Single-Molecule DNA Amplification and Analysis Using Microfluidics

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1. Introduction

The broad availability of amplified DNA molecules enabled the development of many powerful applications in molecular biology techniques. Currently, a variety of DNA amplification techniques have been developed since the mid-1980s, including widely used polymerase chain reaction (PCR),[1] multiple displacement amplification (MDA),[2] rolling circle amplification (RCA),[3] and nucleic acid sequence-based amplification (NASBA).[4] Additionally, other PCR variants are rapidly being developed as the emerging amplification techniques, such as digital PCR[5] and linear-after-the-exponential (LATE)-PCR.[6,7] Among these techniques, PCR has achieved the most widespread research applications, most likely due to its simplicity and cost-effectiveness. In principle, PCR can amplify a single DNA molecule due to its highly efficient exponential amplification (i.e., single-molecule PCR).[8−11] Since its discovery, single-molecule PCR has offered some attractive benefits in the analysis of somatic mutations such as reducing PCR error rate, minimizing template jumping, and neutralizing allelic preference.[12] However, conventional single-molecule PCR often requires critical parameters to be accurately optimized to maximize product yield and minimize false incorporations and, also, reduce the formation of primer dimers or mispriming.[9,11] Moreover, most macroscale single-molecule PCR reactions rely on a thermoelectrically heated metal block holding plastic tubes containing 50 and even 100 μL of PCR mixture.[11] This setup results in a high heat mass, and the PCR run time (typically 1−3 h) is limited by the low heating and cooling rates. In addition, the conventional single-molecule PCR is prone to a high possibility of contamination due to indispensable manual operations, and therefore the proper measures are usually taken to prevent contamination.[9,11]

With the advent of microfluidic technology,[13−20] single-molecule DNA amplification using microfluidics becomes

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possible, and microfluidic technologies have even allowed individual molecules to be manipulated and measured.[13,21−25] However, the visualization and manipulation of single DNA molecules without PCR amplification often requires expensive, complicated, and sophisticated optical analysis systems with high degrees of control of the DNA and capabilities of high-resolution detection,[25] which are not commonly found in a molecular biological laboratory. Moreover, it is unclear how easily these equipments can be scaled. Microfluidics-based single-molecule DNA amplification and analysis offers several advantages over its conventional counterpart. For example, small volumes reduce the time taken to amplify and analyze individual molecules,[26] the unique behavior of reaction liquids at the microscale level allows more precise control of initial molecule concentrations,[27,28] reagent costs and the amount of chemical waste can be reduced, and high throughput allows tens of thousands of individual DNA molecules to be amplified and analyzed,[29−32] and therefore more genetic information can be obtained. Highly integrated devices also allow individual DNA molecules to be amplified and analyzed automatically,[33] and as a result artificial contamination can be reduced, as is critical for successful single-molecule DNA amplification. The development of PCR microfluidic devices has been discussed in recent reviews.[34−40] In this review, instead, we limit our discussion to those experiments in which single-molecule DNA amplification and analysis has been studied within the confines of microfluidic reactors, and most of the reference citations have been published since 2001.

2. Rationale for Single-Molecule DNA Amplification Using Microfluidics

2.1. Why Are Small-Scale Reactors Suitable for Single-Molecule DNA Amplification?

In general, a PCR reaction saturates at about10^{11} product molecules/μL, due in part to rapid reannealing of product strands.[26] With 100% amplification efficiency and under end-point PCR conditions, to reach this product concentration after 30 cycles in a standard 25-μL PCR requires 2.5×10^3 starting target molecules (i.e., $(2.5 \times 10^3 \times 2^{30})/25\ \mu\text{L} = 10^{11}/\mu\text{L}$).[26] Somewhat less than this number of starting molecules can be detected by increasing the number of cycles, and under special conditions even single starting molecules can be detectable,[8−10] but this strategy often fails before getting to the limit of detecting single molecules due to the competition between the desired PCR fragments and spurious PCR products such as primer dimers and the higher possibility of contamination than in multicopy PCR.[12] However, if the PCR volume is reduced to the nanoliter (nL) and even picoliter (pL) level (e.g., 10 nL $= 10 \times 10^{-3}\ \mu$L), then a single target molecule might suffice to produce a saturating concentration of PCR product after ~30 cycles (i.e., $(1 \times 2^{30})/(10 \times 10^{-3}\ \mu\text{L}) = 10^{11}/\mu\text{L}$).[26] Kalinina et al. have shown that volume reductions would allow detection of PCR products generated from single DNA molecules using the TaqMan assay.[26] Subsequently, Li et al. also showed that the sensitivity of laser-induced fluorescence (LIF) could be improved to enable detection of single DNA molecules if reaction volumes were reduced, and as a result, single-molecule DNA amplification could be realized with one-step PCR and one primer pair.[41] In brief, therefore, the advantages of small-scale reactors for single-molecule DNA amplification are compelling, although designing and making these reactors that operate effectively is challenging.

2.2. Poisson Statistics

As the number of target molecules for the PCR reaction is reduced to the single-molecule level, stochastic amplification effect begins to appear.[33] The resulting distribution of amplification event frequencies and amplitudes should conform to a Poisson distribution. Poisson statistics is often used to describe the distribution of DNA or RNA templates in reactors including microreactors etched on a chip and droplet reactors in water-in-oil (w/o) emulsions. This implies that all reactors are of equal volume. The probability that a

Table 1. Materials and Fabrication of Chip-Based Microreactors for Single-Molecule DNA Amplification

substrate material	microfabrication technology			refs
silicon	reactive ion etching (RIE)			44−46
	photolithography			30, 32, 45, 47−49
	anisotropic etching			32, 47, 49
	low-pressure chemical vapor deposition (LPCVD)			45
glass	lithography	photolithography		33, 50−52
	etching	chemical vapor deposition (CVD)	LPCVD	33, 50
			plasma-enhanced chemical vapor deposition (PECVD)	50
		wet chemical etching		33, 50
		RIE		33, 51, 52
		isotropical etching		53, 54
quartz	photolithography and wet chemical etching			55
	a			56
polydimethylsiloxane (PDMS)	soft lithography			42, 57−59
	multilayer soft lithography (MSL)			27, 28, 60
polycarbonate (PC)	patterning using the knife plotter (xurography)			61
epoxy resin (SU-8)	*a*			62
poly(cyclic olefin) (PCO) resin	compression molding			63
polypropylene (PP)	*a*			64
aluminum	end mill process			65, 66
317 stainless steel	double-sided wet-etching and vapor deposition			31, 67−71

[a] Not available.

microreactor has 0, 1, 2, 3, or more DNA or RNA templates can be calculated by $f(n,\lambda) = (e^{-\lambda}\lambda^n)/n!$, where n is the number of DNA or RNA molecules per reactor and λ is the ratio between the number of DNA or RNA templates and the number of reactors on chips or in the emulsions (i.e., the average number of template molecules per reactor before amplification).[42,43] At low λ (<0.3), the large majority of reactors contain no more than a single DNA molecule, and fitting the number of PCR competent reactors to the Poisson equation allows the DNA concentration to be calculated.[42] The Poisson distribution can apply to explain the phenomenon typical of single-molecule PCR that once the starting sample molecules are diluted, decreasing starting molecule should only decrease the number of microreactors that support amplification.

3. Materials Used for Microreactor Construction of Single-Molecule DNA Amplification

The materials used for on-chip reactor construction are important for their success. Several materials have found utility in a variety of microfluidic reactors for single-molecule DNA amplification (Table 1). Ideal materials should be mechanically rigid, optically transparent with low autofluorescence, resistant to the sustained high temperatures associated with PCR, compatible with licensed PCR reagents, and impervious to organic solvents. Such materials can offer a stable microfluidic environment for an extended period of time suitable for on-chip sample preparation, single-molecule amplification, and optical detection of product. Polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), and other polymers have been widely used in microfluidic reactor construction and for on-chip PCR amplification and analysis.[38,40] The versatility of PDMS makes fabrication of various numbers of channels and chambers simple and reduces the time, complexity, and cost of fabricating reaction chambers or channels. The combination of PDMS with glass makes a hybrid microfluidic design suitable for amplification and detection of single DNA molecules.[33,50−52,54,72,73] Unfortunately, PDMS is not fully compatible with organic solvents. For example, emulsion oils, which are widely used to form the emulsion microdroplets for single-molecule DNA amplification (discussed below), will swell PDMS on contact, thus rendering the microfluidic device unusable.[72] In addition, the permeability of PDMS can result in diffusion loss of biological sample, and the surface properties of PDMS can be difficult to control. Compared with polymers, glass has considerably lower autofluorescence, making it ideal for fluorescence microscopy detection. Its electro-osmotic-flow (EOF) property allows the integration of single-molecule PCR and microscale capillary electrophoresis (CE) on a single chip[33,50−52,54,73] Glass is mechanically rigid and resistant to organic and inorganic solvents, and microchambers or -channels constructed of glass can be rigorously cleaned and used repeatedly for multiple single-molecule runs.[51,52,73]

4. On-Chip Microreactors for Single-Molecule DNA Amplification

4.1. Chamber Microreactors for Stationary Single-Molecule DNA Amplification

Microreactors etched on chips using a variety of fabrication techniques[38,40,74] have been well applied to amplify single DNA molecules. These reactors are formed on chips in two different formats: single chamber and multiple chamber (Table 2). Using microfluidic chip-based single-chamber stationary PCR and CE detection of amplification product, Kaigala et al. have successfully assessed risk of BK virus (BKV)-associated nephropathy in renal transplant recipients, with the limit of detection (LOD) as few as one to two viral copies (Figure 1).[57] The single-chamber stationary chips operate effectively in terms of fluidic and thermal controls and offer reduced thermal and fluidic crosstalk between chambers. However, they are not very suitable for applications of single-molecule DNA amplification and analysis, in part because they generally cannot realize high throughput and cannot readily be used for special purposes such as gene expression analysis at the single molecule level[94] and high-throughput genome sequencing.[72,95−104] Recently, Dettloff et al. have developed a microfluidic device that enabled rapid PCR of individual DNA molecules within eight parallel channels.[55] The long narrow PCR channels allowed sufficient separation between neighboring DNA template molecules to enable amplification of discrete DNA molecules. As a result, amplification throughput within a single reaction channel was desirably increased.

Table 2. On-Chip Chamber Microreactors for Single-Molecule DNA Amplification and Analysis[a]

type of chamber	number of chamber (throughput)	features of chamber and its related description	refs
		Single Chamber	
silicon channel chamber	1	microchannel chamber (W × H = 300 × 48 μm^2 or 300 × 64 μm^2)	44
glass channel chamber	1	microchannel chamber (W × H = 150 × 30 μm^2)	53
PDMS chamber	1	PDMS holes (D =1.5 mm) with a maximum volume of ~3.5 μL	57, 59
PCO channel reactor	1	microchannel chamber (W × H = 360 × 50 μm^2), V = 29 nL	63
aluminum channel chamber	1	channel chamber (L × W × H = 35 × 3 × 4 mm^3)	65, 66
		Multiple-Chamber Array	
silicon chamber array	4	microchannel chamber (W × H = 1000 × 200 μm^2), V = 4 μL	46
	4/1	chamber in a diamond shape, V = 1 μL	47, 75
	10	chamber with hexagonal cross section, and tapered PP insert, V = ~20 μL	45
	1248	microchamber (L × W × H = 650 × 650 × 200 μm^3), V = ~50 nL	30, 48
	10000	microchamber with a reversed-pyramidal cross-section (400−2500 μm^2), V = 1.3 pL to 32 μL	32
	10000	microchamber with a reversed-pyramidal cross-section (80 × 80 μm^2), V = 85 pL	49
glass chamber array	4	microchamber with an approximately trapezoidal cross-section, V = 380 nL	52
	8	microchamber of 42 μm deep, V = 280 nL	33, 50, 51
	9	chambers with varying volume capacity of up to 6 μL. The chamber of ~0.7 mm deep gives a volume of V ≈ 3 μL	54
quartz chamber array	8	microchannel chamber (L × W × H = 40 mm × 180 μm × 10 μm), V = 72 nL	55
PDMS chamber array	9	V = 50 nL	27, 28
	72	each chip contains 8 flow channels with 9 microchambers, V = 450 pL	60
PC chamber array	1000	chambers with dimensions of 0.5 mm × 0.5 mm × 0.125 mm, V = 33 nL	61
PP chamber array	1024	chambers with 1125 μm interchamber distance (pitch), V = 500 nL	64
Fluidigm digital array	9180 (12.765 digital array chip)	the array chip has 12 panels, each containing 765 partitions. V = 6 nL	29, 76−88
	14112 (12.1176 array chip)	each panel has 1176 independent chambers, and each device contains 12 such panels. V = 6.25 nL	89
	2304 (48.48 dynamic array chip)	the 48.48 dynamic array chip enables 2304 individual reactions to be analyzed	85
	14400 (12.1200 array chip)	the array chip has 12 sample panels, and each panel contains 1200 isolated reaction chambers	90
BioTrove OpenArray	3072	the through-holes (D = 320 μm) are grouped in 48 subarrays of 64 holes each and spaced on a 4.5 mm pitch, V = 33 nL	31, 67−71, 91
chemically structured chips from Alopex GmbH	60	each chip contains 60 hydrophilic reaction compartments ("anchor spots," D = 1.6 mm) arranged in 12 rows of 5 spots, V = 1 μL	92, 93

[a] In this table, L, W, and H are the length, width, and height of each chamber in the array chip, respectively. And, D is the diameter of chamber, V is the reaction volume of chamber.

During the past decade, great efforts have been made to develop multiple stationary chamber chips for single-molecule DNA amplification applications. The amplification throughput of a single chip ranges from several to several thousands of reactors (Table 2). Early in 2004, Matsubara et al. reported the on-chip single-copy PCR amplification and analysis based on counting fluorescence released microchambers.[30] The chip feature was 1 in. × 3 in. and had 1248 chambers, each of which could accommodate ~50 nL of sample volume (Figure 2A). On the developed array chips, the quantitative analysis of initial DNA concentration has been performed by counting the number of microchambers with positive fluorescence signal versus the number of initial target copies in one chamber (Figure 2B,C).[30] In fact, this is the first multichamber chip-based digital PCR for single-molecule DNA amplification and analysis. The multichamber chip allows generation of standard curves, parallel use of multiple primer pairs or template numbers for different PCR,[30,48] and optimization of single-molecule PCR.[60] In addition, an array of real-time PCR can combine the parallelism of microarrays with the quantification, sensitivity, dynamic range, and specificity of quantitative PCR. As a result, it may become an attractive alternative for single-molecule DNA amplification and analysis. However, for ultrahigh-throughput chips, two challenges need to be overcome. The first is creation of a simple macro-to-micro interface[105] for precise and accurate transfer of reaction liquids from the macroscale systems to the microscale reactors. The second is achieving the accuracy, precision, and sensitivity demanded by conventional PCR but in a substantially reduced reaction volume.[31] At present, several facile methods for rapid world-to-chip handling and processing of liquids have been developed, for example, noncontact piezo/valve dispersing,[64] nanoliter dispenser coupled to

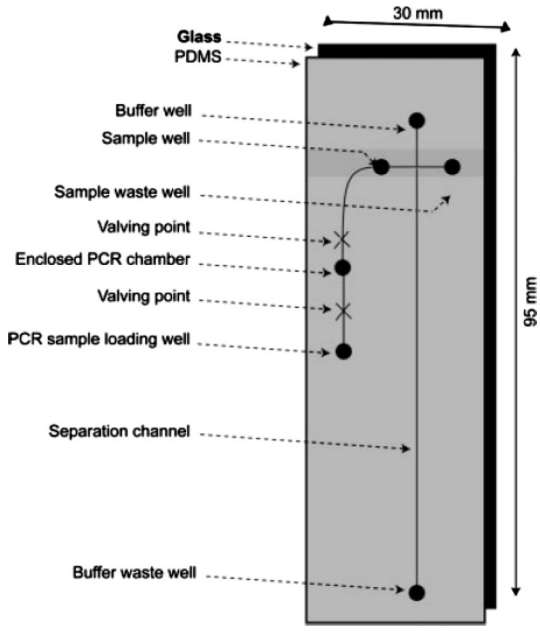

Figure 1. Integrated PCR-CE chip with a single-chamber microreactor (length = 95 mm, width = 30 mm, height = 2.3 mm). The chip is comprised of a layer of glass irreversibly bonded to a layer of PDMS with features for fluidic flow, single-chamber PCR, and holes within them. Reprinted with permission from ref 57. Copyright 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

chemically constructed chips,[30,48] integrated capillary sampling,[55] chip-to-chip nanoliter dispenser using a PDMS−silicon hybrid microfluidic dispenser and a glass microwell array chip,[106] and on-chip microvalving, micropumping, and micromixing.[39]

It is noteworthy that the commercially available array chips have been well applied for high-throughput single-molecule genetic amplification and analysis, such as the BioTrove OpenArray, the Fluidigm digital array, and the chemically structured chips from Alopex GmbH, which were originally developed for single cell analysis and quantification of single genome equivalents (Table 2). For the OpenArray platform, the holes act as capillaries, accurately self-metering reaction mixture added by an automated loader. Hydrophilic inner through-holes and hydrophobic outer-surface coating ensure that the mixture remains isolated through surface tension. As a result, cross-contamination of samples is both theoretically and demonstratively eliminated on the array plates.[71] The workflow for performing quantitative PCR in the OpenArray plate has been described by Brenan and Morrison.[91] The integrated fluidic circuit (IFC)-based Fluidigm digital array is a novel nanofluidic chip in which digital PCR amplifications can be performed. By using nanoscale microvalves and pumps, each 12.765 Digital Array drives 12 reaction mixtures into 12 individual panels, each of which is compartmentalized into 765 6-nL reaction chambers. In addition, the array uses MSL and PDMS to produce nanoscale valves and pumps that can be used in serial or parallel format and is composed of a PDMS IFC, an integrated heat spreader to ensure rapid heat transfer and temperature uniformity within the array, and a Society of Biomolecular Screening (SBS)-formatted carrier with inputs and pressure accumulator to act as an interface between the user and the PDMS chip.[83] After loading and mixing of the reaction mixture, the chip is ready for thermal cycling and detection of the reaction products on the BioMark Real-Time

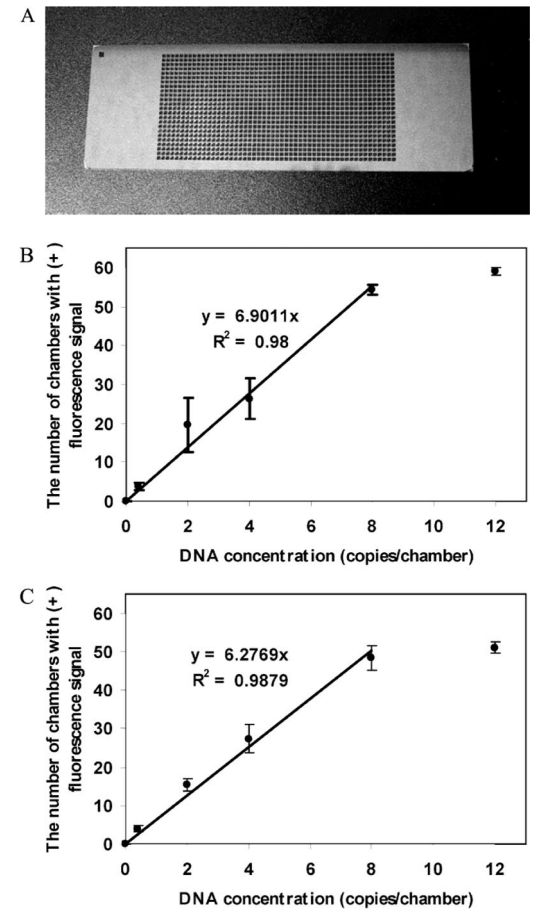

Figure 2. On-chip single-copy PCR by counting fluorescence released microchambers. (A) Photographic image of the silicon chip (1 in. × 3 in.) with 1248 microchambers. (B, C) Number of microchambers with positive fluorescence signal versus the number of initial target copies in one chamber for quantitative analysis ((B) sex-determining region Y (SRY) gene and (C) rhesus D (RhD) gene). Data were obtained from three consecutive experiments of microchamber array PCR, and six different areas of the microchamber array were used, each of which consisted of 60 microchambers. Reprinted with permission from ref 30. Copyright 2004 American Chemical Society.

