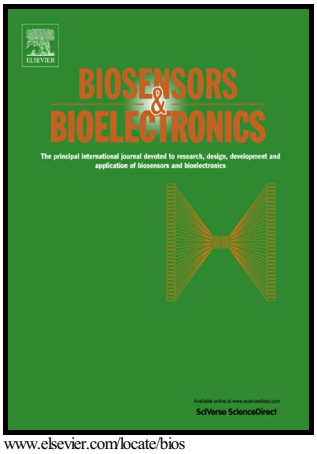

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A novel lab-on-chip platform with integrated solid phase PCR and supercritical angle fluorescence (SAF) microlens array for highly sensitive and multiplexed pathogen detection

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Abstract

Solid-phase PCR (SP-PCR) has become increasingly popular for molecular diagnosis and there have been a few attempts to incorporate SP-PCR into lab-on-a-chip (LOC) devices. However, their applicability for on-line diagnosis is hindered by the lack of sensitive and portable on-chip optical detection technology. In this paper, we addressed this challenge by combining the SP-PCR with super critical angle fluorescence (SAF) microlens array embedded in a microchip. We fabricated miniaturized SAF microlens array as part of a microfluidic chamber in thermoplastic material and performed multiplexed SP-PCR directly on top of the SAF microlens array. Attribute to the high fluorescence collection efficiency of the SAF microlens array, the SP-PCR assay on the LOC platform demonstrated a high sensitivity of 1.6 copies/µl, comparable to off-chip detection using conventional laser scanner. The combination of SP-PCR and SAF microlens array allows for on-chip highly sensitive and multiplexed pathogen detection with low-cost and compact optical components. The LOC platform would be widely used as a high-throughput biosensor to analyze food, clinical and environmental samples.

Keywords: solid phase PCR, microlens array, supercritical angle fluorescence, high sensitivity, lab-on-a-chip

1. Introduction

Multiplex detection methods are essential to conduct DNA-based diagnostics (Gehring et. al., 2011). In order to accurately analyze food, clinical or environmental samples, it is important to detect and differentiate not only different pathogens but also different genus, sub-species and serotypes of the pathogens. At the moment, the most commonly used strategy is multiplex quantitative PCR (qPCR) (Duffy et al., 2013). A qPCR assay can amplify and detect different DNA sequences in the same reaction tube through the use of specific probes, but the techniques is only able to amplify 4 or 5 genes due to the interferences between different primer and probe sets. As an alternative strategy, solid phase PCR (SP-PCR) has attracted enormous interest (Khodakov et. al., 2014). The technique amplifies target nucleic acids on a solid support with one or both primers attached to a surface. Since the spatial separation of the primers minimizes undesirable interactions, numerous primers can be arranged in format of high-density array within a relatively small space. Therefore, SP-PCR provides much higher multiplexing capability than qPCR and holds significant promise in multi-analyte analysis (Sun et. al., 2011).

To date, molecular diagnostic industry is actively seeking simple and cost-effective sensing platforms that can be employed in point-of-use settings(Yoon et. al., 2012). Driven by the demand for rapid online or at site detection, lab-on-a-chip (LOC) technology has been rapidly developed utilizing the capabilities of microfluidic technologies to integrate multiple laboratory processes in a miniaturized device. A few attempts have been done to incorporate the SP-PCR into LOC systems in order to achieve multiplexed analysis (Hoffmann et. al., 2012; Sun et al., 2011). The SP-PCR was performed either in a microarray format within a single chamber (Sun et al., 2011) or in multiple wells with reaction volumes down to pico liters (Hoffmann et al., 2012). However, as the amplification efficiency

of the SP-PCR is lower than conventional liquid PCR, a highly sensitive benchtop laser scanner is often required to detect and quantify the signals of the SP-PCR. Such scanners are bulky, expensive and take extremely long time for scanning an area with high resolution. Therefore, the technique is not suitable for on-site testing.

Recently, a number of portable detection methods were developed for parallel readout in LOC devices (Pires et al., 2014). They were based on various principles, such as magnetic (Gaster et al., 2011), electrochemical (Lu et al., 2012), mechanical (Johnson et al., 2012) or optical properties (Yildirim et al., 2012). Although magnetic, electrochemical and mechanical techniques possess the ability of label-free and real-time detection, their applications in SP-PCR-based microfluidic devices are greatly limited by complex fabrication procedures, strict requirements of assay optimization and precise control on differentiation between nonspecific and specific binding. In contrast, the optical detection is more attractive for LOC systems as it is robust, not affected by the environmental variations, and moreover, the ability to detect multiplexed reactions in SP-PCR could simply be realized by rapidly extracting data from e.g., fluorescence imaging (Pires et al., 2014). On-chip optical sensing has been achieved by either developing portable versions of conventional optical instrumentation (Yao et al., 2012), or by incorporating part of the optical detection system on the microfluidic substrate itself (Yildirim et al., 2012). Despite of the recent advances in compact optical sensors, inherent factors related to on-chip detection, such as the requirements of a short working distance and miniaturized electronic or optical components, have led to small field of view, high optical losses and decreased signal-to-noise ratio. It remains an ongoing challenge for integrating of optical detectors in microfluidic devices for highly sensitive and multi-point detection.

