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Solid-phase PCR in a picowell array for immobilizing and arraying 100 000 PCR products to a microscope slide†

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Received 19th March 2012, Accepted 15th June 2012
DOI: 10.1039/c2lc40534b

We present a method for performing highly parallel PCR reactions in a picowell array (PWA) simultaneously immobilizing generated PCR products in a covalent and spatially-resolved manner onto a microscope slide via solid-phase PCR (SP-PCR). This so called PWA-SP-PCR was performed in picowell arrays featuring 100 000 wells cm⁻² of 19 pL reaction volumes with a surface-to-volume ratio of 0.2 μm⁻¹. Positive signals were obtained in 97.2% of the 110 000 wells in an area of 110 mm². Immobilized DNA was either indirectly detected using streptavidin-Cy5 or directly by molecular hybridisation of Cy3- and/or Cy5-labelled probes. Amplification and immobilization was demonstrated for template DNA ranging from 100 bp up to 1513 bp lengths. Even single DNA molecules were successfully amplified and immobilized demonstrating digital solid-phase PCR. Compared to widely established emulsion based PCR (emPCR) approaches, leading to PCR products immobilized onto bead surfaces in a highly parallel manner, the novel technique results in direct spatial registration of immobilized PCR products in a microarray format. This enables the subsequent use for massively parallel analysis similar to standard microarrays.

Introduction

The first primer-directed PCR published in 1988 by Saiki et al. was performed in a reaction volume of 100 μL.¹ Over the last decades, multiple efforts have been made to scale down PCR reaction volumes and simultaneously increase the number of reactions.²–¹⁰ And for good reason: small volume PCR in large-scale systems drastically decreases costs per reaction. When e.g. loading Saikis 100 μL of reaction mix into an array of 19 pL volumes, more than 5 million individual reactions can be performed in parallel. Additionally, low thermal masses and an enlarged surface-to-volume ratio result in an increased thermal transfer that leads to reduced cycling times. As an example, Neuzil et al. amplified an 82 bp template in less than six minutes within 100 nL droplets.¹¹ Together with ongoing improvements in the sensitivity and resolution of analytical equipment, small volume PCR allows for economical large scale analysis of genomic features, signaling pathways or expression profiles down to the single molecule level. Nevertheless, in most of the small-volume PCR systems products are discarded after PCR,²,³,⁵,⁷,⁹,₁⁰ whereas the literature is full of creative uses of PCR products immobilized to a solid phase (array), for multiplexing,¹²,¹³ SNP analysis,¹⁴,¹⁵ and whole-genome sequencing.¹⁶–¹⁸ Our approach combines picowell array based large-scale liquid-phase PCR amplification with solid-phase PCR. Here, PCR products are immobilized to a standard microscope slide allowing subsequent analysis by commercial microarray equipment.

A first requirement is a simple macro-to-micro interface for distributing the PCR reaction mix homogeneously in the picowell array. For running the PCR reaction, the composition of the PCR reaction mix must be adjusted to ensure successful amplification of DNA in the 19 pL volumes of the picowell array. Due to its large surface-to-volume ratio of 0.2 μm⁻¹, adsorption of reaction components to the surface of the wells is increased.¹⁹ To ensure spatial resolution and prevent evaporation the filled picowells must be sealed with a slide which is elastic (to seal individual wells) and vapour tight (to prevent evaporation of water during thermocycling). For successful immobilization of PCR products, the surface of the slide has to provide thermostable grafted primers and a free 3’ end which is accessible to annealing of complementary template DNA and DNA polymerase.

In literature, compartmentalization of PCR reactions for small-volume PCR is realized either by aliquoting PCR mix into microreactors on chip or by encapsulating reagents in individual droplets. The first enzymatic reactions on single molecules within individual droplets are described by Griffiths and Tawfik.²⁰ This concept has been further developed to amplify single DNA molecules via emulsion PCR (emPCR) in the liquid phase²¹ or
Simultaneously immobilizing PCR products to a solid-phase of a bead.22–24 Over the last decades, PCR microreactors on-chip have been realized by closed-architecture systems where micro-reactors are formed on-demand either by mechanical compartmentalization of PDMS channels5,9 or by segmentation through a second, immiscible liquid phase e.g. mineral oil.5,9 In open architecture systems, arrayed microreactors are enclosed for example by a HIPORA membrane,22 silicon sheet,5,9 mineral oil,10 or formed by the SlipChip system.6 Until 2011, the smallest PCR is performed by Nagai et al. who successfully amplified the pGFP fragment within a microreactor of only 86 pL, whereas this reaction failed in volumes of 23 pL and 1.3 pL.5 Heyries et al. recently presented an emulsion-based PCR system in a chip where the smallest version comprises 1 × 106 of 10 pL reactors with a density of 440,000 reactors cm–2.9

