Application – Microfluidic Biochemical Analysis Devices (I)

Date: 2013/06/07

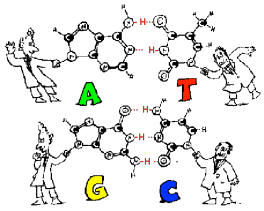
Dr. Yi-Chung Tung

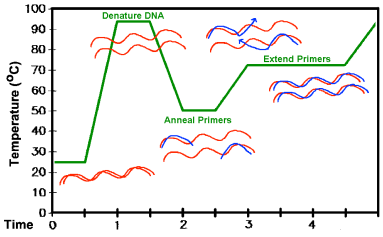
Polymerase Chain Reaction (PCR)

- The **polymerase chain reaction** (**PCR**) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.
- Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.
- In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

Polymerase Chain Reaction (PCR)

- The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.
- **Primers** (short DNA fragments) containing sequences complementary to the target region along with a **DNA polymerase** (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.



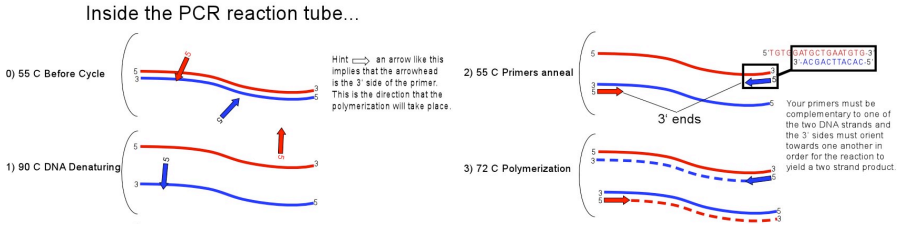


Polymerase Chain Reaction (PCR)

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing step: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme.



Polymerase Chain Reaction (PCR)

- Thermal Cycler.

Continuous Flow PCR

A continuous-flow PCR system can be realized by a time-space conversion in the PCR system—that is, by keeping temperatures constant over time at different locations in the system and moving the sample through the individual temperature zones.

REPORTS

Chemical Amplification: Continuous-Flow PCR on a Chip

Martin U. Kopp, Andrew J. de Mello, Andreas Manz*

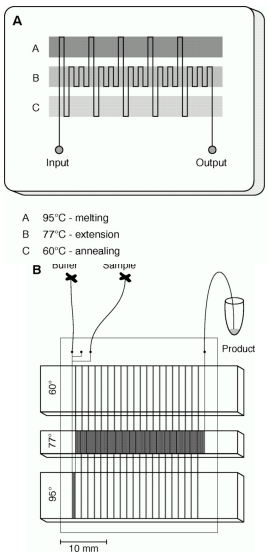
A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is continuous, and amplification is independent of input concentration. A 20-cycle PCR amplification of a 176–base pair fragment from the DNA gyrase gene of *Neisseria gonorrhoeae* was performed at various flow rates, resulting in total reaction times of 90 seconds to 18.7 minutes.

Electronic amplifiers allow weak signals to be increased by a large constant factor with the same time dependency and virtually no capillary, or slide containing the reagent mixture. The product is then analyzed by an endpoint measurement or directly used for performing PCR in a continuous flow at high speed. The results demonstrate the concept of a chemical amplifier for DNA.

The speed of thermal cycling is usually instrument limited, except for a commercial system that uses an air stream to heat and cool sealed glass capillaries containing the PCR mixture; this system has demonstrated high thermal cycling speeds and efficient amplification (*2*). More recently, several groups have reported high cycling speeds for PCR and the ligase chain reaction (LCR) with various designs of micromachined heating chambers (*3*, *4*). Micromachining can be defined as the patterning of silicon and its derivatives to create three-dimensional microstructures. A wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years (*5*).

