



(51) International Patent Classification:

C12Q 1/6806 (2018.01) C12Q 1/6825 (2018.01)

(21) International Application Number:

PCT/US2018/055432

(22) International Filing Date:

11 October 2018 (11.10.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/570,932 11 October 2017 (11.10.2017) US

(71) Applicant: **THE CHARLES STARK DRAPER LABORATORY, INC.** [US/US]; 555 Technology Square, Cambridge, Massachusetts 02139 (US).

(72) Inventors: **MAGYAR, Andrew P.**; 555 Technology Square, Cambridge, Massachusetts 02139 (US). **SPRACHMAN, Melissa M.**; 555 Technology Square, Cambridge,

Massachusetts 02139 (US). **HUANG, Haiyao**; 555 Technology Square, Cambridge, Massachusetts 02139 (US). **HOEFLER, Brian**; 555 Technology Square, Cambridge, Massachusetts 02139 (US). **SWITKES, Michael**; 555 Technology Square, Cambridge, Massachusetts 02139 (US).

(74) Agent: **HOUSTON, J. Grant**; HOUSTONHOGLE LLP, 1666 Massachusetts Avenue, Suite 12, Lexington, Massachusetts 02420 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

(54) Title: GUIDED-DROPLET OLIGONUCLEOTIDE SYNTHESIZER

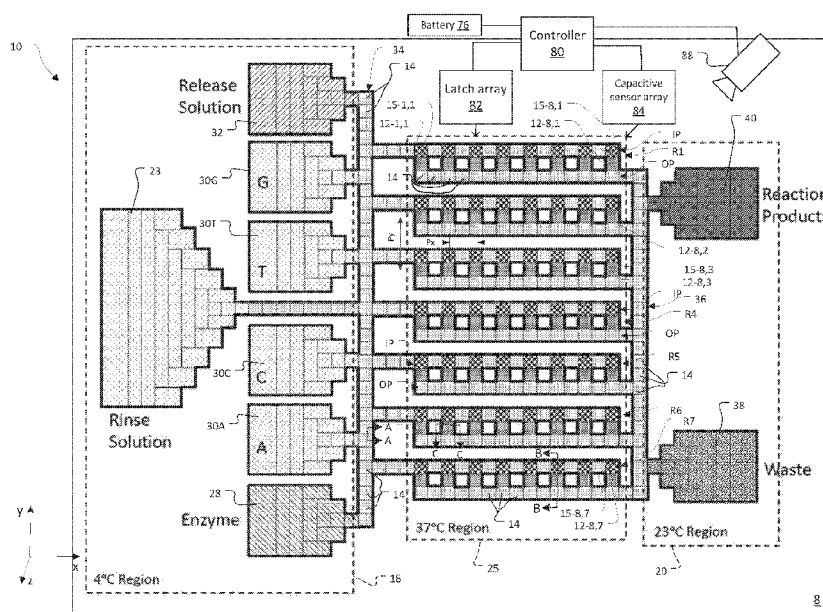


Fig. 1A

(57) Abstract: An oligonucleotide is prepared by the controlled, iterative addition of nucleotides to seed molecules at a functionalized electrode on a digital microfluidic device (DMF). Droplets containing a payload such as specific nucleotides and/or an enzyme, terminal deoxynucleotidyl transferase (TdT), for example, are individually guided, via path electrodes, to the functionalized electrodes, where chains are grown by addition of one nucleotide at a time. The DMF device includes an input section, containing reservoirs for reagents, a reaction section for growing the nucleotide chains and an output section for collecting waste and product. Each unit electrode on the device can be individually and independently addressed.

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

GUIDED-DROPLET OLIGONUCLEOTIDE SYNTHESIZER

RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/570,932, filed on October 11, 2017, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] It has become increasingly feasible to synthesize new strands of DNA with one's own choices for base pairs. Known methods of attaching nucleotides include standard phosphoramidite solid phase synthesis. Also relying on phosphoramidite chemistry are approaches that use printed DNA microarrays for enabling chip-based chemical DNA synthesis with error correction.

[0003] Some recent techniques for synthesizing molecular chains such as single-stranded DNA are described in PCT Application PCT/US17/33770, filed on May 22, 2017 and published on December 28, 2017 as International Publication No. WO 2017/222710A1, the contents of which are incorporated herein by this reference. According to this document, an oligonucleotide product can be prepared by anchoring a first end of a seed oligonucleotide's backbone onto a substrate, the other end remaining free, and attaching one nucleotide at a time to one of these two ends. As a result, the molecule grows progressively longer according to the desire sequence.

[0004] Also described in WO 2017/222710 is an apparatus for optically-verified de novo DNA synthesis. The apparatus includes a microfluidic system with associated pumps and channels leading in and out of a synthesis chamber having a functionalized region on its floor. A single-strand of DNA, to which nucleotides are to be successively attached, can be fixed to this functionalized region. The chamber is in optical communication with both an illumination system, which excites an electron in a fluorophore that is attached to the DNA strand, and a detection system, which detects a signature photon emitted as the excited electron decays into its ground state.

SUMMARY OF THE INVENTION

[0005] A need continues to exist for methods and devices applicable in the synthesis of molecular chains, and, in particular, oligonucleotides. A need also exists for integrated approaches suitable for a wide variety of circumstances.

[0006] Moreover, associated techniques and devices should be appropriate not only in the laboratory but also at remote locations, in the field, at the location of disease out-break. Being able to synthesize DNA chains at a point of need addresses storage and deployment problems raised by the relative short shelf life associated with preassembled DNA sequences currently used for assays, which are undertaken during viral or bacterial outbreaks, for instance. Other attributes desirable for in-field work relate to the simplicity of the device design and ease of its operation.

[0007] In many of its aspects, the invention features technology that integrates various functions to provide a self-contained approach for synthesizing oligonucleotides, possibly even at a point of need. Many embodiments use synthetic methods in which an oligonucleotide is grown by the iterative attachment of one nucleotide at a time, an approach that differs from DNA assembly techniques in which two or more DNA fragments are combined into a larger molecule.

[0008] Typically, the attachment of one nucleotide at a time is conducted on a digital microfluidic (DMF) device using electrowetting-on-dielectric (EWOD) to control reagents and timing of the synthesis. Voltage differences are employed to create and manipulate (combine, divide, move, etc.) droplets along paths of electrodes, with individual electrodes being activated according to a synthesis protocol, for instance. In specific examples, the DMF device is designed to maintain a desired temperature on specific regions of the device. Digital control can be provided by subsystems handling the electric, fluid, and temperature functions. In one implementation the three subsystems are integrated with suitable control software.

[0009] The device can be constructed to include three main sections: the input reservoirs, the reaction region, containing electrodes, typically functionalized with patterned seed oligonucleotides, and the output reservoirs or other fluidics. In one example, a bottom electrode plate is covered by a dielectric layer. Some configurations

also include a top plate preferably made from a transparent material. Hydrophobic coatings can be employed to ease the movement of droplets on electrode pathways.

[0010] The device can be connected to standard or customized microfluidic manifolds. In some situations, the manifold can be reused, whereas the DMF device itself can be disposable or, in some cases, recycled, or partially disposable. Reagents and/or other materials necessary to carry out or a suitable synthetic procedure (protocol), whether currently known or developed in the future, can be provided on the device. In illustrative examples, the device can support controlled DNA syntheses using a template-independent enzyme, terminal deoxynucleotidyl transferase (TdT), a DNA polymerase that catalyzes the addition of nucleotides to the 3' terminus of a growing DNA strand, to add nucleotides, thereby synthesizing an oligonucleotide. Specific embodiments rely on the addition of molecular subunits onto the end of an immobilized seed molecule. In an illustrative example, the synthesis is conducted by the controlled addition of one nucleotide at the time. In many cases, the device serves to synthesize multiple (two or more) DNA sequences in a single run.

[0011] In one aspect, the invention features an apparatus (device) for synthesizing an oligonucleotide product that comprises a plurality of nucleotides. The apparatus includes an input section, a reaction section and an output section. Temperature regulators, thermoelectric coolers and/or heaters, for instance, can be used to maintain a desired temperature, e.g., in the reaction region and/or the input region. Pluralities of unit electrodes perform in different capacities. Some electrodes are functionalized with seed molecules and others provide pathways, e.g., for moving droplets to and from the seed molecules. Some implementations also include sensing electrodes, e.g., for providing information regarding the presence of a droplet at a certain location and/or its properties. The device can be coupled by one or more systems that control formation, movement, and/or removal of droplets, microfluidics, and/or the temperature on different sections of the device.

[0012] In many embodiments, the device is configured to individually propel nucleotide-containing droplets along selected paths to selected functionalized electrodes. A droplet containing a nucleotide can also include an enzyme and/or other materials needed to attach the nucleotide. A blocking group linked directly or indirectly to the nucleotide prevents attachment of more than one of the nucleotides present in the droplet.

Removal of this blocking group renders the chain ready for attachment of the next desired nucleotide, provided in a subsequent droplet.

[0013] In an alternative embodiment no blocking group is used, and the concentration of the reagents and the duration of exposure to the synthesis region controls synthesis.

[0014] In some cases, the electrostatic force applied to pathway electrodes are selected to cause a droplet to arrive at the functionalized electrode. In other cases, the electrostatic forces applied to pathway electrodes are selected to move a droplet from the functionalized electrode to an output region. Hydrophobic surfaces can be used to promote a gliding motion of the droplets. Electrostatic forces applied to functionalized electrodes are selected to immobilize the droplet for a suitable time period and then release the droplet.

[0015] The device can be constructed to include a bottom electrode plate with a dielectric layer that can be patterned for attaching seed nucleotides. Many embodiments of the device also include a top plate. Hydrophobic surfaces can promote the integrity and movement of droplets.

[0016] During operation, droplets of desired compositions (e.g., nucleotide precursor and/or enzyme) are individually directed, via selected electrode pathways, from reservoirs or sources in the input region to specific functionalized electrodes. A droplet is maintained at the functionalized electrode for a time period sufficient to allow attachment of the unit nucleotide. Droplets are then directed to a waste reservoir in the output region. When the synthesis is completed, droplets containing a suitable agent are directed, via pathways electrodes, to the functionalized electrodes to release the product oligonucleotide, which is then collected on the output region.

[0017] In some embodiments, a plurality of functionalized electrodes is grouped into a single logical electrode. In others, adjacent functionalized electrodes are grouped into a single logical electrode.

[0018] Yet other practices include selecting the functionalized electrode to be a virtual functionalized-electrode that comprises at least two physical functionalized-electrodes.

[0019] Further practices include causing the droplet to move along at most one contact surface.

[0020] The apparatus and method described herein can be used in the laboratory or at remote locations and can be adapted for synthesizing molecules (e.g., polymers or oligomers) other than oligonucleotides.

[0021] In many of its aspects, the invention combines advantages associated with synthetic methods that attach one nucleotide at the time with digital microfluidic (DMF) technology in which voltage difference are used to create and/or manipulate (combine, divide, move, etc.) droplets along paths of electrodes.