Two fields of applications arose from the development of nano- and picoliter PCR systems: single-cell analysis7,8,24–26 and digital PCR (dPCR) based analysis.5,27–30 Lindström et al. cultivated single cells in a microwell array followed by PCR based genotyping and minisequencing.8 Gong et al. detected protein translation and thereafter gene expression levels by on-chip RT-PCR from single cells deposited in a microwell array.7 Detection of specific genes from single cells has also been demonstrated with emPCR.24,26 dPCR has been used to quantify DNA libraries for sequencing,31 high throughput gene expression analysis,32 and the identification of SNPs.9

Some shortcomings are inherent to all systems performing PCR in microreactors. (1) Recovery of the PCR product: in current systems, further analysis is either ignored2,3,5–7,9,10 or pooled reaction products are analyzed externally.5 It is especially challenging to maintain the spatial distribution of amplified DNA after PCR when a seal is removed from the array.2,8 (2) Stability of micro-reactors: When the material PDMS is used for the formation of microreactors, special care must be taken to prevent evaporation.9,33,34 In emPCR systems, individual droplets can merge during PCR leaving aggregates of beads containing chimeric DNA sequences.23 (3) Sensitivity: when individual reactions are monitored by TaqMan probes,2,3,5,7,9,10 or intercalating dyes,5 signal-to-noise ratios can be critical due to adsorption of the fluorescence molecules to the surface or surface-induced quenching. Furthermore, when signals are detected within microreactors, signals can be attenuated by the material of the array made from PDMS,5,7,9 polycarbonate,5 or silicon.2

This work describes the setup and protocol for amplification of DNA molecules in picowells arrays16,35 (1000 wells cm–2) with a starting concentration of 20 molecules or < 1 molecule (digital PCR) per 19 pL well and an amplicon length of 100 to ~1500 bp. Generated PCR products are immobilized onto a standard slide format via SP-PCR.36 The aim of this study is to overcome the shortcomings: (1) recovery of the PCR product and (2) stability of microreactors by PWA-SP-PCR. Here, PCR products are recovered by immobilizing individual products to corresponding positions on a microscopic slide, sealing the array during SP-PCR. In contrast to PCR microreactors made of PDMS or formed in an oil matrix, the rigid boundaries of the glassy array promise to keep each reaction mix localized during PCR. Concerning (3) sensitivity: well distinguishable signals are expected since signals are detected on a planar surface by established detection techniques derived from microarrays.

Experimental section

Materials and chemicals

All materials and chemicals were obtained from commercial sources and used as received. Glass slides, bovine serum albumin (BSA Fraktion V), Tween 80 (reagent grade), sodium hydroxide (≥99%), sodium chloride (~99.8%), and sodium dodecyl sulphate (SDS; ultrapure >99.5%) were obtained from Carl Roth (Karlsruhe, Germany). Formamide (≥99.5%, for molecular biology) was purchased from Sigma-Adrich (Schnelldorf, Germany). Polydimethylsiloxane (PDMS Silgard 184) was obtained from Arrow Central Europe (Bietigheim-Bissingen, Germany). HPLC purified, synthetic, lyophilized oligonucleotides were ordered from Biomers (Ulmm, Germany) and rehydrated with DNase/RNase-free water to 100 μM and 10 μM stock solutions. The amplification vector pTYB1 was obtained from New England Biolabs (Hitchin, United Kingdom). GeneFrames® (In situ Rahmen, 25 μL, 1.0 × 1.0 cm) were ordered from PEQLAB Biotechnologie (Erlangen, Germany). DNA polymerase HotStarTaq Plus, reaction buffer, MgCl2 were purchased from Qiagen (Hilden, Germany). dNTPs were purchased from Jena Bioscience (Jena, Germany). biotin-11-dUTP was purchased from Yorkshire Bioscience (York, United Kingdom). For the DNA dilution buffer, TE buffer (0.2 ×) from Applichem (Darmstadt, Germany) and salmon sperm DNA (10 ng μL–1 salmon (sperm DNA) from Invitrogen (Carlsbad, Germany) was used. DNase/RNase-free water for PCR reactions was obtained from Invitrogen (Carlsbad, Germany).

