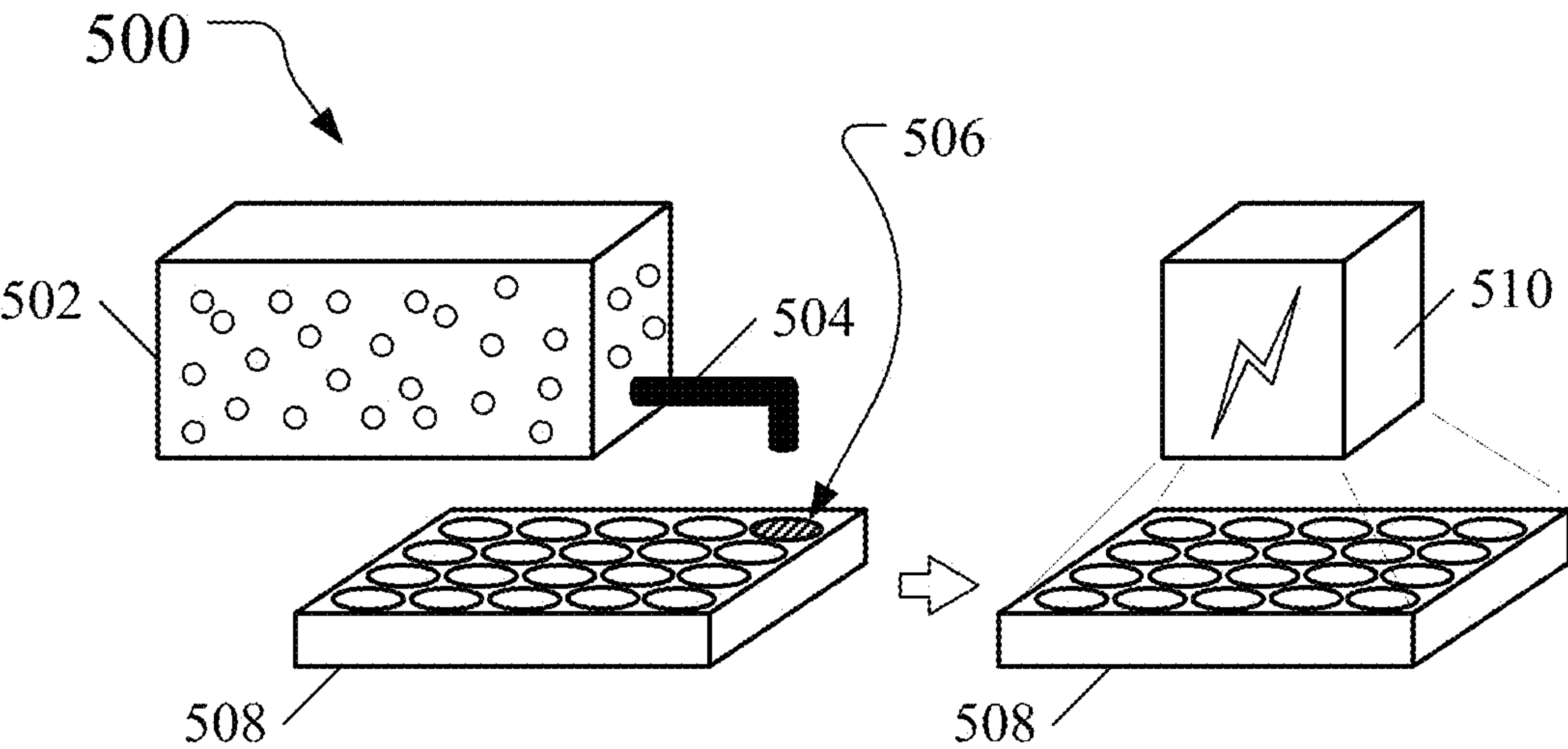


FIG.5A

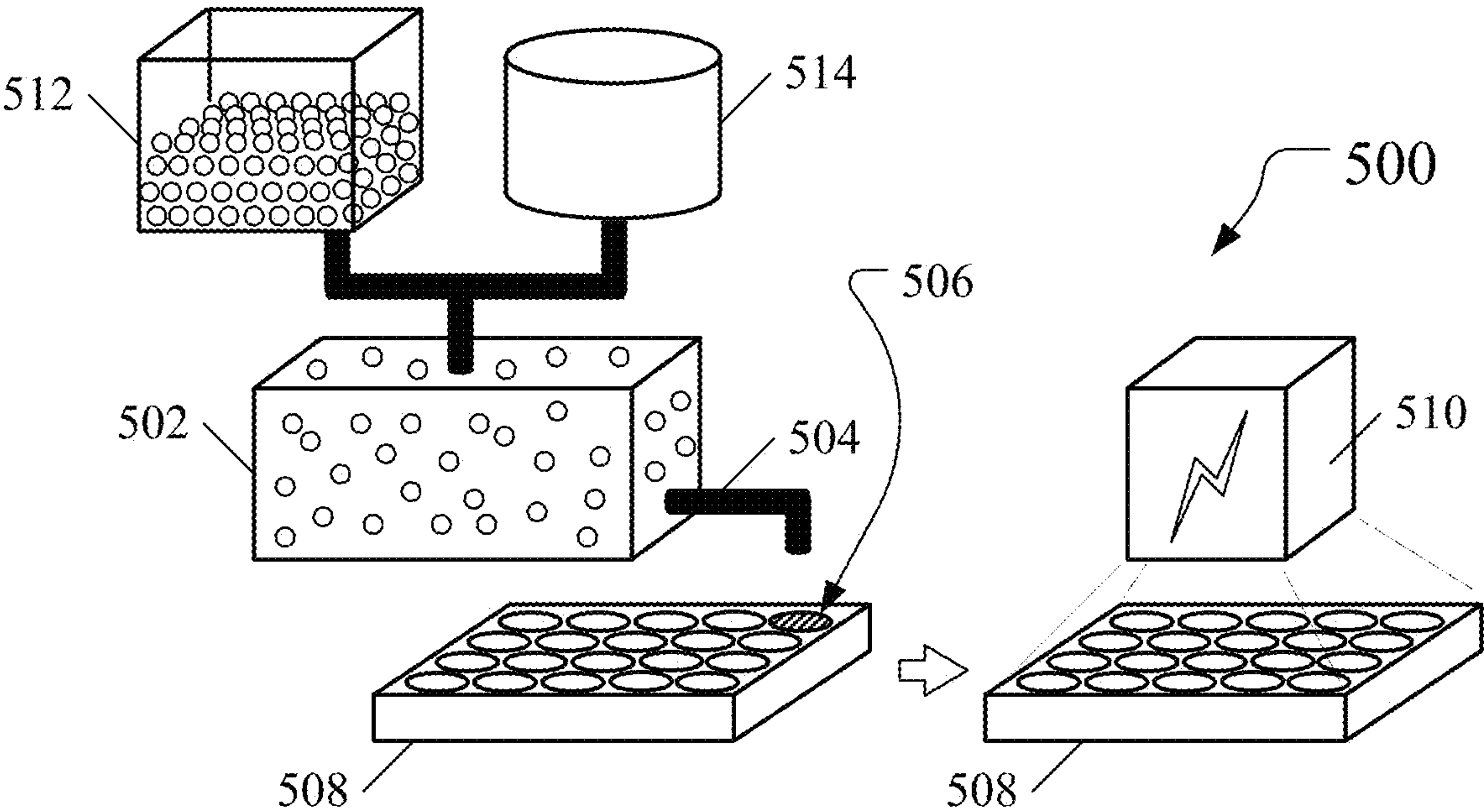


FIG.5B

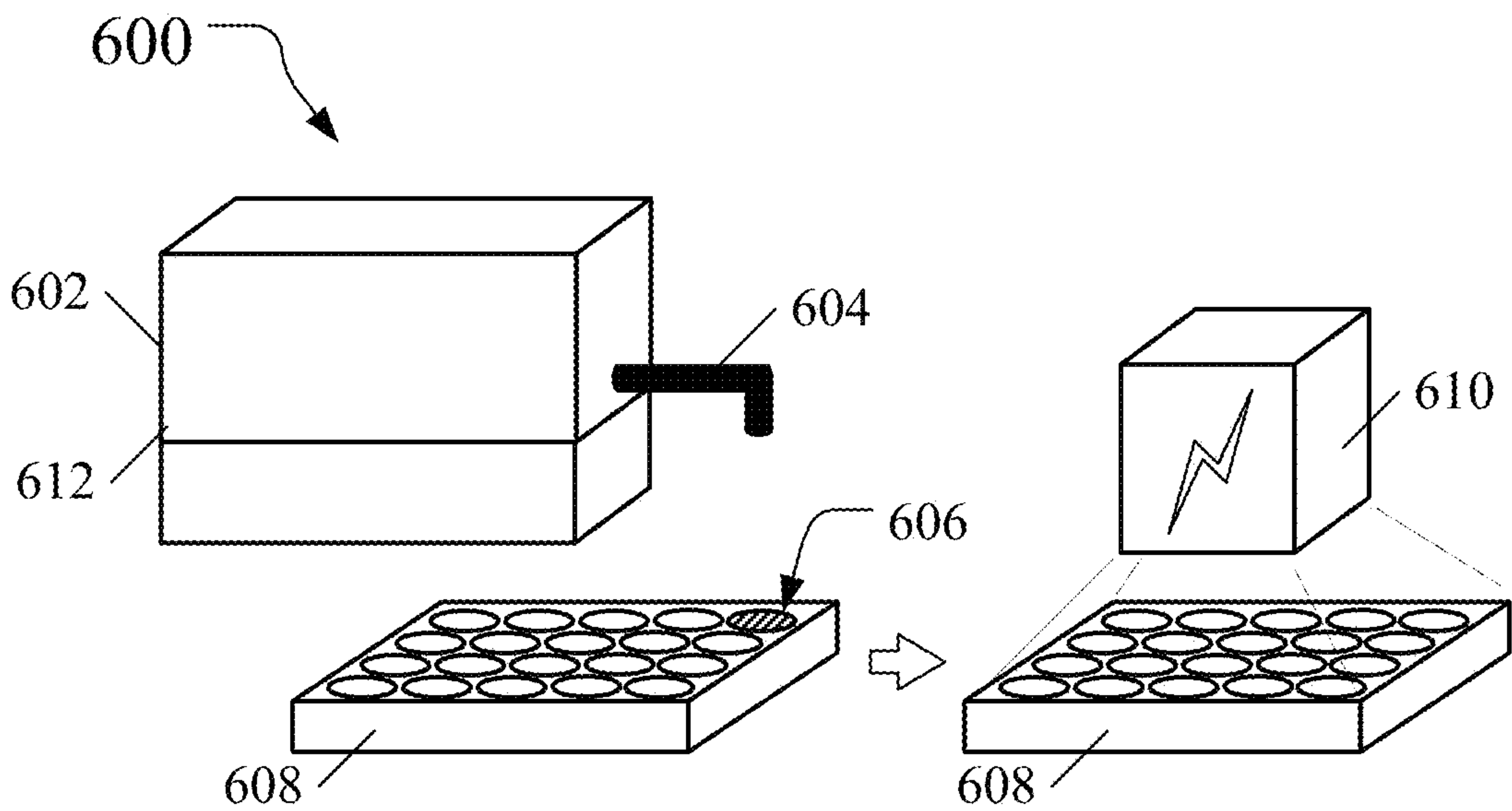


