History of Digital PCR

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Outline

1.1. PCR, the start of a revolution....1
1.2. Limit dilution PCR....1
1.3. Digital PCR, a term coined by Vogestein and Kinzler....2
2.1. PCR meets microfluidics....2
2.2. PCR in micro-droplets....3
3.1. Digital PCR in Microarrays....4
3.2. Digital PCR in Micro-droplets....4
4. Combining microarrays and microdropelts with Crystal™ digital PCR....5
5. Discussion....6

1. The origins of Digital PCR

1.1. PCR, the start of a revolution

The polymerase chain reaction (PCR) was invented by Kary Mulis while he was developing methods for detection of point mutations using oligonucleotides at Cetus Corporation, California. The idea of PCR occurred to him while driving through the Californian mountains on a Friday night in 1983. Contemplating the idea that occurred to him, he reasoned that using two oligonucleotides of different lengths instead of one to bind to the upper and lower strands of the target DNA sequence and copying it several times using a polymerase enzyme would amplify the target sequence manifold, and could then be separated on a gel. He developed the method over the next few months using different temperatures, polymerases, and most importantly cycling the reaction multiple times, 30 cycles, to strengthen the signal without a background[1]. He later patented the technology and in 1987, Thermal Cyclers were made commercially available by a joint venture between Cetus and Perkin-Elmer. PCR forever revolutionized the field of molecular biology and clinical genetics.

A PCR reaction involves the exponential amplification of a target polynucleotide sequence by repeatedly thermocycling a salt-buffered mix of template nucleic acid molecules, oligonucleotide primers, dNTPs, and a thermostable DNA polymerase. Over the years, ongoing development and application of the PCR reaction enabled molecular cloning, engineered transgenic organisms, DNA forensics, clinical diagnostic DNA sequencing, among many additional innovative technologies.

In a traditional PCR reaction, the template DNA is usually several nanograms' worth corresponding to millions of DNA molecules. The template DNA is most often a heterogeneous mixture of mutated alleles, plasmids, or genomes depending on the application. Cancer is one of the clinically relevant examples of this heterogeneity, where a relatively small quantity of template DNA originating from cancer cells may harbor a cancer-causing somatic mutation relative to the wild-type (WT) DNA.

1.2. Limit dilution PCR

In the early 1990s, several research groups began exploring the possibility of diluting the template DNA to an extent such that, on average, any single PCR reaction contained only a single template molecule, a method named "limit dilution PCR". A key advantage of limit dilution PCR is that each DNA molecule is amplified separately, killing interferences between template

molecules during PCR and greatly reducing background noise in complex samples. Additionally, when many reactions are performed at this level of dilution, the frequency of positive and negative reactions follows the Poisson distribution and therefore allows for calculating the abundance of the target molecule based on the dilution factor.

In 1992, Sykes *et al.*[2] first described the concept of digital PCR using the principles of limiting dilution, PCR, and Poisson statistics to quantitate the total number of rare leukemic cells in a population of normal cells. Other researchers reported using versions of limit dilution PCR strategy to study, for example, variation among HIV proviruses[3], human genomic haplotyping[4], and to quantify the fraction of leukemic cells in patient samples[5]. These publications are the first reports of digital PCR, from pioneers who developed the method before it was even called digital PCR.

1.3. Digital PCR, a term coined by Vogestein and Kinzler

In 1999, Bert Vogelstein and Kenneth Kinzler[6] recognized the urgent importance of detecting cancer-causing somatic mutations at an initial stage in clinical samples to enable early cancer diagnosis. Indeed, reliably detecting these rare somatic mutations could help diagnose primary tumors in patients who are asymptomatic and whose disease is still curable. They also observed that detecting these mutations depended on isolating them from a large excess of WT DNA.

