Published in final edited form as:

Lab Chip. 2017 August 08; 17(16): 2821–2830. doi:10.1039/c7lc00211d.

Rapid Real-Time Recirculating PCR Using Localized Surface Plasmon Resonance (LSPR) and Piezo-electric Pumping

J. M. Haber^{a,b}, P. R. Gascoyne^{a,*}, and K. Sokolov^{a,b,*}

^aDepartment of Biomedical Engineering, University of Texas at Austin, Austin, TX 78712

^bDepartment of Imaging Physics, UT MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030

Abstract

Rapid detection and characterization of pathogens in patients with bloodstream infections (BSIs) is a persistent problem for modern medicine, as current techniques are slow or provide incomplete diagnostic information. Real-time polymerase chain reaction (qPCR) allows specific detection of a wide range of targets and quantification of pathogenic burden to aid in treatment planning. However, new technological advances are required for a rapid and multiplex implementation of qPCR in clinical applications. In this paper, feasibility of a novel microfluidic platform for qPCR is presented integrating highly sensitive, label-free localized surface plasmon resonance (LSPR) imaging of DNA hybridization in a recirculating chip design for real-time analysis. Single target and multiplex detection of DNA target amplification are demonstrated, with a limit of detection of 5fg/uL of *E.coli* DNA for single target PCR, correlating to approximately 300 bacteria per mL. The results of this study demonstrate potential of this platform for simultaneous real-time detection of multiple target genes within 15 minutes that could provide live saving benefits in patients with BSIs.

TOC image

Introduction

Bloodstream infections (BSIs) are a critical problem in modern medicine. In addition to patients who present with BSIs, hospital acquired, or nosocomial, BSIs remain a persistent

*Please, send correspondence to ksokolov@mdanderson.org (Dr. Sokolov); petergascoyne@gmail.com (Dr. Gascoyne).

problem. Approximately 5% of all patients in the US get an infection during their hospitalizations¹, and risks increase with duration of hospital stay, catheterization², and low nurse-to-patient ratio³. The likelihood increases dramatically for patients in intensive care^{4, 5}, and exceeds 15% among the immunocompromised⁶, who are also at increased risk for polymicrobial infections⁷ which carry an increased mortality⁸ over an estimated 16 – 40% in ICU patients^{9, 10}.

Time to adequate treatment has a significant impact on survival rate¹¹, but antibacterial resistance increases time that is required to identify the most effective approach^{12, 13}. Rising rates of antibacterial resistance as well as increasing numbers of BSIs caused by fungi and gram positive bacteria have only worsened patient outcomes^{14, 15}. Timely characterization of pathogens that facilitates determination of the most effective course of treatment is critical to decreasing the burden of nosocomial infections. Cell culture-dependent techniques, the current gold standard for pathogen detection and analysis, take a significant amount of time, generally 1–2 days to complete¹⁶. During this time patients are often treated with broad-spectrum antibacterials¹⁷ which is sub-optimal and is associated with an increased emergence of antibiotic-resistant strains¹⁸. Therefore, there is a critical need for an alternate method that would allow rapid detection and characterization of BSI pathogens.

The use of genetic marker analysis for both pathogen identification and characterization of antimicrobial resistance, notably with polymerase chain reaction (PCR), has become a mainstay of clinical microbiology due to high specificity and potential for multiplexing. In addition, real-time PCR (qPCR) allows for both faster detection¹⁹ and quantification of pathogenic burden through the use of standard curves, an additional dimension that is beyond simple PCR^{20, 21}. As a result, qPCR is a powerful tool for the development of a rapid pathogen detection system. However, massive-scale multiplexing would be very difficult to implement in conventional, commercial qPCR systems. Indeed, DNA hybridization arrays, the primary approach for highly multiplexed measurements²², cannot maintain binding during thermocycling due to temperatures exceeding the melting point of probe/DNA complexes, while separating DNA hybridization from the thermocycling system prevents collection of real-time data, and thus qPCR. Due to these factors, multiplexed qPCR integrating DNA hybridization arrays has not been previously demonstrated.