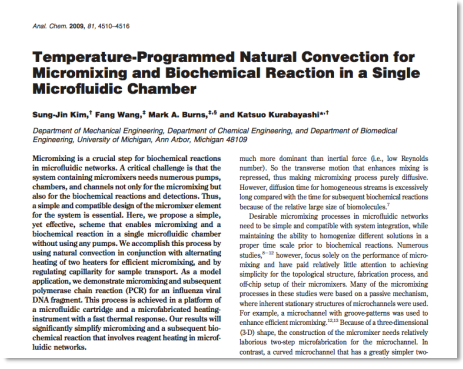
Polymerase Chain Reaction (PCR)

Chip layout. (A) Schematic of a chip for flow-through PCR. Three well-defined zones are kept at 95°, 77°, and 60°C by means of thermostated copper blocks. The sample is hydrostatically pumped through a single channel etched into the glass chip. The channel passing through the three temperature zones defines the thermal cycling process. (B) Layout of the device used in this study. The device has three inlets on the left side of the device and one outlet to the right. Only two inlets are used: one carrying the sample, the other bringing a constant buffer flow. The whole chip incorporates 20 identical cycles, except that the first one includes a threefold increase in DNA melting time.



Polymerase Chain Reaction (PCR)

This paper presents how temperature-programmed natural convection leads to significant mixing in the microfluidic regime. Most important, this study enables micromixing and a subsequent biochemical reaction in a single microfluidic chamber, within a simple pumpless platform. As a model application, we sequentially show micromixing and polymerase chain reaction (PCR) in a single chamber, while maintaining a fast thermal response.



Anal. Chem. **2009**, *81*, 4510–4516

Temperature-Programmed Natural Convection for Micromixing and Biochemical Reaction in a Single Microfluidic Chamber

Sung-Jin Kim,[†] Fang Wang,[‡] Mark A. Burns,[‡,§] and Katsuo Kurabayashi*[,†]

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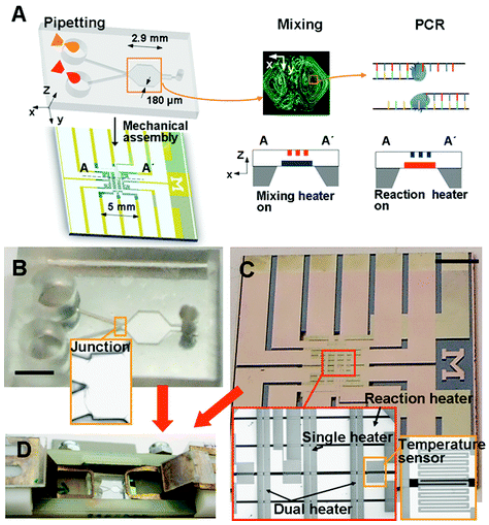
Micromixing is a crucial step for biochemical reactions in microfluidic networks. A critical challenge is that the system containing micromixers needs numerous pumps, chambers, and channels not only for the micromixing but also for the biochemical reactions and detections. Thus, a simple and compatible design of the micromixer element for the system is essential. Here, we propose a simple, yet effective, scheme that enables micromixing and a biochemical reaction in a single microfluidic chamber without using any pumps. We accomplish this process by using natural convection in conjunction with alternating heating of two heaters for efficient micromixing, and by regulating capillarity for sample transport. As a model application, we demonstrate micromixing and subsequent polymerase chain reaction (PCR) for an influenza viral DNA fragment. This process is achieved in a platform of a microfluidic cartridge and a microfabricated heating-instrument with a fast thermal response. Our results will significantly simplify micromixing and a subsequent biochemical reaction that involves reagent heating in microfluidic networks.

much more dominant than inertial force (i.e., low Reynolds number). So the transverse motion that enhances mixing is repressed, thus making micromixing process purely diffusive. However, diffusion time for homogeneous streams is excessively long compared with the time for subsequent biochemical reactions because of the relative large size of biomolecules.[1]

Desirable micromixing processes in microfluidic networks need to be simple and compatible with system integration, while maintaining the ability to homogenize different solutions in a proper time scale prior to biochemical reactions. Numerous studies,[2-10] however, focus solely on the performance of micromixing and have paid relatively little attention to achieving simplicity for the topological structure, fabrication process, and off-chip setup of their micromixers. Many of the micromixing processes in these studies were based on a passive mechanism, where inherent stationary structures of microchannels were used. For example, a microchannel with groove-patterns was used to enhance efficient micromixing.[11,12] Because of a three-dimensional (3-D) shape, the construction of the micromixer needs relatively laborious two-step microfabrication for the microchannel. In contrast, a curved microchannel that has a greatly simpler two-