Vogelstein and Kinzler developed a strategy based on limit dilution PCR to selectively amplify these rare mutations, distinguish them from WT, and quantify the fraction of mutant alleles relative to WT. The dilution PCR strategy allowed one to partition individual template molecules such that the resulting amplification was either completely mutant or completely WT. In practice, they diluted and partitioned the starting DNA template into a 384-well plate, so that any given well contained one-half genome equivalent on average, i.e. half the wells contained one template molecule and half the wells contained zero template molecules. Subsequent amplification by PCR allowed the detection and quantification of the targeted mutant DNA sequences in the partitioned sample

Vogelstein and Kinzler named their method “digital PCR”, in reference to the classification of reactions as “zeros/negatives” or “ones/positives” in a similar fashion to bits classification in computer science.

The two scientists recognized the variety of potential applications of digital PCR, including detecting somatic single nucleotide variants in cancer, chromosomal translocations, changes in gene expression, allelic discrimination, and allelic imbalance. In 1999, they also noted that the limit of detection was defined by the number of single-template reactions that could be analyzed, paving the way for increased sensitivity of the technology by using high-throughput platforms such as 1,536-well plates, microarrays, and beyond.

The scientific community quickly recognized key advantages of digital PCR over traditional end-point or real-time PCR: does not rely on a standard curve; has improved accuracy; provides absolute quantification; and offers improved detection of low copy-number variants. Other key advantages of digital PCR became evident later, as the method was used more broadly[7]: repeatability of assays, over time and across different labs; robustness and tolerance to PCR inhibitors.

2. PCR in Microfluidic Systems

2.1. PCR meets microfluidics

In the mid-1990s, advances in microfabrication techniques had allowed the production of devices with feature sizes as small a few microns. Micromachining, photolithography, and etching silicon and glass substrates produced networks of flow channels that could sort reagents and molecules, partition chemical reactions, and act as droplet generators for mixtures of aqueous and oil-based fluids. These microfluidic devices were well-suited for performing biochemical assays, such as single-cell assays, studies of

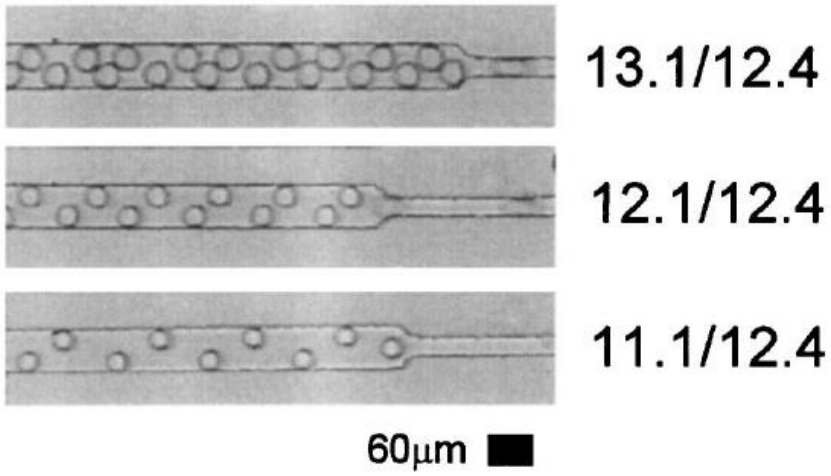

Figure 1. Reverse micelles in square channels. Photomicrographs show the transition from the 30 mm wide channel to the 60 mm wide channel. Respective pressures for the water and oil/surfactant (hexadecane_2% Span 80) are noted in the figure[13].

macromolecules *e.g.* proteins and nucleic acids, and the polymerase chain reaction (PCR).

The partitioning capabilities of microfluidic lab-on-chip devices were particularly useful for PCR, where a microenvironment allows for increased thermocycling speeds and increased reagent concentrations within the reaction mixture[8]. Several groups developed integrated systems utilizing microfabricated components of glass and silicon to perform restriction enzyme digests, PCR, and electrophoresis within a single chip-based device[9,10]. These systems generally relied on thermocapillary pumps to perform PCR in nanoliter-sized droplets within micron-sized channels and included entry ports, heating elements, and DNA detectors. They did not require any novel hardware, biochemistries or DNA detection methods; rather, they simply miniaturized existing components such as heaters and applied well-known PCR methods in a micron-sized environment on a chip.

