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# DESCRIPTION

## BACKGROUND

**[0001]** Separation of analyte components from a more complex analyte mixture on the basis of an inherent quality of the analytes, and providing sets of fractions that are enriched for states of that quality is a key part of analytical chemistry. Simplifying complex mixtures in this manner reduces the complexity of downstream analysis. It can be advantageous to perform two or more enrichment steps that are orthogonal, (e.g., based on different and/or unrelated qualities). In many cases, however, the process of performing orthogonal enrichment steps using known methods and/or devices is cumbersome, and can dilute the analyte beyond the sensitivity of the downstream analytical equipment. In addition, complications can arise when attempting to interface known enrichment methods and/or devices with analytical equipment and/or techniques.

**[0002]** Methods have been used to interface protein sample preparation techniques with downstream detection systems such as mass spectrometers. A common method is to prepare samples using liquid chromatography and collect fractions for mass spectrometry (LC-MS). This has the disadvantage of requiring protein samples to be digested into peptide fragments, leading to large number of sample fractions which must be analyzed and complex data reconstruction post-run. While certain forms of liquid chromatography can be coupled to a mass spectrometer, for example peptide map reversed-phase chromatography, these known techniques are restricted to using peptide fragments, rather than intact proteins, which limit their utility.

**[0003]** Another method to introduce samples into a mass spectrometer is electrospray ionization (ESI). In ESI, small droplets of sample and solution at a distal end of a capillary or microfluidic device are ionized to induce an attraction to the charged plate of a mass spectrometer. The droplet then stretches in this induced electric field to a cone shape ("Taylor cone"), which then releases small droplets into the mass spectrometer for analysis. Typically this is done in a capillary, which provides a convenient volume and size for ESI. Capillaries however, provide a linear flow path that does not allow for multi-step processing.

**[0004]** Other work has been pursued with microfluidic devices. Microfluidic devices may be produced by various known techniques and provide fluidic channels of defined width that can make up a channel network designed to perform different fluid manipulations. These devices offer an additional level of control and complexity than capillaries. In connection with ESI, known devices include outwardly tapered tips and conductive edges in an attempt to enhance the ESI in these devices. The outward taper of known microfluidic devices used for ESI, however, exposes the fragile Taylor cone structure to potential disturbances by turbulent air flow and results in a contact surface geometry that will support only a limited range of cone radii, which limits control over the volume introduced to the mass spectrometer through ESI.

Additionally, electrolysis of water at the conductive edge can lead to gas bubble formation, which interferes with the cone development.

**[0005]** One application for protein mass spectrometry is for characterization during the development and manufacturing of biologic and biosimilar pharmaceuticals. Biologics and biosimilars are a class of drugs which include, for example, recombinant proteins, antibodies, live virus vaccines, human plasma-derived proteins, cell-based medicines, naturally-sourced proteins, antibody-drug conjugates, protein-drug conjugates and other protein drugs.

**[0006]** Regulatory compliance demands that biologics require extensive testing during development and manufacture that is not required for small molecule drugs. This is because the manufacture of biologics has greater complexity due to, for example, using living material to produce the biologic, greater complexity of biologic molecule, greater complexity of the manufacturing process. Characteristics required to be defined include, for example, charge, efficacy, hydrophobic changes, mass, and glycosylation. Currently these tests are done independent of each other leading to a very time consuming and expensive process of characterizing biologics.

**[0007]** US 7,655,477 describes a system and method for the separation of analytes.

## SUMMARY OF THE INVENTION

**[0008]** The present invention provides a method, comprising:

1. a) introducing an analyte mixture into a separation channel of a microfluidic device;
2. b) applying an electric field across the separation channel to separate the analyte mixture into enriched analyte fractions via isoelectric focusing;
3. c) imaging the separation of the analyte mixture and subsequent mobilization of the enriched analyte fractions within the separation channel via a transparent portion of the microfluidic device;
4. d) introducing a sheath fluid electrolyte into the separated analyte mixture to mobilize and expel substantially all of the enriched analyte fractions via electrospray ionization into a mass spectrometer from a single orifice that is in line with the separation channel; and
5. e) correlating an absorbance peak for a specific enriched analyte fraction detected by the imaging of the separation channel in (c) with mass spectrometer data for the specific enriched analyte fraction. Further aspects of the method are defined in claims 2-14.

## SUMMARY OF FURTHER DISCLOSURE

**[0009]** Described herein are devices and methods that can enable the analysis of analytes in an analyte mixture. For example, many specific characterizations of biologic proteins are required by regulatory agencies. Methods and devices described herein can be suitable for characterizing proteins and/or other analytes. Methods and devices described herein can relate to characterizing an analyte mixture that includes one or more enrichment steps performed to separate an analyte mixture into enriched analyte fractions.

**[0010]** In some instances, these analytes can be, for example, glycans, carbohydrates, DNA, RNA, intact proteins, digested proteins, antibody-drug conjugates, protein-drug conjugates, peptides, metabolites or other biologically relevant molecules. In some instances, these analytes can be small molecule drugs. In some instances, these analytes can be protein molecules in a protein mixture, such as a biologic protein pharmaceutical and/or a lysate collected from cells isolated from culture or *in vivo*.

**[0011]** The methods described herein can include a first enrichment step, in which fractions containing a subset of the analyte molecules from the original analyte mixture are eluted one fraction at a time; these enriched analyte fractions are then subjected to another enrichment step. At the final enrichment step, the enriched analyte fractions are expelled for further analysis.

**[0012]** One or more of the enrichment steps may be solid-phase separations. One or more of the enrichment steps may be solution-phase separations.

**[0013]** A final step may concentrate the enriched analyte fractions before expulsion.

**[0014]** Substantially all of the enriched analyte fractions from the final enrichment step may be expelled in a continuous stream. A portion of the analyte mixture (e.g., a fraction of interest) may be expelled from a microfluidic device via an outlet configured to interface with an analytical instrument, such as a mass spectrometer or another device configured to fractionate and/or enrich at least a portion of the sample. Another portion of the analyte mixture (e.g., containing fractions other than the fraction of interest) can be expelled via a waste channel.

**[0015]** The expulsion may be performed using pressure, electric force, or ionization, or a combination of these.

**[0016]** The expulsion may be performed using electrospray ionization (ESI) into, for example, a mass spectrometer. A sheath liquid may be used as an electrolyte for an electrophoretic separation. A nebulizing gas may be provided to reduce the analyte fraction to a fine spray. Other ionization methods may be used, such as inductive coupled laser ionization, fast atom bombardment, soft laser desorption, atmospheric pressure chemical ionization, secondary ion mass spectrometry, spark ionization, thermal ionization, and the like.

**[0017]** The enriched fractions may be deposited on a surface for further analysis by matrix-assisted laser desorption/ionization, surface enhanced laser desorption/ionization, immunoblot,

and the like.

**[0018]** Described herein are devices and methods for visualizing an analyte in an electrophoretic separation before and during the expulsion of enriched fractions.

**[0019]** Described herein are devices and methods for visualizing an analyte during an enrichment step.

**[0020]** Described herein are devices and methods for visualizing an analyte in a channel between enrichment zones.

**[0021]** The visualization of an analyte can be performed via optical detection, such as ultraviolet light absorbance, visible light absorbance, fluorescence, Fourier transform infrared spectroscopy, Fourier transform near infrared spectroscopy, Raman spectroscopy, optical spectroscopy, and the like.

**[0022]** Described herein are devices that can enable the analysis of analyte mixtures, in that they contain one or more enrichment zones and an orifice to expel enriched analyte fractions. These devices may include at least one layer which is not transmissive to light of a specific wavelength, and at least one layer which is transmissive to that specific wavelength. One or more portions of the layer which is not transmissive to light can define the one or more enrichment zones, such that the enrichment zones serve as optical slits.

**[0023]** An analyte mixture can be loaded into a device through a tube or capillary connecting the device to an autosampler. An analyte mixture can be loaded directly into a reservoir on the device.

**[0024]** An orifice through which at least a portion of a sample can be expelled from a device may be countersunk and/or shielded from air flow. This orifice may be not electrically conductive. As used herein, countersunk should be understood to mean that a portion of a substrate defines a recess containing the orifice, irrespective of the geometry of the sides or chamfers of the recess. Similarly stated, countersunk should be understood to include counterbores, conical and/or frustoconical countersinks, hemispherical bores, and the like.

**[0025]** Described herein is an apparatus, such as a microfluidic device that includes a substrate constructed of an opaque material (e.g., soda lime glass, which is opaque to ultraviolet light). The substrate can define a microfluidic separation channel. Similarly stated, the microfluidic separation channel can be etched or otherwise formed within the substrate. The microfluidic separation channel can have a depth equal to the thickness of the substrate. Similarly stated, the microfluidic separation channel can be etched the full depth of the substrate (e.g., from the top all the way through to the bottom). In this way, the microfluidic separation channel can define an optical slit through the substrate. A transparent layer (e.g., a top layer) can be disposed on a top surface of the substrate, for example, sealing the top surface of the substrate. A transparent layer (e.g., a bottom layer) can also be disposed on a

bottom surface of the substrate, such that both the top and the bottom of the microfluidic separation channel are sealed. Only a portion of the top layer and/or the bottom layer may be transparent. For example, the top layer and/or the bottom layer can define a transparent window in an otherwise opaque material; the window can provide optical access to, for example, the microfluidic separation channel.

**[0026]** Described herein is an apparatus, such as a microfluidic device that includes a substrate. The substrate can define one or more enrichment zones or channels. For example, the substrate can define a first enrichment zone containing a media configured to bind to an analyte. Such a first enrichment zone can be suitable to separate an analyte mixture chromatographically. The apparatus can further include two electrodes electrically coupled to opposite end portions of a second enrichment zone. Such a second enrichment zone can be suitable to separate an analyte mixture electrophoretically. The second enrichment zone can intersect the first enrichment zone such that after a fraction of an analyte is separated, concentrated, and/or enriched in the first enrichment zone, it can be further separated, concentrated, and/or enriched in the second enrichment zone. The device can also include a recessed orifice. The orifice can be an outlet of the second enrichment channel and can be disposed on a countersunk or otherwise recessed surface of the substrate. The apparatus can be configured to expel a portion of an analyte mixture from the orifice via ESI. The recess can provide a stable environment for formation of a Taylor cone associated with ESI and/or can be configured to accept an inlet port of a mass spectrometer.

