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Devices and methods for conducting binding reactions are described. The devices comprise a plurality of detection elements affixed to a substrate, where each of the detection elements is confined to a discrete spatial location on the substrate. The detection elements comprise a plurality of binding reagents, where the binding reagents contained within a single detection element can bind to a single target.

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(57) Abstract

Devices and methods for conducting binding reactions are described. The devices comprise a plurality of detection elements affixed to a substrate, where each of the detection elements is confined to a discrete spatial location on the substrate. The detection elements comprise a plurality of binding reagents, where the binding reagents contained within a single detection element can bind to a single target.

ASSAY DEVICE COMPRISING MIXED PROBES

Field of the Invention

The invention provides improved devices and methods for detecting analytes in a sample, such as nucleic acids.

Background of the Invention

Assay arrays have widespread application for efficient and rapid characterization of a test sample. Arrays derive their efficiency from combining a large number of singular, discrete tests into one, complex test. Recently, the value of arrays confined within small dimensions, so called "microarrays," also has been recognized.

The application of microarrays for biological assays has expanded rapidly. The promise of rapid sample analysis by detecting binding between one or more components of a test sample, for example a pool of cDNA, and an array of immobilized binding reagents, such as DNA or oligonucleotides, has led to the development of a variety of methodologies useful in characterizing a test sample. As a result of such development microarrays have been used in protocols for gene discovery, gene expression, gene mapping and mutation detection.

A traditional approach to characterizing a test sample is to perform a series of separate, discrete tests on the sample. The test sample or target compound is divided into separate portions or aliquots, and each portion then is evaluated by a different assay, where each assay utilizes a particular binding reagent with a defined specificity. Blood bank analyzers, for example, typically split each blood sample into small aliquots that are analyzed separately to determine each measured property. This strategy becomes cumbersome as the number of tests increase and suffers from a dilution-by-aliquoting effect. For example, the amount of sample

available for each assay diminishes in direct proportion to the number of assays to be performed on the sample. Therefore, a thorough characterization of a test sample by this strategy requires relatively large sample volumes. This strategy also requires separate analysis channels for each test, thereby increasing the complexity of the analytical instrumentation in proportion to the number of tests examined. In sum, this approach limits the amount of sample available to each test, requires additional sample manipulation and increases apparatus complexity.

An alternative approach to characterizing a test sample is to use microarrays to perform multiple tests on the sample simultaneously. Devices employing this strategy separate specific binding reagents or "probes" into discrete locations in a defined pattern on a surface and expose all of the probes to the sample at the same time. Since all of the binding probes access the sample in a single step, there is no need to divide the sample, and the dilution-by-aliquoting effect discussed in the previous approach is avoided. This strategy simplifies the testing process and reduces the complexity of the apparatus and, consequently, its cost. The benefits of this approach become more apparent as the number of tests performed on a sample increases.

Thus far, assays of binding probes have typically been applied to flat surfaces. The resulting two dimensional microarrays have two major limitations. First, the quantity of binding probe that may be applied to the surface of the substrate is limited by the amount of surface per unit area. This parameter governs the yield of bound target and, thus, the detection sensitivity. Since a goal of such microarrays is to determine the presence of subtle changes in the levels of particular targets in a test sample, the sensitivity of a microarray largely determines its usefulness.

Second, in two dimensional arrays, diffusion is the rate-limiting step of binding. On a flat surface, the time required for target molecules to diffuse over relatively long distances before encountering complementary binding probes can be up to several hours. Such time-consuming hybridizations reduce the efficiency of the assay and preclude use of such microarrays in point-of-care testing. Rapid, on-site analysis, while the patient is still in the office, allows for immediate treatment or counseling decisions.

Microarrays utilizing microchannels overcome some of the inherent limitations observed in two dimensional arrays. Microchannel devices have been described and are defined as comprising a substrate having oppositely facing first and second major surfaces, a multiplicity of discrete channels extending through said substrate from said first major surface to said second major surface and a binding reagent immobilized on the walls of said channels was described. (See Beattie, U.S. Patent No. 5,843,767). Microchannels improve detection sensitivity due to the vastly increased surface area within the channels, which increases the quantity of binding reagent bound per cross sectional area. The use of microchannels also permits a flow-through strategy to be employed which minimizes the rate-limiting diffusion step preceding the hybridization reaction by reducing the time required for the average target molecule to encounter a surface-tethered probe from hours to milliseconds.

While microarrays have been instrumental in decoding complex biological systems and have improved the characterization of target molecules, improvements in accessibility and sensitivity are required to maximize the benefit of microarray technology. An important component in the effectiveness of microarrays is exposure of target molecules to as many binding probes as possible. Consequently, binding probes that are not presented adequately to target molecules

do not add value to the array. Finally, the processes employed to construct arrays are extremely complex, requiring highly technical machinery and lengthy manufacturing times. The high costs associated with these aspects are prohibitive and prevent the widespread use of the technology.

During procedures used for processing nucleic acids from biological samples such as cells or tissues, various methods are employed to fractionate the nucleic acid samples for application to nucleic acid-based microarrays. For example, cDNA may be prepared from cellular mRNA, and then regions of the cDNA subjected to amplification by the polymerase chain reaction. The cDNA may be fractionated by incorporation of a digestible nucleotide during the reverse transcription step from the mRNA. For example, a proportion of the dT nucleotides added to the reverse transcription step can be replaced with dU, providing a reverse transcript containing a controllable proportion of dU residues. Subsequent treatment of the transcript with uracil DNA glycosylase will create abasic sites at some or all of the dU residues, and these abasic sites can act as sites for facile cleavage of the DNA strand, for example by heat and/or basic conditions. Alternatively, so-called "cRNA" may be prepared that provides multiple RNA copies of certain regions of the original nucleic acid of interest. See for example US Patent Nos. 5,545,522 and 5,514,545.