PCR System (Fluidigm). In addition to the 12.765 digital array, the other chips from Fluidigm can also be used for single-molecule DNA amplification and analysis, such as the 12.1176,[89] 48.48,[85] and 12.1200[90] array chip. The Fluidigm digital array chips have some specific advantages or characteristics, such as single-molecule sensitivity, high precision and accuracy, high sample throughput, high number of targets per sample, large dynamic range, short sample preparation and turnaround time, low sample consumption and cost per data point, and simple surface passivation. In addition, these chips do not require the arduous pipetting steps due to integrated channels, chambers, and micropump and valves. However, it is worth noting that a commercial Teflon or fused-silica capillary has been used as the reaction vessel to perform the amplification and analysis of single molecules.[26,41,107,108]

4.2. Channel Microreactors for Dynamic Continuous-Flow Single-Molecule DNA Amplification

Up to now, great attention has focused on developing chamber microreactors for single-molecule DNA amplification, where a stationary reaction mixture in a confined space is alternately heated and cooled, similar to the processes in

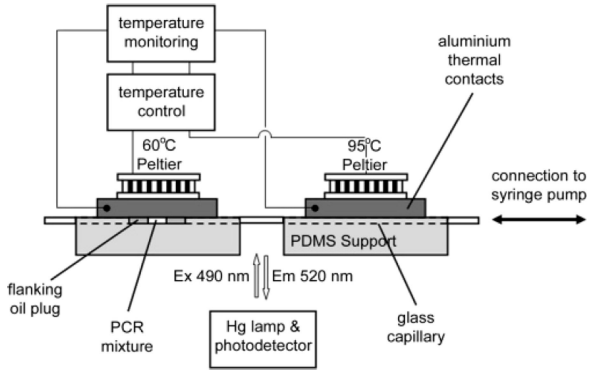

Figure 3. Oscillatory-flow real-time PCR thermocycling system for ultrasensitive detection from human genomic samples. A standard 1.0 mm i.d. glass capillary with a wall thickness of ~100 μm was used and cast within a sheet of PDMS. A 2 μL PCR volume plug that was flanked by the mineral oil was transported back and forth using a syringe pump. Reprinted with permission from ref 116. Copyright 2007 American Chemical Society.

conventional PCR instruments. However, the chamber stationary microreactors generally lack the flexibility to change the amplification speed. The dynamic continuous-flow PCR in channel microreactors can overcome this problem by using the "time−space conversion" concept.[108−115] In continuous-flow PCR, the temperature is kept constant over time at specific zones in the system, and the reaction mixture is moved between the individual temperature zones for cycling. This approach leads to relatively rapid heat transfer because it is not necessary to thermally cycle the entire heat masses associated with most chamber-type devices. The first continuous-flow PCR system for high-throughput and single-molecule DNA amplification was presented in 2003 by Baker and co-workers.[56] The device integrated reaction assembly, thermal cycling, and multi-channel fluorescence detection on a chip. In addition, the device used a slip-and-split design and allowed eight straight channel microreactors to amplify and analyze each sample in a parallel format. The oscillatory-flow approach presented here combines the advantages of both static and dynamic PCR systems, as may be important for dynamic continuous-flow single-molecule DNA amplification and analysis. A similar oscillatory PCR system has been developed by Chen and colleagues to perform ultrasensitive PCR and real-time detection, with a LOD of 24 human genome copies (Figure 3).[116] Recently, Kiss et al. and Schaerli et al. have used serpentine channel-based continuous-flow PCR for single-molecule DNA amplification in microfluidic monodisperse droplets.[58,62] The design of their devices allows droplets to flow through alternative temperature zones, avoiding temperature cycling of the entire device. Kiss et al. used flow focusing to generate monodisperse picoliter reaction droplets,[58] while the nanoliter droplets reported by Schaerli et al. were formed at a T-junction.[62] The channel microreactors reported by Kiss et al. and Schaerli et al. utilize the serpentine channel configuration, similar to the manners introduced by Kopp et al.[112] For serpentine channel microreactors for continuous-flow single-molecule PCR, parallelization is not easily obtained, most likely due to the complexity of chip architecture and to the increased chip footprint. In addition, the number of cycles is generally not adjustable: the number of meanders (or turns) is decided during the design procedure and cannot be modified once the chip is fabricated. This feature is disadvantageous for single-molecule DNA amplification. It is to be noted that a completely different design

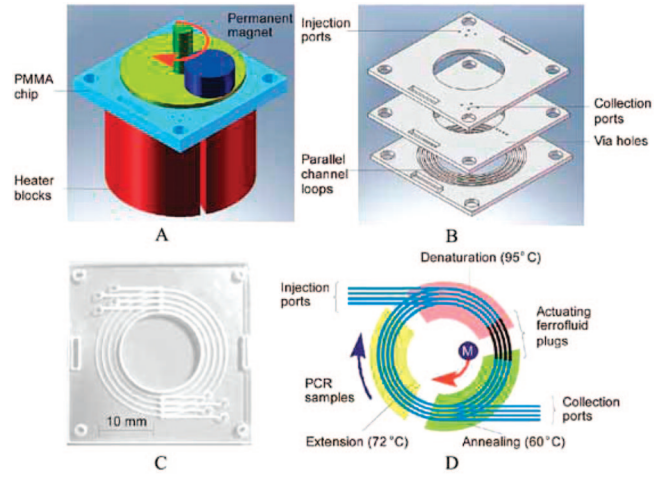

Figure 4. (A) Schematic of the PCR system. (B) Three-layer lamination concept. The first layer has four pairs of straight inlet/outlet channels; the middle layer has eight small via holes; and the third layer has four concentric loops. The layers were fabricated separately in PMMA and thermally bonded together. (C) Photograph of the fabricated microchip. The outer dimension was 30 mm ×30 mm. The four concentric circles are 900 μm wide and 400 μm deep, with diameters ranging from 15 to 20 mm. (D) The microchip was placed on top of three heating blocks. Samples as well as ferrofluid plugs were injected into the different loops. When exposed to the moving magnetic field, the ferrofluid plugs are drawn around the microchannels and continuously propel the PCR samples through the three temperature zones. Reprinted with permission from ref 118. Copyright 2008 American Chemical Society.

has been proposed by Liu et al.,[117] where continuous-flow PCR was performed in a rotary channel device in which the reaction mixture was passed through different heating zones. Recently, high-throughput PCR in parallel rotary channels using magnetic actuation has been reported to simultaneously amplify genetically modified organisms (GMOs) (Figure 4).[118] The use of the rotary channel microreactor for continuous-flow PCR has several obvious advantages. For example, parameters of PCR, such as number of cycles, incubation time, and even temperature of each step, are easily adjusted. In addition, the chip footprint is not too large when parallel PCR reactions are performed. To date, however, this design has rarely been used for single-molecule DNA amplification and analysis. Here, we need to point out that a cylindrical helical thermal-cycling device has been recently established for continuous-flow single-cell amplification and analysis.[108] Although high throughput with this device is obtained by applying aqueous segments embedded in an immiscible inert carrier liquid (perfluoromethyldecaline), high-throughput PCR in parallel is hard to realize with this device.

5. Water-in-Oil (w/o) Droplet Microreactors for Single-Molecule DNA Amplification

5.1. Principle, Advantages, and Prerequisites of w/o Droplet Vesicles As Microreactors for Single-Molecule DNA Amplification, and Other Encapsulated Microreactors Possible for Application of Single-Molecule PCR

Droplet emulsion techniques have become a promising new variation of microfluidics. The w/o droplet microreactors obtained by the emulsion method have found a wide diversity of applications in biological and chemical fields. Some

Table 3. Oil Phase and Surfactants Used within w/o Droplet Microreactors for Single-Molecule PCR Amplification

oil phase	surfactants used in oil phase	refs
mineral oil	Span 80, Tween 80 and Triton X-100 [polyoxyethylene (9)4-(1,1,3,3-tetramethylbutyl)phenyl ether]	136, 140, 142−147
	ABIL EM 90 (cetyl poly(ethylene glycol) (PEG)/poly(propylene glycol) (PPG)-10/1 dimethicone, a silicone-based surfactant)	62, 136, 148−150
	ABIL WE 09 (cetyldimethicone copolyol-polyglyceryl-4-isostearate-hexyllaurate, a silicone-based surfactant), and Tegosoft Liquid (cetearyl ethylhexanoate) [or Tegosoft DEC (diethylhexyl carbonate)]	151, 152
	Sun Soft (polyglycerol esters of interesterified ricinoleic acid)	144, 153
	Lubrizol U (polyisobutylen-succinimide pentamine)	145
HFE-7500 fluorinated oil (2-trifluoromethyl-3-ethoxydodecafluorohexane)	EA surfactant (a PEG-perfluoropolyether (PFPE) amphiphilic block copolymer)	42
Ar20 silicone oil (phenylmethylsilicone Ar 200)	DC 5225C formulation aid [cyclomethicone (and) dimethicone copolyol] and DC 749 fluid [cyclomethicone (and) trimethylsiloxysilicate]	72, 141, 154
Isopar M	Lubrizol U or mixture of surfactants (Span 80, Tween 80 and Triton X-100)	145

excellent reviews are available for a more in-depth appreciation of the development of w/o droplet microreactors for a variety of biochemical applications.[119−135] The principle of single-molecule droplet PCR (or emulsion PCR) is to disperse the PCR solution in a thermostable w/o emulsion at a concentration where each droplet contains on average one template molecule and amplify them *in situ* to reach $>10^3$ clonal copies within each droplet. The advantages of droplet PCR at the single-molecule level include but are not limited to the following: (1) Compared with conventional microfluidic PCR systems,[38,40,74,114,115] PCR amplification using DNA templates confined in droplets would result in a further reduction in the consumption of reagents, shorter reaction times, and more precise amplification due to decreased thermal masses.[129,136] (2) Droplet PCR systems can allow the number of reactors and the reaction volume to be flexibly changed instead of preestablished by the chip design and construction. (3) The w/o droplets on a planar chip can be well manipulated by magnetic,[65,66] electrowetting-on-dielectric (EWOD),[137] or surface acoustic waves (SAW)[138] actuation. The droplet itself becomes a pump, valve, mixer, extractor, and thermocycler.[139] As a result, the sample preparation, single-molecule DNA amplification, and product detection can be organically integrated on a single chip. (4) Ultrahigh throughput "clonal amplification" of DNA templates in droplet systems enables attractive new implications for the field of PCR.[140−142]

In general, the droplet microreactors can be classified into two categories based on the nature of their formation mode: multidisperse and monodisperse. They have been successfully applied to perform single-molecule PCRs in parallel. However, the thermostability of droplets is important for its successful application to single-molecule DNA amplification and analysis, and it becomes the prerequisite of w/o droplet vesicles as microreactors for single-molecule DNA amplification. Droplet stability depends on a number of factors such as oil phase and surfactants contained in oil or aqueous phases. Table 3 has summarized the surfactant-contained oil phases often used within droplet microreactors for single-molecule PCR amplification. It is necessary to emphasize that the ABIL EM 90 in light mineral oil can be used to generate very stable inert emulsions for single-molecule emulsion PCR.[62,136,148−150] Moreover, emulsion PCR generated by this method exhibits reduced competition between template molecules of different lengths, and thus produces fewer chimeric molecules.[136] This can be explained by the presence of a single, or at most a few, DNA template molecule in each emulsion droplet. This segregation of DNA template molecules prevents recombination between homologous or partially homologous gene fragments during PCR, thus eliminating the generation of short, chimeric products and other artifacts.[136] In addition, other continuous oil phases, which are different from the mineral oil, have also been developed for single-molecule PCR applications (Table 3). For example, Margulies et al. reported a novel emulsion oil system (Table 3) to generate the extremely small emulsions, or "microfines", that confer additional stability to the reaction.[141] Recently, Kumaresan et al. also found that the microfines are important for enhancing reaction stability during PCR within monodisperse droplet reactors and that droplets can be formed using the microfine solution instead of the oil and results from both techniques are comparable.[72] The possible reason for this effect is that the small droplets may assemble around the larger droplets during thermal cycling and prevent them from combining with other large droplets to destabilize the emulsion. Sometimes, the addition of surfactant(s) to the aqueous phase can help to stabilize the droplets and to enhance the PCR. For example, only Triton X-100 in PCR or one-step reverse transcription PCR (RT-PCR) mixtures can be used to be sufficient to maintain the w/o droplets in the silicone oil.[155,156] And the w/o emulsion was still stable after 13 cycles (preheat at 95 °C for 5 min; 13 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min). If the aqueous phase contained no Triton X-100 surfactant, the resulting emulsion was unstable. It should be noted that in the case of the particular emulsification strategy, the presence of a bulk protein such as bovine serum albumin (BSA) is also critical for the formation of a stable emulsion and that the bulk protein in the aqueous phase can also prevent the DNA polymerase from becoming trapped and denatured at the oil/water interface of the emulsion droplets.[136] In addition, not all emulsions require surfactants to stably maintain the droplets. For example, the monodisperse emulsion systems developed by Beer et al.[44,53] did not require stabilizing additions. This is most likely due to the high hydrophobicity of the silanization coating obtained by flushing the channel surfaces with SigmaCote (a special silicone solution in heptane) and then baking at 100 °C for 30 min. For the Petri dish PCR in nanoliter droplet arrays reported by Kim et al., it has not been found necessary to add surfactants to improve droplet stability.[157] The hydrophobic polystyrene surface of the Petri dish with the oil/water interface helped to keep the contact area of a droplet very small and the contact angle larger than 150°. Also, it needs to be pointed out that after single-molecule droplet PCR cycling, it is generally necessary to break emulsions, especially for off-line (or further) detection of PCR products.

Table 4. Approaches of Breaking Emulsions for Single-Molecule PCR in Droplets

types of approach	general description	solvent(s) used	refs
extraction	several extractions can remove the remaining oil from the emulsion and cause it to break; however, such extractions may not be suitable for some specific PCR strategies such as two-step PCR	hexane	158
		isopropyl alcohol	72, 141
		water-saturated ether or diethyl ether	43, 62, 136
centrifuging	this method is straightforward; however, it is limited because the oil phase is hard to completely remove		145, 155
vortexing and centrifuging		emulsion destabilizer (RainDance Technologies)	42
		isopropanol	152
Flash-freezing in liquid nitrogen and centrifuging	this approach can be desirably used to break the emulsified droplets; however, it is somewhat complicated		58
breaking buffer to break the emulsion	this method is relatively widely used, and the buffer used is often tailor-made and therefore can effectively break the emulsion	NX or NX2 buffer [NaCl, Triton X-100, Tris-HCl, and ethylene diamine tetraacetic acid (EDTA)]	140, 142, 146, 147
		NX-sodium dodecyl sulfate (SDS) buffer [NaCl, Triton X-100, Tris-HCl, EDTA, SDS]	148−151, 159
		B/W buffer [NaCl, Tris−HCl, and EDTA]	144

Several often used approaches to break w/o emulsions are listed in Table 4.

In addition to w/o droplet microreactors, other encapsulated microreactors could be used for single-molecule DNA amplification. Up to now, various strategies for encapsulating chemical or biological materials in ultrasmall-volume vesicles have been reported, such as lipid vesicles (liposomes or lipid-based microcapsules) that are formed by flow-focusing,[160,161] microfluidic jetting,[162,163] "lipid-coated ice droplet hydration method",[164] on-chip extrusion,[165] rotaevaporative technique[166] or electroformation on a silicon substrate,[167] polymerosomes generated from double-emulsions,[168] and microcapsule compartments fabricated using layer-by-layer (LbL) technology.[169,170] Early in 1995, Oberholzer et al. described the enzymatic RNA replication in self-reproducing oleic acid/oleate vesicles.[171] Subsequently, they reported the encapsulation of PCR reagents into liposomes to perform liposome-based PCR,[172] where liposomes composed of l-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or of a mixture of POPC and phosphatidylserine (PS) are very stable at high temperatures required for PCR, giving a mean volume of 3.3×10^{-18} L. Very recently, Mak et al. demonstrated a novel approach using diffusion-controlled and temperature-stable LbL microcapsule compartments (~110.8 μm average diameter) to perform a high number of individual PCRs, termed "microcapsule-PCR".[169] Because of the limited amount of space, however, we have to confine our referencing to highlight the w/o droplet microreactors (multidisperse and monodisperse) for single-molecule DNA amplification.

5.2. Multidisperse Microreactors in w/o Emulsions

Microreactors in emulsions used for single-molecule PCR amplification first appear in a multidisperse form. An aqueous phase and an oil phase are mixed, with the aid of surfactants, to create thermostable w/o emulsions. The bulky emulsions are produced by conventional mechanical methods, including aggressively stirring[136,140,143−145,156] or vortexing.[146,147,151,159] A w/o emulsion can be well prepared by dropwise addition of the aqueous phase to the oil phase at certain time intervals while stirring is maintained.[72,136,140,143,144,155,156] Microemulsions can also be simply made by stirring PCR solution/oil/surfactant mixes.[140,145] In general, the diameter sizes of the droplets formed in a mechanical way range from 2 to 20 μm, and thus they appear polydisperse.[140,144,146,149,150,155,156] In the "picoliter plate pyrosequencing" approach, which has been widely termed as "454 sequencing",[141] the sizes of the emulsion droplets are up to 100 μm in diameter. Due to the large size distribution, the number of the initial templates may differ among these droplets. If the droplet reactors are too small, there will be little or no amplification. If the reactors are too large, the fraction of reactors with only a single template will be too low to provide a statistically significant result. Fortunately, the droplet sizes can be optimized by varying the mixing time or stirring speed. In addition, the temperature at which an emulsion is generated affects the size distribution of its droplets.[136] Recently, Musyanovych et al. reported a single-molecule PCR performed inside stable and narrowly distributed w/o droplets with a size of 100−300 nm in diameter.[145] The relatively homogeneous droplets were obtained by the sonication-based miniemulsion process.

5.3. Monodisperse Microreactors in w/o Emulsions

Processing microreactors in bulk emulsions limits the uniformity in droplet size and composition, and reaction rate that can be achieved. The extreme polydispersity of the droplets precludes efficient and uniform amplification of the long DNA fragments needed for complex genome assemblies.[141] In addition, bulk emulsion methods typically use end point product detection after breaking the emulsion and recombining all the amplified nucleic acids. As a result, the compartmentalization of the DNA amplification hardly gives rise to a digital readout. To overcome these restrictions, some researchers have capitalized on the compatibility of scale of the small droplets formed in emulsion and on-chip microfluidics so as to produce monodisperse microreactors for single-molecule PCR.[44,53,72,173] For example, Beer et al. recently reported an on-chip real-time PCR instrument for

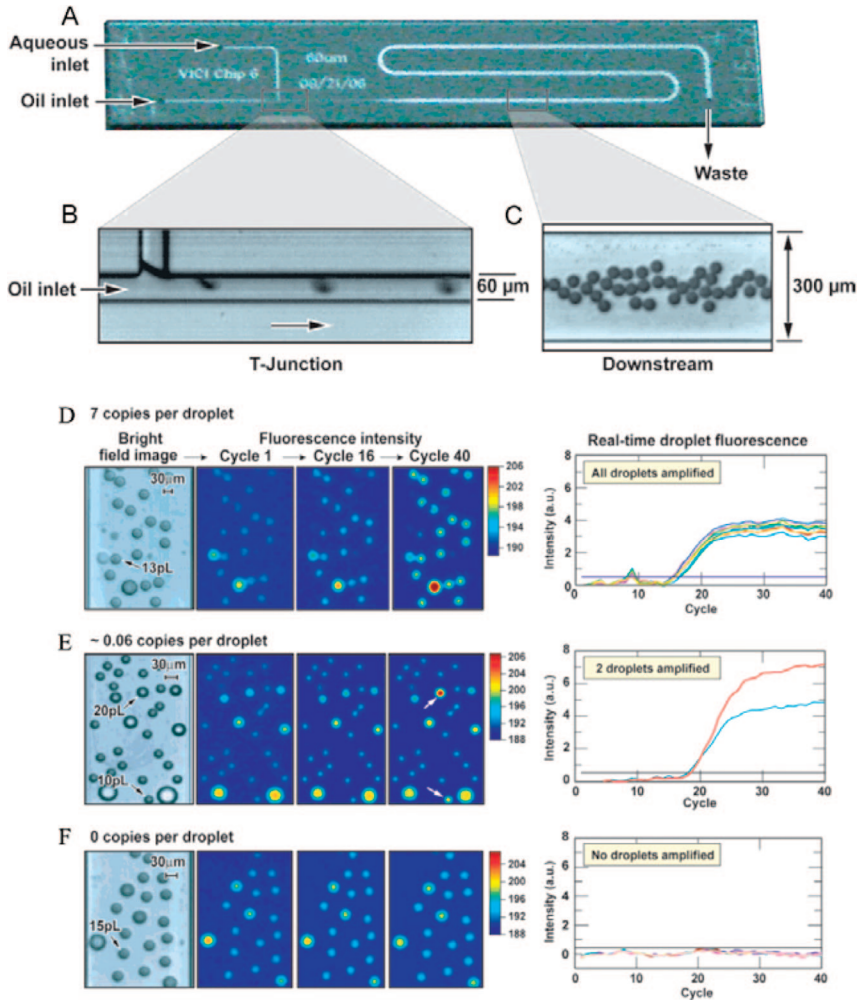

Figure 5. PCR chip for real-time, high-throughput, single-molecule amplification in picoliter droplet reactors. (A) Overall channel and flow configuration. (B) Droplet generation at the T-junction. (C) Monodisperse droplets in the downstream channel. (D−F) Real-time PCR data from picoliter droplets at an estimated 7, ~0.06, and 0 copies of genomic DNA per single droplet, respectively. Droplets were identified from the bright-field image and then monitored at each cycle to generate real-time fluorescence curves. In panel D, all droplets amplified, whereas in panel E, only two droplets amplified (white arrows). No amplification was observed from panel F, the negative control. Reprinted with permission from ref 44. Copyright 2007 American Chemical Society.

generating monodisperse microdroplet reactors in a single channel chamber, thermal cycling them for PCR, and detecting real-time amplification in the individual picoliter droplets (Figure 5).[44] This method utilized a chip with hydrophobic channel surfaces and a shearing cross-flow T-junction to generate w/o microdroplets and allowed detection of a single-molecule DNA at significantly reduced cycle thresholds. Subsequently, they developed a similar system for on-chip single-molecule real-time RT-PCR in monodisperse picoliter droplets.[53] By this means, single-molecule PCR throughput is significantly increased, and thus it can be applied for single-molecule or single-cell genetic analysis and massively parallel genome sequencing. In their work, in addition, Beer et al. have proposed a novel monodisperse droplet trapping method with droplet stopping times of ~38 ms that is applicable to most on-chip droplet generators for single-molecule detection and reaction kinetics measurement.[174] However, they have also observed that on-chip droplet production for w/o emulsion results in downstream droplet velocity fluctuations with a periodicity equivalent to the droplet generation rate, which can affect the accuracy of data collection due to variation in photon integration times.[175] Very recently, Kumaresan et al. developed a high-throughput single-copy genetic amplification (SCGA) process, where a chip-based microdroplet generator (μDG) creates uniform droplets inside which bead PCR is performed on single molecules or cells (Figure 6A,B).[72] For the first time, the research group has shown the generation of attomole quantities of >500 base pair (bp) PCR amplicons inside droplets starting from a single template, and these amplicons are competent for next-generation *de novo* sequencing applications. Figure 6C, D shows the amplification of DNA templates in emulsion droplets as a function of template length. In addition, it is noted that a glass−PDMS hybrid assembly is used to integrate a micropump into the μDG that provides uniform droplet size, controlled generation frequency, and effective bead incorporation. The two excellent examples provided here have demonstrated the possibility of combining high-fidelity manipulation of microdroplets using micropumps, microvalves, and micromixers in microfluidic networks with the ability to perform DNA/RNA amplification in microdroplets. However, the set-ups presented by Beer et al. and Kumaresan et al. have involved thermal cycling of the entire device[44,53] or bulk PCR amplification of the droplets collected off-chip,[72] thus limiting cycling rate and throughput.