**[0027]** Described herein is a method that includes introducing an analyte mixture into a microfluidic device that contains a separation channel. An electric field is applied across the separation channel to effect a separation of the analyte mixture. The analyte mixture is imaged during separation via a transparent portion of the microfluidic device. Similarly stated, a window and/or optical slit can provide optical access to the separation channel such that the whole separation channel or a portion thereof can be imaged while the separation is occurring. A fraction of the analyte mixture is expelled from an orifice that is in fluid communication with the separation channel. The fraction is expelled via ESI. The orifice can be disposed on a countersunk surface of the microfluidic device such that a Taylor cone forms within a recess defined by the countersunk surface.

**[0028]** Described herein is a method that includes injecting an analyte into a microfluidic device containing a first separation channel and a second separation channel. The first separation channel can contain a medium configured to bind an analyte from the analyte mixture. Accordingly, when the analyte mixture is injected into the microfluidic device at least a fraction of the analyte mixture can be bound to the matrix and/or impeded from flowing through the first separation channel. For example, injecting the analyte into the microfluidic device can effect a chromatographic separation in the first separation channel. An eluent can be injected into the microfluidic device such that at least a fraction of the analyte is mobilized from the media. The first separation channel can be imaged while the analyte is mobilized. Imaging the first separation can include whole column (e.g., whole channel) imaging and/or imaging a portion of the channel. An electric field can be applied to the second separation channel when the

imaging detects that the fraction is disposed at an intersection of the first separation channel and the second separation channel such that the fraction is mobilized into the second separation channel. For example, the first separation channel and the second separation channel can form a T-junction. The imaging can detect when a portion of the fraction (e.g., a portion of interest) is at the junction. Applying the electric field can mobilize the portion of the fraction (and, optionally, not other portions of the fraction that are not located at the junction) into the second separation channel for a second stage of separation. At least a portion of the fraction can be expelled from the microfluidic device.

#### BRIEF DESCRIPTION OF THE DRAWINGS

##### [0029]

**FIG. 1** is a schematic illustration of a device for two dimensional separation and ESI of an automatically loaded sample.

**FIG. 2** is a schematic exploded view of a device having three layers.

**FIG. 3** is a schematic of a light path through a microfluidic device.

**FIG. 4** is a schematic illustration of a device for isoelectric focusing (IEF) and ESI of an automatically loaded sample.

**FIG. 5** is a schematic illustration of a microfluidic device.

**FIG. 6** is a flowchart of an exemplary method for analyte characterization.

**FIG. 7** is a schematic of a microfluidic device.

**FIG. 8** is a schematic of a microfluidic device.

#### DETAILED DESCRIPTION

**[0030]** In this application, the use of the singular includes the plural unless specifically stated otherwise. Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising," "include," "includes" and "including" are not intended to be limiting.

##### Devices

**[0031]** **Figure 1** is a schematic illustration of a device for two dimensional separation and ESI of an automatically loaded sample. A microfluidic network, **100**, is defined by a substrate **102**.

The substrate is manufactured out of material which is compatible with the enrichment steps being performed. For example, chemical compatibility, pH stability, temperature, transparency at various wavelengths of light, mechanical strength, and the like are considered in connection with selection of material.

**[0032]** Substrate 102 may be manufactured out of glass, quartz, fused silica, plastic, polycarbonate, polyfluorotetraethylene (PTFE), polydimethylsiloxane (PDMS), silicon, polyfluorinated polyethylene, polymethacrylate, cyclic olefin copolymer, cyclic olefin polymer, polyether ether ketone and/or any other suitable material. Mixtures of materials can be utilized if different properties are desired in different layers of a planar substrate and/or any other suitable material. Mixtures of materials can be utilized if different properties are desired in different layers of a planar substrate.

**[0033]** Channels 106, 110, 114, 116, 118, 124 122, 126, 132, 136 and 140 form the microfluidic network 100 and are fabricated into substrate 102. Similarly stated, the substrate 102 defines channels 106, 110, 114, 116, 118, 124 122, 126, 132, 136 and/or 140.

**[0034]** Channels may be fabricated in the substrate through any channel fabrication method such as, for example, photolithographic etching, molding, machining, additive (3D) printing, and the like.

**[0035]** Analyte mixtures and external reagents can be loaded through tube/conduit 112, and excess reagent / waste can be removed through tube/conduit 130.

**[0036]** Tubes 112 and 130 can be manufactured out of any material compatible with the assay being performed, including, for example, fused silica, fused silica capillary tubes, silicone tubing, and/or PTFE tubing.

**[0037]** Channels 116 and 124 can be used to separate and/or enrich an analyte and/or a portion (e.g., a fraction) of an analyte. Channels 116 and/or 124 can be used to perform chromatographic separations (e.g., reversed-phase, immunoprecipitation, ion exchange, size exclusion, ligand affinity, dye affinity, hydrophobic interaction chromatography, hydrophilic interaction chromatography, pH gradient ion exchange, affinity, capillary electrokinetic chromatography, micellar electrokinetic chromatography, high performance liquid chromatography (HPLC), amino acid analysis-HPLC, ultra performance liquid chromatography, peptide mapping HPLC, field flow fractionation - multi angle light scattering) or electrophoretic separations (e.g., isoelectric focusing, capillary gel electrophoresis, capillary zone electrophoresis, isotachophoresis, capillary electrokinetic chromatography, micellar electrokinetic chromatography, flow counterbalanced capillary electrophoresis, electric field gradient focusing, dynamic field gradient focusing). For example, channel 116 can be derivatized or packed with material to perform a first enrichment step.

**[0038]** The material disposed into channel 116 and/or 124 can be selected to capture analytes based on, for example, hydrophobicity (reversed-phase), immunoaffinity

(immunoprecipitation), affinity (efficacy), size (size exclusion chromatography), charge (ion exchange) or by other forms of liquid chromatography.

**[0039]** Many different methods can be used to dispose the enrichment material within channels **116** and/or **124**. The walls can be directly derivatized with, for example, covalently bound or adsorbed molecules, or beads, glass particles, sol-gel or the like can be derivatized and loaded into these channels.

**[0040]** After sample is loaded into channel **116** wash solution and then elution reagent can be introduced through tube **112** and channel **114**.

**[0041]** The elution process will depend on the enrichment method performed in channel **116**. A suitable eluent can be selected to elute a fraction of the bound analyte. Some enrichment options may not require an elution step (e.g., size exclusion chromatography, electrophoretic separations, etc.).

**[0042]** The eluent or flow-through would then flow through channel **118** into channel **124**. Channel **124** could be used to perform either a chromatographic or electrophoretic enrichment step.

**[0043]** Electrophoretic separations can be performed in channel **124** by using a power supply to apply an electric field between reservoir **108** and reservoir **120**. Similarly stated, the device **100** can include electrodes in electrical contact with reservoir **108** and/or reservoir **120**. The electrical ground of the power supply can be connected to the electrical ground of a mass spectrometer to provide continuity in the electric field from channel **124** to the mass spectrometer.

**[0044]** Any capillary electrophoresis (CE) electrophoretic method can be performed in channel **124** - IEF, isotachophoresis (ITP), capillary gel electrophoresis (CGE), capillary zone electrophoresis (CZE), and the like. Alternately, non-electrophoretic enrichment methods can be performed in the channel **124**.

**[0045]** In the case of IEF or ITP, concentrated purified sample bands would be mobilized by pressure or electrical means towards confluence **126**. Sheath solution from reservoirs **108** and **134** could serve as sheath and catholyte.

**[0046]** The sheath/catholyte can be any basic solution compatible with the electrophoretic separation and mass spectrometry (MeOH/N<sub>4</sub>OH/H<sub>2</sub>O for example). Anolyte can be any acidic solution (e.g., phosphoric acid 10mM).

**[0047]** Alternately, the electric field could be reversed and catholyte (NaOH) could be loaded in reservoir **120**, and anolyte could be used as the sheath solution in reservoirs **108** and **134**.

**[0048]** The confluence **126** is where the enriched analyte fraction mixes with the sheath

solution. As the analyte fractions in channel **124** are mobilized, solution will be pushed through confluence **126** out to orifice **128**.

**[0049]** The orifice **128** can be disposed within a recess defined by surface **127** of substrate **102**. For example, surface **127** can be a countersunk ESI surface. For example, as shown in figure 1, the enriched analyte solution, being electrically grounded through well **108**, can form a Taylor cone emanating from orifice **128**, which is disposed entirely within a recess defined by surface **127**. The orifice **128** and/or surface **127** can be oriented toward a mass spectrometer inlet, which can have a voltage potential difference relative to well **108**. As spray breaks off from the cone structure toward the mass spectrometer, it can be flanked by nebulizing gas provided through channels **106** and **140** before it leaves the substrate **102**. The nebulizing gas can be any inert or non-reactive gas (e.g., Argon, Nitrogen, and the like).

**[0050]** Additionally, using a sheath liquid and/or nebulizing gas can allow for the use of an ion depleting step as the last "on-device" step. The sheath liquid allows for replenishment of ion potential lost during an IEF charge assay concentrating step prior to ESI, and nebulization provides the sample in a fine mist for the offline analysis.

**[0051]** By generating the Taylor cone on surface **127**, the cone is created in a stable pocket or recess and is protected from disturbing air currents. Additionally, the conical geometry surrounding the countersunk orifice has a naturally expanding contact surface that will accommodate a wider range of Taylor cone radial cross sections, allowing for a wider range of flow rates into the mass spectrometer.