[0022] Advantageously, the device described herein can be free-standing (also referred to herein as self-contained and independent), integrating the functions required to carry out oligonucleotide syntheses, e.g., at a point of use. Embodiments of the invention address problems raised by the short shelf life encountered with already-made DNA arrays and can find applications during virus or bacteria outbreaks around the world. For example, the device described herein can increase storage and distribution options relative to premade DNA arrays.

[0023] The device can be designed for compatibility with lab-standard 96-well, 384-well, and other well-plate formats. As such, the pitch between functionalized locations is 4.5 millimeters +/- 1 millimeter in both x and y axes, or the pitch between the pitch between functionalized locations is 9.0 millimeters +/- 1 millimeter in both x and y axes.

[0024] The device and method described herein can be easily adapted to other synthetic procedures, to prepare, for example, polymers or oligomers such as peptides or molecular chains assembled by click chemistry.

[0025] The above and other features of the invention including various novel details of construction and combinations of parts, and other advantages, will now be more particularly described with reference to the accompanying drawings and pointed out in the claims. It will be understood that the particular method and device embodying the invention are shown by way of illustration and not as a limitation of the invention. The principles and features of this invention may be employed in various and numerous embodiments without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] In the accompanying drawings, reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale; emphasis has instead been placed upon illustrating the principles of the invention. Of the drawings:

[0027] FIG. 1A is a top plan view of a DMF device according to one embodiment of the invention;

[0028] FIG. 1B is a schematic side cross-sectional of the DMF device;

[0029] FIG. 2 is a top plan view of a DMF device according to another embodiment of the invention;

[0030] FIG. 3A and 3B are cross-sectional views along A-A and B-B of FIG. 1A, respectively;

[0031] FIG. 4A and 4B are an exploded cross-sectional view and a side cross-sectional view along C-C of FIG. 1 showing components and steps used to assemble a DMF devices at the functionalized unit electrodes 12, according to a printed circuit board embodiment of the invention;

[0032] FIG. 4C and 4D are an exploded cross-sectional view and a side cross-sectional view along C-C of FIG. 1 showing components and steps used to assemble a DMF devices at the functionalized unit electrodes 12, according to glass plate embodiment of the invention;

[0033] FIG. 5 is a block diagram showing the subsystems controlling a device and/or synthesis according to aspects of the invention; and

[0034] FIG. 6 shows a grouping of functionalized regions to form a compound functionalized unit electrode in another arrangement according to embodiments of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0035] The invention now will be described more fully hereinafter with reference to the accompanying drawings, in which illustrative embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are

provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

[0036] As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. Further, the singular forms and the articles "a", "an" and "the" are intended to include the plural forms as well, unless expressly stated otherwise. It will be further understood that the terms: includes, comprises, including and/or comprising, when used in this specification, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof. Further, it will be understood that when an element, including component or subsystem, is referred to and/or shown as being connected or coupled to another element, it can be directly connected or coupled to the other element or intervening elements may be present.

[0037] It will be understood that although terms such as "first" and "second" are used herein to describe various elements, these elements should not be limited by these terms. These terms are only used to distinguish one element from another element. Thus, an element discussed below could be termed a second element, and similarly, a second element may be termed a first element without departing from the teachings of the present invention.

[0038] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

[0039] This invention generally relates to building molecular chains, and in particular, to synthesizing oligonucleotides.

[0040] Various synthetic approaches can be employed, as currently known in the art or as developed in the future. Possible chemistries that can be used are described in Griswold et al., International Publication No. WO 2018/102554 A1, Sprachman et al., International Publication No. WO 2017/222711 A1, and Magyar et al., in International Application No. PCT/US2018/033798 with the title *Modified Template-Independent DNA Polymerase*, filed

on May 22, 2018. An alternative compatible chemistry for non-DNA polymer synthesis is described in Magyar et al., International Publication No. WO 2018/1293281 A1. These documents are incorporated herein by reference in their entirety.

[0041] In many embodiments, a controlled DNA synthesis using a template-independent enzyme, terminal deoxynucleotidyl transferase (TdT), and a DNA polymerase that catalyzes the addition of nucleotides to the 3' terminus of a growing DNA strand, is employed to generate a product oligonucleotide.

[0042] The synthesis is conducted by the controlled, iterative addition of molecular subunits, first onto the end of an immobilized seed molecule and then to a growing chain. In the case of growing a DNA chain, the synthesis involves adding one nucleotide (naturally occurring, a synthetic analog, etc.) at the time. In further embodiments, the synthetic process employed prevents subsequent undesired additions of the same nucleotide. In many cases, the synthetic route uses an enzyme, such as, for instance, terminal deoxynucleotidyl transferase (also commonly known by the abbreviation TdT or TDT) for attaching the nucleotide at the growing end. The nucleotide can be added to the backbone of the strand, typically at the 3' end. For a tethered arrangement, the addition can occur at the free end or at the attached end. As a result, the DNA chain grows progressively longer. The process will eventually be completed, at which point the chain becomes the oligonucleotide product.

[0043] Tethering a seed oligonucleotide can be conducted by forming a covalent or a non-covalent bond between the oligonucleotide and a substrate.

[0044] Typical approaches for forming a covalent bond include those in which the bond is between a 5' amine-modified oligonucleotide and one of a surface-immobilized epoxide, an aldehyde, and a succinimidyl ester, those in which the bond is between a 5'-alkynyl-modified oligonucleotide and a surface-immobilized azide, and those in which the bond arises as a result of a click chemistry reaction, such as 1,3-dipolar cycloaddition between a 5'-alkynyl-modified oligonucleotide and a surface-immobilized azide, or Staudinger ligation.

[0045] An illustrative approach for forming a non-covalent bond involves forming a bond between a 5'-biotinylated oligonucleotide and a surface-immobilized streptavidin. Another practice that involves forming a non-covalent bond is one that includes hybridization.

[0046] The product oligonucleotide can be removed from its tethered configuration by using a moiety that facilitates release of the growing chain, followed by the addition of a final nucleotide required to prepare the oligonucleotide product. Suitable approaches include using a moiety comprising a cleavage site, such as, for instance, a site for photochemical cleavage, a site for enzymatic cleavage, or a site for chemical cleavage. Examples of a site for chemical cleavage include a phosphorothiolate linkage and a site for cationic silver reagent cleavage.

[0047] The invention generally relates to a method and device that combine a synthetic approach such as described above (e.g., the iterative addition of one nucleotide at a time) with digital microfluidics (DMF) to prepare an oligomer or polymer such as, for instance, an oligonucleotide.

[0048] In some embodiments the DMF device is part of a system that includes a suitable microfluidic arrangement, external to the device itself and configured for supplying and removing materials to and from the device, and a control function for conducting various operations, controlling device conditions and so forth.

[0049] Digital microfluidic (DMF) technology is described, for example, by Cho, Sung Kwon, Hyejin Moon, and Chang-Jin Kim, *Creating, transporting, cutting, and merging liquid droplets by electrowetting-based actuation for digital microfluidic circuits*, Journal of Microelectromechanical Systems 12.1, pp. 70-80 (2003). To date, this technology has found applications in PCR (see, e.g., Norian, Haig, et al., *An integrated CMOS quantitative-polymerase-chain-reaction lab-on-chip for point-of-care diagnostics*, Lab on a Chip 14.20, pp. 4076-4084 (2014); DNA sequencing (see, e.g., Boles, Deborah J., et al., *Droplet-based pyrosequencing using digital microfluidics*, Analytical Chemistry 83.22, pp. 8439-8447 (2011); DNA assembly from DNA fragments (see, e.g., Yehezkel, Tuval Ben, et al., *Synthesis and cell-free cloning of DNA libraries using programmable microfluidics*, Nucleic Acids Research 44.4 pp. e35-e35 (2016); and bacterial transformations (see, e.g., Gach, Philip C., et al., *A droplet microfluidic platform for automating genetic engineering*, ACS Synthetic Biology 5.5, pp. 426-433 (2016). These documents are incorporated herein by this reference in their entirety.

[0050] Embodiments of a DMF device for preparing a plurality of product oligonucleotides by starting with a seed oligonucleotide and adding one nucleotide at a

time are described with reference to FIGS. 1A, 1B, and 2. In some cases the product nucleotides formed on the device have different sequences.

[0051] Shown in FIG. 1A, for example, is DMF device or chip 10 fabricated on a substrate 8. The device includes several types of electrodes according, for instance, to their functions. Unit path electrodes 14 (shaded light grey) are used to direct (propel) droplets to and from reservoirs 23, 28, 30A, 30C, 30T, 30G, 32, 38, 40. Functionalized unit electrodes 12 (shaded dark grey) are electrodes functionalized with DNA, while sensing unit electrodes 15 (shaded with a checkerboard pattern) are sensing electrodes that can both propel the droplets but also detect the presence of a droplet or the absence of a droplet.

[0052] In the illustrated embodiment, capacitive and optical droplet sensing is utilized. In more detail, capacitive sensing (capacitive sensor array 84) is used to detect the presence and size of droplets at the sensing unit electrodes 15 and at the functionalized electrodes 12. In addition, detection and tracking of the droplets on the unit path electrodes 14 is further utilized in some example to thus track and monitor the droplets before they are directed to the functionalized electrodes 12 for DNA synthesis and after the droplet have left the functionalized electrodes 12.

[0053] Optical droplet sensing and tracking also can be employed. In this case, A camera 88 captures images of the chip 10 and utilizes image analysis to detect the presence or absence of droplets at a particular location, e.g., unit path electrodes 14, functionalized unit electrodes 12, and/or sensing unit electrodes 15. A camera can also provide information about the size of the droplets as well as the quantities of fluids contained in each of the reservoirs 23, 28, 30A, 30C, 30T, 30G, 38, 40. In the preferred implementation, the camera 88 provides images to the controller and the controller the executes image analysis to separate the background and static portions of the image associated with the device 10 from the foreground portions of the image associated with the moving droplets.

[0054] The functionalized unit electrodes 12 are arranged in an array. In the specific illustrative example, the functionalized unit electrodes 12 are arranged in an 8 by 7 array. Thus, the functionalized unit electrodes 12 are identified by their Cartesian position x, y in the array. In addition, in the illustrated embodiment, the sensing unit electrodes 15 are associated with each of the functionalized unit electrodes 12 and similarly identified by their Cartesian position x, y .

[0055] In general, the size of the arrays typically depends on the overall size of the device, route configurations and/or other parameters. Nevertheless, each unit electrode in the device 10 is independently controlled by a controller 80. In more detail, the controller determines the electrical potential to be applied to each of the unit electrodes: unit path electrodes 14, functionalized unit electrodes 12, and sensing unit electrodes 15 of the device 10. This electrical potential information is loaded into a latch array 82 that contains a latch for each of the electrodes. Each latch in the latch array 82 is electrically connected to its individual electrode 12, 14, 15 and holds the electrical potential for its dedicated electrode. Preferably, the electrical connections between each latch of the latch array and the associated electrode 12, 14, 15 is provided by fabricating electrical traces on a top or bottom surface or an inner surface of the substrate 8 on which the device 10 is fabricated.