This fractionation is used to produce a variety of short nucleic acid targets that are representative of the DNA or RNA pools that are of interest. These shorter nucleic acid targets are detectably labeled and applied to the array which is comprised of a solid support with attached oligonucleotide-based probes. Usually, the probes located within each individual spot have a single nucleic acid sequence that is specific for a particular locus within a gene or mRNA of interest. During the fractionation procedures, the generation of shorter nucleic acid targets is a

random event which produces targets having a variety of sequences derived from the original gene or RNA of interest. These multiple sequences usually are of varying length, and many of the sequences are not perfectly complementary to the nucleic acid probes contained within a spot. As a result, the majority of the labeled targets do not stably hybridize and cannot produce signals for detection.

One solution to this problem is to use longer sequence probes, which can ensure capture of a larger proportion of the target nucleic acids. However, use of longer probe sequences slows hybridization rates and increases the rate of false positive signals observed due to the random production of partial hybrids with non-target nucleic acids.

It is apparent, therefore, that improved arrays for detecting the presence of nucleic acid molecules are greatly to be desired. It also is apparent that improved methods of detecting nucleic acid molecules are desirable.

Summary of the Invention

It is therefore an object of the present invention to provide improved devices for detecting multiple binding reactions.

It is another object of the invention to provide methods of detecting multiple binding reactions using the devices.

In accomplishing these objects, there has been provided, in accordance with one aspect of the present invention, an improved assay device that reduces background and enhances the probability or generating a signal from low abundance target molecules.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Detailed Description

The present invention provides improved devices for detecting the presence of target molecules. The devices use arrays of detection elements disposed in separate and spatially distinct sites, where each detection element can contain a plurality of probe molecules that hybridize to the target molecules. These devices allow improved detection of target molecules compared to conventional arrays.

In conventional arrays used for detecting, for example, nucleic acids, each spatially isolated detection element contains a single probe that is complementary

to a specific target molecule. When such arrays are used to detect, for instance, gene expression in a tissue or cell ("the nucleic acid sources"), a mixture of target nucleic acids first is prepared that is representative of the amount and types of the various mRNA molecules produced from the nucleic acid source. For example, reverse transcription-polymerase techniques (RT-PCR) have been used to prepare double stranded cDNA segments that correspond to portions of the mRNAs produced in the nucleic acid source. Alternatively, cRNA may be prepared that provides multiple RNA copies of certain regions of the original nucleic acid of interest, as described, for example in US Patent Nos. 5,545,522 and 5,514,545.

Ideally, for each mRNA produced in the nucleic acid source, these techniques would provide a single detectable target molecule that is representative of the identity and quantity of that mRNA. However, in practice these techniques tend to produce a mixture of target moieties, each of which can be considered representative of the original mRNA. For example, the mixture may contain target molecules of differing length, representing different regions of the original mRNA. These regions may be overlapping, may only overlap by a few nucleotides, may be juxtaposed, or may not overlap at all and be separated by many nucleotides. Accordingly, a detection element containing a single probe cannot efficiently hybridize to every target molecule that represents an original mRNA molecule, resulting in a potential loss of information.

One potential way to overcome this in a conventional array is to dedicate several different detection elements to probes for detecting target molecules representative of the original mRNA. For example, some probes could be complementary to a 5' region of the mRNA, while others could be complementary to an internal sequence or a 3' sequence. This approach has several disadvantages, however. For example, use of multiple detection elements for

detecting different targets representative of a single mRNA significantly reduces the number of targets that can be assayed with a single substrate. Also, the amount of target bound at each detection element must exceed the lower limit of sensitivity for the detection system used to identify binding. If target molecules representative of a single mRNA are dispersed between several detection elements, the level of binding at each detection element might not be detectable, even where the aggregate amount of target nucleic acid would comfortably exceed this detection limit.

The present invention solves this problem by providing arrays of individual detection elements where each detection element can comprise a plurality of probe molecules. The probe molecules typically are nucleic acids such as DNA or RNA, but the invention is not so limited. For example, the probe molecules can be other binding moieties such as antibodies or their antigens, receptors or their ligands, or other binding molecules known in the art. Accordingly, the resulting arrays of detection elements are effective for carrying out analytical tasks such as analysis of patterns of gene expression by hybridization of mRNA, cDNA, or cRNA to gene-specific probes, immunochemical analysis of protein mixtures, assay of receptor-ligand interactions and profiling of cellular populations involving binding of cell surface molecules to specific ligands or receptors. Other analytical tasks will be apparent to those skilled in the art.

When the probe molecules are nucleic acid molecules, the probes within an individual detection element are each complementary to different sequences within a target molecule representative of a single expressed gene. As a consequence, a variety of target molecules that would be missed by a single probe, but that are target molecules that are representative of an expressed gene, are captured at a single detection element. This enhanced capture reduces the number of detection

elements that are needed to analyze a given set of expressed genes. Moreover, by capturing an increased number of target molecules at a single detection element the method can improve the sensitivity of detection of the target molecules, because the detectable signal from the mixture of target molecules is additive and is more likely to exceed the sensitivity limit of the detector. With this increased signal, the capacity to detect low abundance targets is increased.

Another advantage of the present invention is that it allows relatively short probe molecules to be used, as well as capturing target molecules that are representative of all regions of the expressed gene. When probes such as full length cDNAs or significantly longer nucleic acid probes are used, then there is an increased potential for non-specific hybridization, which increases background or produces false positive results. By using shorter probes, hybridization is specific and random partial hybridization events are less likely to occur, reducing background. In preferred embodiments, the probes are about less than 200 bases long, although the skilled artisan will recognize and determine the optimum length of the probes that would be useful in the present invention using techniques well known to persons in the art.

The skilled artisan will recognize that a target molecule is "representative" of an expressed gene when it contains a contiguous sequence of nucleotides that is identical to a sequence found within the expressed gene. Preferably, the contiguous sequence is at least 15 nucleotides in length. More preferably, the contiguous sequence is at least 30, 50, 100, 200 or 500 bases in length. The target molecule preferably is cDNA or mRNA.

