DNA amplification: does 'small' really mean 'efficient'?

Andrew J. de Mello reviews developments in DNA amplification

The advent of the polymerase chain reaction (PCR) has, without a shadow of a doubt, hugely accelerated the progress of studies on the genetic structure of a diversity of organisms. PCR is an enzyme catalyzed amplification technique that allows any nucleic acid sequence to be generated *in vitro* and in abundance.[1] It was first reported in early 1986 at the 51st Cold Spring Harbour laboratory Symposium on Quantitative Biology by Kary Mullis, and since has become an indispensable tool in basic molecular biology, genome sequencing, clinical research and evolutionary studies.[2]

The reason for the almost immediate acceptance of PCR as a DNA building tool lies in the beautiful simplicity of its underlying mechanism. Briefly, high temperature (normally in excess of 95 °C) is used to separate (denature) double stranded DNA into two single strands. Synthetic sequences of single stranded DNA (normally 20–30 nucleotides long), known as primers, are used to define or bracket the target region to be amplified. One primer is complementary to one DNA strand (at the start of the target region) with the second primer being complementary to the other DNA strand (at the end of the target region). The primers are hybridized (annealed) to the single stands by reducing the local temperature to between 50 and 65 °C. This is followed by an extension step at a slightly elevated temperature (approximately 72 °C) in which a complementary strand from each primer is extended by the catalytic action of a thermostable DNA polymerase enzyme (in the presence of free deoxynucleoside triphosphates) to form complementary strands of the template. This three-step process constitutes one PCR cycle, and if repeated n times will, in theory, lead to 2^n-1 copies of the target duplex. In other words, amplification is exponential, and consequently after only 20 cycles over one million copies of the original target DNA can be synthesized. In reality, amplification is never truly exponential and the copy yield is given by

$$Y = (1 + x)^n \quad (1)$$

where x is the mean efficiency per cycle. Non-ideal amplification is a result of many experimental factors, which may include reagent consumption at higher cycle numbers, poor template–primer hybridization, sample contamination, inefficient thermal cycling and poor temperature control.

Conventional instrumentation

Since its inception, PCR has been performed using a wide variety of instrumental techniques.[3] Laboratory setups for early PCR experiments consisted of large arrays of individual heating blocks or baths (each set at a different temperature) with researchers present to manually move samples from one unit to the next. Fortunately, microprocessor-controlled heating block systems were soon developed. In these thermal cyclers, temperatures and time periods for each part of a cycle could be set in advance, thus allowing the process to run unattended. Today, most conventional amplification instruments are still manufactured to the same basic design and more specifically rely upon Peltier effect thermoelectric heating, in which solid-state devices convert electrical energy into a temperature gradient. Although instruments of this kind have proved enormously popular for many lab-based procedures (due to the high degree of automation that they provide) a number of technical frailties limit the speed and efficiency of the amplification process.

The fundamental requirement for efficient amplification is rapid heat transfer. Consequently, it is desirable to have a system with a low heat capacity that can transfer heat quickly to the sample on heating, and quickly away when the heater is switched off. Almost all conventional thermal cyclers possess large thermal masses. This results in relatively high power requirements and slow heating and cooling rates. Furthermore, since samples are contained in polypropylene microtubes (typically 200–700 μL volume) sunk into depressions in the heating block, the relatively large sample masses combined with the relatively low thermal conductivity of the vessel walls exacerbate the heating/cooling problem. In other words, regardless of how quickly the heating block reaches the target temperature, the temperature within the sample volume will only equilibrate after a further time delay (typically 30–60 s). In addition, due to non-uniformity in the block temperature, the geometry of the microtubes themselves and relatively large sample volumes (>50 μL), temperature gradients are often created within the sample. Average temperature differentials between the top and bottom of standard microtubes as high as 10 °C have been reported.[3] This heterogeneity often results in a loss of specificity, non-ideal strand extension and reduced annealing efficiencies.