[0056] In an illustrative example, each of the unit path electrodes 14, functionalized unit electrodes 12, and sensing unit electrodes 15 are preferably less the 5 millimeters (mm) by 5 mm in their x axis and y axis dimensions. Currently, the electrodes are shaped as square electrodes having dimensions of 1 mm by 1 mm to about 0.7 mm by 0.7 mm, or less. Nevertheless, other dimensions and/or shapes can be employed.

[0057] In some embodiments, the device 10 is designed for physical compatibility with lab-standard 96-well, 384-well, and other commercial well-plate formats. In one such embodiment, the functionalized unit electrodes 12 are arranged in a 12 by 8 array in which the pitch between functionalized unit electrodes 12 is $P_x, P_y = 9.0 \text{ millimeters} \pm 1 \text{ millimeter}$ in both x and y axes. In another such embodiment, the functionalized unit electrodes 12 are arranged in a 24 by 16 array in which the pitch between functionalized unit electrodes 12 is $P_x, P_y = 4.5 \text{ millimeters} \pm 1 \text{ millimeter}$ in both x and y axes.

[0058] DMF device 10 can be thought of as being comprised of sections or regions. Shown in FIG. 1, for example, are: input reservoir region 18; reaction region 25; and output reservoir region 20.

[0059] Input region 18 includes various input reservoirs or sources providing materials required by the synthetic protocol. For preparing, a DNA chain, for example, the sources shown are: reservoirs 30G (guanine), 30T (thymine), 30C (cytosine) and 30A (adenine), optional enzyme reservoir 28 for storing an enzyme (e.g., TdT) that promotes nucleotide attachment, and a release solution reservoir 32, for storing a release agent that releases the oligonucleotide product from the reaction section 18 once the synthesis is completed.

[0060] In some implementations, the enzyme, TdT, for instance, is premixed with one, more or all the G, T, C and A nucleotide solutions, rendering redundant the use of enzyme reservoir 28, or even its presence on the chip.

[0061] Input region 18 can also include rinse reservoir 23 for storing a suitable solution for rinsing remaining traces of a nucleotide prior to introducing the subsequent nucleotide.

[0062] The input reservoirs can be constructed from square and/or rectangular electrodes of different sizes, each reservoir electrode being controlled independently by the controller 80 via the latch array 82. When combined, the reservoir electrodes form the foot print of the reservoir. In an illustrative example, reagent reservoirs 30G, 30T, 30C and 30A are about 6 mm by 5 mm or smaller, with a capacity of 10 microliters (μ l) or smaller. In another illustrative example, reagent reservoirs 30G, 30T, 30C and 30A hold a solution volume of 50 μ l or more. Typically, the rinse reservoir is larger, e.g., 2 to 3 times as large as reservoirs 30G, 30T, 30C and 30A. The size of a reservoir can be increased or decreased by adding or removing unit electrodes.

[0063] An example of a type of electrode pattern that can be used for droplet generation is shown in Jones et al., "Dielectrophoretic liquid actuation and nanodroplet formation", Journal of Applied Physics 89, 1441 (2001).

[0064] The illustrated reaction region 25 includes the 8 by 7 array of functionalized unit electrodes 12 electrodes. The array is arranged in seven (7) rows R1-R7. Each row includes an input pathway IP comprised of alternating unit path electrodes 14 and sensing unit electrodes 15. Each row also includes an output pathway OP that is comprised of unit path electrodes 14, although the output pathway OP further includes its own sensing unit electrodes 15, in other embodiments.

[0065] An input bus 34 of unit path electrodes 14 connects reservoirs or sources 23, 28, 30G, 30C, 30A, 30T, and 32 to each of the input paths IP of each of the rows R1-R7.

[0066] On the other hand, an output region 20 includes waste reservoir 38 for collecting spent materials. In disposable devices, the spent materials can be disposed of with the device. Different approaches can be used to implement recycling schemes. In other examples, the waste reservoir 38 could simply be a fluidic channel conveying the spent materials off of the substrate 8.

[0067] Also present on the output section is reservoir 40 for receiving reaction products. Reservoirs in the output region 20 can have volumes of a few milliliters (ml), for example, within the range of 5 to 10, 15, 20 or more ml.

[0068] An output bus 36 of unit path electrodes 14 connects the output paths OP of each of the rows R1-R7 to the reservoirs 38, 40 of the output region 20.

[0069] Preferably, the input section 18 is maintained at a relatively low temperature to preserve the integrity of input materials, such as, for example, the enzymes in reservoir 28. A suitable temperature in this case is typically less than 20°C, and preferably lower such as less than 10°C, or about 4°C. In contrast, reactions occurring in reaction region 25 benefit from higher temperatures (typically greater than 20°C or more than 30°C or preferably about 37°C, in one example). In some cases, even higher temperatures may be desirable, to promote the release of the oligonucleotide product from the substrate, for instance. Moreover, the temperature can be modulated by the controller 80 depending on the current reaction that is programmed to be taking place on the device 10. An air gap or a different type of insulation or temperature shielding can be provided between the input section 18 and the reaction region 25.

[0070] Fig. 1B is a side view illustrating one way of maintaining the temperature gradients across the substrate 8 of the device 10.

[0071] In more detail, the substrate 8 of the device 10 is divided up into its different regions: input region 18, reaction region 25, and the output region 20.

[0072] Moreover, a number of temperature transducers 92 are attached to, or fabricated upon, the substrate 8. In one embodiment, there are separate thermistors associated with each of the regions such as thermistor 92-1 for the input region, possibly multiple thermistors 92-2, 92-3 for the reaction region 25 and a thermistor 92-3 for the output region 20.

[0073] On the backside of the substrate 8, a thermally-conductive frame 94, such as a heat spreader or heatsink, is in thermal contact with the input region 18 and possibly the output region 20. The frame 94 provides a thermal conduction path to a Peltier or thermoelectric cooler 96. On the other hand, on the backside of the reaction region 25, a resistive heater 98 is in a thermally conductive communication with the reaction region 25.

[0074] Through this arrangement, the controller 80 maintains the desired thermal gradients across the substrate 8. In more detail, the controller 80 determines the temperatures of the input region 18, reaction region 25, and the output region 20 via possibly averaging the temperatures read from the thermistors 92. Based on this information, the controller controls the operation of the thermoelectric cooler 96 and the resistive heater 98 via the thermoelectric cooler driver 72 and the resistive heater driver 74.

[0075] In general, the device 10 can be part of a system that incorporates one or more microfluidic arrangement(s) and additional control function(s). A typical microfluidic arrangement will include external reservoirs for supplying and/or collecting materials to the device, associated pumps and conduits. The controller 80 and any additional controls can be provided by a suitable computer or microcontroller and software platform. In one embodiment, the controller 80 is an embedded system that is integrated with the remaining parts of device 10 as a disposable unit. As such, device can further include its own battery power source 76, with its batteries installed on top or bottom face of the substrate 8.

[0076] The DMF device 10 is constructed using a bottom electrode plate coated with a dielectric layer or film. Preferred designs also include a top plate that can be transparent. In some implementations, the top plate is omitted. Various layers of materials can be cut manually or laser-cut and can be laminated together to form a DMF device.

[0077] FIG. 2 shows another embodiment of a DMF device or chip 10. It includes a laterally extending input bus 34. The bus supplies droplets to input paths IP of three longitudinally extending rows R1, R2, R3. The output paths OP of each of these rows R1, R2, R3 then connect to an output bus 36 of path unit electrodes 14.

[0078] Here, the output region 20 includes several collection reservoirs 40A, 40B, 40C, 40D, 40E. This is useful for cases in which several different oligonucleotide products are being made at the same time, in which case it becomes necessary to sort them at the end of the process.

[0079] Shown in FIG. 3A is a cross sectional view of the device 10 in the region of two electrodes such as unit path electrodes 14, such as along section A-A of Fig. 1A. The figure does not show an optional integrated top plate.

[0080] The substrate 8 of the device 10 supports a droplet 55 and the electrodes 14 of the substrate 8 are being driven to propel the droplet in the direction of the arrow under the control of the controller 80 via the latch array 82.

[0081] The substrate 8 includes an electrode layer 44 containing adjacent first and second path electrodes 14-1, 14-2 separated by an insulating region 55 that separates the successive electrodes 14-1, 14-2.

[0082] The electrode layer 44 lies between a dielectric base layer 50, such as silicon wafer material or printed circuit board material, and a first hydrophobic layer (coating) 52, the latter being the layer that contacts the guided droplets 44. The hydrophobic layer 52 reduces resistance to droplet movement and preserves the integrity of the droplet. It can be constructed from a suitable material, such as, e.g., a polymeric material based on polytetrafluoroethylene. In specific examples, the hydrophobic layer is fabricated from an amorphous fluoroplastic such as Teflon® AF.

[0083] Shown in FIG. 3B, is a cross sectional view of the device 10 showing the top plate 54 in the region of a functionalized unit electrode 12 such as along section B-B of Fig. 1A.

[0084] The dielectric substrate 50 supports a sensing unit electrode 15, followed by a functionalized unit electrode 12 followed by a unit path electrode 14. An insulating layer 55 covers the sequence of sensing unit electrode 15, functionalized unit electrode 12, and unit path electrode 14. The insulating layer might be an epoxy-based photoresist, such as SU-8, which is composed of Bisphenol A Novolac epoxy.

[0085] The functionalized unit electrode 12 supports seed DNA molecules 47 required for synthesis.

[0086] In more detail, in the illustrate embodiment, the seed DNA molecules 47 are located in wells 120-1, 120-2, 120-3 and attached to exposed portions of the functionalized unit electrode 12. As such, the material of the functionalized unit electrode 12 is preferably a nonreactive material such as gold or platinum, for example. Preferably, each functionalized unit electrode includes multiple isolated exposed regions to which separate DNA molecules 47 are attached. In the illustrated embodiment, the wells 120-1, 120-2, 120-3 are fabricated by etching or otherwise patterning the hydrophobic layer 52 and the

insulating layer 55 over the regions of the unit electrode 12 to create discrete exposed regions of the metal of the unit electrode 12.

[0087] In addition to or alternatively to immobilizing the seed molecule on the bottom plate, seed DNA molecules can be immobilized on the inner surface of the top plate.

[0088] Preferably, the top plate 54 is transparent, and made of glass, for example. Support is provided by spacers 56, preferably made of a dielectric material and sized to accommodate the droplets sandwiched between the top and the bottom plates. In one example, the spacer has a height of about 300 μm . These spacers can also provide walls on either side of the path electrodes 14 in order to help confine the droplets 55 and isolate the droplets from the surrounding environment.