By the late 1990s, integrated chips utilizing the continuous flow of nanoliter-sized plugs through microchannels were becoming common. These systems achieved thermocycling by transporting the reaction plugs through a microcapillary etched into a glass chip that traverses heaters at the respective melting, annealing, and primer extension temperatures appropriate for PCR[11,12].

In late 1999, the first commercial lab-on-a-chip, the HP 2100 Bioanalyzer, was introduced by Hewlett-Packard's Chemical Analysis Group, which shortly thereafter became Agilent. Based on Caliper Technologies' LabChip, the 2100 was the first complete instrument that could perform sample handling, separation, fragment sizing, quantitation, and digital data processing for PCR products, restriction enzyme digests, or RNA samples. Following the HP 2100, other lab-on-chip platforms were commercialized by companies including Nanogen Inc., HandyLab Inc., and Micronit Microfluidics among others.

2.2.PCR in micro-droplets

By the early 2000s, water-in-oil emulsions were being studied for potential applications designed to partition biological materials into picoliter-scale droplets[13,14]. A significant advance was the use of surfactants in the oil-based continuous phase to maintain the integrity of droplets formed after the introduction of the discontinuous aqueous phase. Because of surfactants in the oil-filled microchannels, droplets can be pooled in widened channels or reservoirs, touching each other without breaking or fusing[13] (Fig.1). Extensive fluidic networks of oil-and-surfactant filled channels can be designed

to coordinate the intentional mixing of plugs or droplets to mediate biochemical reactions[14].

Fluorinated oils combined with fluorinated surfactants were particularly useful for handling biomolecules within aqueous droplets, as these carrier fluids are inert, stable at high temperatures, compatible with the most common lab-on-chip materials, and can prevent surface adsorption of charged biomolecules[14–16]. Fluorinated oils and surfactants were commonly used and commercially available, *e.g.* Fluorinert™ by 3M and Zonyl™ by Dupont, among other offerings from Fluorochem Ltd., Sigma-Aldrich Inc., and others.

The combination of commercially available lab-on-chip microfluidic networks with advanced picoliter-scaled aqueous droplet generation, stable surfactant-mediated pooling and sorting of droplets provided future opportunities for single-molecule reactions within these droplet microreactors.

3. Next-Generation Digital PCR

3.1. Digital PCR in Microarrays

In the early 2000's, the digital PCR method described by Vogeslstein and Kinzler was largely overshadowed by real-time PCR. Indeed, such early digital PCR experiments, performed using microtubes or 384-well plates, suffered from drawbacks such as the amount of reagents needed, limited capacity for partitioning and automation. However, in the engineering and microfluidics world, digital PCR publications were increasing exponentially. Efforts to enhance sensitivity by partitioning the template molecules into thousands of chambers at nanoliter-scale volumes, along with providing the additional advantages of reagent savings, decreased diffusion distances, and improved statistics were ongoing. A further innovation was multiplexing, *i.e.* targeting multiple alleles within a single PCR mixture by introducing a plurality of primers and fluorescent probes[17,18]. Since target molecules are partitioned individually, a given primer pair will only amplify its particular target in a partition that contains that unique allele. By the mid-2000's, microfluidic digital PCR in chambers had been used for applications as varied as interrogating microbial community diversity[19], human genomic copy number variation[20,21], and fetal aneuploidy[22].

With microfluidic-based digital PCR becoming well-known and more widely practiced, in 2006 Fluidigm Corporation became the first company to commercialize the technology in an integrated microfluidic circuit. The BioMark system was based on the 12.765 Digital Array, a chip of 12 panels, each panel partitioned into 765 6-nL chambers. After loading the PCR reaction mixture through 12 carrier inputs, the chip was thermocycled, fluorescence was detected, and the signal was processed and analyzed by the Digital PCR Analysis software.

However, digital PCR technology during this time was limited by the number of individual partitions (chambers) per sample, volume of the reactions, and increased hands-on time. Moreover, digital PCR was very costly to run, with a reaction costing several hundred dollars compared to a little over a dollar for traditional end-point or real-time PCR.