A number of approaches have since been proposed to overcome both problems with cycling speed and temperature homogeneity. For example, the fabrication of modified ultra-thin walled microwell plates has afforded gains in both heat transfer and temperature homogeneity (through reduced sample volumes). Furthermore, the format allows for facile interfacing with conventional instrumentation for pre-and post-PCR sample processing and for this reason has steadily gained popularity.[4] Nevertheless, possibly the most exciting development in conventional PCR technology has been the conception of systems that incorporate heated air circulation around glass capillaries.[5] Pioneered by Carl Wittwer and colleagues at the University of Utah and Idaho Technology Inc., these thermal cyclers function as temperature-controlled recirculating hair dryers. Rapid heat transfer is achieved by blowing air over large surface-to-volume ratio samples. Glass microcapillary tubes or thin walled microcentrifuge tubes containing 5–100 μL are held at microtitre spacing for simultaneous sample pickup, cycling, and

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delivery. Current commercial systems using this technology provide for cycle times between 30 and 60 s and also allow the formation of amplification products to be monitored in real-time.

Although these approaches have considerably improved the efficiency and applicability of PCR protocols in the lab, significant issues related to sample preparation and handling have yet to be fully addressed. Consequently, the search is still on for instrumentation that affords highly efficient amplification of multiple samples in short times and at low cost.

Going down to another level

A clear message from the development of conventional thermal cycling systems is that miniaturisation of sample volumes affords significant gains in terms of cycle times and amplicon yield. Nevertheless, manipulating and processing extremely small sample volumes (< 1 μL) within conventional instruments is a difficult task, and not surprisingly few bench-top instruments are able to successfully handle reaction volumes below 1 μL.

Over the past decade the application of microfabricated chip technology to a diversity of analytical problems has become an area of huge interest. In particular, the miniaturization of chemical and biological reaction chambers has been shown to afford gains in terms of control, speed, efficiency and functionality.[6] Importantly, due to micron sized feature dimensions and closed fluidic formats, sample volumes down to the picolitre scale can be manipulated and processed with a high degree of control. Accordingly, interest in micromachining high efficiency PCR devices has been a highly visible sub-discipline within lab-on-a-chip science for the past five years.

At a fundamental level the attraction of reducing sample volumes in PCR lies in improvements with respect to the rates of thermal and mass transfer. By reducing the reactor volume, the sample may be heated and cooled extremely quickly, thus negating the large time constants associated with temperature variation on the macroscale. As has been seen, this directly leads to improvements in both the cycle speed and the efficiency of annealing and extension. Allen Northrup (Lawrence Livermore National Laboratory) and Richard White (University of California, Berkeley) used these basic ideas in 1993 to define the first microfabricated device for performing PCR on the microscale.[7] The silicon based microreactor (25–50 μL volume) incorporating a polysilicon thin-film heater provided heating and cooling rates of 13 and 35 °C s^{-1}, respectively. As a result, successful amplification of a 142 base pair region of the CAG gene of the HIV sequence was achieved in times as low as 20 min. Peter Wilding and Larry Kricka of the University of Pennsylvania subsequently reported similar microfabricated silicon chambers capped with Pyrex glass.[8] The chambers, etched to depths of 40 or 80 μm, permitted free flow of fluid through the chamber and defined volumes of 5 or 10 μL. Thermal cycling was effected by placing the device into a Peltier heater–cooler unit, with detection of products performed off-chip. Both studies utilized silicon as the primary substrate material due to its excellent thermal and structuring characteristics.

Development of the basic technology continued at an expeditious rate, with Wilding and co-workers using similar devices to amplify genomic DNA from lymphocytes directly introduced into the microchambers.[9] The Pennsylvania group also realized the fact that surface chemistries play a dominant role in biological reactions performed within microfabricated environments. In comparison to conventional microtube formats the surface-to-volume ratio on chip is normally at least one order of magnitude larger. This means that molecules start to 'see' and 'interact' with the surface on a regular basis. Initial studies demonstrated that both native silicon and silicon nitride inhibit PCR within high surface-to-volume ratio environments. However, an oxidized silicon surface gave consistent amplifications that were comparable to those performed in conventional PCR tubes.[10]

At a similar time Northrup and co-workers refined their integrated device now termed the Miniature Analytical Thermal Cycling Instrument (MATCI) to address the ideas of integrated detection and portability.[11] Refinements to the original design included the use of two heating units to improve thermal uniformity throughout the reaction chamber, active cooling to reduce cycle times (down to as little as seven minutes), polypropylene liners to improve reaction fidelity and a diode based detection to perform real-time PCR product detection. The entire instrument, including a laptop computer for process control and data analysis, was small enough to fit into a brief case (Fig. 1) and thus ideally suited to point-of-care and in-the-field applications. The authors have subsequently demonstrated efficient use of the MATCI in a number of key applications, including the analysis of human genes,[11,12] single nucleotide polymorphisms,[11] pathogenic viruses[11] and bacteria.[13] Moreover, fundamental aspects of the MACTI technology have now been developed into commercial diagnostic test-systems.[14]