The probe molecules contained within a particular detection element are complementary to different sequences within the expressed gene. These different species may have any degree of overlap: they may overlap completely or only

slightly, they may be juxtaposed, or they may not overlap at all. The particular probe sequences used, and the proportion of the individual probes contained within an individual detection element can be optimized depending on the method used to prepare the mixture of target molecules. For example, when the target molecules are cRNA prepared according to the methods of US Patent No. 5,716,785, the composition of the target molecule mixture tends to be weighted towards the 3' end of the expressed gene. Accordingly, the individual detection elements may contain a majority of probes complementary to sequences contained at the 3' end of the expressed gene. Optimization of the mixture of probes at a particular detection element can be carried out, if desired, using target molecule mixtures of known composition, by measuring the detectable signal obtained with particular probe mixtures.

Another example where depositing multiple probes into a single spot is desired is for the detection of genomic DNA contamination in a mRNA sample. Genomic DNA contains the messenger RNA sequence interspersed with intron sections. The presence of genomic DNA in a sample is undesirable because it can diminish the measurable differential gene expression between two samples due to its presence at a constant level in both samples. Probes can be designed for one or more of the intron regions for a selected gene and placed within a single spot, thereby providing a means for detecting genomic DNA contamination in a sample.

The mixed probe sets contained within individual detection elements are useful in essentially any of the known microarray formats used for assaying gene expression, and also in array formats that are yet to be discovered. For example the mixed probe sets are useful in microchannel arrays of the type described in US Patent No. 5,843,767, and also in arrays where the probes are confined to a two-dimensional substrate surface, such as those described in US Patent No.

5,744,305. The substrate may be fabricated from any material that is capable of being stably affixed with nucleic acid probe molecules. For example the substrate may be fabricated from silicon, nylon, or glass, or may be any material that can be coated with a gold film to which thiol-containing nucleic acid probes can be affixed. The method of affixation can be covalent or non-covalent, provided that the probe molecule is retained on the substrate during hybridization conditions. Other suitable substrates are described at columns 7-8 of US Patent No. 5,482,867, which patent is hereby incorporated by reference in its entirety.

Typically, target preparations are derived from the 3' end of mRNAs, although preparations derived from the 5' end, and from internal portions of the mRNA sequence, can be used. A set of probes that is unique to the mRNA is chosen using sequence analysis techniques that are well known in the art. The probes are prepared as single compounds using known methods.

The probes that make up an individual detection element may then be mixed and affixed to the substrate, or may be affixed sequentially or a combination thereof. When the probes are mixed prior to affixation, then each probe typically is affixed using the same process, for example via the same type of covalent bond. For example, each probe may contain a 5' or 3' terminal thiol moiety which can react covalently with a surface that contains N-ethyl maleimide residues. When the probes are applied sequentially, the affixation method can be varied for each probe, if necessary. Sequential application in this fashion permits use of affixation methods that cannot be carried out in a single step. For example, base-catalyzed and acid-catalyzed affixation steps can be carried out sequentially, but cannot be carried out simultaneously. Other methods of affixation are known in the art and also are described in more detail below.

Synthesis of the probes as individual molecules also allows optimization of the binding probes. For example, the probes can be prepared with or without linker molecules used for affixation to the substrate surface, and the nature and length of the linkers can be varied. Alternatively, the probes can be prepared to allow their affixation in either possible orientation (5'→3' or 3'→5'). For example, a thiol group used to affix the probe to a surface may be placed at either terminus of the probe or, indeed, may be placed at an internal site, if desirable. Each probe sequence may be optimized against a target molecule mixture before affixation to the substrate.

Yet another advantage of synthesizing probes individually prior to affixation is that the probes within single detection element can have different chemical structures. For example, the probes may be DNA, RNA, phosphorothioate-linked oligonucleotides, 2'-O-methoxy linked oligonucleotides, or peptide nucleic acids. The skilled artisan will recognize that any molecular structure capable of binding to DNA or RNA in a sequence-specific manner can be used as a probe molecule in the invention.

Probes can be produced from cDNAs by fragmentation and incorporation of a reactive chemistry, for example by incorporation of one or more nucleotides containing a reactive moiety such as an amine group. Probes also can be produced for novel or unknown targets that are isolated from a target mixture by, for example, gel electrophoresis. Reverse transcription ("RT") of an mRNA or PCR amplification of a DNA can be used to prepare a probe from a nucleic acid isolated from a band on a gel (or from a peak detected in a capillary electrophoresis) by extension of a primer. The extended sequences resulting from the RT or PCR steps are randomly terminated by incorporation of a terminating nucleotide (e.g. dideoxyNTP) in the RT or PCR step. This procedure will

produce a variety of probes of varying length, each having a common 5' end (the primer sequence), but where all the probes recognize a single gene.

A. Chemistry of attachment to the substrate

Optimal procedures for attachment of nucleic acids to silicon dioxide surfaces can be based on well-established silicon chemistry (Parkam *et al.*, Biochem. Biophys. Res. Commun., 1:1-6 (1978); Lund *et al.*, Nucl. Acids Res. 16:10861-10880, (1988)). This chemistry is used to introduce a linker group onto the glass bearing a terminal epoxide moiety that specifically reacts with a terminal primary amine group on the oligonucleotide. This versatile approach (using epoxy silane) is inexpensive and provides a dense array of monolayers that can be readily coupled to terminally modified (amino- or thiol-derivatized) oligonucleotides. Alternatively, chemistries that introduce other linker groups, for example an amine or a mercaptan, also can be used. The linker group should be reactive toward moieties that can be incorporated into a biomolecule, or toward a cross-linking agent that is reactive with a moiety that can be incorporated into a biomolecule. See for example U.S. Patent No. 5,077,210, which is herein incorporated by reference. The density of probe attachment may be controlled over a wide range by mixing long chain amino alcohols with the amine-derivatized oligonucleotides during attachment to epoxysilanized glass. This strategy essentially produces a monolayer of tethered DNA, interspersed with shorter chain alcohols, resulting in attachment of oligonucleotides down to about 5 nm apart on the surface. Variable length spacers are introduced optionally onto the ends of the oligonucleotides, by incorporation of triethylene glycol phosphoryl units during the chemical synthesis. These variable linker arms are useful for determining how

far from the substrate surface oligonucleotide probes should be separated to be readily accessible for pairing with the target DNA strands.