Recently, Kiss et al. presented an online detection system in which a range of template concentrations could be detected

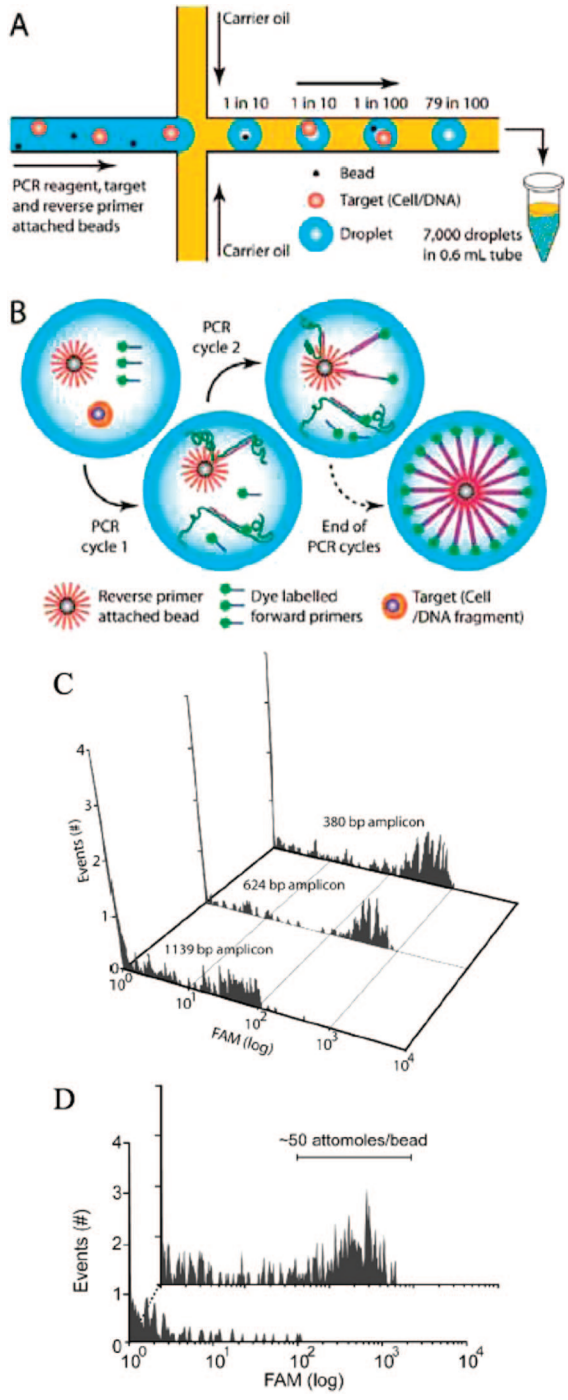

Figure 6. Single copy genetic amplification (SCGA). (A) Target DNA or cells and beads are mixed with the PCR reagent (blue) at very dilute concentrations and pumped through a microfabricated droplet generator (μDG). Monodisperse droplets of the PCR reagent are formed in a carrier oil (yellow) at the cross-injector and routed into a tube for temperature cycling. (B) Each functional PCR droplet contains a bead covalently labeled with the reverse primer, dye-labeled forward primer, and a single target copy. Subsequent steps of PCR generate dye-labeled double-stranded product on the bead surface. Following emulsion PCR, the droplets are broken and the beads are analyzed by flow cytometry to quantify the bound clonal amplified product. (C) Comparison of PCR yields in emulsion droplets for 380 (~175 amol/bead), 624 (~155 amol/bead), and 1139 bp (~10 amol/bead) amplicons from a starting pUC18 template concentration of 10 molecules per droplet. (D) Flow cytometry analysis of beads carrying a 624 bp product amplified from 1 template per droplet (upper) and 0 template per droplet (lower). Here, average reverse primer density per bead is 4.4 fmol. Reprinted with permission from ref 72. Copyright 2008 American Chemical Society.

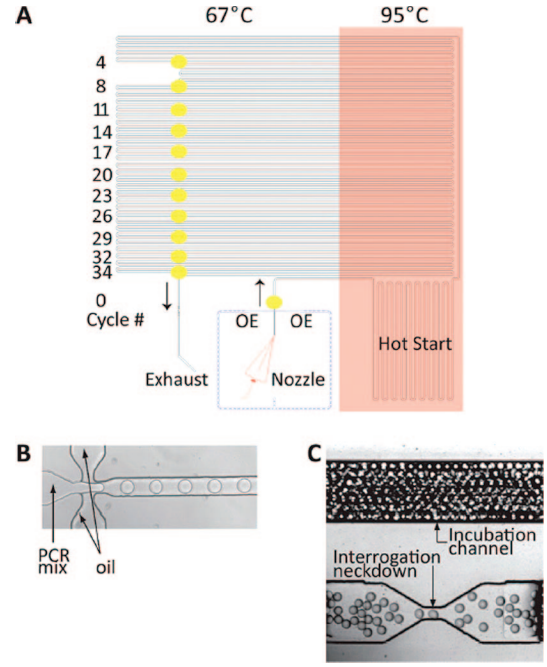

Figure 7. Images of the continuous-flow digital PCR chip. (A) Schematic of the overall flow configuration. Pink-shaded zones of the chip were set at 95 °C, and nonshaded zones were at 67 °C. The yellow regions for the interrogation neckdowns and the corresponding cycle numbers are indicated on the left. The nozzle is highlighted in red, and the oil extractor (OE) is in blue. (B) Optical image of droplet generation at the nozzle. (C) Optical image of uniform droplets in the downstream channel and flowing through one of the neckdowns. Reprinted with permission from ref 58. Copyright 2008 American Chemical Society.

by dynamic continuous-flow digital PCR that agreed closely with the frequencies predicted by Poisson statistics (Figure 7).[58] PCR reagents are encapsulated in millions of picoliter droplets in a continuous-flow oil phase that flows through a serpentine channel, and the amplification process within individual droplets at specific channel locations can be monitored. As a result, amplification of a 245-bp product can be detected and quantified in 35 min at template concentrations as low as 1 molecule/167 droplets. This represents the first successful continuous-flow microfluidic digital PCR chip for high-throughput, single-molecule DNA amplification. Very recently, Schaerli et al. reported another high-throughput microfluidic device for continuous-flow PCR of single-copy DNA in w/o droplets of nanoliter volumes (Figure 8).[62] Fluorescence lifetime imaging (FLIM) of rhodamine B was used to measure the temperature inside the droplets to provide precise temperature control for successful single-copy DNA amplification by PCR. High-throughput amplification from a single molecule of DNA per droplet obtained here is paving the way for microfluidic digital PCR, although current product analysis was completed off-line by gel electrophoresis, sequencing, and real-time PCR.

In addition to the monodisperse droplet microreactor-based PCR microfluidic systems,[44,53,58,62,72,108] other droplet microreaction systems for DNA amplification and analysis have also been reported. For example, Mazutis et al. very recently reported a method for high-throughput isothermal "hyperbranched rolling circle amplification" (HRCA) of single DNA molecules in a droplet microfluidic system.[42] DNA amplification in droplets was analyzed using an intercalating fluorochrome, allowing fast and accurate digital quantifica-

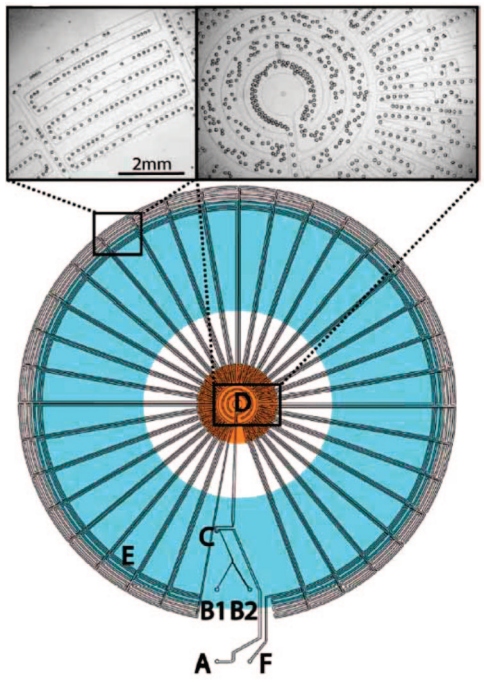

Figure 8. Design of the continuous-flow radial PCR device for single-molecule DNA amplification. The device contains an oil inlet (A) that joins two aqueous inlet channels (B1 and B2) to form droplets at a T-junction (C). The droplets pass through the inner circles (500-μm wide channels) in the hot zone (D) to ensure initial denaturation and travel on to the periphery in 200-μm wide channels where annealing and extension occur (E). The droplets then flow back to the center where the DNA is denatured and a new cycle begins. Finally, the droplets exit the device after 34 cycles (F). All channels are 75 μm deep. The positions of the underlying copper rod and the Peltier module are indicated with orange and blue areas, respectively. Devices are made of SU-8 embedded in PMMA. Reprinted with permission from ref 62. Copyright 2009 American Chemical Society.

tion of the DNA template based on the Poisson distribution of DNA molecules in droplets. The developed microfluidic systems consist of HRCA droplet generation device, HRCA droplet analysis device, droplet fusion device, and fused droplet analysis device, thus allowing the amplified DNA in each 2 pL droplet to be further analyzed by measuring the enzymatic activity of the encoded proteins after fusion with a 15 pL droplet containing an in vitro translation (IVT) system (Figure 9). This is the first droplet microfluidics-based digital RCA for single-molecule DNA amplification and analysis. The use of the isothermal HRCA avoids many complications associated with the PCR thermocycling. In addition, the ability not only to measure single-molecule DNA amplification but also to measure the activity of the encoded enzymes after IVT greatly increases the ranges of potential applications.

5.4. Droplet Microreactors on an Open Planar Surface

Different from the aforementioned multidisperse/monodisperse emulsion microreactors, droplet microreactors on a planar chip surface use a chemically modified surface instead of closed tubing to provide virtual fluid confinement. In other words, the reaction chamber is made by encapsulation of a reaction liquid in oil liquid, forming a virtual reaction chamber/channel (VRC)[138] (Figure 10). The distribution of the liquid on the chip surface is dominated by the surface free energy and not by chamber or channel walls. For forming an open w/o droplet for single-molecule DNA amplification,[138,157,176] the chip surface for the VRC system has to be hydrophobic as well as oleophobic. To this end, chemical modification is needed to produce desired static contact angles with water and oil, respectively. Such modification can be achieved by coating with silanes such as perfluoroalkylsilane (PFAS)[138] and (heptadecafluoro-1,1,2,2-tetrahydrodecyl) trimethoxysilane (FDTES).[139,177,178] Pipper et al. further coated the surface-modified glass substrate with a certain concentration of Teflon AF 1600 (a family of amorphous fluoropolymers) solution in Fluorinert FC-75 [perfluoro(2-butyl-tetrahydrofurane), a fluorocarbon derivative of tetrahydrofuran] or FC-40 (a fluorinated oil containing perfluorotri-*n*-butylamine and perfluoro-*n*-dibutylmethylamine), so as to produce droplet-based microfluidic architectures enabling complex (bio)chemical tasks including sample preparation and PCR or RT-PCR amplification.[139,179] Recently, Kim et al. have explored the VRC concept for a highly parallel single-molecule real-time nanodroplet PCR system with laser-assisted heating.[157,176] In one of their works, they replaced the glass cover slide by the commercial Hybrislip that is a single-use, 0.25-mm-thick, hydrophobic PC cover. After mineral oil was deposited in the chamber (6 mm in height and 20 mm in diameter) placed on top of the Hybrislip, PCR droplets were dispensed by a micropipet into the oil phase to form 20−100 nL droplets. With a typical droplet volume of 30 nL, all the droplets containing only 0.4 copies of human genome were successfully amplified.[176] Note that each human genome has 300−400 copies of the 18S rRNA target gene, and thus the droplets containing 0.4 copies of human genome still contain 120−160 copies of target templates. In this work, however, a micropipet was used to dispense the PCR mixture droplets into the oil phase, and a single dispensing action often produces multiple droplets that have disuniform sizes. In order to overcome this shortcoming, this group has developed a simple contact printing technique to produce a large array of uniform sized nanoliter PCR droplets, which are laser-heated on an unmodified disposable polystyrene Petri dish.[157] Actuation for each droplet took typically less than 2 s to print, and 3 min was enough to print a 10 × 10 array (i.e., 100 droplets). Printed droplets showed variation of less than 10% in volume (the typical volume of the droplet is ∼12 nL), and the oil/water/polystyrene interface formed a compact droplet microreactor approximately spherical in shape. Figure 11A displays the bright filed (a) and fluorescence (b) images of a 4 × 5 array of PCR droplets. Figure 11B demonstrates the images of droplets during PCR amplification at four concentrations of the template DNA. Compared with Guttenberg et al.'s work, which first proposed the VRC concept for single-molecule PCR amplification,[138] the amplification throughput of Kim et al.'s approach is increased up to 100 droplets. In fact, this technology opens the door for VRC-based microfluidic digital PCR. It is worth noting that a similar droplet VRC-based real-time PCR system using IR laser heating has been developed for single-copy DNA amplification and analysis, where droplets (10−30 nL) were made by a micropipet on a glass base dish filled with mineral oil.[180] In comparison with etched reactors on chips, the droplet-based VRC on an open chip surface has several advantages as follows: (1) The open configuration of VRC is beneficial from a simplicity and low-cost fabrication standpoint. In addition, an open system also allows the use

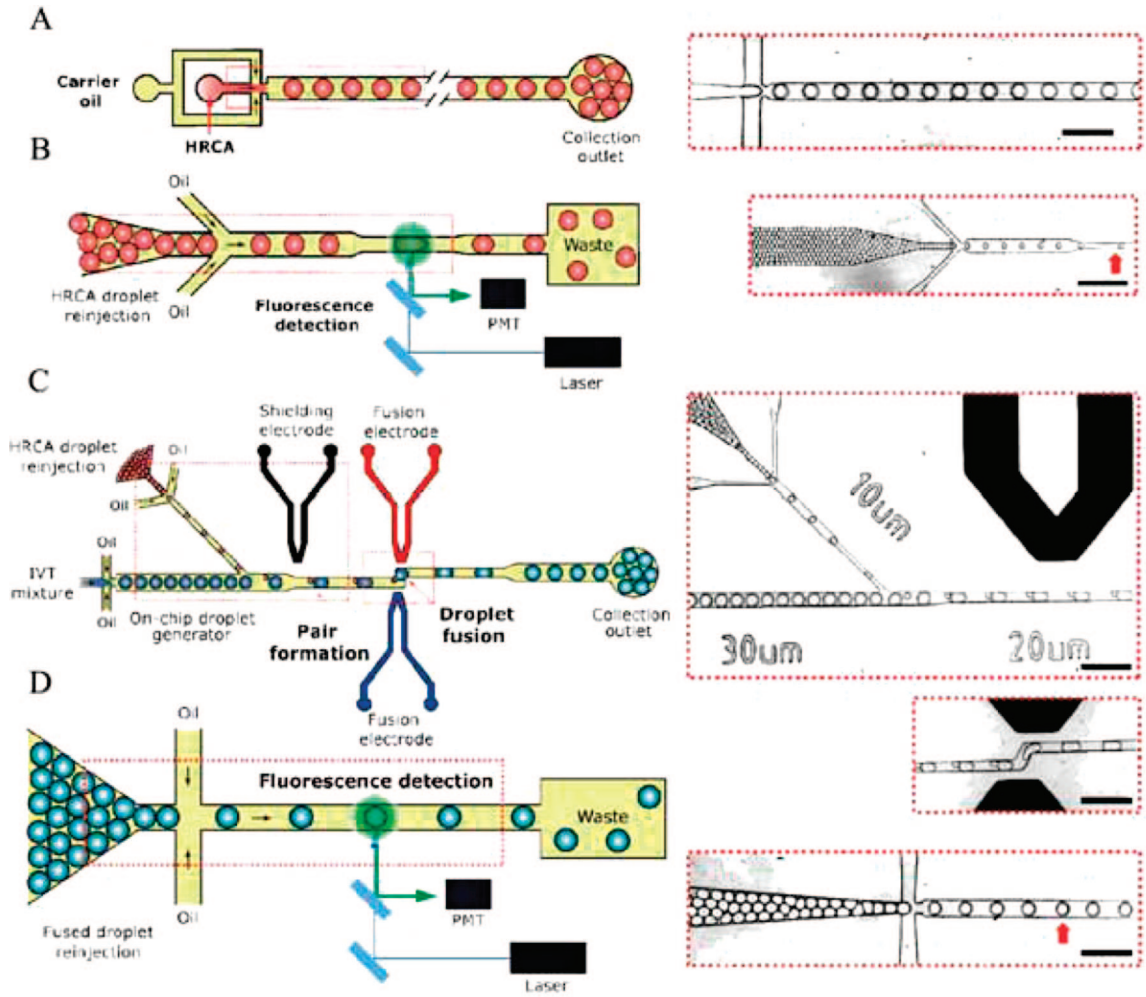

Figure 9. The droplet-based microfluidic devices for high-throughput isothermal amplification and anaysis of single DNA molecules. (A) HRCA droplet generation device. An HRCA system containing the plasmid DNA was emulsified using HFE-7500 fluorinated oil with 2% (w/w) EA surfactant. (B) HRCA droplet analysis device. After incubation off-chip, HRCA droplets were reloaded into the device and spaced with HFE-7500 fluorinated oil, and their fluorescence was monitored using photomultiplier tubes (PMTs). (C) Droplet fusion device. HRCA droplets were reloaded, spaced by HFE-7500 fluorinated oil containing 2% (w/w) EA surfactant, and paired with IVT droplets generated on-chip. Droplet pairs were electrocoalesced between two electrodes (red and blue) by application of an AC field (30 kHz, 600 V), while shielding electrodes (black) prevented unwanted electrocoalescence. (D) Fused droplet analysis device. Fused droplets were reinjected and spaced with HFE-7500 fluorinated oil, and their fluorescence was monitored. Devices were fabricated in PDMS using soft lithography. The scale bar is 100 μm, and red arrows show the detection points. Reprinted with permission from ref 42. Copyright 2009 American Chemical Society.

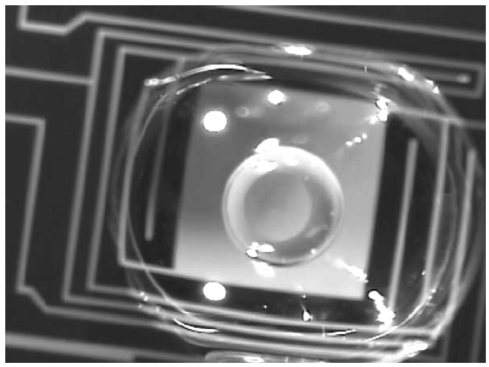

Figure 10. A virtual reaction chamber (VRC) system situated on a chip heater. The chip−PCR chamber consists of a PCR droplet covered by an oil cap that is placed in the middle of the window in the heater structure. The temperature is measured with the sensor line around the window, while resistive heating is provided by the outer line. Reprinted with permission from ref 138. Copyright 2005 The Royal Society of Chemistry.

of conventional reagent distribution approaches, such as a simple pipet or dispensing robot. (2) The manipulation of individual droplets on a planar surface can offer multiple operation functions. Depending on its particular task, a droplet temporarily becomes a pump, valve, mixer, extractor, and thermocycler.[139,179] (3) The VRC system usually has a high temperature ramping rate since no thermal mass of solid cover or chambers or channels is added. So far, the fastest heating rate (175 °C/s) and cooling rate (125 °C/s) have been obtained using the VRC method.[177] Recently, Pipper et al. have combined the VRC and the time−space concept to perform clockwork-like continuous-flow PCR inclusive of sample preparation.[179] Typically, when the angular velocity during a rotation is 90° per second, heating and cooling rates of ±35 °C/s are obtained.

