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A SILICON BASED LAB ON A CHIP FOR DIGITAL DROPLET PCR

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Microfluidics, DNA amplification, Real Time PCR, digital PCR

ABSTRACT

In this work, we present a miniaturized device that performs uniform droplet generation and fast temperature cycling, for digital droplet Polymerase Chain Reaction (ddPCR). The device was fabricated using deep silicon etch for sculpting the microfluidic structures that were then sealed by anodic bonding with a Pyrex wafer. Droplets of an aqueous solution containing PCR reagents and templates were generated in oil using a simple T-junction. Droplet volume and generation rate were respectively 60 pL and 6400 droplets per second. Volume reproducibility in different experiments was about 5%. A standard two-step Taqman protocol was used for DNA amplification. After each PCR cycle a fluorescent image of the reactor was recorded using a conventional inverted microscope equipped with a CCD camera. The analysis of these images allowed to count dark and fluorescent droplets and to reconstruct the real time fluorescence intensity curve for each droplet. To demonstrate the functionality of the device, human genomic DNA with seven nominal starting concentrations ranging from 20 to 13000 copies per µl was amplified. The determined template concentration was in agreement with the nominal one in the full dynamic range of the system (almost three orders of magnitude). In the current design the dynamic range is limited by the reactor volume (3mm x 3mm x 30 µm) that can be extended by a factor of hundred by increasing both depth and footprint.

1. INTRODUCTION

The advance of microfluidics over the years has provided several solutions for fluid handling in small, confined volumes. Mixers, microreactors, filters for purification of clinical samples, integrated capillary electrophoresis devices, miniature columns for liquid chromatography are examples of microfluidic components which were fabricated on a large variety of substrates and using very different technologies. A microsystem integrating a certain number of these components on the same chip and performing some type of biological or diagnostic analysis is commonly named Lab-on–a chip (LoC). LoCs mounted in small, disposable, microfluidic cartridges and controlled by compact bench-top tool, are already used in point of care (PoC) applications to perform clinical tests faster and at lower cost than in conventional laboratories [1,2]. More recently a trend emerged, towards the development of ubiquitous test devices; in this new approach the cartridge and the bench-top tool are combined in a small device (typically with a USB connection), controlled by a portable, non-dedicated device (e.g. laptop, tablet) allowing clinical testing anywhere [3].

Among the microfluidic components a large attention has been attracted by miniaturized PCR systems, that, besides the usual advantages of LoCs, also offer the possibility of a fast temperature cycling, due to their small thermal mass. Single and multiple PCR reactors [4], quantitative PCR (qPCR) [5] and digital PCR [6] have all been realized in form of small chips. Digital PCR (dPCR) is an emerging method, enabling a direct quantification of nucleic acids. The golden standard for nucleic acid quantification is qPCR which, although being a robust and well established technique, suffers from some drawbacks. The most important is the need of calibration curves for accurate quantification, this is a lengthy process and to be accurate requires the

availability of samples identical to those to be analyzed and with known concentration of nucleic acid, which is not always possible. Digital PCR, instead, does not need any preventive calibration. It is based on dividing the sample into small partitions, each initially containing at most a few units of the template. After PCR, the ratio of positive (detected by fluorescence) to total partitions allows, through the use of Poisson statistics, an accurate quantification. The sample partitioning for dPCR can be achieved by using small cavities [6] or by generating droplets of the aqueous PCR solution in oil [7].This second approach is known as digital droplet PCR (ddPCR).

Available commercial solutions for dPCR propose a lengthy workflow using different tools for sample partitioning, temperature cycling, and fluorescence reading [8]. The use of a LoC approach for dPCR would allow to combine partitioning, thermal cycling and eventually analysis in a small single chip, hence removing this limitation.