Thiol chemistry, adapted from the method of Whitesides and coworkers on the generation of monolayers on gold surfaces (Lee *et al.* Pure & Appl. Chem. 63:821-828 (1991) and references cited therein.), may be used for attachment of DNA to gold and platinum surfaces. Dithiols (e.g., 1,10-decanedithiol) are linked at one terminus to the metal surface, and also provide a terminal, reactive thiol moiety for reaction with bromoacetylated oligonucleotides. The density of attachment of DNA to gold or platinum surfaces is controlled at the surface-activation stage by use of defined mixtures of mono- and dithiols. Alternatively, chemistries that introduce other linker groups, for example an amine or a carboxylic acid, can also be used. The linker group should be reactive toward moieties that can be incorporated into a biomolecule (for example aldehydes or amines), or toward a cross-linking agent that is reactive with a moiety that can be incorporated into a biomolecule. Suitable cross-linking agents are known in the art and are available, for example, from Pierce Chemical Co. (Rockford, IL).

For silicon substrates, the silicon surface may be halogenated with, for example, a thionyl halide or a phosphoryl halide, or with an elemental halogen, to produce a halogenated surface. The halogenated surface may then be derivatized, either directly with a nucleic acid probe containing a nucleophilic amine or thiol moiety, or with a linker that then can be used to couple to a probe molecule. See (Bergerson *et al.* J. Am. Chem. Soc. 121:454-455 (1999), which is incorporated herein by reference. In a preferred embodiment, the halogen is chlorine.

B. Application of the probes to the substrate surface

Several previous approaches to the preparation of microarrays have used various techniques to synthesize probes *in situ* at defined sites on the surface of a substrate. Because the synthesis methods necessarily involve reaction with all the putative probe molecules at each defined site, only a single probe can be produced at each site on the substrate surface, and these *in situ* methods cannot be used for preparing arrays of detection elements that each contain controlled mixtures of probe molecules.

The present invention requires precise application of the probes or probe mixtures to discrete locations on the substrate surface of a device. As the variety and replication of probes in a microarray may be very large, their accurate and precise application on the device is a considerable technical challenge.

A variety of devices have been used to make microarrays by spotting probes onto a surface. Mechanical pen-like spotting devices have been developed that deliver the desired probes by contacting the substrate surface. For example, such a device has been used to spot 8,192 oligonucleotide probes onto a flat surface. (See Drmanac *et al.*, *Nature Biotechnology* 16: 54-58 (1998)). Another device used for spotting probes directly onto a surface is an ink jet-like dispensing device. Such a device has been employed to dispense oligonucleotide probes directly onto the surface of a charge-coupled device. The integration of a charge-coupled device and a probe-based assay decreased the detection time for quantitative analysis of bound molecules to a tenth of that required by conventional phosphorimaging and to a hundredth of that required by film. (See Eggers *et al.*, *BioTechniques* 17: 516-524 (1994)). Such methods can be used to place probe mixtures at precise locations on the substrate surface or, alternatively, may be used to reproducibly place probes sequentially at the same location on the surface.

C. Detection of binding

The device may be used in conjunction with detection technologies that are known in the art that are capable of discriminating between regions in which binding has taken place and those in which no binding has occurred. When necessary, the detection methodology is capable of quantitating the relative extent of binding in different regions. In DNA and RNA sequence detection, autoradiography and optical detection advantageously may be used, although the skilled artisan will recognize that other detection methodologies, including methods to be developed in the future, may be used. Autoradiography may be performed, for example, using ^{32}P or ^{35}S labelled samples, although the skilled artisan will recognize that other radioactive isotopes also may be used.

A highly preferred method of detection is a charge-coupled-device array or CCD array. With the CCD array, a individual pixel or group of pixels within the CCD array is placed adjacent to each confined region of the substrate where detection is to be undertaken. Light attenuation, caused by the greater absorption of an illuminating light in test sites with bound molecules, is used to determine the sites where binding has taken place. Lens-based CCD cameras and microscope imaging also can be used.

Alternatively, a detection apparatus can be constructed such that sensing of changes in AC conductance or the dissipation of a capacitor placed contiguous to each conformed region can be measured. Similarly, by forming a transmission line between two electrodes contiguous to each confined region, bound molecules can be measured by the radio-frequency (RF) loss. Methods suitable for use herein are described in, Optical and Electrical Methods and Apparatus for Molecule Detection, PCT Published Application WO 93/22678, published November 11, 1993, and expressly incorporated herein by reference.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention. Each of the examples is directed chiefly to use of microchannel devices, but the skilled artisan will recognize that the invention is not limited to such devices.

EXAMPLE 1: Nanochannel Glass (NCG) Wafers

Nanochannel glass ("NCG") arrays developed at the Naval Research Laboratory can be used in the present invention to provide a high surface area nanochannel substrate to tether binding probes in detection elements for hybridization. NCG materials are glass structures containing a regular geometric array of parallel holes or channels as small as several nm in diameter (e.g. 33 nm) or as large as a hundred micrometers or more in diameter. See Tonucci *et al.*, *Science* 258:783-785 (1992), and US Patent No. 5,234,594 which are incorporated herein by reference in their entireties. These nanochannel glass structures can be fabricated in various array configurations to provide a high surface area to volume ratio, and can possess packing densities in excess of $3x10^{10}$ channels per square centimeter. A variety of materials can be immobilized or fixed to the glass surfaces within the channels of the NCG array.

Nanochannel glass arrays are fabricated by arranging dissimilar glasses in a predetermined configuration where, preferably, at least one glass type is acid etchable. Typically, a two-dimensional hexagonal close packing array is assembled from etchable glass rods (referred to as the channel glass) and an inert glass tube (referred to as the matrix glass). The pair is then drawn under vacuum to reduce the overall cross-section to that of a fine filament. The filaments are then stacked, re-fused and redrawn. This process is continued until appropriate

channel diameters and the desired number of array elements are achieved. By adjusting the ratio of the diameter of the etchable glass rod to that of the outside dimension of the inert glass tubing, the center-to-center spacing of the rods and their diameters in the finished product become independently adjustable parameters. See Tonucci, *supra*.