It needs to be noted that other forms of droplet microreactors on chips can also be used to perform DNA amplification and analysis at the single-molecule level, such as droplets using EWOD-based digital microfluidic technology.[137,181−184] In 2003, Pollack et al. first reported EWOD-based digital microfluidics for real-time PCR applications.[183] It has been shown in their work that the manipulations and the environment inside the EWOD digital microfluidic chip did not

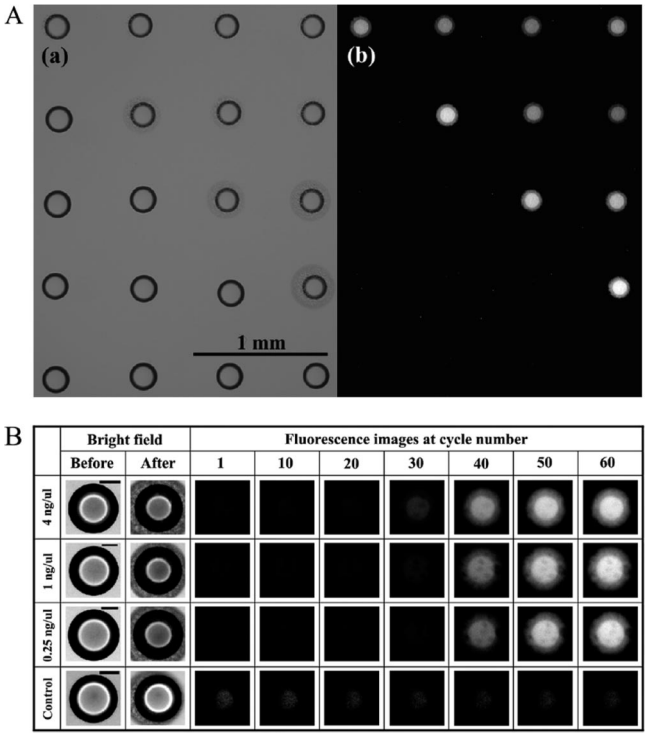

Figure 11. (A) Bright field (a) and fluorescence (b) images of a 4 × 5 array of PCR droplets. Ten droplets in the upper right were amplified, while the rest were not amplified. (B) Images of droplets during PCR at four concentrations of the template DNA. At left are bright field images before and after amplification. At right are fluorescence images at every tenth thermal cycle. In case of the control droplet, the brightness of the fluorescence image was increased ~5 times so that the weak intensity can be distinguished. The black bars in the bright field images are 100 mm long. Reprinted with permission from ref 157. Copyright 2009 The Royal Society of Chemistry.

inhibit PCR and that the real-time PCR assays were successfully performed in 300 nL droplets using the EWOD chip. However, their EWOD system used an additional thermocycler device, including an external thermocouple and heating fans for temperature control. Chang et al. also reported an integrated microfluidic chip for PCR applications utilizing digital microfluidics, where sample transportation, mixing, and DNA amplification were performed on the integrated EWOD chip.[181] An innovative hydrophobic/hydrophilic structure has been successfully used to integrate the digital microfluidic chip with the on-chip PCR device containing a film temperature sensor and two film heaters. However, the total time for the PCR test was 55 min, and the total sample volume consumed was 15 μL. Nearly at the same time, Fouillet et al. used a droplet-based EWOD digital microfluidic chip to amplify 10 human placental DNA copies in a 64 nL droplet,[182] showing the robustness of the EWOD system with small DNA copy numbers. By integrating the heating on the chip, the PCR cycling can be completed within 15 min. Very recently, an electrowetting-based digital microfluidic platform for point-of-care testing has been developed by Sista et al.,[137] with particular emphasis on reducing the time-to-result and integrating sample-to-answer functionality, which are critical for point-of-care devices. Droplet-based magnetic bead immunoassay, droplet-based oscillatory-flow real-time PCR, and on-chip sample preparation using magnetic beads have been integrated on a digital microfluidic cartridge (Figure 12A,B). On such a microfluidic cartridge, the magnetic bead immunoassays could be completed in less than 8 min using whole blood samples, and a 40-cycle real-time PCR was performed within 12 min by shuttling a 600 nL droplet between two thermal zones. In addition to rapid results, high sensitivity of the digital microfluidics PCR system was also obtained using genomic DNA from *Candida albicans*, and the sample with one copy of *Candida* genome was successfully amplified and detected (Figure 12C,D). This is the first EWOD-based digital microfluidic PCR for single-molecule DNA amplification and analysis. It is necessary to note that another EWOD droplet-based digital oscillatory-flow PCR microfluidics system has recently been designed, simulated, and studied experimentally.[184] An enormous advantage of EWOD digital microfluidics is that it only requires electrical switches for fluidic actuation without pumps or any other moving parts. Using EWOD actuation, liquids with a wide range of fluidic properties (for example, interfacial tension, viscosity, ionic strength, and protein content) have been shown to be compatible with digital microfluidic functions such as transporting, mixing, splitting, and dispersing.[137]

6. Single-Molecule DNA Amplification Reaction Volume and Speed

Small volume operation[119,120] and rapid thermal cycling have been the major motivations in the development of single-molecule DNA amplification and analysis using microfluidics. The small volume not only suits single-molecule amplification applications (discussed above) but also conserves reagent and sample and reduces cost. In general, the multidisperse reactors in emulsion have a reaction volume ranging from picoliter[140,144,149,153] to femtoliter,[145,146,155,156] and they are thermally cycled using a conventional thermal cycling system. The single-molecule DNA amplification volume and speed performed on chips will be discussed in detail here. Table 5 is a summary of the literature involving single-molecule DNA amplification on microfluidic chips. Several microfluidic amplification devices with a LOD of less than 5 molecules with high signal-to-noise ratio (SNR) are also included in Table 5, since these devices indicate that amplification from only one starting molecule will be detectable.[50,51,63]

6.1. Reaction Volume

Microfabricated devices offer the ability to react extremely small volumes of PCR solution. So far, the smallest PCR reaction volume reported for single-molecule DNA amplification using reactors etched on chips has come from the work of Nagai et al.[32,49] In their work, the lower limit of the volume for PCR was examined using microchambers of various volumes. The silicon microchamber array was prepared with 21 kinds of chambers of various volume capacities from 1.3 pL to 32 μL, and PCR was performed in each chamber. It has shown that the lower limit of volume for PCR in the silicon-based microchamber was 85 pL. Microchambers with volume equal to or greater than 85 pL gave successful PCR, and the limit of the volume for PCR was for the first time examined using silicon microchambers with various volumes. It should be noted that the droplet reactors smaller than this volume will also result in successful single-molecule PCR, probably due to the decreased surface adsorption (Table 5). For example, Beer et al. recently reported single-molecule real-time PCR and RT-PCR in the

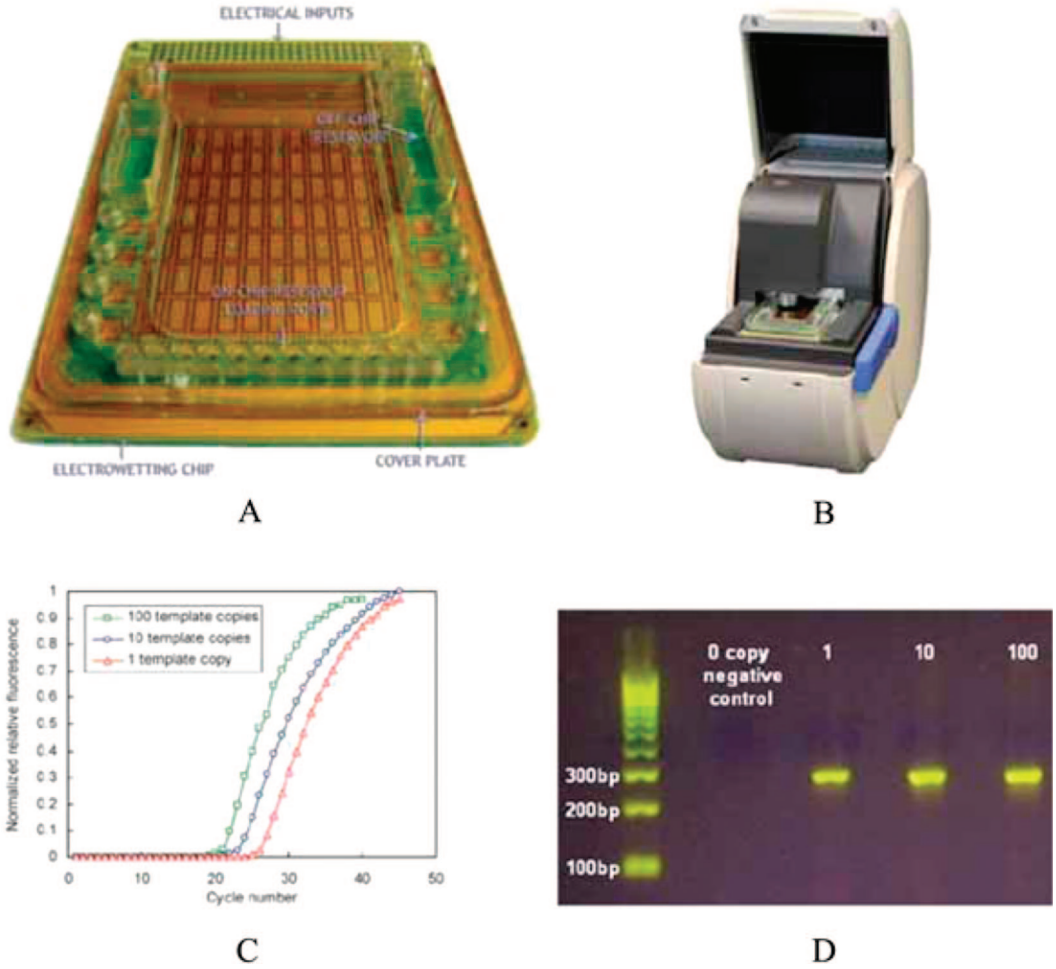

Figure 12. (A) The fully assembled digital microfluidic multiwell plate cartridge (8.55 cm ×12.78 cm), where both immunoassay and DNA amplification experiments were performed. (B) The control instrument (20.32 cm × 33.02 cm × 53.34 cm (W × H × L)). (C) Real-time on-chip PCR titration of samples with different numbers of *Candida* genome copies. (D) Corresponding gel images to panel C. Reprinted with permission from ref 137. Copyright 2008 The Royal Society of Chemistry.

monodisperse picoliter droplets formed in a single microfabricated channel.[44,53] Amplification speed of this work was not the major focus and thermocycling was performed by placing the entire chip on a Peltier device or by using a flexible Kapton resistive heating element. In recent work from the Mathies group,[72] monodisperse droplets formed on chip with volumes of 2.4 ± 0.3 nL were collected off-chip into a PCR tube and then subjected to thermocycling in a conventional device. This method was successful in generating product from single-molecule templates up to 1139 bp long with ~10 amol per amplified bead. This is the longest single-molecule amplicon reported to date using droplet PCR in emulsion. A similar work has been very recently reported by Tiemann-Boege et al.[152] Greater molecule crowding with the longer DNA molecules on the bead surface will cause reduced amplification efficiency and even lead to amplification failure. Guttenberg et al. used the 200-nL VRC on chips, where each PCR droplet was covered with a drop of mineral oil, to explore product length effects on single-molecule DNA amplification.[138] When the length of the PCR amplicon was 150 bp, only one human chromosome was enough to produce a visible band on the electrophoresis gel. Under the same conditions, a successful amplification of the 1382 bp fragment needed at least 6 human genome equivalents. Lagally et al. demonstrated that within the multiplex PCR at the single-molecule level, the difference in product length will result in the difference in amplification efficiency.[33] This is presumably due to competition between the two amplicons for resources during the PCR. A similar result was also reported by Koh et al.[63] These reports indicate that within a certain reaction volume, the choice of product length will affect the single-molecule DNA amplification. In addition, it should be noted that the type of template DNA will also have an effect on the single-molecule DNA amplification. For example, human genomic DNA can be considered a "difficult" template since it has a high sequential and spatial complexity.[138] For single-molecule DNA amplification and analysis within microreactors on chips, the different types of DNA templates and amplicon lengths have been included in Table 5.

Other reports have described single-molecule PCR with nanoliter reaction volumes. For example, Kalinina et al. performed single-molecule PCR in 10-nL capillary reaction chambers.[26] This is the first work concerning single-molecule PCR in nanoliter-scale reactors and digital PCR. Matsubara et al. performed high-throughput single-molecule PCR amplification in 40-nL reaction vessels.[30,48] In their work, stochastic amplification of 0.4 copies per reaction chamber was achieved (i.e., 24 copies of target template were introduced into 60 microchambers), which was determined as the LOD of the current work. It is necessary to note that when 0.4 copies/chamber were dispensed, only 2 microchambers out of 60 chambers reached within the fluorescence level of 1000−1200 arbitrary units (AU),

Table 5. Characteristics of Single-Molecule DNA Amplification and Analysis Performed within Microreactors on Chips

reaction volume	heating/cooling rate, °C/s	amplification times, s/cycle number	template DNA	LOD[a]	amplicon length, bp	refs
			Picoliter			
10 pL	1.8/1.4	6480/40	genomic DNA		*b*	44
65 pL	*b*	2100/34	adenovirus genome		245	58
70 pL	~5/2	*b*/40	MS2 virion		*b*	53
85 pL	16/16	1080/40	pGFP fragment		100	49
85 pL	*b*	*b*/40	pGFP fragment		100	32
131 pL	*b*	1020/34	tyrocidine synthetase 1 gene		85	62
			Nanoliter			
10 nL	*b*	~1800/40	human β actin gene		295	26
10 nL	*b*	*b*	*b*		*b*	56
10−30 nL	32/32	210/50	*Bacillus subtilis* DNA		72	180
12 nL	*b*	~420/40	human genomic DNA	1 human genome	187	157
29 nL	~12/~2	*b*/35	*Escherichia coli* O157/ *Salmonella typhimurium*	~6	232/559 and 429	63
30 nL	*b*	370/40	human genomic DNA	0.4 copies of human gemone	187	176
40 nL	*b*	>3400/40	human genomic DNA	0.4 copies	295	30
40 nL	*b*	~3720/40	human rhesus D	0.4 copies	74	48
72 nL	>5/>7	*b*/30−40	2D6.6 CYP450		*b*	55
200 nL	*b*	*b*	human genomic DNA	0.2 molecules	*b*	64
200 nL	10/5	600/30	human genomic DNA	1 chromosome/6 genome equivalents	150/1382	138
200 nL	*b*	1200/30	intact *E. coli* cells	2−3 cells	280	51
280 nL	~10/~10	900/30	M13/Puc19 cloning vector/ human genomic DNA		136/231	33
280 nL	*b*	600/20	M13/Puc19 cloning vector	5−6 molecules (SN ≈ 7)	136	50
350 nL	*b*	*b*/45	human β-globin gene/HIV-1 DNA		110/142	41
600 nL	*b*	720/40	genomic DNA from *C. albicans*		300−400	137
			Microliter			
1 μL	20/*b*	<1200/40	hepatitis B virus (HBV) DNA		*b*	47
1 μL	10/10	474/30	chromosomal DNA of *Paenibacillus larvae*	4.5 copies	233	75
~2 μL	5−6/3−4	*b*/35	virus	1−2 copies	293	57
2 μL	~6/*b*	*b*/35	MB4-1, MB4-2, and MB4-3	1 cell	438, 275, and 343	59
~3 μL	*b*	*b*/40	*Bordetella pertussis*	genetic DNA from <2 cells	167	54
4 μL	~2/~3	*b*/40	chromatin immunoprecipitation DNA	2 molecules	*b*	46
11 μL	*b*	~1800/45	*Bacillus anthracis* genomic DNA	0.3 copies	*b*	107

[a] Here, LOD is defined as the lowest number of template molecules per microreactor that produces a positive amplification signal. Unless stated, LOD is one DNA molecule per microreactor (for multiple microreactors, LOD is one DNA molecule per microreactor on average). [b] Not available.

where the average fluorescence intensity value of 1000 AU was determined as the threshold (Figure 2). It was reported that the combined negative effects of biochemical (resulting from the TaqMan PCR itself) and instrumental limitations resulted in the appearance of only 2 chambers out of 60 with positive signals after TaqMan PCR on this microsystem.[30] The first integrated microfluidic device for single-molecule DNA amplification and analysis was reported in 2001 by the Mathies group.[33] The glass microdevice consisted of 280-nL PCR chambers that were directly coupled to a microfabricated CE system. This group also reported other integrated PCR-CE microdevices for ultrahigh-sensitivity DNA or RNA detection.[50−52,73,185,186] Finally, it is necessary to note that several microliter-sized PCR assays have also been developed for single-molecule DNA amplification applications (Table 5). It is obvious that these reports did not have small-volume PCR with rapid amplification as the ultimate goal of the project. However, if a complete nucleic acid analysis system for throughput-sensitive diagnosis (e.g., single-molecule genetic expression and high-throughput genomic sequencing) is needed, the PCR must be performed with lower reaction volume.

6.2. Reaction Speed

The low volume reactor, the low thermal mass, and the use of thin film heaters can permit rapid thermocycling. So far, temperature cycling on almost all of microfluidic devices for single-molecule DNA amplification is performed with a contact heating method, which is defined as having heaters fabricated within the chip or in thermal contact with the outside of the chip. The microfabricated film heaters have smaller thermal mass and thus usually allow faster thermal response and high heating rates, up to 10 °C/s.[63,138] A drawback to incorporating heater and even temperature sensors directly into the chip device is the complicated and costly fabrication. Alternatively, the commercially available flexible thin heaters[50,53] and Peltier elements[44,46,48,57,59] can also be used to obtain rapid thermocycling rates. In cases where high-throughput single-molecule genetic and analysis was performed, the thermal cycling can be achieved by placing the chip on a conventional thermocycler[27,28,32,60,89,90,92,93] or on a specific Fluidigm's BioMark system.[77−81,83,84,86] However, when time-sensitive diagnostics (e.g., detection of infectious diseases and related biowarfare agents) is needed, the PCR must be performed with greater reaction speed. It

is worth noting that the use of small-volume PCR vessels does not necessarily coincide with reduced cycling times if the heating method does not exploit the advantages of low-volume design (Table 5). In addition, within a certain reaction time, when the heating or cooling rate(s) increases, the number of cycles will increase (the amplification times will generally decrease). And, amplification at higher cycle numbers will increase the SNR, allowing reduction of starting template concentration to single-copy levels, but linearity may decrease.[50] For single-molecule PCR microreactors on chips, the heating/cooling rates, amplification times, and cycle number have been compared and listed in Table 5. We refer the readers to an excellent review for a further survey on the amplification speed and the PCR reaction volume.[37]

7. Approaches to Increasing Single-Molecule Amplification Efficiency on Microreactors

Amplification efficiency always receives much concern during genetic amplification, and single-molecule DNA amplification using microfluidics is not excluded from this. For microreactors etched on chips, the amplification efficiency is often limited by interactions between the inner surface and the biomolecules, primarily due to increased surface-to-volume ratio (SVR) in a microenvironment. Two surface passivation techniques have been applied to increase single-molecule amplification efficiency: dynamic and static passivation. The widely used dynamic reagents include BSA,[26,31−33,41,42,47,49−51,53,58,59,61−63,67,68,70,71,108,116] Tween-20,[29,55,69,79,89,90,116] and polyvinylpyrrolidone (PVP).[30,48,61,116] Other reagents have also been used as dynamic coating agents, such as betaine,[55,57] glycerol,[31,47,67−71] formamide,[31,47,69−71] Pluronic F38/F68 (a difunctional block copolymer surfactant terminating in primary hydroxyl groups),[67−71] and dimethyl sulfoxide (DMSO).[55] Since the passivation agent(s) are included in the reaction solution and this passivation actually occurs during the operation of single-molecule amplification, this passivation approach is simple and time-saving. The static passivation involves the precoating of chip surface during chip fabrication or before use with one of the following substrates: SiO_2,[30,32,46−49] BSA,[30,48,63] PEG,[31] PVP,[41] parylene C,[65,66] or silanizing agents (e.g., dimethyldichlorosilane (DMDCS),[116] SigmaCote,[44] and 1*H*,1*H*,2*H*,2*H*-perfluorodecyltrichlorosilane (FDTS)).[53] The SiO_2 coating is a reproducible and inexpensive microfabrication process, and can be accomplished in mass production. BSA and other dynamic agents are preferably used in part due to their simplicity, but the reproducibility is argued. Silanization is also a widely used process to prevent surface adsorption of biomolecules, but it is time-consuming, labor-intensive, and even irreproducible. It is necessary to note that the use of small-volume chambers does not necessarily need dynamic or static passivation. For example, Dahl et al. used a dense microwell plate made from PP to detect targets down to single starting molecules in 200-nL reactors.[64] PP not only enables high flexibility in use and high-throughput capacity, but also it is more biocompatible, so it is perfect for single-molecule PCR amplification without any needed surface passivation. In addition, other proper approaches can also be used to improve single-molecule DNA amplification efficiency, such as longer annealing and extension times needed when the reaction is performed in a thin channel chamber,[41] bubble-free sample and reagent loading,[52] digestion of template DNA into short intact target DNA fragments,[76] and sample and reagent loading at low temperature (4 °C) to minimize RNA degradation.[52,90]

For droplet microreactors in emulsion, the composition of the emulsion oil and proper use of surfactants is critical for biocompatibility and for ease of droplet formation, as is stated above. In addition to this, others will also affect the amplification efficiency. For example, the mechanical process during emulsion can affect the existence state of DNA molecules in droplets. The DNA molecule in an expanded form should fit into the picoliter droplets, and the DNA molecule could not be present in the expanded form inside the nanosize droplet.[145] Also, the long molecules are very sensitive to the mechanical emulsion procedure, especially to sonication.[145,146] Therefore, the emulsion process should be optimized to increase single-molecule DNA amplification efficiency within droplet microreactors. In addition, droplet PCR parameters in emulsion can be optimized to improve amplification reactions, including aqueous/oil ratio, nucleotide concentration, $MgCl_2$ concentration, Taq polymerase, extension time, and PCR cycles.[142,144,149] A nested PCR strategy can be used to increase single-molecule PCR yield within droplet microreactors.[149] Finally, it should be noted that a new class of molecules such as PrimeSafe PCR additive can be used to increase single-molecule DNA amplification efficiency within microreactors. These small molecules improve PCR specificity by inhibiting polymerases in a temperature-dependent manner. At lower temperatures, they inhibit polymerase activity, but at higher temperatures the inhibition is completely relieved.[187]

8. Single-Molecule DNA Amplification Strategies on Microreactors

8.1. PCR for Single-Molecule DNA Amplification

8.1.1. Conventional Liquid-Phase PCR

Up to now, the most widely used strategy for the amplification of single DNA molecules at the microscale level is still conventional liquid-phase PCR, probably due to its maturation and clinical application obtained by benchtop PCR or to the relatively easier transfer from macro- to microscale. It can be performed by using on-chip etched microreactors including stationary multiple-chamber[26,29−33,45−52,54,55,61,64,68,77−84,86−88,90] and single-chamber[41,57,59,63] microreactors, and dynamic continuous-flow channel[56,116] microreactors, or w/o droplet[44,58,62,66,108,136−138,145,155,157,176,180] microreactors. However, associated with the development of conventional liquid-phase PCR, its evolved strategies of single-molecule nucleic acid amplification have been developed for specific applications, such as liquid-phase linking emulsion PCR (LE-PCR),[146,147] which combines linking PCR and emulsion PCR to produce a haplotyping technology, and liquid-phase RT-PCR in droplet microreactors[65,108,156] or microreactors etched on chips.[31,52,53,60,73] Single-molecule RT-PCR using batch-generated w/o emulsion is an easier way to detect a single RNA molecule than the conventional single-molecule RT-PCR method,[188] because it does not require very complicated optimization, for example, of the sequence of the primers, the annealing temperature, and concentration of magnesium ions, which is necessary each time the conditions of PCR are changed.[156] In microfabricated compartments, RT-PCR in 450-pL reactors has been demonstrated down to an estimated level of 34 RNA copies.[60] Nanoliter-volume RT-PCR has been integrated to on-chip