Many other research teams have subsequently described similar batch devices for performing high-efficiency PCR in small volumes. For example, Mike Albin and co-workers at PE Applied Biosystems have described real-time sequence specific detection of PCR products in silicon microstructures.[15] Arrays of 48 PCR reactions could be performed and analyzed in real-time and, significantly, volumes as low as 500 nL could be successfully processed with the addition of carrier protein to minimize polymerase binding to the reactor surfaces. Researchers at the University of Cambridge and Defence Evaluation and

Fig. 1 Photograph of the briefcase-sized, rechargeable, battery operated, portable MATCI. Reproduced from ref. 11 with permission.

Research Agency have also described similar silicon microchambers for DNA amplification.[16] These low-power devices incorporate both temperature sensing elements and heaters and afford heating rates of 60–90 °C s^{-1} and cooling rates of 74 °C s^{-1}. Interestingly, the authors note that although many alternative substrate materials are becoming popular for bioanalysis, silicon provides distinct advantages in terms of fabrication and thermal characteristics.

The vast majority of the microfabricated devices discussed up to this point, although demonstrating very real gains in performance through reactor miniaturisation, involve the processing of volumes close to the μL scale. A necessary consequence of creating highly integrated PCR arrays is a dramatic reduction in sample volume into the pL regime. Apart from the increasing significance of surface effects one must consider the actual number of molecules present within the reaction vessel under normal conditions. Hinedori Nagai and associates at the Japan Institute of Science and Technology have recently described large-scale microchamber arrays for picolitre PCR (Fig. 2).[17] The arrays, machined in silicon, are coated with SiO_2 using wet thermal oxidation techniques and yield microchambers with volumes ranging from 1.3 pL to 32 μL. Initial experiments demonstrate successful amplification in chambers with volumes greater than 86 pL. Furthermore, the use of a water repelling membrane allowed effective removal of PCR products from individual microchambers without cross-contamination. Despite the demonstration of high efficiency amplification within the reactor arrays, issues relating to efficient sample delivery to individual microchambers make implementation of high-throughput screening protocols a long-term goal.

Although the vast majority of microfabricated PCR chambers utilize resistive heating elements to effect thermal cycling other highly efficient methods have been reported. For example, James Landers and co-workers at the University of Virginia have recently reported infrared mediated thermal cycling of samples in capillaries down to volumes of 160 nL.[18] This approach does not directly heat the reactor vessel and thus provides for extremely fast heating and cooling rates. In addition, Anne Kopf-Sill and associates at Caliper Technologies have ingeniously used Joule heat generated during electrophoresis to perform PCR within microfabricated channels.[19] To avoid partitioning of reactants and products during the amplification process an alternating (rather than continuous) electric field is applied across the channel. Development of this thermal cycling method should prove particularly significant when fabricating robust and low-cost integrated devices.

Thinking outside the box

One of the most desirable advantages associated with microfabricating analytical instrumentation is the ability to facilitate processes that are either extremely difficult or even impossible to recreate on the macroscale. An elegant illustration in separation science is synchronized cyclic capillary electrophoresis (SCCE). This technique, first demonstrated on-chip by Norbert Burggraf and Andreas Manz, permits extremely efficient electrophoretic separations of molecular species at low applied voltages and in short times.[20] Although James Jorgenson of the University of North Carolina, Chapel Hill, has since reported a macroscale SCCE system that offers improved resolving power and peak capacity, the 'plumbing' problems associated with interfacing capillary tubing will most likely make its widespread application impractical.[21] In a similar fashion, Andreas Manz and colleagues at Imperial College of Science, Technology and Medicine have utilized the inherent flexibility of micromachining technology to address the problem of highly efficient thermal cycling in an unusual way. As has been seen the vast majority of approaches to performing chemical and biological reactions within microfabricated formats have focused on downscaling the dimensions of a conventional batch reactor. Manz and colleagues used the concepts of downscaling and micromachining to realize a continuous flow PCR system where a time–space conversion allows temperature to be kept constant over time at specific locations in the system.[22] Sample is then moved between individual temperature zones to effect the thermal cycling process. A schematic of the concept and experimental set-up is