Microfluidic PCR provides an alternative to conventional approaches²³, and comes with advantages such as improved thermocycling speed and sensitivity and the ability to perform PCR in-line with sample preparation and detection systems. While multiplexed microfluidic qPCR has been demonstrated using PCR chamber array approaches²⁴, separating samples into a number of reactors reduces the effective target copy number for each chamber and thus reduces PCR sensitivity in low concentration samples, as PCR sensitivity is limited by target copy number rather than concentration.

Microfluidic DNA hybridization for simultaneous detection of multiplexed targets in a single reaction chamber has been previously demonstrated in the context of PCR²⁵, however, the measurements in such studies were not carried out in real-time and, therefore, were not suitable for qPCR. One of challenges in implementing on-chip qPCR is real-time detection of DNA hybridization that has been traditionally carried out using fluorescence imaging²²;

this approach requires fluorescent labels that is a significant complicating factor in a design of a real-time microfluidic system. Recently, it was demonstrated that a highly sensitive detection of DNA hybridization events can be achieved using localized surface plasmon resonance (LSPR) detection method^{26–28}. LSPR is a label-free detection scheme that is based on measuring the shift in extinction spectra of plasmonic nanoparticles occurring when the local dielectric constant around the particles changes, such as from DNA binding to probes immobilized on the nanoparticle surface.^{28, 29}

In this paper, we combined advantages of qPCR technology and LSPR detection method to develop a novel microfluidic on-chip system for rapid highly sensitive and label-free multiplex detection of pathogens. This chip design includes a recirculating flow path alternating between hybridization and thermocycling that allows for bulk PCR with real-time detection and improved DNA hybridization kinetics for faster signal response³⁰. Furthermore, we developed LSPR imaging (LSPRi) system that utilizes a digital micromirror device (DMD) for detection of DNA hybridization in a sensor zone consisting of a two-dimensional array of silver nanoprisms functionalized with DNA probes. The use of a DMD provides a cost-effective and flexible solution to controlling spectral characteristics of illumination light that is necessary for quantitation of LSPR spectral shift. Alternative designs such as the use of multiple light emitting diodes (LEDs) could require numerous narrow band-pass filters and/or optical fiber switches that could increase cost and complicate system design. This work aims to demonstrate feasibility for this new “Recirculating PCR/LSPRi” chip and to provide foundation for future expansion of real-time, in-flow LSPR-based detection of PCR output for multiplexed sensitive detection of multiple pathogens in clinical specimen.

Materials and methods

PCR Chip Assembly

Figure 1A shows the overall scheme for a microfluidic PCR thermocycling system with integrated detection of DNA hybridization using surface plasmon resonance imaging. A VT-127-1.0-1.3-7.1 thermoelectric cooler (TEC) (TE Technology, Traverse City, MI) connected to a 24V, 4A DC power source (BioTec NG 60-5, Sweden) was used for rapid PCR thermocycling of a microfluidic chip. Temperature control was handled using custom PID software on an Arduino Uno board controlled by a 1k Ω resistance temperature detector (RTD) (DigiKey, Thief River Falls, MN). Circuit diagram and PCR thermocycling stability data can be found in Fig. S1–S2.

The microfluidic chip shown in Figure 1B was constructed using 200 μ m deep by 2mm wide channel structures milled from Lexan polycarbonate that was adhered to glass and 36-gauge aluminium substrates positioned side-by-side by a 100 μ m thick dry film adhesive etched with the same channel structure pattern (Figure 1B). The combined depths of the channels – the milled polycarbonate plus the adhesive film – was 300 μ m. The milled polycarbonate substrate also contained a 4 $\times$ 5mm zero depth sensor zone that was machined by ramping the milled channels to a depth of zero, so that only the dry film adhesive was providing channel depth of 100 μ m in this zone.