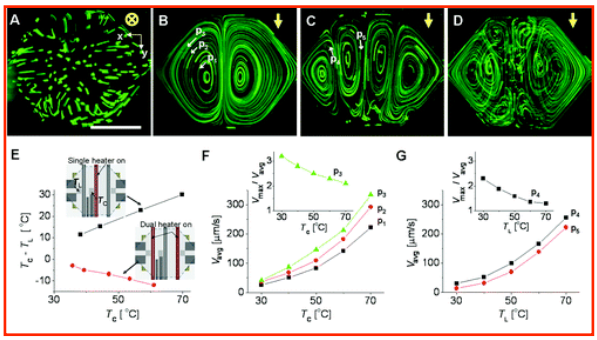
Polymerase Chain Reaction (PCR)

Single-chamber micromixing and PCR. (A) Sequential process of pipetting, micromixing, and PCR. After injecting solutions, their streams fill the chamber by capillarity. Upper- and lower-level heaters embedded in a membrane are used for micromixing and PCR, respectively. Natural convection induces micromixing. (B) Microfluidic cartridge. The microfluidic cartridge is a disposable component, which has simple 2-D shape microchannels and a chamber where the height is 180 μm. Sample transport is driven by capillarity in the microfluidic cartridge. The PDMS cartridge slab is coated with parylene to prevent evaporation of solutions. A stainless-steel film of 8 μm thickness is used as the sealing membrane at the bottom of the chamber. (C) Microfabricated heating-instrument. The microfabricated heating-instrument is a component for repeated use that incorporates temperature sensors and heaters within the membrane. The single and dual heaters are for micromixing, and the reaction heater is for PCR. (D) Assembly of heating instrument and microfluidic cartridge components. Scale bars, 3 mm.



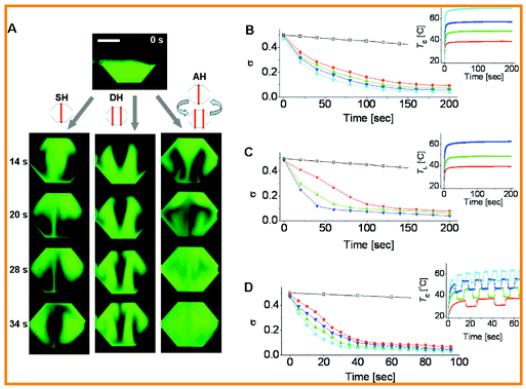
Polymerase Chain Reaction (PCR)

Natural convection-driven flows in the chamber. (A to D) Flow trajectories of fluorescent microparticles taken for 35 s. Measured maximum temperatures, Tmax, in A to D are 52, 51, 46, and 50 °C, respectively. The yellow arrows indicate the gravity direction, and the white arrows depict the flow direction of the individual fluorescent particles of 8 μm diameter. The single heater is turned on in A and B, and the dual heater is turned on in C. The single and dual heaters are alternatively turned on and off in D. To see the vertical image of B to D, we used a 45° inclined mirror. (E) Temperature gradient in the chamber. TC and TL are the fluid temperatures measured at the temperature sensors of the center and the left of the chamber-region, respectively. (F and G) Flow speed of the fluorescent microparticles in B and C. The single heater is turned on in F, and the dual heater is turned on in G. V_{avg} is the flow speed averaged over the individual microparticles in a single loop, whereas V_{max} is the maximum flow speed for the same loop. The height of the chamber is 180 μm. Scale bar, 1 mm.



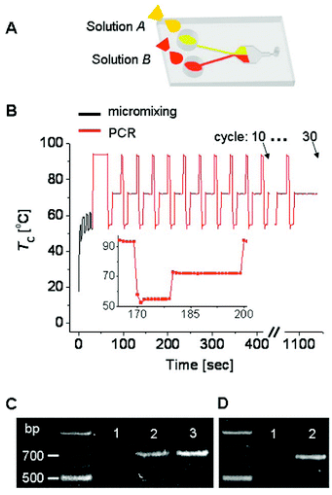
Polymerase Chain Reaction (PCR)

Micromixing performance in different heating modes. (A) Time evolution of micromixing in the single heating (SH), dual heating (DH), and alternating heating (AH) modes. The values of Tmax are 57, 48, and 56 °C in the SH, DH, and AH modes, respectively. (B to D) Time variation of the standard deviation, σ, from the measurement of fluorescent intensity. Shown are data for the SH mode in B, the DH mode in C, and the AH mode in D. The unmixed and perfectly mixed cases are set by σ = 0.5 and σ = 0, respectively. The insets show the fluid temperatures measured using the sensors in Figure 2E (TL or TC). Their line colors correspond to those in the σ vs time plots. For comparison, data for micromixing purely due to diffusion are shown with the points. Scale bar, 1 mm.