3.2. Digital PCR in Micro-droplets

An alternative approach that further enhanced throughput and sensitivity while addressing the cost per reaction limitation was to generate picoliter-sized microdroplet reactors by flow-focusing offering many more partitions than the chamber-based systems. Droplets are generated in a microfluidic system, thermocycled to perform single-molecule digital PCR within the droplets, and end-point amplification is detected and quantified via real-time fluorescence curves[23]. These droplet-based lab-on-chip systems were also adapted to perform reverse transcription PCR (RT-PCR) to detect single copies of RNA genomes[24] and to perform multiplex reactions directed to multiple targets within a single droplet[25].

This technology was first commercialized by QuantaLife, Inc. as the QX100 ddPCR™ System in 2011. The microfluidic consumables used on the ddPCR™ platform could

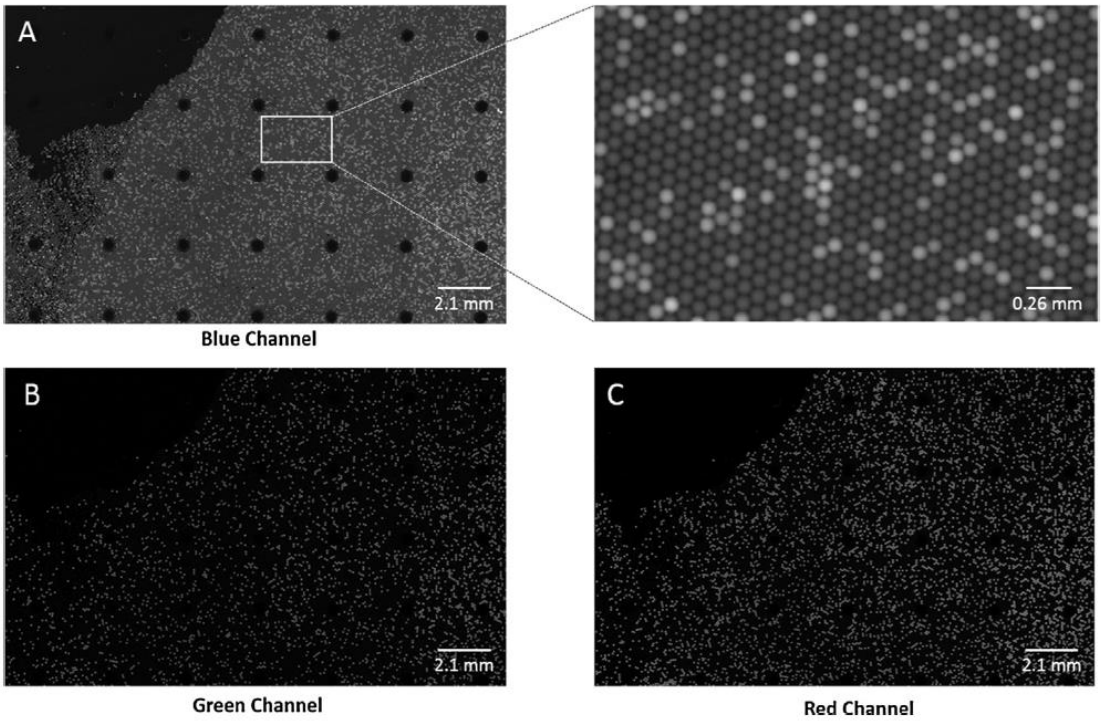

Figure 2. Visualization of droplet crystals. Droplet Crystal containing 29078 analyzable droplets, imaged post PCR using the Blue (A), Green (B) and Red (C) Prism3 acquisition channels. The left insert in A. presents a zoomed-in portion of the droplet crystal. Droplets in which no amplification has taken place remain dark, and droplets in which amplification is observed are lighter in color. Droplets have a higher fluorescent baseline in the Blue channel due to the FITC added to the reaction mix[26].

accommodate up to eight samples per chip, generating 14,000-16,000 droplets per sample.

The reduction in the cost of running digital PCR experiments on the QX100 ddPCR™ System accelerated the adoption of digital PCR for many applications in molecular biology. Nonetheless, when compared to the standard real-time or quantitative PCR, the digital PCR workflow remained cumbersome to run, with high hands-on time, multiple instruments required and long time-to-result. Multiplexing capabilities were also limited to two detection channels.