[0089] The movement and integrity of droplets such as droplet 55 are promoted by hydrophobic layer 52 on the insulating layer 55 and an upper hydrophobic layer 58 deposited on the inner surface of the plate 54. In some embodiments, the inner walls 56-I of the spacers 56 are additionally coated with hydrophobic layer. The hydrophobic layers are relatively thin and can be made of a suitable material such as described above, e.g., Teflon® AF. The material and thickness of layers 52 and 58 can be the same or different. In some embodiments the device is filled with a silicone oil filler fluid (e.g., commercially available 2-5cSt silicone fluid) to enhance droplet transport and decrease evaporation.

[0090] The top and bottom plates (54, 49) can be compressed with rare earth magnets at the edges and the top plate can be provided with injection ports for insertion of reagents and extraction of synthesis products. In specific implementations, the ports are sized for a 10 μL pipette tip.

[0091] The DMF device 10 can be fabricated as separate elements that are bonded together. Some embodiments utilize three elements: a bottom plate, such as silicon wafer for printed circuit board (PCB) material, that includes the electrodes for controlling droplet motion, a middle layer that defines the microfluidic channel, and a top plate that forms the top cover of device. In other embodiments two layers may be used, a bottom plate with patterned electrodes and a top plate that defines the microfluidic channel and encloses the device. In many cases, the device is designed for one-use only, followed by its disposal. However, it can be designed as part of a system in which at least some of the components can be reused while others can be discarded and replaced with fresh components. For instance, a fresh array can be inserted in a chip that is designed for repeated use.

[0092] In an illustrative example, a DMF device 10 is prepared using a PCB as the dielectric substrate 50 (instead of the silicon wafer), polyimide and glass.

[0093] In this example, the array of electrodes that run between the controller 80, latch array 82, capacitive sensor array 84 and the separate sensing unit electrodes 15, functionalized unit electrodes 12, and unit path electrodes 14 and thus used to control electrowetting, to create droplets from the solution reservoirs and to move droplets along the fluid track are formed in the inner layers using standard printed circuit board techniques. The fluidic channels are patterned into a polyimide sheet (300 μm thick, for instance) using a laser cutter. The hydrophobic coating on the top and bottom plates is formed from a spin-on material, such as Cytop® fluoropolymer. The hydrophobic layer can be patterned photolithographically and etched using an Ar/O_2 plasma to expose regions of SiO_2 or an alternative material such as gold (Au) that can be used for seed DNA immobilization.

[0094] FIG. 4A shows an exploded side cross-section view and FIG. 4B shows a side cross-section view along section line C-C shown in FIG. 1A showing a PCB implementation.

[0095] DMF device 10 includes bottom PCB that functions as the substrate 8 and top glass (SiO_2) plate 54, having inner surfaces coated with fluoropolymer layers that functions as upper hydrophobic layer 58. Channels are created (defined) in polyimide layer that functions as the spacers 56 on either side of the functionalized unit electrodes 12. Here the seed DNA molecule 47 is immobilized at an inner surface of the SiO_2 top plate 104 above the functionalized unit electrodes 12. The seed DNA molecule 47 is attached to an exposed portion of the plate created by forming a wells 120 through the upper hydrophobic layer 58.

[0096] To bond the device, the PCB 102, polyimide layer 103, and glass plate 104 are aligned and laminated in a Carver press.

[0097] In many cases, the seed molecule is immobilized prior to laminating (bonding) the layers to form the device. In other cases, a suitable surface region is prepared (modified) in a step preceding bonding, with the final DNA attachment being completed in the bonded device.

[0098] FIG. 4C shows an exploded side cross-section view and FIG. 4D shows a side cross-section view along section line C-C shown in FIG. 1A showing a channelized implementation. This example can be coupled to a reusable manifold that provides a facile interface to pumps and electronic control.

[0099] Here, DMF device 10 is fabricated using patterned electrodes on SiO₂ glass substrate 8 and channel formed in SiO₂ glass of the top plate 54.

[0100] In this example, the metal electrodes, e.g., sensing unit electrodes 15, functionalized unit electrodes 12, and unit path electrodes 14 used to control electrowetting and propel the droplets are patterned lithographically on a bottom glass plate that functions as the substrate 8. A shadow mask or photolithography is used to create patterned metal electrodes on one substrate. After completing the electrode layers, the device is capped with an insulator layer top plate 54, for example SiO₂ deposited by plasma-enhanced chemical vapor deposition.

[0101] To create microfluidic channels 140 in the top glass plate 54, a Cr hard mask is deposited on the SiO₂ patterned photolithographically. A buffered oxide etch is used to transfer the pattern from the Cr into the glass and create the fluidic channels in a timed etch process that leaves the dome-shaped channel 140.

[0102] The top and bottom plate are both treated with a hydrophobic fluoropolymer, e.g., Cytop® coating to form layers 52, 58. The fluoropolymer is patterned on one or both surfaces to allow functionalization with the seed DNA molecule, which can be carried out by modifying a desired surface region to form the well 120 prior to bonding and finalizing the immobilization of the seed molecule in the bonded DMF device.

[0103] FIG. 4D shows the bonded device 10. As seen in this drawing, DMF device includes bottom plate 8, made of glass and electrode material, as well as top plate 54, made of glass. Seed molecule (e.g., seed DNA) 47 is immobilized using a SiO₂ surface formed at the bottom plate by creating a well 120 through layer 52. Channel 140 is formed in top plate 54 and can be sized to accommodate the droplets used during the synthesis. In one implementation, the channel is sized to accommodate a droplet diameter of about 300 μm.

[0104] A fluoropolymer such as the Cytop® used to form the hydrophobic coatings described above can also be used to bond the top plate and bottom plates together by

aligning the substrates and heating to 150 °C, under 70 kg pressure for a few minutes, e.g., about 3 minutes.

[00105] As before, the bonded device can be part of a system that incorporates a microfluidic arrangement and a control function. For example, the device in FIG. 4D can be coupled to a reusable manifold that provides a facile interface to pumps and electronic control.

[00106] As already mentioned, the DMF device is configured for the controlled growth of oligonucleotides by directing droplets carrying payloads such as nucleotide precursors, enzyme, etc., to the functionalized electrodes. Voltages applied for device function will be dependent on the contact angle for wettability, the dielectric, the hydrophobic layer, and so forth.

[00107] Some synthetic approaches that can be employed rely, at least in part on an enzyme to mediate the attachment of nucleotides. In such cases, the enzyme is present in a droplet that is formed and moved (guided) as described herein. Other embodiments rely on a chemical reagent to mediate the attachment of nucleotides, in which case the droplet being guided contains the relevant chemical reagent. Materials utilized in the synthetic procedure are provided by forming droplets from the contents of the relevant reservoirs (sources), e.g., reservoirs 28, 30G, 30T, 30C, 30A, 32 in FIG. 1A.

[00108] In one embodiment, a droplet is first created from the enzyme reservoir 28 and guided along the input bus 34 to a functionalized electrode 12. Enzyme droplets may be placed in reserve at the electrodes adjacent to the functionalized electrodes, and the whole array of droplets may be triggered to move to the functionalized electrodes simultaneously. The enzyme droplet will sit on the functionalized electrode 12 until some enzyme has bound to the seed oligonucleotide, at which point the droplet will be moved off to the output bus 36 to the waste reservoir 38. Droplets of the nucleotide (from reservoirs 30) are then directed to the array of functionalized electrodes 12 and remain there until nucleotide incorporation by the enzyme occurs. The droplets are then also moved to the waste reservoir 38. This repeats until the correct sequence has been synthesized. To remove the synthesized oligonucleotides, droplets of the release solution are created from reservoir 32 and directed over the functionalized array where they are allowed to incubate before being directed to the reaction products reservoir 40. Rinse droplets from reservoir 23 can be used

to remove traces left behind by a given nucleotide and ready the electrodes for the next nucleotide addition.

[00109] In another embodiment, the first droplet is a droplet obtained from a bulk solution that contains the desired nucleotide together with an enzyme that catalyzes bonding of a nucleotide to an oligonucleotide. The nucleotide and enzyme in the bulk solution are present in concentrations that are known to promote bonding of a nucleotide to an oligonucleotide that is present at a functionalized electrode.

[00110] Once the first droplet is formed, it is propelled along the path electrodes 14 to a suitable path such as input bus 34 until it reaches an appropriate functionalized electrode 12. The first droplet remains on the functionalized electrode 12 long enough to permit attachment. After this time lapses, the first droplet is moved towards the waste-disposal area 38.

[00111] In some practices, the nucleotide is provided in combination with a blocking group (moiety), in a complex, for example. Once attached to the tethered oligonucleotide, the blocking group inhibits or prevents bonding of other nucleotides present in the droplet.

[00112] Modified nucleotides with blocking groups—groups that prevent further addition of new nucleotides can also be used, for example in WO2016/128731A4 and Nucleosides, Nucleotides Nucleic Acids, by Hutter et al., NIH Public Access, 2010, doi 10.1080/15257770.2010.536191. The blocking group can be linked to fluorophores, the emission from which can be useful for validating attachment of the nucleotide. The presence of the fluorophore is not required; rather, a droplet carrying a nucleotide bearing a blocking group is delivered to the DNA chain being grown, where it is maintained for a period of time sufficient for attachment to occur, thereby bypassing verification steps based on emission from an attached fluorophore illuminated by interrogation photons.

[00113] To allow attachment of a next nucleotide (provided in a subsequent nucleotide-containing droplet), the blocking group is detached and removed, using, for example, a droplet containing a suitable cleaving agent. This droplet is formed and moved along a suitable path, input bus 34, until it reaches the functionalized electrode 12 with the attached nucleotide-blocking group complex and delivers the agent thus effecting the detachment. The cleaved moiety enters the solution and the droplet is propelled to the waste disposal reservoir 38. Other approaches for detaching the blocking group include

photochemical cleavage, cleavage using chemical reagents, or electrochemically actuated cleavage and others.

[00114] At this point, the cycle can be repeated to add the next nucleotide.

[00115] Eventually, all the desired nucleotides will have been added. To collect the product oligonucleotide, a release droplet is formed at the release solution reservoir and it uses the path electrodes 14 of the input bus 34 to propel it along until it reaches the functionalized electrode 12 that contains the oligonucleotide product and detaches it from the substrate. The detached oligonucleotide product transfers to the release droplet which is moved to the reaction product reservoir 40.

[00116] In one illustrative example a nucleotide chain (ATCG, for instance) is synthesized as described below.

[00117] First, the DMF device can be readied for the synthesis by introducing the necessary solutions, e.g., by pumping them from external reservoirs into the on-chip reagent reservoirs 30G, 30T, 30C and 30A. In one illustration, these solutions include four synthesis mixtures, each containing different nucleotide precursors: deoxyguanosine triphosphate (dGTP), (deoxy)thymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP) or deoxyadenosine triphosphate (dATP). A rinse solution and a release solution are introduced via a pump from an external reservoir into device (on-chip) reservoirs 23 and 32, respectively. In one illustration, the synthesis mix contains a reaction buffer (e.g., 50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, adjusted to a pH 7.9); CoCl_2 (e.g., 0.25 mM); enzyme (TdT, 0.1 – 5 μM); and dATP/dCTP/dGTP/dTTP (50 μM – 500 μM). The rinse solution is the reaction buffer (in this case 50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, adjusted to a pH 7.9). The release solution can be deionized water or a solution of a detergent such as triton-x. In the implementations shown in FIGS. 1A, 1B, and 2, the enzyme is segregated in separate reservoir 28 and enzyme and nucleotide containing droplets are merged together during synthesis.

