Miniaturized detection technology in molecular diagnostics

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Miniaturization of genetic tests represents the convergence of molecular biology and engineering and is leading to a new class of small analyzers and test systems for genetic testing with improved analytical characteristics. Miniaturization initially focused on devices that contained micrometer-sized features designed for a particular analytical purpose (e.g., filters for cell isolation and chips for capillary electrophoresis). Now, the focus is shifting to analytical applications based on nano-sized objects such as nanotubes, nanochannels, nanoparticles, nanopores and nanocapacitors. These nanofabricated objects provide new tools for sequencing of nucleic acids and rapid, multiplexed, nucleic acid detection.

Expert Rev. Mol. Diagn. 5(4), 549–559 (2005)

CONTETNTS

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KEYWORDS:
carbon nanotube, electrochemical detection, microchip CE, multiplexing, nanoparticles, nanopore, nanotechnology, non-PCR testing

Microtechnology encompasses devices that contain micrometer-sized features that are usually fabricated using methods developed in the microelectronics industry (e.g., photolithography and deposition). Nanotechnology is defined as 'the branch of engineering that deals with things smaller than 100 nm (especially with the manipulation of individual molecules)'. Nano-sized structures can be fabricated through a bottom-up process, where the object is built up molecule by molecule or atom by atom. Alternatively, a top-down approach can be utilized in which the structure is fabricated by removal of unwanted material from a larger block of material. Both micro- and nanotechnology are finding applications in many different areas of science and engineering [1–4]. This article reviews recent advances in the application of both micro- and nanotechnology in molecular diagnostics. It surveys the current use of silicon-glass and plastic microchips, and nano-sized structures such as carbon nanotubes, nanopores and nanoparticles, and discusses their prospects and current barriers to implementation in routine molecular diagnostics. Key objectives for innovation in molecular diagnostics include rapid sequencing, multiplexed testing, integrated analysis that combine all steps in the analytical process from sample handling to read-out of the result, and assays that do not require an amplification step. Microchips and a range of nanostructures are gaining in importance as possible technological solutions that will lead to the realization of these ambitious objectives (TABLE 1).

Rapid DNA sequence analysis

A goal in genetic technology is rapid DNA sequence analysis of long fragments of nucleic acids or even whole genomes. The capability to perform such a feat would allow for whole-genome sequencing for purposes of identification of organisms in the case of microbes, viruses or other pathogens, and mutation or identity analysis in the case of humans. Arguably, this could obviate the need for any other mode of nucleic acid analysis. Upon completion of the human genome sequence in 2003, the National Human Genome Research Institute (MD, USA) challenged the scientific community to develop technology that would allow sequencing of a human genome for US$1000. Using current state-of-the-art capillary-based DNA sequence analysis, it still costs well over US$10 million to sequence the 3 billion base pairs comprising the human genome. However, a number of investigators, including those at Microchip Biotechnologies, Agencourt Bioscience, 454 Life Sciences,

10.1586/14737159.5.4.549

ISSN 1473-7159

LI-COR and Caltech, are among the awardees developing near-term methods to sequence a human-sized genome for US$100,000 [101].

One of the novel approaches to performing rapid DNA sequence analysis of nucleic acids involves manipulation of nucleic acid strands through nanoscale pores. This technology has been steadily developed over the last 10 years and utilizes electrophoretic transport through nanoscale pores fabricated in membrane surfaces or in artificial channel structures. Examples of these nanometer diameter pores include ion beam-generated holes in a silicon nitride membrane [5] and self-assembled α-hemolysin pores in a lipid bilayer [6].

In operation, a voltage is applied across a membrane that is embedded with pores several nanometers in diameter. The voltage across the membrane drives nucleic acids through the membrane. The only locations through which the nucleic acids can traverse the membrane are at the pores. At each pore, a current is generated that is disrupted as DNA or RNA molecules traverse the pore. The characteristics of the time and magnitude of disruption of the crosspore current are dependent on the base composition and length of the DNA or RNA molecule. This is analogous to the Coulter counter that is used in routine hematology analysis of peripheral blood cells [7]. The nanometer-sized pores control the processing of nucleic acid strands as size limits the passage to one DNA or RNA specie at a time. Initial studies with 2.6-nm hemolysin pores showed that single-stranded (ss)DNA but not double-stranded (ds)DNA traversed the pore [8]. The cross-sectional size of a dsDNA molecule is approximately 2 nm, and passage of a dsDNA has been demonstrated using an ion beam-fabricated 3 nm diameter silicon nitride nanopore [9]. In contrast, ssDNA but not dsDNA translocated through a 0.5 nm, ion beam-fabricated pore [10]. There is an increasing diversity of sizes of nanopores that are being created and used to process a greater distribution of sizes of DNA [5]. Another type of nanomanipulation uses a nanochannel to stretch genomic DNA and simplify the sequencing. This potential to manipulate genomic DNA directly is at an early stage of development, but is an indication that single-molecule nucleic acid detection and manipulation is a realistic target [11].