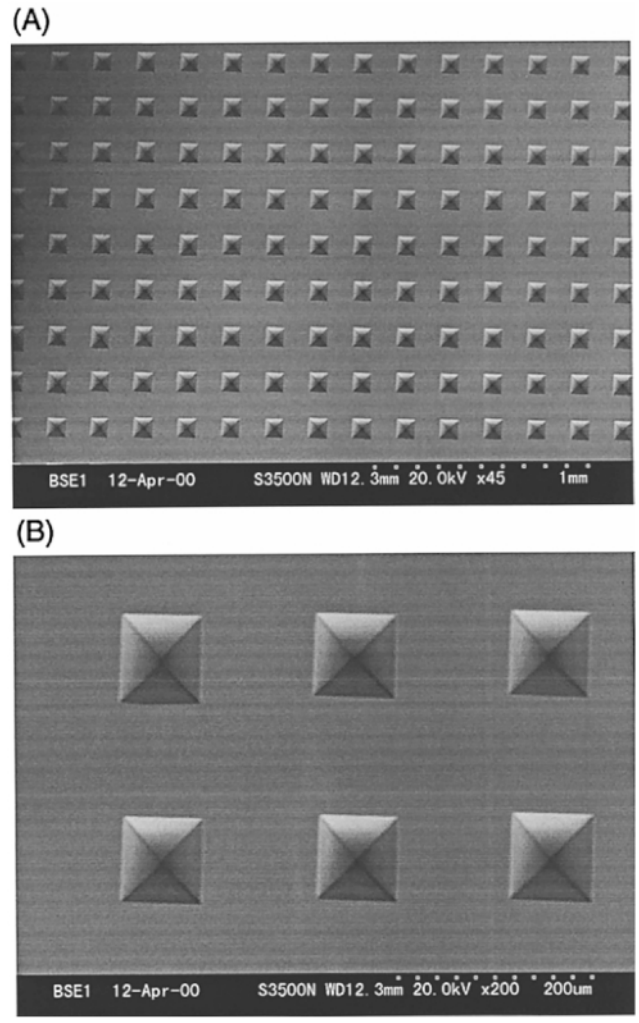

Fig. 2 SEM images of silicon PCR microchambers: (A) expanded view of 80 × 80 μm microchamber array; (B) close up. Reproduced from ref. 17 with permission.

illustrated in Fig. 3. Using a glass microchip containing a single microchannel 2.2 m long a 20-cycle PCR amplification of a 176-base pair fragment from the DNA gyrase gene of *Neisseria gonorrhoeae* was performed in times ranging from 18 min down to 90 s. These initial studies were important in demonstrating dramatic reductions in PCR times, and also potential gains in sample throughput. Subsequently, Johann Michael Köhler and associates have reported similar devices for DNA amplification.[23] Recent studies from Köhler's group have demonstrated silicon–glass devices incorporating heaters and temperature sensors. In addition, the devices have been used to perform both continuous and serial flow PCR with efficiencies equivalent to commercial technologies.

Functional integration

DNA analysis through the use of PCR has undoubtedly advanced biological and clinical research, and the microfabricated structures described in this review have afforded significant gains in efficiency, throughput and reaction time. However, in most research and diagnostic applications DNA analysis ideally incorporates a number of distinct processes in addition to PCR. These may include sample pre-conditioning, reagent addition and electrophoretic analysis and detection of reaction products. Indeed, the ability to extract the required information from a biological system will almost always involve performing more than one of these analytical operations. As a result, many research groups have addressed the ideas of functional integration to allow for high-throughput biological analyses to occur on a large scale.

Richard Mathies at the University of California, Berkeley, was one of the first people to recognize the need for highly integrated microdevices and over the past five years has pioneered the development of integrated PCR/CE microdevices. In collaboration with researchers at the Lawrence Livermore National Laboratory the Berkeley group first presented the integration of ultra-fast PCR and DNA sizing on a single microdevice in 1996.[24] In this study PCR microchambers of the kind described previously were integrated with a planar CE microchip (Fig. 4) to amplify and size a 268 base-pair product from the β-globin gene within 20 min of sample introduction. More recent studies have reported monolithic integrated DNA analysis systems that include microfluidic valves and vents (Fig. 5).[25] These microdevices allow for controlled sample loading into 280 nL PCR chambers, fast thermal cycling and automated electrophoretic sizing of reaction products. The monolithic technology has been further modified and utilized this year to explore the stochastic amplification of single DNA molecule templates.[26] Results demonstrate the ultimate limit of sensitivity for microfabricated PCR devices and bode well for the continued evolution of low-volume, high-density diagnostic devices.