An unidirectional flow was achieved using in-channel piezoelectric pumping³¹ that was implemented by, first, milling a 10mm diameter indentation to 200 μ m depth in the polycarbonate chip to serve as the pumping chamber. Then, the chamber was connected to the channel structures via Diffusion no-moving parts valves (NMPs)^{31, 32}, which were also milled to 200 μ m depth and utilized for flow rectification (Fig. S3). Two valve designs were evaluated – diffuser NMP (D-NMP) and recirculating NMP (R-NMP) (Fig. S3). On the reverse side of the chip, a 12.3mm diameter indentation was milled flush to the pumping chamber to adhere an AB1290B-LW100-R piezoelectric buzzer on a 12.3mm brass disc (PUI Audio, Dayton, OH). Piezoelectric pumping was controlled with an HP 33120A signal generator (Hewlett Packard) and Model 7500 voltage amplifier (Krohn-Hite, Brockton, MA). Pumping flow rates were characterized by filling the chip with 0.1% w/w 45 μ m silicon beads in water and measuring velocity of the beads passing over a ruled backdrop under different power input frequency (Fig. 2A) and voltage conditions (Fig. 2B). Maximal flow rates were achieved at 110Hz and varied with the square of voltage, or linearly with pumping power, in the range from 30 to 80V. D-NMP valve design was chosen due to its larger range of potential flow rates (Fig. S4). For experiments in this paper, input parameters of 40V and 110Hz were chosen to achieve an internal flow rate of 45 μ L/min in the chip.

Before each experiment the polycarbonate chip was incubated in 1mg/mL bovine serum albumin (BSA) for 1 hour and then dried followed by bonding of a nanoprism coated glass coverslip in the sensor zone and a 36-gauge aluminum sheet in the heating zone using 100 μ m dry film adhesive (3M). The boundary between the two materials was sealed with a thin layer of glass for structural support, adhered into place with dry film adhesive and PCR-safe epoxy (NOA68, Thorlabs) as shown in Figure 1B, chip cross-section. A piezoelectric buzzer and PEEK tubing for input and output were bonded to the chip using NOA68 UV-cured adhesive.

Sensor Zone: Silver Nanoprism Monolayer on a Glass Substrate

Specific detection of target DNA sequences was based on the high sensitivity of the plasmon resonance peak of silver nanoprisms to small changes in permittivity of the surrounding media. Figure 3A shows the DNA hybridization scheme on the surface of silver nanoprisms in the sensor zone of the PCR/LSPRi chip. First, silver nanoprisms were prepared using a modified version of the technique described by Li, et al³³. Briefly, a solution was prepared consisting of 16.97mL diH₂O, 1mL trisodium citrate (75mM), 93 μ L silver nitrate (10mM) and 128 μ L hydrogen peroxide (0.5%). Then, 96 μ L sodium borohydride (100mM) was added under vigorous stirring until the solution turned golden yellow indicating formation of small silver seeds. The seed solution was allowed to age overnight prior to addition of 21mL diH₂O and 2mL L-ascorbic acid (10mM). Silver nitrate (10mM) was then added dropwise at a flow rate of 100 μ L/min to initiate growth of nanoprisms until the solution reached an extinction peak at 555nm as observed using UV-Vis spectrometry that required approximately 0.5mL of the silver nitrate (Fig. 3B).

Next, two-dimensional arrays of silver nanoprisms were prepared on glass cover slips. To this end, glass slides were incubated in 0.01% Poly-L-Lysine (PLL) solution for 15 minutes followed by drying under a jet of nitrogen and washing in diH₂O. PLL coated cover slips

were incubated in colloidal nanoprism solutions with $OD > 3$ for 20 minutes and, then, washed in diH_2O and dried under a jet of nitrogen. The nanoprism monolayers formed on glass substrates were tested for stability under PCR temperature cycling and flow conditions by hyperspectral optical imaging on Leica DM6000M microscope equipped with PARISS hyperspectral imaging device (LightForm, Inc.) of the monolayers before and after heating for one hour at $85^\circ C$ and piezoelectric pumping for one hour at 70V and 110Hz. No difference between the measured extinction spectra before and after interventions was detected, indicating no damage had occurred to the arrays (Fig. S5).