FIG.6A

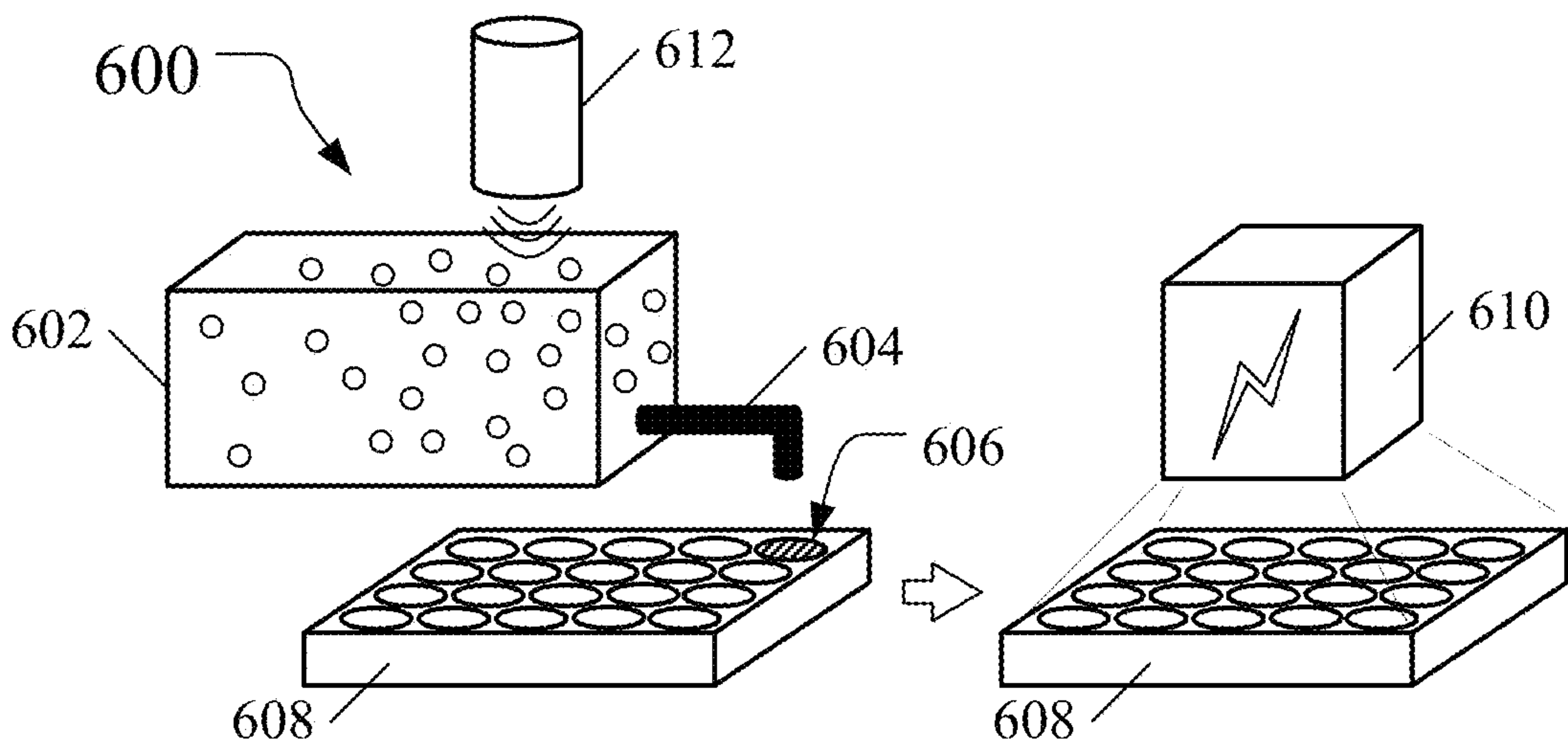


FIG.6B

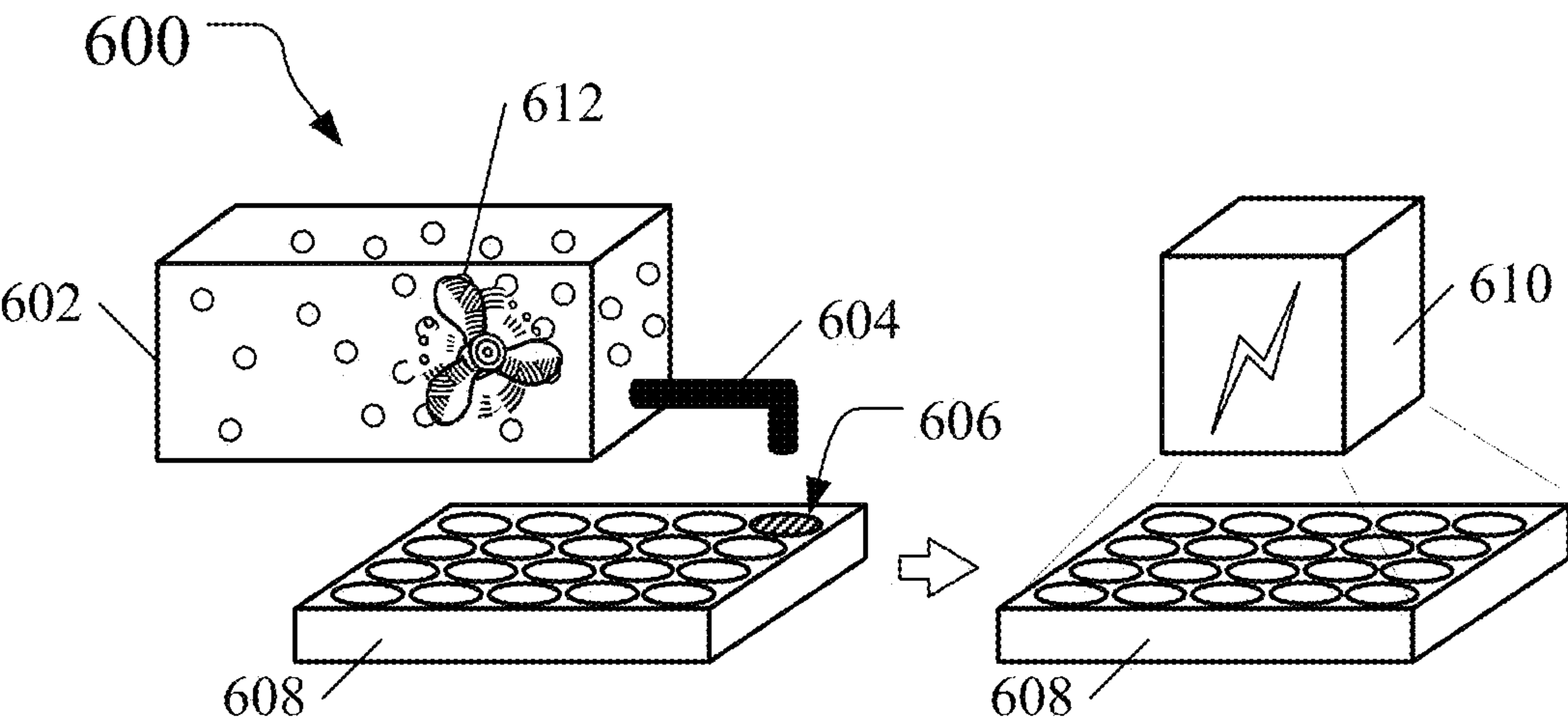


FIG.6C

