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(54) Title: VARIABLE VISCOSITY INKS FOR INKJET DELIVERY OF ENZYME REAGENTS

(57) Abstract: The invention is directed to formulations of terminal deoxynucleotidyl transferases (TdTs) inks employing variable viscosity modifiers and their use in inkjet assisted synthesis of a plurality of polynucleotides at reaction sites on a substrate.

# VARIABLE VISCOSITY INKS FOR INKJET DELIVERY OF ENZYME REAGENTS

**[0001]** Inkjet printing is a low-cost versatile technology for non-contact delivery of defined quantities of liquids to precise locations with minimal wastage. The technology has been applied to synthesis of oligonucleotide microarrays using phosphoramidite chemistry and has been employed to directly print enzymes onto substrates in the production of enzyme-based biosensors In regard to the latter applications of inkjet printing, it has been observed that not only is enzyme activity affected by shear forces and the rheological requirements for droplet formation, but also by the changing enzyme concentration and buffer conditions from evaporative loss when, for example, enzyme-containing fluids are printed to microarrays.

**[0002]** Recently there has been an interest in applying enzyme-based polynucleotide synthesis to problems which are ill-suited for conventional chemically based DNA synthesis, especially because of the mild aqueous reaction conditions of the enzymatic process. It has been observed that a trade-off exists presently between desired viscosity values of inks for printing droplet formation and desired viscosity values for efficient implementation of enzymatic processes after inkjet delivery. That is, ink viscosities optimized for droplet formation are currently suboptimal for enzyme activity and vice versa.

## Summary of the Invention

**[0003]** The present invention is directed to inks comprising variable viscosity modifiers for inkjet delivery of enzymes to reaction sites.

**[0004]** In one aspect, the invention is directed to an inkjet-based method of enzymatically synthesizing a plurality of polynucleotides using such inks, which comprises the steps: (a) providing a substrate having initiators at a plurality of reaction sites, wherein each initiator has a free 3'-hydroxyl and wherein each polynucleotide of the plurality is assigned to a reaction site for synthesis; (b) dispensing through one or more inkjet pumps at least one droplet of at least one synthesis reagent to each reaction site of the plurality to perform a reaction cycle comprising the steps of (i) reacting under elongation conditions the initiator or elongated fragments having free 3'-O-hydroxyls with a 3'-O-protected nucleoside triphosphate and a template-free polymerase so that the initiator or elongated fragments are elongated by incorporation of a 3'-O-protected nucleoside triphosphate to form 3'-O-protected elongated fragments, and (ii) deprotecting the elongated fragments to form elongated fragments having free 3'-hydroxyls, wherein the synthesis reagent comprises a template-free polymerase, a 3'-O-

protected nucleoside triphosphate, a mixture of a template-free polymerase and a 3'-O-protected nucleoside triphosphate, or a deprotection solution; wherein the at least one synthesis reagent comprises a variable viscosity modifier having a first viscosity of greater than 2 mPa.s during formation of the at least one droplet at the one or more inkjet pumps and a second viscosity of less than or equal to 2 mPa.s at each reaction site of the plurality during the reacting step of the reaction cycle; and (c) repeating step (b) until the plurality of polynucleotides is synthesized; wherein the first viscosity and second viscosity are different. In some embodiments, the substrate is a planar substrate.

**[0005]** In another aspect, the invention is directed to a template-free polymerase ink for inkjet printing, comprising: an aqueous solution comprising a template-free polymerase having a concentration of in a range of from 1.0 μM to 30 μM; wherein whenever the ink is printed to a substrate, printed droplets each have a volume in the range of 0.1 pL to 5 nL of the aqueous solution and wherein the ink comprises a variable viscosity modifier having a first viscosity in the range of from 2-20 mPa.s whenever the temperature is in the range of from 5-30°C and a second viscosity in the range of from 1-2 mPa.s whenever the temperature is in the range of from 35-60°C; wherein the first viscosity and second viscosity are not the same.

Brief Description of the Drawings

**[0006]** Fig. 1A contains a schematic representation of an enzymatic synthesis cycle wherein a 3'-O-protected dNTP is added to a nucleic acid strand followed by de-protection.

**[0007]** Fig. 1B illustrates a droplet microarray with a hydrophobic-hydrophilic patterned surface which may be used as a synthesis support.

**[0008]** Figs. 2A-2C illustrate synthesis cycles of four different embodiments of the invention.

**[0009]** Fig. 3 illustrates one embodiment of the invention where the inkjet instrument is used to define reaction sites on an array so to eliminate the problem of aligning the inkjet delivery with pre-formed reaction sites.

**[0010]** Figs. 4A illustrates components of an exemplary inkjet system for use with the invention and 4B illustrates an inkjet instrument for practicing several embodiments of the invention.

**[0011]** Fig. 5 illustrates a technique for assessing the efficiency of inkjet-based enzymatic synthesis which may be used for selecting variable viscosity modifiers.

**[0012]** Fig.6 illustrates the change of viscosity at different temperature for the example.

**[0013]** Fig.7 illustrates the stability of elongation ink stored at different temperatures.

**[0014]** Fig.8 illustrates the results of 10 cycles poly(T) performed with manual EDS.

Detailed description of the invention

**[0015]** While the invention is amenable to various modifications and alternative forms, specifics thereof have been shown by way of example in the drawings and will be described in detail. It should be understood, however, that the intention is not to limit the invention to the particular embodiments described. The intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention. Guidance for selecting materials and components to carry out particular functions may be found in available treatises and references on scientific instrumentation including, but not limited to, Moore et al, Building Scientific Apparatus, Third Edition (Perseus Books, Cambridge, MA); Hermanson, Bioconjugate Techniques, 3rd Edition (Academic Press, 2013); and like references.

**[0016]** In one aspect, the invention is directed to inks for delivering enzyme-containing reagents in droplets formed by an inkjet, and methods using such inks to enzymatically synthesize polynucleotides. Such inks comprise at least one variable viscosity modifier capable of exhibiting a first viscosity at a nozzle of a print head during droplet formation and a second viscosity at a reaction site after delivery for carrying out an enzymatic process. Generally, variable viscosity modifiers exhibit different viscosity values in response to different physical conditions, such as temperature, at a nozzle during droplet formation and at a reaction site when an enzymatic process is implemented after droplet delivery. In some embodiments, a nozzle of a print head is maintained at a lower temperature than that of reaction sites and a variable viscosity modifier is employed which has a viscosity that monotonically decreases with increases in temperature. In other embodiments, a nozzle of a print head is maintained at a higher temperature than that of reaction sites and a variable viscosity modifier is employed which has a viscosity that monotonically increases with increases in temperature. In still other embodiments, a variable viscosity modifier is employed which has a shear-thickening (or dilatant) property so that conditions of droplet formation induce a viscosity in the range of from 2-20 mPa.s and conditions at a reaction site induce a viscosity in the range of 1-2 mPa.s.

**[0017]** Variable modifiers whose viscosity decreases with increases in temperature may comprise conventional viscosity modifiers at selected concentrations and selected temperature ranges. Such conventional viscosity modifiers include, but are not limited to ethylene glycol, polyvinyl alcohol, dimethylsulfoxyde (DMSO), polyethyleneglycol (PEG), polyethyleneglycol

methyl ether (OMe)PEG, polyethyleglycol dimethyl ether $(OMe)_2PEG$, carboxymethyl cellulose, hydroxyethyl cellulose, and the like. . Selected concentrations of the variable viscosity modifier in inks of the invention include, but are not limited to, between 5 to 50 percent in volume of the solution, preferably between 20 to 40 percent in volume of the solution. Selected temperature ranges include, but are not limited to, temperatures at least one inkjet pump of 15 °C or lower, or of 12 °C or lower, or of 10 °C or lower.

**[0018]** Variable viscosity modifiers whose viscosity decreases with increases in temperature may also comprise a thermoreversible polymer, preferentially uncrosslinked, composition incorporating an poly(N-alkyl-substituted-acrylamide), preferentially uncrosslinked, e.g. Sassi et al, Electrophoresis, 17: 1460-1469 (1996); Schild, Prog. Polymer Sci., 17: 163-249 (1992); and the like. For example, variable viscosity properties of poly(N-isopropylacrylamide) polymers may be readily modified by selection of molecular weight, concentration and suitable copolymerization monomers (e.g. SigmaAldrich, Technical Bulletin, "Designing temperature and pH sensitive NIPAM based polymers;" Plate et al, Polymer Journal, 31(1): 21-27 (1999)).

**[0019]** In some embodiments, template-free polymerase inks may be formulated comprising an uncrosslinked poly(N-alkyl-substituted-acrylamide) which has a first viscosity in the range of from 2-20 mPa.s whenever the temperature is in the range of from 5-30°C and a second viscosity in the range of from 1-2 mPa.s whenever the temperature is in the range of from 35-60°C.

**[0020]** Variable viscosity modifiers whose viscosity increases with increases in temperature may comprise a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymer, such as, F127, e.g. Wu et al, Electrophoresis, 19: 231-241 (1998); Rill et al, Proc. Natl. Acad. Sci., 95: 1534-1539 (1998); and the like. Variable viscosity properties of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymers may be readily modified by selection of molecular weight, concentration and suitable copolymerization monomers. In some embodiments, template-free polymerase inks may be formulated comprising an uncrosslinked poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymer which has a first viscosity in the range of from 2-20 mPa.s whenever the temperature is in the range of from 35-60°C and a second viscosity in the range of from 1-2 mPa.s whenever the temperature is in the range of from 5-30°C.

**[0021]** In further embodiments, variable viscosity modifiers may comprise a shear-thickening agent, or a dilatant, including, but not limited to, Rheovis PU 1331, Rheovis PE 1330 (BASF), or the like. Other such variable viscosity modifiers may include poly(N-

isopropylacrylamide). Concentrations and temperatures are selected so that whenever shear force at a nozzle of an inkjet pump is 105 (s-1) or greater, ink viscosity is 3 mPa.s or greater.

**[0022]** In some embodiments, template-free polymerase inks may be formulated comprising a polymer solution that gels upon cooling which has a first viscosity in the range of from 2-20 mPa.s whenever the temperature is in the range of from 5-30°C and a second viscosity in the range of from 1-2 mPa.s whenever the temperature is in the range of from 35-60°C.

**[0023]** In some embodiments, variable viscosity modifier may comprise polymer solutions that gels upon cooling. Such polymer is chosen from the group consisting in hydrophobically modifed polymers having UCST-type phase behavior, natural polymers, deblock copolymer brush grafted silica nanoparticles, poly(PEO-co-styrene) and at least one of their combination. The polymer can be dissolved in an ionic liquid or solutions of PNIPAM microgels and host-guest interactions.

**[0024]** In some embodiments, at least one synthesis reagent solution is glycerol free.

**[0025]** In some embodiments, there is more than one synthesis reagent solution, each solution with a different variable viscosity modifier.

**[0026]** In some embodiments, inks having variable viscosity modifiers may also comprise additional components as described below, including but not limited to conventional viscosity modifiers, surfactants, humectants, aldehyde scavengers, and the like, as described below.

**[0027]** In some embodiments, the invention is directed to methods and compositions for inkjet assisted synthesis of a plurality of polynucleotides each at a distinct reaction site on a substrate using template-free polymerases, such as, terminal deoxynucleotidyl transferases (TdTs). Typically, such synthesis takes place on substrates comprising a planar surface, such as, glass, silica, silicon oxide, plastic, or like surfaces, but it may also take place on other surfaces, such as, for example, biological tissues, or surface-immobilized cDNAs extracted from tissues. As used herein, "inkjet assisted synthesis" means that one or more synthesis reagents are delivered to reaction sites in droplets generated by one or more inkjet pumps.

**[0028]** "Synthesis reagents" include any reagent used in a synthesis cycle to couple a monomer, particularly a 3'-O-protected-nucleoside triphosphate, to an initiator or elongated fragment, such as, buffers comprising a template-free polymerase, buffers comprising 3'-O-protected-nucleotide monomers, buffers comprising a mixture of a template-free polymerase and one or more 3'-O-protected nucleoside triphosphates, deprotection (or deblocking) buffers, and the like. The terms "deprotection" agent, buffer, solution, or the like, are used synonymously herein to the terms "deblocking" agent, buffer, solution, or the like, respectively. Likewise, the term "protected" in reference to compounds, such as, dNTPs, is used

synonymously with the term "blocked" in reference to compounds. As used herein, the term "deprotection solution" (or its equivalent terms) means a reagent that brings about or promotes the removal of a protection group, for example, a 3'-O-protecting group of a nucleotide. As described more fully below, the composition of a deprotection solution (and deprotection reaction conditions) depends on the nature of the protecting group (or blocking group) which is to be removed. In various embodiments, a deprotection solution may contain specific reagents that chemically react with a protection group and/or protected moiety (such as, a reducing agent like TCEP (tris(2-carboxyethyl)phosphine)), enzymes for enzymatic cleavage, scavengers, co-factors, or the like. In some embodiments, a deprotection solution may not contain specific reagents that react with a protection group, but may contain components, e.g. pH buffers, that are compatible with or promote physical cleavage of a protecting group, such as in the case of a photocleavable protecting group. Typically, in a reaction cycle for elongating a polynucleotide fragment, in a deprotecting step a deprotection solution is incubated with 3'-O-protected elongated fragments for a predetermined incubation time. Typical incubation times (i.e. durations of incubating steps) are in the range of from 1 minute to 30 minutes; or in the range of from 3 minutes to 30 minutes; or in the range of from 3 minutes to 15 minutes. Typical elongation reaction temperatures are in the range of from room temperature (RT) to 80°C; or from 20°C to 80°C; or from 20°C to 60°C.

**[0029]** "Synthesis reagents" also include reagents for preparing a substrate for polynucleotide synthesis, such as, reagents for defining reaction sites, initiators, capping reagents, and the like. Usually, a "distinct reaction site" on a substrate is a discrete site in that it is separated from other reaction sites; that is, a discrete site does not have a border with, or overlap with, another reaction site, such as exemplified in Fig. 1B. In other words, a discrete or different reaction site is not contiguous with, or overlapping, other reaction sites. Exceptions to this usual arrangement include "overwriting" embodiments described below for generating high density barcodes on surfaces.

**[0030]** In some embodiments, the plurality of polynucleotides may be in the range of from 2 to 500,000; or in the range of from 100 to 400,000; or in the range of from 100 to 200,000; or in the range of from 100 to 100,000. The plurality of polynucleotides may be the same or different than the plurality of reaction sites. In some embodiments, the plurality of reaction sites may be greater than the plurality of polynucleotides. In some embodiments, the above pluralities of reaction sites each have a density equivalent to that if uniformly deposited on an area equivalent to that of a standard 25 mm x 75 mm microscope slide. In some embodiments, an array of reaction sites formed by uniform deposition may be a rectilinear array; and in other

embodiments, an array of reaction sites formed by uniform deposition may be a hexagonal array.

**[0031]** The basic steps of template-free enzymatic synthesis of polynucleotides is illustrated in Fig. 1A and is described more fully below. Briefly, the synthesis process comprises cycles of steps most involving the delivery to a reaction site at least one of the following reagents: a buffer comprising a template-free polymerase, one or more buffers each comprising one or more 3'-O-protected-dNTPs (i.e. monomers), deprotection buffer, and wash solutions. In various embodiments of the invention, the template-free polymerase buffer, buffers comprising the 3'-O-protected-dNTP monomers, or deprotection buffer may be conveyed to reaction sites by droplets created and delivered by inkjet pumps. To be delivered by inkjet-generated droplets, these reagents must be formulated to meet the rheological requirements for droplet formation. These formulations are referred to as "inks." The key rheological parameters affecting droplet formation are viscosity, density and surface tension, e.g. Derby, Annu. Rev. Mater. Sci., 40: 395-414 (2010); Derby, J. Mater. Chem., 18: 5717-57-21 (2008); Calvert, Chem. Mater., 13: 3299-3305 (2001); Tekin et al, Soft Matter, 4: 703-713 (2008); and like references. Another key parameter relating to droplet volume is the nozzle diameters of the inkjet pumps. In some embodiments, nozzle diameters for use in the present invention may be in the range of from 10 μm to 100 μm. Thus, as described more fully below, one aspect the invention includes reagent inks for template-free enzymatic synthesis of polynucleotides, and in particular, inks comprising a template-free polymerase, particularly, inks comprising a terminal deoxynucleotidyltransferase (TdT) or inks comprising a TdT and one or more 3'-O-protected-dNTP monomers. In some embodiments, inks of the invention may include more than one 3'-O-protected nucleoside triphosphates, in some cases, all four monomer types, for the purpose of synthesizing random sequence segments of polynucleotides, for example, for the creation of polynucleotide tags or barcodes.

**[0032]** In accordance with the invention, inkjet assisted enzymatic synthesis of polynucleotides may be implemented in a variety of embodiments in which different reagents are delivered by inkjet pumps. Some of these embodiments are described in Figs. 2A-2C. Fig. 2A illustrates two cycles in which an ink droplet (or microdrop) comprising a template-free polymerase and a single kind of monomer is delivered to a reaction site enveloped by a buffer droplet on a substrate. The surface of reaction site (204) comprises a layer of initiator oligonucleotides (not shown) and is surrounded by hydrophobic surface of substrate (203), which allows reaction site (204) to be enveloped by a volume (202) of aqueous liquid on surface (203) without spreading or coalescing with liquid from another reaction site. Fig. 2A depicts

the initiators as having a 3'-terminal monomer "A" with a free 3'-hydroxyl (not shown). In Figs 2A-2C, monomers A, B and C are each meant to represent any 3'-O-protected-dNTP monomer. To volume (202) at reaction site (204) (or directly to reaction site (204) if a drying step is implemented), ink droplet (201) is dispensed via an inkjet pump (not shown). Ink droplet (201) comprises a predetermined concentration of a template-free polymerase and a predetermined concentration of monomer B, in addition to salts and buffer components for polymerase activity and viscosity modifiers and surface tension modifiers as needed to meet the rheological requirements for droplet formation. Droplet (201) may also include humectants to minimize evaporation loss. In some embodiments, droplet (201) may further include an aldehyde scavenger whenever 3'-O-amino-NTPs are employed. Droplet (201) is deposited on dried reaction site (207) or coalesces with volume (202) on an undried reaction site (221) to form reaction mixture (206) in droplet (205) which is allowed to incubate (208) for a predetermined time to permit coupling of B monomers to the 3' ends of the initiators (or previously extended or elongated strands after the initial cycle). In some embodiments, such incubation takes place at a higher than ambient humidity to prevent drying during the incubation step.

**[0033]** In some embodiments, a separate step of drying reaction sites may be implemented to prevent fluid accumulation and/or coalescence with reaction mixtures at adjacent reaction sites.

**[0034]** After the incubation time for the coupling reaction has elapsed, the entire substrate surface is immersed or sprayed (209) in deprotection buffer (210) for a predetermined time to permit removal of a protection group, which regenerates free 3'-hydroxyls at the ends of the elongated strands. After the predetermined deprotection time has elapsed, the entire substrate surface is immersed one or more times in one or more wash buffers for predetermined times to give reaction sites (211) with extended or elongated strands or fragments, shown as "—AB", which are ready for the next coupling cycle.

**[0035]** In some embodiments, as mentioned above, a drying step may be implemented after deprotection and washing (213) in order to minimize the chance of droplet (211) spreading or coalescing with adjacent droplets. Conventional drying techniques in inkjet printing may be used, warm air or gas, radiative drying, or the like, e.g. Hoynant et al, U.S. patent 8485096.

**[0036]** The steps of the next cycle are the same as that for attaching the B monomer, except that in the next cycle a C monomer is coupled. Thus, droplet (212) having the same composition as droplet (201), except possibly for the identity of the C monomer, is dispensed to volume (211). As above, reaction mixture (214) in droplet (215) is incubated for a predetermined

amount of time, after which the entire substrate is treated (218) with deprotection buffer and wash solution (210) to give reaction sites (220) having extended strands, illustrated as "—ABC".

**[0037]** Depending on the nature of substrate (203) in this and other embodiments, a coupling cycle may also include a drying step so as to prevent droplet spreading and coalescence between adjacent reaction sites. If the surface of substrate (203) between reaction sites (204) is sufficiently hydrophobic then the possibility of such coalescence is minimized.