**[0052]** Orifice **128** can be positioned in proximity to an inlet port of a mass spectrometer. In some instances, the surface **127** can be configured such that an inlet port of a mass spectrometer can be disposed within a recess defined by the surface **127**.

**[0053]** **Figure 2** a schematic exploded view of a device **212** having three layers. Figure 2A shows a top layer **202** of device **212**. Figure 2B shows a middle layer **206** of device **212**. Figure 2C shows a bottom layer **210** of device **212**. Figure 2D shows the device **212** as assembled. Each of the three layers **202**, **206**, **210** may be made of any material compatible with the assays the device **212** is intended to perform.

**[0054]** Layer **202** may be fabricated from a material which is transparent to a specific wavelength, or wavelength range, of light. As used herein, "transparent" should be understood to mean that a substantial majority light having a specific wavelength or range of wavelengths is transmitted through the material. A transparent material can also be understood to mean the material has sufficient transmittance to allow the amount of light on one side of the material to be quantified by a detector on the other side. A wavelength range of interest may include the middle ultraviolet range (e.g., 200nm - 300nm), and materials such as, for example, glass, quartz, fused silica and UV-transparent plastics such as polycarbonates, polyfluorinated polyethylene, polymethacrylate, cyclic olefin polymer, cyclic olefin copolymer, and other UV-transparent materials can be used as transparent materials. The light spectrum of interest may

be expanded beyond the visible spectrum (e.g., 200-900nm).

**[0055]** Through-holes, **204**, are fabricated in layer **202** to allow pressure and electrical interface to a channel network in a lower layer (e.g., layer **208**) from outside the device.

**[0056]** Figure 2B shows the internal middle layer **206** of device **212** containing the channel network **208**. The channel network is designed to interface with the through-holes fabricated in the top layer **202**. The channel network **208** contains inlet and outlet tubes/conduits **209**, and orifice **205** for expelling enriched analyte fractions, and a viewable enrichment zone **207**. Enrichment zone **207** is fabricated so its depth is the full thickness of the layer **206**. Otherwise, zone **207** can be less than the full thickness of layer **206**.

**[0057]** Layer **206** may be fabricated from a material which is opaque and/or not transparent to a specific wavelength, or wavelength range, of light. As used herein, "opaque" should be understood to mean that a substantial majority light having a specific wavelength or range of wavelengths is not transmitted through the material (e.g., reflected, absorbed, and/or scattered by the material). A material that is not transparent can also be understood to mean the material has insufficient transmittance to allow the amount of light on one side of the material to be quantified by a detector on the other side, and will effectively block this light except in the regions where the zone in the channel network is as deep as the full thickness of layer **206**.

**[0058]** Figure 2C shows a bottom layer **210** of device **212**. Bottom layer **210** can be, for example, a solid substrate. Bottom layer **210** can be fabricated from a material with the same transmittance as layer **202**.

**[0059]** Figure 2D shows the device **212** including top layer **202**, the middle layer **206**, and the bottom layer **210**, as assembled. Inlet and outlet tubes **209**, reservoirs **204** and orifice **205** can still be accessed after the device **210** is assembled. The entire top layer **202** and/or the entire bottom layer **210** can be transparent. A portion of the top layer **202** and/or a portion of the bottom layer **210** can be opaque with another portion of the top layer **202** and/or the bottom layer **210** being transparent. For example, the top layer **210** and/or the bottom layer **210** can define an optical window that aligns with at least a portion of the enrichment zone **207** when the device **212** is assembled.

**[0060]** Figure 3 is a schematic of a light path through a microfluidic device **302**. Figure 3A shows a top view of the microfluidic device **302**. Figure 3B shows the microfluidic device **302** positioned between a light source **306** and a detector **308**. The detector **308** is positioned to measure light passing through the device **302**. While not illustrated in figure 3, the microfluidic device **302** can have a similar channel structure as described in figures 1 and 2, but the channel structure is not shown for ease of reference. A portion of top surface of the microfluidic device **302** may be opaque and completely or substantially obscures light projected from the light source **306** from reaching the detector **308**. The portion of the opaque top surface substantially prevents the transmission of light through the device at those portions where detection of sample properties is not desired. For example, the microfluidic device **302** may be

not opaque (e.g., allows some light to pass through) over one or more channel region(s) 304, as the channel 304 transverses the entire thickness of a non-transparent layer.

**[0061]** This transparent channel region(s) 304, can be an enrichment zone, where optical detection can be used to detect analyte, monitor the progress of the enrichment and/or monitor enriched analyte fraction(s) as they are expelled from the device. Changes in the amount of light passing through transparent channel 304 may be used to measure the absorbance of the analyte fractions while they are in this channel. Thus, channel region(s) 304 may define an optical slit, such that the light source 306 positioned on one side of the microfluidic device 302 effectively illuminates the detector 308 only through the transparent channel region(s) 304. In this way, stray light (e.g., light that does not pass thorough the transparent channel regions(s) and/or a sample) can be effectively blocked from the detector 308, which can reduce noise and improve the ability of the detector 308 to observe sample within the transparent channel region(s) 304. The transparent channel regions(s) 304 may be between two enrichment zones, and can be used to detect analyte fractions as they are eluted from the upstream enrichment zone.

## Methods

**[0062]** **Figure 6** illustrates a method of analyte mixture enrichment. The method includes loading and/or introducing an analyte mixture onto a microfluidic device, at **20**. The microfluidic device can be similar to the microfluidic devices described above with reference to figures 1-3. The analyte mixture can be, for example, glycans, carbohydrates, DNA, RNA, intact proteins, digested proteins, peptides, metabolites, vaccines, viruses and small molecules. The analyte mixture can be a mixture of proteins, such as a lysate of cultured cells, cell-based therapeutics, or tumor or other tissue derived cells, recombinant proteins, including biologic pharmaceuticals, blood derived cells, perfusion or a protein mixture from any other source. The analyte mixture may be loaded directly onto the device, or may be loaded onto an autosampler for serial analysis of multiple mixtures.

**[0063]** The microfluidic device can include a first separation channel and/or enrichment zone. The first separation channel and/or enrichment zone can be configured for chromatographic separation. For example, the first separation channel and/or enrichment zone can contain a media configured to bind an analyte from the analyte mixture and/or otherwise effect a chromatographic separation. At **21**, a first enrichment can be performed; for example, a chromatographic separation can be performed in the first separation channel and/or enrichment zone. For example where the analyte mixture is a protein mixture, the first enrichment, at **21**, can simplify the protein mixture. The first enrichment, at **21**, can be based on any discernable quality of the analyte.

**[0064]** This enriched analyte fraction is then eluted, at **22**. For example, an eluent can be injected into the microfluidic device to mobilize the enriched analyte fraction from media disposed within the first separation channel and/or enrichment zone. The enrichment and/or

mobilization of the enriched analyte fraction can be imaged. For example, as discussed above, the first separation channel and/or enrichment zone can define an optical slit. Light can be projected onto the microfluidic device and a detector can detect light passing through the first separation channel and/or enrichment zone. The sample, or a portion thereof can be detected via absorbance and/or fluorescence imaging techniques.

**[0065]** The microfluidic device can include a second separation channel and/or enrichment zone. The second separation channel and/or enrichment zone can be configured for electrophoretic separation. At **23**, a second enrichment can be performed, for example, on the eluate. For example, an electric field and/or electric potential can be applied across the second separation channel and/or enrichment zone.

**[0066]** The second enrichment can be initiated, at **23**, when a fraction of the analyte mixture is disposed at an intersection of the first separation channel and/or enrichment zone and the second separation channel and/or enrichment zone. For example, the first separation channel and/or enrichment zone can be monitored (e.g., imaged) and a an electric potential, and/or electric filed can be applied when a fraction of interest reaches the intersection.

**[0067]** The second enrichment, at **23**, can provide fractions enriched based on charge characteristics (charge isoforms). Such enrichments can include, for example, gel isoelectric focusing, isoelectric focusing with mobilization, isoelectric focusing with whole column imaging, ion exchange chromatography, pH gradient exchange chromatography, isotachophoresis, capillary zone electrophoresis, capillary gel electrophoresis or other enrichment techniques that are, for example, charge-based.

**[0068]** Although the first enrichment, at **21**, has been described as a chromatographic enrichment and the second enrichment, at **23**, has been described as electrophoretic, it should be understood the any suitable enrichment can be performed in any suitable sequence. For example, the first enrichment, at **21**, and the second enrichment, at **23**, can both be chromatographic or both be electrophoretic. As another example, the first enrichment, at **21**, can be electrophoretic, and the second enrichment, at **23**, can be chromatographic.

**[0069]** One or more enrichments can provide fractions enriched based on hydrophobic changes, such as oxidation. Such enrichments can include, for example, reversed-phase chromatography, hydrophobic interaction chromatography, hydrophilic interaction chromatography, or other enrichment techniques that are, for example, hydrophobicity-based.

**[0070]** One or more enrichments can will provide fractions enriched based on post-translational modifications, glycoforms including galactosylation, fucosylation, sialylation, mannose derivatives and other glycosylations, as well as glycation, oxidation, reduction, phosphorylation, sulphanation, disulfide bond formation, deamidation, acylation, pegylation, cleavage, antibody-drug conjugation (ADC), protein-drug conjugation, C-terminal lysine processing, other naturally and non-naturally occurring post-translational modifications and other chemical and structural modifications introduced after translation of the protein, and the

like. Such enrichments can include, for example, binding assays and the like.

**[0071]** One or more enrichments can provide fractions enriched based on hydrophobic changes, such as oxidation. Such enrichments can include, for example, reversed-phase chromatography, hydrophobic interaction chromatography, hydrophilic interaction chromatography, or other enrichment techniques that are hydrophobicity-based.

**[0072]** One or more enrichments can provide fractions enriched based on primary amino acid sequence, such as caused by mutation, amino acid substitution during manufacture and the like. Such enrichments can include, for example, separating by charge isoforms, hydrophobic changes, or other enrichment techniques that can distinguish between primary amino acid sequence differences.