DNA immobilization

Glass-PDMS slides were fabricated by spincoating a 70 μm thick PDMS layer on a glass slide in a cleanroom to produce a homogeneous and particle-free PDMS layer. PDMS pre-polymer and curing agent were thoroughly mixed at a 10 : 1 (w/w) ratio. 1.5 mL was pipetted onto a glass slide fixed to the vacuum chuck and curing agent were thoroughly mixed at a 10 : 1 (w/w) ratio. 1.5 mL was pipetted onto a glass slide fixed to the vacuum chuck of a spincoater (Spincoater WS-400B-6NPP/LITE, Laurell Technologies, North Wales, USA). After spinning for 20 s at 2200 rpm, slides were cured at 70 °C in an oven cabinet overnight (12 h < t ≤ 16 h).

Activation of glass-PDMS slides with the homobifunctional linker 1,4-phenylene disiothiocyanate (PDITC) for PCR compatible immobilization of PCR primers has been described previously by our group.37 The full-match solid-phase primer 5′–NH2–C6–TTT TT TCT GAG CGG GCT GGC GC–3′ or the full-mismatch primer 5′–NH2–C6–TTT TTT TTT TGA TAC ATG GTG A GT AGT CAT CAA TTA CGA–3′ was reacted with the PDITC-activated surface overnight (12 h < t ≤ 16 h) at room temperature in a 25 mL slide holder containing activated glass-PDMS slides and the primer diluted to 200 nM in 150 mM sodium phosphate buffer (pH = 8.3).

Reaction setup

The reaction mixture contained 40 U HotStarTaq Plus, 1 × reaction buffer, 1.5 mM MgCl2 (in total 3 mM), 300 μM each dATP, dGTP, dCTP, 225 μM dTTP, 75 μM biotin-dUTP, 0.5% (w/v) BSA, 0.05% (v/v) Tween 80, 0.125 μM forward primer 5′–CTG AGC GGG CTG GCA–3′ and 1 μM reverse primer 5′–GCC TCC CTC GCG CCA TCA G–3′, and calculated 20 copies of
template DNA per well. The generation of template DNA in length of 100 bp, 346 bp, and 1513 bp is described in the ESI (1). Each template sequence was flanked at the 3′ and 5′ end by generic primer sequences to amplify all sequences with the same pair of primers. Final volume of the reaction mix was adjusted with ultrapure DNAse/RNase-free distilled water to 16 μL. Thoroughly vortexed reaction mixture was kept on ice until loading into the picowell array.

The loading setup is similar to Fig. 1. The 13 × 13 mm² picowell array (Fig. 1 A) (PTP, Roche Diagnostics, Mannheim, Germany) was placed into the 14 × 14 mm² recess of the in-house fabricated copper holder (Fig. 1 B). A silicone gasket confining a microfluidic loading chamber volume of 15.6 μL was placed on the array with the wells facing upwards. The gasket on the array was pressed to the copper holder (Fig. 1 B) by fastening an in-house micro-milled PMMA loading lid containing an inlet and outlet port with two M3 screws. 16 μL of reaction mix was loaded into the inlet by pipet and uniformly driven through the microfluidic chamber on the array.

When PCR is performed in a reaction compartment containing solid as well as liquid-phase PCR primers, the latter in an asymmetric ratio (Fig. 2 A), two different reaction regimes of the amplification kinetics have to be distinguished. Phase 1: in the beginning of the reaction, liquid-phase PCR kinetically dominates until one liquid-phase primer (green primer in Fig. 2 B) is depleted. This is due to steric restriction and lower efficacy of the surface reaction. Phase 2: after depletion of the limited liquid phase primer and/or a sufficient amount of generated PCR product the generated freely diffusing amplicons predominantly anneal to the solid-phase primers, which can then be extended by a DNA polymerase. A careful balancing of Phase 1 and Phase 2 allows for the amplification and immobilization of the PCR-products to a surface (Fig. 2 C). Validation of successful solid-phase primer extension is performed by binding of streptavidin-Cy5 (Fig. 2 E and F) to incorporated biotinylated dUTP (Fig. 2 D) or by hybridization of a sequence specific fluorescent probe and subsequent fluorescence detection.