**[0038]** An embodiment of Fig. 2A may be carried out by the following steps: (a) providing a substrate having initiators at a plurality of distinct reaction sites, wherein each initiator has a free 3'-hydroxyl and wherein each polynucleotide of the plurality is assigned to a different reaction site for synthesis; (b) dispensing to each reaction site through one or more inkjet pumps at least one droplet a buffer solution comprising a mixture of a template-free polymerase and a 3'-O-blocked-dATP, a 3'-O-blocked-dCTP, a 3'-O-blocked-dGTP, or a 3'-O-blocked-dTTP, wherein the kind of 3'-O-blocked dNTP dispensed to a reaction site depends on the predetermined sequence of the polynucleotide assigned to the reaction site; (c) incubating the template-free polymerase and 3'-O-blocked-dNTPs at each reaction site so that initiators or elongated fragments at the reaction site are elongated by incorporation of a 3'-O-blocked dNTP to form 3'-O-blocked elongated fragments; (d) deblocking the elongated fragments at each reaction site to form elongated fragments having free 3'-hydroxyls by treating the planar support with a deblocking agent; (e) repeating steps (b), (c), and (d) until the plurality of polynucleotides is synthesized. In some embodiments, a plurality of droplets are delivered to each reaction site during each cycle of steps (b), (c) and (d). In some embodiments, the plurality of droplets is in the range of from 2 to 10, or in the range of from 2 to 5, or in the range of from 2 to 3. In other embodiments, the plurality of droplets may be in the range of from 2 to 150, or in the range of from 10 to 120. In some embodiments, a further step is implemented of cleaving the plurality of polynucleotides from the substrate.

**[0039]** In some embodiments, the substrate is a planar substrate.

**[0040]** In some embodiments, a drying step may be included after step (d), or after step (d) and a washing step, to minimize spreading or coalescence of droplets when the next droplet is dispensed.

**[0041]** Fig. 2B illustrates an embodiment in which monomer and template-free polymerase are delivered to reaction sites in separate inkjet-delivered droplets. As above, the surface of reaction site (204) comprises a layer of initiators terminating with an A monomer illustrated as "—A." To volume (202) is dispensed (250) droplet (252) comprising a template-free

polymerase, after which droplet (254) comprising B monomer is dispensed (256). In some embodiments, the order of dispensing template-free polymerase and monomer may be reversed, so that within a cycle the monomer is dispensed before the template-free polymerase is dispensed. After incubation of reaction volume (265) for a predetermined time to permit the coupling of B monomer to the initiators or extended strands at reaction site (204), the entire surface of the substrate is exposed (268) to deprotection buffer (267) and subsequently to one or more wash buffers to give reaction site (268) with extended strands "—AB." The next cycle follows in the same manner, except C monomer is added, namely: droplet comprising template-free polymerase is dispensed (270), droplet comprising C monomer is dispensed (272) to form reaction mixture (273), incubate reaction mixture (273), deprotect and wash (276) to give reaction site (278) with extended strands "—ABC."

**[0042]** An embodiment of Fig. 2B may be carried out by the following steps: (a) providing a substrate having initiators at a plurality of distinct reaction sites, wherein each initiator has a free 3'-hydroxyl and wherein each polynucleotide of the plurality is assigned to a different reaction site for synthesis; (b) dispensing to each reaction site through one or more inkjet pumps at least one droplet a buffer solution comprising a template-free polymerase; (c) dispensing to each reaction site through one or more inkjet pumps at least one droplet a buffer solution comprising a 3'-O-blocked-dATP, a 3'-O-blocked-dCTP, a 3'-O-blocked-dGTP, or a 3'-O-blocked-dTTP, wherein the kind of 3'-O-blocked dNTP dispensed to a reaction site depends on the predetermined sequence of the polynucleotide assigned to the reaction site; (d) incubating the template-free polymerase and 3'-O-blocked-dNTPs at each reaction site so that initiators or elongated fragments at the reaction site are elongated by incorporation of a 3'-O-blocked dNTP to form 3'-O-blocked elongated fragments; (e) deblocking the elongated fragments at each reaction site to form elongated fragments having free 3'-hydroxyls by treating the planar support with a deblocking agent; (f) repeating steps (b), (c), (d) and (e) until the plurality of polynucleotides is synthesized. In some embodiments, a plurality of droplets are delivered to each reaction site during each cycle of steps (b) and (c). In some embodiments, such plurality of droplet may comprise only buffer comprising template-free polymerase, or only buffer comprising a 3'-O-blocked nucleoside triphosphate, or a combination of both. In some embodiments, the plurality of droplets is in the range of from 2 to 10, or in the range of from 2 to 5, or in the range of from 2 to 3. In other embodiments, the plurality of droplets may be in the range of from 2 to 150, or in the range of from 10 to 120. In some embodiments, a further step is implemented of cleaving the plurality of polynucleotides from the substrate.

**[0043]** In some embodiments, the substrate is a planar substrate.

**[0044]** In some embodiments, prior to step (b) a step of drying each reaction site may be implemented, and prior to step (c) a step of drying each reaction site may be implemented.

**[0045]** Fig. 2C illustrates an embodiment in which multiple droplets of buffer containing template-free polymerase and monomer is delivered to a reaction site while a coupling reaction is taking place in a droplet at the reaction site. A significant problem related to carrying out reactions in droplets at a reaction site is fluid loss from evaporation. Such loss changes the concentrations of enzyme, salts, as well as components such as viscosity modifiers, surfactants, and the like, all of which may affect enzyme activity. Furthermore, in some embodiments, the concentration of template-free polymerase in a droplet delivered by an inkjet may have to be lower than that optimal for coupling reactions because at higher concentrations the polymerase increases the viscosity of the ink such that it negatively impacts droplet formation; thus, to be able to deliver by inkjet a sufficient concentration of polymerase two or more droplets of low concentration polymerase are delivered to a reaction site so that the concentration at the reaction site increases in the course of a coupling reaction by such repeated additions while at the same time buffer at the reaction site is continuously evaporating. Fig. 2C illustrates the steps of a single reaction cycle in which two droplets of a buffer comprising a template-free polymerase and a monomer are delivered to a reaction site. In some embodiments, a plurality of droplet containing such reagents may be delivered to each reaction site during synthesis. The number of such droplet deliveries is a design choice depending on factors, such as, reaction site droplet size, delivery droplet size, relative humidity, whether humectants are components of the inks, the duration of the coupling reaction, whether different print heads are used for different reagents, and the like. In some embodiments, reagents, such as, surfactants, viscosity modifiers, detergents, humectants, and the like, to alter droplets on the substrate can be delivered in separate droplets generated by separate inkjet pumps in the inkjet print head. The operation of this embodiment is similar to that of Fig. 2A, except that two or more droplets are delivered to the reaction mixture. As above, the surface of reaction site (204a, containing droplet (202), or 204b, containing no droplet) comprises a layer of initiators terminating with an A monomer illustrated as "—A." To droplet (202) is delivered a first droplet of a buffer comprising a template-free polymerase and B monomer to form droplet (282) of reaction mixture at reaction site (204).

**[0046]** Again, in some embodiments employing a drying step, instead of droplet (202) at reaction site (204), droplet (283) would be delivered directly to reaction site (204b) to form reaction droplet (282).

**[0047]** Reaction droplet (282) continuously loses water by evaporation (285) during incubation (286), which reduces its volume and increases the concentration of all the non-volatile components of the droplet, particularly that of the template-free polymerase, thereby improving coupling activity in reaction droplet (282). Coupling activity is further improved by delivering a second droplet of a buffer comprising template-free polymerase and B monomer, either separately or as a mixture. Although at the initial coalescence of delivery droplet (287) and reaction droplet (282) the concentration of polymerase is reduced, the on-going evaporation rapidly increases the concentration of polymerase in reaction droplet (282) so that it approaches a desired value. After a plurality of droplets are delivered containing buffer with polymerase and monomer, the entire surface of the substrate is immersed with deprotection buffer (288) and one or more wash solutions to give reaction site (294) with extended strands "—AB". The same procedure of delivering pluralities of droplets of reagent solutions to address problems raised by evaporation and the rheological constraints of droplet formation also can be applied to the embodiments of Figs. 2B and 2C.

**[0048]** In some embodiments of Figs. 2A-2C, coupling cycles may further include a washing step after the deprotecting step. In some such embodiments, coupling cycles may further include a drying step after a washing step. As described above, a drying step prior to a successive coupling cycle would prevent the spreading and possible coalescence of reaction droplets at adjacent reaction sites. In some embodiments, washing and drying can be combined by using a volatile wash solution, such as, acetonitrile, methanol, or the like, which is readily evaporated between coupling cycles.

**[0049]** As noted above, embodiments of the method of the invention may include one or more washing steps wherein a wash solution is flowed or sprayed on a substrate comprising an array of reaction sites. Wash solution may comprise a variety of solvents including, but not limited to, water, acetonitrile, methanol, PBS or other buffered salt solutions, or the like. In some embodiments, a wash solution may include one or more proteases, e.g. proteinase K, for the purpose of removing any polymerases that may adhere to the reaction site. That is, the embodiments of Figs. 2A-2C may further include a step of treating reaction sites with one or more proteases to remove or deactivate polymerase that may accumulate at the reaction sites.

**[0050]** Although Figs. 2A-2C show substrates with reaction sites continuously enveloped by, or occupied by, a droplet, this is not a requirement of all embodiments of the invention. In some embodiments substrates with reaction sites may be dried between cycles of steps so that, strictly speaking, the substrate is not always, or not continuously, a droplet microarray throughout a synthesis.

**[0051]** In some embodiments, including those described above, the plurality of polynucleotides enzymatically synthesized (that is, the number of reaction sites) on a substrate with inkjet delivery of reagents is in the range of from 100 to 2 million, or in the range of from 100 to 1 million, or in the range of from 100 to 100 thousand, or in the range of from 100 to 500 thousand, or in the range of from 1000 to 1 million. In some embodiments, such pluralities are synthesized on a substrate having a surface area in the range of from 1 to 500 $cm^2$, or from 1 to 256 $cm^2$, 1 to 30 $cm^2$, or having a surface area in the range of from 1 to 15 $cm^2$, or having a surface area in the range of from 1 to 7 $cm^2$, or having a surface area in the range of from 7 to 20 $cm^2$. In some embodiments, substrates may be prepare and undergo surface treatment after which it is cut, or diced, into smaller pieces for use. In some embodiments, the lengths of the polynucleotides synthesized in accordance with the invention are in the range of from 10 to 500 nucleotides, or in the range of from 50 to 500 nucleotides, or in the range of from 100 to 400 nucleotides, or in the range of from 100 to 500 nucleotides. In some embodiments, the per cycle coupling efficiency in the synthesis of polynucleotides in these length ranges is at least 98%, or is at least 99%, or is at least 99.5%, or is at least 99.8%, or is at least 99.9%. In some embodiments, the coupling cycle time in the synthesis of polynucleotides in these length ranges is less than 15 min per cycle, or less than 10 min per cycle, or less than 7 min per cycle, or less than 5 min per cycle.

**[0052]** In some embodiments, inkjet delivery of droplets may be directed to features on a substrate which have a dimension directly related to its size or area, such as a width of a square reaction site or a diameter of a round reaction site. Thus, in some embodiments, reaction sites have a width or diameter in the range from about 10 μm to about 1.0 cm. In some embodiments droplets can be deposited to reaction sites whose widths, or diameters, are in the range of from about 1.0 μm to about 1.0 mm, usually about 5.0 μm to 500 μm, more usually about 10 μm to 200 μm, and still more usually from about 20 μm to about 100 μm.

**[0053]** In some embodiments, the volume of reagent ink delivered to a reaction site is in the range of 0.1 to 1000 pL, or in the range of from 0.5 to 500 pL, or in the range of from 1.0 to 250 pL, or in the range of from 1.0 to 100 pL, or in the range of from 2 to 50 pL. In some embodiments, reagent ink is delivered to each reaction site in a predetermined number of droplets, or "pulses," generated by a print head wherein, for example, each pulse has about a 2.4 picoliter volume.

Apparatus for Inkjet Synthesis

**[0054]** Delivering fluids by inkjets is a mature technology that has been available for several decades so that extensive literature is available describing it and providing guidance for adapting it to novel applications, as in the present invention. Exemplary references providing guidance for constructing inkjet delivery systems: Lausted et al, Genome Biology, 5: R58 (2004); Le, Recent Progress in Ink Jet Technologies II, chapter 1, pgs. 1-14 (1999); Derby (2010, cited above); Zapka, editor, "Handbook of Industrial Inkjet Printing," (Wiley-VCH, Weinheim, Germany); U.S. patents 5474796; 10384189; 10669304; 6306; 6323043; 5847105; and the like. As noted by Le (1999) inkjet pumps may be classified as "continuous" and "drop-on-demand" (DOD). In some embodiments, DOD inkjet pumps are employed with apparatus of the invention, and in particular, of the various DOD inkjets, piezoelectric inkjet pumps are of interest. For example, droplet formation in DOD inkjets is described in Dong et al, Physics of Fluids, 18: 072102 (2006). Such varieties of inkjet pumps are available banks or assemblies of large numbers of inkjets (e.g. from 10's to 100's) that may be individually programmed for actuation and delivery of droplets. Such inkjets and inkjet assemblies (referred to herein as "inkjet heads") are commercially available from many manufacturers including Epson, Xaar, Fujifilm, and the like. As used herein, "inkjet pump" means a device capable of generating and ejecting droplets of a fluid. In some embodiments, an inkjet pump is a device capable of generating and ejecting droplets of a fluid at a predetermined rate and of a predetermined uniform size. In some embodiments, an inkjet pump is capable of ejecting droplets each having approximately the same size in the range of from 0.1 pL to 5.0 nL, or the same size in the range of from 0.5 pL to 1.0 nL. In some embodiments, an inkjet pump is capable of ejecting droplets at a rate in the range of from 1 to 100 kilohertz.

**[0055]** In some embodiments, components of an inkjet apparatus of the invention may be arranged according to whether they may be moved relative to one another or whether they are fixed, as illustrated in Fig. 4A by components (602) and (600), respectively. Computer and software (604) provide overall control of the system components, either directly or indirectly via controllers. For example, software may provide for single pass reagent deposition in which print head (618) is stationary and synthesis support holder (620) moves to deliver reagents to reaction sites. Alternatively, different software may provide for one or more moving print heads (618) and/or moving synthesis support holder (620) via a variety of components, such as, print controller (606), print head driver (612) and motion controller (610). Typically, computer and software (604) control capping station (622), flush station (624), wiper (626), inspection system (628) and washing and possibly drying functions (630), if any.

**[0056]** Capping station (622) keeps the print head moist and stops drying of ink. Flush station (624) primes and flushes the print head, which helps remove trapped air and debris as well as dried ink. Wiper (626) is used to remove excess ink and prevent cross-contamination. It may be part of the flush station. Inspection system (628) records the presence, absence or size of spots of deposited reagents or incorrectly placed spots of reagents. Inspection system (628) may comprise a camera that takes images of the synthesis support and an image analysis software that extracts process information from the images. Such information may be used in real-time to optimize synthesis or to implement corrective measures. Washing and drying functions (630) are carried out by a fluid delivery system separate from that used for droplet delivery. Washing may include deprotection steps wherein a deprotection reagent is flowed across a synthesis substrate, optionally followed by a drying step. Drying may be accomplished by blowing air or an inert gas, such as argon, over the synthesis support, or by using a volatile solvent, such as methanol, in the washing step.

**[0057]** In some embodiments, cameras or microscopes may be used to capture images of the spots (i.e. reaction sites) and identify missing spots, determine spot size and spot placement. Lighting for image capture may be from above, from the side, from below or integrated into a substrate holder, whichever gives the best contrast in the absence or presence of dye in the inks. Where a dye is used (as described below), it is selected so that it would not interfere with the enzymatic reaction, would not react with the protecting group of the nucleotide and would be compatible with the enzyme and deprotection buffers. In some embodiments, each monomer would have a different distinguishable dye, covering a different part of the visible spectrum. In some embodiments, imaging of an array of reaction site is carried out during incubation (30 s - 10 min) of the elongation reactions and using high enough magnification to see individual spots but not so high that an inordinate length of time would be needed to scan the array. The number of images taken in an imaging step may be 20 to 100 for a standard microscope slide. Images may be captured seamlessly in a video stream by scanning the substrate or captured in a move-stop process. The images captured may be stitched using algorithms and aided by the presence of fiducial markings on the slide. Fiducial markings also help determine whether the slide has moved in the slide holder and help determine spot positions. In some embodiments, real time image analysis allowing the identification of missing spots or poor spot placement could be accompanied by the automatic generation of a new image and an additional print or prints.

**[0058]** An exemplary inkjet apparatus for implementing various embodiments of the invention (e.g. those of Figs 2A-2C) is diagrammatically illustrated in Fig. 4B. A plurality of DOD inkjets are housed in print head (680) which is capable of x-y and z movement relative to

droplet microarray (657). In some embodiments both print head (680) and droplet microarray (657) are capable of x-y movement. In some embodiments, print head (680) is held in a fixed position and droplet array (657) undergoes x-y movement. In this example, "dATP Reagent," "dCTP Reagent," "dGTP Reagent," and "dTTP Reagent," (696) are each buffer formulations, or inks, comprising template-free polymerase, respective 3'-O-protected-dNTPs, salts and cofactors necessary or useful for polymerase activity, as well as viscosity and surface tension modifiers, humectants, and the like, as needed to meet the requirements for desired droplet formation and/or to reduce evaporation loss. Print head (680) includes temperature regulation to maintain the inks at a temperature optimized for delivery and activity. In this embodiment, reagents flowed or delivered to the droplet microarray in bulk are deprotection solution (or buffer) (695) and wash solution (661). Droplet microarray (657), which is formed on substrate (655), sits or is mounted in flow chamber (677) which comprises inlet (652) and outlet (653). Flow chamber (677) defines the flow path of reagents (not delivered by print head (680)) over droplet microarray (657). Such reagents may flow continuously over droplet microarray (657) or reagents may be delivered to flow chamber (677) where they remain for a predetermined incubation time, and then are removed or recycled. Such reagents may be moved by conventional pumps or by pressure heads over reagent reservoirs. Flow chamber (677) includes temperature control elements (not shown) and humidity control elements (not shown) to maintain, or optimize, coupling reaction activity. After exiting, reagents are discarded into a waste container (656) or recycled. Timing of inkjet discharges, positioning of print head (680), actuation of valves (675) and (674) are controlled by fluidics/inkjet controller (665), which may include imaging software that performs analysis of array images obtained by camera (697) and that causes alterations of reagent deposition, for example, when coalescing reaction sites are detected. In some embodiments, print head (680) may be driven by electronics available from Meteor (Meteor Inkjet Ltd, (Cambridge, UK). For example, a Print Controller Card (PCC) synchronizes to the encoder signal from a Thorlabs motion controller. A Head Driver Card (HDC) provides power and a waveform to the printhead. The drive electronics are controlled by Meteor's digital printing front end, which includes MetDrop and MetWave software for optimization of spotting parameters, with printing initiated by the Thorlabs Kinesis software. Overall instrument control can be performed by instrument software, such as LabView.

**[0059]** Typically, the distance between the inkjet nozzles and the substrate surface may be in the range of from about 10 μm to 10 mm, or in the range of from about 100 μm to 2 mm, or in the range of from about 200 μm to 1 mm, or in the range of from 500 μm to 3 mm. Droplet velocities may be in the range 1–10 meters/sec. Print head movement may be in the range of

from 1-30 cm/sec, or 5-30 cm/sec, or 20-30 cm/sec. As described more fully below, print heads may have different droplet delivery modes, for example, single-pass mode, multiple pass mode, and move-stop mode.

**[0060]** As mentioned above, in some embodiments, nozzle diameters for use with the invention may be in the range of from 10 μm to 100 μm. In other embodiments, inkjet nozzle size may be in the range of from 20-30 μm for generating droplet sizes in the range of from 10-20 pL. In some embodiments, nozzle diameter, synthesis reagent density, surface tension and viscosity are selected to dispense droplets to reaction sites having a volume in the range of from 2 pL to 5 nL, or in the range of from 2 pL to 1 nL, or in the range from 2 pL to 500 pL, or in the range from 2 pL to 100pL. In some embodiments, inkjet pumps are DOD inket pump and have a droplet generation rate in the range of from 1 to 100 kHz.

**[0061]** In some embodiments, inkjet-based synthesizers include droplet detection components to monitor and record any anomalies in droplet formation and delivery by the inkjet nozzles. In some embodiments, such droplet monitoring may comprise a laser diode mounted orthogonally to the direction of print-head motion such that the droplet stream of each bank of nozzles intersects the beam, causing the light to scatter if a droplet is present. Before each round of printing, nozzles may be fired in series through the beam and the forward scattering of each droplet is detected by a photodiode. Nozzles failing to fire may be taken off-line during synthesis. Apparatus of the invention may also be equipped with commercially available droplet monitors, such as, a Meteor dropwatcher, available from Meteor Inkjet Ltd, (Cambridge, UK) as well as a camera to image the solid support and array of reaction sites. The latter permits the array of reaction sites to be monitored to detect accuracy in droplet deposition, size and geometry of reaction sites, coalescence of reaction sites, and the like. In some embodiments, software may be provided to provide a full image of an array on a slide or solid support by patching together tiles comprising smaller images, e.g. S. Preibisch, S. Saalfeld, P. Tomancak, Bioinformatics, 2009, 25(11), 1463-1465.