**[0073]** One or more enrichments can provide fractions enriched based on efficacy. Such enrichments can include, for example, bioassays, enzyme inhibition assays, enzyme activation assays, competition assays, fluorescence polarization assays, scintillation proximity assays, or other enrichment techniques that are efficacy-based and the like.

**[0074]** One or more enrichments can provide fractions enriched based on affinity. Such enrichments can include, for example, solution phase binding to target, binding to bead based targets, surface bound target, immunoprecipitation, protein A binding, protein G binding and the like.

**[0075]** One or more enrichments can provide fractions enriched based on mass or size. Such enrichments can include, for example, poly acrylamide gel electrophoresis, capillary gel electrophoresis, size exclusion chromatography, gel permeation chromatography, or other enrichment techniques that are mass-based.

**[0076]** The analyte mixture may go through more than two enrichment before being expelled from the device.

**[0077]** At 24, an enriched analyte fraction can be expelled from the device. The enriched analyte fraction can be expelled via IEF. Expelling the enriched analyte fraction, at 24, can concentrate the analyte fractions before they are expelled from.

**[0078]** The analyte fractions may be expelled, at 24, using an ionization technique, such as electrospray ionization, atmospheric pressure chemical ionization, and the like.

**[0079]** The analyte fractions may be expelled, at 24, using electrokinetic or hydrodynamic forces.

**[0080]** The enriched protein fractions may be expelled, at 24, from the device in a manner coupled to a mass spectrometer.

**[0081]** Mass of an analyte expelled from the microfluidic device (e.g., a biologic or biosimilar) can be measured, for example, through time-of-flight mass spectrometry, quadrupole mass spectrometry, ion trap or orbitrap mass spectrometry, distance-of-flight mass spectrometry, Fourier transform ion cyclotron resonance, resonance mass measurement, and nanomechanical mass spectrometry.

**[0082]** pI markers may be used to map pI ranges in the visualized IEF channel (e.g., the first separation channel and/or enrichment zone and/or the second separation channel and/or enrichment zone). pI markers or ampholytes can be used to determine the pI of the analyte by their presence in downstream mass spectrometry data.

**[0083]** IEF can be monitored during the mobilization and ESI. In this way, mass spectrometry data can be correlated to peaks in the IEF, which can maintain and/or improve peak resolution.

**[0084]** The analyte mixture and/or a portion thereof can be mobilized within the microfluidic device using pressure source. Mobilization may be done with hydrostatic pressure. Mobilization may be chemical immobilization. Mobilization may be electrokinetic mobilization

**[0085]** **Figure 7** is a schematic of a microfluidic device. A microfluidic network, 800, is disposed in and/or defined by a substrate, 802. The substrate is manufactured out of material which is compatible with the enrichment steps being performed. For example, chemical compatibility, pH stability, temperature, transparency at various wavelengths of light, mechanical strength, and the like may be of concern when selecting the material

**[0086]** Substrate 802 may be manufactured out of glass, quartz, fused silica, plastic, polycarbonate, PTFE, PDMS, silicon, polyfluorinated polyethylene, polymethacrylate, cyclic olefin copolymer, cyclic olefin polymer, polyether ether ketone and/or any other suitable material. Mixtures of materials can be utilized if different properties are desired in different layers of a planar substrate.

**[0087]** Channels 806, 808, 810, 811, 817, 814, 812 form a channel network and are fabricated into (e.g., defined by) substrate 802.

**[0088]** Channels may be fabricated in the substrate through any channel fabrication method such as photolithographic etching, molding, machining, additive (3D) printing, and the like.

**[0089]** Analyte mixtures and external reagents can be loaded through tube 804, and excess reagent / waste can be removed through tube 810 and 818.

**[0090]** Tubes 804 and 810 818 can be manufactured out of any material compatible with the assay being performed, including fused silica, fused silica capillary tubes, silicone tubing, PTFE tubing, and the like.

**[0091]** Channels 806 and 814 can be designated as separation/enrichment zones. Either of

channel **806** and/or **814** can be used to perform chromatographic separations (reversed phase, immunoprecipitation, ion exchange, size exclusion, ligand affinity, dye affinity, hydrophobic interaction, affinity, capillary electrokinetic chromatography, micellar electrokinetic chromatography and/or the like) or electrophoretic separations (isoelectric focusing, capillary gel electrophoresis, capillary zone electrophoresis, isotachophoresis, capillary electrokinetic chromatography, micellar electrokinetic chromatography, flow counterbalanced capillary electrophoresis, electric field gradient focusing, dynamic field gradient focusing, and/or the like). For example, channel **806** can be derivatized or packed with material to perform a first enrichment step, represented by darker circles in channel **806**.

**[0092]** The material disposed into channel **806** can be selected to capture analytes based on hydrophobicity (reversed phase), affinity (efficacy), size (size exclusion chromatography), charge (ion exchange), immunoaffinity (immunoprecipitation), protein-protein interaction, DNA-protein interaction, aptamer-base capture, small molecule-base capture or by other forms of liquid chromatography and the like.

**[0093]** Many different methods can be used to dispose the enrichment material within channel **806** and/or **814**. The walls can be directly derivatized with covalently bound or adsorbed molecules, or beads, glass particles, sol-gel or the like can be derivatized and loaded into these channels, or channels can be packed with a sieving material such as - linear polymer solutions such as linear polyacrylamide (LPA), polyvinylpyrrolidone (PVP), polyethylene oxide (PEO), dextran, and the like, cross-linked polymer solutions such as polyacrylamide and the like, matrices for liquid chromatography, or other materials.

**[0094]** Chemically reactive solutions may be added depending on the particular assay performed. In some cases, derivatization of material may occur after it is loaded into channel **806** (or channel **814**), by adding molecules which will adsorb or covalently bond to the loaded material, or can chemically cross link reactive elements to the material. For example, material coated with an antibody-binding molecule such as protein A, protein G, epoxy or the like, could be disposed into channel **806**. Subsequent rinsing with an antibody solution would leave the material coated with antibody and able to participate in immunoaffinity capture. In some cases, the antibody may be mixed with a target analyte or lysate so that the antibody can bind its target in free solution before being coated onto the material.

**[0095]** After enrichment materials are loaded onto device, sample is loaded via tube **804** into channel **806**. Subsequently, wash solutions and elution reagents can be introduced through tube **804** to channel **806**.

**[0096]** In some cases, detection reagents will be added to bind to captured material. Numerous labeling reagents are available that can covalently attach detection moieties such as fluorophores, chromophores or other detection molecules to the target proteins at terminal ends of the polypeptide, and by attachment to amino acid side chains such as lysine, cysteine and other amino acid moieties. Covalently bound detection moieties allow for the protein to be detected through fluorescence excitation, chromophoric assay, or other indirect means. In

some cases, the target protein can remain unlabeled and detected through native absorbance at 220nm, 280nm or any other wavelength at which the protein will absorb light, or native fluorescence. In some cases, the protein will be detected using non-covalently bound fluorogenic, chromogenic, fluorescent or chromophoric labels, such as SYPRO® ruby, Coomassie blue and the like.

**[0097]** In some cases, detection reagents will be added directly to channel **814** to aid detection.

**[0098]** The elution process will depend on the enrichment method performed in channel **806**. It will be selected to elute at least a fraction of the bound analyte. In some cases, this can be accomplished with a combination of heat and sodium dodecyl sulfate (SDS), or other detergents, glycine, urea, or any other method which will induce the release of the captured analyte. Some enrichment options may not require a direct elution step (e.g. size exclusion chromatography). In some cases, elution will be followed by denaturation.

**[0099]** The eluent would then flow through channel **808** into the next separation/enrichment zone, channel **814**. Channel **814** could be used to perform either a chromatographic or electrophoretic enrichment step.

**[0100]** Electrophoretic separations can be performed in channel **814** by using a power supply to apply an electric field between reservoir **812** and reservoir **816**. When eluate from channel **806** passes through the intersection of channels **808** and **814**, the electric field can be enabled, loading analyte into channel **814**. In some case, the analyte will be negatively charged, such as in the standard gel electrophoresis mode where protein analyte is saturated with a negatively charged detergent like SDS. However, the polarity of channel **814** can easily be reversed to accommodate systems where for example, a protein analyte is saturated with a positively charged detergent such as cetyl trimethylammonium bromide (CTAB) or the like. In other cases, a protein analyte may be coated with a neutral detergent, or no detergent - such as in native gel electrophoresis. In this case, polarity will be selected based on the anticipated charge of the protein target in the buffer system selected, so that the protein analyte will migrate into channel **814**.

**[0101]** Any CE electrophoretic method can be performed in channel **814** - IEF, ITP, CGE, CZE, and the like. Alternately, non-electrophoretic enrichment methods can be performed in the channel.

**[0102]** Analyte in channel **814** can be viewed by whole column imaging, partial column imaging, and/or by single point detection.

**[0103]** In some cases, the enrichment material in channels **806**, **814** or both may be removed and replenished with fresh material so that the device can be used on another analyte sample.

**[0104]** In some cases, a channel design such as Figure 7 may be repeated multiple times on a

device, so that more than one analyte sample may be analyzed in parallel.

## EXAMPLES

**[0105]** Aspects of embodiments may be further understood in light of the following examples, which should not be construed as limiting in any way.

### Example 1 - Characterize protein charge on chip before Mass Spectrometry (MS)

**[0106]** For this example, the channel network shown in **Figure 4** is fabricated from a plate of soda lime glass, which has very low transmission of 280nm light using a standard photolithographic etching technique. The depth of the enrichment channel **418** is the same as the thickness of the glass layer **402**, i.e., the enrichment channel **418** passes all the way from the top to bottom of this glass plate **402**. The device **400** can be illuminated by a light source disposed on one side of device **400** and imaged by a detector disposed on an opposite side of device **400**. Because substrate **402** is opaque, but enrichment channel **418** defines an optical slit, the substrate **402** can block light that does not pass through the enrichment channel **418**, blocking stray light and improving resolution of the imaging process.