4. Combining microarrays and microdropelts with Crystal™ digital PCR

An advanced digital PCR equipment, the Naica System™, was launched in 2016 by Stilla Technologies. This system performs digital PCR by partitioning the sample, using a confinement gradient (Figure 3), into a large 2D array of droplets, also called a droplet crystal. A PCR reaction occurs in each of the partitioned 25-30,000 droplets that make up the droplet crystal, and a fluorescence read-out is performed at end point by taking high-resolution images of the crystal. This digital PCR workflow, named Crystal™ digital PCR combining the advantages of a) array-based digital PCR such as an integrated workflow and multiplexing; with b) those of droplet-based PCR such as reduced cost. The ability to partition the sample extensively and uniformly affords the system with superior precision and sensitity for target detection and quantification. Additionally, the flexibility to explore different fluorophores, the capability of performing multiplexing of targets (up to 3-colors[26]; Figure 2), and a fast time to result make it a unique digital PCR system. A recent advancement,as a proof of concept, the capacity to detect multiple targets in Lung cancer patient samples using 6-color Crystal™ digital PCR was reported, opening new avenues for multiple target DNA investingation[27].

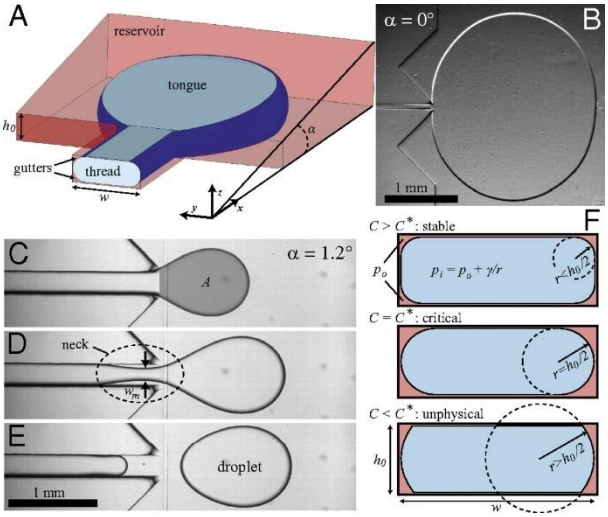

Figure 3. Device geometry and mechanism for drop formation through a confinement gradient. (A) Three-dimensional sketch of a device during operation. The dispersed phase is pushed through the inlet channel (width wand height h_0) into a wide reservoir containing a stationary continuous phase. The top wall of the reservoir is inclined at an angle α. Fluid from the continuous phase remains in the corners of the inlet channel, forming gutters connected to the reservoir. (B) For a flat reservoir (α=0°), the circular tongue grows indefinitely without detaching. (C–E) Even a small slope (α=1.2°) leads to a modification of the tongue shape and to a drop detaching. (C) A tongue of water in oil has a projected surface area A. (D) A neck appears in the inlet channel and its width w_m decreases in time. (E) The thread ruptures, when $w_m=h_0$, releasing a self-propelled droplet. (F) Cross-sectional shape of the confined thread in the inlet channel for different imposed C. If C>C*, the interface flattens against all four walls and the gutter radius of curvature r = 1/C. For C = C*, r = $h_0/2$: The inner fluid is tangent to the side walls. When C<C*, the shape of the interface is unphysical. The curvature in this case must adjust in the out-of-plane direction[28].

Finally, Crystal™ digital PCR reduces the need for specialized training of personnel by integrating the entire digital PCR workflow in a single microfluidic consumable, from partitioning to read-out.

5. Discussion

Early on the challenges faced by the digital PCR community were tremendous. Nevertheless, joint efforts from researchers in the microfluidics, molecular biology, and computer science fields resulted in significant improvements in the digital PCR machines over the past decade. For many applications, digital PCR is beginning to replace the conventional qPCR owing to its ease-of-use, reduced complexity, repeatability, and superior precision. Now, efforts to further enhance the applicability of the digital PCR technique to a wide range of experiments in different research areas are in place, with the ultimate aim of making digital PCR a standard lab technique.

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