**[0062]** In certain embodiments, it may be desirable to prevent evaporation of the synthesis reagents and reaction mixtures following deposition. Evaporation may be prevented in a number of different ways. In some embodiments, synthesis cycles may be carried out in a high humidity environment, such as a relative humidity in the range of from 75-85%. Alternatively or in addition to, one may employ reagents with an evaporation retarding agent or humectant, e.g. glycerol, polyethylene glycol, carboxymethyl cellulose, hydroxyethyl cellulose, and the like.

**[0063]** In some embodiments, recirculating ink print heads are employed because problems of drying and/or clogging of nozzles by enzymes is reduced. Recirculating ink print heads are commercially available, for example, from Fujifilm and are described in U.S. patents 8820899; 8534807; 8752946; 9144993; 9511598; 9457579, which are incorporated herein by reference.

Synthesis Substrates

**[0064]** In some embodiments, substrates for synthesis comprise surfaces that have been patterned with hydrophobic and hydrophilic regions wherein discrete hydrophilic reaction sites are formed. These allow the formation of droplets on hydrophilic reaction sites, for example, after flowing aqueous reagents or reactants of the entire surface. That is, in some embodiments, substrates for synthesis comprise so-called "droplet microarrays," e.g. as disclosed in the following exemplary references, which are incorporated by reference: Brennan, U.S. patent 5474796; Chrisey et al, Nucleic Acids Research, 24(15): 3040-3047 (1996); Fixe et al, Materials Research Society Symposium Proceedings. Volume 723, Molecularly Imprinted Materials - Sensors and Other Devices. Symposia (San Francisco, California on April 2-5, 2002); Goldfarb, U.S. patent publication 2008/0166667; Gopinath et al, ACS Nano, 8(12): 12030-12040 (2014); Hong et al, Microfluid. Nanofluid., 10: 991-997 (2011); Kumar et al, Nucleic Acids Research, 28(14): e71 (2000); Peck et al, U.S. patent 10384189; Indermuhle et al, U.S. patent 10669304; Wu et al, Thin Solid Films, 515: 4203-4208 (2007); Zhang et al, J. Phys. Chem., 111: 14521-14529 (2007): and like references. As used herein, the term "droplet microarray" refers to a substrate, preferentially a planar substrate, whose surface has been treated to create a plurality of discrete hydrophilic regions, which may serve as reaction sites either directly or with further treatment, e.g. attaching initiators. In some embodiments, each of the plurality of discrete hydrophilic regions are surrounded by hydrophobic regions. The discrete hydrophilic regions may have a variety of shapes, but are usually circular or rectangular or square for manufacturing convenience. In some embodiments, reaction sites have areas and capacities to hold an aqueous reaction mixture as described above. Although synthesis substrates of some embodiments may comprise droplet microarrays, in a synthesis process such arrays may undergo a drying step which removes liquid from reaction sites. That is, in some embodiments, a synthesis substrate comprising a droplet microarray may be devoid of droplets from time to time, for example, after an elongation cycle ending in a drying step. The hydrophilic-hydrophobic configurations permit the formation of droplets on the surface of a droplet microarray either after inkjet delivery of a synthesis reagent to the

hydrophilic regions or by flowing a "bulk" aqueous solution, such as a synthesis reagent or wash solution, over the substrate. As disclosed in the above references, the droplets retained by the hydrophilic regions may serve as reaction chambers or vessels. Such a process is illustrated in Fig. 1B. Substrate (150), which is a planar substrate, has a surface with hydrophobic region (152) and discrete hydrophilic regions (154) which may serve as reaction sites. When substrate (150) is flooded (156) with aqueous solution (158) both hydrophobic regions (152) and hydrophilic regions (154) are immersed. When aqueous solution (158) drains off (160) some of the aqueous solution is retained by hydrophilic regions (154) to form droplets (162) of droplet microarray (164). Individual droplets, such as (162), may be referred to as "microarray droplet" to distinguish them from droplets formed by an inkjet pump prior to its delivery to a reaction site, such as (162).

**[0065]** Preparation of substrates with discrete reaction sites can be accomplished by known methods. For example, such methods can involve the creation of hydrophilic reaction sites by first applying a protectant, or resist, over selected areas over the surface of a substrate, such as a silicon oxide, or like material. The unprotected areas are then coated with a hydrophobic agent to yield an unreactive surface. For example, a hydrophobic coating can be created by chemical vapor deposition of (tridecafluorotetrahydrooctyl)-triethoxysilane onto the exposed oxide surrounding the protected circles. Finally, the protectant, or resist, is removed exposing the well regions of the array for further modification and nucleoside synthesis using the high surface tension solvents described herein and procedures known in the art such as those described by Maskos & Southern, Nucl. Acids Res. 20:1679-1684 (1992). Alternatively, the entire surface of a glass plate substrate can be coated with hydrophobic material, such as 3-(1,1-dihydroperfluoroctyloxy)propyltriethoxysilane, which is ablated at desired loci to expose the underlying silicon dioxide glass. The substrate is then coated with glycidyloxypropyl trimethoxysilane, which reacts only with the glass, and which is subsequently "treated" with hexaethylene glycol and sulfuric acid to form an hydroxyl group-bearing linker upon which chemical species can be synthesized (Brennan, U.S. Pat. No. 5,474,796). Arrays produced in such a manner can localize small volumes of solvent within the reaction site by virtue of surface tension effects (Lopez et al., Science 260:647-649 (1993)).

**[0066]** In some embodiments, reaction sites may be formed on a substrate following the photolithographic methods of Brennan, U.S. patent 5474796; Peck et al, U.S. patent 10384189; Indermuhle et al, U.S. patent 10669304; Fixe et al (cited above); or like references cited above. In accordance with these methods, a set of hydrophilic molecules comprising an aminosilane is attached to the surface of a substrate to form reaction sites. Such hydrophilic molecules may

comprise N-(3-triethoxysilylpropyl)-4-hydroxybutyramide (HAPS), 11-acetoxyundecyltriethoxysilane, n-decyltriethoxysilane, (3-aminopropyl)trimethoxysilane, (3-aminopropyl)triethoxysilane, 3-glycidoxypropyltrimethoxysilane (GOPS), or 3-iodo-propyltrimethoxysilane. A set of hydrophobic molecules comprising a fluorosilane is attached to the surface of the substrate in regions outside of the reaction sites. Such hydrophobic molecules may comprise perfluorooctyltrichlorosilane octylchlorosilane, octadecyltrichlorosilane, (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane, or tridecafluoro-1,1,2,2-tetrahydrooctyl)trimethoxysilane. After such attachment, a substrate is prepared for polynucleotide synthesis by coupling initiators to the aminosilanes at the reaction sites. Such coupling may be accomplished using any number of available homo- or heterobifunctional linkers to form covalent bonds between amino groups on the substrate and 5'-thiol groups or 5'-amino groups on the initiators. Such linkers are, for example, available from Sigma-Aldrich (St. Louis, MO) and are described in treatises such as, Hermanson, Bioconjugate Techniques, 3rd Edition (Academic Press, 2013). Synthesis of polynucleotides having 5'-thiol or 5'-amino groups is well-known and is described in Kupihar et al, Nucleosides Nucleotides & Nucleic Acids, 22(5-8): 1297-1299 (2003); Fung et al, U.S. patent 4757141; and like references.

**[0067]** In some embodiments, an array of reaction sites may be formed using click chemistry by depositing under coupling conditions droplets of 5'-DBCO (dibenzocyclooctyl) labeled initiators (e.g. Glen Reseach) on a substrate, preferentially a planar substrate, comprising an azide layer (e.g. PolyAn 2D azide glass slide). In some embodiments, such reactions may be carried out as a copper-free click reaction which is less damaging to the DNA, e.g. Dommerholt et al, Top. Curr. Chem. (Z) 374: 16 (2016).

**[0068]** A wide variety of substrates may be employed for creating arrays of reaction sites for enzymatic synthesis of polynucleotides. Substrates may be a rigid material including, without limitation, glass; fused silica; silicon such as silicon dioxide or silicon nitride; metals such as gold or platinum; plastics such as polytetrafluoroethylene, polypropylene, polystyrene, polycarbonate, and any combination thereof. A rigid surface can be fabricated from a material selected from the group consisting of silicon, polystyrene, agarose, dextran, cellulosic polymers, polyacrylamides, polydimethylsiloxane (PDMS), and glass. Substrates may also comprise flexible materials, which is capable of being bent, folded or similarly manipulated without breakage. Exemplary flexible materials include, without limitation, nylon (unmodified nylon, modified nylon, clear nylon), nitrocellulose, polypropylene, polycarbonate, polyethylene, polyurethane, polystyrene, acetal, acrylic, acrylonitrile, butadiene styrene (ABS),

polyester films such as polyethylene terephthalate, polymethyl methacrylate or other acrylics, polyvinyl chloride or other vinyl resin, transparent PVC foil, transparent foil for printers, Poly(methyl methacrylate) (PMMA), methacrylate copolymers, styrenic polymers, high refractive index polymers, fluorine-containing polymers, polyethersulfone, polyimides containing an alicyclic structure, rubber, fabric, metal foils, and any combination thereof.

**[0069]** In some embodiments, patterned surfaces of superhydrophobic and superhydrophilic regions may be formed on a substrate. Guidance for forming droplet microarrays with such patterned surfaces are described in the following references, which are incorporated by reference: Feng et al, Adv. Mater. Interfaces, 1400269 (2014); Zhan et al, Trends Anal. Chem., 108: 183-194 (2018); Neto et al, Adv. Functional Mater., 201400503 (2014);

**[0070]** Achieving accurate alignment of droplet delivery to reaction sites of a prefabricated droplet microarray is an important aspect of inkjet-assisted synthesis of polynucleotides. In some embodiments, such alignment tasks may be minimized or avoided by creating immediately prior to synthesis an array of reaction sites by depositing droplets of synthesis reagents onto a layer of initiator oligonucleotides on a substrate in order to define the locations of reaction sites. Following this initial deposit of droplets, the initiator layer outside of the droplet-defined sites are treated to render them inert to subsequent extension or to render them inert to extension as well as hydrophobic. After such an initial surface treatment to create reaction sites, further or subsequent inkjet delivery of droplets to the same reaction sites will be accurate because the same inkjet head and pumps that were used to define the locations of the reaction sites will be used to deliver subsequent droplets during synthesis of the polynucleotides. In some embodiments, the synthesis reagents delivered to the initiator layer comprise a mixture of a template-free polymerase and a 3'-O-protected-dNTP. These reagents extend the initiators to define reaction sites or regions on the oligonucleotide layer which is populated by extended fragments having 3'-O-protected ends. The areas outside of these regions are then treated to render them inert to extensions. In some embodiments, after the initial coupling step defining reaction sites, the entire substrate is exposed to a template-free polymerase and a terminator, such as a dideoxynucleoside triphosphate (ddNTP), or like reagent. In some embodiments, such ddNTP could be, for example, a ddNTP conjugated to a hydrophobic moiety, thereby rendering the coating outside of the reaction sites hydrophobic. Such a hydrophobic moiety may be, for example, a dye or quencher molecule, such as, a Black Hole Quencher® molecule. A variety of terminators may be employed for this purpose. In particular, terminators include nucleoside triphosphates that lack a 3'-hydroxyl substituent and

include 2',3'-dideoxyribose, 2',3'-didehydroribose, and 2',3'-dideoxy-3'-haloribose, e.g. 3'-deoxy-3'-fluoro-ribose or 2',3'-dideoxy-3'-fluororibose nucleosides. Alternatively, a ribofuranose analog can be used in terminators, such as 2',3'-dideoxy-β-D-ribofuranosyl, β-D-arabinofuranosyl, 3'-deoxy-β-D-arabinofuranosyl, or the like. Further terminators are disclosed in the following references: Chidgeavadze et al., Nucleic Acids Res., 12: 1671-1686 (1984); Chidgeavadze et al., FEBS Lett., 183: 275-278 (1985); Izuta et al, Nucleosides & Nucleotides, 15: 683-692 (1996); and Krayevsky et al, Nucleosides & Nucleotides, 7: 613-617 (1988). Nucleotide terminators also include reversible nucleotide terminators, e.g. Metzker et al. Nucleic Acids Res., 22(20):4259 (1994).

**[0071]** Thus, in such embodiments, a starting material for a synthesis operation is a surface coated with a layer of initiator oligonucleotides. An exemplary fabrication of reaction site on such starting material is illustrated in Fig. 3. Substrate (400) (e.g. a glass slide) has a layer (402) of initiator oligonucleotides that have free 3'-hydroxyl groups and that are attached by their 5'-ends to the substrate. In some embodiments, initiator densities may be, for example, in the range of from $10^{11}$ to $10^{13}$ strands/$cm^2$. Inkjet pumps in inkjet head (404) are used to deposit droplets (407) in a regular and repeatable pattern on layer (402) that define reaction sites (e.g. 406). For example, the 3'-hydroxyls of such initiators may be unprotected and the droplets may contain a template-free polymerase and an initial 3'-O-protected nucleoside triphosphate, thereby producing 3'-O-protected elongated fragments in each reaction site. After such deposition, the layer (402) of initiators is immersed and incubated (409) in a buffer (408) comprising a template-free polymerase and a terminator, e.g. as described above, to produce droplet microarray (414) having a surface (410) outside of the reaction sites (e.g. 412) inert to extension or inert to extension and hydrophobic depending on the terminator selected.

**[0072]** An embodiment of the invention for synthesizing a plurality of polynucleotides employing reaction site formation as described in Fig. 3 may be carried out by the following steps: (a) providing a substrate, preferentially a planar substrate, having attached a layer initiators, wherein each initiator has a free 3'-hydroxyl; (b) dispensing through one or more inkjet pumps one or more droplets to each of a plurality of sites on the layer of initiators to define an array of reaction sites, wherein each droplet comprises a buffer solution comprising a mixture of a template-free polymerase and a 3'-O-blocked-dATP, a 3'-O-blocked-dCTP, a 3'-O-blocked-dGTP, or a 3'-O-blocked-dTTP, and wherein each polynucleotide of the plurality is assigned to a different reaction site for synthesis; (c) capping the free 3'-hydroxls of initiators outside of the reaction sites; (d) dispensing to each reaction site through one or more inkjet pumps at least one droplet a buffer solution comprising a mixture of a template-free polymerase

and a 3'-O-blocked-dATP, a 3'-O-blocked-dCTP, a 3'-O-blocked-dGTP, or a 3'-O-blocked-dTTP, wherein the kind of 3'-O-blocked dNTP dispensed to a reaction site depends on the predetermined sequence of the polynucleotide assigned to the reaction site; (e) incubating the template-free polymerase and 3'-O-blocked-dNTPs at each reaction site so that initiators or elongated fragments at the reaction site are elongated by incorporation of a 3'-O-blocked dNTP to form 3'-O-blocked elongated fragments; (f) deblocking the elongated fragments at each reaction site to form elongated fragments having free 3'-hydroxyls by treating the planar support with a deblocking agent; (g) repeating steps (d), (e), and (f) until the plurality of polynucleotides is synthesized.

**[0073]** An aspect of the invention is a method for preparing an array of reaction sites for template-free enzymatic synthesis of a plurality of polynucleotides. In some embodiments, such method of array preparation may be carried out by the steps of (a) providing a surface with initiators attached, (b) delivering with one or more inkjet pumps droplets to a plurality of distinct locations on the surface to form a plurality reaction sites, the droplets containing a synthesis reagent that reacts with initiators in the reaction sites to remove 3'-O-protecting groups or to elongate such initiators by addition of a 3'-O-protected nucleoside triphosphate, and (c) capping initiators on the surface outside of the reaction sites. In some embodiments, initiators on the surface of step (a) have free 3'-hydroxyls and the synthesis reagent delivered in step (b) comprises a template-free polymerase and a 3'-O-protected nucleoside triphosphate, so that the template-free polymerase catalyzes the addition of the 3'-O-protected nucleoside triphosphate to produce 3'-O-protected elongated fragments within the reaction sites. Thus, initiators outside of the reaction sites may be capped by immersion of the surface in a capping reagent (such as a mixture containing a dideoxynucleoside triphosphate and template-free polymerase). In some embodiments, initiators on the surface may have 3'-O-protection groups and the synthesis reagent delivered by droplets may contain a deprotection agent that removes the 3'-O-protection groups from initiators to form reaction sites. In the newly formed reaction sites, a reagent is delivered which contains 3'-O-protected nucleoside triphosphates and a template-free polymerase, wherein the protection group of the delivered nucleoside triphosphate is orthogonal to that of the initiators of the surface. Exemplary orthogonal 3'-O-protection groups are described below. For example, such orthogonal protection groups may be azidomethyl and amino.

**[0074]** One of ordinary skill would appreciate that similar reaction site formation can be implemented for other embodiments, such as those described in Figs. 2B and 2C. One of ordinary skill would also appreciate that the optional steps (e.g. washing, drying, treating with

protease, or the like) described for the embodiments of Figs. 2A-2C may also be implemented in the embodiments described above, including those of Fig. 3.

**[0075]** In another embodiment, a beginning layer of initiator oligonucleotides all have 3'-O-amino-protected or 3'-O-azidomethyl-protected ends. The process steps in this embodiment are similar to those of Fig. 3, except that a deprotection buffer is inkjet printed on the substrate to define reaction sites as discrete regions of initiators having free 3'-hydroxyls. After such selective deprotection, the surface is treated with an aqueous solution of an aldehyde or ketone to form a stable non-extendable hydrophilic or hydrophobic 3'-oxime. The aldehyde or ketone could be water soluble, e.g. acetone, or slightly water soluble and hydrophobic (e.g. pentanal, aldehyde-PEG-DBCO, or the like) or very hydrophobic and water insoluble (e.g. heptanal).

**[0076]** In another embodiment, a buffer comprising a template-free polymerase/3'-O-protected-dNTP mixture is printed on the initiator oligonucleotide layer with free 3'-hydroxyls as described above to define reaction sites having extended initiators with 3'-O-protected ends. The surface outside these defined sites is then treated with template-free polymerase and azide or alkyne derivatized ddNTP to block further 3' extensions. A hydrophobic molecule with a complementary click chemistry group (e.g. DBCO, benzyl-azide) may then be reacted with the ddNTP terminator to render the surface outside of the reaction sites hydrophobic. Exemplary click chemistry pairs are described in Feng et al, Adv. Mater. Interfaces, 1400269 (2014).

**[0077]** In still another embodiment, to a substrate surface without a layer of initiator oligonucleotides, a buffer comprising initiator oligonucleotides having 5' linker groups is inkjet printed on the surface derivatized with a complementary reactive group (e.g. epoxy, azide/alkyne) so that the initiators are attached to the surface by their 5'-ends. To these attached initiators, cycles of coupling reactions can take place in accordance with the invention. Also, unreacted complementary reactive groups may be quenched by reacting them with an inert group (e.g. ethanolamine for epoxy) and the inert group may be selected to have a hydrophobic character.

Methods of Template-Free Enzymatic Synthesis

**[0078]** Generally, methods of template-free (or equivalently, "template-independent") enzymatic polynucleotide synthesis comprise repeated cycles of steps, such as are illustrated in Fig. 1A, in which a predetermined nucleotide is coupled to an initiator or growing chain in each cycle. The general elements of template-free enzymatic synthesis are described in the following references: Champion et al, U.S. patent 10752887; Ybert et al, International patent publication WO/2015/159023; Ybert et al, International patent publication WO/2017/216472; Godron et al, International patent publication WO/2020/120442; Hyman, U.S. patent 5436143; Hiatt et al, U.S. patent 5763594; Jensen et al, Biochemistry, 57: 1821-1832 (2018); Mathews et al, Organic & Biomolecular Chemistry, DOI: 0.1039/c6ob01371f (2016); Schmitz et al, Organic Lett., 1(11): 1729-1731 (1999).

**[0079]** In the present invention synthesis reagents delivered by inkjet pumps must be formulated to satisfy at least two constraints: (i) the need to preserve the elongation activity of the template-free polymerase (in the case of template-free polymerase inks), and (ii) the need to meet the rheological requirements for droplet formation. The key solution parameters affecting droplet formation by inkjets are viscosity, surface tension, liquid density and the diameter of the inkjet nozzle. For particular embodiments of the invention, synthesis reagents prepared for non-droplet delivery to a reaction mixture may be reformulated by adding viscosity modifiers, surface tension modifiers and density modifiers, and the like, in order to form "printable inks" that may be delivered in droplets generated by inkjet pumps. "Printable" in reference to a reagent ink means repeatable droplets are able to be ejected from the nozzle, with uniform velocities and volumes and without satellite droplets.