**[0107]** The glass layer **402** is sandwiched between two fused silica plates, which are transmissive (e.g., transparent) to 280nm light. As in figure 2, the top plate contains through holes for the instrument and user to interface with the channel network, while the bottom plate is solid. The 3 plates are bonded together at 520°C for 30 minutes. The inlet and outlet tubing is manufactured from cleaved capillary (100µm ID, polymicro), bonded to the channel network.

**[0108]** The device is mounted on an instrument containing a nitrogen gas source, heater, positive pressure pump (e.g., Parker, T5-1IC-03-1EEP), electrophoresis power supply (Gamm High Voltage, MC30) terminating in two platinum-iridium electrodes (e.g., Sigma-Aldrich, 357383), UV light source (e.g., LED, qphotonics, UVTOP280), CCD camera (e.g., ThorLabs, 340UV-GE) and an autosampler for loading samples onto the device. The power supply shares a common earth ground with the mass spectrometer. The instrument is controlled through software (e.g., labView).

**[0109]** Protein samples are pre-mixed with ampholyte pH gradient and pI markers before placing into vials and loading onto the autosampler. They are serially loaded from an autosampler via the inlet **412** onto the microfluidic device **400** through the enrichment channel **418** and out of the device to waste **430** through the outlet **434**.

**[0110]** The sheath/catholyte fluid (50% MeOH, N<sub>4</sub>OH/H<sub>2</sub>O) is loaded onto the two catholyte wells **404**, **436**, anolyte (10mM H<sub>3</sub>PO<sub>4</sub>) onto the anolyte well **426**, and the source of heated nitrogen gas is attached to the two gas wells **408**, **440**.

[0111] After all reagents are loaded, an electric field of +600V/cm is applied from anolyte well 426 to catholyte wells 404, 436 by connecting the electrodes to the anolyte well 426 and catholyte wells 404, 436 to initiate isoelectric focusing. The UV light source is aligned under the enrichment channel 418, and the camera is placed above the enrichment channel 418 to measure the light that passes through the enrichment channel 418, thereby detecting the focusing proteins by means of their absorbance. The glass plate 402, being constructed of soda-lime glass, acts to block any stray light from the camera, so light not passing through the enrichment channel 418 is inhibited from reaching the camera, increasing sensitivity of the measurement.

[0112] Images of the focusing proteins can be captured continuously and/or periodically during IEF. When focusing is complete, low pressure will be applied from the inlet 412, mobilizing the pH gradient toward the orifice 424. The electric field can be maintained at this time to maintain the high resolution IEF separation. Continuing to image the enrichment channel 418 during the ESI process can be used to determine the pI of each protein as it is expelled from the orifice 424.

[0113] As the enriched protein fraction moves from the enrichment channel 418 into the confluence 420, it will mix with the sheath fluid, which will put the protein fraction in a mass spectrometry compatible solution, and restore charge to the focused protein (IEF drives proteins to an uncharged state), improving the ionization.

[0114] The enriched protein fraction then continues on to the orifice 424, which can be defined by a countersunk surface 422 of the glass plate 402. The enriched protein fraction can create a Taylor cone once caught in the electric field between the sheath fluid well ground and mass spectrometer negative pole.

[0115] As solution continues to push at the Taylor cone from the enrichment channel 418, small droplets of fluid will be expelled from the Taylor cone and fly towards the mass spectrometer inlet. Nitrogen gas (e.g., at 150°C) can flow from the gas wells 408, 440, down gas channels 410, 432 and form nitrogen gas jets which flank the Taylor cone which can convert droplets emanating from the Taylor cone to a fine mist before leaving the microfluidic device, which can aid detection in the mass spectrometer. Adjusting pressure from the inlet 412 can adapt Taylor cone size as needed to improve detection in mass spectrometer.

#### Example 2 - Reversed-Phase -> IEF -> MS

[0116] Example 2 can be similar to example 1, but is described with reference to **Figure 1**. The channel 116 can be a first enrichment zone loaded with sol-gel derivatized with C18. After loading protein, a volume of eluent (MeCN/H<sub>2</sub>O with IEF ampholytes and standards) can be loaded into channel 116 to elute the least hydrophobic proteins trapped on the sol gel. The eluate is directed to channel 124, which can be a second enrichment zone where IEF, UV

absorbance monitoring and finally ESI take place as described in example 1. Once the ESI of the first eluate is complete, a volume of higher MeCN concentration is used to elute the next lowest hydrophobic protein fraction.

#### Example 3 - Efficacy -> IEF-> MS

**[0117]** Example 3 can be similar to example 2, but biologic drug target derivatized beads can be loaded into channel 116 and used to capture protein. Affinity of reaction is characterized through elution by solution phase target (competitive), salt, pH, or the like.

#### Example 4 - Reversed-phase -> Capillary zone electrophoresis -> MS

**[0118]** Example 4 can be similar to example 2, but is described with reference to **figure 5**. A protein mixture can be loaded through inlet **521** and pass through to enrichment zone **510**, which can contain beads derivatized with C18 for reversed-phase chromatography. During loading, fluid passes through the zone **510**, through viewing region **511** and out outlet **522** to waste. Viewing region **510** transverses an internal layer made of soda-lime glass, which is opaque to 280nm UV light, while the top and bottom layers are made of fused silica, which are transparent to 280nm light.

**[0119]** A 280nm light source is positioned below viewing region **511** and a CCD detector is placed above viewing region **511**.

**[0120]** A solution of 20% MeCN/H<sub>2</sub>O is loaded through inlet **521** through enrichment zone **510**. This solution will elute a fraction enriched for the least hydrophobic proteins in the mixture. Viewing region **511** is monitored for the absorbance of the enriched protein fraction at 280nm as it moves from enrichment zone **510** to the outlet **522**. When the fraction is positioned at the intersection of enrichment zone **510** and enrichment zone **515**, a power supply is turned on creating an electric field between a positive electrode in reservoir **514** and ground at reservoir **504**. This polarity can easily be reversed by switching the polarity of the power supply. Once the electric field is present, the enriched protein fraction will migrate down enrichment zone **515** separating proteins by capillary zone electrophoresis. The separated proteins will mix with the sheath, electrolyte solution at confluence **516**, and form a Taylor cone on surface **518**. Nebulizing Nitrogen gas line is connected to the device at ports **508** and **528**, and moves through channels **512** and **530** to flank material from the electrospray as it exits the device via orifice **520**.

**[0121]** Alternatively, hydrodynamic pressure could be used to load the enriched protein fraction into enrichment zone **515**.

#### Reference Example 5 - Immunoprecipitation -> Capillary Gel Electrophoresis of protein lysates

**[0122]** In this example, a microfluidic channel layer represented by the layout in **Figure 7** is fabricated from a cyclic olefin copolymer. Similarly stated, substrate **802** of microfluidic device **800** defines a channel network. For many applications, for example, if fluorescent detection is employed, microfluidic device **800** could be manufactured using a single material, provided that this material will transmit the wavelength range of light needed to detect the analyte.

**[0123]** Protein A coated beads are loaded into channel **806**. These beads are rinsed with a solution of antibody raised against a target of interest, which will bind to the protein A beads. To reduce antibody shedding interfering with analyte detection, the antibody is then covalently cross-linked to the antibody to the bead using commercially available cross linking reagents, such as Dimethyl pimelimidate (DMP), Bis(sulfosuccinimidyl)suberate (BS3) and the like. After immunoprecipitation beads are prepared and loaded in channel **806**, lysate analyte sample can be loaded via tube **804**. After analyte is given sufficient time to be captured by immobilized antibody, unbound proteins are washed and cleared to waste via tube **822**.

**[0124]** Next, the protein is eluted from the antibody beads so it can be analyzed. Elution is accomplished by loading solution of sodium dodecyl sulfate (SDS) and heating to 50C for 10 minutes. Once released, the eluted analyte is flowed through channel **808** toward the intersection of channel **808** and **814**. When the analyte plug reaches the intersection of channel **808** and **814**, an electric field is turned on between a negative pole at reservoir **812** and a positive pole at reservoir **816**, causing the negatively charged protein to migrate through a dextran linear polymer solution in channel **814**, which has been loaded with the fluorogenic protein dye SYPRO® ruby.

**[0125]** Fluorescently labeled target protein can be visualized during CGE in channel **814** using whole column imaging. Similarly stated, the entirety of channel **814** can be imaged while the SYPRO® ruby dye is excited with 280nm light and emitted light, at 618nm, is measured by a detector.

#### **Reference Example 6 - Variations of microfluidic design without mass spectrometer interface**

**[0126]** In some cases, it will be advantageous to have two designs of a microfluidic layer, that differ by presence or absence of the mass spectrometer interface. Once an analyte is characterized, confirmatory characterization may be done in the absence of the mass spectrometry data. By doing the confirmatory characterization in nearly the same microfluidic design, when an anomaly is identified, it will be simple to transfer the assay back to the chip with the mass spec interface for mass identification. This can eliminate the work otherwise needed to show that the anomaly in the confirmatory data is being analyzed in the mass spectrometry data.

**[0127]** As an example, **Figure 8** shows a microfluidic design similar to microfluidic device **400** shown in Figure 4, without orifice **424** and countersunk surface **422**. Analyte is still introduced to the chip through an inlet **904** and channel **906** to an enrichment channel **908**, but after analysis the sample will be flushed out through an outlet channel **910**, rather than conducting electrospray ionization at an orifice. This design could be run for general operation, and then at times when mass identification is required, the same enrichment can be performed on the microfluidic device **400**, shown in Figure 4, ensuring identification of the analyte variants see on microfluidic device **900** of Figure 8.

**[0128]** The foregoing descriptions of specific embodiments have been presented for purposes of illustration and description. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application, to thereby enable others skilled in the art to best utilize the invention and various embodiments with various modifications as are suited to the particular use contemplated. The scope of the invention is defined by the claims appended hereto.