Table 1. Applications of micro- and nanotechnology in nucleic acid analysis.

Application	Technology
Sequencing	Nanopores
Labels and detection reactions	Nanoparticles, nanotubes
Multiplexing	Micro- and nanoarrays, nanocapacitors, nanocantilevers, micro- and nanorods, quantum dots, nanotube-modified atomic-force microscopy scanning
Nonamplification	Nanotube and nanoparticle labels, nanotube-modified electrodes, molecular inversion probe assay
Integrated analysis	Microchip analyzers, bioelectronic chips

The sequencing rate that may be achieved using nanopores has been estimated in the range of 1000 to 10,000 bases/s, which dwarfs the 30,000 bases/day throughput of conventional sequencers. An additional advantage of nanopore-based sequencing is the capacity for miniaturization. Miniaturization is particularly interesting because not only does a nanoscale pore lend itself to submicron sizing, but utilizing electrical detection will also provide independence from the size constraints of optical detectors.

One of the notable efforts in terms of development and commercialization of this technology is a collaboration between Agilent Technologies, Inc. and a Harvard group. Hypothetically, with single-base resolution, a nanopore sequencer could decode patients' genomes in 24–48 h with a 500-nanopore device costing approximately US$20,000. Even at the lower resolution, nanopores could be used to detect important differences in a patient's DNA much more easily than can be achieved with gene chips [12]. In its current state of development, a nanopore sequencer can only distinguish signatures caused by long stretches of differing nucleotides, and a key challenge is to refine the technique to distinguish individual nucleotides.

Improved labels & detection reactions

Most nucleic acid assays rely on a label for detection and quantitation. Nanotechnology in particular has provided a new crop of labels and detection methods for sensitive detection and for multiplexing nucleic acid assays.

Nanoparticle labels

Nanometer-sized particles of different shape and composition are emerging as an important type of label for nucleic acid assays. However, as yet, they have not displaced the labels currently used in routine hybridization assays (northern or Southern blotting or microarray analysis) such as enzymes (e.g., alkaline phosphatase and horseradish peroxidase), chemiluminescent compounds (e.g., acridinium esters) or fluorophores (e.g., cyanine dyes). The lack of adoption may be due to the significant implementation barrier a replacement technology encounters as it attempts to displace established technologies for which equipment and protocols are tried and tested. Nevertheless, nanoparticle labels have a number of intriguing properties that facilitate multiplexing and direct visual detection that may ultimately propel these labels to the forefront of testing. The two principal means for detecting and quantitating nanoparticles are based on optical and electrochemical measurements.

Optical detection

Colloidal gold (gold granules) has been used for electron microscopic localization of nucleic acids since the 1960s [13]. The renaissance in the use of gold and decorated gold nanoparticles as oligonucleotide labels can be traced to the work of Mirkin and his associates in the mid-1990s [14]. At first, the use of gold

nanoparticles in DNA assays relied on colorimetric changes that occurred when gold nanoparticles aggregate (e.g., color changes from red to purple) [15]. The introduction of new scanning methods and the re-emergence of surface-enhanced Raman spectroscopy (SERS) have provided considerable impetus to this rapidly evolving field of nanoparticle labels.

Fluorescent detection, although the norm, has some disadvantages, such as the high cost of fluorescent scanners, the broad emission spectrum and the photoinstability of the dyes commonly used. Metal nanoparticles have distinct advantages over fluorescent dyes as labels. They have narrow Raman emission peaks, and the surface plasmon resonance (SPR) is directly related to nanoparticle size, which makes for simple multiplexing strategies based on size [16]. The SPR of an object depends on small changes in the refractive index that occur near or at the surface of a metal such as gold, silver or copper [17]. Metal nanoparticles are stabilized by surface ligands (such as citrate) in order to maintain solubility, and the variation in the size of the nanoparticles creates the variation in SPR (thus a 40-nm gold nanoparticle gives off a different SPR than a 15-nm gold nanoparticle, even though they are both made of the same metal).