Previously, we managed to fabricate a high-quality micro-optical lens array as part of polymeric chip to improve the sensitivity of fluorescence detection (Hung et al., 2015). It was based on one important phenomenon called super critical angle fluorescence (SAF), which reveals that for a fluorophore sitting at the surface interface, majority of the light is emitted into the material with higher refractive index, mostly around the supercritical angle (Winterflood et al., 2013). To collect this main part of the fluorescent light which is normally lost when using conventional microcopy, we designed and micromilled cone-shaped structures in aluminum master and then replicated the structures in polystyrene substrates by injection molding. The SAF microlens array presented great advantages for on-chip detection. We showed by collecting light at the critical angle, the sensitivity of the test was dramatically increased by 46 folds. Moreover, the SAF structure offers a large field of view as the special arrangement of optical pathway makes light collection efficiency independent of the numerical aperture of the optical system. Last but not least, the SAF-microlens array could be fabricated in a high density, which increases the multiplexing capability for detection of biological targets.

In this paper, we present an innovative LOC platform by combining the SP-PCR with the SAF microlens array in a polymeric microfluidic chip, so that signals from highly multiplexed SP-PCR reactions could be effectively detected on chip with low-cost and compact optical components. Our experimental results demonstrated that with the SAF-microlens array, the limit of detection of the LOC system was 0.8 fluorophores/μm^2. The sensitivity of the SP-PCR assay on the SAF-microlens array was determined as low as 1.6 copies/μL. This detection sensitivity is comparable to that obtained by a conventional microarray with data acquisition from a high-end laser scanner. The LOC system presented in this work is advantageous in terms of having a small equipment footprint, whilst retaining high sensitivity and multiplexing capability. In addition, incorporation of the SAF microlens array

could easily be realized by injection molding for mass production. The new technologies would result in a portable, high-throughput biosensors that are ideal for online or onsite food safety control, clinical diagnosis as well as environmental monitoring.

2. Concept

The principle of the LOC system is illustrated in Fig. 1. The cone-shaped SAF microlens array is located at the bottom layout of a microchamber. DNA probes specific to different pathogens are immobilized directly on top of the SAF array. The SP-PCR is then carried out on the SAF array. Briefly, during initial thermal cycle, if the probes match the DNA sequence in the sample, they will be extended by the DNA polymerase. In the next cycle, the extended probes served as templates for the second strand elongation, thus generating new templates for the next round amplification. After the reaction, PCR products remain covalently attached to the substrate and fluorescence signals are generated as one of the primers is labelled with fluorescent dye. The signals are then collected through the SAF structures, and the pathogens can be identified from the pattern of fluorescence on the SAF array. In this study, the SP-PCR reactions for multiplexed detection of Salmonella spp. at subspecies level were developed and performed on the SAF microlens arrays. Attributed to the low fabrication cost and the ease of integration, the utilization of the SAF array in the SP-PCR detection will allow the SP-PCR to be widely applied for on-site multiplexed pathogen detection.

3. Materials and Methods

3.1. Fabrication of injection moulded chip with SAF microlens array

The microchip with dimension of a microscope slide (76 mm × 25 mm × 1 mm) was fabricated in cyclic polyolefin copolymer (COC) (TOPAS 5013-10, Topas Advanced Polymers GmbH, Germany) by injection molding (Engel Victory 80/45 Tech, PA, USA). To make the injection molding insert, we use a computer controlled micro-milling system (Folken Ind., Glendale, California, USA). The inverted master of microfluidic chambers and SAF arrays was milled using a 60^{o} engraving tip (DIXI 7006, Le Locle, Switzerland) in hard aluminium (alloy 2017, MetalCentret, Denmark). The master was polished to get smooth surface. As shown in Fig. 2, the polymeric chip had eight chambers located in parallel with a pitch of 9 mm. Each chamber had dimension of 5 mm (length) × 3.6 mm (width) × 0.4 mm (height), corresponding to a volume of 12 µL. In the middle of each chamber, there was a miniaturized SAF microlens array of 32 truncated cone-shape structures (diameter of top surface 150 µm; diameter of rear surface 300 µm; height 130 µm and pitch 680 µm). Microfluidic channels with width of 350 µm and depth of 400 µm were connected the chambers for sample processing. The surface roughness of the SAF structure was measured by a PLu Neox 3D optical profiler (Sensorfar, USA).

3.2. Chemicals

Chemicals and reagents used in this study were of analytical grade and purchased from Pierce Inc., USA and Sigma-Aldrich, USA unless otherwise specified.

3.3. Bacterial strains and culture conditions

Salmonella reference strains Salmonella Enteritidis (CCUG 92243) and Salmonella Typhimurium (Jeo 3979 Jgt.110) were from a strain collection of National Food Institute, Technical University of Denmark (DTU-Food). The S. Enteritidis reference strain was originated from a culture collection at

University of Goteborg. The strains were resuscitated and selected on Xylose Lysine Desoxycholate Agar (XLD) and grown overnight on Blood Agar (BA, 40 g/L of blood agar base no.2 (CM271, Oxoid, Basingstoke, UK) supplemented with 5% calf blood at 37 °C before use.