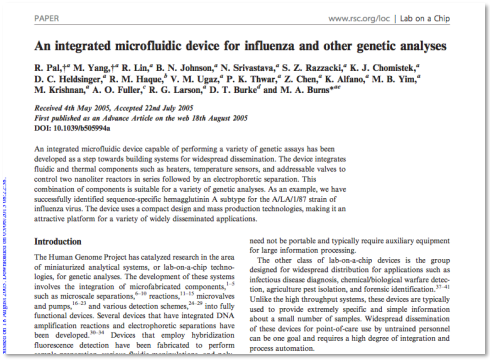
Polymerase Chain Reaction (PCR)

Natural convection-driven micromixing and PCR in a single microfluidic chamber. (A) Pumpless sample transport by capillarity. Solution A is a DNA template and solution B is a reaction mixture of primer, enzyme, and dNTPs. (B) Time sequence of heating used for the AH mode micromixing and the PCR process. The inset shows a single heat cycle of the PCR process with a time interval of 1 s between the adjacent data points. PCR-based amplification of a DNA fragment from the influenza viral strain A/LA/1/87 is performed for 10, 20, and 30 cycles. (C) Influence of PCR cycles. Lanes 1, 2, and 3 correspond to the amplified PCR products after the microfluidic mixing and the subsequent 10, 20, and 30 PCR cycles, respectively. (D) Control experiment showing improvement of PCR by microfluidic mixing. The comparison between PCR products after 20 cycles without (lane 1) and with (lane 2) a microfluidic mixing process in the AH mode clearly shows the mixing effect.



Polymerase Chain Reaction (PCR)

An integrated microfluidic device capable of performing a variety of genetic assays has been developed as a step towards building systems for widespread dissemination. The device integrates fluidic and thermal components such as heaters, temperature sensors, and addressable valves to control two nanoliter reactors in series followed by an electrophoretic separation. This combination of components is suitable for a variety of genetic analyses.



PAPER www.rsc.org/loc | Lab on a Chip

An integrated microfluidic device for influenza and other genetic analyses

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An integrated microfluidic device capable of performing a variety of genetic assays has been developed as a step towards building systems for widespread dissemination. The device integrates fluidic and thermal components such as heaters, temperature sensors, and addressable valves to control two nanoliter reactors in series followed by an electrophoretic separation. This combination of components is suitable for a variety of genetic analyses. As an example, we have successfully identified sequence-specific hemagglutinin A subtype for the A/LA/1/87 strain of influenza virus. The device uses a compact design and mass production technologies, making it an attractive platform for a variety of widely disseminated applications.

Introduction

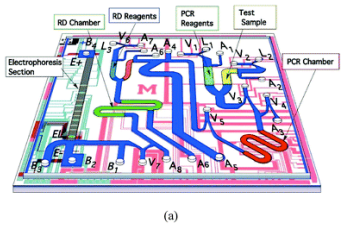
The Human Genome Project has catalyzed research in the area of miniaturized analytical systems, or lab-on-a-chip technologies, for genetic analyses. The development of these systems involves the integration of microfabricated components,[1–7] such as microscale separations,[8–10] reactions,[11–13] microvalves and pumps,[14–22] and various detection schemes,[24–26] into fully functional devices. Several devices that have integrated DNA amplification reactions and electrophoretic separations have been developed.[30–34] Devices that employ hybridization fluorescence detection have been fabricated to perform

need not be portable and typically require auxiliary equipment for large information processing.

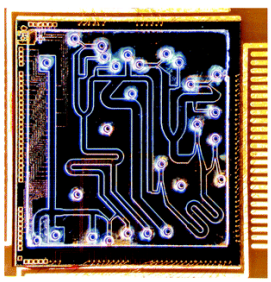
The other class of lab-on-a-chip devices is the group designed for widespread distribution for applications such as infectious disease diagnosis, chemical/biological warfare detection, agriculture pest isolation, and forensic identification.[37–41] Unlike the high throughput systems, these devices are typically used to provide extremely specific and simple information about a small number of samples. Widespread dissemination of these devices for point-of-care use by untrained personnel can be one goal and requires a high degree of integration and process automation.