CE to detect <11 RNA copies in 45 min[52] or analyze single-cell gene expression in <75 min.[73] Recently, high-throughput real-time quantitative PCR in 33-nL reactors has detected single cDNA molecules that are reverse transcribed from the corresponding RNAs.[31] Most recently, another on-chip microfluidic real-time RT-PCR instrument has been developed for generating monodisperse picoliter reactors for real-time detection of single RNA genome copies.[53] It is worth noting that other PCR strategies have been developed to further improve single-molecule PCR within microreactors, including nested or seminested PCR[26,43,158,189−191] and touch-down PCR.[51,149−151,191] The nested PCR[192] has the added advantage of mitigating against primer-dimer and single-primer artifacts and thus increases the specificity of PCR. Touch-down PCR[193] can be used to circumvent spurious priming during single-molecule amplification and is suitable to multiplex single-molecule amplification reactions because several primer sets may have slightly different melting temperatures.[51]

8.1.2. Solid-Phase PCR

8.1.2.1. On-Bead Solid-Phase PCR. Although liquid-phase single-molecule PCR (or RT-PCR) using microfluidics has been well developed, the downstream detection and further applications for other purposes of its products are usually limited. In order to circumvent these drawbacks, great efforts have been made to develop solid-phase single-molecule PCR using microfluidics. In solid-phase PCR, one of the primers is immobilized on a solid support, and thus it allows clonal amplification of each template molecule onto a solid support. To date, solid-phase PCR on a bead has been well studied.[72,140−144,148−154,158,159] Many beads can be used for this procedure, such as sepharose[72,154] and polystyrene,[158] and the mean diameter of beads ranges from 1 μm[140,142,149,158,159] to 28 μm[141] or ever higher.[72,154] Dynal MyOne beads[142,149−151,159] or Dynabeads M-270[152] are uniform, which is especially advantageous for flow cytometry. Other magnetic beads (such as Sera-Mag particles from Seradyn) have more surface streptavidin molecules than MyOne and can be used when surface density is more important than uniformity.[149] Larger beads have even more surface streptavidin molecules but are much more expensive, and the emulsion formulation needs to be changed to make them efficient supports.[149] In addition, nonmagnetic beads can be used,[141,142] though they are more difficult to handle because centrifugation rather than magnets must be used to manipulate them. Streptavidin beads have been widely used because of the simplicity of coupling biotinylated oligonucleotides to them.[140,141,149,158] Of course, it is likely that amino-, sulfhydryl-, or carboxyl-modified oligonucleotides covalently coupled to beads modified with corresponding groups can also function as bead-bound primers for bead solid-phase PCR.[140] It is noted here that oligonucleotides with just a single 5′-biotin group will dissociate from the beads during temperature cycling. In order to overcome this, oligonucleotides labeled with dual biotin groups at their 5′ end can be used to be stable to cycling.[140,159] In addition, it should be noted that the slide-based solid-phase PCR has been integrated onto an emulsion droplet system for detecting many DNA fragments, where a set of primers are immobilized on a slide. Different free primer sets and PCR components are dispersed in different emulsion droplets before thermocycling, and then they will be released to the surface of the slide with previously immobilized primers, which is followed by PCR amplification on the slide.[194] Within the newly proposed method, thermal instability of the emulsion droplet system is utilized as a positive factor for multiplex detection of DNA fragments, which eliminates the problem of primer interference that is common to conventional multiplex PCR.

The beads generated by emulsion PCR have highly desirable characteristics: high signal density, geometric uniformity, strong feature separation, and a size that is small but still resolvable by inexpensive optics.[142] They have enabled several novel applications, such as BEAMing[140,144,148−151,159] or its improved version BEA (bead-emulsion amplification)[152,154] strategy, and ultrahigh-throughput DNA sequencing.[141−143] BEAMing stands for beads, emulsion, amplification, and magnetics. After PCR preamplification of DNA samples, single DNA molecules are amplified within w/o emulsion droplets and bound to magnetic beads precoated with oligonucleotide primers. Each bead then contains multiple copies of the DNA fragment identical in sequence to the original. This strategy makes use of paramagnetic DNA capture beads permitting bulk enrichment of the total bead population including both PCR-positive and PCR-negative beads. Figure 13 is the schematic of the BEAMing strategy. Recovery of the magnetic beads allows hybridization of fluorescently labeled oligonucleotide probes capable of specific detection and quantification of genetic sequence variants,[140,148] the direct determination of polymerase error rates,[159] and the identification of transcription factor targets.[144] Although BEAMing is a robust technology and becomes an advancement of the current amplification methods, there are some limitations to this technology, such as limitation of the size of the PCR products (usually <200 bp), the need to prepare a different emulsion for each amplicon to be queried, and very inefficient PCR priming by oligonucleotides coupled to beads compared with the priming by the same oligonucleotides when free in solution.[140] However, these limitations can be overcome by the development and use of the novel functionalized magnetic beads,[195] thermostable DNA polymerases[196] and emulsion-making procedures. For example, Tiemann-Boege et al. have identified BEA reaction conditions that efficiently produce longer PCR products of up to 450 bp, including the use of a Titanium Taq amplification system.[152] Alternatively, for solid-phase bead PCR described by Margulies et al.,[141] enrichment of amplified beads is accomplished by hybridization to larger, less dense non-magnetic "capture" beads bearing a DNA sequence complementary to the emulsion PCR amplicon sequence. Once hybridized, this density difference between the amplified bead/capture bead complexes and unamplified beads is exploited by centrifugation. The less-dense complexes in the supernatant recovered are melted with NaOH, and enriched amplified beads are recovered by magnetic separation. Because PCR primers are utilized as the hybridization target, any PCR amplicon can be enriched without *a priori* sequence knowledge, allowing true *de novo* sequencing or analysis of complex genomic mixtures.

With on-bead solid-phase PCR to produce the template for sequencing, two ultrahigh-throughput DNA sequencing methods have been described recently, such as picoliter plate pyrosequencing (454 sequencing)[141] and multiplex polymerase colony (polony) sequencing.[142,143] However, the sequencing systems themselves are very different. For the 454 sequencing, the emulsion compartmentation is combined with a readout on microfabricated arrays of wells. Genomic DNA is first fragmented and ligated to adaptor DNA

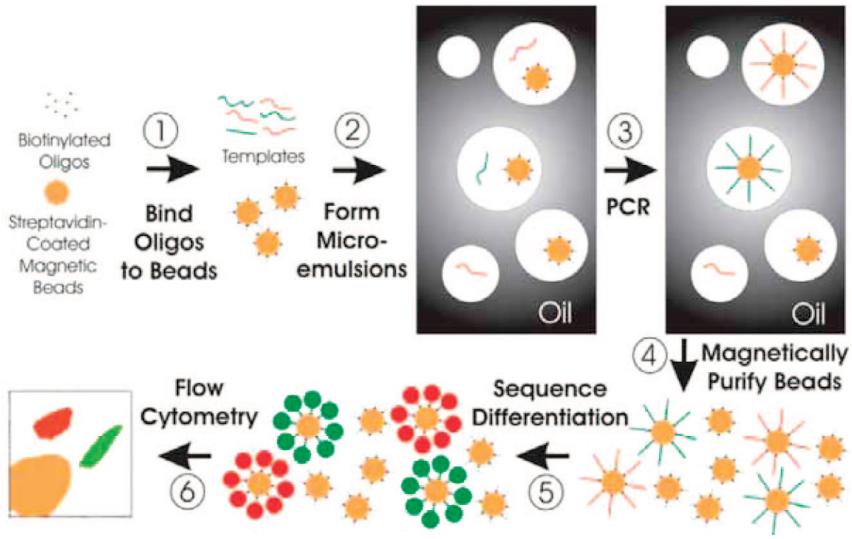

Figure 13. Schematic of BEAMing. Step 1, magnetic beads covalently coated with streptavidin are bound to biotinylated oligonucleotides (oligos). Step 2,: A solution containing all PCR components and primer-bound beads is stirred together with an oil/detergent mix to create microemulsions. The aqueous compartments (white circles in the gray oil layer) contain <1 template molecule and <1 bead on average. Red and green templates represent two template molecules, the sequences of which differ by one or many nucleotides. Step 3, The microemulsions are cycled in a conventional cycler. If a DNA template and a bead are present in a single compartment, the bead-bound oligonucleotides act as primers. The red and green lines connected to the beads represent products from two different templates. Step 4, The emulsions are broken, and the beads are purified with a magnet. Step 5, After denaturation, the beads are incubated with oligonucleotides that can distinguish between the sequences of the different templates. Fluorescently labeled antibodies then are used to label the bound hybridization probes, which renders the beads containing PCR product red or green after appropriate laser excitation. Step 6, Flow cytometry is used to count the red and green beads. Reprinted with permission from ref 140. Copyright 2003 National Academy of Sciences, U.S.A.

sequences. Single-stranded DNA fragments are then bonded to ~28 μm diameter beads so that the majority of beads contain at most a single DNA fragment. The beads are then compartmentalized within the emulsion droplets (the majority of aqueous droplets contain no more than one bead), and PCR amplification occurs within each droplet. After thermocycling, each bead contains roughly 10 million copies of the initial DNA fragment. To perform the sequencing, the beads carrying single-stranded DNA clones are subsequently loaded into picoliter-volume wells etched on the surface of a fiber-optic slide, which is imaged using a charge-coupled device (CCD) camera that can detect light emitted from the well during the pyrosequencing reaction. Figure 14 describes the picoliter-plate pyrosequencing technique. This technique is capable of sequencing a whole bacterial genome (25 million bases) at 99% or better accuracy in a 4-h period, which is approximately 100-fold increase in throughput over current Sanger sequencing technology. Within the multiplex polony sequencing approach reported by Church and colleagues,[142] a cell-free, mate-paired library provided single DNA molecules that were amplified in parallel to beads (~1 mm in diameter) by PCR in emulsion droplets. Then, template DNA carrying beads are immobilized in a polyacrylamide gel and mounted in a flow cell to allow automated cycles of sequencing by ligation and four-color imaging (Figure 15). This technique has been applied to resequence the genome of an evolved *E. coli* strain at less than one error per million consensus bases. Cost per base was roughly one-ninth as much as that of conventional sequencing. In addition, the reported protocols were implemented with off-the-shelf instrumentation and reagents.

8.1.2.2. In-Gel Solid-Phase PCR. The in-gel solid-phase amplification approach is sometimes also termed the molecule colony technique (MCT)[197] or nanocolony,[198] which was first invented by Chetverin and Chetverina, and used to reliably detect and quantify nucleic acids.[197−204] MCT amplifications are performed in a thin gel solid medium, resulting in the formation of molecular colonies in a 2-D pattern. Each colony comprises a molecule clone; namely, many copies of a single parental molecule. That is, the solid gel retards diffusion such that the resulting PCR product is localized in a spherical region surrounding the original template molecule. Very similar to this approach, the DNA polony was subsequently performed in 1999 by the Church group.[191] By including an Acrydite modification on the 5′ end of one of the primers, the PCR-amplified DNA in each polony can be covalently attached by one of its ends to a thin layer of heat-resistant polyacrylamide (PAA) attached to a standard microscopy slide (Figure 16). These localized polonies can subsequently be queried in parallel by *in situ* probing[205−211] or sequencing.[142,212] It should be noted that when using the multiplex polony sequencing reported by Shendure et al., cost per base is roughly one-ninth as much as that of conventional sequencing.[142] Compared with solid-phase PCR on bead, solid-phase PCR in a gel is performed in a 2-D pattern, and digital nucleic acid amplification (e.g., digital PCR) is easy obtained. In addition, the intrinsic resolving power of the PCR-MCT gel is extremely high, and it can readily detect a low quantity of molecules of interest in samples containing a huge amount of nontarget molecules. Moreover, the PCR polony technique in a gel possesses all the advantages of conventional digital PCR but is technically simpler and more economical.[197] It needs to be noted that in-gel MDA at the single-molecule level has very recently been applied to amplify long DNA fragments in order to determine the haplotypes of seven single nucleotide polymorphisms (SNPs) spanning 240 kb of the DNA surrounding the human ataxia telangiectasia mutated gene region on chromosome 11.[213] In addition, the loop-mediated isothermal amplification (LAMP) reaction of a single DNA molecule has been performed and directly observed in the PAA gel-based microchamber using an integrated, localized heating system.[214]

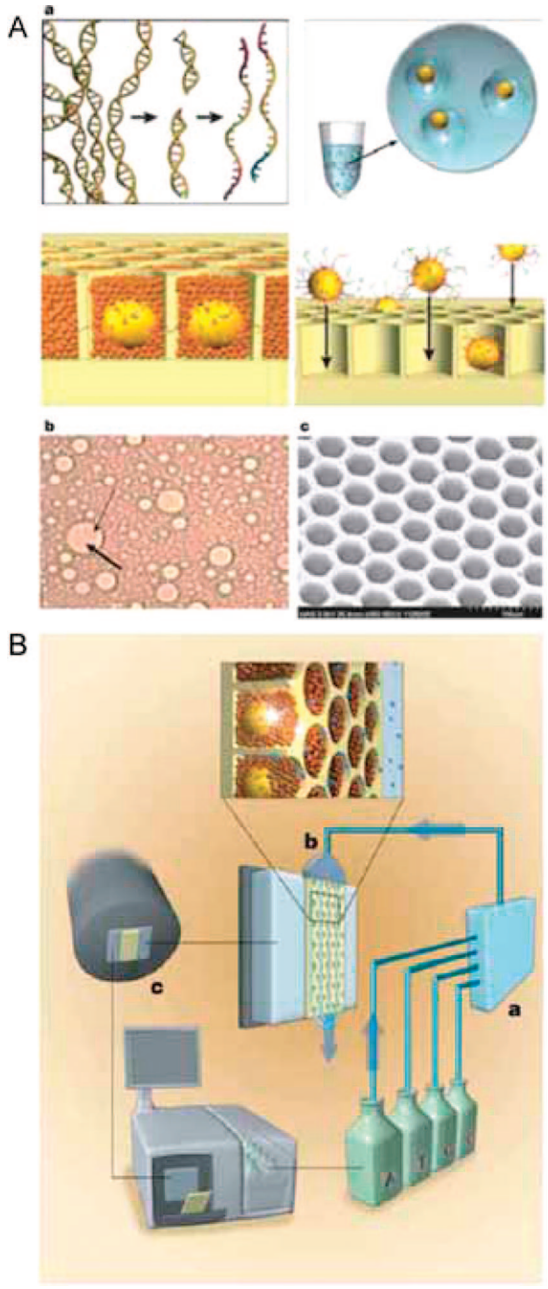

Figure 14. Picoliter-plate pyrosequencing. (A) Sample preparation: (a) Genomic DNA is fragmented, ligated to adapter DNA sequences, and separated into single strands (top left). Fragments are bound to beads under conditions that favor one fragment per bead, the beads are captured in the droplets of a PCR-reaction-mixture-in-oil emulsion, and PCR amplification occurs within each droplet, resulting in beads each carrying ten million copies of a unique DNA template (top right). The emulsion is broken, the DNA strands are denatured, and beads carrying single-stranded DNA clones are deposited into wells of a fiber-optic slide (bottom right). Smaller beads carrying immobilized enzymes required for pyrophosphate sequencing are deposited into each well (bottom left). (b) Microscope photograph of emulsion showing droplets containing a bead and empty droplets. The thin arrow points to a 28-mm bead; the thick arrow points to an approximately 100-mm droplet. (c) Scanning electron micrograph of a portion of a fiber-optic slide, showing fiber-optic cladding and wells before bead deposition. (B) Sequencing instrument. It consists of the following major subsystems: a fluidic assembly (a), a flow chamber that includes the well-containing fiber-optic slide (b), a CCD camera-based imaging assembly (c), and a computer that provides the necessary user interface and instrument control. Reprinted with permission from ref 141. Copyright 2005 MacMillan Publishers Ltd.

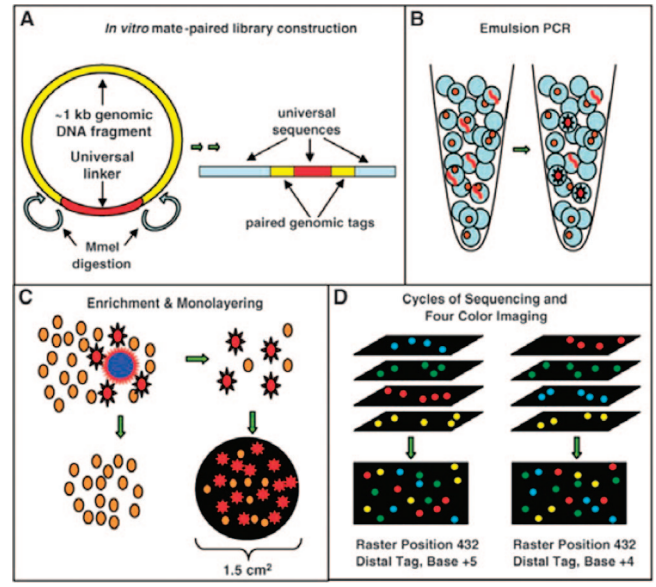

Figure 15. A multiplex approach to genome sequencing. (A) Sheared, size-selected genomic fragments (yellow) are circularized with a linker (red) bearing *Mme* I recognition sites. Subsequent steps, which include a RCA, yield the 134−136-bp mate-paired library molecules shown at right. (B) Emulsion PCR yields clonal template amplification on 1-mm beads. (C) Hybridization to nonmagnetic, low-density "capture beads" (dark blue) permits enrichment of the amplified fraction (red) of magnetic ePCR beads by centrifugation. Beads are immobilized and mounted in a flow cell for automated sequencing. (D) At each sequencing cycle, four-color imaging is performed across several hundred raster positions to determine the sequence of each amplified bead at a specific position in one of the tags. Reprinted with permission from ref 142. Copyright 2005 the American Association for the Advancement of Science.

8.1.3. Digital PCR Using Microfluidics

8.1.3.1. Development and Principle of Digital PCR. Let us trace the development history of conventional digital PCR. In 1992, Sykes et al. presented a method for quantification of PCR target samples by using the limiting dilution analysis and Poisson statistics.[215] An optimized two-round PCR strategy was developed to detect the rearranged immunoglobulin heavy chain (IgH) gene derived from a leukemic clone against an excess of rearranged IgH genes from normal lymphocytes. The remarkable feature of this research was that amplification took place in an "all-or-none" fashion, and that the number of input template molecules was determined by simply counting the number of positive PCR results. Therefore, this study opened the door for digital PCR by the use of a plus/minus readout per reaction. However, to achieve single-molecule sensitivity in this study, two sequential PCRs were performed by using nested sets of primers, and this easily results in problems of carry-over contamination. In 1997, Kalinina et al. described a novel nanoliter PCR method using 10-nL glass capillaries as PCR reaction systems.[26] In their study, to determine the number of template molecules in a sample may be feasible by counting the number of positive PCRs in a set of replicate reactions using terminally diluted sample, mainly because of the small reaction volumes and the use of TaqMan probes.[26] In addition, the potential of a microfluidic approach to limiting dilution PCR has been demonstrated for the first time by using limiting dilution target templates that were separated spatially in a capillary. Such an approach allowed discrete amplifications of targets to be detected; however,

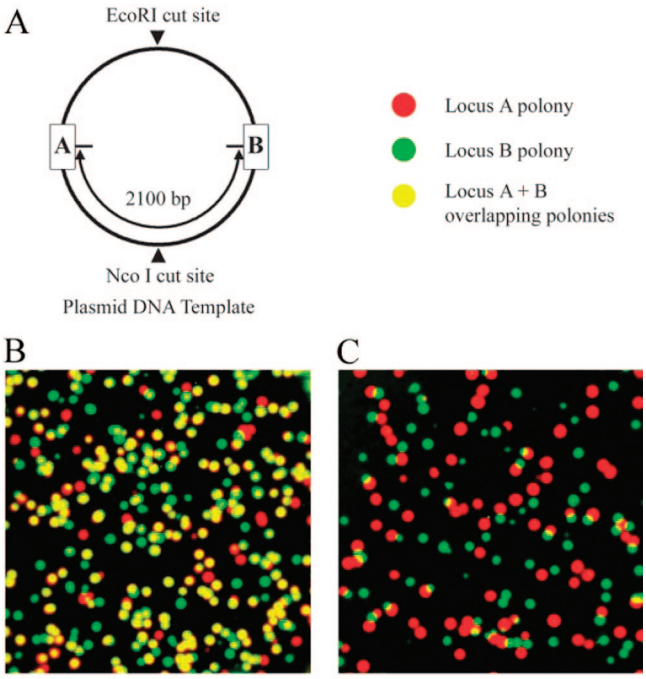

Figure 16. Amplification of two polonies from a single DNA molecule. (A) Plasmid DNA was cut with *Eco*RI, leaving locus A and locus B on the same template molecule or with *Eco*RI and *Nco*I, separating the loci. Polony amplification was performed with two pairs of primers designed to amplify loci A and B and hybridized cy5-labeled probe (red, locus A) and cy3-labeled probe (green, locus B) to the polonies. (B) Eighty-six percent of locus A polonies overlapped a locus B polony (and 80% of locus B polonies overlapped a locus A polony) when the plasmid was singly cut. (C) <2% of locus A polonies significantly overlapped a locus B polony when the plasmid was doubly cut. Reprinted with permission from ref 208. Copyright 2003 National Academy of Sciences, U.S.A.

throughput was limited and the detection system was labor-intensive. Subsequently, Vogelstein and Kinzler extended these searches to develop a new version of digital PCR using a 96-well plate for the PCR amplification system.[5] In fact, the groundbreaking "digital PCR" term was first put forward in this study. Up to now, this approach of digital amplification has been used for a number of applications.[216−219] However, the utility of this type of digital PCR is severely limited by the low number of reactions that can be performed by conventional PCR cyclers, the labor-intensiveness of performing hundreds to thousands of reactions for each sample, and the high costs associated with the large volumes of reagents and large numbers of microwell plates.