An early application for gold nanoparticles was as labels for probes used in DNA microarray assays. Nanoparticle probes were shown to produce a sharper and higher temperature melting profile than fluorescence-based techniques [18]. It was also noted that after silver enhancement, the sensitivity was increased 100-fold compared with fluorescence-based methodology.

Several detection systems are available for nanoparticles. One detection method employs a fiber optic illuminator known as a Darklite Illuminator; in this detection scheme, the array slides are mounted on a microscope stage and illuminated in the plane of the slide by a fiber optic illuminator [16]. Another method uses a flatbed scanner to detect gold nanoparticles after silver enhancement [19]. The build-up of the silver coating on the gold nanoparticles is also visible to the naked eye, thus allowing for quick signal detection on an array. A commercial scanner, the Verigene ID™ (Nanosphere, Inc. [102]), measures scattered light from silver-enhanced gold nanoparticles and acquires visual records of the experimental data [19]. Another scanner, the NIS 2000 image analyzer, measures the Rayleigh scatter signal of silver-enhanced gold nanoparticles; this scanner can detect up to approximately 0.001 gold probes/μm^2 [20].

The most promising and versatile detection scheme for nanoparticles is SERS. This was developed to alleviate the problem of background fluorescence from the sample. Raman spectroscopy relies on detecting a signal that is only 0.0001% of the light that is scattered from the sample, thus the combination of a low signal intensity and fluorescence background could be problematic [21]. SERS solves the problem of autofluorescence and background fluorescence through metal adsorption of the sample with metals such as silver and gold. The resonant excitation of surface plasmons on the metal surface results in a Raman signal enhancement of 10^2–10^4 compared with the Raman signal generated from the sample alone [22]. The importance of SERS is evident from its diverse applications in genomic and proteomic assays [23,24]. Another important aspect of SERS is its application in a DNA–gold nanoparticle-based method of detecting unamplified genomic DNA sequences by measuring scattered light [25], as opposed to the flatbed scanner method that measures reflected light [20]. This unamplified DNA assay relied on monitoring the plasmon shift that occurs when 40–50-nm diameter gold nanoparticles aggregate; the process allows for the detection of aggregates in the presence of a large amount of nonaggregated material and a detection limit of just 333 zmol of target was achieved [25].

Nanoparticles have also been exploited in more complex analytical strategies, such as the bio-barcode amplification (BCA) technique. This approach uses gold nanoparticles derivatized with single-stranded oligonucleotide barcodes and an oligonucleotide complementary to a portion of the target sequence under interrogation, as well as magnetic microparticles that are modified with multiple copies of an oligonucleotide complementary to a second portion of the same target sequence under interrogation. Oligonucleotide complementary to the single-stranded, bound bio-barcodes are hybridized to the gold nanoparticle. The target under interrogation is hybridized to both the nanoparticle and magnetic microparticles. If complementation occurs to both single-stranded, bound oligonucleotide on the nanoparticles as well as the second oligonucleotide from a different region of a target bound to the magnetic microparticle, then the two particles become bridged by hybridization to two different regions in the target DNA. This complex is then trapped on a magnetic column, washed and the single-stranded hybridized nanoparticle barcode is released by heat denaturation and annealed to an array containing one register that is complementary to the released bio-barcode strand. Since there are many bio-barcodes on each gold nanoparticle, hybridization of one target molecule results in release of many bio-barcodes per nanoparticle, which greatly amplifies the detection signal. The released bio-barcode contains a region complementary to one of the array-capture barcodes as well as sequence complementary to an oligonucleotide-bound, gold nanoparticle probe. The bio-barcode is captured on the array and hybridized to the universal oligonucleotide-bound nanoparticle. Detection is facilitated by silver enhancement of the bound gold nanoparticle and scanning with a flatbed scanner. The BCA technique allows for the detection of 500 zmol of target DNA, which translates to approximately ten copies in a 30-µl reaction mixture [26].

