

Microfluidic Systems Being Adapted for Microbial, Molecular Biological Analyses

Microfluidic systems are shrinking, speeding PCR and DNA sequencing, and headed toward personalized diagnostic devices

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Microfluidic analysis depends on devices containing micrometer- to millimeter-scale components capable of handling microliter-to-nanoliter quantities of liquids and gases. Soon after microfluidic inkjet printers came into use in 1987, microfluidic analytic devices with chemical and microbiological endpoints became available in the early 1990s. However, the pace of development remained slow until 1997, when research in microfluidics analytical systems began to grow geometrically. Dozens of examples now illustrate the value of microfluidics in molecular biology, especially for genetic analyses at the DNA level.

With microfluidic systems, investigators can follow chemical reactions at a scale that is 100 to 10,000-fold smaller than traditional assays. Hence, sample and reagent volumes are much

smaller, costs per assay are lower, assays are faster, and the potential for automation, portability, and high sample throughput is greatly enhanced. Various components used in such systems can be miniaturized, including pumps, heaters, valves, mixers, coolers, and detection devices. To provide control and reporting, the overall system generally depends on microcontrollers and microprocessors already developed and miniaturized by the computer industry.

The scale of microfluidic systems offers many benefits but also poses problems, including surface tension, capillary effects, evaporation, diffusion, mixing, and hydraulic resistance. In assays that depend on detecting particles such as cells, viruses, or DNA fragments, the very small volumes of microfluidic reactions often mean that very few target molecules are available for detection, requiring efficient amplification techniques or more sensitive detectors.

Materials such as silicon, glass, and quartz are widely used for fabricating reaction chambers in microfluidics systems. However, alternative materials, including polydimethylsiloxane and polycarbonate, are coming into wider use. Each such material comes with benefits and drawbacks. For instance, plastic-like materials, despite their ease of construction and low cost, may not be compatible with widely used reagents. For example, polydimethylsiloxane inhibits PCR.

In general, microfluidics components are fabricated using the same techniques that the computer manufacturing industry harnessed 40 years ago. Hence, microfluidics manufacturing processes are readily automated, yielding large quantities of devices

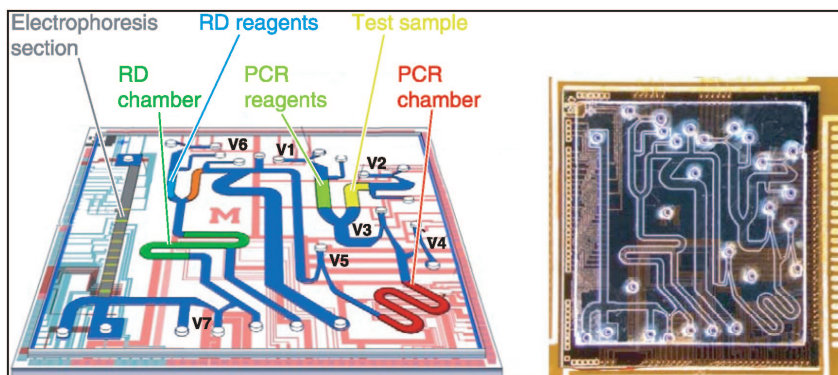
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Summary

- Microfluidic systems are helping to shrink analytic formats, leading toward personalized diagnostic devices.
- Examples of microbiologically oriented microfluidics devices include an influenza virus genotyper and an oligonucleotide probe-based pathogen detector.
- Although few microfluidics devices are validated, some are comparable in performance to conventional analytic devices; meanwhile, biological sample preparation problems are being solved.
- Market forces continue to shape development of microfluidics devices, favoring clinical diagnostic over environmental applications.



FIGURE 1



A microfluidic chip to detect influenza virus. It integrates many molecular biology processes including reverse transcriptase, PCR amplification, and gel electrophoresis to accomplish the task of detection.

with high levels of precision. Moreover, those components can be readily integrated with electronics and control processes.

Microfluidics Genetic Analysis Systems for Genotyping, PCR, and DNA Sequencing

Miniaturization and microfluidic systems are pushing the envelope of genetic analysis. Ruggedness, speed, cost, sample and assay throughput, specificity, and size are key parameters that are being substantially improved. Analytical sensitivity is often as good as for conventional assays—but not always. Although the small volumes used in microfluidics assays impose limits on the numbers of molecules being measured, systems for detecting those few molecules are being miniaturized at a slower pace. To accommodate such differences, overall systems often come in large packages.