Once the fabrication process is complete, the NCG material is wafered perpendicular to the direction of the channels with a diamond saw and then polished to produce sections of material having a defined thickness, for example, about 0.1 mm to about 1.0 mm. The channel glass of the array structure is then etched away with an acid solution. The skilled artisan will recognize that other geometries of the substrate are possible. For example, the opposing faces of the substrate need not be parallel, and the substrate may be thinner or thicker than about 0.1 mm to about 1.0 mm. For example, the thickness of the substrate also can range from about 10 μm to about 250 μm, from about 50 to about 500 μm, from about 250 μm to about 1.5 mm, or about 500 μm to about 2 mm thick. Moreover, the skilled artisan will appreciate that the cross-sectional configuration of the channels may be varied. For example, the geometry of the channels may include, but is not limited to, a circular or hexagonal cross-section.

In one particular example, a hexagonal close packing arrangement of channel glasses is used which, after acid etching, contains typically 10^7 channels that are uniformly dispersed in the substrate. The channel diameter is typically 450 nm and the center-to-center spacing is approximately 750 nm. The skilled artisan will recognize, however, that the channel diameter can be wider or narrower than 450 nm, and the center-to-center spacing also may be varied. Variation in the channel geometry allows for design of variation in the density of the channels in the substrate.

A second example of hexagonal array structure is one in which separated clusters of channels are formed during the fabrication process. For example, an open array structure with typical channel diameters of 300 nm in which the overall glass structure consists of an array of 18 μm diameter subarrays, spaced typically 25 μm apart from neighboring arrays. Once again, the skilled artisan will recognize that the diameters of the channels and the subarrays and their spacing can be varied without departing from the spirit of the invention.

EXAMPLE 2: Silicon Wafers

Two illustrative general types of silicon devices can be prepared according to the process are described herein below.

Silicon designs containing channels are advantageously employed because of their adaptability to low cost mass production processes and their ability to incorporate in the fabrication process structural elements that function in fluidic entry and exit from the hybridization site and structures (e.g., electrodes) that may function in hybridization detection. Stable, open-cell materials containing channels between first and second surfaces of the material are used to accomplish enhancements and to introduce qualitatively new features in these devices, whereby the surface area of discrete and isolated binding regions comprises groups of channels that are increased by a factor of 100 to 1000 relative to a two-dimensional surface.

Thin-film processing technology can be used to deposit chemically inert and thermally stable microchannel materials. Materials and processing methods are selected to achieve low-cost semiconductor batch fabrication of integrated semiconductor detectors. The microchip device provides in situ multi-site analysis of binding strength as ambient conditions are varied. Silicon materials containing

channels are fabricated in oriented arrays with channel diameters selected over the range from 2 nm to several micrometers. Random, interconnected pore arrays also can be made.

Porous silicon is produced most easily through electrochemical etching. It can be processed into two important channel structures, interconnected networks and oriented arrays. The channel diameter is tailored from approximately 2 nm to micrometer dimensions by selection of doping and electrochemical conditions. For n-type material, etching is thought to proceed through a tunneling mechanism in which electrons are injected into the channel surface through field concentration effects. In the case of p-material the mechanism seems to be through moderation of carrier supply at the electrolyte/silicon interface. In practice, the following structures can be fabricated for use as suitable substrates for the present invention:

i) dense oriented arrays of channels oriented with axis along <100> direction and with channel diameters in the range of 10 to 100 nm. Obtained in p-type material with resistivity less than 10-2 Ω-cm.

ii) dense oriented arrays of channels oriented along <100> direction and with channel diameters in the range less than 10 nm. Obtained in n-type material with resistivity between 10-1 and 10-2 Ω-cm.

iii) dense oriented arrays of rectangular channels oriented with axis along <100> direction, rectangle side defined by {001} planes, and with channel diameters in range less than 100 nm. Obtained in p-type material with resistivity between 10-1 and 10-2 Ω-cm.

Characterization can be undertaken by scanning electron microscopy. The surface wetting properties are varied using vapor treatment with silylation materials and chlorocarbons.

Oriented porous silicon also can be produced by track etching similar to that used to produce high channel-density dielectrics which function as molecular sieves are produced by nuclear track etching. While nuclear track etching is used to produce these molecular sieves in a wide range of inorganic materials, it is most often used with dielectrics such as mica and sapphire. In this method, described in U.S. Patent No. 3,303,085 (Price, *et al.*, which is hereby incorporated by reference in its entirety), a substrate is first bombarded with nuclear particles (typically several MeV alpha particles) to produce disturbances or "tracks" within the normal lattice structure of the material and then wet-etched to produce channels which follow the tracks caused by the nuclear particles. More specifically, Price *et al.* disclose that the exposure of a mica substrate to heavy, energetic charged particles will result in the formation of a plurality of substantially straight tracks in its lattice structure and that these tracks can be converted into channels by wet etching the substrate. The same approach can be used to form oriented channels in silicon substrates.

Channel sizes and density of the channels are variably controllable using track etching techniques with channels typically 0.2 μm in diameter and densities on the order of $10^9/cm^2$, although narrower or broader channels can be generated, leading to greater or smaller channel densities. Particle track depths are energy dependent on the incident particle beam. With typical beam energy, channels can be extended, for example, through an entire 500 μm-thick substrate.

Incorporation of these materials on the device discussed above is readily accomplished. In addition, the use of implantation-etched dielectrics as the sensor element has advantages versus the silicon approach since the material is hydrophilic.

The size of the silicon array wafers may be modified in a variety of ways without departing from the spirit of the invention.

EXAMPLE 3: Robotic Fluid Delivery

Delivery of binding reagent to defined locations within or upon a substrate is accomplished in certain embodiments using micro-spotting devices, as illustrated below.