**[0080]** As illustrated in Fig. 1A, initiator polynucleotides (100) with free 3'-hydroxyl groups (130) are provided, for example, attached to synthesis support (120). To the initiator polynucleotides (100) (or elongated initiator polynucleotides in subsequent cycles) are added a 3'-O-protected-dNTP and a template-free polymerase, such as a terminal deoxynucleotidyltransferase (TdT) or variant thereof (e.g. Ybert et al, WO/2017/216472; Champion et al, WO2019/135007) under conditions (140) effective for the enzymatic incorporation of the 3'-O-protected-dNTP onto the 3' end of the initiator polynucleotides (100) (or elongated initiator polynucleotides). This reaction produces elongated initiator polynucleotides whose 3'-hydroxyls are protected (160). If the elongated sequence is not complete, then another cycle of addition is implemented (180). If the elongated initiator polynucleotide contains a competed sequence, then the 3'-O-protection group may be removed,

or deprotected, and the desired sequence may be cleaved from the original initiator polynucleotide (182). Such cleavage may be carried out using any of a variety of single strand cleavage techniques, for example, by inserting a cleavable nucleotide at a predetermined location within the original initiator polynucleotide. An exemplary cleavable nucleotide may be a uracil nucleotide which is cleaved by uracil DNA glycosylase. In accordance with some embodiments of the invention, cleavage reagents may be delivered to reaction sites in droplets generated by inkjet pumps. In such embodiments, polynucleotides at reaction sites known to be incomplete or otherwise defective can be separated from fully competed polynucleotides or can be selectively re-synthesized either by cleaving and re-synthesizing the entire polynucleotide, or by cleaving or otherwise removing incorrect sequences and re-synthesizing only the defective part of the polynucleotide.

**[0081]** If the elongated initiator polynucleotide is not a completed sequence (i.e. the end product), then the 3'-O-protection groups are removed to expose free 3'-hydroxyls (130) and the elongated initiator polynucleotides are subjected to another cycle of nucleotide addition and deprotection.

**[0082]** As used herein, an "initiator" (or equivalent terms, such as, "initiating fragment," "initiator nucleic acid," "initiator oligonucleotide," or the like) usually refers to a short oligonucleotide sequence with a free 3'-hydroxyl at its end, which can be further elongated by a template-free polymerase, such as TdT. In one embodiment, the initiating fragment is a DNA initiating fragment. In an alternative embodiment, the initiating fragment is an RNA initiating fragment. In some embodiments, an initiating fragment possesses between 3 and 100 nucleotides, in particular between 3 and 20 nucleotides. In some embodiments, the initiating fragment is single-stranded. In alternative embodiments, the initiating fragment may be double-stranded. In some embodiments, an initiator oligonucleotide may be attached to a synthesis support by its 5'end; and in other embodiments, an initiator oligonucleotide may be attached indirectly to a synthesis support by forming a duplex with a complementary oligonucleotide that is directly attached to the synthesis support, e.g. through a covalent bond. In some embodiments a synthesis support is a solid support which may be a discrete region of a solid planar solid, or may be a bead.

**[0083]** In some embodiments, an initiator may comprise a non-nucleic acid compound having a free hydroxyl to which a TdT may couple a 3'-O-protected dNTP, e.g. Baiga, U.S. patent publications US2019/0078065 and US2019/0078126.

**[0084]** After synthesis is completed polynucleotides with the desired nucleotide sequence may be released from initiators and the synthesis supports by cleavage.

**[0085]** A wide variety of cleavable linkages or cleavable nucleotides may be used for this purpose. In some embodiments, cleaving the desired polynucleotide leaves a natural free 5'-hydroxyl on a cleaved strand; however, in alternative embodiments, a cleaving step may leave a moiety, e.g. a 5'-phosphate, that may be removed in a subsequent step, e.g. by phosphatase treatment. Cleaving steps may be carried out chemically, thermally, enzymatically or by photochemical methods. In some embodiments, cleavable nucleotides may be nucleotide analogs such as deoxyuridine or 8-oxo-deoxyguanosine that are recognized by specific glycosylases (e.g. uracil deoxyglycosylase followed by endonuclease VIII, and 8-oxoguanine DNA glycosylase, respectively). In some embodiments, cleavage may be accomplished by providing initiators with a deoxyinosine as the penultimate 3' nucleotide, which may be cleaved by endonuclease V at the 3' end of the initiator leaving a 5'-phosphate on the released polynucleotide. In some embodiments, an initiator may contain a terminal uridine so that after synthesis the desired polynucleotide may be cleaved from the initiator by treatment with KOH, or like base. Further methods for cleaving single stranded polynucleotides are disclosed in the following references, which are incorporated by reference: U.S. Pat. Nos. 5,739,386, 5,700,642 and 5,830,655; and U.S. Patent Publication Nos. 2003/0186226 and 2004/0106728; and in Urdea and Horn, U.S. patent 5367066.

**[0086]** Returning to Fig. 1A, in some embodiments, an ordered sequence of nucleotides are coupled to an initiator nucleic acid using a template-free polymerase, such as TdT, in the presence of 3'-O-protected dNTPs in each synthesis step. In some embodiments, the method of synthesizing an oligonucleotide comprises the steps of (a) providing an initiator having a free 3'-hydroxyl; (b) reacting under extension (or elongation) conditions the initiator or an extension intermediate having a free 3'-hydroxyl with a template-free polymerase in the presence of a 3'-O-protected nucleoside triphosphate to produce a 3'-O-protected extension intermediate; (c) deprotecting the extension intermediate to produce an extension intermediate with a free 3'-hydroxyl; and (d) repeating steps (b) and (c) until the polynucleotide is synthesized. (Sometimes the terms "extension intermediate" or "elongation fragment" or "growing chain" are used interchangeably). As used herein, the term "elongation conditions" means physical and chemical conditions of a reaction mixture necessary for a template-free polymerase to catalyze an elongation reaction wherein a 3'-O-protected nucleoside triphosphate monomer is coupled (by formation of a phosphodiester bond) to a free 3'-hydroxy of a nucleic acid fragment which, for example, may be an initiator or an elongated fragment. Exemplary elongation conditions include selections of reaction temperature, reaction duration, pH, concentrations of various salts, scavengers of undesired reaction components, agents to reduce

nucleic acid secondary structures, and the like. In some embodiments, an initiator is provided as an oligonucleotide attached to a solid support, e.g. by its 5' end. The above method may also include washing steps after the reaction, or extension, step, as well as after the de-protecting step. For example, the step of reacting may include a sub-step of removing unincorporated nucleoside triphosphates, e.g. by washing, after a predetermined incubation period, or reaction time. In some embodiments, such predetermined incubation periods or reaction times may be in the range of from 30 seconds to 30 minutes, or from 1 min to 30 min, or from 1 min to 15 min, or from 1 min to 10 min, or from 30 sec to 5 min.

**[0087]** In some embodiments, after the synthesis cycles of Fig. 1A are completed further steps may be performed to cleave the completed polynucleotides from the solid supports. Such further steps may be performed at the reaction sites of the array. Additionally, some cleavage methods may result in a released product that still requires modification to convert it into a useable product. For example, in the "endonuclease V-inosine" cleavage (described below) leaves a 5'-phosphate that must be removed for some applications. Thus, a further step of phosphatase treatment may be required.

**[0088]** When the predetermined sequences of polynucleotides on a synthesis support includes reverse complementary subsequences, secondary intra-molecular or cross-molecular structures may be created by the formation of hydrogen bonds between the reverse complementary regions. In some embodiments, base protecting moieties for exocyclic amines are selected so that hydrogens of the protected nitrogens cannot participate in hydrogen bonding, thereby preventing the formation of such secondary structures. That is, base protecting moieties may be employed to prevent the formation of hydrogen bonds, such as are formed in normal base pairing, for example, between nucleosides A and T and between G and C. At the end of a synthesis, the base protecting moieties may be removed and the polynucleotide product may be cleaved from the solid support, for example, by cleaving it from its initiator.

**[0089]** In addition to providing 3'-O-blocked dNTP monomers with base protection groups, elongation reactions may be performed at higher temperatures using thermal stable template-free polymerases. For example, a thermal stable template-free polymerase having activity above 40°C may be employed; or, in some embodiments, a thermal stable template-free polymerase having activity in the range of from 40-85°C may be employed; or, in some embodiments, a thermal stable template-free polymerase having activity in the range of from 40-65°C may be employed.

**[0090]** In some embodiments, elongation conditions may include adding solvents to an elongation reaction mixture that inhibit hydrogen bonding or base stacking. Such solvents include water miscible solvents with low dielectric constants, such as dimethyl sulfoxide (DMSO), methanol, and the like. Likewise, in some embodiments, elongation conditions may include the provision of chaotropic agents that include, but are not limited to, n-butanol, ethanol, guanidinium chloride, lithium perchlorate, lithium acetate, magnesium chloride, phenol, 2-propanol, sodium dodecyl sulfate, thiourea, urea, and the like. In some embodiments, elongation conditions include the presence of a secondary-structure-suppressing amount of DMSO. In some embodiments, elongation conditions may include the provision of DNA binding proteins that inhibit the formation of secondary structures, wherein such proteins include, but are not limited to, single-stranded binding proteins, helicases, DNA glycolases, and the like.

**[0091]** When base-protected dNTPs are employed, the above method of Fig. 1A may further include a step (e) removing base protecting moieties, which in the case of acyl or amidine protection groups may (for example) include treating with concentrated ammonia.

**[0092]** The above method may also include capping step(s) as well as washing steps after the reacting, or extending, step, as well as after the deprotecting step. As mentioned above, in some embodiments, capping steps may be included in which non-extended free 3'-hydroxyls are reacted with compounds that prevents any further extensions of the capped strand. In some embodiments, such compound may be a dideoxynucleoside triphosphate. In other embodiments, non-extended strands with free 3'-hydroxyls may be degraded by treating them with a 3'-exonuclease activity, e.g. Exo I. For example, see Hyman, U.S. patent 5436143. Likewise, in some embodiments, strands that fail to be deblocked may be treated to either remove the strand or render it inert to further extensions. When a capping agent, such as ddNTPs, are used, the buffer or synthesis reagents containing such agents may be delivered by flowing or spraying such reagent over substrate containing the reaction sites.

**[0093]** In some embodiments, reaction conditions for an elongation step (also sometimes referred to as an extension step or a coupling step) may comprising the following: 2.0 μM purified TdT; 125-600 μM 3'-O-blocked dNTP (e.g. 3'-O-$NH_2$-blocked dNTP); about 10 to about 500 mM potassium cacodylate buffer (pH between 6.5 and 7.5) and from about 0.01 to about 10 mM of a divalent cation (e.g. $CoC1_2$ or $MnC1_2$), where the elongation reaction may be carried out at a temperature within the range RT to 45°C, for 3 minutes. It is understood that whenever the foregoing coupling reagent is delivered by inkjet-produced droplets its viscosity, density and surface tension must be adjusted so that it becomes a printable ink. In this

connection, the invention in part includes the recognition and appreciation that an ink for delivering TdT to a reaction site may have its viscosity modified for droplet formation and activity preserved by selection of a viscosity modifier, such as, when carboxymethyl cellulose is selected as the viscosity modifying agent.

**[0094]** In embodiments, in which the 3'-O-blocked dNTPs are 3'-O-$NH_2$-blocked dNTPs, reaction conditions for a deblocking step may comprise the following: 700 mM $NaNO_2$; 1 M sodium acetate (adjusted with acetic acid to pH in the range of 4.8-6.5), where the deblocking reaction may be carried out at a temperature within the range of RT to 45°C for 30 seconds to several minutes. Washes may be performed with the cacodylate buffer without the components of the coupling reaction (e.g. enzyme, monomer, divalent cations). If the above reagent compositions are delivered to reaction sites by inkjet delivery, it is understood that the compositions would be altered to meet the rheological requirements for droplet formation by the nozzles of the inkjet print heads used.

**[0095]** In some embodiments, RNA synthesis may be accomplished by similar steps as described above but with template-free polymerases and monomers specifically selected for RNA synthesis, such as, polyA polymerase (PAP), polyU polymerase (PUP), or the like, e.g. International patent publication WO2020/077227. For example, systems, apparatus and kits of the invention may implement methods of synthesizing a polyribonucleotide having a predetermined sequence comprising the steps of: a) providing an initiator having a 3'-terminal nucleotide having a free 3'-hydroxyl; and b) repeating, until the polyribonucleotide is formed, cycles of (i) contacting under elongation conditions the initiator or elongated fragments having free 3'-hydroxyls with a 3'- O-blocked-nucleoside triphosphate and a template-free polymerase so that the initiator or elongated fragments are elongated by incorporation of a 3'- O-blocked-nucleoside triphosphate to form 3'-O-blocked-elongated fragments, and (ii) deblocking the elongated fragments to form elongated fragments having free 3'-hydroxyls; wherein the template-free polymerase is a poly(A) polymerase (PAP) or a poly(U) polymerase. In further embodiments, the initiator may be attached to a support by a 5' end, the support may be a solid support, and the above method may include a step of cleaving the polynucleotide from the initiator. In some embodiments, reaction conditions for an extension or elongation step using PAP or PUP may comprising the following: Reaction conditions 1 (for primer+AM-rATP): 250 uM AM-rATP, 0.1 uM ATTO488-(rA)5, 1 uM PAP, 1x ATP buffer (20 mM Tris-HCl, 0.6 mM MnCl2, 0.02 mM EDTA, 0.1% BSA, 10% glycerol, 100 mM imidazole, pH 7-8), 37 C, 30 min. Reaction condition 2 (for primer+AM-rGTP): 250 uM rGTP, 0.1 uM ATTO488-(rA)5, 1 uM PAP, 1x GTP buffer (0.6 mM MnCl2, 0.1% BSA, 10 mM imidazole, pH 6), 37 C, 30 min.

In the foregoing, "AM-rNTP" refers to 3'-azidomethyl-O-ribonucleoside triphosphate. Many of the 3'-O-blocked rNTPs employed in the invention may be purchased from commercial vendors (e.g. Jena Bioscience, MyChemLabs, or the like) or synthesized using published techniques, e.g. U.S. patent 7057026; International patent publications WO2004/005667, WO91/06678; Canard et al, Gene (cited above); Metzker et al, Nucleic Acids Research, 22: 4259-4267 (1994); Meng et al, J. Org. Chem., 14: 3248-3252 (3006); U.S. patent publication 2005/037991; Zavgorodny et al, Tetrahedron Letters, 32(51): 7593-7596 (1991). In a further particular embodiments, the 3'-blocked nucleotide triphosphate is blocked by either 3'-O-propargyl, a 3'-O-azidomethyl, 3'-O-$NH_2$ or 3'-O-allyl group. In still other embodiments, 3'-O-blocking groups of the invention include 3'-O-methyl, 3'-O-(2-nitrobenzyl), 3'-O-allyl, 3'-O-amine, 3'-O-azidomethyl, 3'-O-tert-butoxy ethoxy, 3'-O-(2-cyanoethyl), and 3'-O-propargyl. As above, if the above reagent compositions are delivered to reaction sites by inkjet delivery, it is understood that the compositions would be altered to meet the rheological requirements for droplet formation by the nozzles of the inkjet print heads used.

3'-O-Protected Nucleoside Triphosphates

**[0096]** Depending on particular applications, the steps of deblocking and/or cleaving may include a variety of chemical or physical conditions, e.g. light, heat, pH, presence of specific reagents, such as enzymes, which are able to cleave a specified chemical bond. Guidance in selecting 3'-O-blocking groups and corresponding de-blocking conditions may be found in the following references, which are incorporated by reference: Benner, U.S. patents 7544794 and 8212020; U.S. patent 5808045; U.S. patent 8808988; International patent publication WO91/06678; and references cited below. In some embodiments, the cleaving agent (also sometimes referred to as a de-blocking reagent or agent) is a chemical cleaving agent, such as, for example, dithiothreitol (DTT). In alternative embodiments, a cleaving agent may be an enzymatic cleaving agent, such as, for example, a phosphatase, which may cleave a 3'-phosphate blocking group. It will be understood by the person skilled in the art that the selection of deblocking agent depends on the type of 3'-nucleotide blocking group used, whether one or multiple blocking groups are being used, whether initiators are attached to living cells or organisms or to solid supports, and the like, that necessitate mild treatment. For example, a phosphine, such as tris(2-carboxyethyl)phosphine (TCEP) can be used to cleave a 3'O-azidomethyl groups, palladium complexes can be used to cleave a 3'O-allyl groups, or sodium

nitrite can be used to cleave a 3'O-amino group. In particular embodiments, the cleaving reaction involves TCEP, a palladium complex or sodium nitrite.

**[0097]** As noted above, in some embodiments it is desirable to employ two or more blocking groups that may be removed using orthogonal de-blocking conditions. The following exemplary pairs of blocking groups may be used in parallel synthesis embodiments. It is understood that other blocking group pairs, or groups containing more than two, may be available for use in these embodiments of the invention.

| | |
|---|---|
| 3'-O-NH2 | 3'-O-azidomethyl |
| 3'-O-NH2 | 3'-O-allyl, 3'-O-propargyl |
| 3'-O-NH2 | 3'-O-phosphate |
| 3'-O-azidomethyl | 3'-O-allyl, 3'-O-propargyl |
| 3'-O-azidomethyl | 3'-O-phosphate |
| 3'-O-allyl, 3'-O-propargyl | 3'-O-phosphate |

**[0098]** In some embodiments, specific enzymatically removable blocking groups are required specific enzymes for their removal. For example, ester- or acyl-based blocking groups may be removed with an esterase, such as acetylesterase, or like enzyme, and a phosphate blocking group may be removed with a 3' phosphatase, such as T4 polynucleotide kinase. By way of example, 3'-O-phosphates may be removed by treatment with as solution of 100 mM Tris-HCl (pH 6.5) 10 mM $MgC1_2$ , 5 mM 2-mercaptoethanol, and one Unit T4 polynucleotide kinase. The reaction proceeds for one minute at a temperature of 37°C. As above, if the foregoing compositions are delivered to reaction sites by inkjet delivery, it is understood that the compositions would be altered to meet the rheological requirements for droplet formation by the nozzles of the inkjet print heads used.

**[0099]** Further examples of synthesis and enzymatic deprotection of 3'-O-ester-protected dNTPs or 3'-O-phosphate-protected dNTPs are described in the following references: Canard et al, Proc. Natl. Acad. Sci., 92:10859-10863 (1995); Canard et al, Gene, 148: 1-6 (1994); Cameron et al, Biochemistry, 16(23): 5120-5126 (1977); Rasolonjatovo et al, Nucleosides & Nucleotides, 18(4&5): 1021-1022 (1999); Ferrero et al, Monatshefte fur Chemie, 131: 585-616 (2000); Taunton-Rigby et al, J. Org. Chem., 38(5): 977-985 (1973); Uemura et al, Tetrahedron Lett., 30(29): 3819-3820 (1989); Becker et al, J. Biol. Chem., 242(5): 936-950 (1967); Tsien, International patent publication WO1991/006678.

**[00100]** In some embodiments, the modified nucleotides comprise a modified nucleotide or nucleoside molecule comprising a purine or pyrimidine base and a ribose or deoxyribose sugar moiety having a removable 3'-OH blocking group covalently attached thereto, such that the 3' carbon atom has attached a group of the structure:

-O-Z

wherein –Z is any of –C(R')$_2$-O-R", -C(R')$_2$-N(R")$_2$, -C(R')$_2$-N(H)R", -C(R')$_2$-S-R" and –C(R')$_2$-F, wherein each R" is or is part of a removable protecting group; each R' is independently a hydrogen atom, an alkyl, substituted alkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclic, acyl, cyano, alkoxy, aryloxy, heteroaryloxy or amido group, or a detectable label attached through a linking group; with the proviso that in some embodiments such substituents have up to 10 carbon atoms and/or up to 5 oxygen or nitrogen heteroatoms; or (R')$_2$ represents a group of formula =C(R"')$_2$ wherein each R"' may be the same or different and is selected from the group comprising hydrogen and halogen atoms and alkyl groups, with the proviso that in some embodiments the alkyl of each R"' has from 1 to 3 carbon atoms; and wherein the molecule may be reacted to yield an intermediate in which each R" is exchanged for H or, where Z is –(R')$_2$-F, the F is exchanged for OH, SH or $NH_2$, preferably OH, which intermediate dissociates under aqueous conditions to afford a molecule with a free 3'-OH; with the proviso that where Z is –C(R')$_2$-S-R", both R' groups are not H. In certain embodiments, R' of the modified nucleotide or nucleoside is an alkyl or substituted alkyl, with the proviso that such alkyl or substituted alkyl has from 1 to 10 carbon atoms and from 0 to 4 oxygen or nitrogen heteroatoms. In certain embodiments, -Z of the modified nucleotide or nucleoside is of formula –C(R')$_2$-N3. In certain embodiments, Z is an azidomethyl group.