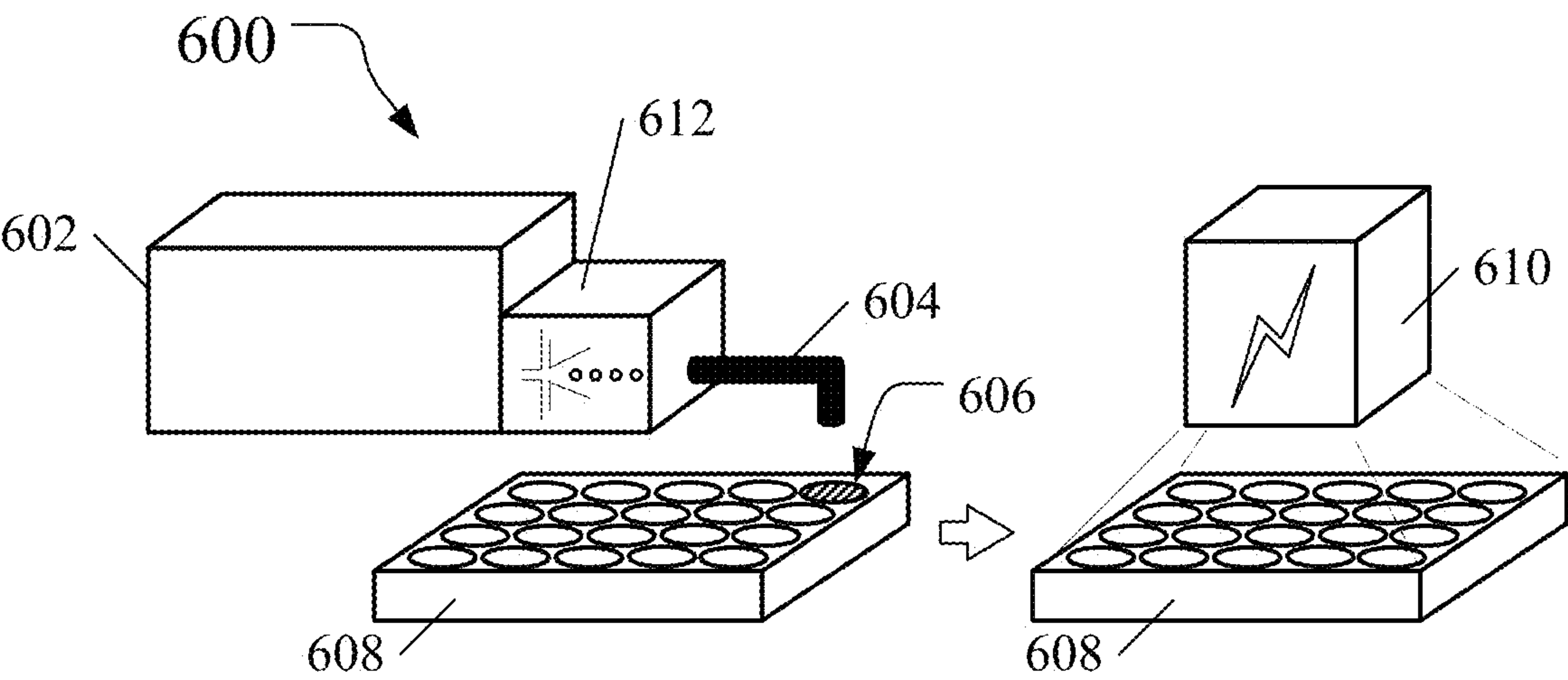
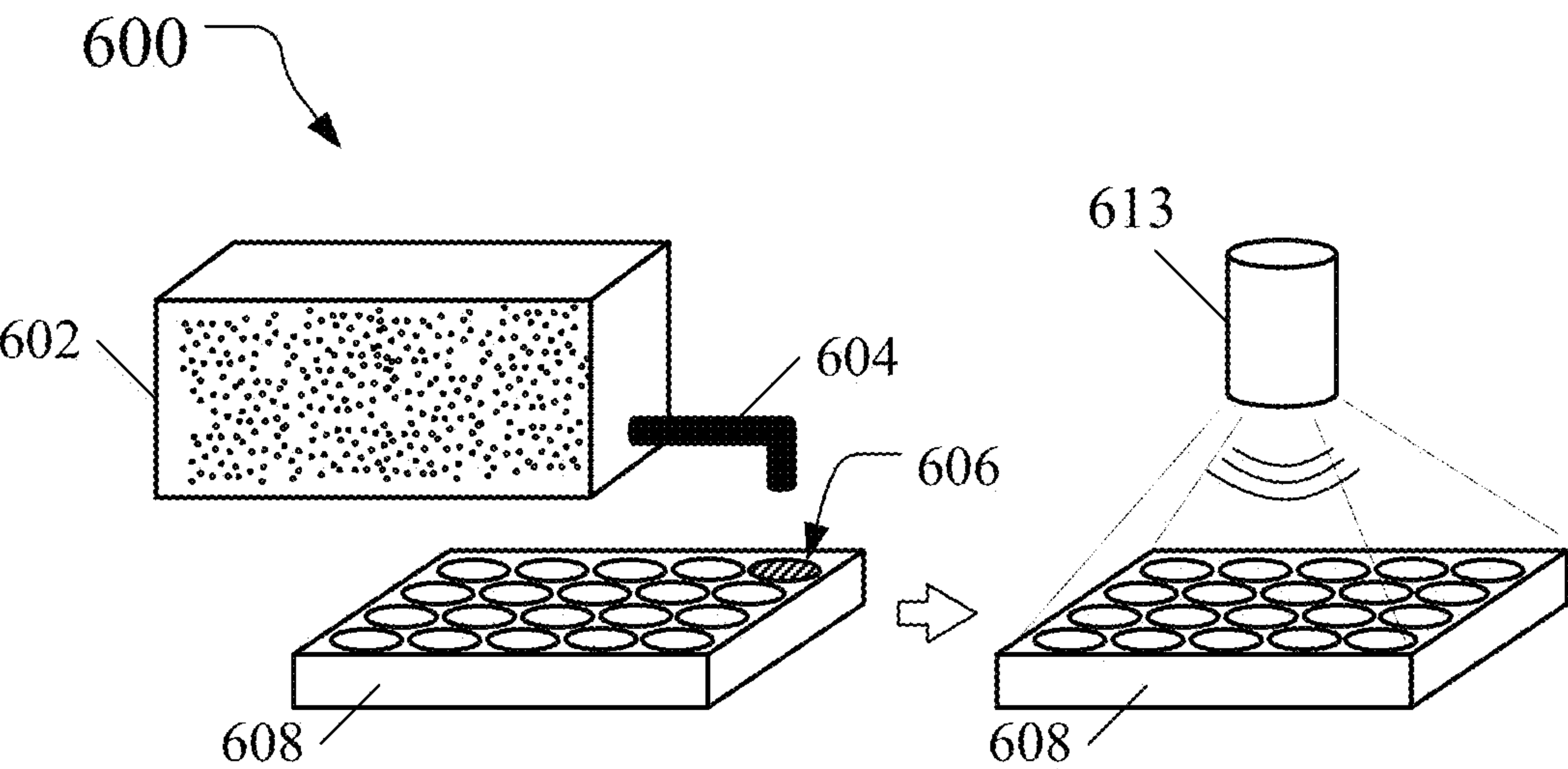
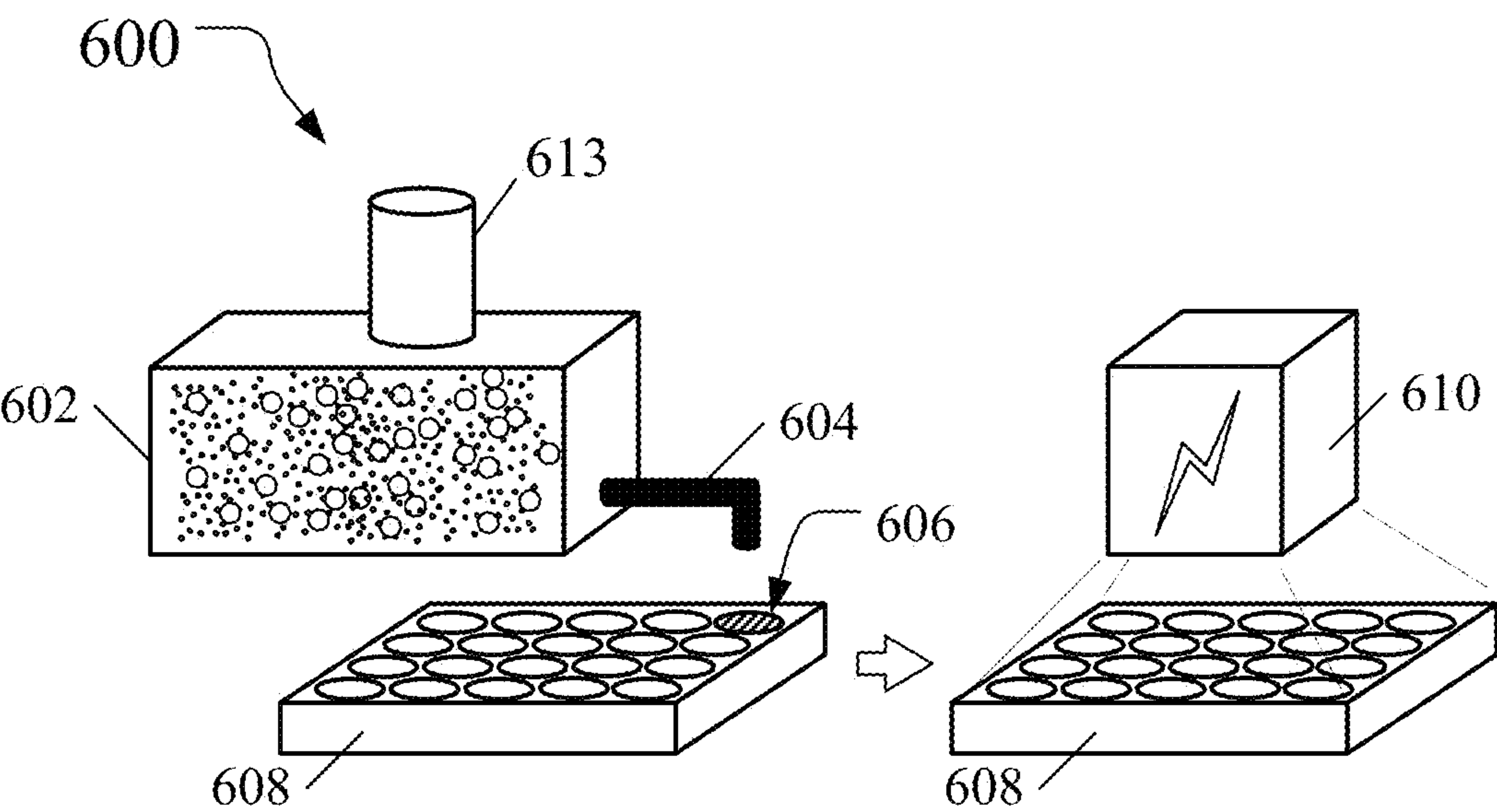