Electrochemical detection

Gold-coated copper nanoparticles functionalized with oligonucleotides have been tested in an electrochemical DNA hybridization assay. Target oligonucleotides were electrostatically adsorbed onto a conducting polypyrrole surface at a glassy carbon electrode. These were then hybridized to the oligonucleotide-coated nanoparticles. The hybridization was monitored by the release of the copper metal atoms and detection of the copper by anodic stripping voltammetry (detection limit 5.0 pmol/l) [27]. In the form of microrods, other metals such as indium can also

be used as labels for DNA hybridization assays (detection limit 250 zmol). This metal is not normally present in biologic samples, and it can be quantitated by solid-state derivative-chronopotentiometric measurements at a thick-film electrode transducer. An extension of this technique could be to multiplex assays using an electrical coding and identification system based on different metal labels [28]. Silver nanoparticles can also be used as oligonucleotide labels in DNA hybridization assays and detected using an electrochemical assay [29]. Following hybridization of the target DNA with the silver nanoparticle-labeled oligonucleotide probe, silver ions are released from the nanoparticle labels by oxidative metal dissolution, and the silver ions measured by anodic stripping voltammetry at a carbon fiber ultramicroelectrode. A large number of silver ions are released from each DNA hybrid and this allows detection of 0.5 pmol/l of target oligonucleotide.

More complicated systems employ gold nanoparticle/streptavidin conjugates covered with 6-ferrocenylhexanethiol that are attached to a biotinylated DNA-detection probe [30]. These hybridize to a target captured by a DNA capture probe/hexanethiol self-assembled monolayer on an electrode. The ferrocene component of the label is detected voltametrically and contributes an amplification factor due to the large number of ferrocene markers on each DNA duplex. A detection limit of 2.0 pM for oligodeoxynucleotide samples (e.g., PCR products) was achieved with good linear range (6.9–150.0 pM) and reproducibility (3.0 and 13.0%).

Nanoparticles doped with electroactive tris(2,2´-bipyridyl)-cobalt(III) provide another variation on nanoparticle labels for DNA hybridization assays. This label can be detected voltametrically at a glassy carbon electrode. The large number of electroactive molecules inside each silica nanoparticle provides an amplification factor, and detection of the target oligonucleotide down to 2.0×10^{-10} mol/l was possible [31].

Multiplexed testing for nucleic acids

The capability for multiplex analysis of nucleic acids is vital in order to accomplish the scale of analysis required in both research (e.g., gene expression monitoring and single nucleotide polymorphism [SNP] profiling) and in routine clinical practice (e.g., screening for multiple cystic fibrosis mutations). Various strategies have been developed for multiplex analysis. These include 2D arrays and monitoring of a binding event, and single-color detection by the location in the array. Alternatively, tagging of unknown targets can be accomplished with a large set of unique labels and subsequent label-by-label recognition in a flow system.

2D microarrays are now a mature technology and the first clinical test, the AmpliChip™ Cytochrome P450 Genotyping Test made by Roche Molecular Systems, Inc. was approved by the US Food and Drug Administration (FDA) in December 2004 [103] following some interesting exchanges on the route by which the product should be approved [104]. This will pave the way for other microarray-based tests, although the issue of quality control of such massively parallel testing devices poses some interesting issues not previously encountered in routine clinical laboratory testing [32]. Microarrays typically have feature sizes in the 10–50 µm range but nanoscale arrays are also technically feasible. For example, 830–860 nm diameter spots of biotinylated ssDNA can be deposited on a streptavidin-coated glass surface by means of a nanopipette [33]. The deposited ssDNA is then detected using complementary Alexa dye-labeled ssDNA. Currently, there is no urgent need for nanoscale miniaturization of DNA arrays, but the prospect of having one array for an entire genome on an ultrasmall chip is appealing (e.g., 1 million 10 × 10-nm features can fit on a 10 × 10-µm chip).

Other forms of 2D arrays are based on arrays of nanogap capacitors and nanocantilevers. Nanogap capacitors (50 nm electrode spacing) are fabricated by means of silicon nanolithography and then sensitized with a ssDNA probe [34]. The change in capacitance resulting from hybridization with specific target DNA provides a quantitative measure of the degree of binding. Flexing of a micromachined cantilever can also be used to detect binding events such as DNA hybridization [35]. A ssDNA probe is immobilized on the cantilever surface and then exposed to target DNA. Probe–target hybridization alters the surface stress on the cantilever and the change in its deflection characteristics is measured optically [36,37]. Multiplex analysis is possible using microcantilever arrays, each coated with a different DNA probe.

Rare earth-doped 20 × 20 × 100-µm glass microrods serve as microbarcodes to facilitate multiplexing. The rare earth ions (lanthanides) have high quantum efficiencies, and emission line widths of approximately 10–20 nm, which is less than that observed for quantum dots (QDs; 25–40 nm) or organic dyes (30–50 nm). The narrow emission line widths allow a large number of resolvable bands to be packed into the same spectral bandwidth, and this enables a larger number of uniquely distinguishable combinations (>1 million). The bands of dyes in the silicate glass microrods act as signal generators and produce a characteristic signature (the barcode) that is detected using a spectral imager. Initial studies have confirmed the effectiveness of this multiplexing strategy in DNA hybridization assays [38].