One of the best examples of a device in which several molecular biology-based analytic processes were integrated into a microfluidics device is the influenza virus genotyper (Fig. 1). Its development entailed a team effort that included chemical engineer Ronald Larson, chemical and biochemical engineer Mark Burns, and a molecular biologist specializing in genomics, David Burke, all at the University of Michigan in Ann Arbor. The device converts RNA from clinical or other specimens of influenza virus, such as from throat swabs, into billions of copies of

complementary DNA, which are readily detected. The device miniaturizes and integrates three key molecular biology processes: reverse transcription, polymerase chain reaction (PCR), and gel electrophoresis. A geographical network of such influenza virus genotypers could be extremely useful to study the epidemiology of this virus, according to the developers of this analytic device.

Another example of miniaturization using microfluidic principles is the 6-minute micro-PCR developed by Pavel Neuzil of the Institute of Bioengineering and Nanotechnology in Singapore and his collaborators (Fig. 2). PCR generally requires about 35 cycles during which temperatures move from annealing the target-primer DNA strands

at 55°C to melting them at 95°C. Conventional thermal cyclers require 1 to 2 hours to complete this cycling process, with most of this time used to change the reaction temperatures using Peltier units, in which current flowing through metal junctions heats or cools the reaction vessel. Peltier units typically heat and cool in the range of 3 to 6°C/sec, thus taking 60 to 90 seconds for each cycle, whereas Taq polymerase copies 100 basepairs in only 2 to 3 seconds. More recently developed PCR thermal cyclers with higher heating and cooling rates complete the amplification reaction cycles in less than 20 minutes, and this time was reduced by Neuzil's group to less than 6 minutes by using integrated silicon heaters and small reagent volumes.

An alternative approach, named flow-through PCR, eliminates temperature cycling altogether by making the PCR mixture flow repeatedly over the three temperature regimes, allowing PCR to complete in approximately 1.5 minutes, which is close to the time required by Taq polymerase to amplify DNA samples during the 30–35 cycles. There are numerous efforts to develop low-cost, on-chip PCR systems for various applications, with some of them already on the market. The above example was chosen to illustrate the concept of microPCR rather than to exclude others.

Miniaturization and microfluidics are playing a key role in reducing the cost of sequencing as well. One such example is a system that integrates cell capture, PCR, and DNA sequencing

on a silicon wafer. This system, designed to analyze 96 samples in parallel (Fig. 3), is being developed by chemist Richard A. Mathies of the University of California, Berkeley, and his collaborators. Their system is akin to the commercially available 454 Life Sciences/Roche Applied Sciences sequencing system that uses PCR followed by pyrosequencing in proprietary plates containing millions of miniaturized wells. In these cases, miniaturization greatly improves sequencing throughput. However, because the overall system is not small, it is not portable.

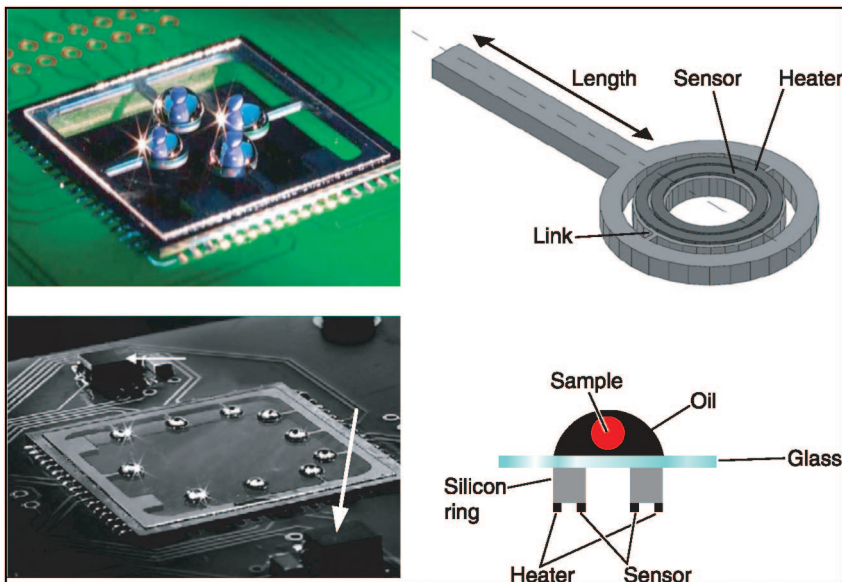
Microfluidics Systems Can Be Adapted to Detecting Pathogens

Microfluidics-based assays are also used for detecting and quantifying infectious agents by hybridizing PCR-amplified products to oligonucleotide probes. For example, we developed and validated a chip (Fig. 4) containing 8,000 microreactors, each with a diameter of 50 μm . Each reactor has oligonucleotide probes synthesized in situ using a low-cost, light-directed DNA synthesis technology. The chip can be used to screen 20 different pathogens per run, based on their respective virulence and marker genes.