FIG.6D



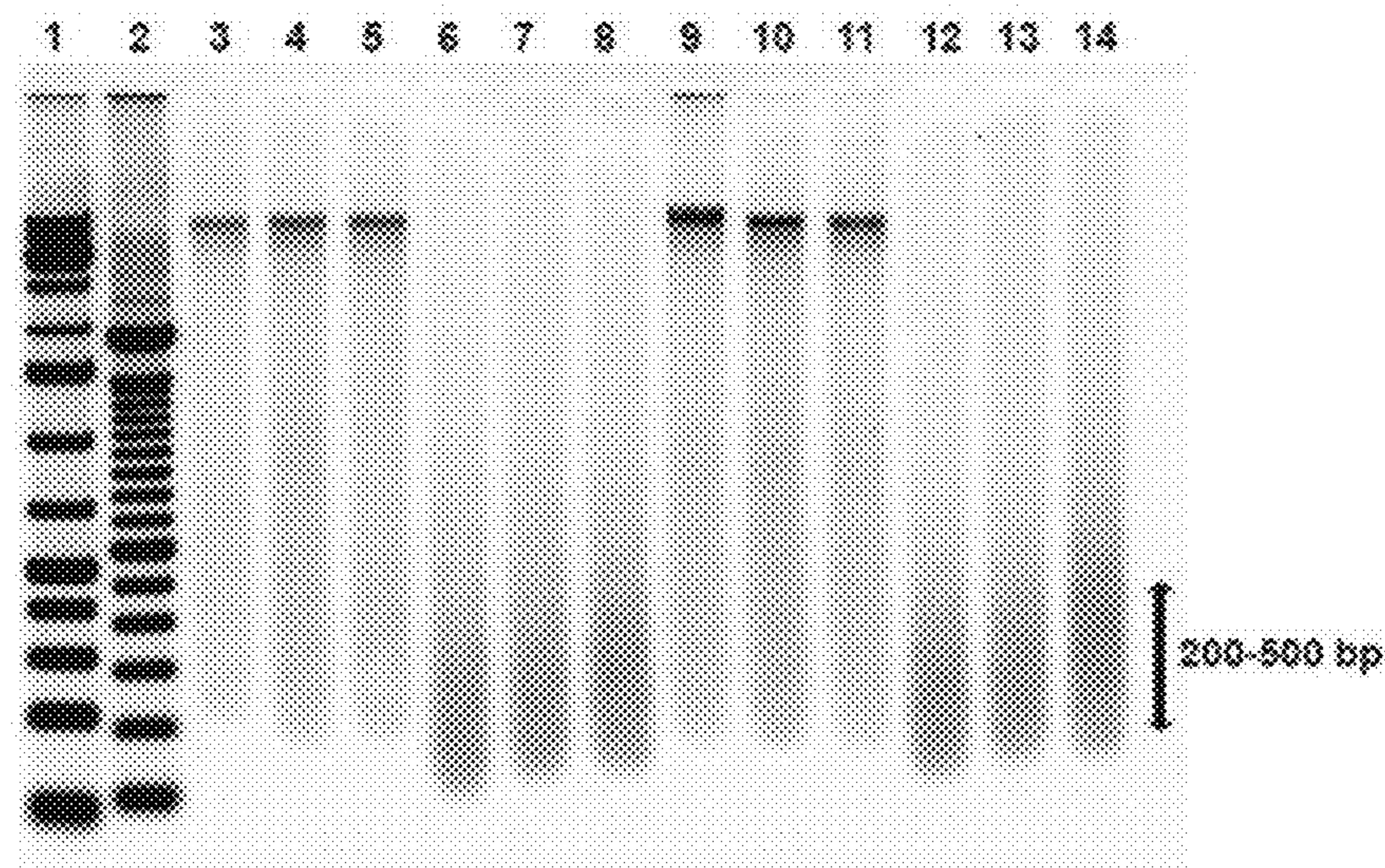


FIG.7

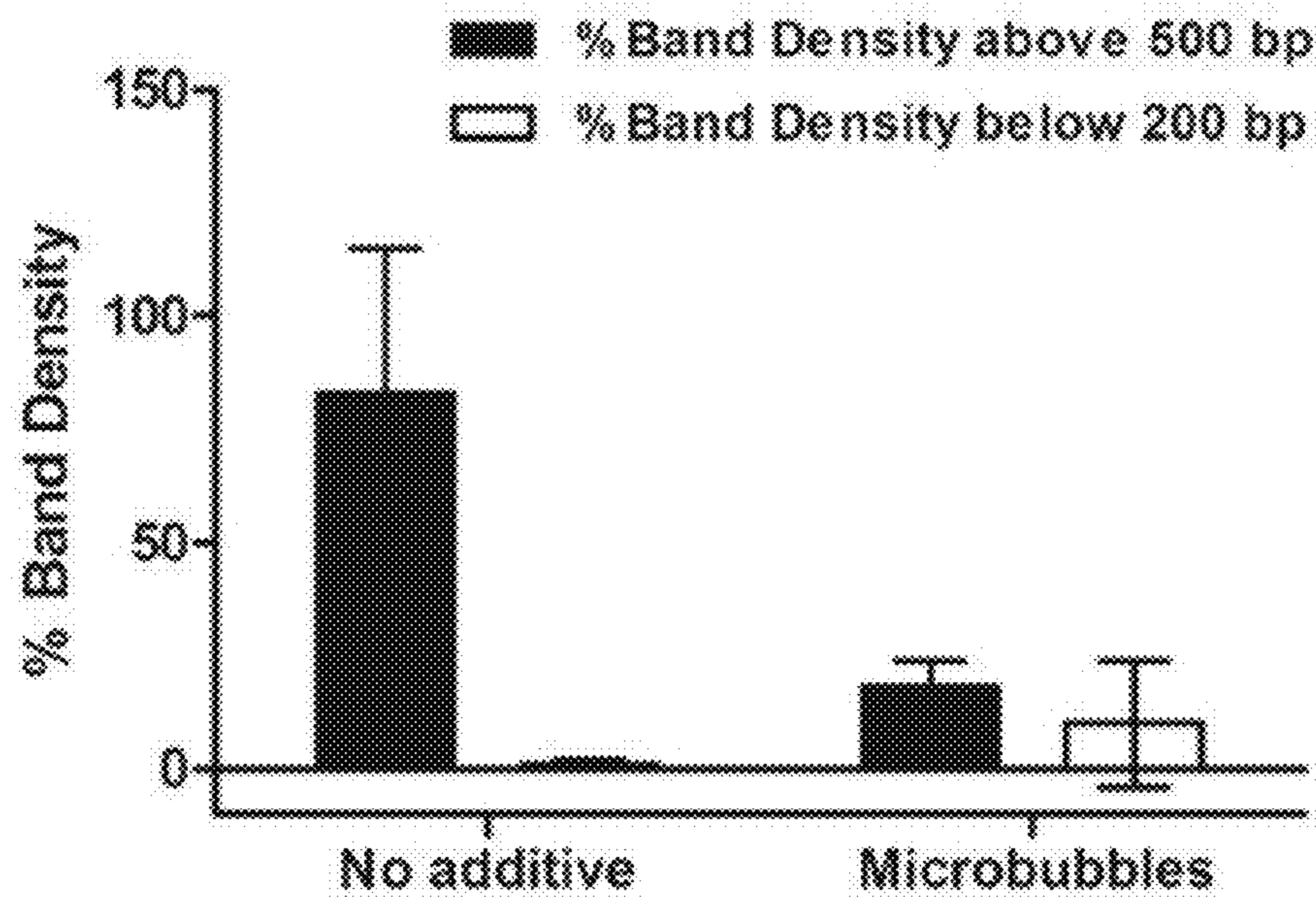


FIG.8

METHODS AND SYSTEMS FOR USING ENCAPSULATED MICROBUBBLES TO PROCESS BIOLOGICAL SAMPLES

PRIORITY CLAIM

[0001] This application is a divisional of U.S. patent application Ser. No. 15/990,171, filed May 25, 2018, which is a divisional of U.S. patent application Ser. No. 14/432,747, filed Mar. 31, 2015, and which issued as U.S. Pat. No. 9,982,290 on May 29, 2018, which is a National Stage Entry of International Patent Application No. PCT/US2013/063397, filed Oct. 4, 2013, which claims the benefit of U.S. Provisional Patent Application Ser. No. 61/709,488, filed Oct. 4, 2012.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under Grant Numbers EB008733 and EB009066 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

[0003] The subject matter described herein relates to methods and systems for processing biological samples. More particularly, the subject matter described herein relates to methods and systems for using encapsulated microbubbles to process biological samples.

BACKGROUND

[0004] The transformation of a normal cell into a cancer cell is the result of an accumulation of genetic mutations or epigenetic changes that lead to aberrant gene expression. Understanding where and when these changes occur in any given tumor type is a primary focus of cancer research. Since cellular transformation is often unique for each patient, a diagnostic test that provides a customized genome-based profile of a tumor is very desirable and is likely to be a standard in the future of oncological medicine. Genetic mutations in transformed cells are now very commonly profiled using next-generation sequencing (NGS). Incorporation of NGS technology into routine clinical diagnostics, however, will require overcoming a number of hurdles including streamlining of sample processing.

[0005] Random, unbiased fragmentation of DNA is necessary for NGS and is a key step in building any genomic library for sequencing. When NGS is used to determine genetic changes, the desired length of each DNA fragment in base pairs (bp) depends on the maximum possible read length of the sequencer. If fragments exceed the maximum length, they will be incompletely read. If they are too small, or degraded, then they will be excluded from the read. Therefore, consistent DNA fragmentation is required for quality NGS data. DNA shearing is also used in processes such as chromatin immunoprecipitation (ChIP), formaldehyde-assisted interrogation of regulatory elements (FAIRE), and DNA sequencing. For each of these techniques, it is important that DNA is sheared to a consistent size in the shortest time possible. Chromatin from cells that have been subjected to formaldehyde crosslinking (e.g., from the ChIP and FAIRE techniques), are particularly resistant to conventional DNA shearing techniques. Thus, the time required to

shear formaldehyde cross-linked DNA makes it difficult to integrate this step into automated protocols with large sample sets.

[0006] Conventional methods for fragmentation of DNA include enzymatic digestion, sonication, nebulization, and hydrodynamic shearing. While all of these techniques are widely used, each has advantages and disadvantages. Enzymatic digestion using DNase I, MNase, or restriction enzymes is very efficient, but introduces an enzyme bias. Regions of transcriptionally silent, tightly packed (heterochromatic) DNA and DNA with high G-C content can be refractive to enzymatic digestion and many enzymes only create nicks in the DNA instead of cutting completely through both strands. The nebulization process shears solubilized DNA by forcing it through a pressurized nozzle (atomization). This method is fast, but requires large quantities of DNA and often results in a large distribution in the DNA fragment size and cross-contamination between samples. Hydrodynamic shearing involves forcing solubilized DNA through a mesh. It has the advantage of rapidly producing small DNA fragments of nearly uniform length. This method, however, is costly and the screen used for shearing is prone to clogging and cross-contamination between samples. Similar to enzymatic digestion, heterochromatic DNA or DNA with high G-C content are very difficult to shear, which creates a bias toward better shearing efficiency in euchromatic and A-T rich regions. Although NGS involves fragmentation of purified genomic DNA, ChIP requires fragmentation of DNA from the nuclei of intact, formaldehyde cross-linked cells. Formaldehyde crosslinks protein to DNA, and as a result cells are very rigid and particularly resistant to lysis. Therefore, DNA fragmentation for fixed samples such as ChIP or FFPE tissue is even more challenging. The summary is that current DNA fragmentation methods are a bottleneck for diagnostic assays such as NGS and ChIP, and that a substantial improvement in methods for DNA fragmentation would have a significant impact.

[0007] Presently, DNA fragmentation in academic laboratories is commonly performed using a probe-based or acoustic sonicator. Sonication uses uncontrolled cavitation to shear DNA. Conventional DNA sonicators range from a single probe to multi-sample acoustic water bath sonicators. The method typically produces inconsistent results and is time consuming, thereby limiting its utility. DNA extracted from cells or tissue must be optimized each time to ensure that fragmentation occurs to the desired size range. In the case of formaldehyde cross-linked samples, checking DNA fragment size involves an overnight incubation, so optimization can take several days for each sample type. Therefore, an inexpensive method that provides shearing consistency independent of cell or tissue type and that can be performed rapidly would be very valuable and would have a significant impact on the use of technologies like NGS as a companion diagnostic for cancer.

[0008] There are few methods currently available to increase DNA fragmentation efficiency by an acoustic or probe sonicator. Borosilicate glass beads are sometimes added to the DNA mix during sonication, but these beads provide limited improvement and will reduce the lifetime of a probe sonicator by causing pitting in the probe. Another technique uses a vial that contains a rod that enucleates bubbles during sonication. Cavitation nucleated from this rod help to shear DNA. While these microbubble nucleating

rods improve consistency results in the sonicator, they release plastic residue that can clog columns in downstream applications, and are very costly (five dollars per sample). Thus, current DNA fragmentation methods are a bottleneck for the creation of NGS libraries.

[0009] Accordingly, in light of these disadvantages associated with conventional techniques for DNA shearing, there exists a need for more consistent and efficient techniques for DNA shearing. More specifically, there exists a need for methods and systems for using encapsulated microbubbles to process biological samples.

SUMMARY

[0010] According to one aspect, the subject matter described herein includes a method for using encapsulated microbubbles to process a biological sample according to an embodiment of the subject matter described herein. The method includes creating a mixture comprising encapsulated microbubbles mixed with a biological sample and adding activation energy to the mixture to cause at least some of the microbubbles to oscillate or burst and thereby process the sample.

[0011] According to another aspect, the subject matter described herein includes a system for delivering a solution of microbubbles to a biological sample according to an embodiment of the subject matter described herein. The system includes a container for holding the solution of microbubbles and at least one outlet for delivering the solution of microbubbles to a biological sample.