Moving to the nanoscale, the range of nanoscale tags for multiplexed assays includes QDs [39] and metallic barcodes [40], and these labels have several advantages over standard fluorophore tags [41,42]. QDs (e.g., semiconductor nanocrystals consisting of a CdSe core and a ZnS cap) can be excited at the same wavelength to produce multiple colors with narrow emission bands (e.g., 20–40 nm) and minimal photobleaching. Differentiation by emission color, intensity and spectral width can provide several thousand unique signatures and a million signatures have been projected [43]. Nanorods (30–200 nm diameter and 0.4–8 µm long) with stripes composed of different metals (e.g., Au, Ag, Pt, Pd, Ni, Cu and Co) can be recognized using reflectivity measurements, and this could be performed in parallel with optical tagging, thus further adding to the possible scale of the multiplexing [44]. The application of this type of nanoparticle has been proposed for various genomics applications, such as gene expression and SNP analysis [201].

Nanocoding for multiplexed assays can also be achieved using different sized nanospheres that are then distinguished using atomic force microscopy (AFM) [45]. Nanoscale properties apart from particle size, such as particle shape, can also facilitate multiplexing. This leads to the prospect of generating different signals from nanoparticle labels with different shapes but made of the same substance. One of the first examples has been a nanoprism (100 nm edge) made of silver [46,47]. Nanoprisms interact with light (three surface plasmon bands at λ_{max} = 335 nm [weak], 470 nm [medium] and 670 nm [strong]) differently from spherical particles, and hence have a different color. This difference provides the basis for multiplexed assays in which the nanoparticle labels are all made from exactly the same material but rely on differences in shape to achieve unique optical signals [48]. There is a burgeoning interest in the control of particle shape and prisms, plates, rods, disks, hexagons, multipods, cubes, arrows, teardrops, tetrapods and branched-tetrapods made from a variety of materials (e.g., gold or silver) [49–55]. Differently shaped particles interact with light in different ways. For example, nanoprisms Rayleigh light scatter in the red range whereas comparably dimensioned spheres scatter in the blue. The prospect for different shaped labels has been explored in a preliminary way in immunoassays, but their potential in molecular diagnostics is uncharted.

Another way of achieving multiplexing is by direct scanning of a DNA strand. The utilization of carbon nanotubes as probe tips for atomic force microscopes has expanded the resolution and utility of atomic force microscopes. Carbon nanotubes are a significant advance in design and manufacturing for atomic force microscope probe tips. They can be manufactured to tight specifications with size and ruggedness qualities ideal for high-resolution imaging [56,57]. Multi- or single-walled carbon nanotubes (SWNT) can be manufactured and then attached to the probe tip surface. Alternatively, SWNT can be directly synthesized at the probe tip.

SWNT probe tips have been used to image a variety of biologic molecules with high resolution. However, the most interesting and potentially revolutionary is the direct detection of multiple polymorphism sites on a single strand of DNA [58]. Most clinically available genetic tests only test for single sites of genetic polymorphism. If multiple sites of interest are tested, these sites are each in isolation; there is no easy way to determine if the multiple sites of interest are located on the same chromosome. Short of sequencing long expanses of DNA or indirectly calculating gene assortment by linkage analysis, there has been no previous method for directly determining the assortment of genetic variation on the same chromosome. In a study utilizing the resolution of SWNT probe tips, two polymorphisms were specifically localized to the same strand of DNA. In brief, the two polymorphisms were hybridized with complementary oligonucleotides linked to markers of differing size. When the AFM directly imaged the presence of the two markers of differing size on the same strand of DNA, this was evidence that the two polymorphisms of interest were linked together on the same chromosome. The implications of this study are meaningful for the direct detection and diagnosis of genetic diversity for purposes of genetic testing for inheritance as well as cancer risk determination.

In addition, the authors of the study speculate that the advance in carbon nanotubes of smaller radii may allow AFM to achieve single-nucleotide resolution. With this advance, multicantilevered atomic force microscopes may have the ability to perform rapid sequencing of DNA in the order of 10 kbp in size [58].