Results and discussion

The enlarged surface-to-volume ratio is challenging when performing PCR within picoliter wells because significant amounts of PCR components are adsorbed by surfaces. Krishnan et al. investigated the influence of magnesium ions and polymerase concentration on the PCR performance within microchannels with surface-to-volume ratios of

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<td>Fig. 1 Exploded view of the cycling cartridge for performing PWA-SP-PCR. (A) Picowell array in a recess of the holder made from copper (B). The thickness of the copper beneath the array is 0.5 mm. (C) glass slide covered by a 70 μm thin layer of PDMS. (D) 3 mm thick elastic stamp made of silicone for an equal distribution of pressure induced by screwing together sealing lid (E) and the holder (A). To load the sample into the array via centrifugation, parts C–D are replaced by a micromilled loading lid made of PMMA defining a 150 μm thick microfluidic chamber on the array.</td>
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The reaction mix, we increased the concentration of MgCl2 from 1.5 mM to 2.5 mM, resulting in a 19 pL well having a surface-to-volume ratio of 0.209. For example, a 10 μL PCR (performed in a standard 100 μL PCR tube) has a surface-to-volume ratio of 0.002 μm−1. In contrast, a 19 pL well has a surface-to-volume ratio of 0.209 μm−1, which is ~100 times higher. Starting from a functional 10 μL PCR reaction mix, we increased the concentration of MgCl2 from 1.5 mM to 3 mM, polymerase from 0.1 U μL−1 to 2.5 U μL−1, and added 0.5% (w/v) BSA and 0.05% (v/v) Tween 80 to reduce non-specific adsorption of PCR components to the surfaces of a picowell to obtain a PCR mix suitable for 19 pL volumes.

Firstly, sequence specificity of the reaction in the picowell array was investigated. Therefore, two picowell arrays filled with the reaction mix with a concentration of 20 DNA copies per well were sealed by a slide coated with a full-mismatch and two arrays with a full-match primer. After PCR, slides were imaged and stained with streptavidin-Cy5 yielding a signal-to-noise ratio of up to 100 (Fig. 3 D) from the slides containing the complementary primer, whereas no detectable signals were obtained from the slides with the non-complementary primer (Fig. 3 B).

Secondly, we investigated the reaction with a 1513 bp template and directly detected the immobilized PCR product via hybridization. Therefore, two arrays were filled with a PCR reaction mix containing the 1513 bp template (PTC) with a concentration of 20 DNA copies per well, and two other arrays with a PCR reaction mix containing no template DNA (NTC). After hybridization with a Cy3 labeled complementary probe, no signals were observed from the NTC reactions (Fig. 3 A) whereas a signal-to-noise of up to 100 was measured from PTC reactions (Fig. 3 C). This shows that template DNA even in the length of 1.5 kb can be bound to a surface via solid-phase PCR and specifically detected via hybridization.

Thirdly, for analysis of the length dependency of the reaction three different DNA templates with lengths of 100 bp, 346 bp and 1513 bp were used. All templates comprised identical 3′ and 5′-ends, to ensure identical binding properties to the surface primers. Again, the concentration is 20 DNA copies per well. After PCR, slides were stained with streptavidin-Cy5 and scanned. For the determination of the yield, the number of positive signals from 10 000 wells on an area of 10 mm2 was determined. Yield for the 100 bp template was 95.3 ± 1.8%, for the 346 bp template 92.1 ± 1.9%, and for the 1513 bp template 90.9 ± 3.5% of positive wells. Taking standard deviations into account, no significant dependency between yield and length of amplicon could be observed.

Fourthly, it was demonstrated that each well can serve as an individual PCR reactor. Therefore, PCR reaction was performed with calculated < 1 molecule per well each of the 346 bp and 1513 bp template DNA. After hybridization with the Cy3 labeled probe (detecting the 1513 bp template) and the Cy5 labeled probe (detecting the 346 bp template), a distinct pattern of green, red, and yellow signals was obtained (Fig. 3 E). Yellow signals resulting from wells where both template sequences were initially present. This experiment confirmed that wells are leak-tightly sealed during PCR and that there is no transfer of PCR products between each well, demonstrating digital solid-phase PCR.

Fifthly, the maximum number of reactions was performed in parallel on an area of 110 mm2. Therefore, >110 000 wells of the picowell array were filled with a reaction mix containing 20 DNA copies of the 100 bp template per well. After PCR, the slide was stained by streptavidin-Cy5 and scanned. Fig. 4 shows the binary image created by the evaluation macro in ImageJ as described in the ESI (3). With the macro, 106 898 signals of 110 000 theoretical signals were counted, which means that in 97.2% of all the 19 pL wells SP-PCR was successfully performed and detected. The Poisson distribution describes the distribution of DNA molecules in microreactors as in eqn (1), where \( P(n) \) is the probability that all reactors of a chip contain \( n \) molecules after filling, and \( \lambda \) being the ratio between the number of DNA molecules and the number of reactors.

\[
P(n) = \frac{\lambda^n}{n!} \times e^{-\lambda}
\]  

(1)

According to eqn (1), the probability that a well contains no DNA molecule for amplification is \( P(0) = 2.1 \times 10^{-7} \) which is ~0 out of 110 000 wells. In this experiment, no signals were generated in 2.8% of all wells, which can therefore not be explained by statistics but a failure of PCR or the adsorption of
DNA template molecules to surfaces. Most importantly, this result shows that arrays can be homogeneously and leak-tight sealed by the employed glass-PDMS slides.