The most used materials for LoC fabrications are polymers, but silicon, as used here, has several generic advantages, among them a well-established fabrication technology developed in decades of research in the semiconductor industry, the ease of mass production, the resistance to high temperature. In the specific field of dPCR microsystems, silicon technology could open the road towards: i) partial or complete integration of detection optics, by leveraging on silicon photonics capabilities and ii) integration of sample preparation by exploiting the capability of fabricating structures with small critical dimensions. Arrays of silicon micropillars coated with oxide having 10 µm diameter and 2-3 µm spacing, have already been used for DNA purification after direct PCR from blood [9] and for DNA extraction [10].

On this report we present the development and testing of a miniaturized ddPCR based on silicon that currently combines uniform droplet generation and fast temperature cycling, while fluorescence readout is performed using an external optical setup. To evaluate the device performance, human genomic DNA with seven starting concentrations ranging from 20 to 13300 copies per µl was amplified. The obtained concentration was in agreement with the nominal one in the full dynamic range of the system (almost three orders of magnitude).

2 FABRICATION AND EXPERIMENTAL

The ddPCR chips were fabricated using deep reactive ion etching. First the microfluidic structures were etched on the front side of the silicon substrate and then sealed by anodic bonding of Pyrex to ensure optical detection. After bonding, a second silicon etch was performed on the backside of the wafer to define holes for fluidics access and trenches for thermal insulation of the microreactor. Details of the fabrication process have been published before [11]. Figure 1a shows the layout of the chip, the violet structures are etched from the front side, the green structures from both front and back side, thus obtaining a through silicon etch. By means of this double etch the reactor has a very small thermal mass as it is completely insulated from the remaining chip, except for the fluidic connections. This can be clearly seen in the photograph of Fig. 1b. The microreactor is S-shaped, its width is 900 µm and it does not occupy the regions close to the inlet and outlet channel, where temperature is slightly non-uniform. Reactors occupying an area of 3 x 3 mm^2 or of 10 x 10 mm^2 have been fabricated. T-junction channels, 30 or 50 µm wide, are used to generate droplets. Devices with etch depth of 30 µm or 60 µm were fabricated, corresponding to a microreactor volume of 0.2 µL or 0.4 µL in the small design and to 1.6 µL or 3.2 µL in the large design. Data reported here refers to 3 x 3 mm^2 and 30 µm deep devices.

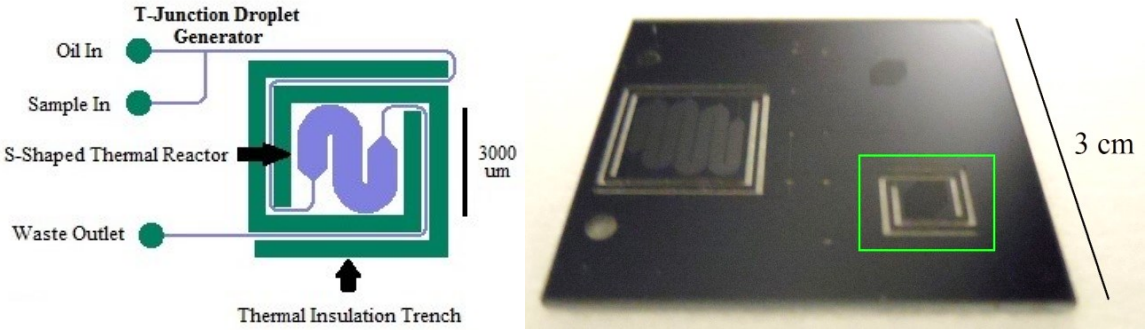


Figure 1. *(a) Mask design used to fabricate a single device composed of a droplet generator and a thermally insulated cavity. The violet regions are etched from the front side only, the green regions from both front and back side. (b) Photograph of a chip containing two ddPCR devices. The rector footprint is 3x3 mm^2 for the small device and 10 x10 mm^2 for the large one.*

The temperature of the ddPCR reaction chamber was controlled using a fine wire thermocouple and a Peltier element that can generate or extract heat depending on the applied current direction. The thermocouple is glued to a silicon interposer having a groove specially designed for this purpose. The thermoelectric cooler is then glued to the interposer. The formed stack is glued to a copper heat spreader, in its turn bonded to a fin radiator. A thermally conductive epoxy is used as a glue in all above steps. The reaction chamber is positioned onto the silicon interposer and kept in place by appropriate springs. To improve thermal contact between the reaction chamber and the heating element a thin layer of conductive thermal grease is used. Thanks to the combination of this effective thermal solution with the small reactor thermal mass fast thermal cycling can be achieved. The aqueous mixture containing templates and reagents necessary to perform PCR, and the oil are injected on the microchip through two independent inlets using syringe pumps, capillary tubes and nanoport fluidic connectors. The inlet flow rates are defined using the pump controller.