## REFERENCES CITED IN THE DESCRIPTION

### Cited references

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### Patent documents cited in the description

- US7655477B [0007]

PATENTKRAV

- 1. Fremgangsmåde, der omfatter:**
  - a) indføring af en analytblanding i en mikrofluidanordnings separationskanal;
  - 5 b) påføring af et elektrisk felt over separationskanalen for at separere analytblandingen i berigede analytfaktioner via isoelektrisk fokusering;
  - c) billeddannelse af separationen af analytblandingen og efterfølgende mobilisering af de berigede analytfaktioner inde i separationskanalen via en transparent del af mikrofluidanordningen;
- 10 **kendetegnet ved**
  - d) indføring af en kappefluidelektrolyt i den separerede analytblanding for at mobilisere og i alt væsentligt uddrive samtlige af de berigede analytfaktioner via elektrosprayionisering ind i et massespektrometer fra en enkelt åbning, der flugter med separationskanalen; og
  - 15 e) korrelering af et absorbanstoppunkt for en specifik, beriget analytfaktion påvist ved billeddannelse af separationskanalen i (c) med massespektrometerdata for den specifikke berigede analytfaktion.
- 2. Fremgangsmåde ifølge krav 1, hvor mikrofluidanordningen omfatter en første separationskanal og en anden separationskanal.**
- 20 **3. Fremgangsmåde ifølge krav 2, der endvidere omfatter: kromatografisk berigelse af analytblandingen i den første separationskanal før påføring af det elektriske felt for udførelse af den isoelektriske fokuseringsseparation af analytblandingen i den anden separationskanal.**
- 4. Fremgangsmåde ifølge krav 1, hvor i alt væsentligt samtlige af de berigede analytfaktioner uddrives fra åbningen i en kontinuerlig strøm.**
- 25 **5. Fremgangsmåde ifølge krav 1, der endvidere omfatter blanding af amfolytter og isoelektrisk punkt- (pl) markører med analytblandingen før indføring i**

separationskanalen for at generere en pH-gradient i separationskanalen og for at kortlægge pl-intervallet i separationskanalen.

- 6.** Fremgangsmåde ifølge krav 1, hvor analytblandingen omfatter intakte proteiner.
- 5      **7.** Fremgangsmåde ifølge krav 1, hvor indføringen af kappefluidelektrolytten sker ved at lade en kappefluidelektrolytopløsning strømme fra en elektrolytkanal, der står i fluidforbindelse med et sammenstrømningsområde på nedstrømssiden af separationskanalen.
- 10     **8.** Fremgangsmåde ifølge krav 1, hvor efterfyldning af ionpotentiale sker på mikrofluidanordningen via indføring af kappefluidelektrolytten.
- 15     **9.** Fremgangsmåde ifølge krav 7, hvor mikrofluidanordningen omfatter separationskanalen, åbningen og kappefluidelektrolytkanal.
- 20     **10.** Fremgangsmåde ifølge krav 9, hvor separationskanalen og kappefluidelektrolytkanal skærer hinanden sammenstrømningsområdet.
- 15     **11.** Fremgangsmåde ifølge krav 10, hvor sammenstrømningsområdet er indbefattet i det elektriske felt påført separationskanalen.
- 20     **12.** Fremgangsmåde ifølge krav 10, hvor sammenstrømningsområdet flytter med separationskanalen og åbningen.
- 20     **13.** Fremgangsmåde ifølge krav 7, hvor mikrofluidanordningen omfatter to elektroder, der genererer et elektrisk felt langs kappefluidelektrolytkanalen.
- 20     **14.** Fremgangsmåde ifølge krav 9, hvor mikrofluidanordningen endvidere omfatter en analytindføringskanal og forstørningsgastilførselskanaler til ionisering.

## DRAWINGS

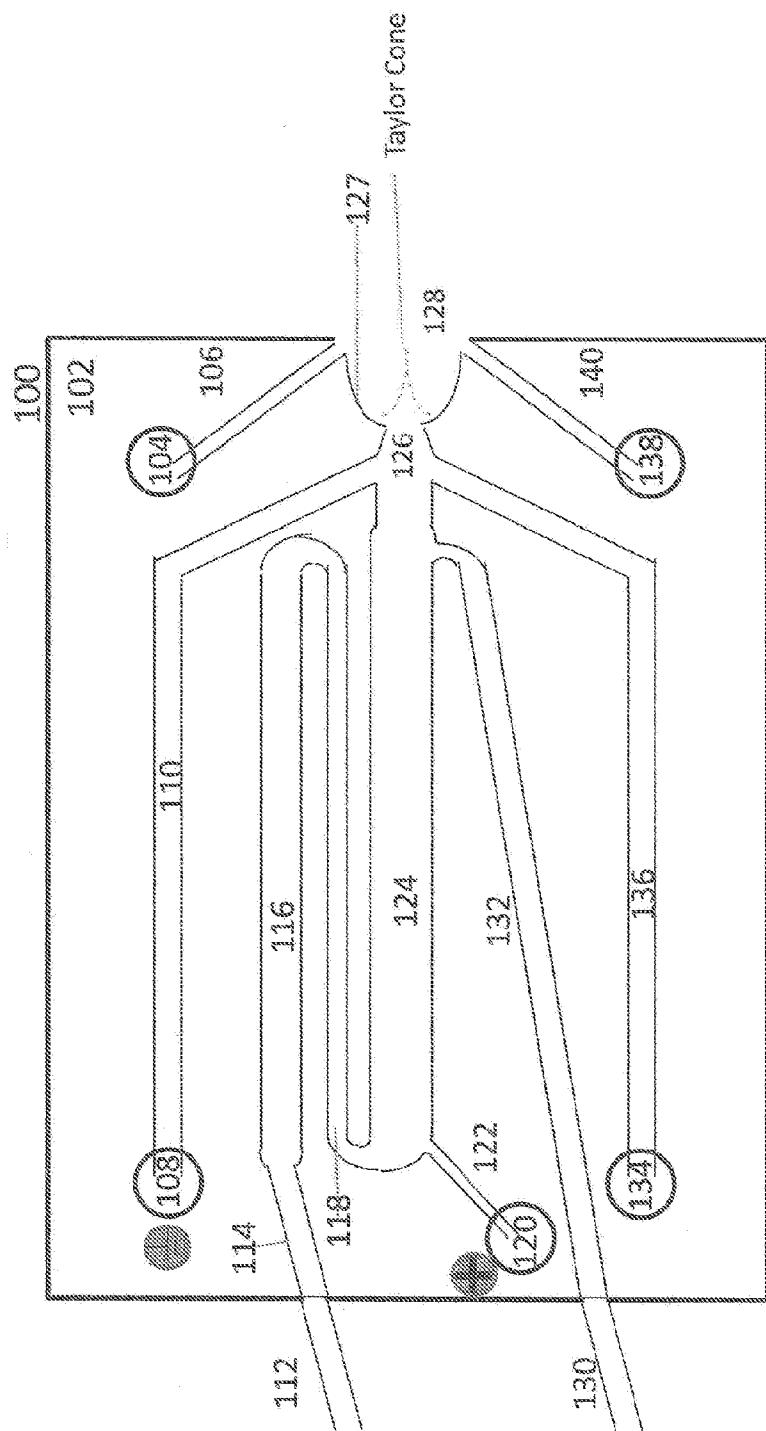
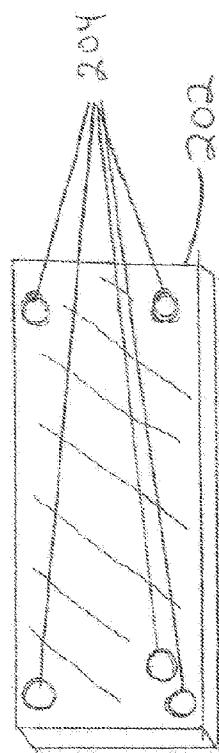
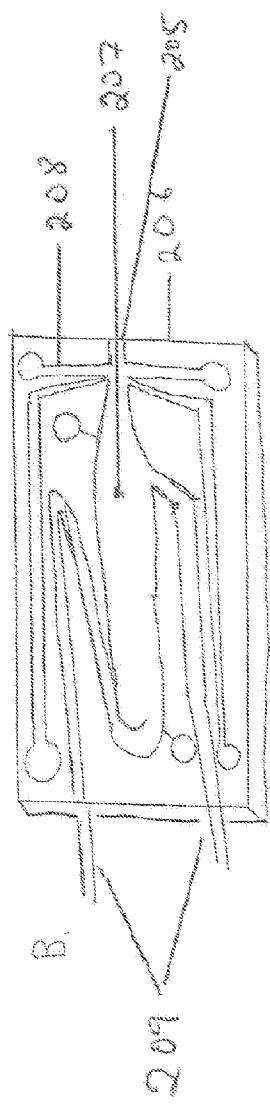


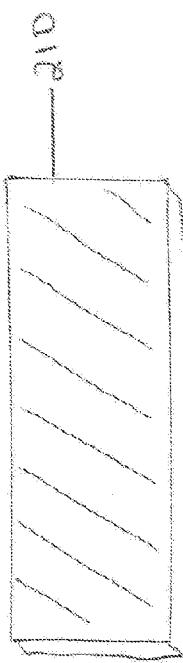
Figure 1



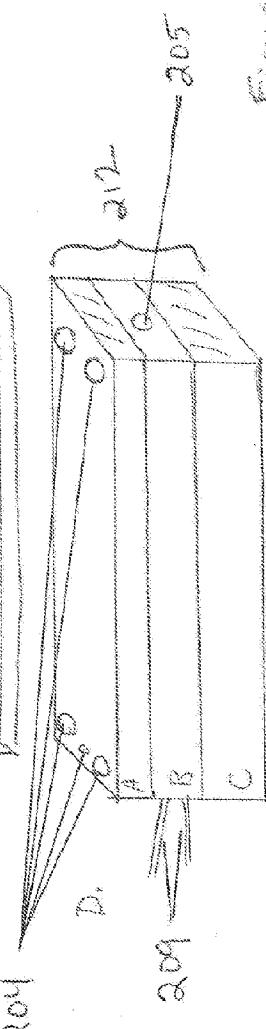
A.