Nonamplification assays for nucleic acids

A long-held goal has been to develop direct assays for nucleic acids that do not require amplification of the target in the sample. An earlier assay based on branched DNA probes can be considered a nanotechnological solution to this problem, as the probes and resulting branched structure are nanoscale. Other nanostructures also promise routes to the level of sensitivity required for a nonamplification assay. Nanotubes are emerging as a versatile and useful tool in analysis. They can be fabricated from a variety of materials including carbon, silica, BN, GaN, BC and organic polymers. Both single- and multiwall varieties of nanotube have been fabricated by a variety of techniques. The initial technique described by Iijima has been further refined to produce nanotubes of varying chemistries, purities and dimension [59]. The principal techniques of manufacturing include a sol-gel process, membrane wetting, chemical vapor deposition and self-assembly.

A route to improved sensitivity of DNA detection may be through the use of electrically conductive, carbon nanotube-based electrodes [60]. Vertically aligned multiwalled carbon nanotubes (MWNTs) are fabricated on 20 × 20-µm microelectrode pads to form a nanoelectrode array. DNA probe molecules are then covalently attached to the end of the MWNTs. Mediator-amplified guanine oxidation is used to directly measure the electrochemical signal associated with target bound to probes on the microelectrode pads, and over 1000 target nucleic acid molecules can be measured at a single microelectrode pad.

A carbon nanotube can function as a carrier for a large number of labels and hence provide a source of amplification in a nucleic acid assay. This strategy has been tested in a sandwich hybridization assay [61]. Nanotubes were coated or filled with the label molecules (alkaline phosphatase) and the filled nanotube attached to a detector probe. More than 9600 alkaline phosphatase molecules were associated with each nanotube. Electroactive phenolic products produced in the alkaline phosphatase-catalyzed reaction were detected at a nanotube-modified electrode. This amplification strategy allowed detection of DNA down to 1.3 zmol.

Yet another route to achieving the sensitivity required for nonamplification (PCR-free) assays for nucleic acids is by multiple labeling. One of these methods is the molecular inversion probe (MIP) genotyping approach that integrates a gap-filling by a polymerase followed by a padlock probe circularization by a ligase that is then linearized by a uracil DNA glycosylase [62]. The MIP method has recently been applied in a large-scale

genotyping of over 10,000 SNPs through a molecular barcoding strategy that enabled the use of over 12,000 oligonucleotide probes to simultaneously examine an unamplified human genomic DNA sample followed only by a single PCR reaction that is detected through a single universal tag DNA chip array [63]. This assay is soon to be commercialized and will employ four different QD labels [PERS. COMMUN.].

Silver-enhanced gold nanoparticles (15 nm diameter) can also be used for SNP identification in unamplified human genomic DNA. A hurdle in SNP identification is the complexity of the human genome, and this is usually overcome through either an adapter-mediated genome complexity reduction PCR reaction or simply by amplification of the target through PCR or multiplex PCR. Bao and his colleagues managed to interrogate SNPs for three genes involved in thrombotic disorders without genome complexity reduction by using allele-specific barcodes for either mutant or wild-type SNPs using 50 fmol of genomic DNA [64].

Microchip analyzers & integrated analysis: from bleed to read

An important goal is to integrate all of the steps in a genetic test onto a small single chip device – a so-called lab-on-a-chip. The advantages of this approach are faster and more reliable assays that could eventually be used in the field or at the point of care.

In genetic testing, sample preparation is a labor-intensive step and several microchip-based strategies have been developed. For example, solid-phase extraction of DNA from whole human blood has been completed in less than 15 min using silica beads immobilized with sol-gel in a glass microchip [65,66].

Quantitative and qualitative analysis of extracted genomic DNA also has been improved by the recent introduction of novel instrumentation for microscale samples, such as the NanoDrop® ND-1000 [105], an ultraviolet-based spectrophotometer that functions as a full-spectrum spectrophotometer over the range of 220 to 750 nm. A 0.2-mm path capability allows the ND-1000 to measure absorbance 50-times greater than that of conventional instruments. It enables highly accurate analyses of extremely small samples (1 µl) with remarkable reproducibility. A proprietary sample-retention system eliminates the need for cuvettes and capillaries, which decreases the measurement cycle time. In addition, the instrument software facilitates the measurement of DNA concentration and dye-labeling effectiveness. The instrument can measure the absorbance of fluorescent dyes, thereby allowing detection at dye concentrations as low as 0.2 pmol/µl.