3.4. DNA preparation

Bacterial stock culture was grown at 37°C for 18 h in Buffered Peptone Water (BPW). One ml of the bacteria mixture in BPW was collected and centrifuged at 15,000× g for 5 min at 5°C. The bacterial pellet was suspended in 1 mL phosphate buffered saline (PBS, Oxoid) by vortex and centrifuges again in 15,000× g for 5 min at 5°C. The bacterial pellet was subjected to the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The extracted chromosomal DNA was stored at -20°C. DNA concentration was determined by Nano drop (Thermo Scientific, USA).

3.5. Primers and probes

PCR primers and probes were designed for specific detection of Salmonella spp. at subtype level. Four different genes were selected to specifically identify different Salmonella genus. In our previous study, the specificity of the probes was confirmed by testing 57 different Salmonella strains and 30 non-Salmonella strains (Chin et. al., 2016, submitted). The hilA gene is specific for Salmonella genus while the sdf gene is specific for serotypes Salmonella Enteritidis; the sefA gene is specific for serotypes Salmonella Dublin and Gallinarum; and the fliC gene is specific for Salmonella Typhimurium and Kentucky. A Campylobacter universal primer namely UC primer set that was originated from 16S Ribosomal DNA gene of Campylobacter genus was used as a negative control. For each gene, one pair of forward and Cy3 labelled reverse primers was designed for the liquid phase amplification and one nested probe was designed as solid support primer. The probe was modified at the 5’ end with a poly

(T)10-poly (C)10 tail to facilitate the attachment to the plastic substrates. All of the primers and probes were synthesized and purchased by DNA technology (Aarhus, Denmark) and the sequences are listed in Table 1.

3.6. Immobilization of DNA probes on SAF microlens array

A simple UV cross-linking technique was used to directly immobilize the poly(T)poly(C)-tagged DNA oligonucleotide probes on the SAF array without any surface modification (Sun et al., 2012). The probes were diluted in 150 mM sodium phosphate buffer (pH 8.5) containing 0.004% Triton X to a final concentration of 60 µM. The DNA probes were deposited on top of the SAF array using a non-contact nano-plotter 2.1 (GeSim, Dresden, Germany). The Picoliter pin deposited 60 pL/droplet and the spot size was around 100 µm. Each probe was spotted in six consecutive spots for confirmation of the results. After drying the spots, the microchip was exposed to UV irradiation at the wavelength of 254 nm with power of 3 mW/cm^2 for 10 min (Stratalinker 2400, Stragtagene, CA, USA). Subsequently, the chip was washed in 0.1× standard saline citrate (SSC) with 0.1% (w/v) sodium dodecyl sulfate (SDS) (Promega, WI, USA) solution, then gently rinsed in deionized water and dried by nitrogen.

3.7. SP-PCR on SAF microlens array

Recently, we investigated the effect of DNA probe length, concentration and the annealing position on the efficiency of SP-PCR, and have developed a highly efficient multiplex SP-PCR for detection and identification of Salmonella spp. at sub species level. The optimized condition was employed in this study. After immobilization of DNA probe at optimized concentration, the microchip was bonded with a 254 µm COC foil using an ultrasonic bonder (USP 4700, Techsonic, Herstad-Piber, Denmark) at trigger force of 750 N, energy of 70 Ws and holding time of 0.35 s. Twelve µL of PCR master mixture

was then loaded into each chamber through the inlet hole. The master mix contained DNA template, 1 × Phusion® Human Specimen PCR Buffer (Thermo Fisher Scientific), 400 nM of hila forward and 1600 nM hila reverse primers, 200 nM of sdf forward and 800 nM sdf reverse primers, 200 nM of sefA forward and 800 nM sefA reverse primer, 600 nM of fliC forward and 2400 nM fliC reverse primer, 16 ng/µL bovine serum albumin (BSA) and 0.125 U Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific). After loading, the inlet and outlet holes were sealed by PCR compatible tape (Applied Biosystems, USA). The SP-PCR was carried out using a bench-top thermal cycler (Thermo Scientific, USA) at 94 °C for 5 min then followed by 30 cycles of 94 °C for 10 sec, 60 °C for 20 sec and 72 °C for 20 sec, and another 15 cycles of 94 °C for 10 sec, 65 °C for 20 sec and 72 °C for 20 sec. After reaction, the microchambers were washed 3 times by 0.1 % SDS and 0.1× SSC buffer solution followed by MQ water.