Polymerase Chain Reaction (PCR)

Schematic representation of an integrated microfluidic device. There are three liquid entry channels (“L”, sample, PCR reagents and RD reagents), several metering channels, drop mixing intersections, a sealed PCR chamber, an open RD chamber, and an electrophoresis channel. Each valve (“V”) is individually and electronically addressable. (b) Photograph of an assembled device (1.5 cm by 1.6 cm). The discrete liquid drops controlled in this device are 100 to 240 nl, with fluidic channel dimensions of 200–600 μm wide and 50 μm deep. The electrodes and diodes shown in the figure were not used in this work.



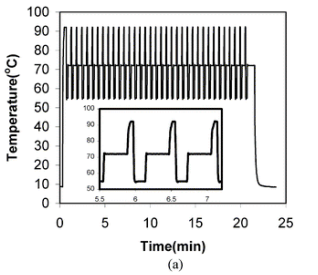
(a)

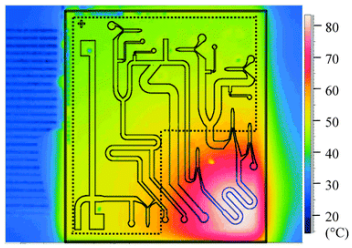


(b)

Polymerase Chain Reaction (PCR)

Thermal control of the integrated device. (a) Temperature profile from the on-chip temperature sensors for 35 cycles controlled by on-chip heaters. The insert shows a blowup of three cycles. (b) Thermal image taken by an infrared camera of a device showing the temperature profile during a PCR thermocycling. The PCR reaction chamber area is heated to 90 °C, while the opposite corner of device remains below 40 °C. The channel layout is overlaid on top of the device to show the position of each component. The dotted line indicates where the underlying PCB has been replaced with a thermally conductive grease.





(b)

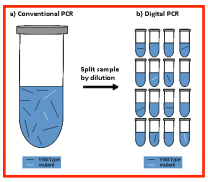
Digital PCR (dPCR)

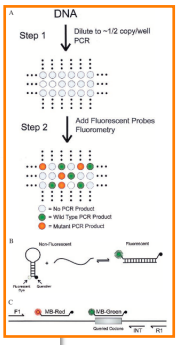
- **Digital polymerase chain reaction** (digital PCR, DigitalPCR, dPCR, or dePCR) is a refinement of conventional PCR methods that can be used to directly quantify and clonally amplify nucleic acids including DNA, cDNA or RNA. The key difference between dPCR and traditional PCR lies in the method of measuring nucleic acids amounts, with the former being a more precise method than PCR. PCR carries out one reaction per single sample. dPCR also carries out a single reaction within a sample, however the sample is separated into a large number of partitions and the reaction is carried out in each partition individually. This separation allows a more reliable collection and sensitive measurement of nucleic acid amounts. The method has been demonstrated as useful for studying variations in gene sequences — such as copy number variants and point mutations — and it is routinely used for clonal amplification of samples for “next-generation sequencing.”

Digital PCR (dPCR)

A sample is partitioned so that individual nucleic acid molecules within the sample are localized and concentrated within many separate regions. (The capture or isolation of individual nucleic acid molecules has been effected in micro well plates, capillaries, the dispersed phase of an emulsion, and arrays of miniaturized chambers, as well as on nucleic acid binding surfaces.) The partitioning of the sample allows one to estimate the number of different molecules by assuming that the molecule population follows the Poisson distribution. As a result, each part will contain "0" or "1" molecules, or a negative or positive reaction, respectively. After PCR amplification, nucleic acids may be quantified by counting the regions that contain PCR end-product, positive reactions. In conventional PCR, the number of PCR amplification cycles is proportional to the starting copy number. dPCR, however, is not dependent on the number of amplification cycles to determine the initial sample amount, eliminating the reliance on uncertain exponential data to quantify target nucleic acids and therefore provides absolute quantification.