The next step after sample preparation in a genetic test is usually PCR to amplify the target. This reaction has become central to most, if not all, genetic testing. Miniaturization of PCR and the development of PCR chips has been a goal since it is a route to rapid PCR that uses less reagent, and it could form part of an integrated miniature genetic testing device. Numerous strategies for miniaturized PCR have been developed that differ in the geometry of the PCR device (e.g., chambers and channels), fabrication material (e.g., glass, silicon-glass or plastic) and the mode of heating/cooling (e.g., external Peltier heater/cooler or thin-film surface heater) [67]. One key issue that has arisen is the adverse effect of the surface of microchip devices on PCR, and this has necessitated development of passivation procedures to eliminate surface-based inhibition of PCR [68]. The goal of integration of the different steps in a genetic test onto a microchip has been achieved by a number of groups. Scientists at Motorola have described a self-contained, fully integrated device for sample preparation, PCR and DNA microarray detection [69]. The 60 × 100 × 2-mm device was used to test for the pathogenic bacterium *Escherichia coli* K12 in blood. On-chip magnetic beads captured bacteria from the blood and thermal cell lysis released DNA for amplification. PCR product was then pumped to a detection chamber where sandwich hybridization occurred with an electroactive ferrocene detection probe and capture probes immobilized on an electrode array (an eSensor chip). The same device was also successfully adapted for a hemochromatosis-associated SNP assay. Other microchip assay devices have integrated PCR and detection of PCR product by DNA probes immobilized on electrodes patterned on the inside surface of the 8-µl volume, silicon-glass PCR chamber [70]. Another analysis strategy has been to transfer PCR products to an adjacent electrophoretic chip [71]. An integrated system comprising of a silicon-glass PCR chamber and a glass electrophoresis chip has been used to amplify genomic DNA and analyze the PCR product. An on-chip aluminum heater/temperature sensor achieved 16-s PCR cycles, and product was transferred by pressure to the glass electrophoresis channel filled with a hydroxypropylmethylcellulose sieving solution for a 180-s analysis step.

Another area in which integration had been a priority is nucleic acid sequencing – a sequencing lab-on-a-chip. A microfluidic chip-fabricated from polydimethylsiloxane (PDMS) containing micromechanical valves has been validated for sequencing-by-synthesis [72]. An alternative strategy uses ultradense channel arrays on a 150-mm glass wafer for processing nanoliter volumes. This has evolved to a 96-lane compact disc-sized device for high-throughput DNA sequencing that integrates clone isolation, PCR amplification, Sanger extension, purification and electrophoretic analysis in each microfluidic circuit [73].

Microchip capillary electrophoresis

Since its development in the early 1990s [74,75], capillary electrophoresis (CE) on a chip (or microchip CE) has been one of the most active areas of research in chemical and biochemical analysis. Microchip CE has many potential advantages for analysis of DNA or RNA mixtures, including miniaturization, integration, high speed and reduced reagent consumption. The application of microchip CE has been facilitated by the commercialization of microchip CE systems, first by Agilent (Agilent Bioanalyzer 2100) and subsequently by Hitachi (Hitachi SV 1100 and SV 1210) and Shimadzu (MCE-2010).

Microchip CE has been applied for the diagnosis of cancer susceptibility genes in methods based on single-strand conformation polymorphism (SSCP) [76] and a combination of allele-specific DNA amplification with heteroduplex analysis [77–80].

For example, Landers and coworkers investigated the use of microchip CE for the analysis of breast cancer susceptibility genes, *BRCA1* and *BRCA2* [72]. Common mutations in these genes show strong correlations with breast cancer, particularly in the Ashkenazi Jewish population. SSCP analysis of the denatured PCR products was performed in glass microchannels coated with polyvinyl pyrrolidone (PVP), in a buffer containing 2.5% (w/v) hydroxyethylcellulose and 10% (v/v) glycerol. Separated DNA was detected by laser-induced fluorescence (excitation wavelength 488 nm, emission wavelength 520 nm). Microchip CE was found to decrease SSCP analysis time 100-fold compared with conventional methods.

Microchip CE has also been used in testing for T- and B-cell lymphoproliferative disorders [81]. PCR-amplified fragments from the variable region of the T-cell receptor gene (150–250 bp) and the immunoglobulin heavy chain gene (80–140 bp) were analyzed. It was found that microchip CE provided the same information as obtained from slab-gel electrophoresis and conventional CE, except with much faster analysis, reducing the time needed for electrophoresis from 2.5 h on slab gel to 15 min on capillary, and to 160 s on microchip.

