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High-throughput droplet PCR

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ABSTRACT

The polymerase chain reaction has facilitated the ready analysis of nucleic acids. A next challenge requires the development of means to unravel the complexity of heterogeneous tissues. This has presented the task of producing massively parallelized quantitative nucleic acid data from the cellular constituents of tissues. The production of aqueous droplets in a two phase flow is shown to be readily and routinely facilitated by miniaturized fluidic devices. Droplets serve as ideal means to package a future generation of PCR, offering an enhanced handling potential by virtue of reactant containment, to concurrently eliminate both contamination and sample loss. This containment also enables the measurement of nucleic acids from populations of cells, or molecules by means of high throughput, single cell analysis. Details are provided for the production of a prototype micro-fluidic device which shows the production and stable flow of droplets which we suggest will be suitable for droplet-based continuous flow microfluidic PCR. Suggestions are also made as to the optimal fabrication techniques and the importance of device calibration.

1. Introduction to the polymerase chain reaction

The polymerase chain reaction (PCR) is an enabling technology which has allowed major advancements in biomedical research as a tool for amplifying nucleic acid samples. However despite the many advantages of conventional PCR there are limitations which are inherent to both the PCR process itself and the requirement of multiple sample preparation steps [1–5] This article seeks to explore these caveats and may provide a novel solution by means of high-throughput droplet PCR in integrated miniaturized microfluidic devices [1,2,5]. Furthermore, the choices made in the design and fabrication of this device are discussed and justified with reference to some of the advantages and disadvantages of previously published miniaturized PCR devices. Several vital calibration experiments are also discussed which are needed in order to establish an efficient, reliable and reproducible PCR which can be comparable to that of conventional thermocyclers. Finally some of the applications are discussed which reflect the rationale behind the development of this technology.

1.1. Limitations of conventional PCR

The throughput of conventional PCR is limited by both spatial constraints and a slow thermal ramp rate between temperatures caused by a large thermal mass [5]. In addition, detection of amplified species can be achieved at much lower reagent volumes than are currently used in standard thermocycling machines [5,6]. However despite these two limitations, the majority of problems are caused by the steps of sample preparation that are necessary to isolate purified nucleic acid templates from a population of cells [4]. The separation of the various steps of sample preparation, from cell lysis to nucleic acid extraction and purification, can lead to both loss and contamination of the sample.

The current methods of sample preparation, and the following qPCR analysis treat heterogeneous sample populations as though they were homogeneous by applying the averaged result of the population to represent each individual cell [4]. This resultant averaged data, caused by bulk sample preparation and analysis of a heterogeneous population of cells, is also commonly normalised relative to a chosen reference gene when examining a quantitative measure of gene expression. Appropriate choice of reference genes aside [7], this result would carry more meaning if expressed in absolute units of molecules per cell for each cell in the sample. The generation of such an unit can only result from the preparation and analysis of each cell on an individual basis. However to make single cell analysis a feasible, and not laborious

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task, a sensitive and high-throughput technique is needed where each cell can be enclosed, within an essentially contained analysis system, thus eliminating factors of sample loss and contamination [5]. Furthermore, the comprehension of the original position of specific cells within solid tissues would greatly assist in the assessment of cell–cell communication and the special context to cell activity.

1.2. Introduction to droplet-based micro-fluidics

1.2.1. History and rationale behind micro-fluidic miniaturization

Micro-fluidics and microfabrication have the potential to significantly change the way modern chemistry and biology are performed. There are several fundamental reasons supporting the scaling-down of fluid handling. First, in a microchannel, the reduction of sample size accelerates chemical reactions and heat transfer due to shorter diffusion length and an increased surface to volume ratio. Second, the smaller volume size of reactants offers the opportunity to produce portable devices, particularly relevant within the field of healthcare, environmental monitoring and forensics, where point-of-care analysis and remote sampling, respectively, would be particularly desirable. Other advantages of smaller reactant volumes are a consequent reduction in the amount of sample which has major clinical impact, and a pro-rata reduction in reagent costs. Miniaturization also offers safety benefits, including containment, where hazardous materials are being used.