A. Hamilton Microlab 2000

A Hamilton Microlab 2200 robotic fluid delivery system, equipped with special low volume syringes and 8-position fluid heads, capable of delivering volumes of 10-100 nl at 500 μm xyz stepping and a few percent precision. Using this equipment 40-nl samples of probe solution are placed into the wells of the high density NCG wafer. A piezoelectrically controlled substage custom fitted for the Microlab 2200 permits xy positioning down to submicron resolution. Custom fabricated needles are employed. The eight-needle linear fluid head is operated in staggered repetitive steps to generate the desired close spacing across the wafer. The system has a large stage area and rapid motion control, providing capacity to produce hundreds of replicate hybridization wafers.

B: Microfab microfluidic jets

Methods are known in the art and devices are commercially available (Microfab Technologies, Inc. and Packard Instrument Company) for delivering microdroplets of fluids to a surface with great precision. A microjet system capable of delivering subnanoliter probe solutions to the wafer surface is employed as follows to form oligonucleotides and other assays: For placement of probe into individual hybridization sites within ultra-high density wafers, with volumes of one nl (corresponding to a 130 μm sphere diameter or 100 μm sphere or 100 μm

cube) commercially available dispensing equipment using ink-jet technology as the microdispensing method for fluid volume below is employed.

The droplets produced using ink-jet technology are highly reproducible and can be controlled so that a droplet may be placed on a specific location at a specific time according to digitally stored image data. Typical droplet diameters for demand mode ink-jet devices are 30-100 μm, which translates to droplet volumes of 14-520 pl. Droplet creation rates for demand mode ink-jet devices are typically 2,000-5,000 droplets per second. Thus, both the resolution and throughput of demand mode ink-jet microdispensing are in the ranges suitable for the ultrahigh density hybridization wafer.

C: Microdispensing System

The microdispensing system is modified from a MicroFab drop-on-demand ink-jet type device, hereafter called a MicroJet device such that this type of device can produce 50 μm diameter droplets at a rate of 2000 per second. The operating principles of this type of device are known (Wallace, "A Method of Characteristics Model of a Drop-On-Demand Ink-Jet Device Using an Integral Drop Formation Method," ASME publication 89-WA/FE-4, December 1989) and used to effect the modification. To increase throughput, eight of these devices are integrated into a line array less than 1 inch (25mm) long. The eight devices are loaded with reagent simultaneously, dispense sequentially, and flush simultaneously. This protocol is repeated until all of the reagents are dispensed. Most of the cycle time is associated with loading and flushing reagents, limiting the advantages of a complex of parallel dispensing capability. Typical cycle time required is as on the following order: 1 minute for flush and load of 8 reagents; 30 seconds to calibrate the landing location of each reagent; 15 seconds to dispense

each reagent on one location of each of the 16 genosensors, or 2 minutes to dispense all 8 reagents. Total time to load and dispense 8 reagents onto 16 sensors is thus 3.5 minutes. Total time for 64 reagents onto 16 sensors would be 28 minutes. The microdispensing system will consist of the subsystems listed below:

1. Microjet Dispense Head –

An assembly of 8 MicroJet devices and the required drive electronics. The system cost and complexity are minimized by using a single channel of drive electronics to multiplex the 8 dispensing devices. Drive waveform requirements for each individual device are downloaded from the system controller. The drive electronics are constructed using conventional methods.

2. Fluid Delivery System –

A Beckman Biomec is modified to act as the multiple reagent input system. Between it and the MicroJet dispense head are a system of solenoid valves, controlled by the system controller. They provide pressurized flushing fluid (deionized water or saline) and air to purge reagent from the system and vacuum to load reagent into the system.

3. X-Y Positioning System - A commercially available precision X-Y positioning system, with controller, is used. Resolution of 0.2 μm and accuracy of 2 μm are readily obtainable. The positioning system is sized to accommodate 16 sensors, but MicroJet dispense head size, purge station, and the calibration station represent the main factors in determining overall size requirements.

4. Vision System - A vision system is used to calibrate the "landing zone" of each MicroJet device relative to the positioning system. Calibration occurs after each reagent loading cycle. Also, the vision system locates each dispensing site on each sensor when the 16 sensor tray is first loaded via fiducial marks on the sensors. For economy, a software based system is used, although a hardware based vision system can be advantageously employed.

5. System Controller - A standard PC is used as the overall system controller. The vision system image capture and processing also reside on the system controller.

EXAMPLE 4: Oligonucleotide Attachment to Glass/Si02

Part A: Epoxysilane treatment of glass

A stock solution of epoxysilane is freshly prepared with the following proportions: 4 ml 3-glycidoxypropyl-trimethoxysilane, 12 ml xylene, 0.5 ml N,N-diisopropylethylamine (Hünig's base). This solution is flowed into the channels of the wafer, followed by soaking for 5 hours in the solution at 80°C, followed by flushing with tetrahydrofuran, drying at 80°C, and drying in a vacuum desiccator over Drierite or in a desiccator under dry argon.

Part B: Attachment of Oligonucleotide

Oligonucleotide, bearing 5'- or 3'-alkylamine (introduced during the chemical synthesis) is dissolved at 10μM - 50μM in water and flowed into the channels of the silica wafer. After reaction at 65°C overnight the surface is briefly flushed with water at 65°C, then with 10mM triethylamine to cap off the unreacted epoxy groups on the surface, then flushed again with water at 65°C and air dried.

As an alternative to attachment in water, amine-derivatized oligonucleotides can be attached to epoxysilane-derivatized glass in dilute (eg., 10mM - 50mM) KOH at 37°C for several hours, although a higher background of nonspecific binding of target sample DNA to the surface (independent of base pairing) may occur during hybridization reaction.

Part C: Mercaptosilane treatment of glass

A stock solution of mercaptosilane was freshly prepared with the following proportions: 1 ml 3-mercaptopropyl-trimethoxysilane, 49 ml toluene. The wafer was incubated in this solution for 1 hour under ambient conditions, followed by rinsing with toluene and ethanol, drying at 80°C for 4 hours. Derivatized wafers were stored in a desiccator cabinent until oligonucleotides are attached.