3.8. Image acquisition and data analysis

A compact optical module was built for detection of fluorescent dye Cy3. This module included a green LED array light source (525 nm, Thorlabs, NJ, USA), a cy3 filter cube (excitation 513-556 nm, emission 570-613 nm, dichroic cut-on wavelength 562 nm, Edmund Optics, UK), and an USB digital microscope (ScopeEye, NKS Tech ApS, Denmark). Working principle of this optical system can be summarized as follows: Cy 3 flourophores were excited by the green LED light after passing through the excitation filter 513-556 nm and the 45º dicroic mirror. The emission light of Cy 3 flourophors after collection by the SAF array was filtered by the dicroic mirror and the emission filter 570-613 nm. Then the emission light was recorded and imaging by the USB digital microscope. Fluorescence intensity was quantified using ImageJ software (Schneider et al., 2012). A circle was adjusted to the size of the spot and the mean value of grey levels of the pixels inside a fluorescent spot was calculated. A square

was drawn surrounding the circle, and the mean signal was taken as background. The signal to noise ratio (S/N) in this study was defined as the mean signal intensity of the feature subtracted by the mean background, and divided by the variation of the background. The limit of detection was determined at S/N of 3.

4. Results and discussion

4.1. Characterization of the SAF microlens array

Based on the theories of SAF emission proposed by Seeger's group (Ruckstuhl et al., 2000), the angular emission distribution is anisotropic for a surface-bound fluorescent molecule. The distribution of light intensity of fluorophores sitting at an air-COC interface was calculated and illustrated in Fig. 3a. It shows that a significant emission maximum around the direction of the critical angle of total internal reflection, which is given by $\theta = \arcsin(1/1.53) = 40°$. Using the known angular distribution of the fluorescence emission, the dimensions of the microlens elements were carefully designed. The design principle was explained in details in our previous study (Hung et al., 2015). Parameters considered include height, angle of the cone, as well as inner and outer diameter. The SAF structure with 60° angle was chosen to ensure total internal reflection at the side wall.

The high-precision micro-milling and polishing provided low surface roughness down to 60 nm, which warranted the high-quality optical property of the SAF microlens array. The scattering light at the sloped wall of the SAF element due to the surface roughness was estimated from Equation 1 as

$$P_{\text{scat}} = \left(1 - e^{-\left(\frac{4\pi\sigma\cos\theta_i}{\lambda}\right)^2}\right) P_{\text{tot}} \qquad (1)$$

where P_{tot} is the total flux of incident light, P_{scat} is the flux of light scattered, λ is the wavelength, θ_i is the incident angle, and σ is the surface roughness (Su et al., 2005). Assuming that the scattering light accounts for the major optical power loss, the insertion loss caused by the surface roughness was approximately 0.25 dB at the wavelength of 525 nm.

4.2. Detection limit of the integrated LOC system

To quantify the light collection efficiency of the SAF structure made in COC, 10 μM Cy3-labeled DNA probe was spotted on the SAF microlens array. Fluorescent images were taken from both the top (air side) and the bottom (COC side) of the SAF microlens array. As shown in Fig. 3b, when taking the image directly from the top, only a small dot could be seen; while for the image taken from bottom, a brighter dot surrounded by a light ring as a "donut" was observed and the signal increased approximately 40 folds. The light ring corresponded to the fluorescence emission reflected at the side-wall of the SAF structure.

To determine the sensitivity of the system, Cy3-labeled probe was spotted at concentrations ranging from 2 pM to 20 μM on the SAF microlens array. Intensity of the fluorescent light measured with the SAF structure was plotted versus probe concentration as shown in Fig. 3c. The detection limit was 0.2 nM, corresponding to 0.8 fluorophores per μm^2, whereas the detection limit of the signal measured without the SAF structure using the same optical system was determined to be 8 nM, corresponding to 32 fluorophores per μm^2. Thus, the SAF structures enhanced the sensitivity of the system by 40 folds.

In conventional optical detection, it is common to collect surface-generated fluorescence from the air side. However, as demonstrated here, the luminescence collection efficiency was poor since only a small amount of surface fluorescence went into air, and it may be subject to further optical loss

depending on the numeric aperture of the lens (Hung et al., 2015). In contrast, substantial enhancement in fluorescence collection efficiency was achieved with the SAF microlens array, and the low detection limit clearly indicated the high sensitivity of the system. Moreover, the miniaturized dimension of the microlens array allows the benefits of SAF microscopy to be fully utilized in multiplexed detection.

4.3 Specificity and sensitivity of SP-PCR assay on SAF microlens array

Four DNA probes targeting hilA, sdf, sefA and fliC genes were spotted on the SAF microlens array for multiplex SP-PCR reaction. The layout of the array is shown in Fig. 4a. Genomic DNA of S. Enteritidis and S. Typhimurium were used to investigate the specificity of the SP-PCR assay. The distinct fluorescence patterns were shown in Fig. 4a and 4b, indicating that the two Salmonella strains could be accurately identified based on the specific combinations of amplified products. No fluorescence signal was obtained from the UC Campylobacter negative control probe. In particular, unspecific amplification was almost negligible as the probes in SP-PCR were spatially separated, showing that the assay was highly specific. In addition, the throughput of the SP-PCR can easily be increased by further expanding the size of the SAF microlens array, offering the flexibility of interrogating targets ranging from a few to hundreds.