B.



C.



D.

Figure 2

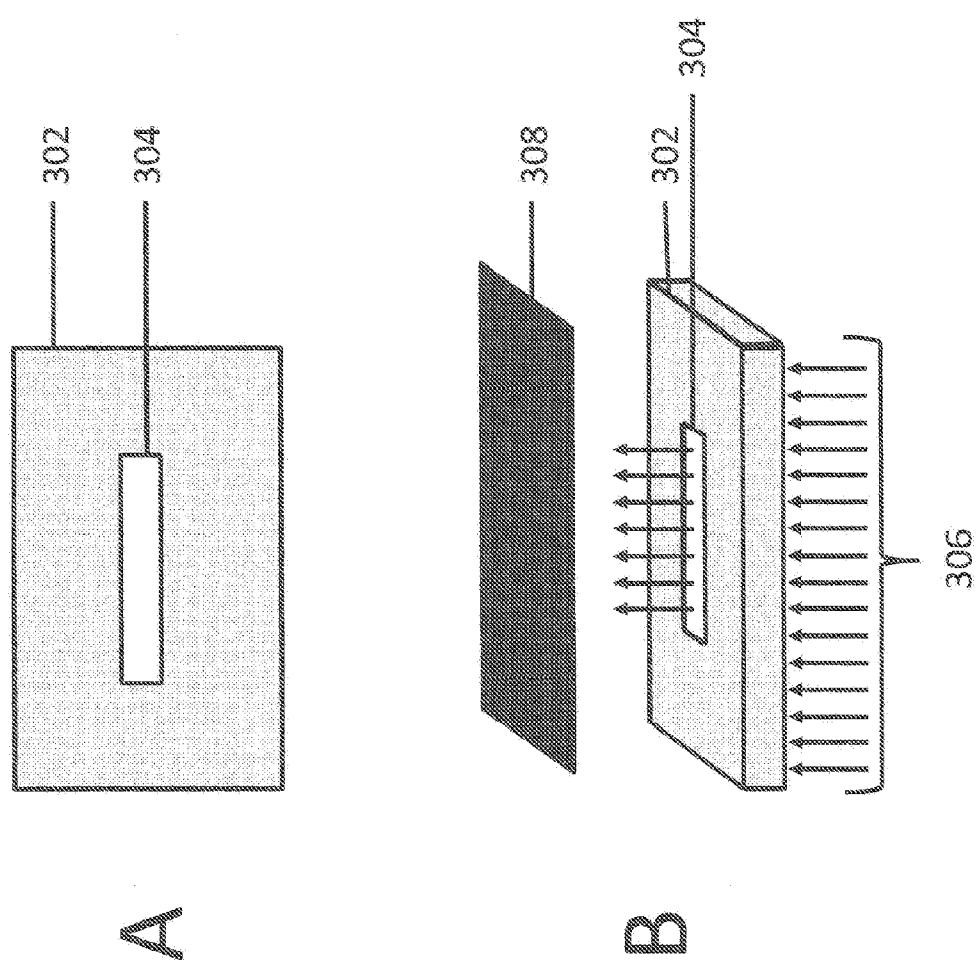


Figure 3

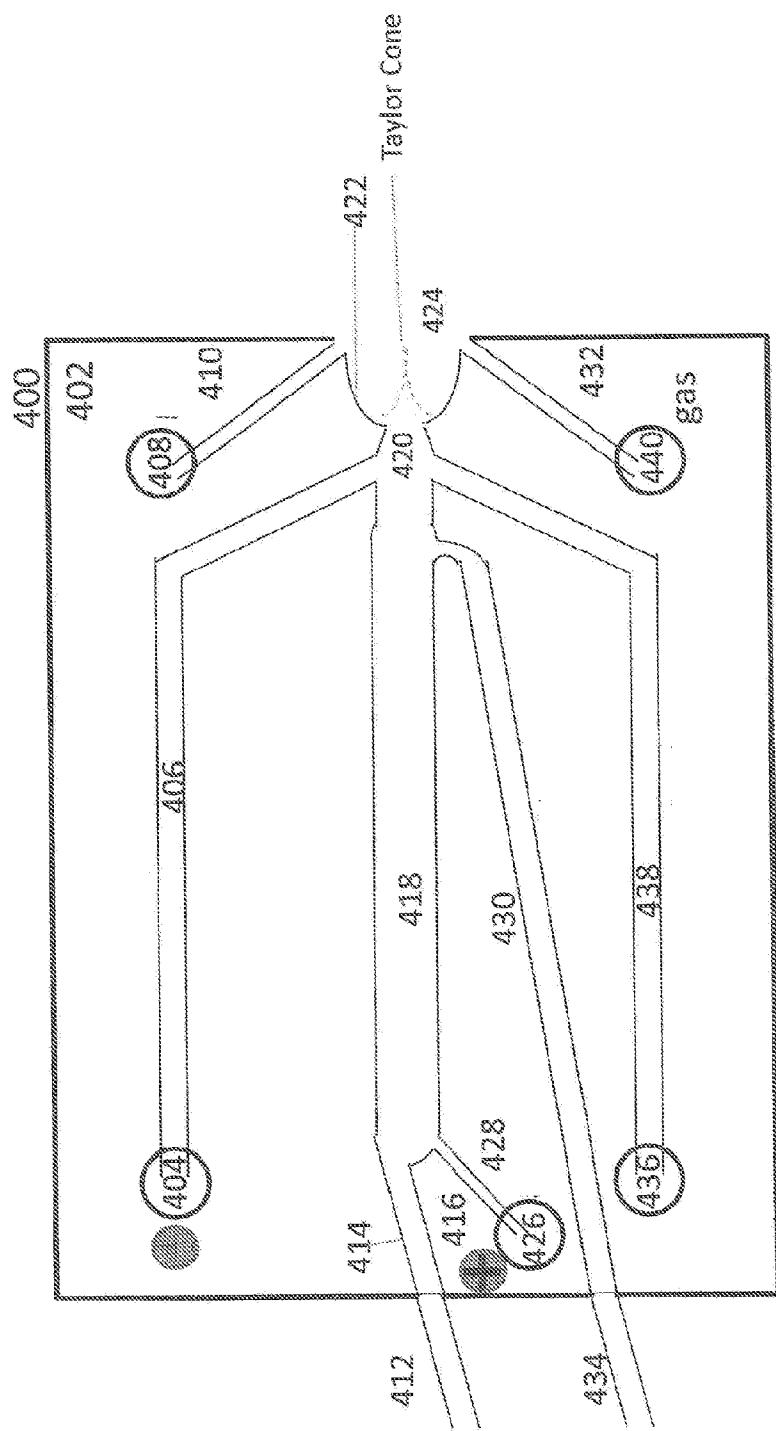


Figure 4

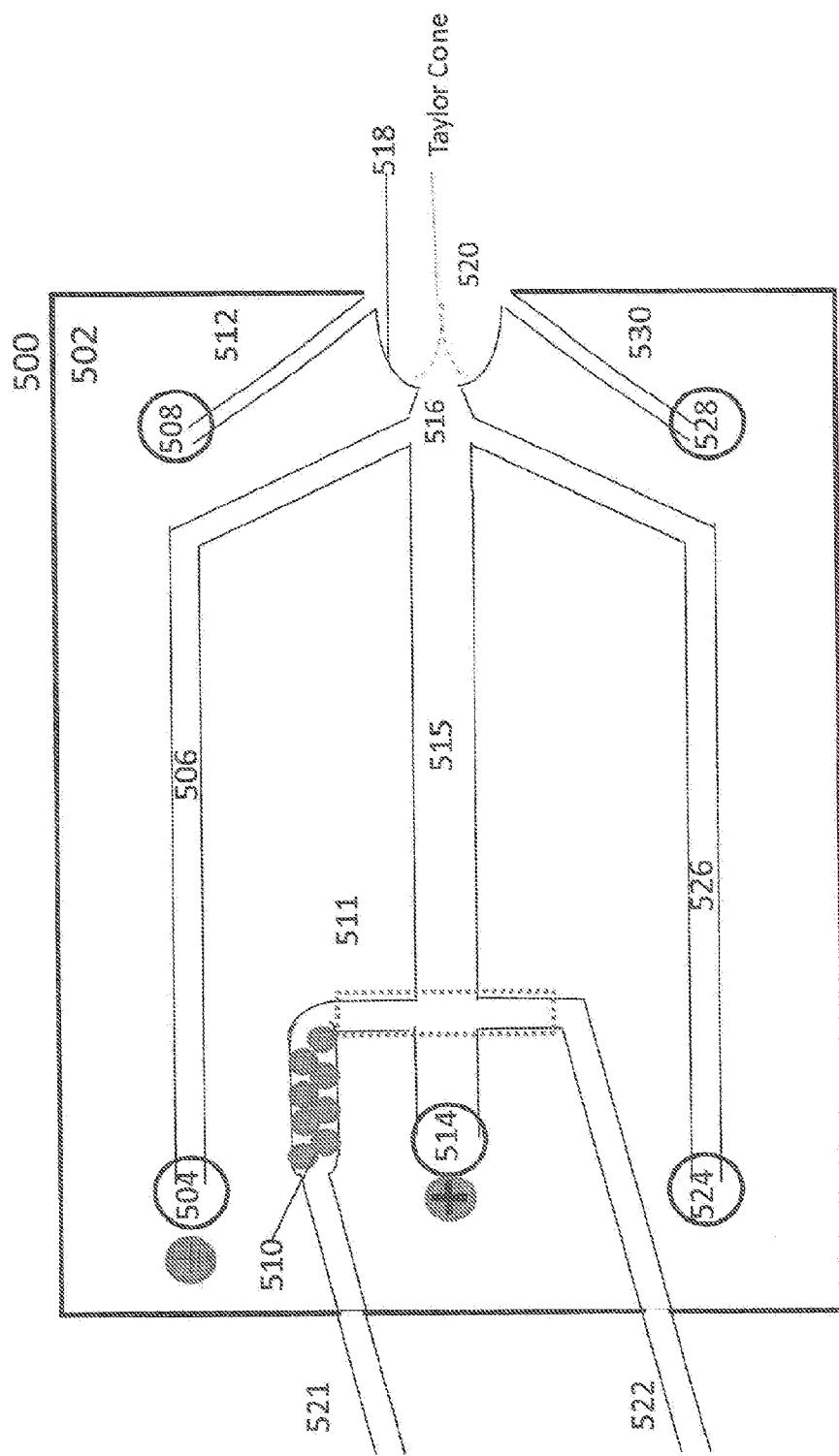