Digital PCR (dPCR)





Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9236–9241, August 1999
Genetics

Digital PCR

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The Howard Hughes Medical Institute and the Johns Hopkins Oncology Center, Baltimore, MD 21231

Contributed by Bert Vogelstein, June 9, 1999

ABSTRACT **The identification of predefined mutations expected to be present in a minor fraction of a cell population is important for a variety of basic research and clinical applications. Here, we describe an approach for transforming the exponential, analog nature of the PCR into a linear, digital signal suitable for this purpose. Single molecules are isolated by dilution and individually amplified by PCR; each product is then analyzed separately for the presence of mutations by using fluorescent probes. The feasibility of the approach is demonstrated through the detection of a mutant *ras* oncogene in the stool of patients with colorectal cancer. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.**

We therefore sought to develop an approach to the problem that would overcome some of the aforementioned difficulties. The strategy described in this paper involves separately amplifying individual template molecules so that the resultant PCR products are completely mutant or completely WT. The homogeneity of these PCR products makes them easy to distinguish with existing techniques. Such separate amplifications are only useful in a practical sense, however, if a large number of them can be assessed simply and reliably. Techniques for such assessments were developed, with the output providing a digital readout of the fraction of mutant alleles in the analyzed population. A variety of applications for this technology are foreseeable.

MATERIALS AND METHODS

Digital PCR (dPCR)

Microfluidic devices allow control and manipulation of small volumes of liquid, in this case allowing for rapid separation and partitioning of single cells from a complex parent sample.

REPORTS

Microfluidic Digital PCR Enables Multigene Analysis of Individual Environmental Bacteria

Elizabeth A. Ottesen,[1] Jong Wook Hong,[2] Stephen R. Quake,[3] Jared R. Leadbetter[4]*

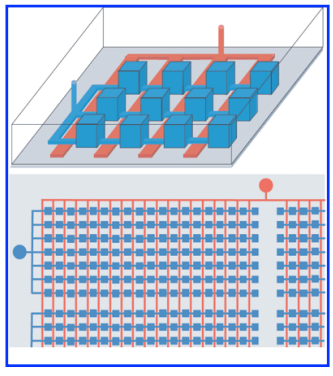
Gene inventory and metagenomic techniques have allowed rapid exploration of bacterial diversity and the potential physiologies present within microbial communities. However, it remains nontrivial to discover the identities of environmental bacteria carrying two or more genes of interest. We have used microfluidic digital polymerase chain reaction (PCR) to amplify and analyze multiple, different genes obtained from single bacterial cells harvested from nature. A gene encoding a key enzyme involved in the mutualistic symbiosis occurring between termites and their gut microbiota was used as an experimental hook to discover the previously unknown ribosomal RNA–based species identity of several symbionts. The ability to systematically identify bacteria carrying a particular gene and to link any two or more genes of interest to single species residing in complex ecosystems opens up new opportunities for research on the environment.

ly, because microfluidic digital PCR yields fluorescent signal upon amplification of a gene regardless of the number of copies present in the cell, this approach can yield estimates of the fraction represented by a given species within the general microbial community. The number of *rrn* operons present in a genome can vary widely, ranging from 1 [e.g., *Rickettsia prowazekii* (*19*)] to 15 [e.g., *Clostridium paradoxum* (*20*)], confounding the interpretation of traditional environmental gene inventories. Moreover, the use of single-cell PCR to prepare clone libraries avoids complications and PCR artifacts such as amplification biases and unresolvable chimeric products (*21*).

We used this technique to examine a complex, species-rich environment: the lignocellulose-decomposing microbial community resident in the hindguts of wood-feeding termites. Therein, the bacterial metabolism known as CO_2-reductive homoacetogenesis is one of the major sources

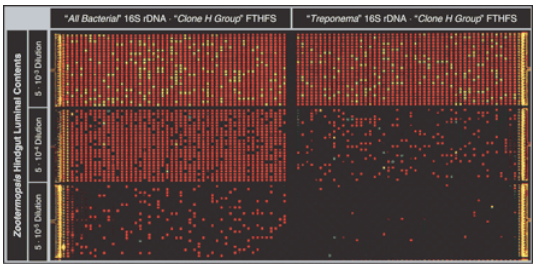
Digital PCR (dPCR)

Microfluidic digital PCR chip. Top: Schematic diagram showing many parallel chambers (blue) connected by channels to a single input. When pneumatic or hydraulic pressure is applied to the control channel network (red), the membranes between the red and blue channels are deflected upward, creating micromechanical valves. When the valves are closed, the continuous blue network is partitioned into independent PCR reactors. Bottom: Schematic showing how a single valve connection can be used to partition thousands of chambers. In the device used, each experimental sample could be partitioned into 1176 chambers, and each device contained 12 such sample panels.