[0012] According to yet another aspect, the subject matter described herein includes a system for delivering microbubbles to a biological sample according to an embodiment of the subject matter described herein. The system includes a container for holding a solution that will formulate encapsulated microbubbles when processed and at least one outlet for delivering the solution to a biological sample.

[0013] The subject matter described herein can be implemented in software in combination with hardware and/or firmware. For example, the subject matter described herein can be implemented in software executed by a processor. In one exemplary implementation, the subject matter described herein can be implemented using a non-transitory computer readable medium having stored thereon computer executable instructions that when executed by the processor of a computer control the computer to perform steps. Exemplary computer readable media suitable for implementing the subject matter described herein include non-transitory computer-readable media, such as disk memory devices, chip memory devices, programmable logic devices, and application specific integrated circuits. In addition, a computer readable medium that implements the subject matter described herein may be located on a single device or computing platform or may be distributed across multiple devices or computing platforms.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Preferred embodiments of the subject matter described herein will now be explained with reference to the accompanying drawings, wherein like reference numerals represent like parts, of which:

[0015] FIG. 1 is a flow chart illustrating an exemplary process for using encapsulated microbubbles to process a

biological sample according to yet another embodiment of the subject matter described herein;

[0016] FIG. 2 is a flow chart illustrating a method for creating the biological sample and microbubble mixture according to an embodiment of the subject matter described herein;

[0017] FIG. 3 is a flow chart illustrating a method for creating the biological sample and microbubble mixture according to another embodiment of the subject matter described herein;

[0018] FIG. 4 is a flow chart illustrating a method for creating the biological sample and microbubble mixture according to another embodiment of the subject matter described herein;

[0019] FIGS. 5A and 5B are block diagrams illustrating exemplary systems for delivering microbubbles to a biological sample according to embodiments of the subject matter described herein;

[0020] FIGS. 6A through 6F are block diagrams illustrating exemplary systems for delivering microbubbles to a biological sample according to other embodiments of the subject matter described herein;

[0021] FIG. 7 shows the results of DNA fragmentation in the presence and absence of microbubbles; and

[0022] FIG. 8 shows the results of densitometry performed on three independent biological replicates after 30 seconds sonication time.

DETAILED DESCRIPTION

[0023] In accordance with the subject matter disclosed herein, methods and systems for using encapsulated microbubbles to process biological samples are provided. The addition of controlled sized lipid microbubbles to an acoustic sonication process significantly improves the efficiency and consistency of DNA fragmentation and the presence of the lipid microbubbles does not interfere with downstream fluorescence-based analyses such as quantitative PCR. This technique can be used to greatly improve sample processing efficiency and robustness. Microbubbles may also be used to enhance other sample processes, such as to effect cell lysis, to perform tissue dispersion, and to detach tissues from tissue culture (TC) plates in preparation for transfer of tissue to polymerase chain reaction (PCR) plates.

[0024] Reference will now be made in detail to exemplary embodiments of the present invention, examples of which are illustrated in the accompanying drawings. Wherever possible, the same reference numbers will be used throughout the drawings to refer to the same or like parts.

[0025] Microbubbles, which are bubbles that are typically in the 1-10 micron diameter size range, have been used in medical diagnostics as contrast agents for ultrasound imaging for approximately two decades. For medical applications, these microbubbles utilize an encapsulating shell, typically a lipid, and high molecular weight gas core to maximize stability. The highly compressible core of a gas filled microbubble enables it to compress and expand in a pressure field. In an acoustic field, this oscillation happens at the frequency of the sound wave. For medical acoustics, this is on the order of megahertz (MHz). It has been shown through the use of high-speed photography that expansion and compression velocity of microbubbles in an acoustic field can be on the order of 350 meters per second even at only moderate acoustic pressures (2.4 MHz, 1.2 megapascals (MPa)). When the bubble remains intact, this phenom-

enon is called stable cavitation. At higher acoustic energies, these microbubbles can oscillate to such a violent extent that the bubbles will break up in a process called transient cavitation.

[0026] Cavitation in general is a very mechanically vigorous process, and the intensity typically increases as acoustic pressure is increased and acoustic center frequency is decreased. At sufficient acoustic parameters microbubble-mediated cavitation is well known to cause various effects such as the disruption of cell membranes, the breakup up of blood clots, and the permeabilization of vascular endothelium. The exact mechanism is still poorly understood, however. Furthermore, because microbubbles are orders of magnitude larger in size than DNA base pairs, it was not known whether microbubbles would perform as an effective reagent for DNA fragmentation in an acoustic sonicator. Since the microbubbles consist of a lipid layer surrounding an inert gas, and they were used in small quantities (10 micrograms (μg) per sample), they were not expected to interfere with downstream analyses such as the fluorescent-based quantitative polymerase chain reaction (qPCR), NGS, or ChIP, however, and were therefore a good candidate for study.

[0027] FIG. 1 is a flow chart illustrating an exemplary process for using encapsulated microbubbles to process a biological sample according to yet another embodiment of the subject matter described herein. In the embodiment illustrated in FIG. 1, step 100 includes creating a mixture that contains encapsulated microbubbles mixed with a biological sample, and step 102 includes adding activation energy to the mixture to cause at least some of the microbubbles to oscillate or burst, thereby processing the sample. In one embodiment, a majority of the microbubbles have a diameter in the range from 0.1 microns to 10 microns.

[0028] The inclusion of microbubbles into a biological sample and their subsequent activation has been found to increase the efficiency of a number of processes. For example, the biological sample may include DNA or DNA that has been cross-linked to protein, in which case processing the sample may include shearing the DNA. The biological sample may include cells, including but not limited to bacteria or yeast cells, where processing the sample may include effecting cell lysis. The biological sample may include tissue, in which case processing the sample includes performing tissue dispersion. The method may be successfully applied to a variety of types of tissues, including fresh tissue, cryogenically preserved tissue, and fixed and paraffin embedded tissue.

[0029] There are a number of ways in which activation energy may be added to the mixture, including but not limited to sonicating the mixture, exposing the mixture to laser light, and exposing the mixture to heat. Sonicating the mixture may include applying sonic energy in the 0.01 MHz to 10.0 MHz frequency range.

[0030] There are a number of ways to create the biological sample and microbubble mixture. Some of these are illustrated in FIGS. 2, 3, and 4.

[0031] FIG. 2 is a flow chart illustrating a method for creating the biological sample and microbubble mixture according to an embodiment of the subject matter described herein. In the embodiment illustrated in FIG. 2, the biological sample and microbubble mixture is created by adding a solution of encapsulated microbubbles to the sample (step 200) and mixing them together (step 202.) The solution of encapsulated microbubbles may be pre-prepared, or may be

prepared by mixing dehydrated microbubbles with a hydrating solution that re-suspends the microbubbles and mixing the resulting solution of encapsulated microbubbles with the biological sample.

[0032] FIG. 3 is a flow chart illustrating a method for creating the biological sample and microbubble mixture according to another embodiment of the subject matter described herein. In the embodiment illustrated in FIG. 3, the biological sample and microbubble mixture is created by adding a solution of encapsulated droplets, which may be nanodroplets, to the sample (step 300), mixing the solution with the sample (step 302), and converting at least some of the droplets to encapsulated microbubbles by the addition of conversion energy (step 303.) In one embodiment, these nanodroplets are identical in composition to the microbubbles, yet remain metastable in liquid form until the addition of acoustic energy, after which they vaporize into microbubbles.

[0033] Both standard microbubbles and vaporized phase change nanodroplets would have identical acoustically active properties once in the gas form. The benefit of the nanodroplets is that they can be prepared in sub-micron form, such as 100 to 750 nanometers, small sizes which makes them nearly neutrally buoyant and more likely to impregnate intracellular spaces for enhanced dispersion. Once vaporized, they grow in diameter by approximately a factor of 6 due to the liquid to gas volume change. The resulting bubble could play a significant role in tissue dispersion, with the additional advantage of enhanced penetration of tissue while in the liquid nanodroplet precursor stage compared to the larger and more buoyant microbubbles. The phase-change droplets have the advantage that they sink rather than float in solution, which might increase interaction with materials at the bottom of a sample chamber.

[0034] Liquid perfluorocarbon-based micro- and nanodroplets may be created with the same type of solution that is used for lipid-coated microbubbles. Microbubbles are mechanically agitated in a 3 mL vial to form a polydisperse size distribution. Due to the low boiling point of the perfluorocarbon gas, transition to the liquid phase is achievable. By increasing the pressure on the headspace of the sealed vial using a custom apparatus, the gas core will transition from the gas phase to the liquid phase, thus creating a polydisperse distribution of lipid-coated perfluorocarbon-based micro- and nano-droplets. By supplying enough acoustic energy, the droplets may transition back to the gas phase and subsequently cavitate to provide the necessary mechanism to expedite the lysis of cells.

[0035] The droplets may be converted to microbubbles before the solution is added to the biological tissue, or they may not be converted until after the solution is added to the biological tissue, which provides additional benefits in some circumstances. For example, the conversion of droplets to microbubbles after the droplets have been added to the biological tissue can effect cell lysis and can help detach tissues from a tissue culture plate in preparation for transfer to PCR plates, which may help avoid a scraping step.

[0036] In one embodiment, the droplets may include a shell surrounding a liquid core which converts to a gas upon the addition of acoustic, thermal, or optical energy. The liquid core may include a hydrocarbon or a perfluorocarbon.

Examples of liquids include but are not limited to isopentane, perfluoropentane, perfluorohexane, perfluorobutane, and perfluoropropane.

[0037] Conversion energy may be added using a number of techniques, including sonicating the mixture, exposing the mixture to laser light, etc. In one embodiment, for example, the droplets may be converted into microbubbles by sonicating the mixture using ultrasound in the 0.01 MHz to 10.0 MHz frequency range.

[0038] FIG. 4 is a flow chart illustrating a method for creating the biological sample and microbubble mixture according to another embodiment of the subject matter described herein. In the embodiment illustrated in FIG. 4, the biological sample and microbubble mixture is created by adding to the biological sample a solution containing reagents that will formulate encapsulated microbubbles upon the addition of energy (step 400), then adding formation energy to a biological sample to induce the formation of encapsulated microbubbles in the biological sample (step 402.)

[0039] In one embodiment, the reagent solution may be added to the biological sample and the resulting mixture is subjected to the formulation energy, which creates the microbubbles. In one example, the reagent solution may be mixed with the biological sample and placed into a test tube or other container that contains air or some other gas. The test tube is then sealed and subjected to vigorous shaking, which creates the microbubbles. A surfactant, emulsifier, polymer, or protein may be added to the sample prior to or during the addition of the formation energy to enhance bubble stability so that the microbubbles persist until the addition of the activation energy. In another example, nanodroplets may be added to the biological sample, and the resulting mixture may be subjected to sonication or other form of formation energy, which causes the nanodroplets to vaporize into microbubbles. Sonication may include using ultrasound having a frequency in the range from 0.01 MHz to 10.0 MHz. Other types of formation energy include light, such as laser light, and heat.