Conclusions and outlook

We successfully performed highly parallel solid-phase PCR in 19 pL wells of a picowell array (PWA-SP-PCR) yielding a maximum of $1.07 \times 10^5$ positive reactions out of $1.10 \times 10^5$ wells (97.2%) from 16 mL reaction mix, only. PCR products were generated from template DNA in length of 100 bp, 346 bp, as well as up to 1513 bp and covalently and spatially-resolved immobilized to a PDMS covered glass slide sealing the picowell array during reaction. Even single DNA molecules were successfully amplified and immobilized demonstrating for the first time digital solid-phase PCR in a picowell array. Surface-bound amplification products (or amplicons) are indirectly detected by staining with Cy5-conjugated streptavidin or directly detected following the molecular hybridization of Cy3- and/or Cy5-labelled probes. To the best of our knowledge, this is currently the smallest successful PCR reaction in a non-emulsion format.

Compared to the presented state-of-the-art, results have demonstrated that PWA-SP-PCR is a promising approach to perform small volume PCR with the following unique features: individual PCR products are spatially-resolved and immobilized onto a microscope slide for further usage. Results from digital solid-phase PCR suggest that there is no cross-talk between

Fig. 3 Binary images of reactions with 20 DNA template molecules per well (A–D) and <1 template molecule per well (E) detected on glass-PDMS slides. (A) Non-specificity control: the PDMS covered glass slide containing an immobilized solid-phase primer with a sequence mismatching the template DNA generated no detectable signals. (B) No-template control: the reaction mix containing no template DNA generated no detectable signals. (C) Direct detection by molecular hybridization. The specificity of the reaction was detected by hybridizing a Cy3-labeled probe against a solid-phase bound 1513 bp sequence. A signal-to-noise ratio of up to 100 was obtained between wells where the reaction failed and positive signals were received from adjacent wells. (D) Indirect detection by reacting streptavidin-Cy5 with biotin molecules which have been incorporated into the 346 bp template during solid-phase primer extension reactions. (E) Digital solid-phase PCR: the reaction mix contained <1 template molecule per well each of the 346 bp and 1513 bp template DNA. The reaction was detected by hybridization. Green color represents the 1513 bp template, red color the 346 bp template, and yellow mixed signals from the 1513 bp and 346 bp template DNA. Scale bars are 200 μm (A–D) and 100 μm (E).

Fig. 4 Binary image analysis of a PCR reaction within 110 000 wells of 19 pL volume each detected on the glass-PDMS slide. In 97.2% of all cavities PCR products were generated and immobilized to the solid-phase primers via SP-PCR. Detection was realized by staining with streptavidin-Cy5.
adjacent wells addressing the issue of instable microreactors.\textsuperscript{9,33,34} Positive signals are easily detectable on the surface of a microscope slide since they feature a signal-to-noise ratio of up to 100. In many systems from literature, reactions are detected in arbitrary array formats either by fluorescence microscopes or application-specific scanners.\textsuperscript{2,3,5,7,10,31} In our system, immobilized PCR products can be processed and detected on standard microscopic slide format and further used in any standard laboratory workflow. Additionally, the solid-phase primer coated slides can be practically applied to all published open-architecture laboratory workflow. Additionally, the solid-phase primer coated immobilized PCR products can be processed and detected on standard slides can be practically applied to all published open-architecture arrays for immobilizing PCR products.\textsuperscript{2,4,7,8,10} As we have shown previously, the coating protocol is applicable for glass, COP, COC, PP, and PDMS.\textsuperscript{37}

Compared to emPCR approaches for the amplification and immobilization of long template DNA molecules, the use of prolonged annealing and extension times is required. As a consequence, single droplets can merge leaving aggregates of beads containing chimeric DNA sequences.\textsuperscript{28} Because of the rigid boundaries of the wells of the picowell array, reactions remain localized even at elevated temperatures. Most interestingly, our system is capable of amplifying and immobilizing PCR products derived from a single molecular level, enabling digital solid-phase PCR. In the future, PWA-SP-PCR may enable highly-parallel single-cell analysis by isolating single cells in different wells and immobilizing specific gene products onto a microscope slide for further analysis.\textsuperscript{7,8}

Acknowledgements

We gratefully thank the Hans L. Merkle Foundation for Excellency in Science and Technology for financial support of this project.

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