The fast growing number of applications in micro-fluidics are driven by so-called 'Lab-on a Chip' (LOC) systems, a concept based on the initial work of Andreas Manz in the early 1990s [8]. Lab-on-a-chip devices are a subset of microelectromechanical systems (MEMS) devices and integrated processes are often described as Micro total analysis systems (μTAS). The concept of μTAS eludes to the fact that integration of laboratory steps, such as sample preparation and analysis, on the same device can advance conventionally isolated sample handling procedures towards a complete laboratory analysis, with the aim of replacing existing laboratory devices.

1.3. Droplet-based micro-fluidic miniaturization

A subsection of micro-fluidics is the rapidly emergent field of droplet-based micro-fluidics, with droplets as discrete fluidic volumes created by two immiscible phases. These micro-fluidic systems are typically aqueous droplets held within a non-aqueous carrier fluid such as oil or solvent, and normally are not required to exchange material at their boundary. However exchanges can occur which can be eliminated through the use of surfactants. The use of surfactants have been shown to aid in droplet stability by preventing the fusion of nearby droplets [9]. In contrast to traditional micro-fluidics, droplets offer the possibility of serialisation and isolation of individual samples, therefore offering potentially a much higher scope of automation.

The produced droplets are normally in the nano to micrometre range and can be produced at rates of tens of thousands per hour [10]. Droplets in small channels also allow fluid flows with no dispersion, which is a general problem with single-phase fluidics. In addition, when such droplets are surrounded by an immiscible fluid this can prevent contact between the surfaces of the channels and the sample within the droplet, eliminating adverse effects due to the large surface to volume ratios. Since identical droplets are produced at a very high frequency within one experiment, parallel processing is achievable to produce large data sets, offering a higher degree of confidence following analysis. Droplets have also been manipulated by electric fields to perform merging, sorting [11,12] or splitting operations[13]. These advantages offer the potential of higher throughput and the possibility to create a true "Lab on the chip".

Applications of droplet-based micro-fluidics range from studies of enzyme kinetics [14–16], protein crystallization [17,18] and particle polymerisation [19–21]. Droplets also offer the possibility to encapsulate and cultivate biological cells in order to study specific dynamics between different cells and their metabolites [22–24].

1.4. Why dropletize PCR?

PCR has a near omni-presence in the biomedical sciences. Its applications are therefore unsurprisingly widespread which is mainly attributable to the ease of execution and the exquisite sensitivity offered by PCR which encompasses amplification from single template molecule up to some nine orders of magnitude. Recent demands of PCR have seen a move away from qualitative assessment of template sequences to a requirement to perform accurate, quantitative measurement of analyte.

PCR in droplets would offer substantial advantages compared to other micro-fluidic-based PCR systems, such as well-based or single-phase continuous flow devices [25], as they still suffer from adsorption of reactants such as template, deoxyribonucleotide triphosphates (dNTPs), and also DNA polymerase enzyme to the channel walls. Droplet PCR would offer a further sample volume reduction and shorter amplification times due to the reduction in thermal mass. Single cell dropletized PCR has the potential to obviate the problems discussed above relating to sample preparation such as avoiding the 'averaging effect' seen in a heterogeneous population by permitting the use of the more meaningful units of molecules per cell. Provided care is taken to ensure that order of cells entering the stream of droplets is maintained, the original position of specific cells within solid tissues can be reconstructed in virtual space which will assist in the assessment of space on cell activity.

2. Description of methods

Over the last few years a trend is emerging using polymers for micro-fluidic devices rather than the more traditional materials such as silicon, glass, ceramics and metal. There are a number of advantages, where primarily large numbers of polymer devices can be made quickly and at low costs using mass fabrication techniques. The most common fabrication methods applied for the manufacture of polymer microchips are: soft lithography [26], hot embossing [27], laser ablation [28], injection moulding [29], and direct Computer Numerical Control (CNC) machining [19].