[00118] To add the sequence ATCG to a seed nucleotide, for example, a droplet (e.g., 0.1 to 1 μl) is generated at the reservoir 30A, which, in this illustrative example, also includes the TdT enzyme. The electrodes are used to route the droplet to the target synthesis region provided with the seed DNA. The droplet is incubated at 37 °C at the reaction region 25 for a suitable period of time determined, for example, by routine

experimentation, prior experience and so forth. In many cases, the incubation lasts for a period within the range of from about 0.2 seconds and 30 seconds. After synthesis the droplet is routed to the waste channel and a rinse droplet is created and routed to the electrode. A rinse droplet is generated at the rinse reservoir and used to rinse both the track and the synthesis region to remove any residual nucleotide. Sequentially, T, C, and G droplets are created at the respective reservoirs 30T, 30C and 30G in FIGS. 1A, 1B, and 2 (also containing TdT), routed to the synthesis region and allowed to extend the oligonucleotide strand. Each nucleotide droplet is followed by a rinse droplet. Synthesis can be continued as specified to produce sequences of arbitrary length. In one example, the length of the DNA product is up to about 10,000 nt.

[00119] To release the DNA from the surface a release solution droplet is created at the release reservoir 32 and routed to the reaction region 25. In many cases, the reaction region is heated, e.g., to a temperature as high as 90 °C, for example, to facilitate release of the product DNA from the substrate. The oligonucleotide product is routed to the reaction products reservoir.

[00120] In many implementations, different nucleotide sequences are synthesized at different functionalized electrodes 12.

[00121] As already mentioned, the functions of the DMF device are digitally controlled. At least three subsystems are of interest: electric, fluid, and temperature. In specific implementations, the control of these subsystems is integrated with custom control software.

[00122] Shown in FIG. 5 is a block diagram showing the control system executing to the controller 80 of the system 10 including DMF device 10, such as described above with reference to FIGS. 1A, 1B and 2, for example

[00123] The electrode control subsystem 202 executes on the controller 80 determines the voltage of each electrode in the device and saves these voltages in the latch array 82. In many embodiments, the electrode control subsystem 202 also measures the capacitance of each of the sensing unit electrodes 15 in addition to optionally also measuring the capacitance of each of the unit path electrodes 14 and functionalized unit electrodes 12 via the capacitive sensor array 84. The capacitive sensors detect the presence of droplets on the electrodes.

[00124] These voltages applied by the latch array cause droplets containing various substances to be guided along the various paths, e.g., as shown in FIGS. 1A, 1B and 2. For example, for a negatively-charged droplet (see, for example, droplet 55 in FIG. 3A), applying a negative voltage to a first electrode and a positive voltage to a second electrode adjacent to the first would tend to urge the droplet towards the second electrode. By suitably timing the voltages, a droplet can be made to move anywhere. The voltages required depend on the factors such as, for example, contact angle for wettability, the nature of the dielectric, the hydrophobic layers (e.g., layers 52 and 58 in FIG. 3B), and others.

[00125] Since electrode control subsystem 202 (using, for example, platform controller 80 in FIGS. 1A and 2) serves to create and move each droplet, it can be designed to also keep track of the location and composition of each droplet and any given time. Variations in impedance, for example, can be exploited. Thus, electrode control subsystem 202 can propel each droplet to where it is needed and can function as a traffic director and dispatcher that simultaneously moves droplets to different locations. In general, the system can guide many droplets present on the device at the same time, with some or all of the droplets carrying different payloads to different destinations. For example, the programmable platform-controller 80 in FIGS. 1A and 2 can be directed to execute a program for transporting droplets along the paths to particular functionalized electrodes 12 according to a programmed sequence algorithm.

[00126] The fluid control subsystem 204 also preferably executing on the controller 80 dispenses reagents from external reservoirs to the device and removes reaction products and waste from the device. The fluid control subsystem is designed to handle external reservoirs for reagents, reaction products, and waste with volumes in the ml range, for instance. In specific embodiments, fluid control subsystem 204 controls one or more pumps dispensing reagents into the on-chip reservoirs for use in the synthesis reactions and collection of reactions products and waste from the on-chip output reservoirs.

[00127] The temperature control subsystem 206 executing on the controller 80 provides and maintains a steady temperature gradient on the device, e.g., from 4°C in the input region to 37°C in the functionalized electrode array, as discussed in connection with Fig. 1B.

[00128] The control subsystem 208 can be integrated with a suitable custom software package that allows for automation of the synthesis process. The subsystem would integrate, for example, the results from the capacitive sensors and the temperature control into the formation and routing of droplets in the device. Under ideal conditions, the control subsystem will automate and optimize the synthesis process for reaction time and reaction conditions given the sequence to be synthesized.

[00129] In some embodiments, algorithms are designed to detect and address droplets that may become stuck at undesired locations, whereby electric control subsystem 202 can turn electrodes on or off to release the droplet from its stuck state, for example.

[00130] In some cases, it is possible to define virtual functionalized electrodes 60 out of multiple adjacent functionalized electrodes 62, 64, 66 as shown in FIG. 6. This is particularly useful for generating arrays for TAQMAN™ assays.

[00131] As seen in FIG. 6, shows a compound functionalized unit electrode 12'. This compound functionalized unit electrode 12' would replace each of the functionalized unit electrodes 12 of FIG. 1A.

[00132] The compound functionalized unit electrode 12' includes three separate the physical functionalized subelectrodes 62, 64, 66, which are grouped into the single logical compound functionalized electrode 12'. This is useful to include sets of forward/reverse primers and probes. Array synthesis begins with a seed oligonucleotide patterned in an array of “growth spots” on a hydrophobic surface. Subelectrodes 62, 64, 66 below the surface, control liquid flow by modifying surface wettability, allowing each functionalized spot of each subelectrode 62, 64, 66, to be addressed individually by the controller 80. Three droplets, each containing a single type of nucleotide, are made to flow separately to each of the subelectrodes 62, 64, 66 by a compound sense electrode 15', which includes three sub-sense-electrodes 15-A, 15-B, 15-C. These three sub-sense-electrodes 15-A, 15-B, 15-C provide three separate droplets to the respective subelectrodes 62, 64, 66. An electric pulse causes a droplet containing the desired nucleotide to flow onto the growth spot, where the TdT enzyme catalyzes its addition to the growing oligonucleotide. Unincorporated nucleotides are rinsed away into a waste stream. The process is repeated for each nucleotide to be added to each spot.

[00133] For certain applications, the DMF device is designed to be compatible with lab-standard 96-well, 384-well, and other well-plate formats. In this embodiment, the

synthesis regions will be distributed in a manner in which one or more synthesis regions are aligned with each well of the plate. Preferably, spots are sized according to a standard lab plate. In one design, the top plate will be removable, so that, after synthesis, the top plate is removed and replaced with a bottom-less well plate which can be attached to the DMF device with adhesive. Subsequent assays using the synthesized DNA can then take place in situ in the well plate. To promote facile production of arrays for the TAQMAN™ quantitative PCR assay three individual sequences corresponding to the forward primer, reverse primer, and probe sequence can be prepared in a single well simultaneously.

[00134] The device, systems and/or methods described herein can be used to synthesize DNA strands on demand, e.g., at a point of need, for example at remote locations during viral or bacterial outbreaks. In some embodiments, once the product oligonucleotides are prepared, they can be used in assay techniques that can be conducted on the same device.

[00135] The following papers are incorporated herein by reference in their entirety:

[00136] Cho, Sung Kwon, Hyejin Moon, and Chang-Jin Kim. "Creating, transporting, cutting, and merging liquid droplets by electrowetting-based actuation for digital microfluidic circuits." *Journal of microelectromechanical systems* 12.1 (2003): 70-80.

[00137] Norian, Haig, et al. "An integrated CMOS quantitative-polymerase-chain-reaction lab-on-chip for point-of-care diagnostics." *Lab on a Chip* 14.20 (2014): 4076-4084.

[00138] Boles, Deborah J., et al. "Droplet-based pyrosequencing using digital microfluidics." *Analytical chemistry* 83.22 (2011): 8439-8447.

[00139] Yehezkel, Tuval Ben, et al. "Synthesis and cell-free cloning of DNA libraries using programmable microfluidics." *Nucleic acids research* 44.4 (2016): e35-e35.

[00140] Gach, Philip C., et al. "A droplet microfluidic platform for automating genetic engineering." *ACS synthetic biology* 5.5 (2016): 426-433.

[00141] Khilko, Yuliya, et al. "DNA assembly with error correction on a droplet digital microfluidics platform." *BMC biotechnology* 18.1 (2018): 37.

[00142] Coelho, Beatriz, et al. "Digital Microfluidics for Nucleic Acid Amplification." *Sensors* 17.7 (2017): /1495.

[00143] While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method for synthesizing an oligonucleotide, the method comprising:
 - guiding a first droplet containing a first nucleotide to a functionalized electrode;
 - attaching the first nucleotide to a seed molecule tethered at the functionalized electrode;
 - guiding a second droplet containing a second nucleotide to the functionalized electrode;
 - attaching the second nucleotide to an end of a chain obtained by attaching the first nucleotide,
 - wherein the first and second droplets are guided by applying voltages to electrodes.
2. The method of claim 1, wherein attachment of the first and/or second nucleotide is conducted in the presence of an enzyme.
3. The method of claim 1, wherein attachment of the first and/or second nucleotide is mediated by terminal deoxynucleotidyl transferase.
4. The method of claim 1, further comprising guiding a rinse droplet to the functionalized electrode between the first droplet and the second droplet.
5. The method of claim 1, wherein the tethered seed molecule is one of a plurality of seed molecules provided on a digital microfluidic device.
6. The method of claim 1, wherein a blocking group prevents attachment of another nucleotide present in the first or the second droplet.
7. A method for synthesizing an oligonucleotide, the method comprising:

guiding a first droplet containing a first nucleotide to a functionalized electrode;

attaching the first nucleotide to a seed molecule tethered at the functionalized electrode;

guiding a second droplet containing a second nucleotide to the functionalized electrode;

attaching the second nucleotide to an end of a chain obtained by attaching the first nucleotide,

wherein the method is conducted on a microfluidic device comprising functionalized electrodes and path electrodes.