**[00101]** In some embodiments, Z is a cleavable organic moiety with or without heteroatoms having a molecular weight of 200 or less. In other embodiments, Z is a cleavable organic moiety with or without heteroatoms having a molecular weight of 100 or less. In other embodiments, Z is a cleavable organic moiety with or without heteroatoms having a molecular weight of 50 or less. In some embodiments, Z is an enzymatically cleavable organic moiety with or without heteroatoms having a molecular weight of 200 or less. In other embodiments, Z is an enzymatically cleavable organic moiety with or without heteroatoms having a molecular weight of 100 or less. In other embodiments, Z is an enzymatically cleavable organic moiety with or without heteroatoms having a molecular weight of 50 or less. In other embodiments, Z is an enzymatically cleavable ester group having a molecular weight of 200 or less. In other embodiments, Z is a phosphate group removable by a 3'-phosphatase. In some embodiments,

one or more of the following 3'-phosphatases may be used with the manufacturer's recommended protocols: T4 polynucleotide kinase, calf intestinal alkaline phosphatase, recombinant shrimp alkaline phosphatase (e.g. available from New England Biolabs, Beverly, MA)

**[00102]** In some embodiments, the 3'-blocked nucleotide triphosphate is blocked by either a 3'-O-azidomethyl, 3'-O-$NH_2$ or 3'-O-allyl group.

**[00103]** In some embodiments, 3'-O-blocking groups of the invention include 3'-O-methyl, 3'-O-(2-nitrobenzyl), 3'-O-allyl, 3'-O-amine, 3'-O-azidomethyl, 3'-O-tert-butoxy ethoxy, 3'-O-(2-cyanoethyl), and 3'-O-propargyl.

**[00104]** 3'-O-blocked dNTPs without base protection may be purchased from commercial vendors or synthesized using published techniques, e.g. U.S. patent 7057026; Guo et al, Proc. Natl. Acad. Sci., 105(27): 9145-9150 (2008); Benner, U.S. patents 7544794 and 8212020; International patent publications WO2004/005667, WO91/06678; Canard et al, Gene (cited herein); Metzker et al, Nucleic Acids Research, 22: 4259-4267 (1994); Meng et al, J. Org. Chem., 14: 3248-3252 (2006); U.S. patent publication 2005/037991. 3'-O-blocked dNTPs with base protection may be synthesized as described below.

Template-Free Polymerases for Polynucleotide Synthesis

**[00105]** A variety of different template-free polymerases are available for use in methods of the invention. Template-free polymerases include, but are not limited to, polX family polymerases (including DNA polymerases β, λ and μ), poly(A) polymerases (PAPs), poly(U) polymerases (PUPs), DNA polymerase θ, and the like, for example, described in the following references: Ybert et al, International patent publication WO2017/216472; Champion et al, U.S. patent 10435676; Champion et al, International patent publication WO2020/099451; Champion et al, International patent publication WO/2021/116270; Heinisch et al, International patent publication WO2021/018919. In particular, terminal deoxynucleotidyltransferases (TdTs) and variants thereof are useful in template-free DNA synthesis.

**[00106]** In some embodiments, TdT variants are employed with the invention which display increased incorporation activity with respect to 3'-O-amino nucleoside triphosphates. For example, such TdT variants may be produced using techniques described in Champion et al, U.S. patent 10435676, which is incorporated herein by reference.

Template-Free Polymerase Inks

**[00107]** As mentioned above, synthesis reagents delivered by inkjet pumps must be formulated so as to preserve activity of synthesis reagents and to meet the rheological requirements for droplet formation, which may be carry out by use of variable viscosity modifiers. Such formulations are referred to herein as "inks." For example, satisfying the first constraint (activity) may require that a template-free polymerase be present in a reaction mixture at a certain minimal concentration. However, because of high protein viscosity, the concentration for the desired activity may prevent satisfaction of the second constraint, i.e. capability of droplet formation. In such cases, embodiments of the invention may call for delivery of a plurality of droplets each with lower concentrations of polymerase, which coupled with evaporation permit the build-up of polymerase concentration for a desired level of activity. Such an embodiment is illustrated in Fig. 2C.

**[00108]** As also mentioned above, the key solution parameters affecting droplet formation by inkjets are viscosity, surface tension, density and diameter of the inkjet nozzle, which are related through the formula: $Z=[(\rho\gamma a)^{(0.5)}]/\eta$, where $\rho$ is the density of the fluid, $\gamma$ is surface tension, $\eta$ is viscosity, a is the radius of the inkjet pump nozzle and Z is in the range of from 1 to 10 for reliable droplet formation, e.g. Derby, J. Mater. Chem., 18: 5717-5721 (2008). This relationship applies to any of the synthesis reagents delivered by inkjet-generated droplets, including (i) template-free polymerase in its coupling buffer, (ii) a mixture of template-free polymerase in its coupling buffer and a 3'-O-protected-dNTP, (iii) a 3'-O-protected-dNTP in a buffer, (iv) a deprotection buffer, and (v) a buffer containing a 5'-linker-derivatized initiator. Applying this relationship to determine ink compositions that are capable of forming desired droplets for particular embodiments may be carried out by one of ordinary skill in the art by adjusting densities of reactants, viscosity modifiers, surface tension modifiers, and the like.

**[00109]** Thus, for particular embodiments of the invention, synthesis reagents prepared for non-droplet delivery to a reaction mixture may be reformulated by adding viscosity modifiers, surface tension modifiers and density modifiers, and the like, in order to form "printable inks" that may be delivered in droplets generated by inkjet pumps. "Printable" in reference to a reagent ink means repeatable droplets are able to be ejected from the nozzle, with uniform velocities and volumes and no satellite droplets.

**[00110]** In some embodiments, if the specific activity of a template-free polymerase is relatively low, so that a relatively large amount of protein must be delivered to reaction sites to complete a coupling step, then the delivery of the polymerase may be carried out by dispensing a plurality of droplets in each coupling cycle together with allowing a controlled amount of

evaporation to maintain a reaction volume within a prescribed range, e.g. 10-100 pL. In some embodiments, the plurality of droplets delivered is in the range of from 2 to 10, or in the range of from 2 to 5, or in the range of from 2 to 3. In other embodiments, the plurality of droplets may be in the range of from 2 to 150, or in the range of from 10 to 120. In some embodiments, whenever the template-free polymerase is a TdT, the plurality of droplets is the number required to bring the concentration of TdT in the reaction mixture at a reaction site to a value in the range of from 1μM to 30μM, or in the range of from 2μM to 20μM. In some embodiments, a concentration of TdT in an ink is the concentration that produces an approximate 1:1 stoichiometry between TdT molecules and polynucleotides at a reaction site. In other embodiments, a concentration of TdT in an ink is a concentration that produces a stoichiometry between TdT molecules and polynucleotides at a reaction site that is 1:1 or greater.

**[00111]** In some embodiments, the invention includes a printable ink comprising a TdT variant and a viscosity modifier, such as a variable viscosity modifier. In some embodiments, such TdT in a concentration in the range of from 1 μM to 20 μM/mg in a buffer suitable for coupling activity. In some embodiments, such buffer comprises about 10 to about 500 mM potassium cacodylate buffer (pH between 6.5 and 7.5) and from about 0.01 to about 10 mM of a divalent cation (e.g. $CoC1_2$ or $MnC1_2$). In some embodiments, an elongation reaction buffer is an acetate buffer, for example, 0.1 M acetate, 0.5 M NaCl, pH 4.5.

**[00112]** In some embodiments, a non-variable viscosity modifier may be used along with a variable viscosity modifier. Such non-variable viscosity modifier may be selected from the group consisting of ethylene glycol, polyethylene glycol of different molecular weights, polyethyleneglycol methyl ether, polyethyleglycol dimethyl ether, poly(vinyl alcohol), carboxymethyl cellulose and hydroxyethyl cellulose.

**[00113]** In some embodiments, besides a viscosity modifier, the printable template-free polymerase ink, such as a TdT ink, comprises a surface tension modifier. Such surface tension modifier may be a detergent. Such detergent may be selected from Tween 20, Triton X-100, CHAPS, NP-40, octyl thioglucoside, octyl glucoside or dodecyl maltoside. Of particular interest is Triton X-100. Also of particular interest is Tween 20. Additional surface tension modifiers (i.e. surfactants) are disclosed in Buret, LabChip, 12: 422-433 (2012).

**[00114]** In some embodiments, the invention includes a printable ink comprising a TdT variant, a 3'-O-protected-dNTP and a variable viscosity modifier. In some embodiments, such TdT is in a concentration in the range of from 1 μM to 50 μM/mg, or 1 μM to 20 μM/mg, in a buffer suitable for coupling activity. In some embodiments, such buffer comprises about 10 to

about 500 mM potassium cacodylate buffer (pH between 6.5 and 7.5) and from about 0.01 to about 10 mM of a divalent cation (e.g. $CoC1_2$ or $MnC1_2$); such 3'-O-protected-dNTP is in a concentration in the range of 125-600 μM. In some embodiments, the variable viscosity modifier is selected from the group consisting of an uncrosslinked poly(N-alkyl-substituted-acrylamide) and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymer. In some embodiments, besides a variable viscosity modifier, the printable TdT ink comprises a surface tension modifier. Such surface tension modifier may be a detergent. Such detergent may be selected from Triton X-100, CHAPS, NP-40, octyl thioglucoside, octyl glucoside or dodecyl maltoside. Of particular interest are Tween 20 and Triton X-100.

**[00115]** In some embodiments, a printable ink comprising a template-free polymerase, such as a TdT variant, includes a humectant for reducing droplet evaporation. Suitable humectants include, but are not limited to, glycerol, alcohol sugars, ethylhexylglycerin, panthenol, sorbitol, xylitol, maltitol, propylene glycol, hexylene glycol, butylene glycol, sodium lactate, hyaluronic acid, and polydextrose,

**[00116]** In some embodiments, a TdT ink of the invention is delivered in a droplet in range of from 1 pL to 200 pL, or from 1 pL to 100 pL, or from 1 pL-50pL.

**[00117]** In some embodiments, the invention is directed to a terminal deoxynucleotidyl transferase (TdT) composition comprising a droplet of an aqueous solution having a volume in the range of from 2 pL to 5 nL and comprising (i) a TdT or variant thereof in a concentration in the range of from 1.0 μM to 30 μM, or in the range of from 2.0 μM to 20 μM, a divalent cation in a concentration in the range of from 0.01 to 10 mM, and a variable viscosity modifier. In some embodiments, the divalent cation is cobalt or manganese and such composition further comprises a surface tension modifier.

**[00118]** In some embodiments, such variable viscosity modifier is selected from the group consisting of an uncrosslinked poly(N-alkyl-substituted-acrylamide) and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymer. In some embodiments, any of the above compositions may further comprise an aldehyde scavenger (described more fully below) whenever the printable template-free polymerase ink, such as a printable TdT ink, comprises a 3'O-amino-nucleotide. In some embodiments, any of the above compositions may further comprise a 3'-O-protected-2'-deoxynucleoside triphosphate monomer in a concentration in the range of from 100-1000 μM, or from 125-600 μM.

**[00119]** In some embodiments, variable viscosity modifier may comprise polymer solutions that gels upon cooling. Such polymer is chosen from the group consisting in hydrophobically

modifed polymers having UCST-type phase behavior, natural polymers, deblock copolymer brush grafted silica nanoparticles, poly(PEO-co-styrene) and at least one of their combination. The polymer can be dissolved in an ionic liquid or solutions of PNIPAM microgels and host-guest interactions.

**[00120]** In some embodiments, the invention is directed to a 3'-O-protected-2'-deoxynucleoside triphosphate composition comprising a droplet of an aqueous solution having a volume in the range of from 2 pL to 5 nL and comprising (i) a 3'-O-protected-2'-deoxynucleoside triphosphate in a concentration in the range of from 125-600 μM. and a variable viscosity modifier. In some embodiments, the foregoing 3'-O-protected-2'-deoxynucleoside triphosphate composition further comprising a surface tension modifier.

**[00121]** In some embodiments of the foregoing compositions, the variable viscosity modifier is selected from the group consisting of an uncrosslinked poly(N-alkyl-substituted-acrylamide) and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymer. In some embodiments, the 3'-O-protected-2'-deoxynucleoside triphosphate of the foregoing composition is a 3'-O-protected-2'-deoxyadenosine triphosphate, a 3'-O-protected-2'-deoxyguanosine triphosphate, a 3'-O-protected-2'-deoxycytidine triphosphate, or a 3'-O-protected-2'-deoxythymidine triphosphate. In some embodiments of the foregoing composition, the 3'-O-protection group is selected from the group consisting of 3'-O-methyl, 3'-O-(2-nitrobenzyl), 3'-O-allyl, 3'-O-amine, 3'-O-azidomethyl, 3'-O-tert-butoxy ethoxy, 3'-O-(2-cyanoethyl), and 3'-O-propargyl.

**[00122]** As mentioned above, in some embodiments of the invention where 3'-O-amino-dNTP monomers are employed, the presence of an aldehyde scavenger in the template-free polymerase inks reduces spurious capping of the 3'-amines by reaction with adventitious aldehydes or ketones, such as formaldehyde, which are pervasive in the environment. This is a special problem with inkjet synthesis because droplets of ink have very high surface-to-volume ratios that enhances absorption of environmental aldehydes. Thus, in embodiments of the invention employing 3'-O-amino-dNTP monomers, template-free polymerase inks as described above further include an effective amount of at least one aldehyde scavenger. As used herein, "effective amount" in reference to an aldehyde scavenger means an amount (or concentration) sufficient to produce a measureable decrease in spuriously capped polynucleotides in a product. Such measurements may be made readily using conventional techniques, e.g. DNA sequence analysis of a sample of a product, gel electrophoresis, or the like. As used herein, the term "aldehyde scavenger" includes ketone scavengers. In some embodiments, aldehyde scavengers are agents that react with compounds having chemical

groups of the formula R-C(=O)H or $R^1$-C(=O)-$R^2$, where R, $R^1$ and $R^2$ are typically alkyl or aryl. More particularly, in some embodiments, aldehyde scavengers are agents that react with R-C(=O)H or $R^1$-C(=O)-$R^2$ groups on compounds at a sufficiently high rate that such compounds do not react with (or react only negligibly with) the 3'-amine group of 3'-O-amino-nucleotides. As used herein, the term "scavenger" means a chemical substance added to a mixture in order to remove or de-activate impurities or compounds that lead unwanted reaction products. In various embodiments, aldehyde scavengers may be in solution, immobilized on the materials used for storage or synthesis or coupled to reagents employed in method of the invention, for example, template-free polymerases, such as TdTs.

**[00123]** As noted above, enzymatic synthesis may be carried out using a variety of reagents (referred to herein as "synthesis reagents") that may contain or consist of reactants, wash solutions, deprotection buffers, enzymes, and the like. ( The term "synthesis reagent" means any reagent used in a synthesis cycle to couple a monomer, particularly a 3'-O-amino-nucleoside triphosphate, to an initiator or elongated fragment, such as, buffers comprising a template-free polymerase, buffers comprising 3'-O-protected-nucleotide monomers, deprotection (or deblocking) buffers, and the like.) In various embodiments, an aldehyde scavenger may be a component of one or more of the synthesis reagents. In some embodiments, an aldehyde scavenger may be added to a reaction mixture as a separate synthesis reagent (without other reactants, wash buffers or enzymes). In some embodiments, an aldehyde scavenger is added to a reaction mixture as a component of a synthesis reagent comprising a template-free polymerase.

**[00124]** In some embodiments, e.g. employing aldehyde scavengers disclosed by Sudo et al, U.S. patent publication US2020/0061225 or listed in Figs. 8A-8B, an effective amount is provided by a concentration in the range of from 1 to 500 mM, or in other embodiments in the range of from 1 to 200 mM, or in other embodiments in the range of from 1 to 100 mM.

**[00125]** In some embodiments, aldehyde scavengers employed in the invention comprise O-substituted hydroxylamines or polyhydroxylamines. In some embodiments, O-substituted hydroxylamines used in the invention are defined by the formula:

$$R^1\text{-}ONH_2$$

such as disclosed by Sudo et al, U.S. patent publication US2020/0061225, or Kitasaka et al, U.S. patent 7241625, which are incorporated herein by reference. In some embodiments, $R^1$ is a $C_{1\text{-}18}$ linear, branched or cyclic alkyl group which may be substituted by at least one substituent selected from the group consisting of a halogen atom; a $C_{1\text{-}6}$ alkyloxy group; a $C_{1\text{-}6}$ haloalkyl group; a $C_{1\text{-}6}$ haloalkyloxy group; a carboxy group; a hydroxy group; a mercapto

group; a cyano group; a nitro group; a $C_{6-14}$ aryl group which may be substituted by a halogen atom, a $C_{1-6}$ alkyl group, a $C_{1-6}$ alkyloxy group, a $C_{1-6}$ haloalkyl group, a $C_{1-6}$ haloalkyloxy group, a carboxy group, a hydroxy group, a mercapto group, a cyano group or a nitro group; a $C_{4-14}$ heteroaryl group which may be substituted by a halogen atom, a $C_{1-6}$ alkyl group, a $C_{1-6}$ alkyloxy group, a $C_{1-6}$ haloalkyl group, a $C_{1-6}$ haloalkyloxy group, a carboxy group, a hydroxy group, a mercapto group, a cyano group or a nitro group; an alkoxycarbonyl group represented by the following formula:

$$-(C=O)-O-R^2$$

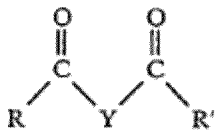
and a carbamoyl group represented by the following formula:

$$-(C=O)-NR^3(R^3)$$

wherein $R^2$ is a $C_{1-18}$ linear, branched or cyclic alkyl group which may be substituted, at a chemically acceptable optional position, by at least one substituent selected from the group consisting of a carboxy group; a hydroxy group; a mercapto group; a halogen atom; a $C_{1-6}$ alkyloxy group; a $C_{1-6}$ haloalkyloxy group; a $C_{6-14}$ aryl group; and a $C_{4-14}$ heteroaryl group; and wherein each $R^3$ may be the same or different and each independently a $C_{1-18}$ linear, branched or cyclic alkyl group which may be substituted by at least one substituent selected from the group consisting of a carboxy group; a hydroxy group; a mercapto group; a halogen atom; a $C_{1-6}$ alkyloxy group; a $C_{1-6}$ haloalkyloxy group; a $C_{6-14}$ aryl group; and a $C_{4-14}$ heteroaryl group; a $C_{6-14}$ aryl group, a $C_{4-14}$ heteroaryl group, or a hydrogen atom.

**[00126]** In particular, exemplary O-substituted hydroxylamines or polyhydroxylamines which may be used in the invention are shown as compounds (1)-(14) in Figs. 3A and 3B, wherein compound (1) is also referred to herein as the "BOX" reagent.

**[00127]** In some embodiments aldehyde scavengers comprise carbonyl compounds disclosed by Pacifici, U.S. patent 5446195 or Burdeniuc et al, U.S. patent publication, US20160369035; which are incorporated herein by reference, and are defined by the formula:




wherein R and R' are $CH_3$ or $H[O(CH_2)_m]_nO$- and wherein m and n are selected from the group of combinations of m and n consisting of: m=1 and n=1, 3-19; m=2 and n=2-19; or m=3 and n=1-19, Y is --$CH_2$- or --$CH_2$ -CO-$CH_2$ --.

**[00128]** In some embodiments of the invention, template-free polymerase inks as described above further include a dye to permit monitoring of the location, size, shape and possible overlap of reaction sites, either at an initial dispensing of reagents to define the reaction sites or at subsequent droplet dispensations during synthesis, particularly to monitor possible coalescence of reaction mixtures at adjacent sites. A large selection of fluorescent and non-fluorescent dyes are available for this purpose. The main criteria for use is that the dye (i) not adversely affect the performance of any reaction component, (ii) be bright or concentrated enough to make droplets or reaction sites readily detectable, (iii) be spectrally distinct if more than one is used, and (iv) not affect the rheological properties of the ink. In some embodiments, food dyes are used in inks of the invention. In other embodiments, pH indicator dyes are used in inks of the invention. In other embodiments, fluorescent dyes are used in inks of the invention. Exemplary dyes for use with template-free polymerase inks include Brilliant Blue FCF, Fast Green FCF, Ponceau 4R and Sunset Yellow FCF. In some embodiments, food dyes are used at a concentration in the range of from 1 to 20 mM, or at a concentration in the range of from 1 to 10 mM.