8.1.3.2. Microfluidic Digital PCR. Recent advances in digital microfluidic technology[123,190] have made possible that digital PCR can be performed in a high-throughput, automated, and low-sample-consumption format, where the single-molecule PCR or RT-PCR amplification and analysis are accomplished by partitioning the bulk sample into thousands of discrete reaction vessels. As a result, conventional digital PCR (typically in a 96- or 384-well plate) has evolved into microfluidic digital PCR. So far, the majority of on-chip microfluidic digital PCR systems are performed in an array chip format ("parallel format"). That is, the chip architecture allows samples to be partitioned into on-chip array reaction chambers such as the Fluidigm digital array,[29,76−90] the BioTrove OpenArray,[31,67−71] the silicon chamber array reported by Matsubara et al.,[30,48] and the PC chamber array arranged on a spiraling channel.[61] This microfluidic digital PCR format is performed very well in many aspects. However, the number of reactions and reaction volumes are hardwired into the chip, and thus there is no flexibility to adjust these parameters unless a different chip design is utilized. In addition, random and independent distribution of the target DNA molecules throughout the partitions of the digital panel and successful amplification from single molecules are critical to the validity of this microfluidic digital PCR format for accurately estimating target DNA copy number.[76] Very recently, Bhat et al. have investigated the major causes of uncertainty and bias with this array-based digital PCR, such as partition volume, amplification efficiency in the initial cycle(s), and length of intact target DNA fragments.[76] In order to overcome these shortcomings of the array chip system, some nonarray systems have been developed for microfluidic digital PCR reactions,[44,53,55,58,108] where the PCR mixtures flow through a reaction channel path as a "droplet train" ("serial format") and the thermal cycling is performed using a stationary[44,53,55] or continuous-flow[58,108] approach. Such systems can allow the number of reactions and the reaction volume to be flexibly selected during run time instead of preestablished by the chip architecture. However, such serial systems easily suffer from cross talk between molecules and molecule dispersions, and consequently appropriate two-phase flow systems are often required. In addition, these systems appear difficult to independently manipulate every droplet in terms of droplet specific reagent addition or when droplet specific protocols necessitating feedback loops are used. Thus, a droplet-based serial system is well adapted when droplets can be managed collectively in a batch mode all having the same protocol and reagents.[182] This limitation of a droplet-based serial system is mainly due to the global actuation mode, which is governed externally by pressure conditions existing at the channel entries or exits.[182] In order to overcome this, digitized droplets on a planar surface from one functional zone to another have been applied for single-molecule DNA amplification and analysis. Several techniques have been reported for manipulating individual droplets for microfluidic digital PCR on a planar chip, such as EWOD,[137] SAW,[138] and magnetic forces acting on magnetic beads in a droplet.[65,66]

It is necessary to point out that the use of high-throughput, small-volume, single-molecule PCR does not necessarily fall into the category of microfluidic digital PCR unless the optical interrogation of individual reactors, which is key to the digital PCR concept, can be performed.[62,72] On the contrary, microfluidic digital PCR does not necessarily perform single-molecule DNA amplification and analysis unless the average number of starting templates per reactor is about 1 according to Poisson statistics (in most cases, the average number of template molecules per reactor is less than 1, since many empty reactions are required to maximize the number of reactions containing only single-molecule targets.). In short, microfluidics allows control and manipulation of small volumes of reaction mixture, and therefore it is capable of rapid partitioning of single molecules and even single cells from a complex parent sample, offering the hope of extending the level of amplification precision and efficiency. However, on-chip microfluidic digital PCR is sometimes problematic. For example, due to random microscale defects of the interior surface coating or contamination by random interfering microparticles, the reaction uniformity and efficiency may be reduced.

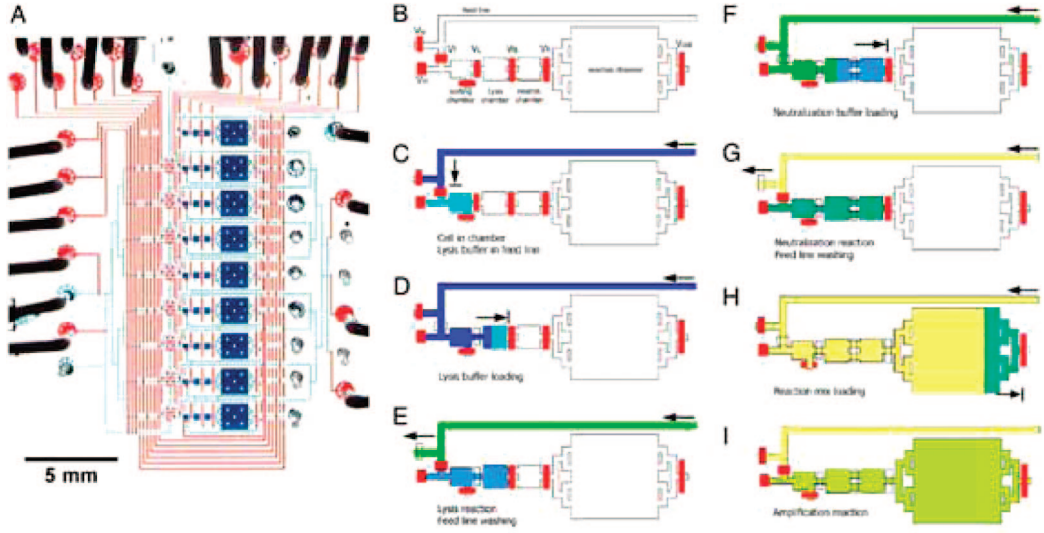

Figure 17. Single-cell genome amplification device. (A) Photograph of a single-cell isolation and MDA-based WGA chip capable of processing eight samples in parallel. To visualize the architecture, the channels and chambers have been filled with blue food coloring, and the control lines to actuate the valves have been filled with red food coloring (Scale bar, 5 mm.). (B) Schematic of a single amplification unit. The feed line is used to bring reagents into the chambers when the V_R valve is open and to the waste when the V_w valve is open. The V_{in} valve allows deposition of a single bacterium into the sorting chamber. The lysis (3.5 nL), neutralization (3.5 nL), and reaction chambers (50 nL) are utilized in sequence and are separated by individual valves V_L, V_N, and V_R, respectively. Valve V_{out} allows recovery of the amplified genomic material from the chip into an individual microfuge tube. (C) After a cell is trapped in the chamber, the feed line is filled with lysis buffer. (D) The lysis buffer is used to push the cell into the lysis chamber. (E) While the lysis buffer is mixing with the cell solution by diffusion, the feed line is flushed. (F) Neutralization buffer is loaded into the feed line and used to push the cell lysate into the neutralization chamber. (G) While the neutralization reaction is mixing by diffusion, the feed line is flushed. (H) The WGA reagents are loaded into the feed line and used to push the neutralized cell lysate into the reaction chamber. (I) The amplification reaction proceeds in a closed system comprising sorting, lysis, neutralization, and reaction chambers. Reprinted with permission from ref 28. Copyright 2007 National Academy of Sciences, U.S.A.

8.2. MDA for Single-Molecule DNA Amplification

MDA using Φ 29 DNA polymerase is an isothermal, non-PCR-based method for whole genome amplification (WGA) that was first developed in 2002 by Dean and colleagues.[2] This technique is an isothermal strand displacement amplification (SDA)[220] capable of yielding large numbers of amplification products from small biological samples. The high processivity and low error rate of Φ 29 DNA polymerase provides an advantage to MDA in terms of yield, accuracy, and coverage over the other PCR-based WGA methods. In this case, it may be especially important for single-cell WGA, since erroneous coping of the single template molecule in the first stages of the reaction may cause a false genotype. However, the conventional bulky MDA approach remains difficult for two primary reasons: (1) the confidence needed to assert the presence of single cells in microliter volumes and (2) the meticulous reagent cleaning and sample handling required to suppress background amplification in microliter-volume MDA.[28] In addition, formation of chimeras is an important limitation to the MDA method, particularly for whole genome sequencing.[221] These difficulties become even greater when complex environmental samples are used. In order to overcome these problems, an on-chip MDA method for single-molecule DNA analysis has been developed. Recently, Marcy and colleagues have developed a microfluidic device that allows the isolation and MDA-based WGA of single bacteria cells from human oral microbes, thereby enabling organism-level genomic analysis of complex microbial ecosystems without the need for culture (Figure 17).[28] This on-chip approach promises genetic analysis of any uncultivated minority member of a microbial community at the single-cell level. Subsequently, this group used a similar microfluidic device to isolate individual *E. coli* and amplify genomic DNA by MDA in 60-nL reactions, and it has been shown that reduced MDA reaction volume lowers nonspecific synthesis that can result from contaminant DNA templates and unfavorable interaction between primers.[27] One possible explanation for the fact that nanoliter reactors improve MDA of genomes from single cells is that the small volume reactions can reduce competition with contaminant or endogenously generated background, thus providing more DNA polymerase molecules per template and ensuring more uniform amplification. It is also possible that damage to the DNA template is reduced in the microfluidic system, and as a result it causes lower amplification bias.[27] Very recently, digital MDA in a gel has been developed for the isolation and multiple genotyping of long individual DNA fragments.[213] It was reported that the efficiency of in-gel MDA increases as the amount of target DNA is reduced most likely due to the saturation effect. This property is both fortuitous and necessary for molecular haplotyping because dilution of the target samples to a single copy number is required.

8.3. RCA for Single-Molecule DNA Amplification

RCA is a new isothermal amplification method that is useful both for signal and for target amplification.[3] It has been developed in two forms: linear and exponential. In linear RCA, a DNA circle is amplified by polymerase extension of a complementary primer, whereas exponential RCA uses a second DNA primer of identical sequence to the DNA circle. This amplification technique is attractive for single-molecule amplification from DNA or RNA samples in microliter volumes[3,222,223] since it is highly sensitive, highly specific, prone to be multiplex and high-

throughput, and easy to manipulate. Recently, small-scale RCA has attracted great interest.[159,224−226] For example, a RCA study using Φ29 DNA polymerase showed single-molecule amplification of circular 5−7-kb DNA templates and demonstrated that improved specificity could be achieved by reducing the volume of the RCA reaction from the standard 30 μL down to 600 nL.[225] The background amplification is dramatically suppressed by reducing Φ29 polymerase RCA volume, which often occurs when template is omitted or at low template concentrations. However, the effect of the lower volume on RCA amplification bias is still not determined. Recently, RCA has been used to increase the copy number bound to beads extended in single-molecule emulsion PCR by at least 2 orders of magnitude.[159] The 3′ ends of the PCR products attached to the beads were used as primers in RCA with Φ29 polymerase. In addition to the increased signal per bead, the SNR obtained upon analysis of beads was increased to 9000-fold. It is necessary to note that Mahmoudian et al. have recently developed an integrated platform for RCA and circle-to-circle (C2CA) of circular probe and downstream microchip CE analysis of a specific gene on a PMMA chip.[226] Although the detection of a clinical sample can be completed in less than 65 min (including RCA and microchip CE), a large amount of bacterial genomic DNA (25 ng) would be required, and thus the detection sensitivity is lower. In addition, digital HRCA in a droplet-based microfluidic device has very recently been developed for high-throughput single-molecule DNA amplification and analysis.[42] A microfluidic flow-focusing device with a 10 μm nozzle was used to produce a highly monodisperse emulsion of 2 pL droplets (Figure 9A). The results revealed that the HRCA yield was almost identical in droplets and in bulk at DNA concentrations corresponding to an average number of template molecules per droplet (λ) >1. However, at $\lambda < 1$, as expected, the yield of amplified DNA in droplets decreases rapidly compared with that in bulk due to the presence of droplets that contained no template DNA, which reduces the quantity of available dNTPs and primers.[42]

8.4. NASBA for Single-Molecule DNA Amplification

NASBA, initially introduced by Compton in 1991,[4] is an isothermal (41 °C) method specifically designed for amplification of any single-stranded RNA (including genomic RNA, mRNA, rRNA, and viriods) and DNA sequence, by using a combination of three enzymes: the avian myeloblastosis virus's reverse transcriptase/DNA polymerase, T7 RNA polymerase, and RNase H. This technology can produce more than 10^9 copies in 90 min and has been widely used for clinical diagnosis of human bacterial and viral pathogens, as well as for quantification and detection of microbes in environmental and food samples.[227] The first successful microfluidic NASBA chip, demonstrated in 2004 by Gulliksen et al.[228] using 10- and 50-nL silicon-glass chambers, performed real-time detection with the oligonucleotide target sample (human papillomavirus (HPV) 16 and artificial 118-bp single-stranded DNA) at a concentration of 1.0 and 0.1 μM, respectively. Subsequently, they demonstrated an improvement to the first design,[229] where real-time NASBA was used for detection of artificial HPV 16 sequences and SiHa cell line samples in cyclic olefin copolymer (COC) chips incorporating supply channels and parallel reaction channels with a detection volume of 80 nL. The presented system reached a sensitivity limit of 10^{-6} μM for artificial HPV 16 sequences, and 20 cells/μL for the SiHa cell line. Most recently, Dimov et al. reported the first monolithic integration of a microfluidic transfer-mRNA (tmRNA) analysis system that incorporates RNA purification, NASBA amplification, and real-time detection.[230] Device efficacy was demonstrated with real-time detection of 100 *E. coli* bacteria in 100 μL of crude lysates in less than 30 min from sample loading to answer. However, the detection sensitivity obtained on these three NASBA microchip platforms is still very low and is not close to a single-molecule level. Most recently, Morisset et al. successfully developed a novel NAIMA-based target amplification strategy allowing quantitative on-chip detection of GMOs with detection sensitivity down to two target copies.[227] NAIMA stands for NASBA implemented microarray analysis. It should be noted that the detection sensitivity of the NASBA is dependent on the target of interest and the quality of the targets and influenced by the design of the primers and the detection probe.[229] It should be possible that a single DNA or RNA molecule is amplified by using the NASBA technology under optimized conditions.

8.5. LAMP for Single-Molecule DNA Amplification

A novel isothermal nucleic acid amplification approach called LAMP has been reported in 2000 by Notomi et al..[231] It has attracted much attention as a potentially rapid, accurate, and cost-effective nucleic acid amplification method.[232] LAMP utilizes a set of specially designed primers (inner and outer primers) and a DNA polymerase with strand displacement activity to recognize and amplify the specific gene sequences. Large amounts of amplified products (>10^9 copies) are generally produced when subjected to a constant temperature between 60 and 65 °C within approximately 1 h. Although conventional bulk LAMP is currently still widely developed, the microfluidics-based LAMP has attracted great interest.[214,233−237] Hataoka et al. have been believed to be the first to miniaturize this amplification method by integrating LAMP and the subsequent electrophoresis analysis of amplification fragments on PMMA microchips.[233] Recently, the Lin group has demonstrated the feasibility of LAMP for the amplification and optical detection of HBV viral DNA in a multilayer PMMA microreactor, which includes an external temperature controller and an optical detection unit for quantitative output of a byproduct turbidity signal.[235−237] Although HBV DNA can be successfully and efficiently amplified and detected by using these compact devices within 1 h, a total of 25 μL of LAMP reaction volume is used due to practical limitations of turbidity detection. In addition, the sensitivity for the byproduct turbidity detection protocol is 50 copies/reaction.[236,237] The sensitivity of HBV LAMP reaction determined by agarose gel electrophoresis is 150 copies/reaction.[235] The low sensitivity obtained on these microdevices may denigrate the effectiveness of performing LAMP reaction in miniaturized reactors. Most recently, Lam et al. have successfully performed LAMP of a single DNA molecule in a PAA gel-based microchamber, where the integrated, localized heating system comprised of circular heater and temperature sensor, and optical microscopy was used for the real-time observation of LAMP reaction (Figure 18).[214] To date, this is the highest detection sensitivity obtained in LAMP microreactors reported previously. In addition, this device allowed amplification and detection to be completed within 50 min, which proved to be an advantageous improvement in detection time compared with a typical bulky LAMP assay. Here, it is worth noting that in

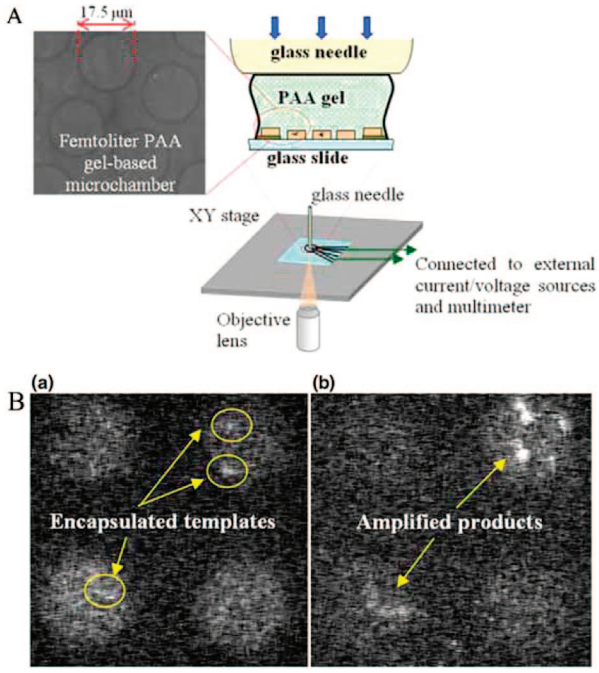

Figure 18. (A) Experimental setup with microscopic view of PAA gel-based microchamber used for LAMP of a single DNA molecule. (B) Fluorescent images of LAMP reaction in PAA gel-based microchambers. (a) Four microchambers under observation at room temperature (0 min). A single λ DNA template and two λ DNA templates, suspended in LAMP sample solution, were trapped in the bottom-left and top-right PAA gel-based microchambers, respectively. (b) Amplified products after 50 min of incubation at 65 °C. Because the current microscope system supported the imaging of fragments with sizes larger than 300 bp, the fragments clearly seen in panel b were DNA molecules with high molecular weight. Reprinted with permission from ref 214. Copyright 2008 Springer Science+Business Media, LLC.

addition to LAMP, MDA, RCA, and NASBA, other isothermal nucleic acid amplification techniques using microfluidics can also be used to perform single-molecule DNA amplification and analysis, such as recombinase polymerase amplification (RPA). RPA is a new isothermal DNA amplification method that runs at 37 °C, which was first introduced by Piepenburg et al. in 2006.[238] Very recently, RPA in a centrifugal microfluidic foil cartridge has been developed to amplify single copies of the antibiotic resistance gene *mecA* within <15 min.[239]

9. Single-Molecule Genetic Amplification from Single Cells on Chips

Many aspects of both normal development and disease result from changes in the expression of multiple genes or gene mutation in individual cells.[240] Moreover, it is widely believed that cancer is a clonal disease (i.e., it arises from a single cell) and that tumor progression occurs due to molecular changes in individual cells.[241] Therefore, genetic amplification and analysis from individual cells at the single-molecule level is very important in many cases. Fortunately, the use of microfluidic devices offers promise for developing rapid and ultrahigh-sensitivity genetic analysis at the single-cell level. For "real" sample (containing target cells, rather than purified DNA) analysis, the first sensitive silicon microstructure, which could perform rapid real-time PCR analysis from a starting template concentration as low as 5 *Erwinia herbicola* vegetative cells, was reported by Belgrader and colleagues.[45] A positive amplicon signal was detected in less than 35 cycles after only 9 min. Lagally et al. reported an integrated portable genetic analysis instrument capable of detecting 2−3 intact *E. coli* cells within 200-nL reactors in less than 30 min.[51] Recently, single-cell DNA amplification and analysis from "real" cell samples using microfluidics have been developed by several groups.[27,28,72,89,90] It is also necessary to point out that Kaigala et al. have used microfluidic chip-based PCR amplification and CE analysis to detect as few as 1−2 viral particles from unprocessed urine samples that were applied directly onto the chip.[57] This is the first demonstration of a microfluidic diagnostic procedure for high-sensitivity BKV screening. In addition, the ability of an instrument and assay to detect target samples in the presence of high commensal backgrounds is critical.[29,41,51,59,185,186] Microfluidic digital PCR reported by Fan and Quake is able to detect aneuploidy in the presence of mosaics or contaminating maternal DNA.[29] VanDijken et al. used the integrated PCR-CE chip to consistently detect in clinical samples as few as 0.1% t(4;14)(p16;q32) chromosomal reciprocal translocation-positive KMS-18 (a human myeloma cell line) cells within the normal patient blood cells (1/1000).[59] For clinical diagnosis, PCR detection of human immunodeficiency virus (HIV)-1 often requires finding low-copy-number blood-borne infectious agents in the presence of high-copy-number host DNAs. Li et al. performed detection of individual copies of HIV-1 virus DNA, in which HIV-1 negative control DNA (human placenta DNA) was present to mimic the abundant host nucleic acid.[41] Here, it is necessary to note that single-cell RT-PCR using microfluidics has been used to perform gene expression analysis for studying diverse biological systems.[65,73,108,242] In addition, microfluidic technology has been well utilized to isolate picogram and subpicogram mRNA templates from single cells, as well as to synthesize cDNA from these templates.[243] However, PCR amplification was carried out off-line using conventional thermal cycling. Very recently, a high-density microcavity array has been developed for isolation and detection of target cells from limited biological samples, where thousands of single cells were neatly arrayed onto 10 000 microcavities with high efficiency at approximately 90% of the loaded cells.[244] However, single-cell RT-PCR is not integrated onto the array chip, and single cells are manually isolated from the microcavities for RT-PCR. Recently, Bontoux et al. have integrated cell capture, lysis, and efficient RT in a 10 nL PDMS rotary mixer, followed by template-switching PCR (TS-PCR) amplification on chip, which allows for sensitive whole transcriptome analysis of a single cell amount of total RNA (10 pg).[242]

10. Sample Handling for Single-Molecule DNA Amplification within Microreactors

10.1. Technical Challenges Associated with the Integration of Sample Handling into Microfluidic DNA Amplification

One of the main challenges in miniaturization of nucleic acid amplification and analysis is the integration of sample handling (cell concentration and DNA/RNA extraction[245,246]) to perform a serial of online operations without the need for external macroapparatus or manual operation for nucleic acid extraction. Whether routine DNA amplification or single-molecule DNA amplification is performed by using microreactor technology, extraction of nucleic acid from a raw

sample (tissue, blood, saliva, urine, etc.) is considered the very first stage. Despite the importance of this stage, on-chip sample handling or its integration with other analytical components has attracted little attention from the research community in comparison with the downstream on-chip amplification and detection stages.[34,35,38,40] This could be attributed to technical challenges due to the following factors: (1) Diversity and complexity of raw biological samples. Such raw samples probably contain (bio)chemicals, air bubbles, particulate substances, food residues, cell debris, etc. that have to be removed in order not to hamper the microfluidic operations or the subsequent PCR. Such a front-end task is highly specific and thus cannot always be done on the (sub)microscale.[179] (2) Variety of (bio)chemical approaches to be performed with the diverse samples for nucleic acid extraction.[247] In this case, it is relatively difficult to tailor an existing bench-scale nucleic acid extraction methodology (to be miniaturized onto a chip) to both the analyte of interest and the analytical technique employed. (3) Low initial concentration of the nucleic acid of interest in a given biological sample (e.g., cell-free fetal DNA in the plasma of pregnant woman). Surface absorption of biological samples on the microscale will exacerbate the sample preparation, and even result in a completely unsuccessful sample preparation. (4) Many benefits afforded through miniaturization lie in improved performance characteristics of the analytical principle.[248] For example, downsizing of PCR has repeatedly been shown to yield distinct advantages when compared with conventional PCR formats (such as reduced amplification time and extremely low reagent consumption). Consequently, it is perhaps unsurprising that little research has focused on the integration of sample handling with subsequent other functional components.