The sensitivity of the SP-PCR on the SAF microlens array was tested using 10-fold dilution series of S. Enteritidis genomic DNA ranging from to 0.16 copies/μL to 1.6×10^5 copies/μL. For comparison, the same SP-PCR reactions were carried out on a microarray spotted on plain COC substrates. In the latter case, fluorescence signals were detected using a conventional scanner (LaVision BioTech GmbH, Bielefeld, Germany).

As shown in Fig. 5, the signal-to-noise ratio (S/N) of the on-chip multiplexed SP-PCR for hilA, sdf, and sefA genes were determined separately. The lowest template concentration at which positive fluorescence signals could be detected for all three genes was 1.6 copies/μL. The S/N increased with template concentration, implying that more amplification occurred on the surface at higher amount of initial template. The signals almost reached plateau when the DNA template was 1.6×10^4 copies/μL, indicating the surface became saturated with the PCR products. The coefficient of variation of the replicate reactions was 9% on average. It was noticed that the S/N varied a lot among different genes. As suggested by Khan et al., this was owing to the inherent difference in hybridization efficiencies between oligonucleotides (Khan, Poetter, & Park, 2008). Despite of the variation, high sensitivity was achieved for all of the three genes. When compared to the conventional SP-PCR that utilized laser scanner to measure fluorescent signals, the LOC system with the SAF microlens array showed comparable detection limit and linear range, though the S/Ns obtained were generally lower. The results suggested that highly sensitive and multi-point detection could be achieved on chip without the use of benchtop sensor systems. As high-density SAF microlens arrays can be easily fabricated by injection molding, they are readily to be incorporated into low-cost and portable microchip devices. The excellent light collection power of the SAF microlens array and the high specificity and multiplexing capability of the SP-PCR are a perfect combination for high-throughput detection in point-of-care settings.

Conclusions

We developed a novel LOC platform that allowed the performance of multiplexed SP-PCR on an injection-molded polymeric SAF microlens array. The enhanced fluorescence collection efficiency and the miniaturized dimension of the SAF structures enabled highly sensitive and multi-point optical

detection on chip. Using an inexpensive and portable optical module for measuring fluorescent signals, detection limit of 0.8 fluorophores per μm^2 was achieved. The SP-PCR reactions developed for multiplexed detection of Salmonella spp. at subspecies level were performed on the SAF microlens arrays in microfluidic chambers. The minimum detectable target concentration was 1.6 copies/μL, which was comparable to those obtained using conventional microarray scanner. As the LOC platform is easy to fabricate and exhibits significant analytical performance features, it has great potential to become a basic tool for point-of-care applications for parallel analysis of molecular interactions, such as gene expression, pathogen detection or single nucleotide polymorphism (SNP) analysis.

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Conflict of interests

The authors declare no competing financial interests.

Contribution

Tran Quang Hung, Yi Sun, Anders Wolff and Dang Duong Bang designed the research plan; Tran Quang Hung, Yi Sun and Wai Hoe Chin performed the experiments; Yi Sun and Tran Quang Hung wrote the paper. The paper was revised by all the co-authors.

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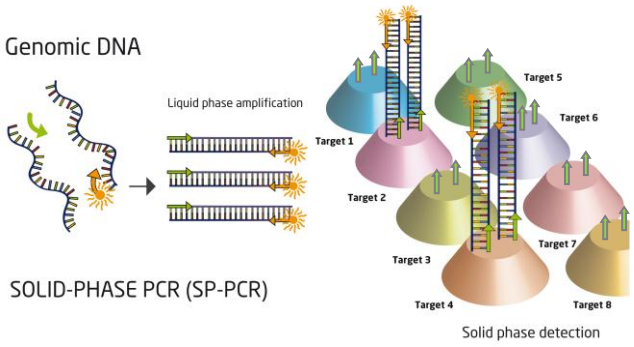


Fig. 1. Concept of SP-PCR on SAF microlens array. Pathogen-specific DNA probes are directly immobilized on the surface of SAF array located at the bottom of the microchamber. PCR reaction mixture is placed in solution with small amount of forward and Cy3-labeled reverse primers. The pathogen DNA is initially amplified in the liquid phase to increase the copy number of the starting template. The target DNA then binds to the immobilized probes on the SAF microlens array, and the matched probes are extended by the polymerase. By this way, highly-multiplexed amplification can occur in a miniature space with thousands of different probes immobilized at discrete spots. After the reaction, PCR products remain attached to the surface through covalent binding. The signals are then collected through the SAF structures, and detected using a CCD sensor.