[0040] All of the steps described above may be applied in parallel for the purpose of performing high throughput processing of multiple biological samples. For example, adding the solution of encapsulated microbubbles or encapsulated droplets to a sample may involve adding the solution multiple biological samples located in individual wells of a multi-well sample plate and mixing the solution with the samples in the wells. Likewise, adding the formation or activation energies may involve adding the formation or activation energies to multiple biological samples located in individual wells of a multi-well sample plate.

[0041] FIG. 5A illustrates a system for delivering microbubbles to a biological sample according to an embodiment of the subject matter described herein. In the embodiment illustrated in FIG. 5A, system 500 includes a container 502 for holding a solution of microbubbles, and at least one outlet 504 for delivering the solution of microbubbles to a biological sample 506. In the embodiment illustrated in FIG. 5A, biological sample 506 is contained in one or more wells of a multi-well plate 508. In one embodiment, container 502 may have multiple outlets 504 for delivering the solution of microbubbles to different wells of multi-well plate 508 in parallel for high throughput processing. In other embodiments, sample 506 may be contained in a single well plate, a test tube, or other container. In one

embodiment, the solution of microbubbles may be mixed with the sample 506 in a mixing step.

[0042] In one embodiment, system 500 may include an energy source 510 for providing activation energy to the microbubbles within the sample(s) such that the microbubbles oscillate or burst and thereby process the sample. Examples of activation energy include, but are not limited to, thermal energy, sonic or ultrasonic energy, and light energy. In one embodiment, for example, activation energy may be provided using ultrasound having a frequency in the range from 0.01 MHz to 10 MHz.

[0043] System 500 may be used to perform different processes, which may be performed on different types of samples. The cavitation caused by oscillating or rupturing microbubbles can detach biological samples from a TC plate in preparation for transferal of the sample to a PCR, for example. In one embodiment, sample 506 may include DNA or DNA that has been cross-linked to protein, in which case processing sample 506 may include shearing the DNA. In another embodiment, sample 506 may include cells, such as (but not limited to) bacteria and yeast cells, in which case processing sample 506 may include effecting cell lysis. In another embodiment, sample 506 may include tissue, in which case processing sample 506 may include performing tissue dispersion. System 500 may be used to process various kinds of tissue, including fresh tissue, cryogenically preserved tissue, and fixed and paraffin embedded tissue. In one embodiment, microbubbles having a diameter in the range from 0.1 microns to 10 microns may be used for DNA shearing or other processes.

[0044] FIG. 5B illustrates a variation of system 500. In the embodiment shown in FIG. 5B, system 500 may also include a second container 512 for holding dehydrated microbubbles, which are mixed with a solution 514 and thus re-suspended before being provided to container 502.

[0045] FIG. 6A illustrates a system for delivering microbubbles to a biological sample according to an embodiment of the subject matter described herein. In the embodiment illustrated in FIG. 6A, system 600 includes a container 602 for holding a solution that will formulate encapsulated microbubbles when processed, and at least one outlet 604 for delivering the solution to a biological sample 606. In one embodiment, container 602 may have multiple outlets 604 for delivering the solution of microbubbles to different wells of multi-well plate 608 in parallel for high throughput processing. In other embodiments, sample 606 may be contained in a single well plate, a test tube, or other container. In one embodiment, the solution of microbubbles may be mixed with the sample 606 in a mixing step. In one embodiment, system 600 may include an energy source 610 for providing activation energy to the microbubbles within the sample(s) such that the microbubbles oscillate or burst and thereby process the sample. Examples of activation energy include, but are not limited to, thermal energy, sonic or ultrasonic energy, and light energy. In one embodiment, for example, activation energy may be provided using ultrasound having a frequency in the range from 0.01 MHz to 10 MHz.

[0046] Like system 500, system 600 may be used to perform different processes, which may be performed on different types of samples. The cavitation caused by oscillating or rupturing microbubbles can detach biological samples from a TC plate in preparation for transferal of the sample to a PCR, for example. In one embodiment, sample

606 may include DNA or DNA that has been cross-linked to protein, in which case processing sample **606** may include shearing the DNA. In another embodiment, sample **606** may include cells, such as (but not limited to) bacteria and yeast cells, in which case processing sample **606** may include effecting cell lysis. In another embodiment, sample **606** may include tissue, in which case processing sample **606** may include performing tissue dispersion. System **600** may be used to process various kinds of tissue, including fresh tissue, cryogenically preserved tissue, and fixed and paraffin embedded tissue. In one embodiment, microbubbles having a diameter in the range from 0.1 microns to 10 microns may be used for DNA shearing or other processes.

[0047] Unlike system **500**, however, system **600** includes a microbubble-forming apparatus **612** for processing the solution to form encapsulated microbubbles within the solution. As will be described in more detail below, in one embodiment the encapsulated microbubbles are formed prior to providing the solution to sample **606** and in another embodiment the encapsulated microbubbles are formed after the solution has been provided to sample **606**.

[0048] FIG. 6B illustrates a variation of system **600**. In the embodiment illustrated in FIG. 6B, microbubble forming apparatus **612** includes a probe for adding sonic energy to the solution in the presence of gas to induce the formation of the microbubbles in the solution before it is added to the biological sample.

[0049] FIG. 6C illustrates another variation of system **600**. In the embodiment illustrated in FIG. 6C, microbubble forming apparatus **612** includes a mechanical mixer or impeller that rapidly agitates the solution in the presence of gas, forming the microbubbles in the solution, before the solution is added to the biological sample.

[0050] FIG. 6D illustrates yet another variation of system **600**. In the embodiment illustrated in FIG. 6D, microbubble forming apparatus **612** includes one or more fluid systems that force gas surrounded by liquid through an opening or that force gas through a porous membrane, which produces microbubbles.

[0051] In one embodiment, the solution that will formulate encapsulated microbubbles when processed includes encapsulated liquid droplets or nanodroplets. In one embodiment, the droplets comprise a shell surrounding a liquid core which converts to a gas upon the addition of acoustic, thermal, or optical energy. The liquid core can include, but is not limited to including, a hydrocarbon or perfluorocarbon. In one embodiment, the liquid core includes isopentane, perfluoropentane, perfluorohexane, perfluorobutane, and/or perfluoropropane. In one embodiment, some or all of the droplets have a diameter in the range from 100 nanometers to 750 nanometers.

[0052] FIGS. 6E and 6F illustrate yet other variations of system **600**. In the embodiments illustrated in FIG. 6E and 6F, container **602** holds a solution that contains nanodroplets, and system **600** includes an energy source **613** for converting the droplets into microbubbles. In FIG. 6E, energy source **613** converts the droplets into microbubbles prior to mixing the resulting solution with biological sample **606**.

[0053] In FIG. 6F, energy source **613** converts the droplets into microbubbles after the droplets and solution have been mixed with biological sample **606**. In this manner, the expansion of the droplet into a microbubble in response to being subjected to the conversion energy provided by energy

source **612** may be used to preprocess sample **606** prior to the further processing that will occur when activation energy is added to the microbubbles so created. In these embodiments, energy source **613** may be the same as or different from energy source **610**, depending on what types of energy are being used for conversion and activation, respectively. In any of the embodiments illustrated herein, the solution being supplied to the biological sample may include other substances that may contribute to the creation of or stabilization of droplets or bubbles, including but not limited to surfactants, emulsifiers, polymers, or proteins.

Specific Embodiments

[0054] In one embodiment, lipid monolayer-coated microbubbles were created using 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)(Avanti Polar Lipids, Alabaster, Ala.) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (polyethylene-glycol)-2000 (DSPE-PEG2000) (Avanti Polar Lipids, Alabaster, Ala.) in a 9 to 1 molar ratio as previously described. The lipids were dissolved in a buffer solution comprised of phosphate-buffered saline (PBS), propylene glycol, and glycerol (16:3:1) for a total lipid concentration of 1.0 mg/mL. The resulting lipid solution was placed into 3 mL glass vials in 1.5 mL aliquots. The vials were sealed with rubber septa and 5 capped. Finally, the air in the vial headspace was removed via a custom vacuum apparatus and replaced with decafluorobutane (Fluoromed, Round Rock, Tex.). The vial was shaken vigorously for 45 seconds using a high-speed mixer (Vialmix, Bristol-Myers Squibb Medical Imaging, North Billerica, Mass.) to produce a polydisperse distribution (Mean Diameter: 1.07 ± 0.9 , Concentration: 1.0×10^{10} #/mL) of lipid-coated microbubbles.

[0055] Sonication was performed in a Covaris E110 sonicator in a 96-well polypropylene plate (Thermo Scientific, AB-0900). Genomic DNA (gDNA) was extracted from HEK293T cells and distributed at 10 μ g per well in a final volume of 150 microliters (uL) in 10 mM Tris-HCl, pH 8.0. Our target fragment range was 300-500 base pairs (bp). Ten μ L of the 1 mg/mL microbubble suspension was added to the appropriate wells. Fragmentation was confirmed by running 1 μ g of DNA on a 2% agarose gel containing SYBR Green and visualizing on a Typhoon Trio+imager.

[0056] FIG. 7 shows the results of DNA fragmentation in the presence and absence of microbubbles. Ten micrograms of DNA purified from human HEK293T cells was resuspended in 150 uL final volume per well of a 96-well plate and sonicated in a Covaris E110 sonicator for 30 seconds (Lanes 3-8) or 1 minute (Lanes 9-14) in triplicate. DNA fragmentation without any additive is inefficient (Lanes 3-5 and 9-11). Addition of 10 uL of one micron lipid encapsulated microbubbles significantly increased the efficiency of fragmentation (Lanes 6-8 and 12-14). DNA ladders are: 1 Kb plus (Fermentas, Lane 1), and 100 bp (Invitrogen, Lane 2). This image represents one of three biological replicates. The samples with microbubbles were efficiently sheared to the desired 300-500 bp range within 30 seconds (Lanes 6-8), while the samples without microbubbles showed very little fragmentation (Lanes 3-5 and 9-11).

[0057] FIG. 8 shows the results of densitometry performed on three independent biological replicates after 30 seconds sonication time. Densitometry analysis indicates that DNA fragmentation is centered in the 200-500 bp range for samples sonicated for 30 seconds in the presence of lipid-

encapsulated microbubbles. Percent band density was calculated using the rolling ball method (ImageQuant TL, GE) by dividing the density for the indicated size range by the density for the entire lane. Error bars represent the standard deviation for 3 biological replicates with 3 technical replicates each. The addition of microbubble reagent to the acoustic sonication process significantly improved the consistency of DNA fragmentation by producing fragments within a narrow size distribution.

[0058] Analysis to assess DNA integrity confirmed that the presence of microbubbles does not interfere with downstream fluorescent-based analyses. Three biological replicates of gDNA fragmented in the presence of microbubbles from FIG. 7 were removed from the sonication vessel and directly used for qPCR using a SYBR green master mix and the absolute quantification method with primers representing various genomic loci. When compared to a standard curve of unsonicated human genomic DNA, 10-fold serial dilutions of fragmented DNA could be linearly amplified. The PCR products were subjected to dissociation curve analysis to confirm that only a single PCR product was present. Both the standards, and the microbubble fragmented DNA produced a single amplification product. In short, it was confirmed that the presence of lipid microbubbles in the fragmented DNA does not interfere with SYBR green-based qPCR analysis.