Mike Ramsey and co-workers at the Oak Ridge National Laboratory (ORNL) have used similar ideas to address the ideas of functional integration. In 1998 the ORNL group demonstrated cell lysis, multiplex DNA amplification and electrophoretic analysis on a monolithic chip device.[27–29] In a first generation device, cycling was achieved by placing the entire chip within a conventional thermal cycler. Although the fluidic network afforded efficient integration of processing steps, the use of conventional technology for thermal cycling meant that total analysis times were excessively long when compared to other microchip approaches. More recent studies by the same group have addressed this issue by coupling the chip system with a compact thermal cycling assembly consisting of dual Peltier elements. Through efficient detection and on-chip DNA concentration as few as ten cycles are required for analysis in times of less than 20 min.[30]

An intricate example of total system integration has been reported by Mark Burns and co-workers at the University of Michigan, Ann Arbor. Since 1996 they have been developing and refining numerous micromachined tools for DNA analysis and have gone some way to achieving complete system integration.[31] Recently, they reported the functioning of a nanolitre DNA analysis chip that incorporates fluidic channels, heaters, temperature sensors and fluorescence detectors.[32] The device can measure and mix reagents, amplify or digest DNA, and separate and detect products. As can be seen in Fig. 6, no external optics, pumps or actuators are utilized and power requirements are extremely low. More recently, Rolfe Anderson and colleagues at Affymetrix described a similarly complex system for multi-step genetic assays.[33] A microstructured polycarbonate

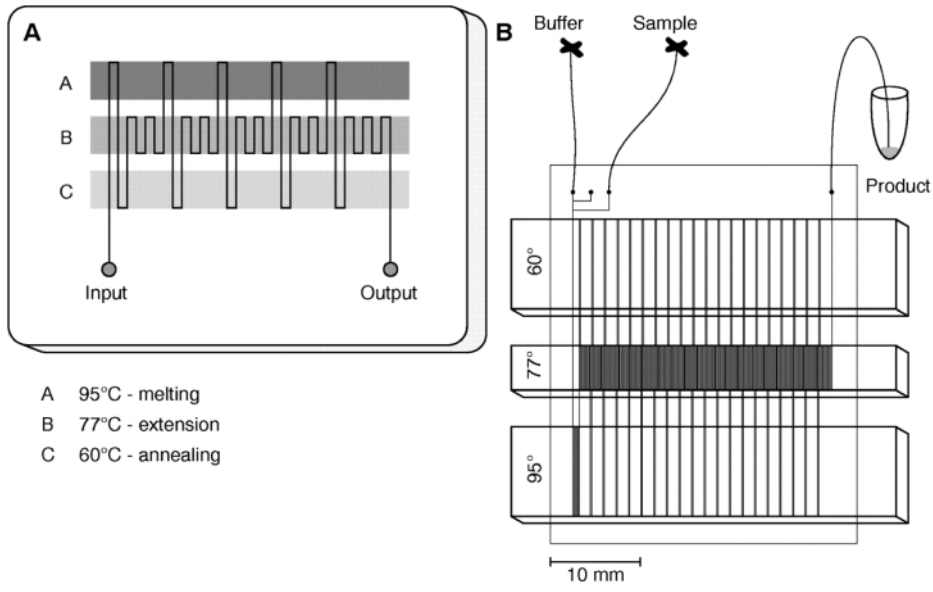

Fig. 3 Continuous-flow PCR on a chip: (A) schematic of chip layout; (B) schematic of experimental set-up. Reprinted with permission from *Science (Washington, D. C.)*, 1998, **280**, 1046. Copyright 1998 American Association for the Advancement of Science.