2.1. Fabrication and machining

The prototype micro-fluidic PCR device presented here was fabricated using CNC precision machining and will be briefly described. The poly(methyl methacrylate) (PMMA) planar chip shown in Fig. 1 was made from a sheet of 4 mm thickness (RS, Corby, UK) and cut to 75 × 75 mm squares. The 3D flow pattern was designed using AUTODESK INVENTOR (Autodesk Inc., San Rafael, California, USA) and translated into CNC machine code by EDGECAM software (EdgeCam, Reading, UK). The micromachining was carried out using a CAT3D M6 CNC milling machine (Datron Technologies Ltd., Milton Keynes, UK) with 0.1–0.4 mm diameter tungsten-carbide milling tools (Toolex Ltd., Trowbridge, UK). The channels were sealed with a 100 μm thick acetate foil, which was attached to the PMMA chip using a thin film of Ultraviolet-curable epoxy (Norland 68, Norland Products Inc., New Brunswick, NJ, USA). To avoid blocking the channels, a hot roll laminator was used

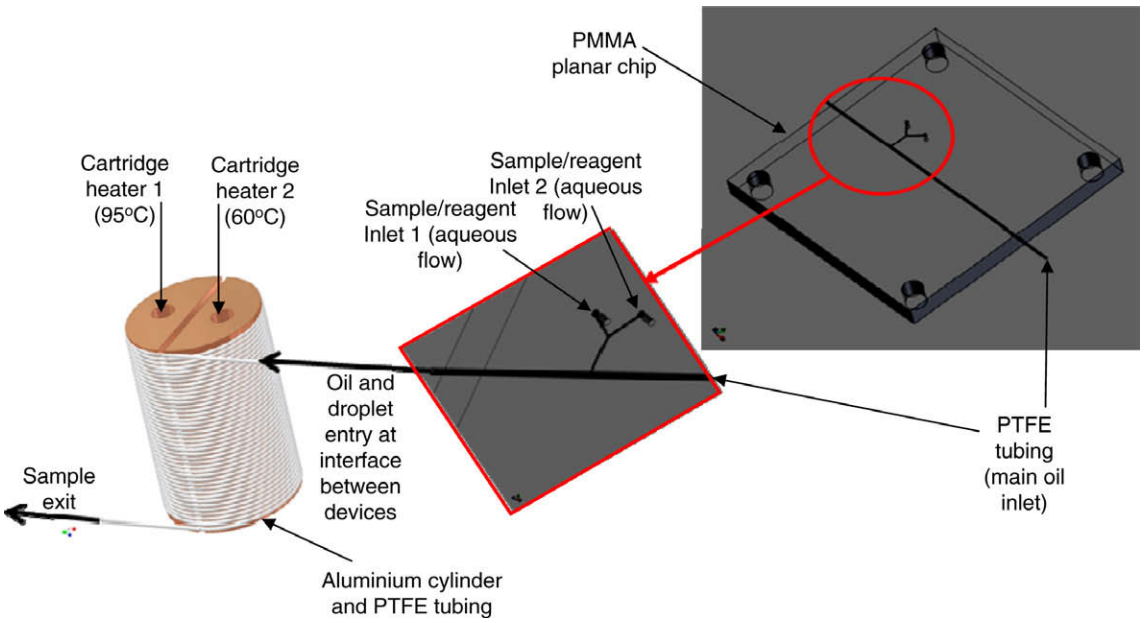

Fig. 1. Design of the device. This figure shows the schematic designs for both the aluminium cylinder device (far left) and the PMMA planar chip (far right, with zoomed image of the channels in the centre) which were both at 90°. Due to the shear forces the flowing aqueous phase is sheared off into droplets which are then suspended in the immiscible oil carrier fluid.

to spread the epoxy to a thickness of several microns between two sheets of acetate. The sheets were then placed on dry ice and cooled for 5 min before being separated. The solidified epoxy stays attached to one of the sheets allowing a seal with the chip. The chip and seal were then brought into contact with the use of the laminator and finally the epoxy was cured using an Ultraviolet (UV) light source (60–80 mW/cm^2) for 2 min at a peak wavelength of 365 nm as shown in Fig. 1.