Part D: Attachment of Oligonucleotide

Oligonucleotides, bearing 5'- or 3'- or internal alkylamine (introduced during the chemical synthesis) were dissolved at 10nM - 50μM in 1xSSC buffer and deposited into a group of channels on the wafer using one of the microdispensing methods described above. After reaction at room temperature in a light-tight dry-box for at least 4 hours, typically 12 – 24 hours, the surface was blocked with a 1% solution of polyvinylpyrollidone and Ficoll™ and dried at 80°C for 1 hour.

EXAMPLE 5: Liquid Flow-Through

In order to bind DNA probes or targets within the channels of the microfabricated hybridization support, carry out the hybridization and washing steps, process the material for re-use, and potentially recover bound materials for

further analysis, a method of flowing the liquids through the wafer was used. To enable flow of liquid through the hybridization wafer, the wafer was packaged within a 2 mm x 4 mm polypropylene frame, which served as an upper reservoir and structure for handling. A polypropylene vacuum chamber with a Delrin o-ring around its upper edge permitted clamping of the wafer onto the vacuum manifold to form a seal. For control of fluid flow through the wafer a screw-drive device with feedback control was provided. The liquid delivery cartridge contained a fluid inlet and outlet, a window for *in situ* observation of the hybridization event, permitted temperature control by either an intrinsic or extrinsic mechanism, and optionally contained a mechanism for moving fluids within the chip-containing cavity (e.g. electrodes, valves or bladders).

EXAMPLE 6: Synthesis and derivatization of oligonucleotides

Oligonucleotide probes were synthesized by phosphoramidite chemistry (Beaucage *et al.* Tet. Lett. 22:1859-1862 (1981)) using an segmented synthesis strategy that is capable of producing over a hundred oligonucleotides simultaneously (Beattie *et al.*, Biotechnol. Appl. Biochem. 10:510-521 (1988); Beattie *et al.*, Nature 352:548-549 (1991)). The oligonucleotides were derivatized with the alkylamino function during the chemical synthesis, either at the 5'-end or the 3'-end.

Based upon quantitative measurements of the attachment of labeled oligonucleotides to flat glass and gold surfaces, the end attachment placed the probes 50-100 nm apart on the surface, corresponding to up to 10^8 probes in a 50 μm x 50 μm area. Approximately 10^{10}-10^{11} oligonucleotide probes were tethered within a 50 μm cube of silicon in the nanofabricated wafer. The density of bound oligonucleotides per cross sectional area was estimated by end-labeling prior to the

attachment reaction, then quantitating the radioactivity using the phosphorimager. Known quantities of labeled oligonucleotides dried onto the surface were used to calibrate the measurements of binding density.

EXAMPLE 7: Hybridization Conditions

Part A: Sample preparation

The target DNA (analyte) was prepared by the polymerase chain reaction, incorporating [^{32}P]nucleotides into the product during the amplification or by using gamma-^{32}P[ATP] + polynucleotide kinase to 5'-label the amplification product. Unincorporated label was removed by Centricon filtration. Preferably, one of the PCR fragments was 5'-biotin-labeled to enable preparation of single strands by streptavidin affinity chromatography. The target DNA was dissolved in hybridization buffer (50mM Tris-HCl, pH 8, 2mM EDTA, 3.3M tetramethylammonium chloride) at a concentration of at least 5nM (5 fmol/μl) and specific activity of at least 5,000 cpm/fmol. PCR fragments of a few hundred bases in length are suitable for hybridization with surface-tethered oligonucleotides of at least octamer length.

Target cRNA also was prepared according to the methods described in US Patent No. 5,716,785. The target cRNA was dissolved in 5x SSPE (50mM phosphate, pH 7.4, 0.75M sodium chloride, 5mM EDTA) at concentrations of 5 to 25 μg/ml.

Part B: Hybridization.

The PCR generated target DNA sample was flowed into the channels of the chip and incubated at 6°C for 5-15 minutes, then washed by flowing hybridization solution through the chip at 18°C for a similar time. Alternatively,

hybridization can be carried out in buffer containing 1M KCl or NaCl or 5.2M Betaine, in place of tetramethylammonium chloride.

The cRNA sample was flowed into the channels of the chip and incubated at 20 to 50°C for 15 minutes to 12 hours, then washed by flowing hybridization solution through the chip at room temperature for 15 minutes.

Part C: Optimization of hybridization selectivity (discrimination against mismatch-containing hybrids)

Although the experimental conditions described above generally yield acceptable discrimination between perfect hybrids and mismatch-containing hybrids, some optimization of conditions may be desirable for certain analyses. For example, the temperature of hybridization and washing can be varied over the range 5°C to **50°C** for hybridization with short oligonucleotides. Higher temperatures may be desired for hybridization using longer probes.

EXAMPLE 8: Quantitative Detection of Hybridization

Part A: Phosphorimager and film detection

The detection and quantitation of hybridization intensities was carried out using methods that are widely available: phosphorimager and film. A Cyclone phosphorimager (Packard Instruments) was used. The Biorad phosphorimager, having a sample resolution of about 100 μm that is capable of registering both beta emission and light emission from chemiluminescent tags also could be used. Reagent kits for chemiluminescence detection available from Millipore and New England Nuclear, which produce light of 477 and 428 nm, respectively, also may be with the Biorad instrument. Chemiluminescent tags are introduced into the target DNA samples (random-primed vector DNA or PCR fragments) using the

procedures recommended by the supplier. Thereafter, the DNA is hybridized to the nanochannel wafers bearing oligonucleotide probes. Radioactive tags (^{32}P and ^{33}P, incorporated by random priming and PCR reaction) are also used in these experiments. Film exposure is used for comparison. In the case of hybridization of labeled oligonucleotides with surface immobilized target DNAs, most preferably the radioactive tags (incorporated using polynucleotide kinase) are used.

Part B: CCD Detection Devices

CCD genosensor devices are capable of maximum resolution and sensitivity and were used with chemiluminescent, fluorescent and radioactive tags (Lamture *et al. supra*.