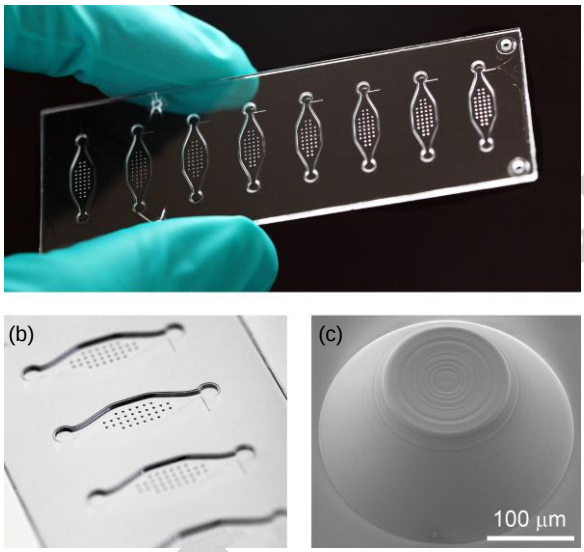


Fig. 2 (a) Image of an injection moulded chip in a microscope slide format. The pitch of the chambers was 9 mm, which was made to fit a multichannel pipette. (b) Image of chambers with integrated SAF arrays. One chamber had a volume of 12 µL, pitch of SAF structures was 0.68 mm. The chamber was surrounded by an energy director for bonding to a COC foil using an ultrasonic welder. (c) SEM image of an injection moulded SAF structure.

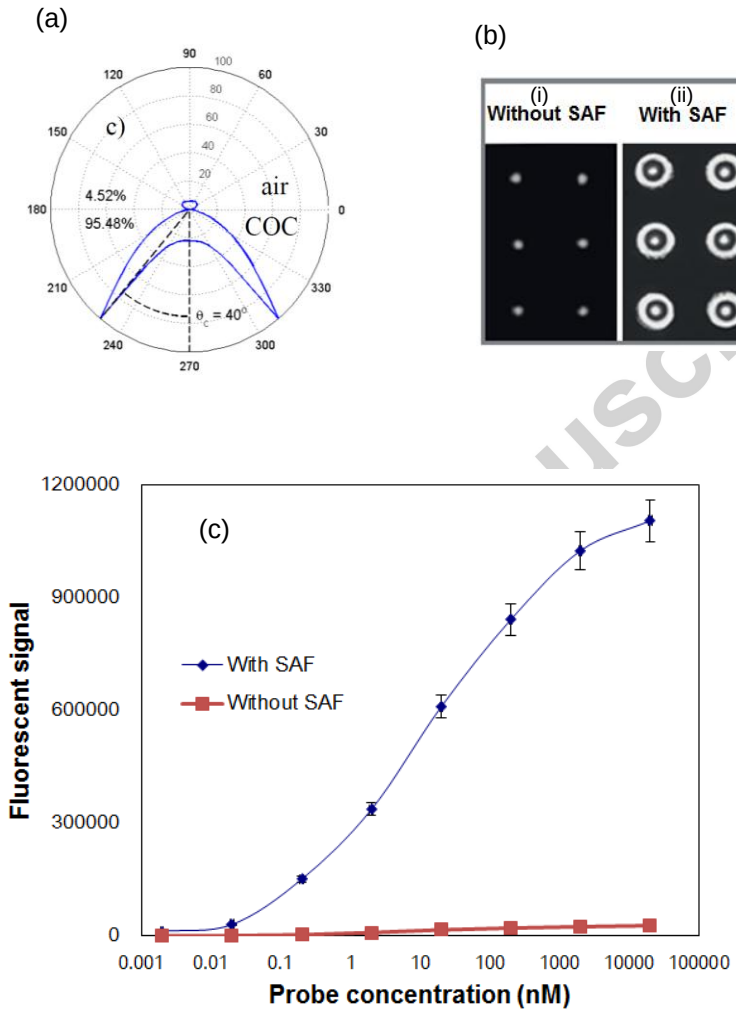
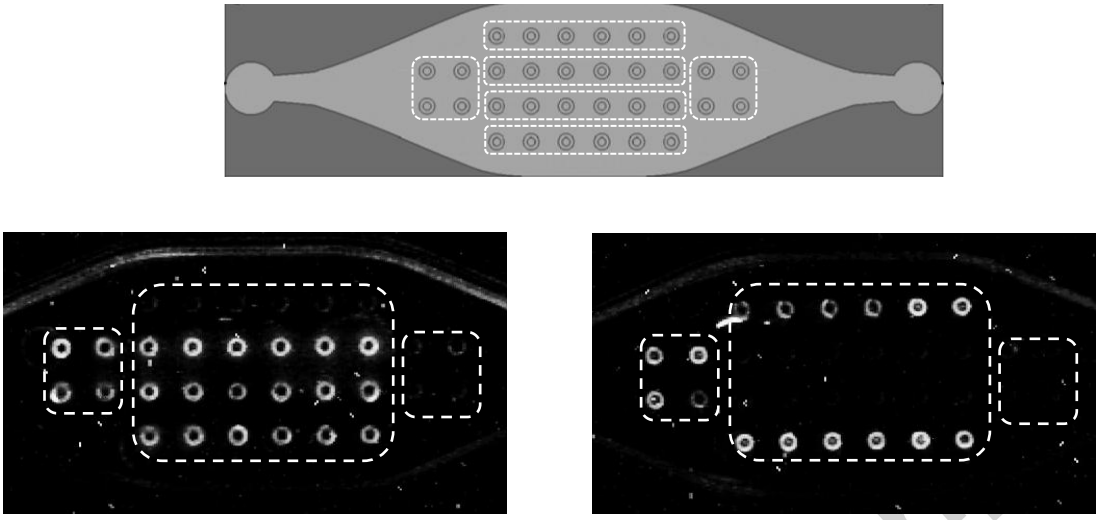
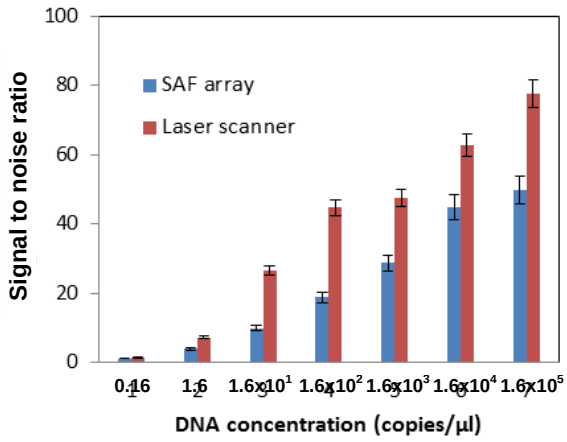