Figure 5

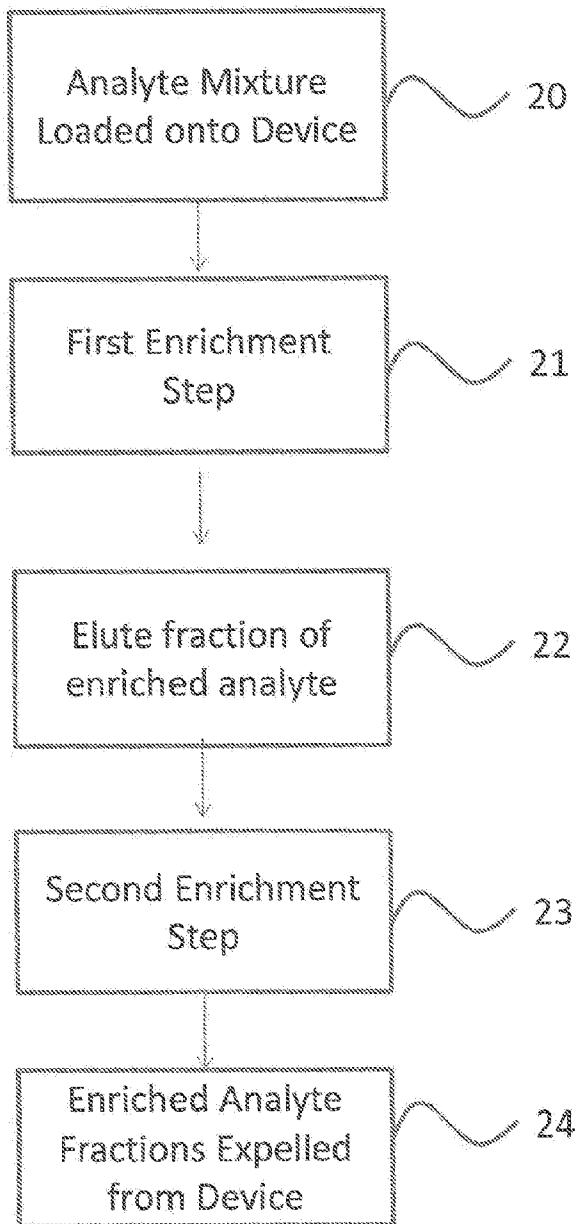


Figure 6

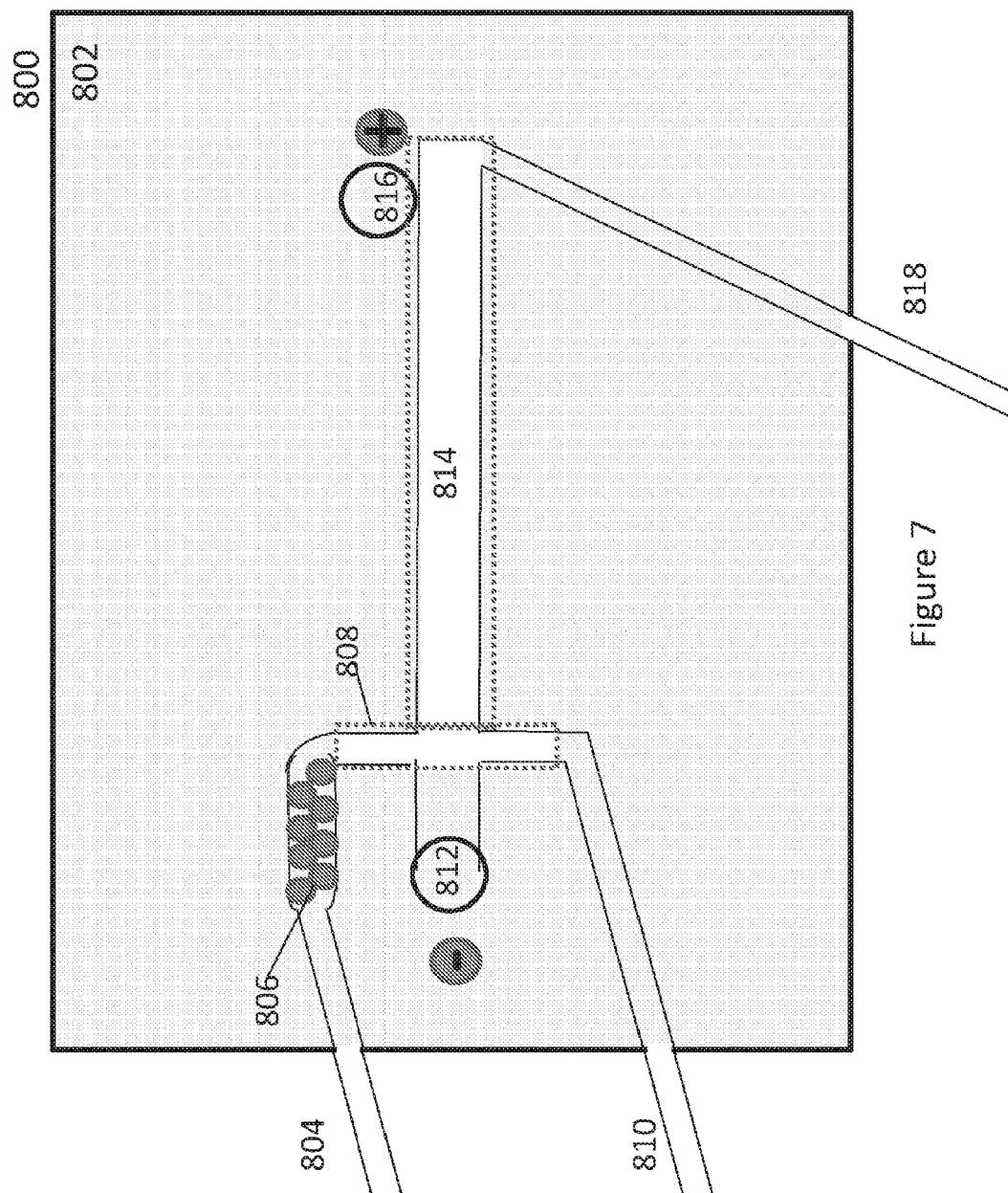


Figure 7

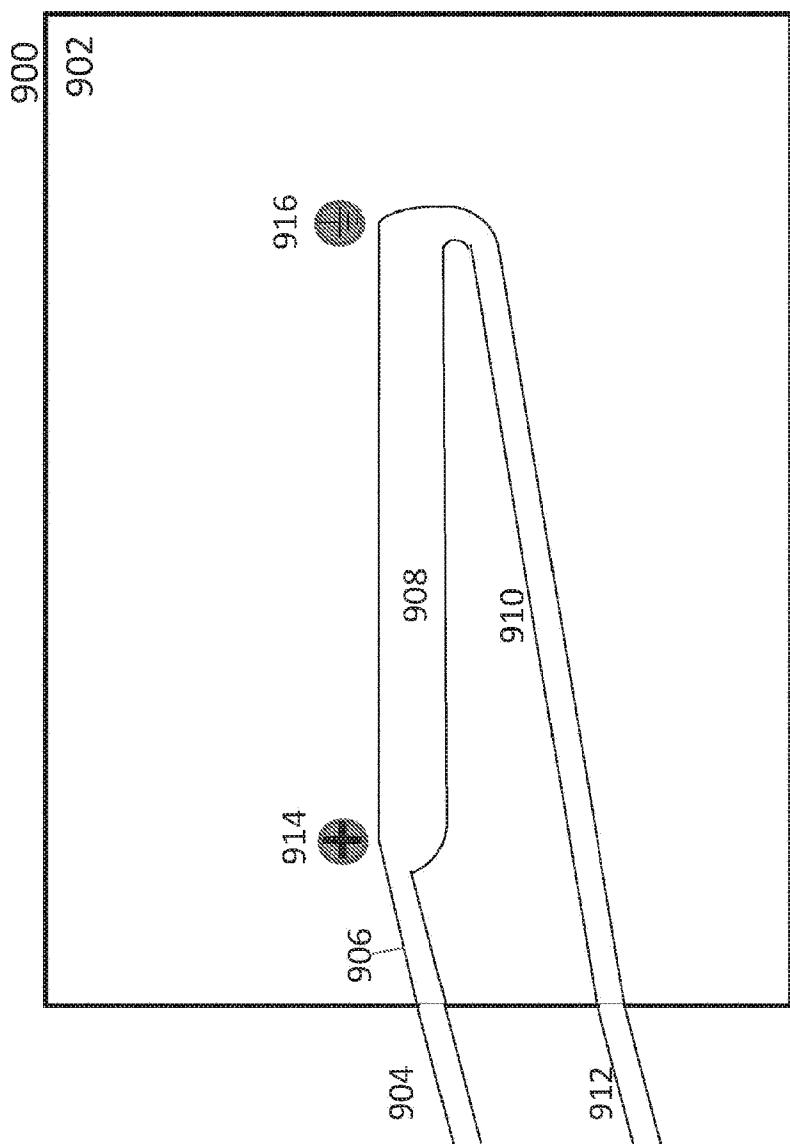


Figure 8