8. The method of claim 7, wherein the microfluidic device further comprises sensing electrodes.
9. The method of claim 7, wherein the microfluidic device includes an input section having reagent reservoirs, a reaction region for growing an oligonucleotide chain, and an output section for collecting an oligonucleotide product and/or waste materials.
10. The method of claim 7, wherein the microfluidic device includes a bottom electrode plate, coated with a hydrophobic layer, and an optional top plate.
11. The method of claim 7, wherein the first droplet is one in a plurality of first droplets being guided on the microfluidic device simultaneously.
12. The method of claim 11, wherein the first droplets in the plurality of first droplets contain the same or different first nucleotides.
13. The method of claim 7, wherein the second droplet is one in a plurality of second droplets being guided on the microfluidic device simultaneously.
14. The method of claim 13, wherein second droplets in the plurality of second droplets contain the same or different second nucleotides.

15. The method of claim 7, wherein the microfluidic device is part of a system, wherein the system includes microfluidics, temperature regulators and/or control subsystems.
16. The method of claim 7, further comprising attaching the seed molecule to the functionalized electrode.
17. The method of claim 7, wherein synthesis of the oligonucleotide is automated.
18. The method of claim 7, wherein an input section of the microfluidic device is cooled by a Peltier cooler and/or a reaction section of the microfluidic device is heated by a Peltier heater.
19. A system for synthesizing oligonucleotides by a controlled, iterative addition of one nucleotide at a time, the system comprising:
a microfluidic device, wherein the microfluidic device includes:
functionalized electrodes for receiving seed molecules;
path electrodes for guiding droplets to and from the seed molecules;
input reservoirs for supplying reagents for synthesizing the oligonucleotide;
output reservoirs for collecting product nucleotides and/or waste; and
controller for controlling movement of the droplets.
20. The system of claim 19, further comprising temperature regulators and/or microfluidics.
21. The system of claim 19, further comprising microfluidic controls and/or temperature controls.
22. The system of claim 19, further comprising pumps and conduits for transferring reagents from external reservoirs to the input reservoirs.
23. A microfluidic device, comprising:

a bottom plate having a dielectric layer patterned for attachment of a seed molecule to a functionalized electrode;

path electrodes formed on the bottom plate:

a top plate supported by spacers, the spacers having a height selected to accommodate a droplet moving between the bottom plate and a top plate; and

a hydrophobic layer coating an inner surface of the bottom electrode plate and/or an inner surface of the top plate.

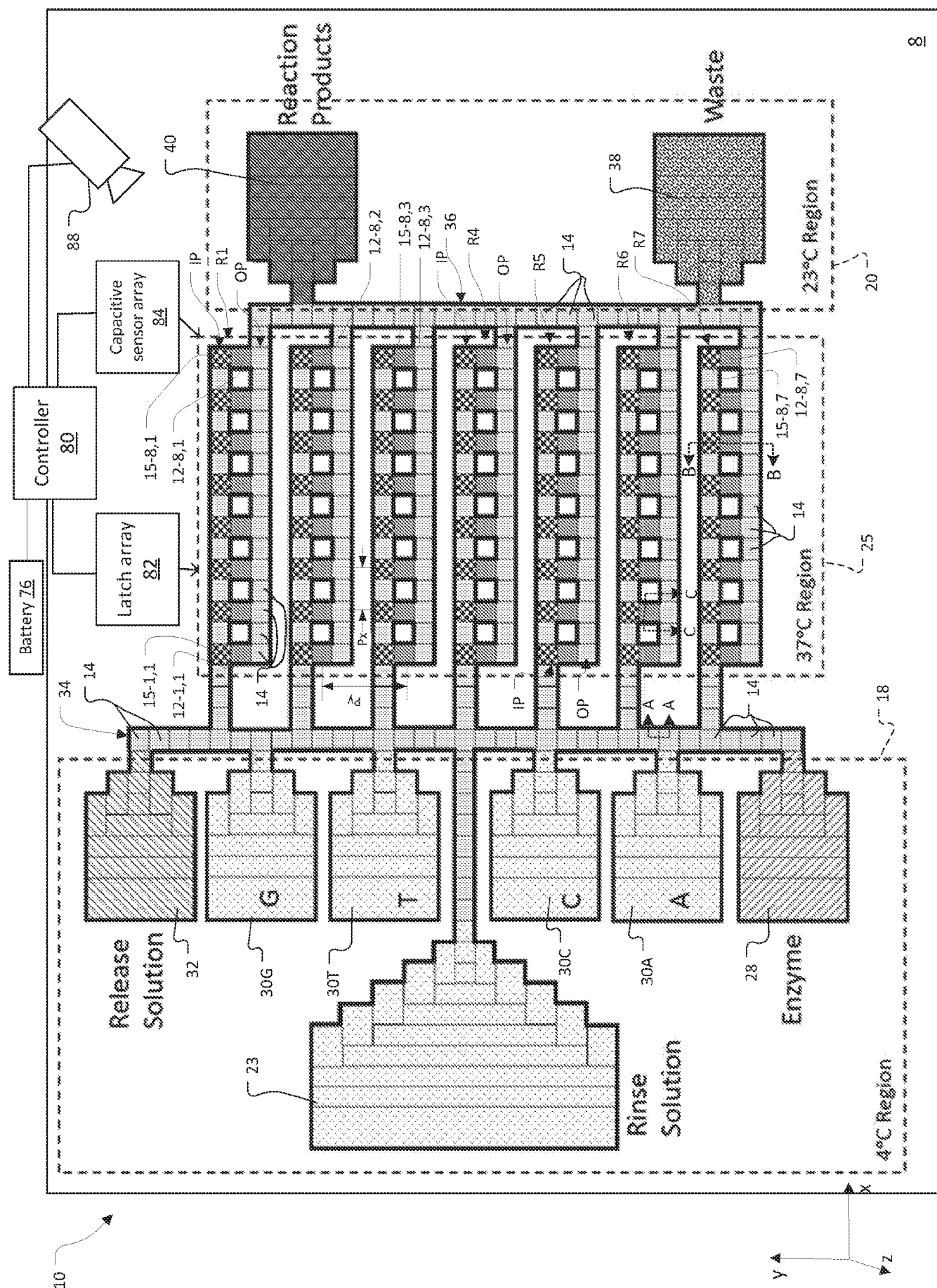
24. The device of claim 23, wherein the top plate is made of glass.
25. The device of claim 23, wherein the top plate includes a microfluidic channel.
26. The device of claim 23, wherein the top plate includes ports for introducing and/or removing materials into and out of the device.
27. A device according to claim 23, wherein the device is compatible with a standard well plate format.
28. A system comprising the microfluidic device of claim 23 and a controller configured to individually guide droplets along selected paths to and from selected functionalized electrodes.
29. The system of claim 28, wherein the controller is configured to group multiple functionalized electrodes into a single logical electrode.
30. The system of claim 28, wherein the controller is configured to group adjacent functionalized electrodes into a single logical electrode.
31. An apparatus for assembling an oligonucleotide product that comprises a plurality of nucleotides, said apparatus comprising a controller and a platform, wherein said platform comprises a floor and a reaction region, wherein said floor has a hydrophobic surface, wherein said reaction region comprises electrodes under said hydrophobic surface, wherein said controller is connected to individually address

each of said electrodes, wherein said electrodes comprise functionalized electrodes and path electrodes, wherein regions around said functionalized electrodes are configured to be seeded by a seed oligonucleotide to which said nucleotides are to be individually attached, wherein said path electrodes define paths that connect to said functionalized electrodes, and wherein said controller is configured to individually propel nucleotide-containing droplets along selected paths to selected functionalized electrodes.

32. A method comprising forming an oligonucleotide product, wherein forming said oligonucleotide product comprises:
- tethering a seed oligonucleotide to a functionalized electrode;
 - attaching a first nucleotide to said seed oligonucleotide, and
 - after having attached said first nucleotide, attaching a second nucleotide to said seed oligonucleotide to which said first nucleotide has been attached,
- wherein attaching said first nucleotide comprises forming a first droplet containing a first mediator for causing said first nucleotide to become attached to said tethered oligonucleotide and exerting electrostatic forces to move said droplet along a path on a surface, said path leading to said functionalized electrode, and wherein attaching said second nucleotide comprises forming a second droplet containing a second mediator for causing said second nucleotide to become attached to said tethered oligonucleotide and exerting electrostatic forces to move said second droplet along a path on said surface, said path leading to said functionalized electrode.
33. A system for synthesizing oligonucleotides by a controlled, iterative addition of nucleotides, the system comprising:
- a microfluidic device, wherein the microfluidic device includes:
 - functionalized compound electrodes for receiving seed molecules;
 - path electrodes for guiding droplets to and from the seed molecules;
 - controller for controlling movement of the droplets;

wherein each of the compound electrodes comprises functionalized subelectrodes.

34. A system as claimed in claim 33, wherein three different nucleotides are synthesized at each of the subelectrodes.
35. A system for synthesizing molecules by a controlled, iterative addition, the system comprising:
a microfluidic device, wherein the microfluidic device includes:
functionalized compound electrodes for receiving seed molecules;
path electrodes for guiding droplets to and from the seed molecules;
sense electrodes for detecting droplets;
controller for controlling movement of the droplets in response to information from the sense electrodes.
36. A system for synthesizing molecules by a controlled, iterative addition, the system comprising:
a microfluidic device, wherein the microfluidic device includes:
functionalized compound electrodes for receiving seed molecules;
path electrodes for guiding droplets to and from the seed molecules;
a camera for taking images of the device; and
controller for controlling movement of the droplets in response to an analysis of the images from the camera.