[0059] Overall, the addition of microbubbles to the DNA fragmentation process is an innovative approach that allows purified gDNA to be consistently fragmented in a manner of seconds. The same techniques may be applied to extraction and fragmentation of DNA from formalin fixed paraffin embedded (FFPE) tissue slices and cryopreserved biopsy samples. The short sonication times that are possible with microbubble addition could greatly improve sample integrity by decreasing sample handling while increasing processing efficiency and robustness.

[0060] An Example DNA Shearing Protocol:

[0061] 1. Grow human cell line on 10 cm plates ($\sim 1 \times 10^6$ cells per plate).

[0062] 2. For formaldehyde fixed cells, fix cells with 1% formaldehyde at room temperature with gentle shaking for 5 minutes. For non-fixed cells, proceed directly to step 4.

[0063] 3. Stop cross-linking reaction with 125 mM glycine for 5 minutes at room temperature with gentle shaking.

[0064] 4. Wash cells on ice 2 times with 10 mL phosphate buffered saline, pH 7.5 (PBS).

[0065] 5. Scrape cells into 1 mL PBS, transfer to 1.7 mL tube, and centrifuge at 500 \times g for 5 minutes. Remove supernatant.

[0066] 6. Wash plates with 1 mL PBS, scrape again, and add to tubes with pellets. Centrifuge at 500 \times g for 5 minutes in Eppendorf 5430 table top centrifuge. Remove supernatant.

[0067] 7. Flash-freeze pellets in liquid nitrogen. Store at -80°C until needed. Each pellet contains $\sim 1 \times 10^6$ cells.

[0068] 8. Re-suspend cell pellet in 150 μL lysis buffer (10 mM Tris, pH 8.0/100 mM NaCl/1 mM EDTA/2% Triton X-100/1% SDS) with 1 \times protease inhibitor cocktail (Roche #05056489001).

[0069] 9. Transfer 150 μL of cell suspension to one well of a 96-well PCR plate (ABgene #SP-0410).

[0070] 10. Add 10 μL of polydispersed mechanically agitated lipid microbubbles to each well.

[0071] 11. Seal plate with heat sealer.

[0072] 12. Sonication is performed using a Covaris model E110 acoustic sonicator. Sonicate each well for 1 minute using the following settings: 20% duty cycle/8 intensity/200 cycles per burst.

[0073] 13. Remove cover. Add 10 μL more of polydispersed lipid microbubbles to each well.

[0074] 14. Seal plate with heat sealer.

[0075] 15. Sonicate each well for 4 minutes using the following settings: 20% duty cycle/8 intensity/200 cycles per burst.

[0076] 16. Centrifuge plate at 1,300 \times g (1,500 rpm) for 5 minutes at 4°C to remove crude cellular debris.

[0077] 17. Remove seal.

[0078] 18. Transfer sonicated lysates to 1.7 mL tubes. Centrifuge at high speed for one minute to pellet cell debris.

[0079] 19. Transfer 10 μL from each tube to a fresh tube. Add 190 μL TE, pH 8.0 and 2 μL 5 mg/mL RNase. Incubate tubes at 37°C for 1 hour. Add 200 μL 2 \times proteinase K buffer (50 mM Tris, pH 8.0/12.5 mM EDTA, pH 8.0/300 mM NaCl/1% SDS) and 2 μL 10 mg/mL proteinase K. Incubate at 55°C overnight. Extract samples by phenol:chloroform and confirm fragment size by gel electrophoresis.

[0080] 20. Use the remaining 140 μL sonicated DNA in downstream applications.

[0081] An Example Ablation Process:

[0082] 1. Removal of formaldehyde cross-linked cells from a 96-well tissue culture plate. Once cells have been formaldehyde cross-linked, they are particularly difficult to remove from a surface without manual scraping. To remove these cells we will do the following:

[0083] a. Add formaldehyde to 1% directly to the cell culture media in each well of a 96-well plate for 5 minutes followed by the addition of glycine to 125 mM final concentration to stop crosslinking reaction.

[0084] b. Wash cells with PBS. Add 150 μL lysis buffer (10 mM Tris, pH 8.0/100 mM NaCl/1 mM EDTA/2% Triton X-100/1% SDS) with 1 \times protease inhibitor cocktail (Roche #05056489001).

[0085] c. Add 100 μL (Not sure about the exact amount) of lipid-coated perfluorocarbon droplets. The droplets sink to the bottom of the well where the fixed cells are located. Seal plate.

[0086] d. Place plate in Covaris E110 sonicator. Sonicate for 1 minute per well.

[0087] e. Remove seal, transfer resuspended cells to 96-well PCR plate (ABGene #SP-0410). Proceed with sonication as described in Step #13 in tested protocol, above. The droplets will “activate,” by vaporizing and subsequently cavitate in the presence of sonication.

[0088] Other Permutations:

[0089] Use of microbubbles with polymer or protein shells

[0090] Use of microbubbles with different gas cores—such as oxygen, air, or different perfluorocarbons

[0091] Use of phase-change droplets which convert to microbubbles upon acoustic energy

[0092] Use of microbubbles with different sizes

- [0093] Use of varying acoustic energies, duty cycles, and frequencies to optimize interaction with microbubbles
- [0094] Use of microfluidics systems to produce the microbubbles directly at the site of use
- [0095] Other Embodiments:
- [0096] Other embodiments contemplated by the subject matter described herein include, but are not limited to, the following list:
- [0097] 1. A method for using encapsulated microbubbles to process a biological sample, the method comprising: adding a pre-prepared solution of encapsulated microbubbles to a biological sample; creating a mixture by mixing the pre-prepared solution of encapsulated microbubbles with the biological sample; and adding energy to the mixture to cause at least some of the microbubbles to oscillate and/or burst and thereby process the sample.
- [0098] 2. The method of list item 1 wherein the biological sample comprises DNA or DNA that has been cross linked to protein and wherein processing the sample comprises shearing the DNA.
- [0099] 3. The method of list item 1 wherein the biological sample comprises cells and wherein processing the sample comprises effecting cell lysis.
- [0100] 4. The method of list item 3 wherein the cells comprise bacteria or yeast cells.
- [0101] 5. The method of list item 1 wherein the sample comprises tissue, and wherein processing the sample comprises performing tissue dispersion.
- [0102] 6. The method of list item 5 wherein the tissue is fresh, or cryogenically preserved, or fixed and paraffin embedded
- [0103] 7. The method of list item 1 wherein adding energy to the mixture comprises sonicating the mixture
- [0104] 8. The method of list item 7 wherein sonicating the mixture includes applying energy of a frequency between 0.01 MHz and 10.0 MHz.
- [0105] 9. The method of list item 1 wherein adding energy to the mixture comprises exposing the sample to laser light.
- [0106] 10. The method of list item 1 wherein adding a pre-prepared solution of encapsulated microbubbles to a sample includes adding the solution to a plurality of biological samples located in individual wells of a multi-well sample plate, wherein mixing the pre-prepared solution includes mixing the solution with the samples in the wells, and wherein adding energy to the mixtures includes adding the energy to each of the mixtures in the wells to perform high throughput processing of a plurality of biological samples.
- [0107] 11. The method of list item 1 wherein a majority of the microbubbles are between 0.1 microns and 10 microns in diameter.
- [0108] 12. A method for using encapsulated droplets to process a biological sample, the method comprising: adding a pre-prepared solution of encapsulated droplets to a biological sample; creating a mixture by mixing the pre-prepared solution of encapsulated droplets with the biological sample; and adding energy to the mixture to convert at least some of the droplets to gas bubbles, and adding further energy to the gas bubbles to oscillate and/or burst the gas bubbles, and thereby process the sample.
- [0109] 13. The method of list item 12 wherein the droplets consist of a shell surrounding a liquid core which converts to a gas upon the addition of acoustic, thermal, or optical energy.
- [0110] 14. The method of list item 13 wherein the liquid core includes a hydrocarbon or a perfluorocarbon.
- [0111] 15. The system of list item 14 wherein the liquid core includes isopentane, perfluoropentane, perfluorohexane, perfluorobutane, or perfluoropropane.
- [0112] 16. The method of list item 12 wherein the biological sample comprises DNA or DNA that has been cross linked to protein, and wherein processing the sample comprises shearing the DNA.
- [0113] 17. The method of list item 12 wherein the biological sample comprises cells and wherein processing the sample comprises effecting cell lysis
- [0114] 18. The method of list item 17 wherein the cells comprise bacteria or yeast cells.
- [0115] 19. The method of list item 12 wherein the sample comprises tissue and wherein processing the sample comprises performing tissue dispersion.
- [0116] 20. The method of list item 12 wherein the tissue is fresh, or cryogenically preserved, or fixed and paraffin embedded
- [0117] 21. The method of list item 12 wherein adding energy to the mixture comprises sonicating the mixture.
- [0118] 22. The method of list item 21 wherein sonicating the mixture includes adding energy having a frequency range from 0.01 MHz and 10.0 MHz.
- [0119] 23. The method of list item 12 wherein adding energy to the mixture comprises exposing the sample to laser light.
- [0120] 24. The method of list item 12 wherein adding a pre-prepared solution of encapsulated droplets to a sample includes applying the solution to a plurality of biological samples located in individual wells of a multi-well sample plate, wherein mixing the pre-prepared solution includes mixing the solution with the samples in the wells and wherein adding energy to the mixture includes adding energy to the mixtures in the wells to perform high throughput processing of a plurality of biological samples.
- [0121] 25. The method of list item 12 where a majority of droplets are between 0.1 and 10 microns in diameter.
- [0122] 26. A method for using encapsulated microbubbles to process a biological sample, the method comprising: adding first energy to a biological sample to first induce the formation of encapsulated microbubbles in the biological sample; and adding second energy to the sample to oscillate and/or burst the microbubbles and thereby process the sample.
- [0123] 27. The method of list item 26 comprising adding a surfactant, emulsifier, polymer, or protein to the biological sample prior to or during the addition of the first energy to enhance bubble stability so that the microbubbles persist until the addition of the second energy.
- [0124] 28. the method of list item 26 where droplets of liquid are added to the biological sample, prior to or during the addition of first energy, so that said liquid droplets vaporize into bubbles during administration of first energy, and resulting bubbles which oscillate and/or burst in response to the addition of the second energy.