The scope of application for microchip CE is now extensive and some recent applications include: rapid analysis of ligase detection reaction products in the identification of point mutations in the human *K-Ras* oncogene in colorectal cancers [82]; evaluating RNA messengers involved in lipid trafficking of human intestinal cells [83]; detection of severe acute respiratory syndrome coronavirus [84], hepatitis C virus [85,86], herpes simplex encephalitis [87], Staphylococcus [88] and Salmonella [89]; as well as diagnosis of Duchene muscular dystrophy [90] and hemochromatosis [91]. The use of polymers, as opposed to glass, as fabrication materials for CE chips is gaining in importance, although several issues such as thermal conductivity and dissipation of Joule heating during electrophoresis remain problematic [92].

Bioelectronic chips

Bioelectronic chips in which an electronic component, such as an electrode, provides an analytical function are making progress as tools for nucleic acid analysis. Arrays of microelectrodes inside microfluidic chips (e.g., the NanoChip® from Nanogen [106]) provide sites for probe immobilization and can control hybridization via electronic stringency. Chips with arrays of 100 or 400 microelectrodes are available and an open-system strategy has been adopted in order to allow customization of assays as well as the use of analyte-specific reagents. A recent example of this approach is the multiplexed hybridization for mutation detection in β-thalasemia [93].

Future of microchip analyzers

The scope of application of integrated microchip devices that has already been documented provides ample evidence of their utility as analyzers suitable for nucleic acid analysis. However, commercialization of these devices is proceeding slowly. A notable exception is the Cepheid GeneXpert® module for DNA detection [107]. This automated real-time device provides fully integrated cartridge-based sample preparation with PCR amplification in a microchamber and detection, and delivers results from unprocessed sample in less than 30 min. A version of this module that identifies the presence of anthrax from air samples had been installed in US Postal Services sorting centers nationwide. This, together with the success of the microchip CE analyzers, should provide the necessary impetus for investment in the commercialization of more types of microchip analyzers in the future.

Expert commentary

The clinical laboratory has a history of quickly replacing old technologies with new technologies that offer advantages in terms of scope of testing, improved sensitivity and specificity, and convenience and adaptability to automation. Microtechnology has many beneficial attributes that recommend it for use in the clinical laboratory. Integration of the individual steps in multistep genetic tests into a single easy-to-use microchip device is one of the most appealing advantages. However, microtechnology is complex and requires sophisticated manufacturing techniques, and the same is true for nanotechnology. Thus, the clinical laboratory must rely on the medical diagnostics industry to commercialize microchip-based assays and analyzers. Until now, most microchip companies have focused on the needs of the pharmaceutical industry in massively parallel assays for drug discovery. There are now signs that finally, the potential of microtechnology in medical diagnostics is being seriously evaluated, and the first steps are being taken in the development of diagnostic microchip-based products.

Five-year view

Making predictions has always been both unwise and error-prone, as the early prognosticators on computers discovered (see [94] for an extensive list of past failures). By 2010, there should be a firm conclusion on the viability of nucleic acid assays based on microchip technology. Most, if not all, of the proof-of-principle microchip experiments have been completed, and the FDA approval of the AmpliChip Cytochrome P450 Genotyping Test, provides a basis for optimism for further expansion of microarray-based genetic tests. Some other factors also must be considered. The current range of routine genetic tests is limited and just a fraction of what might eventually be possible or needed as the genome yields its secrets. However, the full implementation of a wider range of genetic testing services will require appropriate coverage of such testing and reimbursement to a level appropriate for the cost of performing this testing in a clinical laboratory. Generally, reimbursement and the American Medical Association Current Procedural Terminology (CPT) codes have not kept up with genetic testing, and much of the billing is based instead on the generic molecular diagnostic codes, 83890–83912, from the Pathology and Laboratory Chemistry subsection of the CPT [95,96].

The greatest area of uncertainty is the impact of nanotechnology on molecular diagnosis. This science is expanding at a rapid rate, and has already demonstrated an exciting range of

new analytical possibilities. Possible scenarios are that nanotechnology will overtake microtechnology or that hybrid nano/microsystems will emerge for the molecular diagnostic tests of the future.

Acknowledgements

This work was supported in part by grants from the National Institutes of Health (National Cancer Institute [NCI] R33-CA83220 to P Fortina and NCI RO1-CA 78848–04 to LJ Kricka).

Key issues

- Despite experimental verification of the feasibility of lab-on-a-chip devices in molecular diagnosis, commercialization of such devices has been slow.
- The interface between the human operator and the microchip and surface chemistry effects in microchips are two obstacles to the success of this technology.
- The potential of nanostructures in molecular diagnosis is large, but concern over the safety of nanotechnology may present barriers to implementation.
- The more rapidly developing field of nanotechnology may overtake microtechnology, and the winning technologies of the future should be sought in the nanotechnology area.

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