Example

Methods For Evaluating Reaction Conditions in Enzymatic Inkjet Synthesis

**[00129]** In this example, a test bed was created for evaluating different inkjet reaction conditions. Because the very small amounts of materials at individual reaction sites are difficult to analyze, slides were prepared that allowed regions containing large numbers of reaction sites (e.g. 10 or more) to be processed together and pooled for analysis by gel electrophoresis. The regions were created by depositing equal volumes of alkali- or photo-cleavable initiators in each of the regions. An exemplary slide (900) (which may be a PolyAn 3D-epoxy coated glass slide) is shown in Fig. 5. It contains 24 circular regions (902) each of which contains approximately the same number of reaction sites (not shown). Regions (902) were created on slide (900) by hand spotting 20 μM 5'-amino-derivatized photo-cleavable initiators to the 24 locations (following the slide manufacturer's recommended protocol). Briefly, after incubation overnight at 70% relative humidity, the slide is then heated to 80°C for 5 min, washed for 1 h in 1M ethanolamine pH 8, 30 min in SSC 4X, 30 min in SSC 2X 0.1% SDS, 30 min in SSC 0.2X, 30 min in MQ. Exemplary initiators may have the following sequence: 5'-amino-C12-10T(PC)4T(FAM-T)18T-3', where C12 is a 12-carbon alkyl linker, T is thymidine, "PC" is a photo-cleavable linker (e.g. Horgan, WO2021/048142) and "FAM-T" is a fluorescein-labeled thymidine. After an experiment is carried out, sequences are photo-cleaved from specific

regions by immersing the region(s) in PBS (e.g. 40 µL/region) and illuminating the region(s) with 365 nm light (e.g. Analytik Jena, 95-0252-02, UVLMS-38, 8W-intensity a 3" is 1500 µW/cm$^2$ for 365 nm), after which the pooled sequences are loaded on a gel as diagrammed in Fig. 5. In the example of Fig. 5, regions are grouped into eight groups of 3 regions each. In groups 1-4 23 cycles of elongation reactions are carried out, after which the resulting product in each group of three are photo-cleaved, pooled and loaded (909) on their respective lanes 1-4 (914). In groups 5-8 no synthesis reactions are carried out. The non-elongated initiators are processed exactly the same as groups 1-4, but are loaded into lanes 5-8 (916). The gel provides a convenient and sensitive measure of the effects of changes in various reaction parameters, including but not limited to, reactant concentrations, the presence, absence and concentration of rheological ingredients (such as, surfactants, viscosity modifiers, and so on), different template-free polymerases, secondary structure modifiers, and the like.

Evaluation of the impact of temperature on viscosity and Enzymatic Inkjet Synthesis

**[00130]** In this example, the impact of temperature on viscosity of the ink was assessed. The elongation ink was prepared as described in Table 1 below.

| Components | Elongation ink |
|---|---|
| **Cacodylic acid** (2M stock at pH = 7.4) | 0.5 M |
| **DMSO** | 15 v% |
| **Tween20** | 0.05 v% |
| **$(OMe)_2$PEG500** | 7 v% |
| **HEPES** | 500 µM |
| **Tris·HCl** | 2 mM |
| **NaCl** | 40 mM |
| **TdT** | 20 µM |
| **dNTP** | 500 µM |

***Table 1 Ink composition***

**[00131]** As shown in Fig. 6, it is seen that there is a significant decrease in viscosity with the increase of temperature which was expected.

**[00132]** The stability of the elongation ink was also assessed at different temperatures. As shown in Fig.7, it is seen the benefit of storing the ink at low temperature and the impossibility to perform synthesis with inks stored in printheads at 37 °C as the ink precipitates in less than 24 h.

**[00133]** In this example, a manual synthesis test was performed for evaluating the reaction efficacy at different temperatures.

**[00134]** The slide was fully covered with photocleavable initiators. An exemplary slide (900) (which may be a PolyAn 2D-azide coated glass slide) is shown in Fig. 5. Immobilization was performed incubating 5 µM of 5' DBCO photocleavable initiator in NaOAc/NaCl buffer pH =

4.5 for 1 h at 70% RH. The slide was then washed with a series of buffers, rinse with MQ, and dried.

**[00135]** Exemplary initiators may have the following sequence: 5'-DBCO-TEG-10T(PC)4T(FAM-T)18T-3', where DBCO is dibenzocyclooctyne, TEG is triethyleneglycol, T is thymidine, "PC" is a photo-cleavable linker (e.g. Horgan, WO2021/048142) and "FAM-T" is a fluorescein-labeled thymidine.

**[00136]** Manual synthesis is then carried out at 22, 37, or 4 °C (one slide) in a silicon gasket as follows: i) incubation with elongation ink (70% RH, 5 min at 4, 20 or 37 °C), ii) incubation with deprotection buffer (70% RH, 3 min), iii) rinse with H2O milli-Q, iv) dry with compressed air.

**[00137]** After an experiment is carried out, sequences are photo-cleaved from specific regions filling the gasket with PBS and illuminating the region(s) with 365 nm light (e.g. Analytik Jena, 95-0252-02, UVLMS-38, 8W-intensity a 3" is 1500 mW/cm2 for 365 nm).

**[00138]** Elongated oligos at 4, 20 and 37 °C, were submitted to gel electrophoresis along with non-elongated oligos used as reference.

**[00139]** The gel provides a convenient and sensitive measure of the effects of changes in various reaction parameters, including but not limited to, reactant concentrations, the presence, absence and concentration of rheological ingredients, temperature of elongation, different template-free polymerases, secondary structure modifiers, and the like.

**[00140]** It is seen on Fig.8 that the amount of impurity decreases with a higher temperature given a better synthesis at 37°C then at 4°C. With Fig.6 and Fig. 7, it is seen that there is a need to have a high viscosity before the synthesis and a lower viscosity during the synthesis to obtain better results.

## Definitions

**[00141]** Unless otherwise specifically defined herein, terms and symbols of nucleic acid chemistry, biochemistry, genetics, and molecular biology used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Le, Recent Progress in Ink Jet Technologies II, chapter 1, pgs. 1-14 (1999); Zapka, editor, "Handbook of Industrial Inkjet Printing," (Wiley-VCH, Weinheim, Germany).

**[00142]** "Humectant" is any hygroscopic substance that attracts and retains moisture. Exemplary humectants include, but are not limited to, glycerol, alcohol sugars, ethylhexylglycerin, panthenol, sorbitol, xylitol, maltitol, propylene glycol, hexylene glycol, butylene glycol, sodium lactate, hyaluronic acid, polydextrose, or the like.

**[00143]** "Polynucleotide" means a linear polymer of nucleotide monomers or analogs thereof. Monomers making up polynucleotides may be capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include PNAs, phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of a polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, to several thousand monomeric units. Whenever a polynucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York, 1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references. Likewise, the polynucleotide

may refer to either a single stranded form or a double stranded form (i.e. duplexes of an polynucleotide and its respective complement). It will be clear to one of ordinary skill which form or whether both forms are intended from the context of the terms usage.

Claims:

1. A method of enzymatically synthesizing a plurality of polynucleotides at reaction sites on a substrate for inkjet printing, the method comprising the steps of:

(a) providing a substrate having initiators at a plurality of reaction sites, wherein each initiator has a free 3'-hydroxyl and wherein each polynucleotide of the plurality is assigned to a reaction site for synthesis;

(b) dispensing at least one droplet of at least one synthesis reagent to each reaction site of the plurality to perform a reaction cycle comprising the steps of (i) reacting under elongation conditions the initiator or elongated fragments having free 3'-O-hydroxyls with a 3'-O-protected nucleoside triphosphate and a template-free polymerase so that the initiator or elongated fragments are elongated by incorporation of a 3'-O-protected nucleoside triphosphate to form 3'-O-protected elongated fragments, and (ii) deprotecting the elongated fragments to form elongated fragments having free 3'-hydroxyls, wherein the synthesis reagent comprises a template-free polymerase, a 3'-O-protected nucleoside triphosphate, a mixture of a template-free polymerase and a 3'-O-protected nucleoside triphosphate, or a deprotection solution; wherein the at least one synthesis reagent comprises a variable viscosity modifier having a first viscosity during formation of the at least one droplet and a second viscosity at each reaction site of the plurality during the reacting step of the reaction cycle;

(c) repeating step (b) until the plurality of polynucleotides is synthesized;

wherein the first viscosity and second viscosity are different.

2. The method according to claim 1, wherein said first viscosity is greater than 2 mPa.s and the second viscosity is lower than or equal to 2 mPa.s.

3. The method according to any of the preceding claims, wherein said first viscosity is in the range of from 2 to 20 mPa.s, exclusive, and said second viscosity is in the range of from 1 to 2 mPa.s, inclusive.

4. The method according to any of the preceding claims, wherein said at least one synthesis reagent is a template-free polymerase ink.

5. The method according to any of the preceding claims, wherein said variable viscosity modifier comprises a thermoreversible polymer having a viscosity that decreases with increases in temperature.

6. The method according to claims 1 to 4, wherein said variable viscosity modifier comprises a polymer solution that gels upon cooling.

7. The method according to any of the preceding claims, wherein said variable viscosity modifier comprises polyvinyl alcohol, dimethylsulfoxyde (DMSO), polyethylene glycol (PEG), polyethyleneglycol methyl ether (OMe)PEG, polyethyleglycol dimethyl ether $(OMe)_2PEG$, carboxymethyl cellulose, hydroxyethyl cellulose, or at least one of their combinations.

8. The method according to any of the preceding claims, wherein said step (b) further includes washing said elongated fragments after said step of deprotecting.

9. The method according to any of the preceding claims, wherein each of said reaction sites are distinct and non-overlapping with other said reaction sites.

10. The method according to any of the preceding claims, wherein said step of reacting includes incubating said reaction mixture for a predetermine duration.

11. The method according to any of the preceding claims, comprising after said step of reacting a step of capping said initiators or elongated fragments with free 3'-O-hydroxyls that failed to be elongated.

12. The method according to any of the preceding claims, wherein each of said polynucleotides of said plurality is assigned to a different reaction site for synthesis.

13. The method according to any of the preceding claims, wherein the at least one synthesis reagent solution is glycerol free.

14. The method according to any of the preceding claims, wherein said variable viscosity modifier is between 5 to 50 percent in volume of the at least one synthesis reagent solution, preferably 20 to 40 percent in volume of the at least one synthesis reagent solution.

15. The method according to any of the preceding claims, wherein there is more than one synthesis reagent solution, each solution with a different variable viscosity modifier.

16. A template-free polymerase ink for inkjet printing comprising: an aqueous solution comprising a template-free polymerase having a concentration of in a range of from 1.0 μM to 30 μM; wherein whenever the ink is printed as droplets to a substrate, printed droplets each have a volume in the range of 0.1 pL to 5 nL of the aqueous solution and wherein the ink comprises a variable viscosity modifier having a first viscosity in the range of from 2-20 mPa.s whenever the temperature is in the range of from 5-30°C and a second viscosity in the range of from 2 to 3 mPa.s whenever the temperature is in the range of from 35-60°C; wherein the first viscosity and second viscosity are not the same.

17. The template-free polymerase ink according to the preceding claim, wherein said variable viscosity modifier comprises polyvinyl alcohol, carboxymethyl cellulose, hydroxyethyl cellulose, dimethylsulfoxyde, polyethylene glycol (PEG), polyethyleneglycol methyl ether (OMe)PEG, polyethyleglycol dimethyl ether $(OMe)_2$PEG,or at least one of their combinations.

18. The template-free polymerase ink according to any of the claims 16 to 17, wherein said variable viscosity modifier is between 5 to 50 percent in volume of the at least one synthesis reagent solution, preferably 20 to 40 percent in volume of the at least one synthesis reagent solution.

19. The template-free polymerase ink according to any of the claims 16 to 18, wherein said variable viscosity modifier comprises a polymer solution that gels upon cooling.

20. The template-free polymerase ink according to any of the claims 16 to 19, wherein said ink is glycerol free.

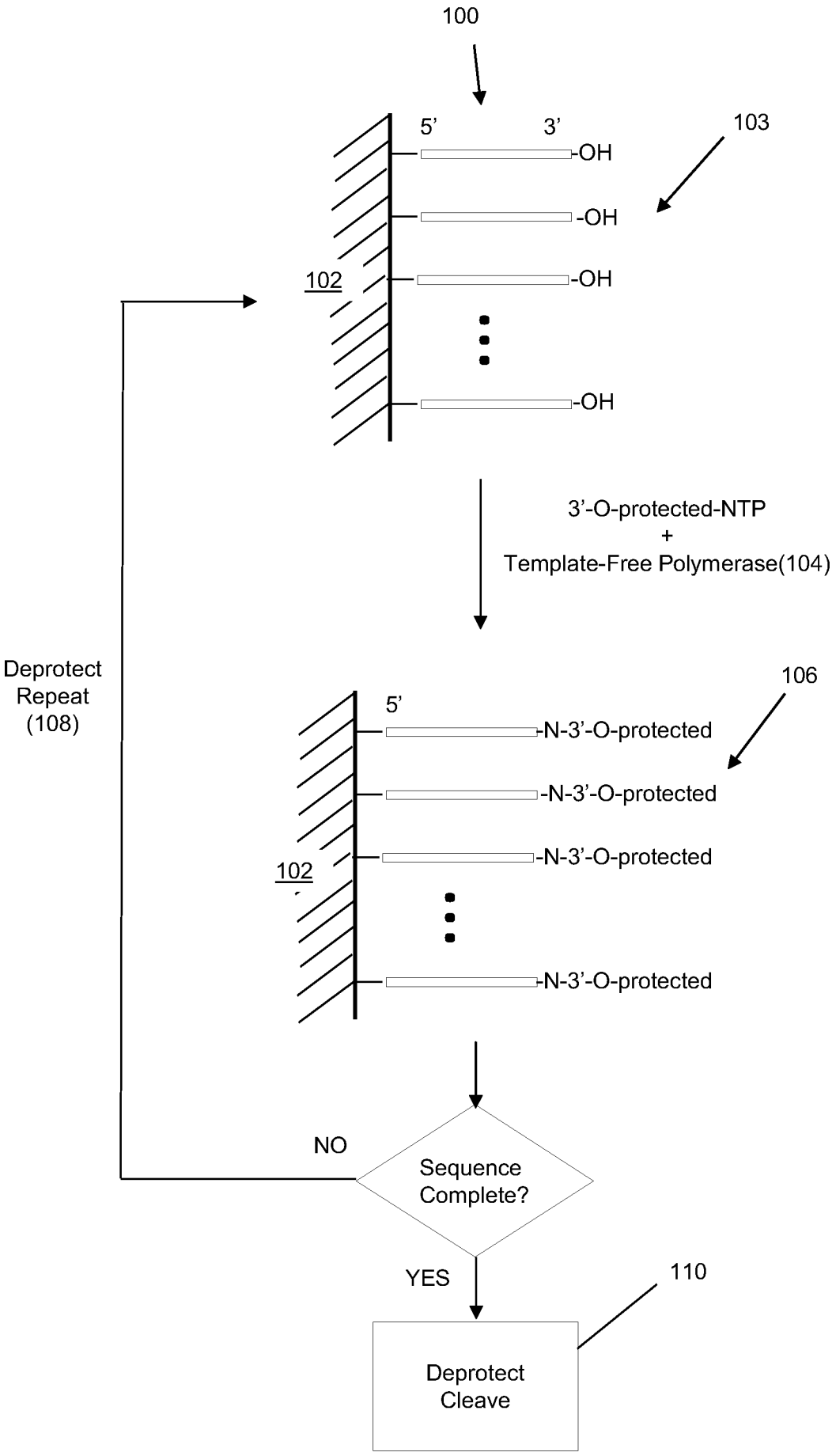

100
5'
3'
-OH
103
102
3'-O-protected-NTP
+
Template-Free Polymerase(104)
Deprotect
Repeat
(108)
106
-N-3'-O-protected
NO
Sequence
Complete?
YES
110
Deprotect
Cleave