10.2. Sample Handling Protocols on Chips

Despite these challenges, great attempts have been made to explore the simple and inexpensive on-chip sample handing protocols for (quasi-) real-world biological samples. Here, great attention has been focused on microfluidic devices coupling pre-PCR sample handling to downstream PCR. Literature reporting pre-PCR sample operations integrated with PCR on single chip devices have been summarized in Table 6. The reported monolithic PCR systems can directly receive relatively raw samples, such as whole blood,[249−253,255,259,179] serum,[255,259] human nasopharyngeal/buccal swat sample,[139,253,255] and bacterial or viral cells.[65,185,256−258,260−262] Typical sample handling techniques employed include 3-D-based mechanical filtration,[249−251,257] SPE,[139,253,255] immunomagnetic separation,[252,258,259,179,185] and dielectrophoresis.[262] The principle of filtration is relatively simple. Particulates can be trapped within the structure when the diameter of the particulate is larger than the feature dimensions of the microstructures, and flow is unimpeded. Among the approaches creating the microfilters, a popular approach is to create pillar structures within fluidic channels to mimic bench-scale filters. Kricka's group has used this type of microfilter with different designs to isolate white blood cells from human whole blood in silicon−glass microchips.[249−251] Genomic DNA from the white blood cells isolated on the microfilters was directly amplified using PCR. Cady et al. used silica-coated pillar microstructures to selectively bind DNA from the lysate of *Listeria monocytogenes* cells in the presence of the chaotropic salt guanidinium isothiocyanate.[257] In principle, this sample processing is a method of SPE that involves coating channel walls with a high affinity stationary phase. SPE is a broad technique in which a target molecule is retained by a stationary phase material and then eluted in an appropriate solvent. In general, SPE acts as both a sample washing method and a sample concentration method. Recently, Easley et al. have reported an integrated SPE-PCR device in which SPE was performed using silica beads packed in the SPE domain against the etched weir.[253] Since the sample handling processes were integrated on a single chip, along with the online product detection techniques, the total analysis process could be completed within less than 25 min (<10 min for DNA extraction, 11 min for PCR, and <3 min for separation detection). Nearly at the same time, Legendre et al. reported a simple, valveless microfluidic sample preparation device for SPE extraction and amplification of DNA from 600-nL blood samples, in which the stationary phase material is hybrid silica bead/sol−gel.[255] More recently, Pipper et al. presented a completely different approach to integrate the SPE of RNA with subsequent RT-PCR on a single chip.[139] The SPE is based on on-chip free droplets manipulated with magnetic forces, in which the processing sequence of droplets (merge, mix, and then split) emulates a two-dimensional SPE. It was reported that the extraction of low copies of RNA from a 100-μL throat swab sample with a 100-nL suspension of superparamagnetic particles corresponds to a preconcentration by 500-fold. In addition to filtration and SPE, immunomagnetic microscale separation integrated with PCR/RT-PCR has also attracted significant interest.[179,185,252,258,259] Large-scale permanent magnets or electromagnets or microfabricated electromagnets[258,259] are used to collect the magnetic beads so that the bound DNA or proteins can be enriched and separated. The principle behind this process is to utilize specific DNA probes or antibody-bound magnetic beads to bind with the sample of interest, which is followed by the application of a magnetic field to collect and to extract the magnetic beads from the raw samples. It is noteworthy that in addition to pre-PCR sample handling integrated with PCR, the post-PCR purification process of amplified DNA can also be integrated onto a single microfluidic device for further applications.[66,186,263] For example, Tsuchiya et al. employed the droplet-based handling system with magnetic manipulation to integrate sample preparation, PCR or RT-PCR, and purification of amplified DNA on a single chip.[66] It was reported that the developed device could perform nucleic acid amplification and analysis from a number of templates that are equivalent to single cells. Very recently, Huang et al. reported an integrated microfluidic device that enables two-step gene synthesis (polymerase chain assembly (PCA) and PCR) and thermally enhanced solid-phase PCR purification using magnetic beads.[263] It has been reported that double-stranded PCR products generated in a 250 nL PCR microreactor are captured, purified, and preconcentrated by an oligonucleotide probe immobilized in an *in situ* polymerized gel matrix followed by thermal release and injection into the CE-separation channel.[186] In addition, sample handling also can be integrated with other nucleic acid amplification technologies on a single chip.[27,28] For example, Marcy et al. have developed a microfluidic device that allows the isolation and WGA of individual microbial cells, thereby enabling organism-level genomic analysis of complex microbial ecosystems without the need for culture.[28] Subsequently, they used a similar microfluidic device to isolate single *E. coli* cells and amplify genomic DNA by MDA in 60-nL reactions.[27]

Table 6. Microscale Sample Handling Integrated with PCR (RT-PCR) on a Single Chip

PCR type	substrate	source/volume, μL	target DNA	sample handing technique	reaction volume, μL	LOD	refs
stationary chamber	silicon/glass	human whole blood/3.5	202-bp DNA fragment of dystrophin gene	on-chip "weir-type" filter for white blood cell isolation	50		249
stationary chamber	silicon/glass	human whole blood/1	379-bp endothelial nitric oxide synthase gene fragment	on-chip 667 high-aspect ratio pillars for white blood cell isolation	15		250
stationary chamber	silicon/glass	human whole blood/<3	226-bp region of human coagulation Factor V gene	on-chip "weir-type" filter for white blood cell isolation	12		251
stationary chamber	PC/PC	rabbit whole blood/10^3	221-bp *E. coli* K12-specific gene fragment	immunomagnetic cell capture and cell preconcentration/ purification/lysis for sample preparation	20	1000 cells	252
stationary chamber	glass/PDMS	mouse whole blood/0.75 or human nasopharyngeal wash/1	211-bp fragment of the virulence B gene (*B. anthracis*) or 181-bp fragment of the IS481 repeated insertion sequence (*B. pertussis*)	solid-phase extraction (SPE) using silica beads packed in the SPE domain against the etched weir	0.55	1500−2000 cfu (mouse whole blood)	253
stationary chamber	Zeone 690R (a polyolefin plastic)/ Zeone 690R	*B. subtilis* cells/50−400	90-bp *ftsA* gene fragement	on-chip SPE with a porous polymer monolith (PPM) embedded with silica particles, and bacterial chemical lysis	50		254
stationary chamber	glass/glass	human whole blood/4, diluted semen/500, or buccal epithelial cells settled/10	139-bp gelsolin-specific fragment, 212/218-bp amelogenin gene fragments, or 80-bp p16 gene fragment	hybrid silica bead/sol−gel SPE	0.33	600-nL blood	255
stationary chamber	silicon/glass	*E. coli* BL21/9	16S rRNA region of bacterial genome	cell lysis by laser-irradiated magnetic bead system (LIMBS), and magnetic beads for the removal of denatured proteins	4	200 cells	256
stationary chamber	PDMS/silicon	lysate of *L. monocytogenes* cells/10	544-bp fragment from the *L. monocytogenes* hlyA gene	DNA bound to silica-coated pillars in the presence of the chaotropic salt guanidinium isothiocyanate	50	10000 cells	257
stationary chamber	PDMS/PDMS	dengue virus serotype 2 and enterovirus (EV) 71 viruses/5	511-bp fragment of dengue virus and 331-bp fragment of the EV71-VP1 region	immunomagnetic bead-based virus capture, enrichment by microelectromagnets or permanent magnets, and virus RNA extraction	50	100 pfu/mL	258
stationary chamber	PDMS/PDMS	dengue virus serotype 2 in serum, EV 71, *Streptococcus pneumoniae* in blood, or group A streptococcus/ 100	171-bp DF-DR, 331-bp EV2449-EV2780, 273-bp lytA fw-rw, or 777-bp speB fw-rw	immunomagnetic bead-based virus capture, enrichment using by microelectromagnets or permanent magnets, and viral RNA extraction	25	100 pfu/mL	259
stationary chamber	silicon/glass	*E. coli* cells/0.1	209-bp fragment of β-lactamase gene	chemical/thermal lysis treatment	3.5		260
stationary chamber	PDMS/glass	*S. pneumoniae* cells	273-bp *lytA* gene fragment	thermal lysis of the cells	10		261
stationary chamber	silicon/glass	*L. monocytogenes* V7 cells/0.6	508-bp *prfA* gene fragment	dielectrophoresis for active cell trapping and concentration	0.6	10^5 cfu/mL (60 cells)	262
stationary chamber	PDMS/glass	mouse embryonic neuronal cells caudal ganglionic eminence (CGE) explants/<0.01	glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene fragments	cell capture using microvalves and micropumps, as well as chemical lysis	0.05	1 cell	242
stationary chamber	PDMS/glass	T lymphocyte Jurkat cells/<0.2	200-bp GAPDH and 247-bp 18S rRNA gene fragments	DNA-mediated cell capture and freeze−thaw lysis	0.2	1 cell	73

Table 6. Continued

PCR type	substrate	source/volume, μL	target DNA	sample handing technique	reaction volume, μL	LOD	refs
stationary chamber	PDMS/glass	*E. coli* K12 and O157 targets/ 10−100	259-bp KI#128 island (K12) and 191-bp OI#43 island (O157)	immunomagnetic cell capture and cell preconcentration	0.1	0.2 cfu/μL (10 cells)	185
stationary chamber	w/o droplet	K562 cells as the leukemia model/<1	A WT1 gene fragment	chemical lysis with magnetic force-based-droplet-handling system		1 cell	65
stationary chamber	w/o droplet	throat swat sample/100	114-bp fragment of the hemagglutinin segment of highly pathogenic avian influenza virus (HPAIV) H5N1	on-chip free droplet-based SPE of RNA	0.5	12 copies	139
continuous-flow	w/o droplet	human whole blood containing the GFP-transfected THP-1 cells/25	99-bp fragment of the transfected pmaxGFP vector	on-chip droplet-based immunomagnetic cell isolation, preconcentration, purification, and lysis by external permanent magnet	1.5	30	179

11. Product Detection Methods for Single-Molecule Genetic Amplification Using Microfluidics

11.1. Off-Line Detection

All existing nucleic acid detection methods can be used for off-line detection of single-molecule amplification products. Due in part to cost-effectiveness, the use of intercalators in combination with gel electrophoresis is still the most popular method for the detection of postamplification products.[32,41,42,58,62,65,66,93,116,137,138,144,145,155,191,214] In this method, the DNA products are effectively labeled with an intercalator dye and subsequently separated according to their sizes. This technique is very simple and highly versatile. However, it can hardly determine the amplification state of individual microreactors in emulsion unless the products within the droplet reactors are physically separated before gel electrophoresis. With the bead solid-phase PCR technique, the physical separation of individual products can be accomplished with antibodies to modified nucleotides incorporated into the amplified products on bead. In addition, by this technique it is difficult to differentiate between specific and nonspecific amplification products. It should be noted that for in-gel solid-phase amplification, other off-line detection methods can be used, such as radioactive probes,[197] the standard curve method of real-time quantitative PCR,[213] *in situ* probing,[205−211] and sequencing.[142,212]

Flow cytometry is another widely used off-line technique for single-molecule amplification product detection. After bead PCR amplification, the droplets are broken, and the beads are recovered and rapidly analyzed via flow cytometry.[72,140,144,148−151,153,159] In addition, a water-in-oil-in-water (w/o/w) double emulsion can also be sorted by flow cytometry for product detection.[189] Flow cytometers equipped with multiple lasers and filters have the capacity to distinguish multiallelic loci and perform multiplex analysis of several genes simultaneously and thus provide extensive biomedical applications in combination with single-molecule DNA amplification. In addition to the analytical power of flow cytometry, fluorescence-activated cell sorter (FACS) instruments can separate specific populations of beads for further analysis.[140] It is necessary to note that FACS technology has been recently used for sample enrichment and titration in 454 sequencing so as to determine the optimal DNA-to-bead ratio prior to sequencing.[264] In recent years, flow cytometry on microfluidic chips has been in intensive study,[265] and this is creating new opportunities for integrating single-molecule DNA amplification or other functional components and flow cytometry analysis on a single device. In addition to flow cytometry and gel electrophoresis, other techniques have also found wide applications in product detection of single-molecule DNA amplification using microfluidics, such as sequencing[27,57,62,72,93,142,143] and CE separation.[46,47,58,92]

11.2. Online Detection

Off-line product detection is usually time-consuming and labor intensive. Moreover, it likely increases the risk of cross-contamination due to the manual sample loading. In order to overcome these drawbacks, great efforts have been made to develop online product detection methods, including fluorescence-based real-time and end-point detection, integrated PCR-CE detection, and integrated on-chip PCR−DNA hybridization detection (Table 7). Fluorescence-based real-time and end-point DNA detection is a powerful and important detection technique, and it has been widely used to online detect single-molecule amplification product using microfluidics. The TaqMan probe, which is complementary to an internal segment of the target DNA to be amplified, is labeled with a fluorescence reporter dye at the 5′ end and a fluorescence quencher dye at the 3′ end, where the fluorescence from the reporter dye is largely quenched by the quencher dye as a result of fluorescence resonance energy transfer (FRET). Due to its high specificity and sensitivity, as well as its ease of use and automation,[26] the TaqMan assay has become the commonly used approach for online amplicon detection (Table 7). However, the use of sequence-nonspecific fluorescence intercalating dyes (e.g., SYBR Green) makes specific and nonspecific products difficult to differentiate. Now, Invitrogen supplies an improved version of their SYBR Green dye, called SYBR GreenER, which may eliminate some background fluorescence problems associated with the old version and reduce the risk of primer-dimer formation at the beginning of the reaction due to the new enzyme formulation in SYBR GreenER.[187] It is necessary to note that various types of fluorescence dyes such as

Table 7. Online Detection Techniques for Single-Molecule Genetic Amplification Using Microfluidics

detection technique	refs
Real-Time	
TaqMan probe	44, 45, 53, 58, 64, 76, 77, 82−87, 107, 116, 157, 176
SYBR green dye	31, 46, 47, 67−71, 75, 138, 180
LCGreen Plus dye	61
EvaGreen dye	137
End Point	
TaqMan probe	26, 30, 32, 48, 49, 55, 60, 77−84, 86−90
SYBR green II dye	214
RDT-D1dye (ds-DNA intercalating dye)	42
Chip-Based CE	33, 41, 50−52, 54, 57, 59, 63
Integrated On-Chip PCR−DNA Hybridization Detection	138

SYBR gold, SYBR green I, SYBR green II, and EVAGreen may have different effects on the single-molecule DNA amplification reaction. For example, SYBR gold, SYBR green I, and EVAGreen inhibit the LAMP reaction; however, SYBR green II did not inhibit this reaction.[214] In addition, fluorescence-based real-time detection is difficult to apply to single-molecule PCR in bulky multidisperse emulsion droplets. To perform real-time detection, the droplets must be focused into a channel so background fluorescence from droplets outside the focal plane does not affect the fluorescence intensity measurement, allowing optical interrogation of individual reactors.[53] Another important online detection technique for single-molecule amplification products is CE integrated with PCR. CE is first demonstrated on-chip by the Mathies group for single-molecule DNA amplification and analysis,[33] and in recent years, an improved version of the integrated PCR-CE device has been reported.[50−52,63,73,185,186] The integration of PCR and CE on chip can allow the total analysis process at the single-molecule limit to be accomplished in less than 30 min.[51]

12. Integrated Systems for Single-Molecule DNA Amplification and Analysis

One of the most challenging issues that single-molecule DNA amplification and analysis using microfluidics has faced in recent and future years is the integration of functional compounds to automate multiple operations without the need for external bulky apparatus or manual operation. The benefits of microfluidics have always been the ability to integrate multiple functionalities on a single device, reduce analysis time, and minimize sample and reagent consumption. Therefore, an ideal single-molecule genetic analysis system should accept a complex biological fluid (e.g., blood, serum, or semen), remove PCR inhibitors, amplify a single DNA or RNA molecule, and, finally, detect the amplified products. A few groups have started to address this issue. For example, Mathies group has reported several monolithic integrated microfluidic DNA amplification and CE analysis systems containing microfabricated heaters, temperature sensors, and membrane microvalves to provide sample positioning and immobilization (Figure 19).[33,50−52,73,185,186] Recently, the Quake group has developed a microfluidic chip device that allows the sorting, lysis and genome amplification of individual cells, and is capable of processing nine samples in parallel.[27,28] Some examples of on-chip integrated systems for single-molecule DNA amplification and analysis have been summarized in Table 8. When compared with continuous-flow PCR, the stationary chamber PCR seems to be preferred for integrated systems for single-molecule DNA amplification and analysis. This is most likely due to its increased simplicity in sample handling and structural design. The most commonly integrated physical components are film resistive heaters, film temperature sensors, microvalves, and micropumps (see Table 8).

Although the partially integrated single-molecule DNA amplification and analysis systems have been successfully developed, the "complete" micro-total analysis system (μTAS) still requires further development. The bottlenecks blocking realization of a truly and highly integrated system include preamplification sample preparation and postamplification product detection. Since the source of raw biological samples is varied and the sample preparation methods are diverse, the miniaturization of conventional sample preparation and functionalities using microfluidics remains a challenge. As for online product detection, bulky optical detection systems such as CCD camera and LIF are difficult to miniaturize onto a chip. However, with the development of micro-electro-mechanical systems (MEMS) technology, optical microdevices such as light-emitting diode (LED) and photodiode can be applied onto the integrated system to realize a portable single-molecule DNA amplification and analysis device.

13. Applications of Single-Molecule Genetic Amplification Based on Microfluidics

In nature, single-molecule DNA amplification and analysis is well carried out in micrometer-sized spaces such as in

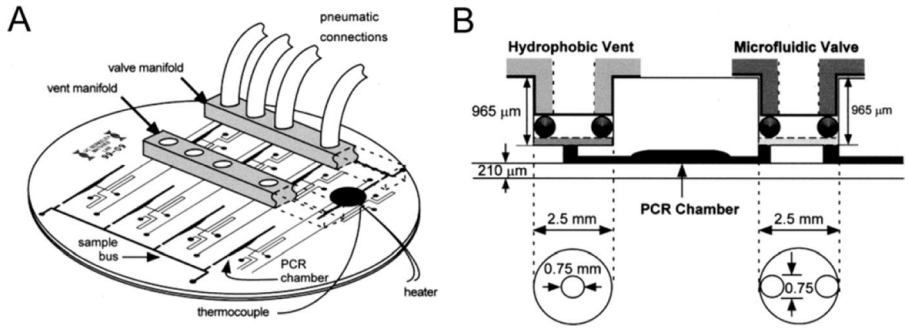

Figure 19. (A) Integrated microfluidic PCR-CE device for single-molecule DNA amplification and analysis. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. (B) Detailed schematic of microfluidic valves and hydrophobic vents. Reprinted with permission from ref 33. Copyright 2001 American Chemical Society.