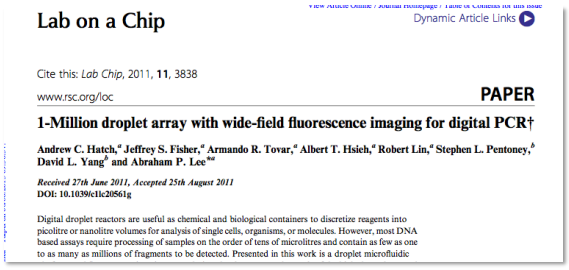
Digital PCR (dPCR)

Multiplex microfluidic digital PCR of single cells in environmental samples. Six panels from a representative experiment show microfluidic digital PCR on diluted hindgut contents harvested from a single Z. nevadensis individual. Left: Multiplex PCR using “all-bacterial” 16S rRNA gene (red fluorescence) and “clone H group” FTHFS gene (green fluorescence) primers and probes. Reaction chambers that contained both genes in 1/500,000 dilutions from this and other on-chip experiments were sampled and the PCR products were analyzed. Right: The same, except that 16S rRNA primers specifically targeted members of the “termite cluster” of the spirochetal genus Treponema.



1-Million droplet array with wide-field fluorescence imaging for dPCR

Mmost DNA based assays require processing of samples on the order of tens of microlitres and contain as few as one to as many as millions of fragments to be detected. Presented in this work is a droplet microfluidic platform and fluorescence imaging setup designed to better meet the needs of the high-throughput and high-dynamic-range by integrating multiple high-throughput droplet processing schemes on the chip. The design is capable of generating over 1-million, monodisperse, 50 picolitre droplets in 2–7 minutes that then self-assemble into high density 3-dimensional sphere packing configurations in a large viewing chamber for visualization and analysis.



Lab on a Chip

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PAPER

1-Million droplet array with wide-field fluorescence imaging for digital PCR†

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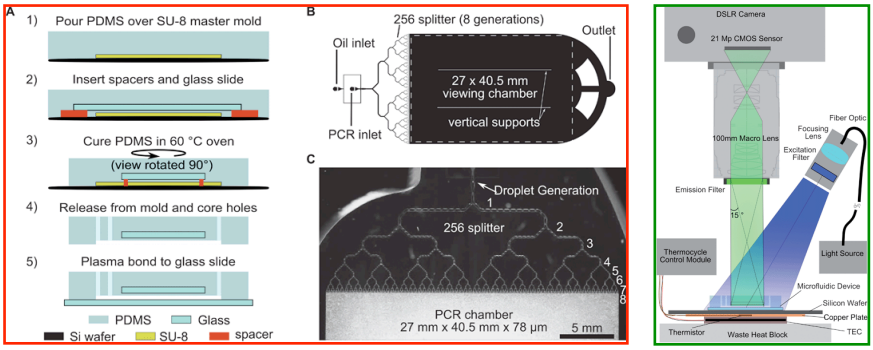
Digital droplet reactors are useful as chemical and biological containers to discretize reagents into picolitre or nanolitre volumes for analysis of single cells, organisms, or molecules. However, most DNA based assays require processing of samples on the order of tens of microlitres and contain as few as one to as many as millions of fragments to be detected. Presented in this work is a droplet microfluidic

1-Million droplet array with wide-field fluorescence imaging for dPCR

Schematic illustration and images of the PCR microdevice: (A) fabrication process, (B) mask design of the droplet array with sample inlets, droplet generator, 256 splitter, viewing chamber and outlet. (C) The picture of droplet generation and 256 splitter filling the 27 mm × 40.5 mm viewing chamber.



1-Million droplet array with wide-field fluorescence imaging for dPCR

Macro-fluorescence image of 1-million PCR amplified droplets in a 27 mm by 40.5 mm by 78–80 µm droplet chamber (highlighted by dashed white line) imaged on a 21-megapixel dSLR camera. The inset is an enlarged section for enhanced visualization.