Fig. 1A

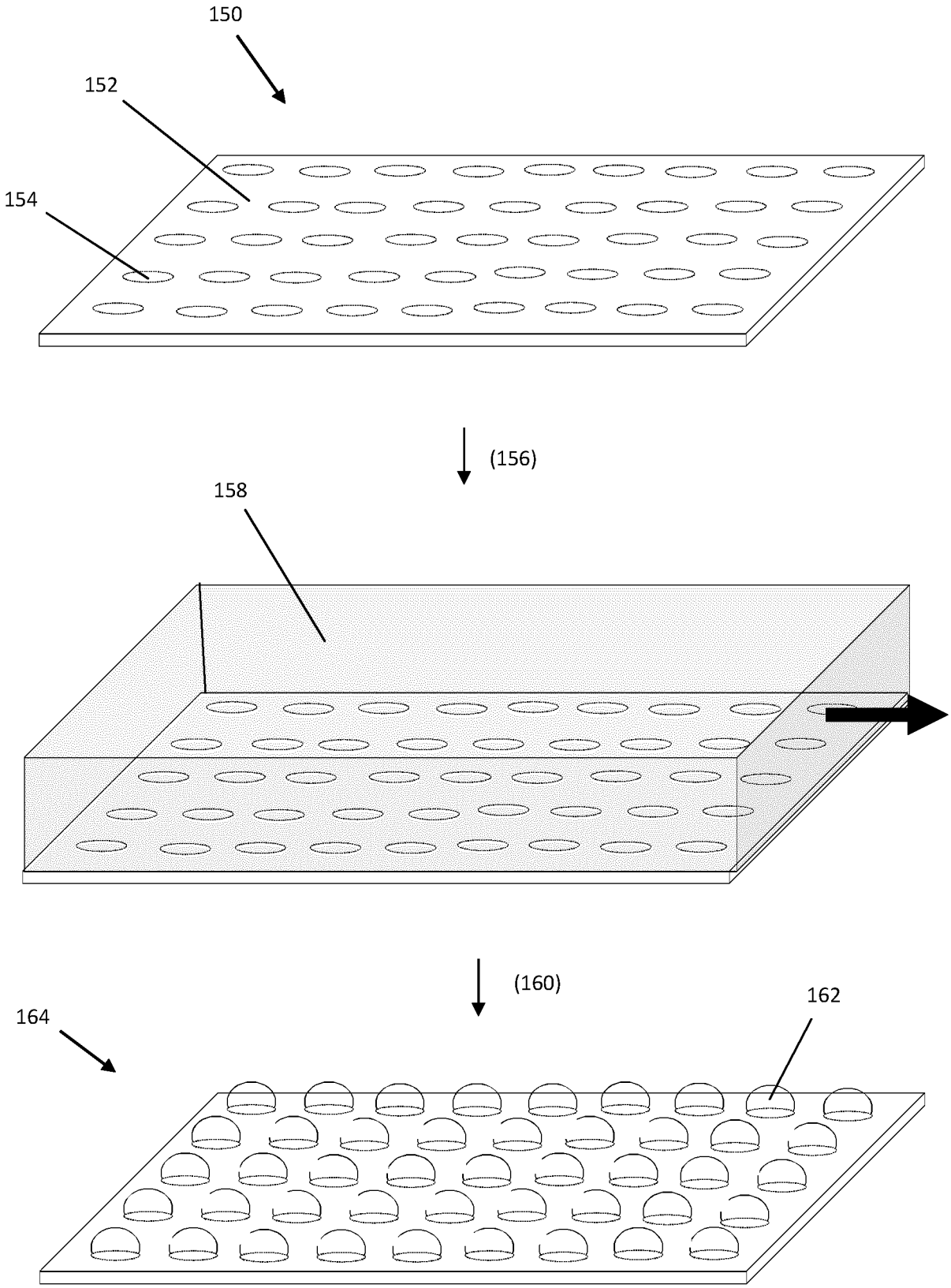



Fig. 1B

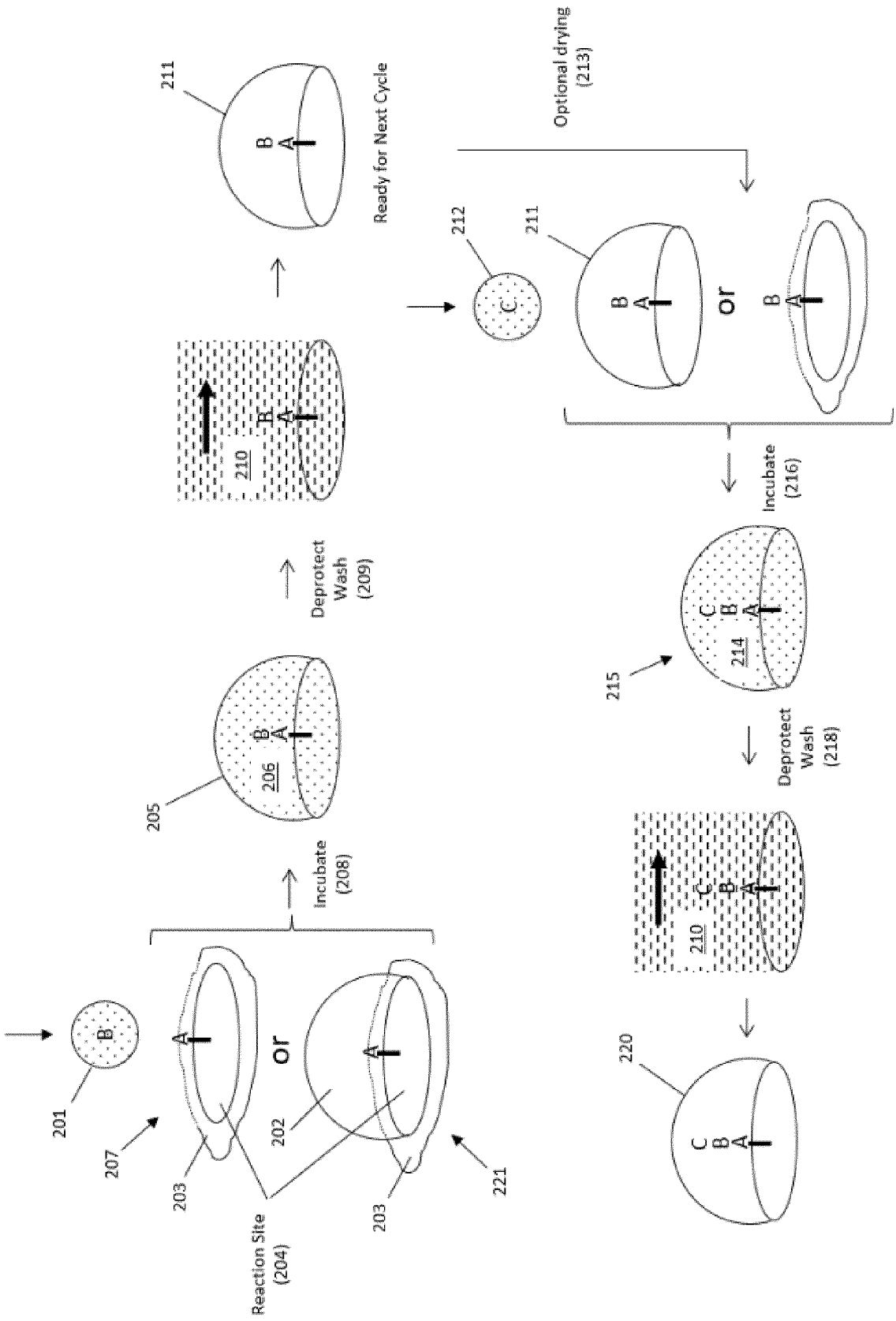

201
207
203
202
or
A
B
C
Reaction Site (204)
Incubate (208)
205
206
Deprotect Wash (209)
210
211
Ready for Next Cycle
Optional drying (213)
212
Incubate (216)
214
215
Deprotect Wash (218)
220
221

Fig. 2A

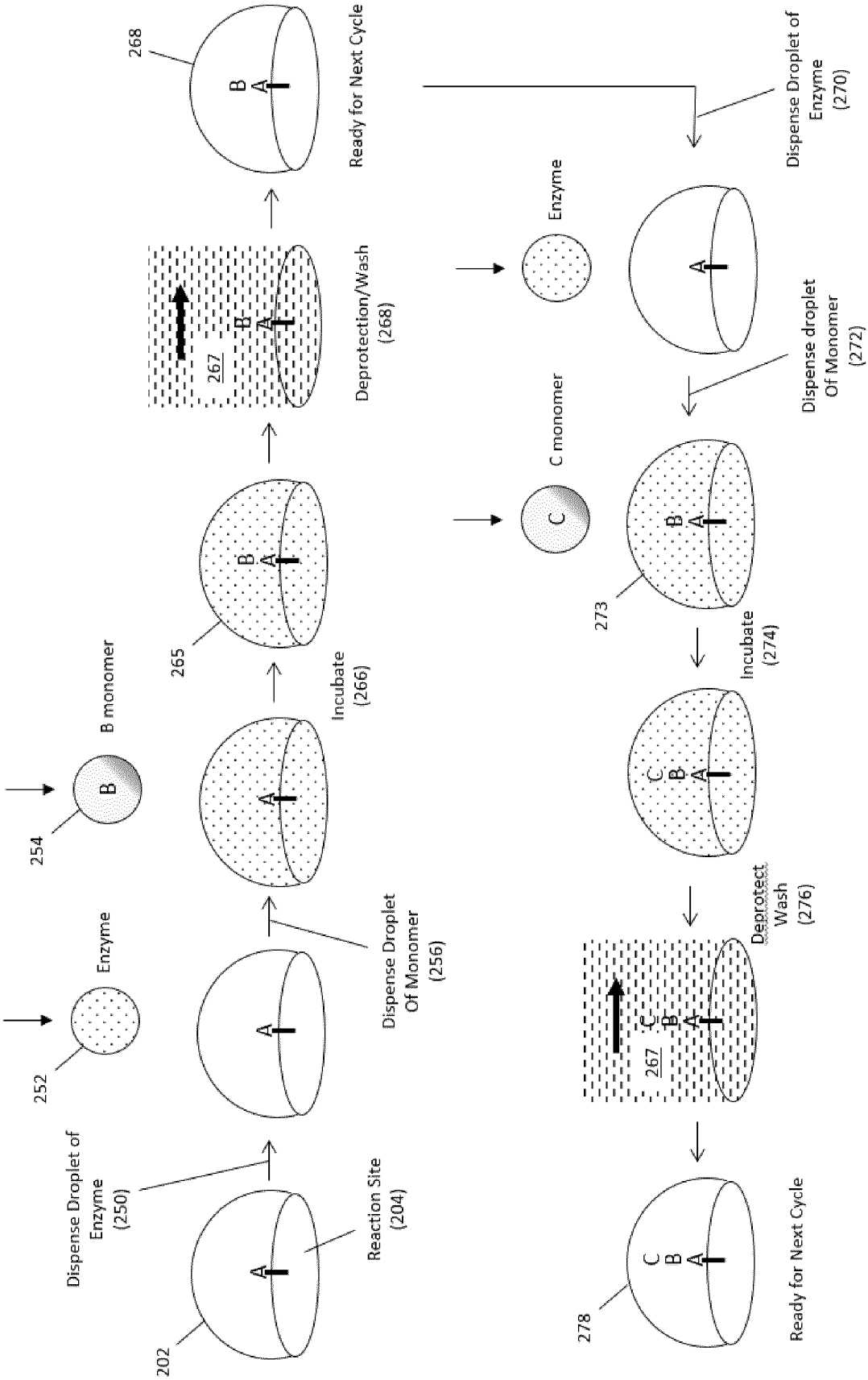

Dispense Droplet of Enzyme (250)
Reaction Site (204)
202
Enzyme
252
Dispense Droplet Of Monomer (256)
B monomer
254
Incubate (266)
265
Deprotection/Wash (268)
267
268
Ready for Next Cycle
Dispense Droplet of Enzyme (270)
Enzyme
Dispense droplet Of Monomer (272)
C monomer
273
Incubate (274)
Deprotect Wash (276)
278
Ready for Next Cycle

Fig. 2B

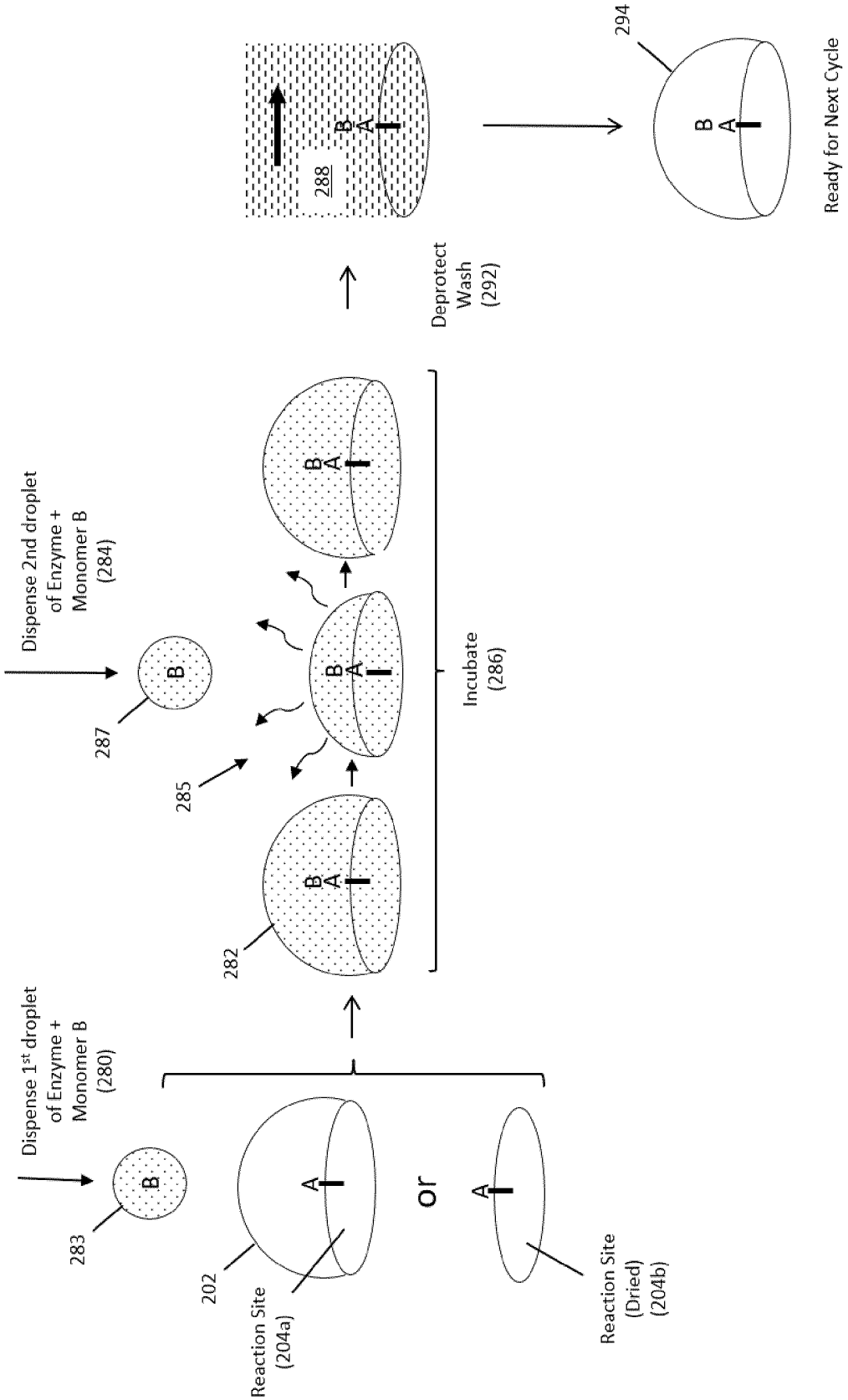

Dispense 1st droplet of Enzyme + Monomer B (280)
283
B
202
A
Reaction Site (204a)
or
A
Reaction Site (Dried) (204b)
282
B
A
Incubate (286)
285
Dispense 2nd droplet of Enzyme + Monomer B (284)
287
B
Deprotect Wash (292)
288
294
Ready for Next Cycle

Fig. 2C

Formation of Synthesis Ar ays

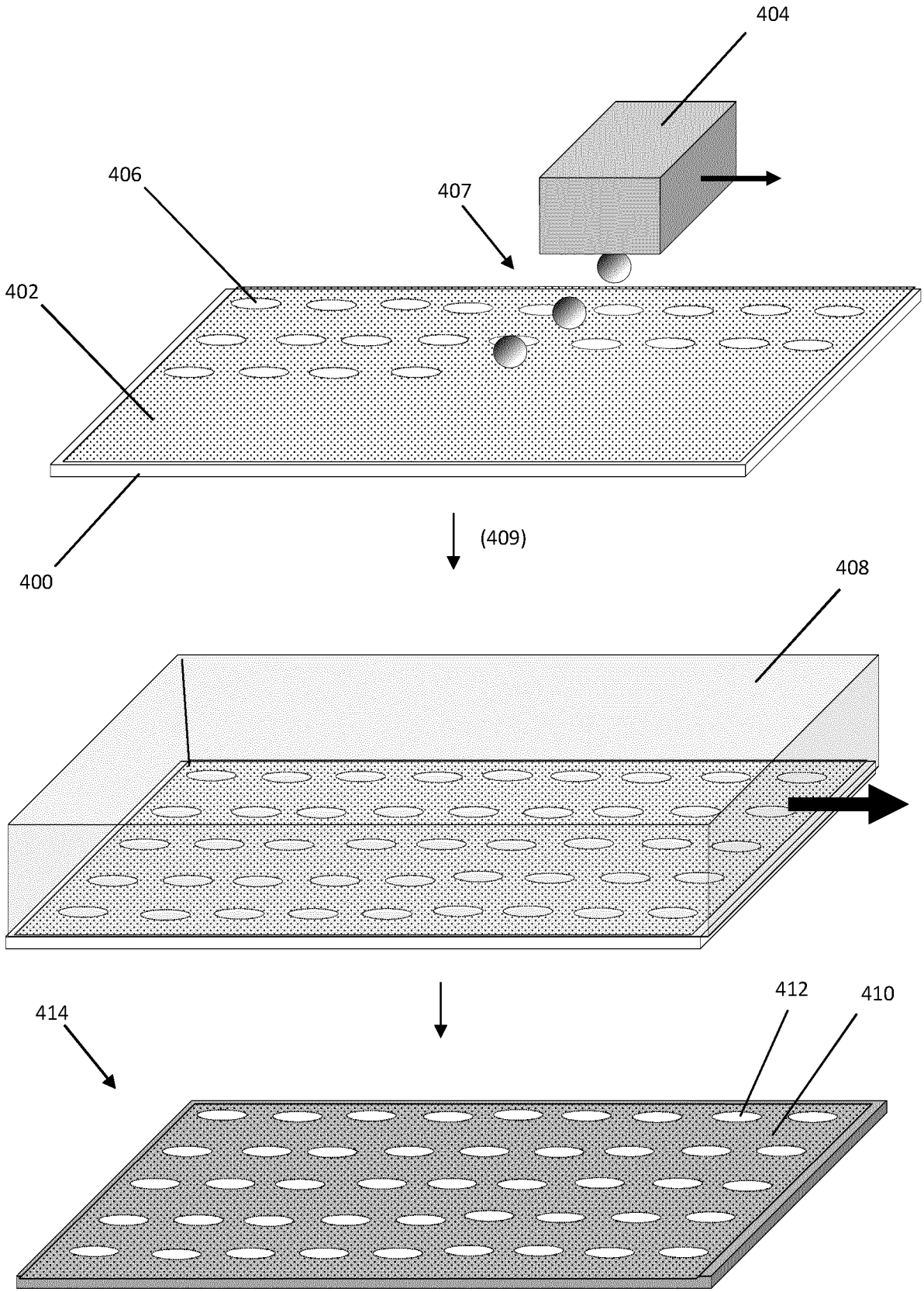



Fig. 3

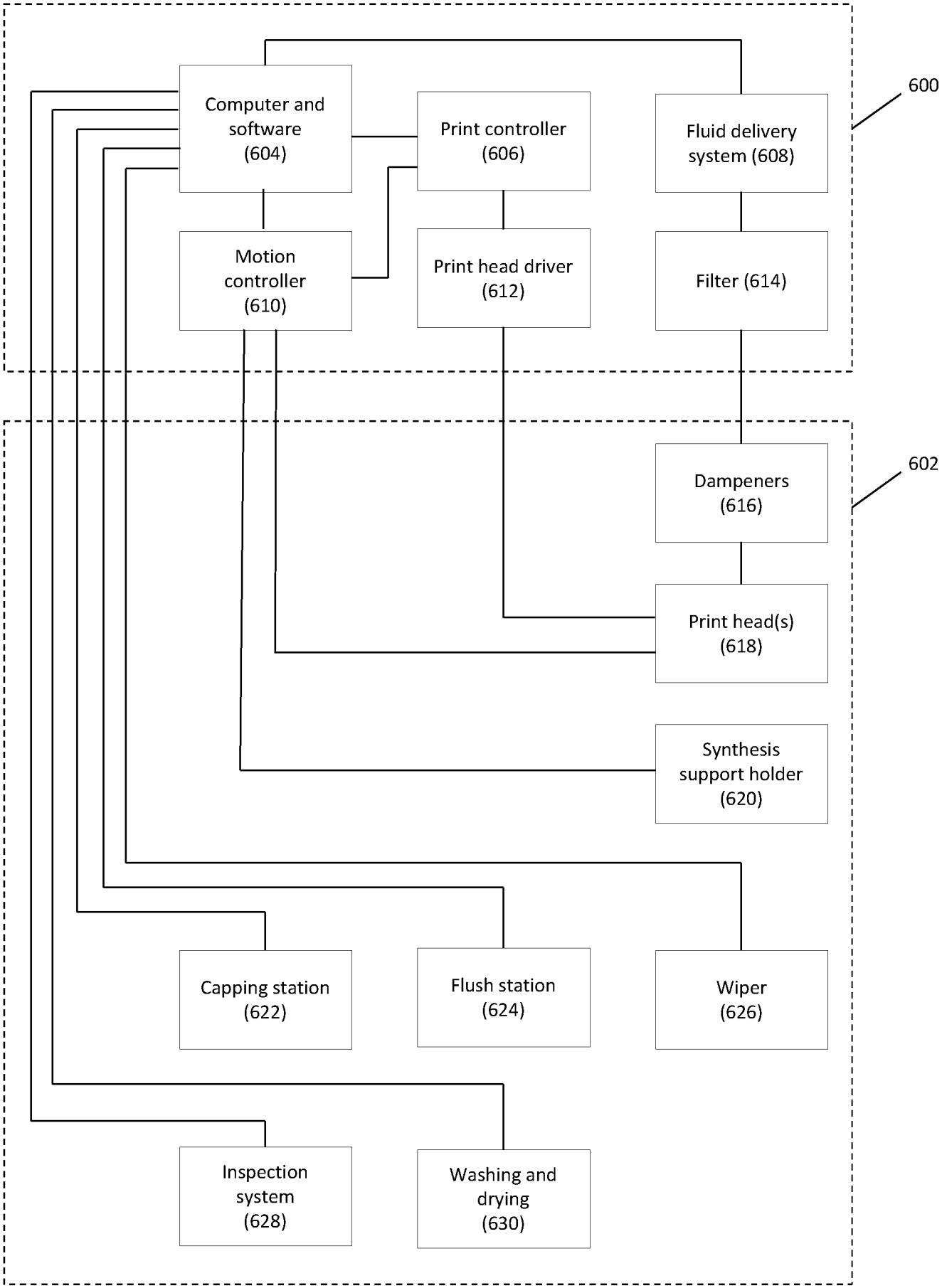

600
Computer and software (604)
Print controller (606)
Fluid delivery system (608)
Motion controller (610)
Print head driver (612)
Filter (614)
602
Dampeners (616)
Print head(s) (618)
Synthesis support holder (620)
Capping station (622)
Flush station (624)
Wiper (626)
Inspection system (628)
Washing and drying (630)