Presence of each pathogen is confirmed by targeting at least 3 to 6 genes. Positive signals are confirmed by hybridizing amplicons for such genes to 5 to 20 standardized probes, resulting in fewer false positives than approaches that rely on single markers. This approach can be easily translated to on-chip PCR and microPCR platforms, making a sensitive and cost-effective tool for screening many genetic elements obtained from microbial or other sources.

On-chip, real-time PCR systems eliminate the need for amplification of target prior to hybridization. One such example is the OpenArray™ system commercialized by BioTrove Corporation (Boston, MA). Each OpenArray™ of the size of a glass slide contains approximately 3,000 small wells of 33 nl arranged in 48 subarrays with customer-dictated primer pairs already suspended in the wells. Using this system, we have developed an assay for simultaneously screening up to 120 virulence gene targets be-

FIGURE 2



A micro-PCR device with integrated heaters. Due to very small reagent volume, the rate of heating can be as high as 165°C per second reducing the time to PCR from hours to less than 6 minutes.

longing to 30 pathogens relevant to water, food, and clinical diagnostics with a sensitivity of 10 to 100 copies per reaction well. Similar assays are being developed for tracking antibiotic resistance genes, genes responsible for cyanotoxins, and microbial communities involved in energy production.

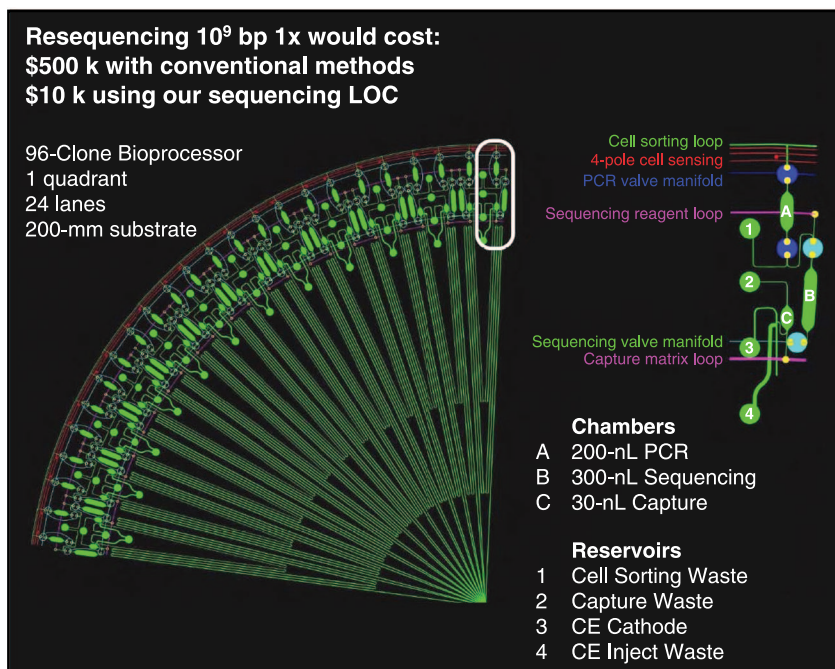
It is worth noting that miniaturization of the assay and miniaturization of the total system are often two separate objectives. If both objectives can be achieved in a cost-effective manner, then the resulting products are expected to readily find applications in many diagnostic laboratories. Many such systems are expected to become available in the near future.

Challenges and Bottlenecks

Microfluidic devices face the same performance challenges that other diagnostic and analytical approaches face regarding sensitivity, specificity, ruggedness, user-friendliness, speed, cost, reliability, and accuracy. Although PCR is capable of detecting a single copy of a gene target under ideal conditions, its sensitivity for now is limited to 10 to 100 copies for microfluidic systems. However, specificity for microfluidics



FIGURE 3



Highly parallel sequencing on a wafer. Technologies like this have the potential to reduce the cost of resequencing and SNP detection significantly in a clinical setting.

PCR may be comparable or better than conventional PCR systems because of the use of multiple targets followed by sequencing or genotyping.