Droplets were generated by setting the flows of PCR reagents and carrier oil to 0.4 µl/min and 0.8 µl/min respectively. To generate droplets of uniform size at a uniform rate the surfaces of the droplet generator and of the PCR cavity need to have a water repealing coating to avoid droplets to stick to the walls causing flow instabilities. In order to deposit a uniform hydrophobic organic monolayer on the surface of the channels, we used a 'PCR compatible' coating, which does not degrade up to 100°C and does not inhibit the PCR reaction [7]. A solution is prepared by dissolving 250 µl of Octadecyltrichlorosilane (104817-25G Sigma Aldrich, Germany) in 25 ml of Toluene (34866-1L, Sigma Aldrich, Germany). The solution is injected in the chip using a syringe pump, at 10 µl/min flow rate during 10 minutes. The chip is then flushed with isopropyl alcohol to remove the toluene residues and baked in a conventional oven at 100°C during 4 hours for cross linkage of the silanes. To avoid droplet merging during the temperature cycling appropriate PCR master mix and oil supplemented with surfactant (BioRad droplet generation oil,1863005) were used. In all measurements performed no droplet merging was observed.

To excite and collect droplet fluorescence during the amplification process an inverted microscope was used. The field of view was such that the full 3 x 3 mm^2 reactor could be visualized A high power broadband light source (Excite 120, Lumen Dynamics), attached to the microscope, was used for excitation. The excitation wavelength was selected using an optical filter transmitting in the 460-500 nm region placed in front of the light source. The filtered light goes through a dichroic mirror and impinges over the sample. The dichroic mirror reflects only in the wavelength region of the emitted light, hence does not perturb the excitation path. The emitted light, collected by the objective, is reflected by the dichroic mirror and directed towards a CCD camera (Orca R2, Hamamatsu) used as detector. To ensure that only the fluorescence light will reach the detector a second filter**,** transmitting in the 515nm to 555 nm region, was installed in the optical path. The fluorescent image of each individual droplet stored on the cavity was analyzed using ImageJ, an open source image processing software package used to count droplets and to measure their size and fluorescence intensity.

In order to perform PCR the following protocol has been used. 10 µL of 2x dPCR Master Mix for Probes (BioRad, 186-3010) were mixed with 1 µL of 20x primers from BioRad (dPCR EGFR, FAM probe). The template (Human Gen DNA Template, 100 ng/µL) was mixed with DNA free water in different proportion to obtain the desired DNA concentration that ranged from 20 to 13000 copies per µl, 9 µL of the mix were aliquot and added to the Mastermix and primers. The following thermal cycle was used: enzyme activation for 5 minutes at 95 °C, 70 cycles of 95 °C for 15 s with a ramp time of 6s to 60 °C, held for 45 s, with ramp time of 12 s to reach back the 95 °C.

3. RESULTS AND DISCUSSION

The droplets generated on the T-junction of the microdevice are presented on Fig. 2a after reaching the reactor, the inset is a magnified view of some of the droplets. The diameter of each droplet is measured using the outer contour, indicated in red in the figure, as it does not change with the objective magnification, while the inner contour, in green, depends on magnification. The droplet volume is estimated assuming that they have a cylindrical shape, which is reasonable as the typical diameter is about twice larger than the cavity depth. The volume of droplets in eight experiments, performed using different chips, is shown in Figure 3b. The average volume is about 63pL per droplet and the volume variation is less than ±5%. The droplet generation rate, calculated as the ratio of the volumetric flowrate to the droplet volume, is about 6400 droplets per minute.