Fig. 3. (a) Analytical calculation of light intensity distribution at the air/COC interface. (b) Fluorescent images of 10 μM Cy3-labeled DNA probe taken from (i) the air side and (ii) the COC side. The light ring corresponded to the emission reflected at the side-wall of the SAF structure. (c) Sensitivity with and without SAF. Cy3-labeled DNA probes with concentrations ranging from 2 pM to 20 μM were spotted on the SAF microlens array and the spot intensities were measured.

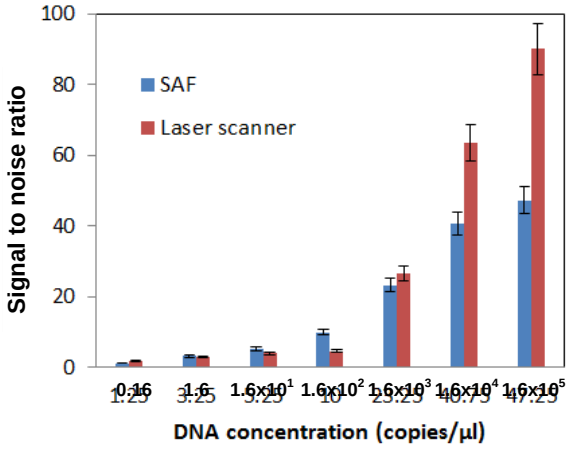


(b) *S.* Enteritidis (c) *S.* Typhimurium

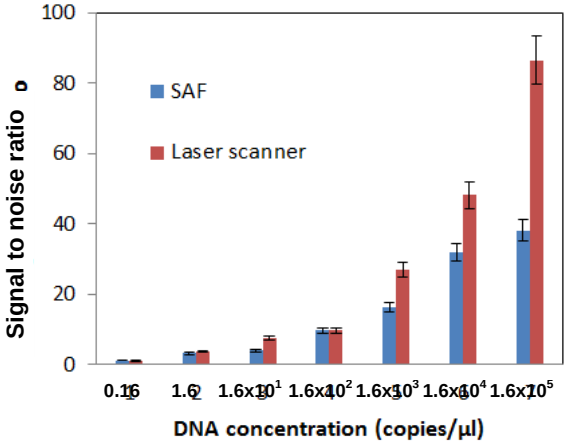
Fig. 4. Specificity of SP-PCR on the SAF microlens array. (a) Microarray layout. The DNA probes targeting hilA, sdf, sefA and fliC genes were deposited in 6 consecutive spots for multiplex SP-PCR reaction. The positive control probe was 5'Cy3 –TTT TTT TTT TCC CCC CCC CC– 3'. The UC gene probe for detection of Campylobacter spp. was used as a negative control. SP-PCR was carried out in the microchamber with a volume of 12 µL. (b) Fluorescent image of the SAF microlens array after SP-PCR using S. Enteritidis template. (c) Fluorescent image of the SAF microlens array after SP-PCR using S. Typhimurium template.