Integrating the steps of sample concentration, cell lysing, and DNA extraction is a major challenge for microfluidic devices focusing on genetic analysis. Concentrating samples also concentrates inhibitors, complicating microfluidic analysis and sometimes yielding false-negative values. However, some elegant solutions are on the horizon.

For example, Andre Marziali, a physicist at the University of British Columbia in Vancouver, British Columbia, Canada, developed a gel electrophoretic technique known as synchronous alternating drag perturbation to prove that as few as 10 molecules of PhiX174 phage DNA in 5 ml (equivalent to a DNA concentration of 3 zeptomolar; $1 \text{ zM} = 10^{-21} \text{ M}$) could be recovered on gels within minutes to hours without concentrating the inhibitors. This modified electrophoresis technique directs DNA molecules into the center of a gel while separating them from common PCR inhibitors and other con-

taminants. In principle, this approach can be incorporated into microfluidic devices to solve this otherwise major challenge.

Another bright spot is the laser-based cell lysis and automated washing and extraction system being developed by various companies. Miniaturized systems still have problems in dealing with large-volume environmental and water samples, as there is no miniaturized replacement for centrifugation or filtration to concentrate particulates in large volumes.

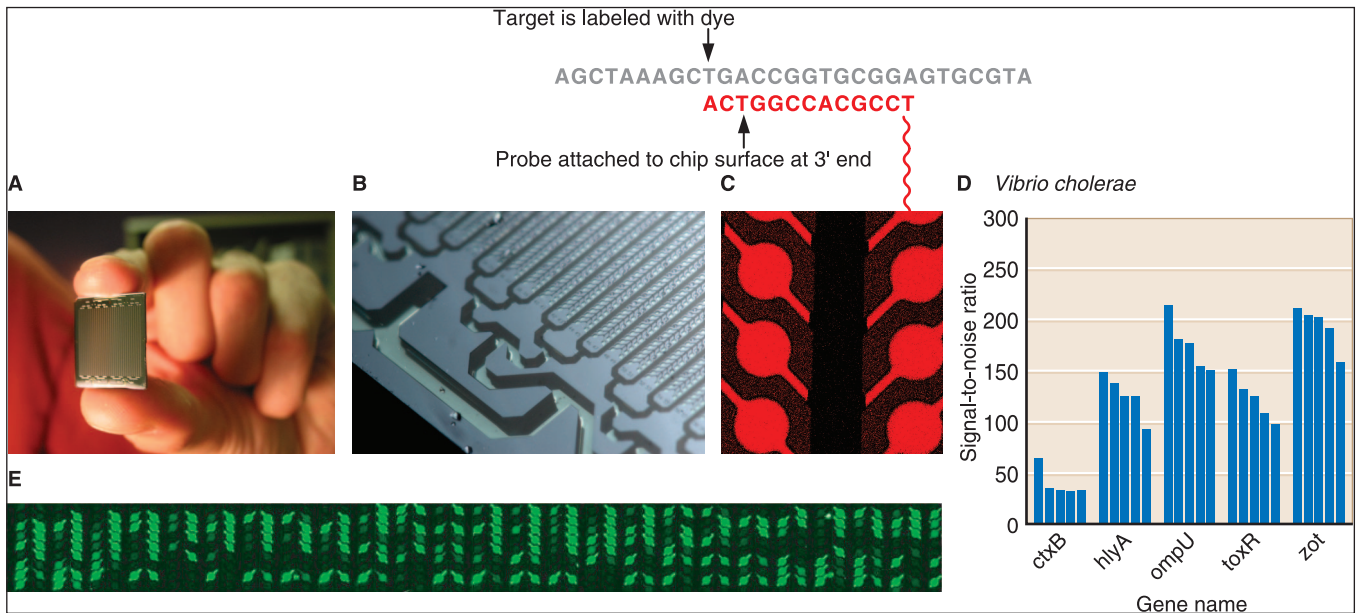
A continuing challenge is the accelerating growth of complex microbial genetics data sets. Increases in DNA sequencing rates lead to more and more changes in databases, in turn leading to changes of detection probes and primers. Even sophisticated analysts may have trouble keeping up. For such reasons, developers of analytic devices must try to use only those genetic markers that are extremely robust and take other steps to minimize false-positive and -negative test results. The U.S. Department of Energy initiative to se-

quence all type strains and other diverse phenotypes would greatly aid in building this database in a more comprehensive and sustained way.