Table 8. Examples of Integrated Systems for DNA Amplification and Analysis at the Single-Molecule Level

functional compounds	refs																														
	76-88			176, 157,	180	63	55	66	54	46	47, 75	72	44	57	107	33, 50	51	138	53	65, 242	52	73	108	58	62	137	27, 28	214
sample loading	•						•																					
sample preparation								•																		•		
cell capture, isolation, and/or lysis																				•		•					•	
microdroplet generator (μDG)												•	•						•				•	•	•	•		
RNA isolation																			•									
RT																			•	•	•	•	•					
stationary chamber PCR	•			•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•						
continuous-flow PCR																							•	•	•	•		
MDA																											•	
LAMP																												•
product capture/purification/concentration								•													•							
CE separation						•								•		•	•			•	•							
hybridization																		•										
sample injection						•																						
on-column labeling						•																						
infrared (IR) laser heating				•																								
film resistive heater						•	•				•				•		•	•			•	•						•
film temperature sensor											•						•	•			•	•						•
microfluidic pumping	•							•				•		•				•		•	•	•				•	•	
microfluidic valving	•					•			•			•		•		•	•				•	•					•	
microfluidic mixing	•						•	•												•						•	•	
microfluidic separating								•																				
cross or T-injector												•	•						•				•		•			
micro nozzle																								•				
mercury lamp				•						•			•						•									•
xenon lamp	•																											
bulky laser (or LIF)						•	•		•					•		•	•				•	•		•				
light emitting diode (LED)											•				•			•					•			•		
photodiode											•				•			•					•			•		
CCD camera	•			•			•			•			•					•	•									
spectrometer																							•					
image intensifier unit																												•
PMT						•										•	•							•				

reactors etched on chips and droplet reactors in emulsion. Microfluidics offers the capability to form femto- to picoliter sized reactors and to perform fast amplification reactions. Therefore, this clonal DNA amplification technology has enabled many novel applications in chemistry, biology, and medicine. Moreover, it has produced some new analytical technologies that have in turn very wide applications, including but not limited to ultrahigh-throughput 454 sequencing (Table 9), polony (Table 10), BEAMing or BEA (Table 11), microfluidic digital PCR (Table 12), or other microfluidic single-molecule DNA amplification and analysis technologies (Table 13). Among these applications, single-molecule emulsion PCR has been commercially developed by 454 Life Sciences with the aim of generating clonal templates from complex populations to enable sequencing.[141] The PCR products served as a template for sequencing DNA ranging from metagenomics to paleogenomics.[351] Currently, the GS20 Sequencer is being replaced by a second generation sequencer (FLX)[95] that has a longer read-length of 230 nt, a lower error rate, and a higher sequencing capacity. The lower error rate and higher sequencing capacity would both be likely to improve sensitivity.[341] In addition, routine 454 sequencing applications still require microgram quantities of initial material. Recently, Meyer et al. have reported a 454 sequencing library quantification method based on quantitative PCR that reduces the material demands of high-

Table 9. A Sea of Applications of 454 Sequencing Technology Using Single-Molecule Emulsion PCR for Sample Preparation

fields of application	refs	fields of application	refs
	Studies in Plant Biology		
analysis of total plant RNA sequences in a search for viruses associated with decline symptoms of Syrah grapevines	266	global analysis of gene expression in the SAMs of maize (*Zea mays* L.)	281
comparative sequencing of transcripts from two olive (Coratina and Tendellone) genotypes during fruit development	267	cell type-specific gene expression profiling in plants	282
sequencing of the transcriptomes of shoot apical meristems (SAMs) isolated from two inbred lines of maize for the high-throughput acquisition of gene-associated SNPs	268	assessment of the arbuscular mycorrhizal fungi (AMF) diversity in a boreonemoral forest to reveal partner specificity in AM symbiosis at the level of ecological groups	283
integration with microarray for large-scale gene expression analysis during berry maturation in nonmodel species (*Vitis vinifera*)	269	genome-wide analysis of nonribosomal peptide synthetase (NRPS) gene clusters and their peptides in a *Planktothrix rubescens* strain	284
assessment of the fungal diversity in different forest soils	270	*de novo* sequencing of barcoded bacterial artificial chromosome (BAC) pools for comprehensive gene survey and genome analysis in the complex genome of barley	285
targeted SNP discovery in a highly polyploid plant species such as sugar cane	271	global repeat discovery and estimation of genomic copy number in a large, complex soybean genome	286
sequencing *Medicago truncatula* expressed sequenced tags to address whether a 454 sequencing run provides new gene discovery from a normalized cDNA library	272	characterization of microsatellites and gene contents from high-throughput genome shotgun sequencing of the mungbean (*Vigna radiata* (L.) Wilczek) genomic DNA	287
generation of expressed sequence tags (ESTs) from the transcriptome of *Pythium ultimum*	273	integration with computational subtraction for characterization of unknown genetic modifications	288
sequencing of cDNA isolated using laser capture microdissection (LCM) from the developmentally important SAM of maize (*Zea mays* L.) for gene discovery and annotation	274	deep sampling of the *Palomero* maize transcriptome	289
transcript profiling in wild-type and *viviparous-1* mutant maize (*Zea mays*) plants	275	global characterization of *Artemisia annua* (*A. annua*) glandular trichome transcriptome	290
comparison of the fungal species richness, diversity and community composition among trees located within and outside a small urban center	276	sequencing of the complex genome of barley	291
sampling the waterhemp (*Amaranthus tuberculatus*) genome	277	whole-genome snapshot to discover the composition of the barley genome and to further provide evidence for parallel evolution of genome size in wheat and barley	292
comprehensive characterization of repetitive DNA in the pea (*Pisum sativum* L.) genome and its comparison to soybean and *Medicago truncatula*	278	definition of intergenic locations of rice centromeric chromatin	293
sequencing of the whole plastid genomes of the basal eudicot angiosperms *Nandina domestica* (Berberidaceae) and *Platanus occidentalis* (Platanaceae)	279	american beech ESTs produced by 454 sequencing used to evaluate if sim4 cm^3 is suitable for the annotation of plant genomes	294
high-throughput gene and SNP discovery in *Eucalyptus grandis*, an uncharacterized genome	280		
	Studies in Metagenomics		
metagenomic analyses of the marine viromes of four oceanic regions	295	analysis of nematode diversity from metagenomic samples	303
metagenomic analyses of environmental genome sequences from two sites in the Soudan Mine (Minnesota, USA)	296	accurate determination of microbial diversity in a lake	304
taxonomic estimation of metagenomic sequences	297	analysis of the metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter	305
evaluation on a metagenome of a bacterial community isolated from the environment	298	investigation of the complexity of anaerobic marine protistan communities by parallel tag sequencing (PTS)	306
phylogenetic characterization of a microbial community residing in a fermentation sample from a production-scale biogas plant fed with maize silage, green rye and liquid manure	299	insight into the plasmid metagenome of wastewater treatment plant (WWTP) bacteria showing reduced susceptibility to antimicrobial drugs	307
genetic analysis of the uncultivated minority member of a microbial community	28	analysis of an obesity-associated gut microbiome with increased capacity for energy harvest	308
metagenomic analysis of lysogeny in Tampa Bay with the implication for prophage gene expression	300	metagenomic analysis of the phylogenetic and functional diversity of microbial communities	309
revealment of major roles for horizontal gene transfer and plasmids in population diversity by coastal *Synechococcus* metagenome	301	definition of the healthy "core microbiome" of oral microbial communities	310
revealment of the presence of metagenomic islands of hyperhalophiles (*Salinibacter ruber*)	302		

Table 9. Continued

fields of application	refs	fields of application	refs
Studies in Medicine Diagnosis			
detection of low-frequency pretherapy chemokine (CXC motif) receptor 4 (CXCR4)-using HIV-1 virus	311	determination of detection frequency of minority HIV type 1 variants in antiretroviral-naive persons with reverse transcriptase codon 215 revertant mutations	328
description of profile of the circulating DNA in apparently healthy individuals	312	investigation of the sequence integrity of 4q24 candidate tumor suppressor gene *TET2* in myelodysplastic syndrome (MDS) patients with UPD on chromosome 4	329
high-resolution, high-throughput genotyping of the human leukocyte antigen (HLA) class I and class II loci	313	characterization and analysis of the transcriptome of human breast carcinoma cells and mapping p53 binding sites in the genome of human colorectal carcinoma cells	330
revealment of chromosome sorting and local allelic variants using whole genome sequencing of a natural recombinant *Toxoplasma gondii* strain	314	revealment of genomic diversity and evolution of *Mycobacterium ulcerans* (*M. ulcerans*)	331
analysis of HIV integration site distributions in resting and activated $CD4^+$ T cells infected in culture	315	sequencing of the full-length of the phosphatase and tensin homologue (PTEN) gene in hepatocellular carcinoma (HCC)	332
genome sequencing, annotation and analysis of *Mycoplasma conjunctivae* leading to infectious keratoconjunctivitis (IKC) in domestic sheep and wild caprinae	316	culture-independent identification of gut bacteria correlated with the onset of type 1 diabetes in a rat model	333
identification of nucleotide polymorphisms in a diverse sampling of Shiga toxin-producing *E. coli* O157:H7 strains of clinically ill human and bovine origin	317	expression analysis of the overlapping genes in a human breast cancer cDNA library	334
identification of differentially expressed alternatively spliced genes in malignant pleural mesothelioma (MPM)	318	ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters for individual human cancers and for the epigenetic classification of tumor subtypes	335
sequencing of cell-free plasma DNA for noninvasive prenatal diagnosis	79	sensitive mutation detection in heterogeneous lung cancer samples	336
draft genome sequencing of *Giardia intestinalis* assemblage B isolate GS for the better understanding of the cause of human giardiasis	319	sequencing of 273 amplificons from a cancerous human sample for evaluating data handling strategies	337
rapid high-throughput HLA genotyping for high-resolution allele identification	320	high-throughput isolation and sequencing of retroviral insertion sites using a splinkerette-PCR method coupled with capillary or 454 sequencing	338
investigation of a transcriptional sketch of a primary human breast cancer	321	characterization of circulating DNA by PTS	339
revealment of community-wide response of the gut microbiota to enteropathogenic *Citrobacter rodentium* infection	322	pyrosequencing of DNA from the cell-associated herpesvirus Marek's disease virus (MDV) purified using micrococcal nuclease digestion and poly(ethylene glycol) precipitation	340
insights to genome variation and evolution in *Salmonella typhi* that causes typhoid	323	detection of minor sequence variants in HIV-1 protease and reverse transcriptase genes from clinical plasma samples	341
sequencing of complete H5N1 HPAIV genomes	324	comprehensive resequence analysis (SNP and haplotype analysis) of a 136 kb region of human chromosome 8q24 associated with prostate and colon cancers	342
combinatorial algorithms for structural variation (SV) detection among individual genomes including cancer patients and others suffering from diseases of genomic origin	325	bisulfite sequencing of CG-rich DNA fragments, which reveals that methylation of many X-chromosomal CpG islands in female blood DNA is incomplete	343
sequencing of the genomes of two pediatric USA300 isolates (one community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) and one community-associated methicillin-susceptible *Staphylococcus aureus* (CA-MSSA)) for pathogenesis study	326	Studies of Ancient DNA (Paleobiology)	344−353
optical mapping and 454 sequencing of *E. coli* O157:H7 isolates linked to the US 2006 spinach-associated outbreak	327		
Studies in Small RNAs (smRNA) (Including MicroRNAs)			
microRNA discovery and profiling in human embryonic stem cells (hESC)	354	one-step identification of conserved microRNAs, their targets, potential transcription factors and effector genes of complete secondary metabolism pathways after pyrosequencing of calyx cDNAs from the Labiate *Salvia sclarea* L	363
identification of microRNAs from a basal eudicot (*Eschscholzia californica*) and conservation in flowering plants	355	systematic analysis of microRNAs in *Aedes aegypti* and multispecies survey of novel mosquito microRNAs	364
identification of MDV-encoded microRNAs	356	identification and analysis of microRNAs in human breast cancer and teratoma samples	365

Table 9. Continued

fields of application	refs	fields of application	refs
characterization of highly dynamic and sex-specific expression profiles of microRNAs during early embryonic stem (ES) cell differentiation	357	evaluation of the changes in *Nicotiana attenuate* (*N. attenuate*)'s smRNA profiles after RdR3 silencing in comparison with wild-type profiles	366
survey of microRNA and trans-acting siRNA (tasiRNA) diversity in mature pollen of *Arabidopsis thaliana*	358	characterization of *N. attenuate*'s smRNA transcriptome before and after insect-specific elicitation in wild-type and RdR1-silenced (irRdR1) plants	367
identification of microRNAs and other small regulatory RNAs	359	identification and expression analysis of infectious laryngotracheitis virus (ILTV) microRNAs	368
bioinformatics analysis of RNA base modifications in tRNAs and microRNAs using large numbers of cDNA sequences of smRNAs	360	discovery of new zebrafish microRNAs	369
large-scale sequence analyses of the nuclear genome, the mitochondrial genome and genome-wide microRNA identification in the Atlantic cod	361	deep evolution of metazoan microRNAs	370
investigation of the smRNA population of the transcriptome of green leaves from the *Populus trichocarpa*	362	discovery and characterization of novel and known microRNAs expressed in primary cultures of normal human ovarian surface epithelium (HOSE) and in tissue from three of the most common histotypes of ovarian cancer	371
	Studies in Other Fields		
evaluation of the power of microarray-based direct selection methods for enrichment of targeted sequences	372	identification of allele specific gene expression	397
identification or discovery of microsatellite(s)	344, 373	identification of extensive SV in the human genome	398
production of high quality draft sequences for prokaryotic genomes using the integrated sequencing technologies	374	assessment of the utility of reaction modifications	27, 221
genotyping of complex major histocompatibility complex (MHC) systems in nonmodel vertebrates.	375	assessment of mitochondrial genome sequence of the arbuscular mycorrhizal fungus *Glomus intraradices* isolate 494 and implications for the phylogenetic placement of Glomus	399
genomic DNA sequence comparison (identification of potential SNPs) between two inbred soybean cyst nematode (SCN) biotypes	376	sequencing of the *Mycoplasma genitalium* genome	141
sequencing of the whole 1.84 Mbp genome of *Prochlorococcus marinus* bacteria, and whole genome assembly from 454 sequencing output by modified DNA graph concept	377	PTS of six human mtDNA genomes	400
SNP detection with variants for low coverage, high coverage, and PCR amplicons	378	sequencing and *de novo* analysis of the transcriptome of planulae larvae from the coral *Acropora millepora*	401
investigation of the polyadenylation site of mimivirus transcripts obeying a stringent "hairpin rule"	379	PTS of human mitochondrial genomes	402
high-resolution analysis of human genome structure for identifying normal/pathogenic genome alteration	380	analysis of transcription during nerve-dependent limb regeneration in salamanders	403
determination of the complete mitochondrial genome sequence to understand the systematic status of of the large yellow croaker, *Larimichthys crocea* (Perciformes, Sciaenidae)	381	454 pyrosequencing-tailored nucleotide barcode design for large-scale sample multiplexing	404
analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces	382	definition of the *Manduca sexta* (*M. sexta*) larval midgut transcriptome with messages for digestion, detoxification and defense	405
integration with other sequencing technologies for *de novo* genome sequence assembly of a filamentous fungus (*Grosmannia clavigera*)	383	assessment of WGA-induced bias	406
identification of physical interactions between genomic elements with chromosome conformation capture carbon copy (5C) technology based on microarrays or quantitative 454 DNA sequencing	384	integration with sequence-independent amplification for sequencing and analysis of complete segmented double-stranded RNA (dsRNA) virus genomes	407
integration with other sequencing technologies for genome resequencing, especially in reconstructing large SVs at maximum accuracy and low cost	385	large-scale phylogenomic analysis of the common emperor scorpion (*Pandinus imperator*)	408
determination of the synchronous adenosine-to-inosine (A-to-I) RNA editing events	386	sequencing of multiple strains of *L. monocytogenes*	409
closing gaps in the human genome	387	gene expression profiling using *Drosophila melanogaster*	410
comparison of Sanger and 454 technologies for whole-genome sequencing of six marine bacterial trains	388	analysis of wasp gene expression for supporting an evolutionary link between maternal behavior and eusociality	411
gene discovery using massively parallel pyrosequencing to produce a substantial ESTs for the flesh fly *Sarcophaga crassipalpis*	389	integration with other approaches (size-selected genomic library and cross-species amplification of a mammal-wide set of conserved microsatellites) for development of microsatellite markers for the short-beaked echidna	412

Table 9. Continued

fields of application	refs
characterization of gonad transcriptomes in the polyploid lake sturgeon (*Acipenser fulvescens*)	390
generation of human sequence to evaluate applications of next-generation sequencing platforms for sequence-based association studies	391
transcriptomic and proteomic profiling of two porcine tissue samples (heart and skeletal muscle)	392
data of *Helicobacter pylori* genome 454 resequencing reads for evaluating EagleView tool	393
complete sequence determination of a novel reptile iridovirus isolated from soft-shelled turtle and evolutionary analysis of *Iridoviridae*	394
long-PCR based sequencing of the complete mitochondrial genome from single *Hemonchus contortus* (Nematoda)	395
analysis of nucleosome core positions in *Caenorhabditis elegans* (*C. elegans*) chromatin	396
characterization of the *Melitaea cinxia* transcriptome	413
determination of global regulation of RNA editing during brain development	414
comparison of next-generation sequencing technologies for transcriptome characterization	415
sequencing of picogram quantities of bacterial and mammalian DNA samples	87
characterization of poly A-transcripts expressed in HeLa cells	416
analysis of ESTs for *Manduca sexta* hemolymph proteins involved in immune responses	417

Table 10. Applications of Polony Technology (or MCT) Based on in-Gel Single-Molecule Genetic Amplification

fields of application	refs
detection of allelic variations of human gene expression	206
characterization of mutations and loss of heterozygosity of p53 and K-ras2 in pancreatic cancer cell lines	205
in vitro studies on chemical reactions between single RNA molecules	199, 202
simultaneous assay of DNA and RNA targets in the whole blood	203
detection and quantitation of viral nucleic acids	197
polony multiplex analysis of gene expression (PMAGE)	143
parallel analysis of *Saccharomyces cerevisiae* strains of single nucleotide mutations	207
haplotyping of long DNA fragments	210, 213
genotyping and haplotyping	208
sample preparation for fluorescence in situ sequencing	212
quantification of known point mutations resistant to ABL kinase inhibitors	209
expressible molecule colonies (DNA clones are transcribed and translated within their home colonies)	204
sample preparation for multiplex polony sequencing, and resequencing of an evolved strain of *E. coli*	142
single-molecule profiling of alternative pre-mRNA splicing	211

Table 11. Applications of BEAMing or BEA Technology Based on on-Bead Single-Molecule Genetic Amplification

fields of application	refs
quantification of mutations in plasma samples of cancer patients	148
quantification of circulating tumor DNA (ctDNA) in serially collected plasma samples from subjects with colorectal cancers for assessing the dynamics of tumor burden	150
detection and enumeration of genetic variants of human *MID42*	140
simple, sensitive and accurate assay for detecting low levels of mutants	154
high-throughput screening of transcription factor targets	144
selecting of a DNA binding sequence for transcription from a random oligonucleotide library	153
direct determination of polymerase error rates	159
quantification of cancer-associated methylation in DNA from clinical plasma or fecal samples	151
compartmentalized *in vitro* transcription-translation reactions	158
highly efficient and specific detection of individual nucleotides of the amplified materials	152

Table 12. Applications of Microfluidic Digital PCR

fields of application	refs
accurate measurement of DNA copy number	76
high-throughput gene expression analysis	29, 31, 67, 90
distinguishing between wild-type and mutant sequences	55
analysis of copy-number variation (CNV)	77, 83, 217
noninvasive prenatal diagnosis	78−81
adenovirus detection	58
absolute quantitative detection of ABL tyrosine kinase domain point mutations in chronic myeloid leukemia (CML)	82
identification of bacteria carrying a particular gene and linking any two or more genes of interest to single species residing in complex ecosystems	89
large-scale SNP genotyping	68
measurement of intracellular expression profiles	84
quantification of virulence and marker genes	69
quantification of the DNA concentrations	61
quantitative multiplex detection of plant pathogen	70
toxicogenomics screening of small molecules	71
accurate, sensitive, and absolute quantification of 454 and Solexa sequencing libraries	87
quantitative detection of epidermal growth factor receptor (EGFR) mutations in the plasma and tumor tissues of patients suffering from nonsmall cell lung cancers	88
DNA methylation analysis	86

throughput sequencing from micrograms to picograms.[350] Maricic and Pääbo have investigated the efficiency of each step in the 454 library preparation to increase the yield of DNA sequence generated by the 454 sequencing from small amounts of initial DNA.[349] It has been found that the last step, when the single-stranded library is released by NaOH, is inefficient and highly variable. When this step is replaced with heat treatment, library amounts dramatically increase. Another commercially available microfluidic chip platform for single-molecule DNA amplification applications is from Fluidigm.[29,85,89,90] Using the Fluidigm array chip, single-molecule DNA amplification has been applied for large-scale gene expression analysis that has become an essential tool for many biological and medical investigations such as detection of aneuploidy,[29] multigene analysis of individual environmental bacteria,[89] and transcription factor profiling in individual hematopoietic progenitors.[90] Finally, it should be noted that the applications of single-molecule DNA amplifications using microfluidics are not limited to the fields mentioned here. In principle, it can be applied to any cases where single-molecule DNA or RNA need to be amplified and analyzed.

14. Outlook

The methods and applications presented here highlight the potential of combinations of single-molecule DNA amplification technique with microfluidics. Over the past several years, approaches have evolved from relatively simple ones, allowing limited control over DNA amplification with respect to reaction speed and volume, to more advanced ones, enabling "full" control over multiple parameters using microfluidics, including

Table 13. Applications of Other Microfluidic Single-Molecule DNA Amplification and Analysis Technologies

fields of application		refs
gene expression analysis	high-throughput gene expression analysis	52, 138
directed evolution	directed evolution by *in vitro* compartmentalization (IVC)	43, 153, 158, 190
disease diagnosis	mutant detection of K-ras for colon cancer	56
	clinical detection of HBV	47
	assessment of risk of BK virus-associated nephropathy in renal transplant recipients	57
	detection of *P. larvae*, the causative agent of American foulbrood (AFB)	75
	detection of HIV	41, 156
	detection of the t(4;14) chromosomal translocation in multiple myeloma	59
	molecular haplotyping by LE-PCR	146, 147
research on the environment	continuous monitoring of the environment for infectious diseases and related biowarfare agents (*B. anthracis*)	107
other applications	SNP	67, 138
	bacterial detection and identification	63
	chromatin immunoprecipitation assays	46
	forensic identification	92
	splice variant biomarker analysis	52

integration, automatization, throughput, and application. With respect to new applications of single-molecule DNA amplification using microfluidics, the most immediately appealing perhaps lies in the field of sequencing. By use of small-sized monodisperse droplets engineered on chips, the existing ultra-high-throughput sequencing technology can be further improved, and the applications of droplet reactors in single-molecule nucleic acid amplification can be further opened up. It is even possible to envision microfluidic platforms capable of performing the entire process of single-molecule amplification and analysis: sample preparation of starting template, single-molecule amplification, sequence-specific or non-sequence-specific detection, and data analysis. We expect to be only at the beginning of this technical development that will have great impact on the field of single-molecule nucleic acid amplification in further years.

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