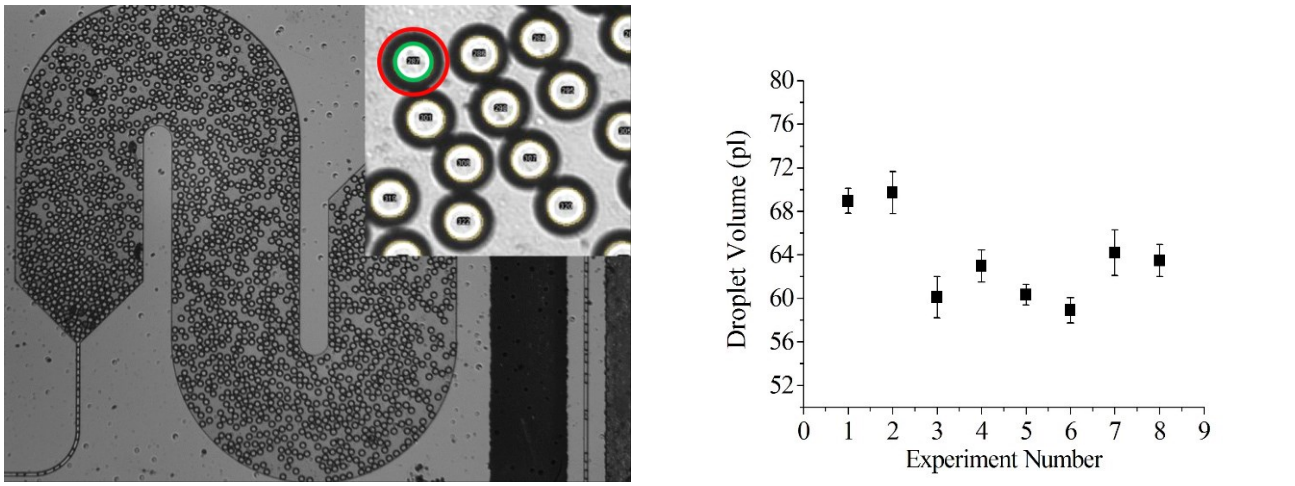
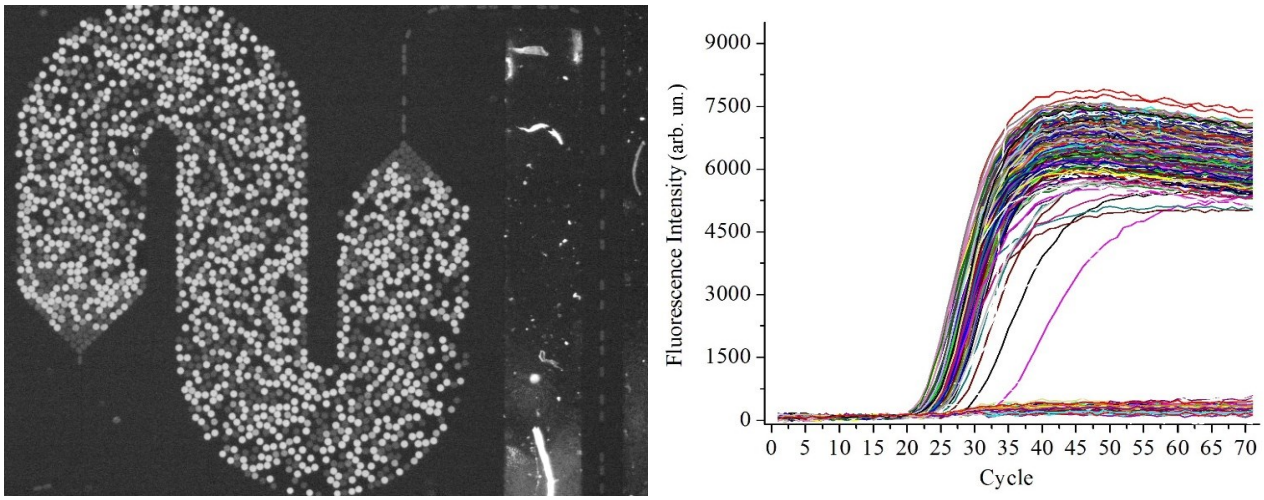