- [0125] 29. The method of list item 26 wherein adding energy to the sample to induce the formation of encapsulated microbubbles includes applying laser light to the sample to form the microbubbles.
- [0126] 30. The method of list item 26 wherein adding energy to the sample to induce the formation of encapsulated microbubbles includes sonicating the sample to form the microbubbles.
- [0127] 31. The method of list item 30 wherein sonicating the sample includes applying sonic energy of a frequency 0.01 and 10.0 MHz.
- [0128] 32. The method of list item 26 wherein the biological sample comprises DNA or DNA that has been cross linked to protein and wherein processing the sample comprises shearing the DNA.
- [0129] 33. The method of list item 26 wherein the biological sample comprises cells and wherein processing the sample comprises effecting cell lysis.
- [0130] 34. The method of list item 33 wherein the cells comprise bacteria or yeast cells.
- [0131] 35. The method of list item 26 wherein the sample comprises tissue and wherein processing the sample comprises performing tissue dispersion.
- [0132] 36. The method of list item 35 wherein the tissue is fresh, or cryogenically preserved, or fixed and paraffin embedded.
- [0133] 37. The method of list item 26 wherein adding the first and second energies to a biological sample includes adding the first and second energies to a plurality of biological samples located in individual wells of a multi-well sample plate to perform high throughput processing of a plurality of biological samples.
- [0134] 38. A system for delivering a solution for delivering microbubbles to a biological sample, the system comprising: a container for holding a solution, the solution comprising reagents that will formulate encapsulated microbubbles upon the addition of energy; and at least one outlet for delivering the pre-prepared solution of to a biological sample.
- [0135] 39. The system of list item 38 comprising a probe for adding sonic energy to the solution in the presence of gas to induce the formation of the microbubbles in the solution before it is added to the biological sample.
- [0136] 40. The system of list item 38 comprising a mechanical mixer/impeller that rapidly agitates the solution in the presence of gas, forming the microbubbles in the solution, before the solution is added to the biological sample.
- [0137] 41. The system of list item 38 comprising at least one fluid system which forces gas surrounded by liquid through an opening to result in microbubble production.
- [0138] 42. The system of list item 38 comprising at least one fluid system that forces gas through a porous membrane to form the microbubbles.
- [0139] 43. The system of list item 38 wherein the biological sample will then be exposed to additional thermal, sonic, or light energy for processing.
- [0140] 44. The system of list item 43 wherein the additional energy is ultrasound with a frequency between 0.01 to 10 MHz.
- [0141] 45. The system of list item 38 wherein the biological sample comprises DNA or DNA that has been cross linked to protein and wherein processing the sample comprises shearing the DNA.
- [0142] 46. The system of list item 38 wherein the biological sample comprises cells and wherein processing the sample comprises effecting cell lysis.
- [0143] 47. The system of list item 46 wherein the cells comprise bacteria or yeast cells.
- [0144] 48. The system of list item 38 wherein the sample comprises tissue and wherein processing the sample comprises performing tissue dispersion.
- [0145] 49. The system of list item 48 wherein the tissue is fresh, or cryogenically preserved, or fixed and paraffin embedded
- [0146] 50. The system of list item 38 wherein the container includes a plurality of outlets for delivering the solution of microbubbles to different wells of a multi-well plate.
- [0147] 51. The system of list item 38 where the solution of reagents includes surfactants, emulsifiers, polymers, or proteins
- [0148] 52. A system for delivering a pre-prepared solution of microbubbles to a biological sample, the system comprising: a container for holding the solution of microbubbles; and at least one outlet for delivering the pre-prepared solution of microbubbles to a biological sample.
- [0149] 53. The system of list item 52 comprising an energy source for exposing the biological sample to thermal, sonic, or light energy for processing
- [0150] 54. The system of list item 53 wherein the additional energy is ultrasound with a frequency between 0.01 to 10 MHz.
- [0151] 55. The system of list item 52 wherein the biological sample comprises DNA or DNA that has been cross linked to protein and wherein processing the sample comprises shearing the DNA.
- [0152] 56. The system of list item 52 wherein the biological sample comprises cells and wherein processing the sample comprises effecting cell lysis.
- [0153] 57. The system of list item 56 wherein the cells comprise bacteria or yeast cells.
- [0154] 58. The system of list item 52 wherein the sample comprises tissue and wherein processing the sample comprises performing tissue dispersion.
- [0155] 59. The system of list item 58 wherein the tissue is fresh, or cryogenically preserved, or fixed and paraffin embedded.
- [0156] 60. The system of list item 52 wherein the container includes a plurality of outlets for delivering the solution of microbubbles to different wells of a multi-well plate.
- [0157] 61. The system of list item 52 wherein a majority of the microbubbles are between 0.1 and 10 microns in diameter.
- [0158] 62. A system for delivering a pre-prepared solution of encapsulated liquid droplets to a biological sample, the system comprising: a container for holding the solution of encapsulated liquid droplets; and at least one outlet for delivering the pre-prepared solution of encapsulated liquid droplets to a biological sample.

- [0159] 63. The system of list item 62 where the droplet consists of a shell surrounding a liquid core which converts to a gas upon the addition of acoustic, thermal, or optical energy.
- [0160] 64. The system of list item 63 where the liquid core is a hydrocarbon or perfluorocarbon.
- [0161] 65. The system of list item 64 wherein the liquid core comprises isopentane, perfluoropentane, perfluorohexane, perfluorobutane, or perfluoropropane.
- [0162] 66. The system of list item 62 comprising an energy source for adding thermal, sonic, or light energy to the sample for processing.
- [0163] 67. The system of list item 66 wherein the additional energy is ultrasound with a frequency between 0.01 to 10 MHz.
- [0164] 68. The system of list item 62 wherein the biological sample comprises DNA or DNA that has been cross linked to protein and wherein processing the sample comprises shearing the DNA.
- [0165] 69. The system of list item 62 wherein the biological sample comprises cells and wherein processing the sample comprises effecting cell lysis.
- [0166] 70. The system of list item 69 wherein the cells comprise bacteria or yeast cells.
- [0167] 71. The system of list item 62 wherein the sample comprises tissue and wherein processing the sample comprises performing tissue dispersion.
- [0168] 72. The method of list item 71 wherein the tissue is fresh, or cryogenically preserved, or fixed and paraffin embedded.
- [0169] 73. The system of list item 62 wherein the container includes a plurality of outlets for delivering the solution of encapsulated liquid droplets to different wells of a multi-well plate.
- [0170] 74. The system of list item 62 wherein a majority of encapsulated liquid droplets are between 0.1 and 10 microns in diameter.
- [0171] 75. A system for delivering a pre-prepared solution of microbubbles to a biological sample, the system comprising: a container which holds dehydrated microbubbles which are mixed with a solution and thus resuspended; and at least one outlet for delivering the resuspended microbubbles to a biological sample.
- [0172] 76. The system of list item 75 wherein the biological sample will then be exposed to additional thermal, sonic, or light energy for processing
- [0173] 77. The system of list item 76 wherein the additional energy is ultrasound with a frequency between 0.01 to 10 MHz.
- [0174] 78. The system of list item 75 wherein the biological sample comprises DNA or DNA that has been cross linked to protein and wherein processing the sample comprises shearing the DNA.
- [0175] 79. The system of list item 75 wherein the biological sample comprises cells and wherein processing the sample comprises effecting cell lysis.
- [0176] 80. The system of list item 75 wherein the cells comprise bacteria or yeast cells.
- [0177] 81. The system of list item 75 wherein the sample comprises tissue and wherein processing the sample comprises performing tissue dispersion.
- [0178] 82. The method of list item 81 wherein the tissue is fresh, or cryogenically preserved, or fixed and paraffin embedded.

- [0179] 83. The system of list item 75 wherein the container includes a plurality of outlets for delivering the solution of encapsulated liquid droplets to different wells of a multi-well plate.

- [0180] 84. The system of list item 75 wherein a majority of the microbubbles are between 0.1 and 10 microns in diameter.

[0181] It will be understood that various details of the subject matter described herein may be changed without departing from the scope of the subject matter described herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

1. A system for delivering microbubbles to a biological sample, the system comprising:

a container for holding a solution of microbubbles or a solution that will formulate encapsulated microbubbles when processed; and

at least one outlet for delivering the solution of microbubbles to a biological sample.

2. The system of claim 1 comprising an energy source for providing activation energy to the microbubbles that causes the microbubbles to oscillate or burst and thereby process the sample.

3. The system of claim 2 wherein the activation energy comprises thermal, sonic, or light energy, optionally wherein the activation energy is ultrasound having a frequency in the range from 0.01 MHz to 10 MHz.

4. (canceled)

5. The system of claim 2 wherein the biological sample comprises DNA or DNA that has been cross-linked to protein and wherein processing the sample comprises shearing the DNA.

6. The system of claim 2 wherein the biological sample comprises cells and wherein processing the sample comprises effecting cell lysis, optionally wherein the cells comprise bacteria or yeast cells.

7. (canceled)

8. The system of 2 wherein the sample comprises tissue and wherein processing the sample comprises performing tissue dispersion.

9. The system of claim 1 wherein the sample comprises at least one of fresh tissue, cryogenically preserved tissue, and fixed and paraffin embedded tissue.

10. The system of claim 1 wherein the container includes a plurality of outlets for delivering the solution of microbubbles or the solution that will formulate encapsulated microbubbles when processed to different wells of a multi-well plate.

11. The system of claim 1 wherein a majority of the microbubbles have a diameter in the range from 0.1 microns to 10 microns.

12. The system of claim 1 comprising a second container for holding dehydrated microbubbles, which are mixed with a solution and thus re-suspended, and that provides the solution of microbubbles to the container for holding the solution of microbubbles.

13. (canceled)

14. The system of claim 1 comprising a microbubble forming apparatus for processing the solution to form encapsulated microbubbles within the solution that will formulate encapsulated microbubbles when processed.

15-24. (canceled)

25. The system of claim **1** where the solution that will formulate encapsulated microbubbles when processed includes surfactants, emulsifiers, polymers, or proteins.

26. The system of claim **14** wherein the microbubble forming apparatus comprises a probe for adding sonic energy to the solution that will formulate encapsulated microbubbles when processed in the presence of gas to induce the formation of the microbubbles in the solution before it is added to the biological sample.

27. The system of claim **14** wherein the microbubble forming apparatus comprises a mechanical mixer or impeller that rapidly agitates the solution that will formulate encapsulated microbubbles when processed in the presence of gas, forming the microbubbles in the solution, before the solution is added to the biological sample.

28. The system of claim **14** wherein the microbubble forming apparatus comprises at least one fluid system that forces gas surrounded by liquid through an opening to result in microbubble production.

29. The system of claim **14** wherein the microbubble forming apparatus comprises at least one fluid system that forces gas through a porous membrane to form the microbubbles.

30. The system of claim **1** wherein the solution that will formulate encapsulated microbubbles when processed comprises encapsulated liquid droplets, optionally wherein a majority of the droplets have a diameter in the range from 100 nanometers to 750 nanometers.

31. The system of claim **30** wherein the droplets comprise a shell surrounding a liquid core that converts to a gas upon the addition of acoustic, thermal, or optical energy.

32. The system of claim **31** wherein the liquid core comprises a hydrocarbon or perfluorocarbon, optionally at least one of isopentane, perfluoropentane, perfluorohexane, perfluorobutane, and perfluoropropane.

33-34. (canceled)

35. The system of claim **30** comprising an energy source for converting the droplets into microbubbles, optionally wherein the energy source is configured to convert the droplets into microbubbles before or after the solution is delivered to the biological sample.

36. (canceled)

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