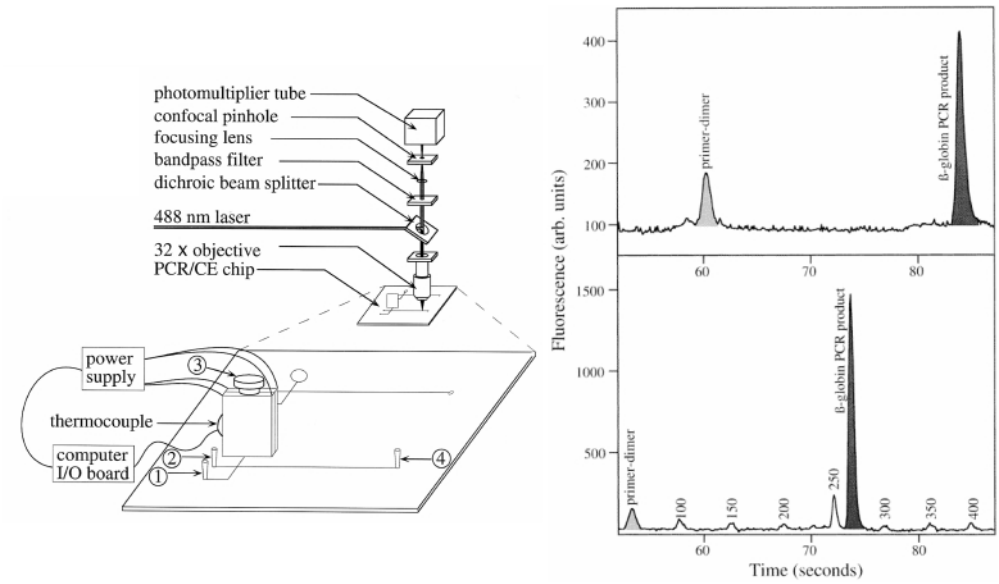

Fig. 4 Schematic of a PCR/CE microdevice with demonstration of high-speed, integrated analysis of a 268 base pair β-globin fragment. Reproduced from ref. 24 with permission.

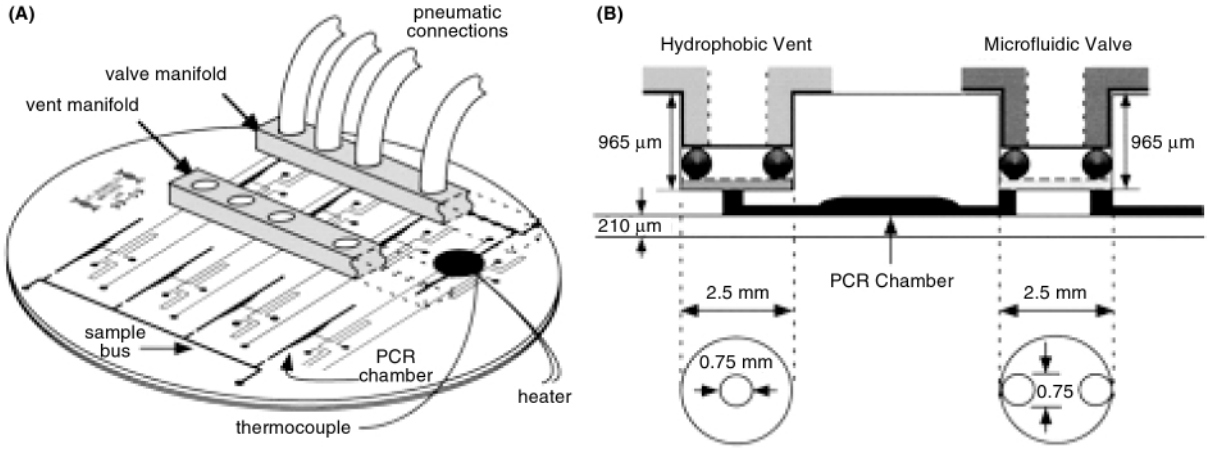

Fig. 5 Schematic of a PCR/CE microdevice incorporating microfluidic vents and valves. Reproduced from ref. 26 with permission.

cartridge was used to automatically perform extraction and pre-concentration of DNA, PCR, reagent mixing and metering, and nucleic acid hybridization.

What next?

As we have seen a wide variety of microfabricated PCR instruments have been reported over the past eight years. Their structure, function and applications have ranged widely; however the gains afforded through miniaturization have been unfailing. Almost all research efforts have been driven by and resulted in gains such as enhanced analytical performance, superior component integration, increased throughput and improved automation.