The cylinder device uses narrow bore polytetrafluoroethylene (PTFE) tubing (Microbore PTFE tubing, Cole-Parmer Instrument Co. Ltd., UK) coiled around an aluminium cylinder which is divided into two temperature zones (Fig. 1). This device was designed using AUTODESK INVENTOR (Autodesk Inc.) and machined in-house. The cylinder was cut in half to enable these two temperature zones to be separated. The cylinder was supported by brackets separating each half by approximately 2 mm air gap, to prevent the transfer of heat from one half to the other. A thread (pitch = 1.5 mm, depth = 1.05/2 mm) was cut into the aluminium cylinder (diameter = 40 mm, length = 65 mm) to accommodate 40 winds of the PTFE tubing (outer diameter = 1.07 mm, inner diameter = 0.55 mm) with one circumference of the cylinder representing one cycle of the PCR reaction. The two temperatures were created by two 50 W cartridge heaters (RS Components Ltd., Corby, UK) which in turn were controlled by proportional-integral-derivative (PID) digital controllers (CAL 9900, RS Components Ltd.).

The PTFE tubing was interfaced with a planar chip, in which droplets could be produced. A rectangular bedding groove, which would accommodate the tubing, was machined into the PMMA planar chip (RS Components Ltd.). Ultra Violet (UV)-curable epoxy (Norland 68, Norland Products Inc., New Brunswick, NJ, USA) was used to fill the gap between the bedding groove and the tubing. Finally a hole was drilled into the centre of the PTFE tubing with a small drill (100 μm diameter) to allow the micro-fluidic channels to join the PTFE tubing.

2.2. Justification of design

The design of the aluminium cylinder core with PTFE tubing used to carry out the PCR amplification and analysis stage is unconventional compared to most previously published devices. The design of the aluminium cylinder and PTFE tubing device was intended to circumvent some of the problems seen in more conventional micro-machined devices. Devices based on reaction wells [30], oscillatory systems [31–33], or closed-loop systems [34,35] are restricted to a fixed throughput which is only a marginal improvement over the conventional thermal cycler. However devices, similar to the one described here, that are based on a fixed-loop system [5,36–38] have the advantage of being high throughput where samples can be continuously injected and collected in an automated fashion. This fixed-loop system does restrict the flexibility of the cycle number and the timings of the cycles within an individual experiment. However, the cycle timings can be directly controlled by adjusting the flow rate in order to optimise the PCR for a particular experiment.

PTFE has a low coefficient of friction allowing the oil carrier fluid and aqueous droplets to pass smoothly through the tubing with minimal resistance or backpressure. Not only does this allow for faster flow rates and thus a higher throughput of reaction but breakages and adsorption of reagents onto the inner walls of the channels is also minimised. Adsorption of samples onto the inner walls of channels is a potential problem of many of the micro-machined devices that are based on a single-phase flow of the reaction mixture [31,32,34,36,37]. To overcome the problems of sample adsorption onto the inner walls of the channels, and the possible inhibition of the channel materials, many groups have used the technique of silanization [39–42] or the inclusion of passivating reagents such as PEG or BSA in the PCR reaction mixture [2]. Such techniques are not required with the use of the hydrophobic, chemically inert and virtually non-porous PTFE tubing. Similarly PTFE tubing can withstand extreme temperatures whilst being crack-resistant and stress-resistant making this material ideal for PCR. Tubing of similar material has been effectively used to contain samples in other miniaturized PCR devices [43] and has been shown to be a favourable material for use in micro-fluidic devices [44].

The use of a fluoro-carbon oil (FC-40, 3M) as an immiscible carrier fluid further improves the design of this device as this oil has a good heat transfer (specific heat = 1050 J kg^{-1} °C^{-1}) [45] The low

viscosity of FC-40 (3.4 centipoise) also aids in the generation of faster flow rates and reduced resistance. [45] An additional benefit of using this design of device, where PTFE tubing is wrapped around a central core, is that the PTFE tubing can be easily removed and replaced with new tubing if required. However the isolation of both the sample and the reagents within an individual droplet should greatly reduce, if not eliminate, sample loss and contamination.