(a) *hilA* gene



(b) *sdf* gene

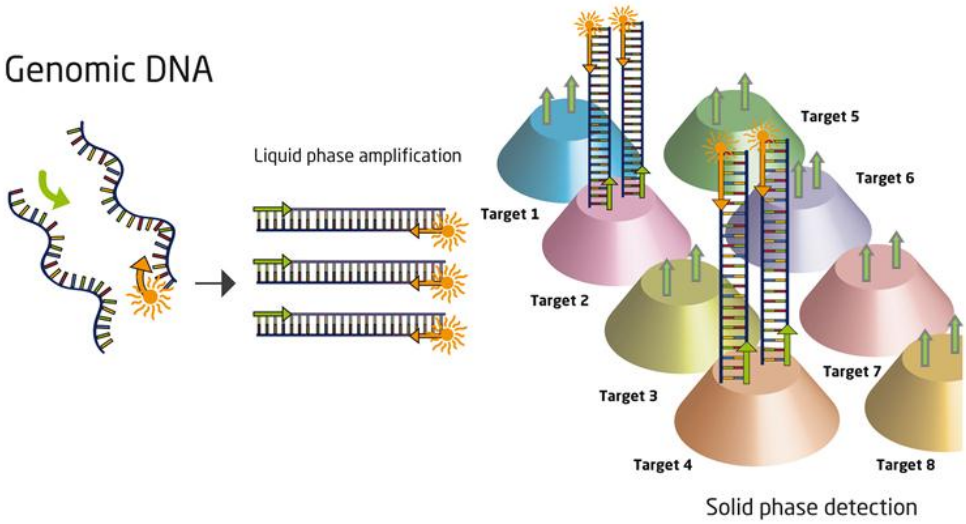


(c) *sefA* gene

Fig. 5. Comparison of sensitivity of the SP-PCR amplification performed on the SAF microlens array and detected on chip vs. those performed on plain COC microarray and detected using a conventional laser scanner. Initial concentration of S. Enteritidis DNA template varied from 0.16 to 1.6×10^5 copies/µL with 10-fold dilution. Signal-to-noise ratio of the SP-PCR products for (a) hilA, (b) sdf, and (c) sefA genes were determined separately. The mean value and standard variation were calculated based on signals from 6 spots. The lowest template concentration at which positive fluorescence signals could be detected for all the three genes was 1.6 copies/µL. The linear range for hilA gene is from 1.6 to 1.6 $x10^4$ (R^2 = 0.9672), for sdf gene is from 1.6 $x10^2$ to 1.6 $x10^4$ (R^2 = 0.9937), and for sefA gene is from 1.6 $x10^1$ to 1.6 $x10^4$ (R^2 = 0.9684).

Table 1. List of primers and probes used in this study.

Species	Target gene	PCR primers' sequences (5'-3')
Salmonella spp.	hilA	hilA -F: GCGACGCGGAAGTTAACGAAG A hilA –R Cy3-CACGATAGAGTAATGCAGACTCTCGGATTGAACCTGATC hilA-probe TTTTTTTTTTCCCCCCCCCCCAAGAGCATCGTTACATTGAAACACTGTACGGACAGGGCTA TCGGTTTAATCGTCCGGTCG
S.Enteritidis, S.Dublin and S.Gallinarum	sefA	sefA-F: GTGGTTCAGGCAGCAGTTACT sefA-R: Cy3-TGTGACAGGGACATTTAGCGTTTCTTGAG sefA-probe TTTTTTTTTTCCCCCCCCCCCCGTATTCAGGGAGCCAATATTAATGACCAAGCAAATACTG GAATTGACGGGCTTGCAGGT
S. Enteritidis	sdf	sdf-F: AAATGTGTTTTATCTGATGCAAGA GG sdf-R: Cy3-TCTAATGAACTACGTTCGTTCTTCTGGTACTTACGATGAC sdf-probe TTTTTTTTTTCCCCCCCCCCCATCAAAAAGGTTTAGTAAATCAGCCTGTTGTCTGCTCACCA TTCGCCAGCCACCACCTTC
S.Typhimurium and S.Kentucky	fliC	fliC-F: CCCCGCTTACAGGTGGACTAC fliC-R: Cy3-CTGCAGCGGGTTTTCGGTGGTTGT fliC-probe TTTTTTTTTTCCCCCCCCCCCACTTACGCTGCAAGTAAAGCCGAAGGTCACAACTTTAAAG CACAGCCTGATCTGGCGGAA
Campylobacter spp.	16S ribosomal RNA	UC-probe TTTTTTTTTTCCCCCCCCCCCAGGAAGGTGTGGACGACGTCAAGTCATCATGGCCCTTATG CCCAGGGCGACACACGTGCT



Multiplexed detection is essential in molecular diagnosis. As a promising technique, solid-phase polymerase chain reaction (SP-PCR) has become increasingly popular. It amplifies target nucleic acids on a solid support where numerous primers are arranged in format of high-density array, thereby providing enormously high throughput. However, the applicability of SP-PCR for point-of-care (POC) diagnosis or on-site testing is hindered by the lack of sensitive and portable multi-point detection technology.

In this paper, we present an innovative Lab-on-a-Chip (LOC) system where SP-PCR is performed on an ultrasensitive microlens array integrated in a plastic microfluidic chip. The micro-optical lens array was designed and fabricated based on principle of super critical angle fluorescence (SAF). The cone-shaped micro-optical structure was able to collect fluorescence at super critical angle, thus dramatically increasing the sensitivity by 40 folds. Compared to the conventional SP-PCR that utilized laser scanner to measure fluorescent signals, the LOC system with the SAF microlens array showed comparable detection limit and linear range.

We demonstrated that highly sensitive and multi-point detection could be achieved on chip using low-cost and portable optical components. The excellent light collection power of the SAF microlens array and the high specificity and multiplexing capability of the SP-PCR are a perfect combination for high-throughput detection in POC settings.