Fig. 4A

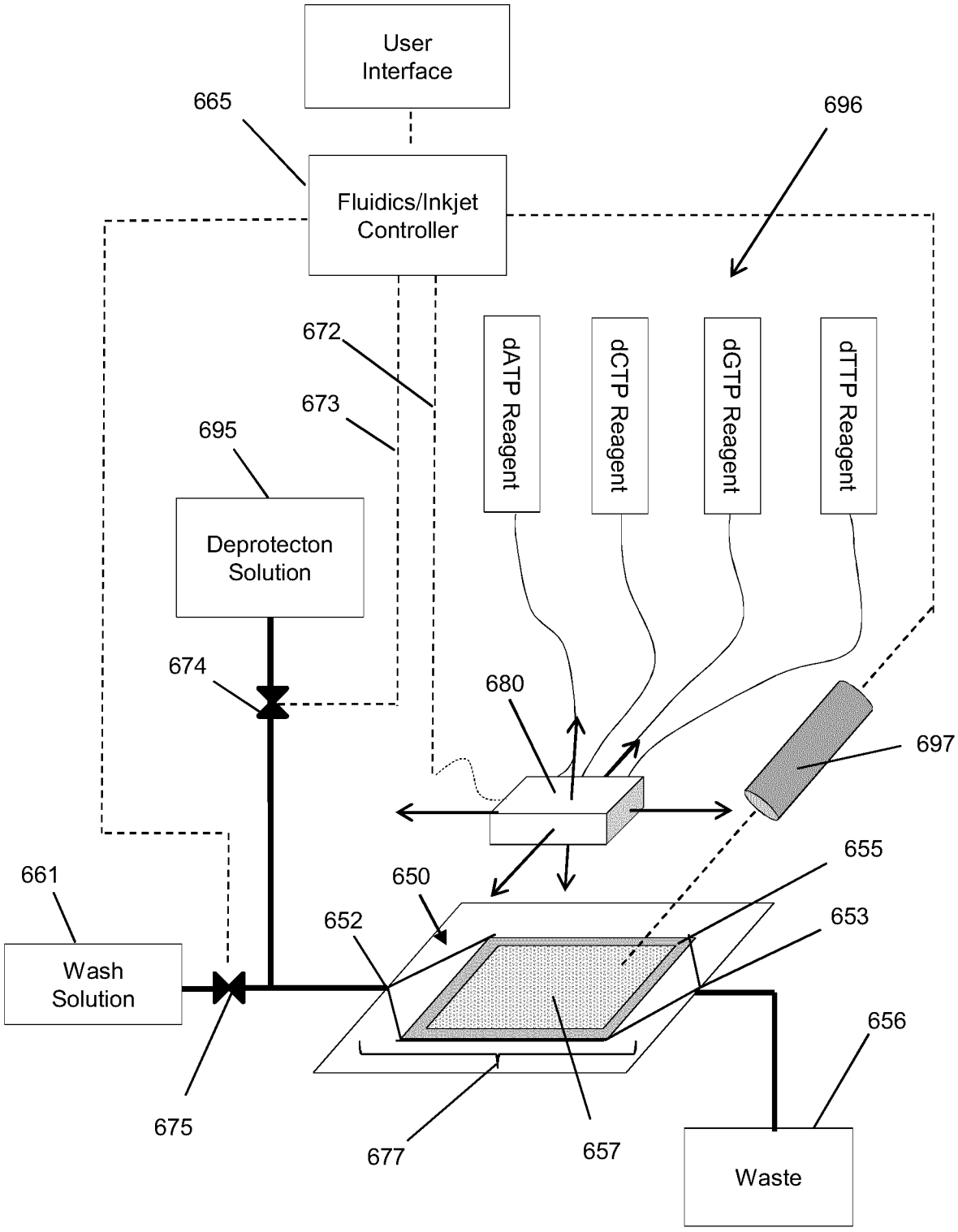

User Interface
665
696
Fluidics/Inkjet Controller
672
673
695
dATP Reagent
dCTP Reagent
dGTP Reagent
dTTP Reagent
Deprotecton Solution
674
680
697
655
661
650
652
653
Wash Solution
656
675
677
657
Waste

Fig. 4B

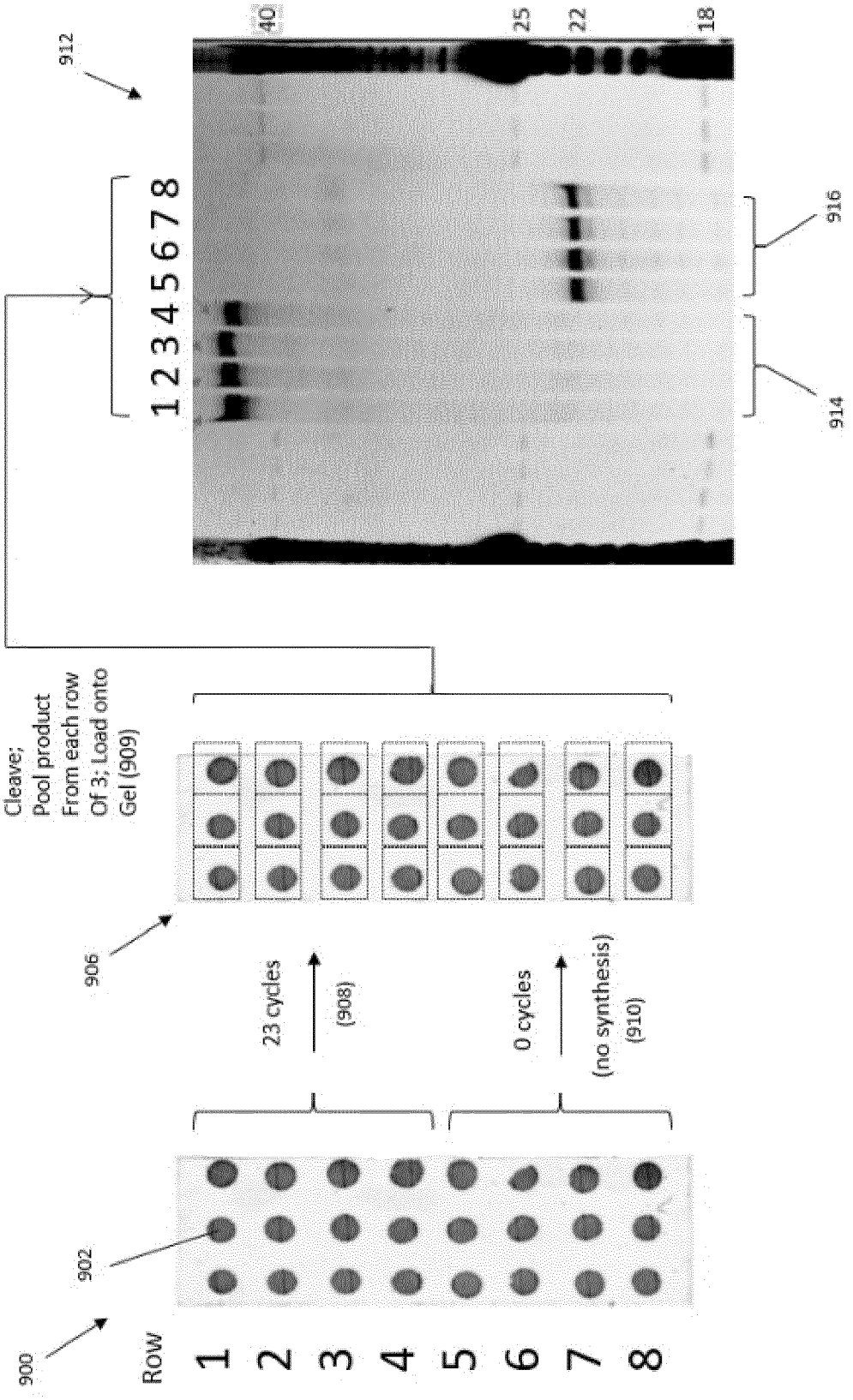

912
40
25
22
18
1 2 3 4 5 6 7 8
916
914
Cleave;
Pool product
From each row
Of 3; Load onto
Gel (909)
906
23 cycles
(908)
0 cycles
(no synthesis)
(910)
902
900
Row
1 2 3 4 5 6 7 8

Fig. 5

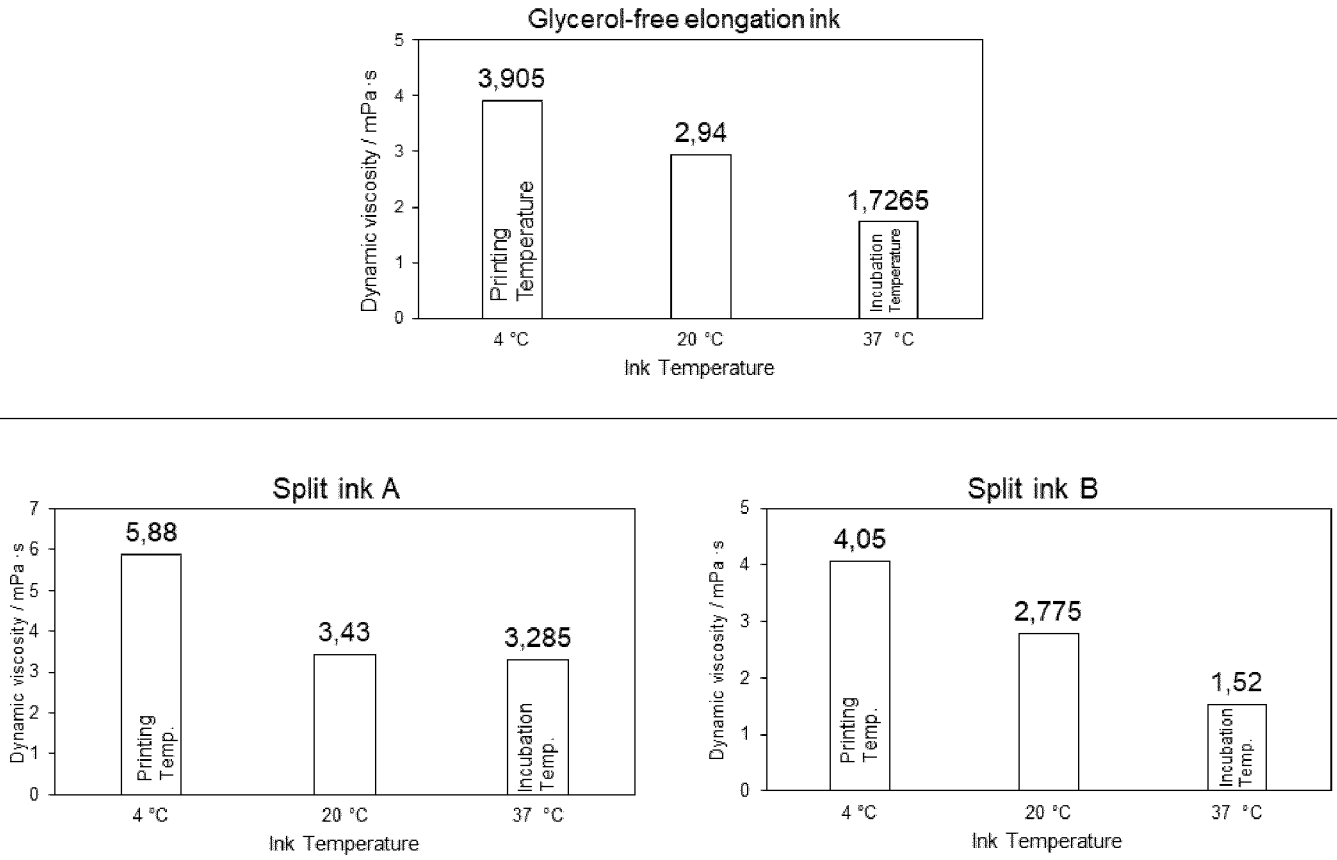

Glycerol-free elongation ink
Dynamic viscosity / mPa·s
3,905
2,94
1,7265
Printing Temperature
Incubation Temperature
4 °C
20 °C
37 °C
Ink Temperature
Split ink A
5,88
3,43
3,285
Printing Temp.
Incubation Temp.
Split ink B
4,05
2,775
1,52

Fig. 6

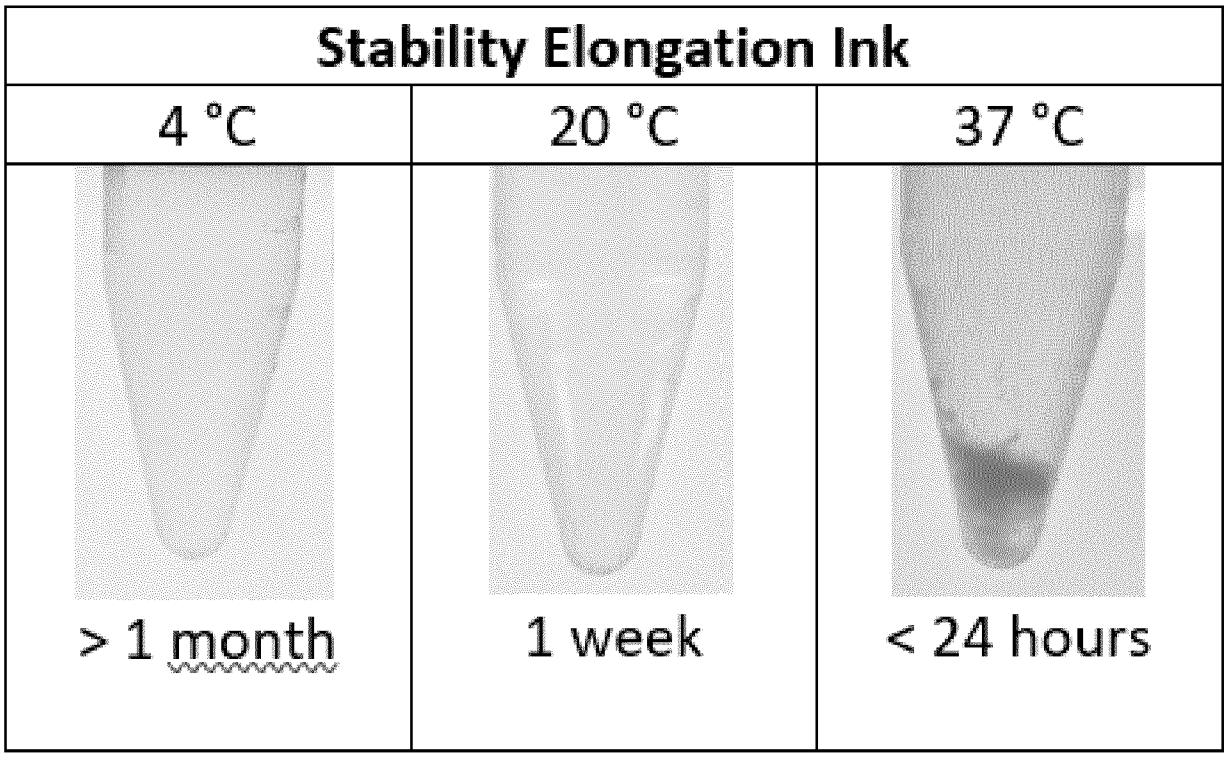

Stability Elongation Ink
4 °C
20 °C
37 °C
> 1 month
1 week
< 24 hours

Fig. 7

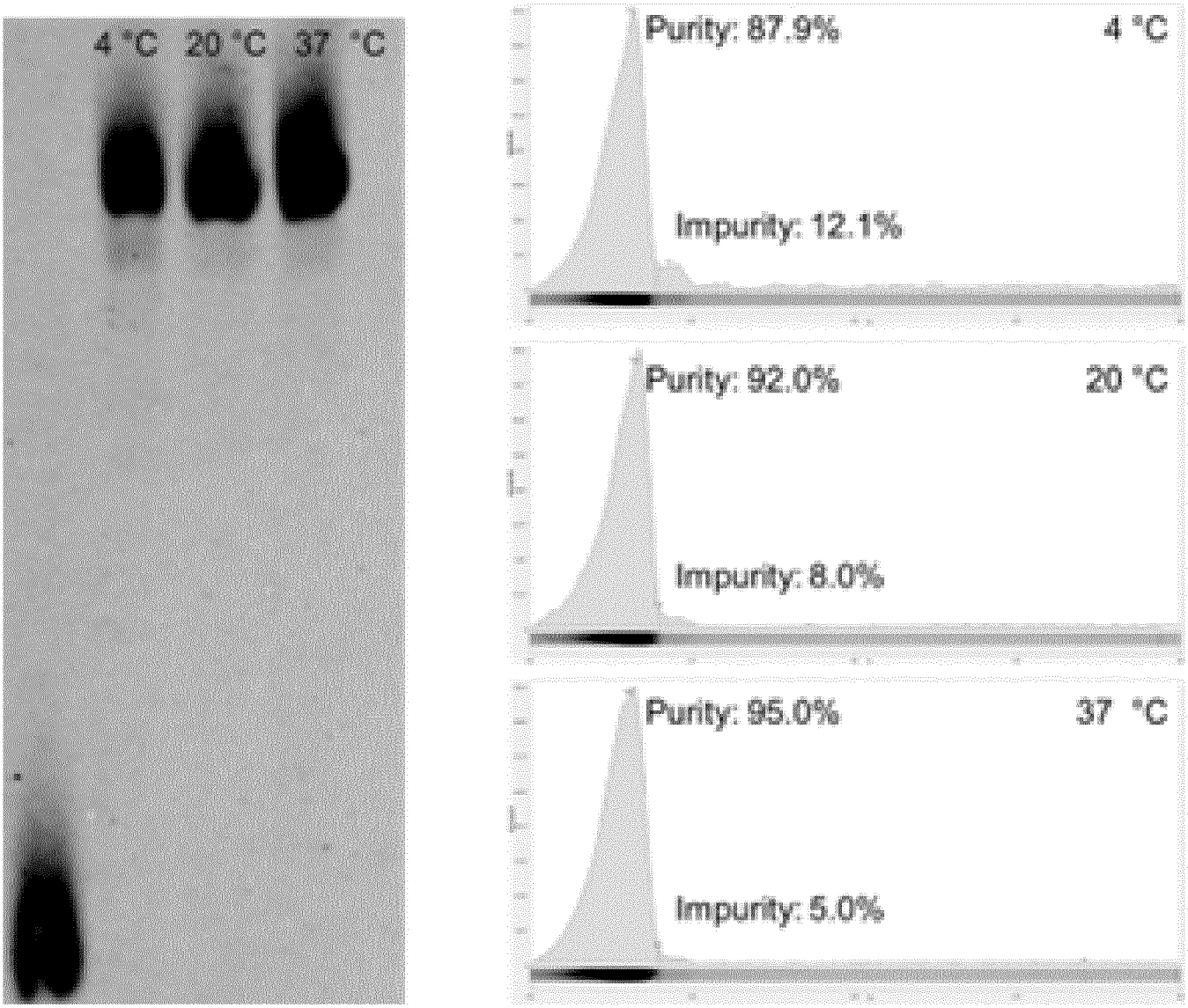

4 °C
20 °C
37 °C
Purity: 87.9%
4 °C
Impurity: 12.1%
Purity: 92.0%
20 °C
Impurity: 8.0%
Purity: 95.0%
37 °C
Impurity: 5.0%

Fig. 8

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/087965

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. C12P19/34 B01J19/00 B41M5/00 C09D11/38 B41M1/00
C09D11/328
ADD.

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)

C09D C40B B41M B01J C12P

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2022/013094 A1 (DNA SCRIPT [FR])<br>20 January 2022 (2022-01-20)<br>cited in the application<br>[0085]-[0088],[00117],[00125],[00129][0005<br>],[0023]-[0024], [0035];<br>page 37, line 19 - line 20; figures 2a,2b | 1-20 |
| A | US 2021/285036 A1 (YIN YIFENG [US] ET AL)<br>16 September 2021 (2021-09-16)<br>[0573],[0910]-[0918] | 1-20 |

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

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"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

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"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

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 May 2024 | 03/06/2024 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Schönwasser, D |

3

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/087965

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Hutchings Ian M. ET AL: "Fundamentals of Inkjet Printing The Science of Inkjet and Droplets (p. 1-364)", , 1 January 2016 (2016-01-01), pages 1-364, XP093057727, ISBN: 978-3-527-68473-1 Retrieved from the Internet: URL:https://elfit.ssru.ac.th/weera_ch/pluginfile.php/48/block_html/content/Inkjet_Printing.pdf [retrieved on 2023-06-26] page 89, paragraph 3; figure 3.24 ----- | 1-20 |
| A | Hutchings Ian M. ET AL: "Fundamentals of Inkjet Printing The Science of Inkjet and Droplets (p. 365-449)", , 1 January 2016 (2016-01-01), pages 365-449, XP093057736, ISBN: 978-3-527-68473-1 Retrieved from the Internet: URL:https://elfit.ssru.ac.th/weera_ch/pluginfile.php/48/block_html/content/Inkjet_Printing.pdf [retrieved on 2023-06-26] ----- | 1-20 |

3

# INTERNATIONAL SEARCH REPORT

**Information on patent family members**

**International application No**
PCT/EP2023/087965

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2022013094 A1 | 20-01-2022 | EP 4182472 A1 | 24-05-2023 |
| | | US 2023313255 A1 | 05-10-2023 |
| | | WO 2022013094 A1 | 20-01-2022 |
| US 2021285036 A1 | 16-09-2021 | US 2021285036 A1 | 16-09-2021 |
| | | US 2023129552 A1 | 27-04-2023 |