Few microfluidics devices for molecular biology and microbiology uses are fully validated. Developers in academic settings tend to focus on proof-of-principle, while those in commercial settings tend to lack the patience to validate analytic devices for use on complex biological samples. The development of new devices is also hampered by the challenges in bringing together scientists and engineers who are working in disparate areas, such as microfluidics, printed circuit board design, programming, and molecular biology and microbiology.

A further challenge microsystem designers have to overcome is simplicity of use and reliability of results. Most of the current microfluidic devices such as PCR arrays and microarrays can be a lot more user-friendly than they currently are. The goal should be to emulate the pregnancy tests which sell for \sim \$10, are very simple to use, and the output of which is easily recognizable in any language without the need for computers or other electronic equipment.

FIGURE 4



A microfluidic DNA biochip with recirculation capabilities: (a) The chip is approximately 1 cm², (b) close-up of microfluidic channels and a portion of the approximately 8,000 reactors on the chip, (c) close of 6 reactors, each with 50 μm diameter, (d) signal to noise ratio for 5 genes belonging to one of the 20 organisms that may be tested on the chip, and (e) laser scanned signal intensities for part of the chip. [Photo credits: (a) V. Deneff & W. Donckerwolcke, (b) Kurt Stepnitz, (c, d, and e) Robert Stedfeld and Dieter Tournousse]

Market Potential Often Affects Technical Developments

The global in vitro diagnostics market is more than \$25 billion in sales per year. Nucleic acid-based analytic methods, however, constitute only about 4% of this market. In contrast, the annual global market for blood-glucose testing is \$4 billion.

Is it possible that microfluidics will lead to development of point-of-care, low-cost diagnostic devices for many other clinical applications, including tests for infectious agents? There are certainly no insurmountable technical barriers to this goal. However, there are financial barriers. For instance, does a \$100 diagnostic device need to be as popular as an iPod or as essential as a blood-glucose meter to survive market forces? It might be easier to satisfy investors by selling limited numbers of analytical units for \$100,000 than to sell huge numbers of units for \$100. Because of financial incentives, initial microfluidic development efforts tend to focus on devices for analyzing clinical samples instead of

less-lucrative diagnostic testing niches such as coliforms in water and at waste treatment plants.

Low-cost microfluidic devices also face the challenge of competing with well-established tests. For example, hundreds of commercial kits are available for detecting or diagnosing *Escherichia coli*. If a microfluidic-based assay were developed for *E. coli* and other similar bacterial agents, will laboratories switch to such high-tech devices from doing plate counts or PCR? Similarly, replacing existing and well-tested diagnostic assays by microfluidic-based assays will require extensive validation of the latter to demonstrate their reliability in addition to savings in cost and ease of use. It is possible that in the initial stages of being adopted, some microfluidic assays may not compete well with existing detection kits due to inertia. Some such devices might fail to gain a market share.

What To Expect in 5 to 10 Years

In the next 5 to 10 years, we can expect a number of analytic devices emanating from the



merger of molecular biology with microfluidics and miniaturized operating systems. Applications are expected in a variety of areas, including clinical diagnostics, water and food testing, produce supply-chain monitoring, paternity screening, and antibiotic resistance tracking. Assays will be based on several kinds of testing, including DNA abundance and sequence analysis, genotyping, and single-nucleotide-polymorphism analysis. In microbiology, microfluidic systems will be especially useful for analyzing organisms that are difficult to culture, such as multidrug-resistant *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus* (MRSA). Many of these microfluidic devices will harness the genetic markers that are accumulating in DNA sequencing databases.

Mature operating systems, such as those used in miniature data storage devices, hand-held computers, and cell phones, can be adapted for use in microfluidics-based diagnostic devices. For example, smaller and simpler microfluidic devices might be integrated with USB key data

storage devices that have built-in operating systems and data analysis software to create a “lab-on-a-USB key” device. Such a device could also be attached to other computational devices such as a cell phone or laptop computer to control molecular assays being done on the microfluidic biochip. Analysis using such devices can be transmitted to central databases for shared use and metaprocessing.

The scale of reactions in microfluidic systems is still 100- to 1,000-fold greater than reactions inside microbial cells, suggesting that microfluidic devices eventually might be miniaturized to follow reactions occurring among only a few molecules. Nanometer-scale features in some microfluidic components—albeit not involving molecular biology and genetic analysis—are being developed using techniques such as extreme UV lithography, lithography galvanoplasty, and nanotechnology. A critical question is whether micro- and nanofluidics will simplify molecular biology and genetic assays enough to make them broadly available, including to developing countries.

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