EXAMPLE 9: Profiling of Gene Expression Using Probes Arrayed in Channels in Silicon Wafers

Part A: Fabrication of Porous Silicon Wafer

The procedure outlined above for fabrication of a porous silicon wafer with integral wells is followed, to yield a wafer with a 50x50 array of 200 μm square patches of channels, spaced 400 μm apart (center-to-center) over the surface of the wafer. The channels of the wafer are activated to bind amine-derivatized oligonucleotides by reaction with epoxysilane, as described above.

Part B: Formation of cDNA Array

A set of oligonucleotide probes is synthesized incorporating primary amines into the strands. The probes corresponding to a single expressed gene are applied to single discrete locations of the wafer using a Hamilton Microlab 2200 fluid delivery system equipped with an 8-needle dispensing head. After all probes

are dispensed, a slight vacuum is briefly applied from below to ensure that fluid has occupied the channels. Following incubation at room temperature overnight or at 60°C for 30-60 minutes, the wafer is flushed with warm water, then reacted with 50mM triethylamine to cap off the unreacted epoxy groups on the surface, then flushed again with warm water and air dried.

Part C: Preparation of Labeled PCR Fragments Representing the 3'-regions of Expressed Genes

Cytoplasmic RNA is extracted from cultured cells by the method of Chomczynski *et al.*, (Anal. Biochem. 162:156-159 (1993)), treated with DNAse I to remove DNA contamination, then extracted with phenol/chloroform and ethanol precipitated. Reverse transcriptions and PCR are performed as described in the "differential display" protocol of Nishio *et al.*, (FASEB J., 8:103-106 (1994)). Prior to hybridization, PCR products are labeled by random priming in the presence of [A-^{32}P]dNTPs, and unincorporated label is removed by Centricon filtration.

Part D: Hybridization of Expressed Sequences to cDNA Array

Prior to hybridization, a solution of 1% "Blotto" or 50mM tripolyphosphate is flowed through the channels of the wafer to minimize the nonspecific binding of target DNA, then the porous silicon array is washed with hybridization solution (50mM Tris-HCl, pH 7.5, 1mM EDTA, 1M NaCl). Labeled PCR fragments representing the 3'-end of expressed genes are recovered from the Centricon filtration units in hybridization buffer, and the entire wafer is flooded with this DNA solution. The hybridization module is placed at 65°C and a peristaltic pump, connected to the lower vacuum chamber, is used to gradually

flow the labeled DNA through the channels of the wafer over the course of 30-60 minutes. The wafer is washed three times with hybridization buffer at 65°C.

Part E: Quantitation of Hybridization Signals

Following hybridization and washing, the wafer is briefly dried, then placed onto the phosphor screen of a phosphorimager and kept in the dark for a period of time determined by the intensity of label. The phosphor screen is then placed into the phosphorimager reader for quantitation of individual hybridization signals arising from each channel region in the array.

Total cytoplasmic mRNA is isolated from cells cultured under two conditions and subjected to the "differential display" procedure described above to prepare fragments representative of individual mRNA species present under the two conditions. These samples are hybridized to two identical probe arrays, to yield two hybridization signal patterns that are compared to determine the relative extent of hybridization at each detection element in the array. The resulting hybridization patterns represent the profile of expressed genes under the two different culture conditions (for example in the presence and absence of a drug or chemical that induces a change in the expression of some genes).

The invention has been disclosed broadly and illustrated in reference to representative embodiments described above. Those skilled in the art will recognize that various modifications can be made to the present invention without departing from the spirit and scope thereof.

The disclosure of all publications cited above are expressly incorporated herein by reference in their entireties to the same extent as if each were incorporated by reference individually.

What Is Claimed Is:

1. A device for binding a target, comprising:

a plurality of detection elements affixed to a substrate,

wherein each of said detection elements is affixed to a discrete spatial location on said substrate,

wherein at least one of said detection elements comprises a plurality of binding reagents, and

wherein the binding reagents contained within a single detection element can bind to a single target.

2. A device according to claim 1, wherein said binding reagents are nucleic acid molecules, and wherein the binding reagents contained within single detection element are complementary to a single gene.

3. The device according to claim 1, wherein said substrate has oppositely facing first and second major surfaces and a multiplicity of discrete channels extending through said substrate from said first major surface to said second major surface, wherein said detection elements are disposed within a multiplicity of discrete channels extending through said substrate from said first major surface to said second major surface.

4. The device according to claim 1, wherein said detection elements comprise 2-10 different binding reagents.

5. The device according to claim 4, wherein said detection elements comprise 3-8 different binding reagents.

6. The device according to claim 5, wherein said detection elements comprises 3-6 different binding reagents.

7. The device according to claim 2, wherein at least two of said nucleic acid probes are complementary to overlapping sequences of said gene.

8. The device according to claim 2, wherein at least two of said nucleic acid probes are complementary to non-overlapping sequences of said gene.

9. The device according to claim 2, wherein at least two of said detection elements comprise probes that are complementary to the same gene.

10. The device according to claim 2, wherein at least one of said detection elements comprises a plurality of probes complementary to a unique gene.

11. The device according to claim 2, wherein each of said nucleic probes is selected from the group consisting of DNA, RNA, phosphorothioate DNA, peptide nucleic acids, and 2'-methoxy DNA.

12. The device according to claim 11, wherein said probes comprise DNA.

13. The device according to claim 1, wherein said detection elements are disposed on a substantially planar surface.

14. The device according to claim 1, wherein said probes are covalently affixed to said substrate.

15. The device according to claim 1, wherein said probes are non-covalently affixed to said substrate.

16. The device according to claim 1, wherein said probes are affixed to said substrate via a linker molecule.

17. A method of detecting a target molecule, comprising contacting a solution suspected of containing said target molecule or a fragment of said molecule with a device according to claim 1, wherein at least one of said detection elements comprises binding reagents that are complementary to said target molecule.

18. A method of detecting a plurality of nucleic acid target molecules, comprising contacting a solution suspected of containing said target molecules or fragments of said molecules with a device according to claim 2, wherein at least one of said detection elements comprises nucleic acid probes that are complementary to each of said target molecules.