A more conventional PMMA chip has been designed for the stage of droplet production. The micro-machined channels have been created to carry the aqueous phase which will go onto form the droplets. These channels meet the oil phase, which is carried in the PTFE tubing, at 90°. The device was made to integrate the PTFE tubing into the PMMA chip so that the oil could remain in the PTFE tubing and move smoothly from one part of the device to the next. Previously the oil channel was also micro-machined into the PMMA substrate and then transferred to the PTFE tubing via a 90° connector. This sharp angle caused a slight change in the flow rate which led to the merging of droplets in the oil carrier fluid at this point. The current design of the device, including both the PMMA chip and the aluminium cylinder, is ideal for a smooth and consistent flow rate as there are no sharp turns or connections. The PMMA chip was also designed with two inlets so that the chip would have the potential of acting as a droplet merging device. The fusion of two droplets, one containing the sample and the other containing reagents, by the application of an electric field [46], would form the basis of sample preparation on this device. This fused droplet can then be passed across a heater, which can be incorporated onto the PMMA planar chip, to both lyse the cell and activate the polymerase enzyme thus forming the basis of single cell integrated preparation and analysis. The droplet will then be transported, in the oil carrier fluid, through the thermocycles of the cylinder device allowing the generation of data, expressed in an absolute unit of molecules, for each individual cell in the sample.

2.3. Droplet formation

A feature of this miniaturized device that differs from many previously developed devices is that it is based on a system of aqueous droplets suspended in an immiscible oil carrier fluid. This system allows both the sample and reagents to be contained within the droplet isolating them from factors such as sample loss by adsorption onto the channel walls, or cross-contamination from adsorption of previous samples. The aqueous droplets are formed in this device at a T-junction where the inlet carrying the aqueous phase meets the oil flow at 90°. Due to the shear force acting on this aqueous phase, by the flow of the immiscible oil carrier fluid, the aqueous solution is sheared off into droplets that quickly become spherical due to surface tension.

This can be controlled in a passive manner by adjusting the flow rates of the oil carrier fluid and the aqueous phase, which will form the droplets, until a balance is reached such that droplets can be produced. However adjustments to the flow rates of the oil and aqueous phase also have an impact on various other factors such as the rate of droplet production, the size of droplets produced, the amount of time each droplet spends in each cycle of the PCR, and the throughput of the reaction.

2.4. Impact of flow rates

Changing the flow rates of both the oil carrier fluid and the aqueous phase in a systematic way will permit the optimisation of droplet sizes allowing reagent costs to be reduced to a minimum. Optimisation of the rate of droplet production is also necessary so that a balance between throughput of the reaction and prevention of cross-contamination by the merging of droplets can be met. A thorough understanding of the effect of flow rates on the time a droplet spends in each cycle of the cylinder is also important for optimisation of the PCR to maintain efficient amplification whilst increasing the throughput of sample analysis. The consistency of the flow rates, measured by the time each droplet spends in each cycle of the device, is also vital to ensure homogeneity across the entire device.

2.5. Calibration of temperatures

One of the main limitations of conventional PCR is the throughput of the technique as this is hindered by both spatial constraints and the slow temperature ramp rate. The temperature ramp rate of this device was explored using Chromazone slurry (Thermographic Measurement Ltd., Flintshire, UK) which undergoes a colour change from black to white at 67.3 ± 2.1 °C. This chromazone slurry was injected into the PTFE tubing of the device and allowed to break into small droplets which were dispersed throughout the tubing. The temperatures were set to 75 and 40 °C and a video was recorded at 30 frames, per second using a Lumenera LU125 M-IO camera, of one of these droplets passing between the two temperature zones. This video was then edited into single frame images using Adobe Premier Pro software. The number of frames taken for the droplet to change from black to white at a known frame rate can then be used to calculate the ramp rate of the device. The temperature ramp rate of this device (5.83 °C/s) was found to be superior to that of a conventional thermal cycler (4.8 °C/s). Also of importance is that this temperature change is consistent across the entire device so that each cycle of the PCR is equivalent. This can be seen by allowing the Chromazone slurry to fill the entire cylinder and observing whether the colour change is homogeneous across each cycle of the device.