81

Fig. 1A

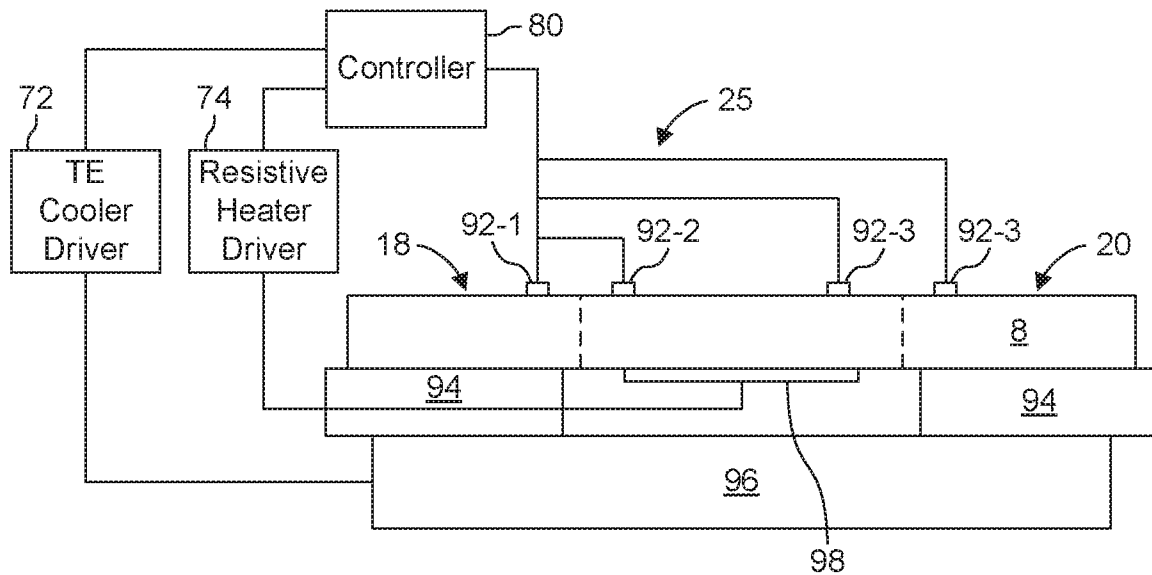


Fig. 1B

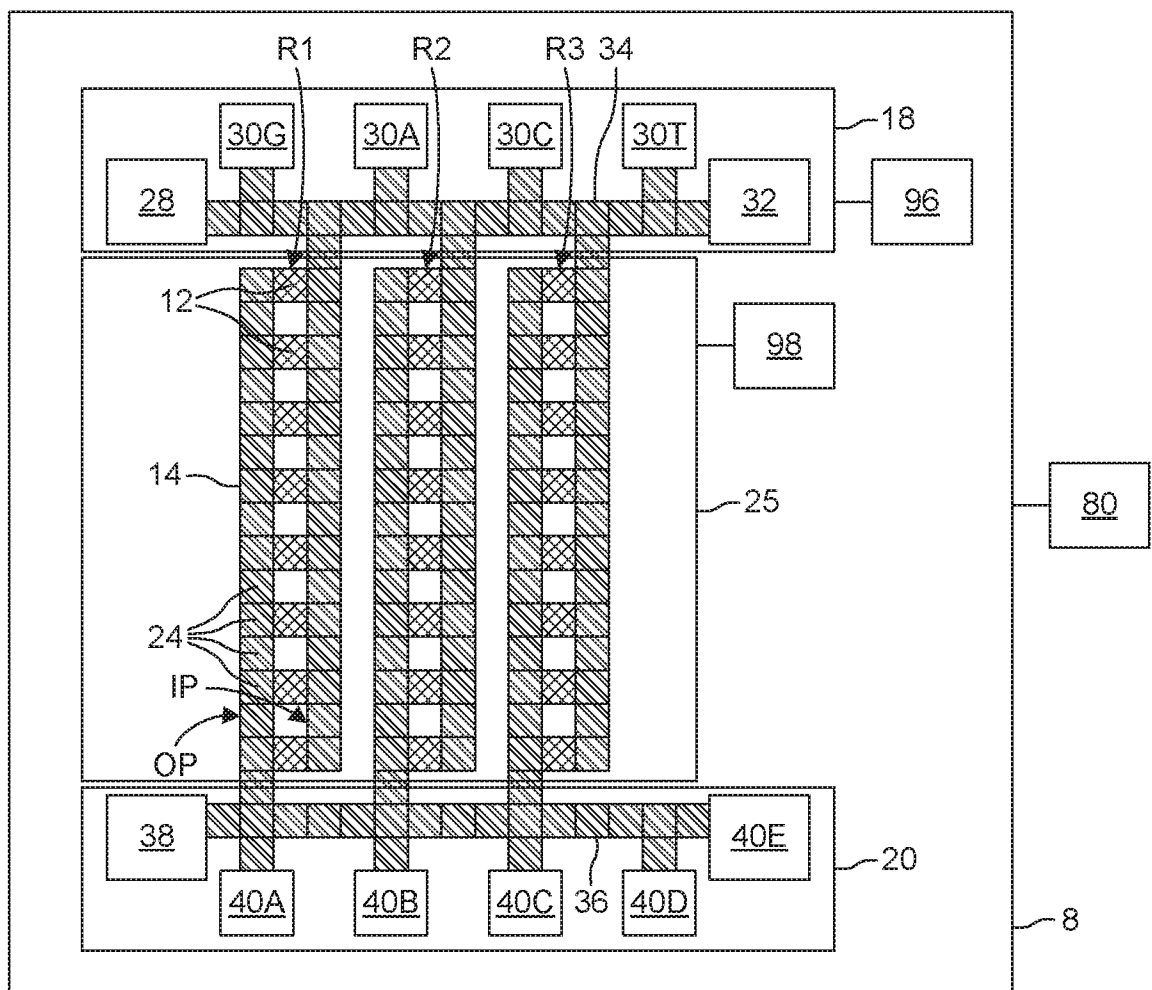


Fig. 2

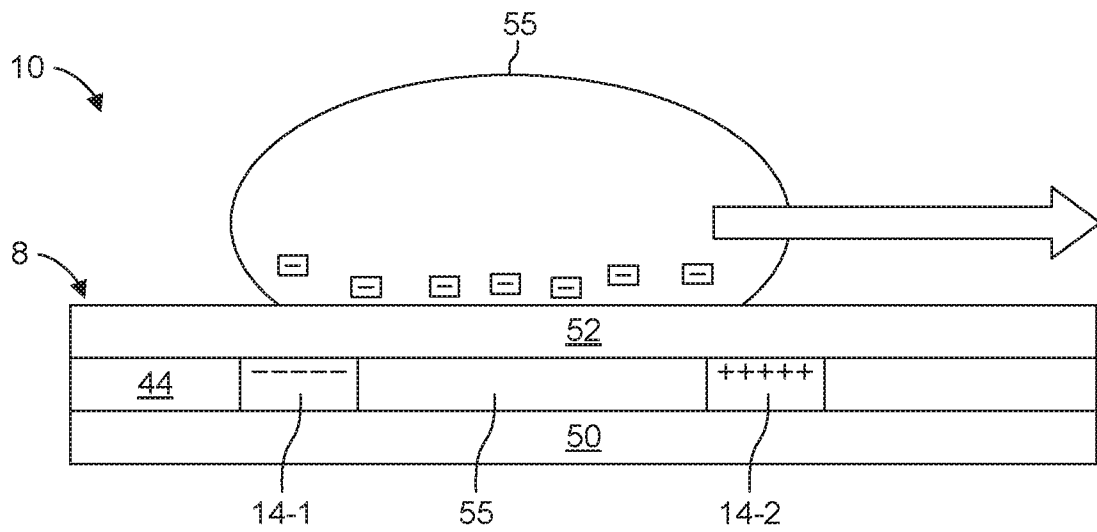


Fig. 3A

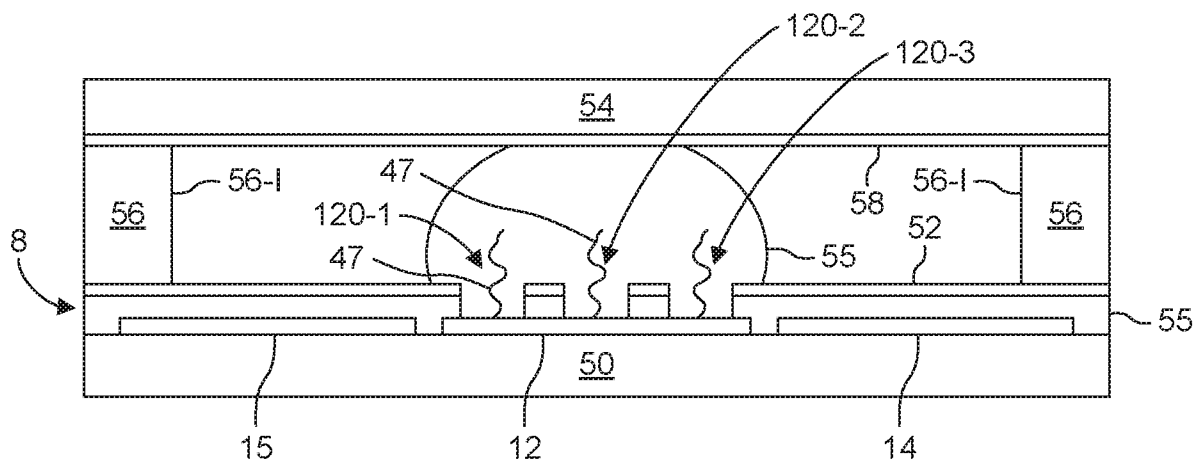


Fig. 3B

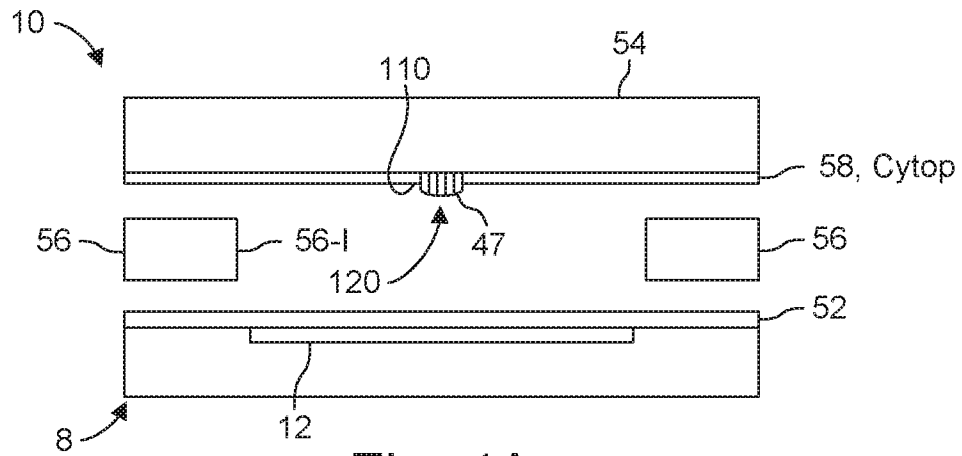


Fig. 4A

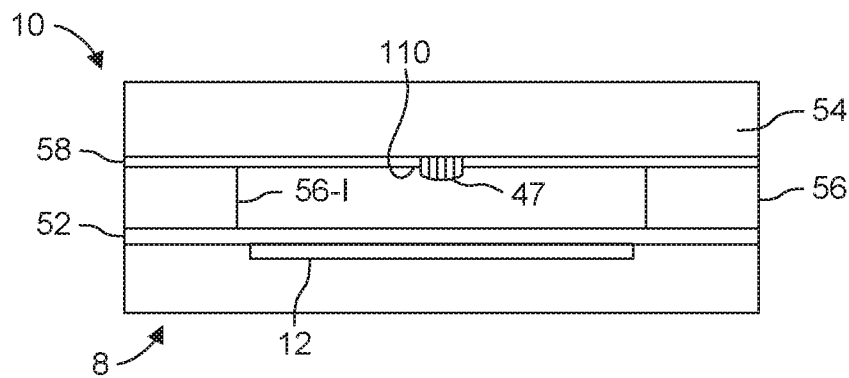


Fig. 4B

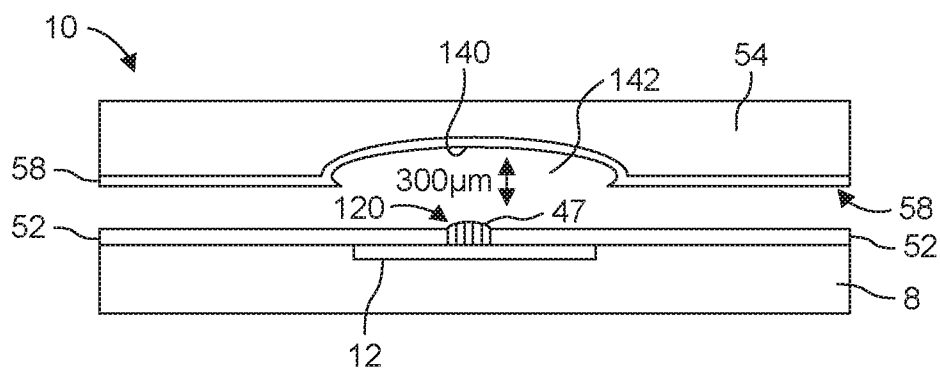


Fig. 4C

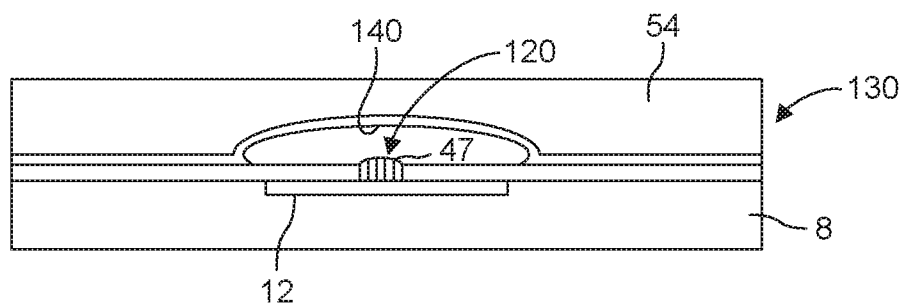


Fig. 4D

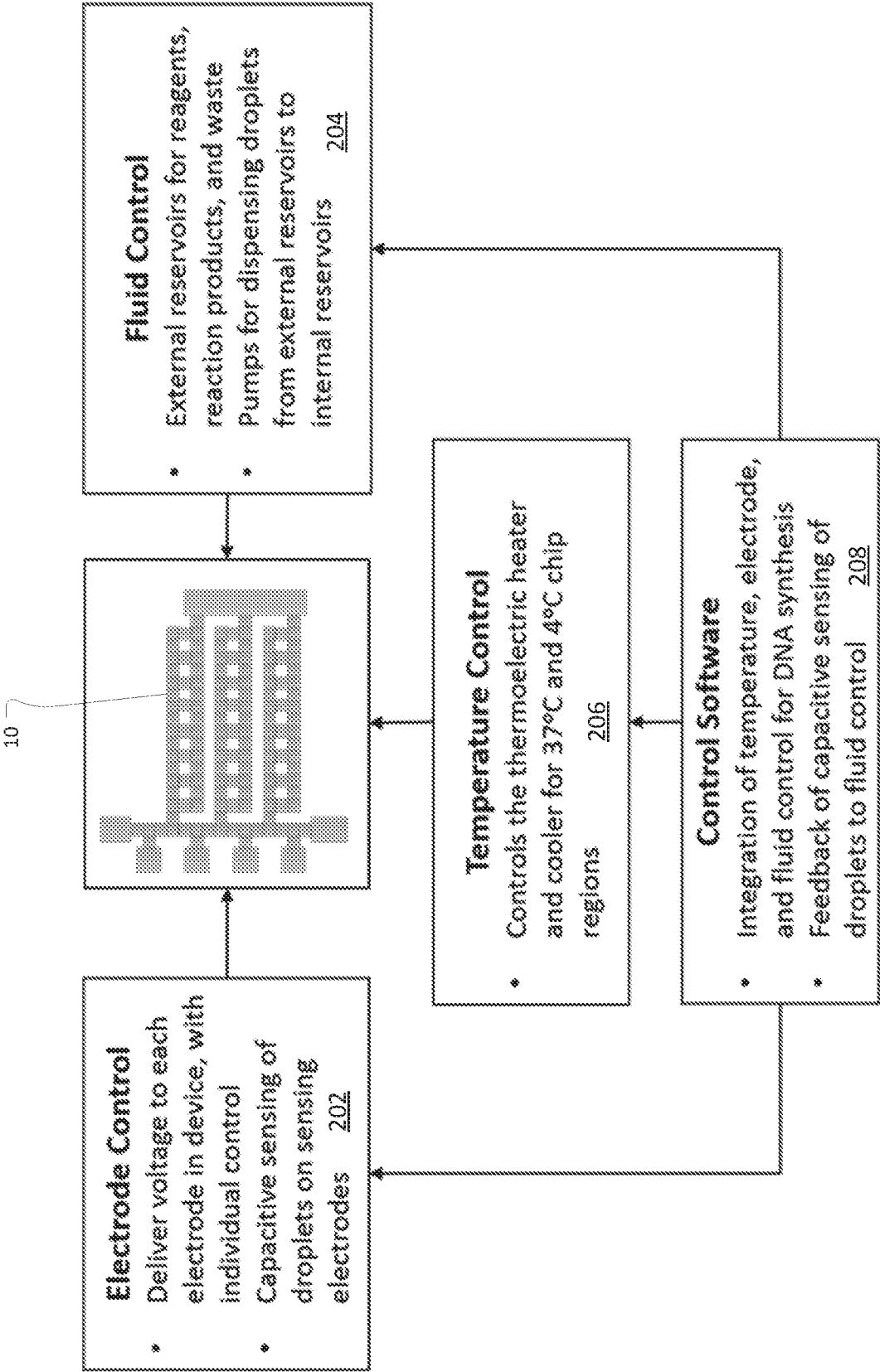


Fig. 5

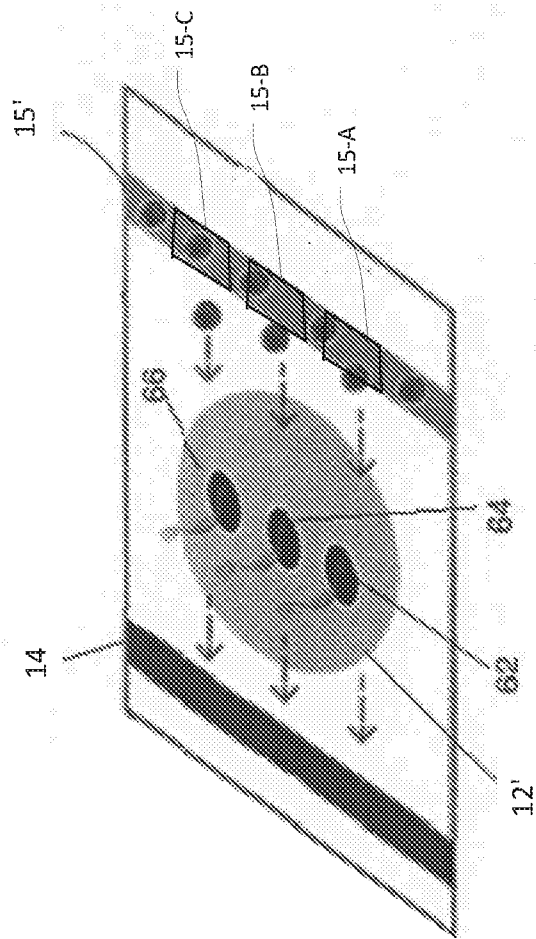


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/055432

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6806 C12Q1/6825
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)
C12Q

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, CHEM ABS Data, 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 03/057010 A2 (UNIV TEXAS [US]; GASCOYNE PETER R C [US]; VYKOUKAL JODY V [US]; SCHWAR) 17 July 2003 (2003-07-17)	1,2,4-18
Y	abstract page 2, line 15 - line 19 page 3, line 32 - line 33 page 4, line 31 - line 33 page 7, line 27 - line 29 page 8, line 7 - line 9 page 11, line 4 - line 11 page 12, line 7 - line 9 page 13, line 5 - line 20 page 15, line 20 - line 25 page 16, line 2 - line 6 page 17, line 6 - page 18, line 30 page 19, line 25 - line 26 ----- -/--	1-18

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

10 December 2018

Date of mailing of the international search report

08/02/2019

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Celler, Jakub

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/055432

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/076390 A1 (APPLERA CORP [US]; ERMAKOV SERGEY V [US]) 20 July 2006 (2006-07-20) abstract; claims 1,2 paragraphs [0078], [0081], [0086], [0093], [0102], [0103], [0135] -----	1-18
Y	WO 2016/064880 A1 (MOLECULAR ASSEMBLIES INC [US]) 28 April 2016 (2016-04-28)	3
A	abstract; claim 24 -----	1,2,4-18
Y	BONANNI A ET AL: "Application of the avidin-biotin interaction to immobilize DNA in the development of electrochemical impedance genosensors", ANALYTICAL AND BIOANALYTICAL CHEMISTRY, SPRINGER, BERLIN, DE, vol. 389, no. 3, 4 August 2007 (2007-08-04), pages 851-861, XP019537650, ISSN: 1618-2650, DOI: 10.1007/S00216-007-1490-X abstract -----	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2018/055432