Figure 2 *a) Bright field image of the reactor. The inset is the magnified image of a few droplets. b) Average droplet volume in eight different experiments.*

Fig. 3a shows a typical fluorescent image after the amplification is completed. Real time fluorescence curves are determined by analyzing the fluorescent image of the reactor at each cycle. An example of these curves is reported Figures 4b which refers to a concentration of 13300 copies/µL. We notice that amplification starts for all droplets between cycle 23 and 25 and that only a few droplets show late amplification.



a) b)

Figure 3. *(a) Fluorescent image of the reactor at the amplification endpoint (b) RT fluorescent curves obtained for a template concentration of 13300 copies/µL*

To demonstrate functionality of the integrated device, human genomic DNA with concentrations ranging from 0.06 to 40 ng/µl (or 20 to 13300 copies/µl) were amplified. A negative control experiment (no template) was performed and no fluorescent droplets were observed, indicating the absence of contamination. The concentration values obtained by analyzing data similar to those in Fig. 3b for each concentration are plotted against the nominal values in Fig. 4. The good linearity on almost three orders of magnitudes illustrates the power of dPCR for accurate DNA quantitation.

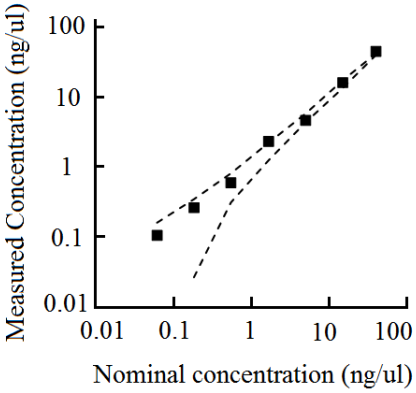


Figure 4. *Measured concentration vs. nominal one. The dotted lines are the 95% confidene interval.*

One of the issues in developing a ddPCR system on chip is the reduced sample volume, which adversely affects the low limit of detection. In the device characterized in this paper, the droplets are arranged in a monolayer in a cavity occupying a footprint of 3mm x 3mm, the depth of the cavity is similar to the droplet diameter, about 50 um, hence the total sample volume is small. A ten times larger volume can be obtained by increasing the footprint to 10 mm x 10 mm, a further increase of one order of magnitude can be achieved by etching a 10 times deeper cavity in silicon.. The volumes of these three devices are approximately 0.5, 5 and 50 µL, of which 50% is oil. The largest system can analyze a sample volume typical of conventional qPCR tools, with this increased volume the low limit of detection shown in figure 4 can be decreased by two orders of magnitude, thus giving a 5 orders of magnitude dynamic range..

4. CONCLUSIONS

An on chip droplet dPCR microsystem based on Si-Pyrex technology was demonstrated. Uniform volume droplets were generated at rates close to 10,000 droplets per minutes. The dynamic range of the tested device spans three orders of magnitudes and the lower limit of detection is 20 copies/µl. the system can be upgraded to ten or hundred times larger sample volume, allowing a lower limit of detection and extended dynamic range. The extension to large volumes is incompatible with the determination of real time fluorescent curves. To implement this approach the droplets have to be flown out of the cavity through a capillary channel and their fluorescence intensity measured at a single point by means of a detector like a photomultiplier tube. The system described here has the potential to perform fast amplification, as thermal transients are in the 1-2 s ranges. In the presented experiments the PCR time was limited by the assay and not by the system, hence assays optimization is necessary, but not straightforward, as it has to combine short time and high amplification efficiency to avoid late amplification of droplets, In addition, droplet stability may be compromised by fast temperature cycling. The use of silicon for ddPCR chips, has the potential for integration of sample preparation and optical detection on the same ddPCR chip, thus allowing the realization of compact PoC or even ubiquitous systems for quantitative molecular analysis.

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