Whilst a rapid and consistent temperature ramp rate is important to generate a high-throughput PCR device this temperature change should not disrupt the flow of droplets within the oil carrier fluid. In order to test this the time taken for a droplet to pass through one temperature zone can be recorded, in triplicate, at five cycle intervals in the presence and absence of heat. This experiment was carried out using two sample injection systems. First, both the aqueous phase and oil were allowed to flow into the chip via gravity from two 3 mL open syringes connected to the inlets. A Gilson Minipuls 3 peristaltic pump was connected to the outlet and set at 2 rpm. This reduced the resistive pressure allowing the oil and aqueous phase to flow into the tubing via gravity. The second system involved both the removal of the peristaltic pump and the connection of the two syringes to a Harvard "33" syringe pump system. The flow rate of the oil and aqueous phase were set to 6 μL/min and 3 μL/min, respectively, which were arbitrarily chosen for ease of droplet counting. Although the peristaltic pump was shown to aid in decreasing the resistance and backpressure in the system the pump had an adverse effect on the reproducibility of the flow of droplets. The flow of the droplets was inconsistent both within and between cycles with the first system and was affected by the heat of thermocycling. However no change in the flow rate of the droplets was seen between or within cycles when using the Harvard syringe pump system and this consistent flow remained unaffected by the thermocycling process.

2.6. Limitations and future work

The device presented here is a prototype, designed to potentially address some limitations of conventional PCR, such as sample loss and contamination, through the integration of sample preparation and analysis steps and the isolation of the samples within individual droplets. Furthermore dropletized PCR may allow a ready solution to the "averaging" problem seen when measuring

analytes within heterogeneous biological samples, by creating single cell high-throughput PCR. This will generate a quantitative result for each individual cell of a population, and simultaneously enable the cell to be used as the denominator for assessing expression activity and predict likely function. The described calibration experiments showed that the flow of droplets within this system is both stable and responsive to the changes in flow rate yet unaffected by the thermal cycling process. An improved temperature ramp rate has also been achieved on this device which will directly increase the throughput of PCRs by allowing a faster transition between the two temperatures of each PCR cycle. However, despite the potential advantages of this novel device, additional work is required to fully optimise the device for an efficient PCR along with eventual improvements to enhance its automation and ease of use.

3. Concluding remarks

The increased density of microtitre plate formats for PCR are positively correlated to the evolving applications of PCR. Specifically, the heightened requirement for throughput has been attributed to both a genuine requirement to increase throughput of routine gene-based assays, and also to enable the generation of high clarity measurements of nucleic acid copies. To a greater extent, the huge increase in throughput is necessitated to overcome heterogeneity of cells in tissues, causing the bulk sample preparation 'averaging' phenomena that preclude accurate measurement and inability to closely associate multiple gene regulation and decipher pathogenesis. A similar scenario exists for the measurement of nucleic acids within a single cell. The gigantic advances seen recently in 3rd Generation Sequencing platforms (such as Genome Sequencer FLX System, Roche; SOLiD™ System, AB, and Genome Analyzer$_{IIx}$, Illumina) permit the accurate assessment of nucleic acid copy numbers by simply counting the number of individually sequenced molecules. Indeed, whilst the dropletized μTAS devices are under development using similar approaches to those described in this article, it is most noteworthy to reflect that the new sequencing platforms are based on emulsified droplets in which PCR is performed. Therefore, the justification and evidence that PCR can be encapsulated in droplets is well founded (as seen with RainDance Technologies). The rationale for the production of streams of PCRs in a micro-fluidic device is more a function of enhancing sample handling, to enable PCR-based nucleic acid measurements from complex biological matter.

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