In many ways, PCR itself has become a unique test reaction for the assessment of novel microfabricated analysis systems. Its importance in molecular biology, its mechanistic simplicity and its dependence on the strict control of experimental parameters means that PCR is ideally suited to optimization in miniaturized formats. The heart of performance gains lie in improved thermal and mass transfer on a small scale. Heat can be dumped into and removed from microreactors exceptionally quickly and temperatures can be controlled uniformly throughout the sample volume. Nevertheless, miniaturization does not always help us. As has been noted, surface effects become highly significant within microfabricated environments. Due to elevated surface-to-volume ratios the morphology and chemistry of vessel walls are key in defining amplification efficiencies. Indeed, trends in commercialization demonstrate a clear push towards polymeric substrate materials.[6] Consequently, the development of cost-effective materials possessing superior bio- and chemi-compatibility will be crucial in mass production of commercial devices.

More recent studies have demonstrated a clear focus on the functional integration of analytical components within monolithic devices. This is particularly important for DNA analysis where a large proportion of future markets will be in the areas of medical diagnostics, environmental monitoring and process control. However, it is certain that future work in this area will need to address integration in a more general sense, and solve problems associated with materials compatibility and, more importantly, the handling of real-world samples.[34]

Since its first report PCR has attracted almost endless interest. Most scientists, on seeing the mechanism for the first time, remark on its magnificent simplicity, and indeed it is this simplicity that has enabled PCR to infiltrate many diverse fields within the biological sciences. The recent advent of miniaturized PCR instruments has without doubt extended the efficiency of the basic technique but,

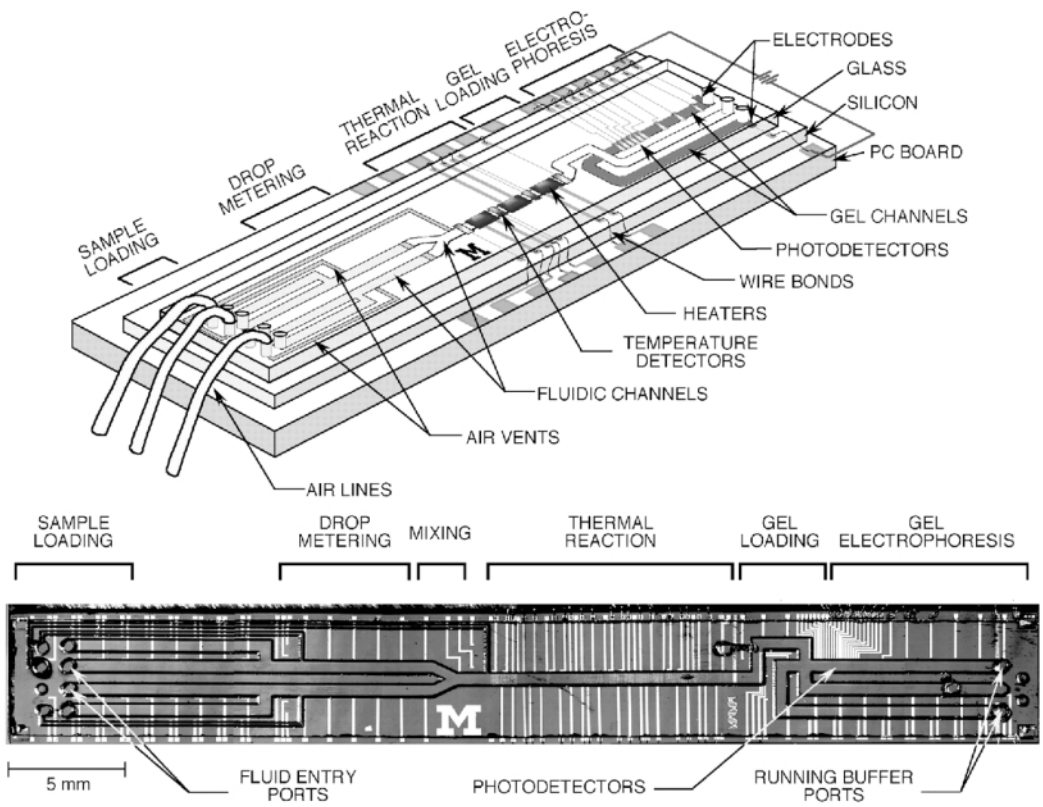

Fig. 6 Schematic of an integrated nanolitre DNA microdevice incorporating fluidic channels, heaters, temperature sensors and detectors. Reprinted with permission from *Science (Washington, D. C.)*, 1998, **282**, 484. Copyright 1998 American Association for the Advancement of Science.

more importantly, the potential applications of this unique assay.

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