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03057010	A2	17-07-2003	AU 2003210438 A1 24-07-2003
			US 2003170698 A1 11-09-2003
			WO 03057010 A2 17-07-2003
-----			-----
WO 2006076390	A1	20-07-2006	US 2006153745 A1 13-07-2006
			US 2006165565 A1 27-07-2006
			US 2010200094 A1 12-08-2010
			US 2011124524 A1 26-05-2011
			US 2012097257 A1 26-04-2012
			US 2012298235 A1 29-11-2012
			US 2014102571 A1 17-04-2014
			US 2014249057 A1 04-09-2014
			US 2015298125 A1 22-10-2015
			WO 2006076390 A1 20-07-2006
-----			-----
WO 2016064880	A1	28-04-2016	EP 3209781 A1 30-08-2017
			US 2016108382 A1 21-04-2016
			WO 2016064880 A1 28-04-2016
-----			-----

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/055432

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-18

Remark on Protest

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

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-18

Subject matter of the above claims, as characterised by the constellations of features identified in present claims 1 and 7

2. claims: 19-22

Subject matter of the above claims, as characterised by the constellation of features identified in present claim 19

3. claims: 23-30

Subject matter of the above claims, as characterised by the constellation of features identified in present claim 23

4. claim: 31

Subject matter of the above claims, as characterised by the constellation of features identified in present claim 31

5. claim: 32

Subject matter of the above claims, as characterised by the constellation of features identified in present claim 32

6. claims: 33, 34

Subject matter of the above claims, as characterised by the constellation of features identified in present claim 33

7. claim: 35

Subject matter of the above claims, as characterised by the constellation of features identified in present claim 35

8. claim: 36

Subject matter of the above claims, as characterised by the constellation of features identified in present claim 36