The final step in preparation of the sensor zone was attachment of probe DNA molecules to the silver nanoprism array. 5'-Thiol capped DNA probes targeted to the DXS and *tufB* genes of *E. coli* were obtained from Sigma Aldrich (sequences in supporting information). In order to optimize DNA probe loading on the nanoprism array, a series of 10 μ L droplets containing thiol-capped DNA probes at 0, 7, 17.5, 35 and 70 μ M concentrations were placed on a nanoprism functionalized glass coverslip for one hour followed by washing with diH_2O and drying under nitrogen. Then, hyperspectral measurements were taken on the Leica microscope to determine spectral changes associated with DNA probe deposition on the surface of silver nanoprisms. DNA probe binding to the particle surface results in a red-shift in their absorption spectra due to changes in the local dielectric properties that can be used as an indicator of functional molecule loading.²⁸ The red spectral shift reached maximum value at 35 μ M concentration, indicating saturation of DNA probe binding. Therefore, this concentration was chosen for nanoprism functionalization in all LSPR experiments. Following functionalization, the glass coverslips with silver nanoprism monolayers were bonded to the sensor zone of the PCR/LSPRi chip as described above (Figs. 1B and 3C).

Localized Surface Plasmon Resonance Imaging (LSPRi)

To detect optical changes associated with DNA hybridization in the sensor zone we have designed a spectral imaging system that utilizes a digital micromirror device (DMD) for adjustable wavelength illumination and a monochromatic CCD camera for detection (Fig. 4). Broad white light illumination was provided by a CREE XP-G2 5W LED (RapidLED, Burlingame, CA). Light from the LED passed through a 1mm pinhole, after which it was collimated using an 18mm plano-convex (PCX) lens ($f=20$ mm) and a 12mm asphere (effective focal length (EFL)=8mm) through a 0.3mm slit aperture onto a 600 groove/mm diffraction grating. The diffracted light was then directed through an 18mm PCX lens ($f=25$ mm) onto a DLP3000 digital micromirror device (DMD) (Texas Instruments, Dallas, TX) creating a spatially resolved pattern of the visible spectrum on the DMD device. The DMD was used to reflect a series of specific predefined spectral bands in the 560–640 nm region, which were focused by a 25mm PCX lens pair ($f=125$ mm and $f=50$ mm) into a 1mm multimode optical fiber (NA=0.5). The light from the distal fiber end was collimated into a 3.3mm diameter beam passing through the sensor zone of the PCR/LSPRi chip and was detected using a Retiga EXi CCD camera (QiCam). The DLP LightCrafter software was used to control the DMD.

In order to optimize spectral resolution of the system and to carry out wavelength calibration, a series of bandpass filters with known parameters were placed in the light path

between the diffraction grating and DMD, and the profile of light reflected from the DMD was compared with the actual optical transmission of the filters (Fig. S7). The full-width at half-maximum of the filter transmittance spectra reflected from DMD closely matched the actual filter spectral bands when the aperture (A in Fig. 3A) was set to width of 0.3mm; this aperture was chosen for all experiments as the best compromise between spectral resolution and light throughput of the imaging system. The measured spatial center points of the bandpass filters were used to correlate spatial coordinates on the DMD with wavelengths, and the spatial distance between these center points was used to calculate the spectral range of the system. Using these measurements, the spectral range of the optical system was determined to be from 540 to 660nm.

Optical changes associated with specific binding of target DNA sequences were detected as follows. Prior to running PCR, an initial spectral sweep was performed from 560 to 640nm through the sensor zone. Images were recorded for 40 evenly spaced spectral bands in this range for a spectral resolution of 2nm, creating a data cube with x- and y-axes representing physical dimensions of 5 by 3.5mm, respectively, and a z-axis representing 40 consecutive 2nm spectral bands from 560 to 640nm. This spectral imaging data set was used as the baseline for determining optical changes associated with DNA hybridization on nanoprisms. Then PCR thermocycling was initiated with amplified target DNA constantly recirculating through the nanoprism sensor zone. During PCR thermocycling, spectral image series were taken every three minutes over the duration of the PCR experiment (~30 min) for the same 40 spectral bands in the 560-640nm range.

DNA Isolation Protocol

DNA solutions were prepared from Stbl3 *E. coli* (ThermoFisher, Waltham, MA) transformed with the pUC19 plasmid. Bacteria were grown in an incubator at 37C for two days in LB Broth containing 100µM ampicillin prior to harvesting and centrifugation. The resulting bacteria were then lysed and DNA was isolated using a DNEasy Blood and Tissue Kit (Qiagen). DNA samples were analysed using a NanoDrop (Thermo Scientific) to determine concentration and verify purity.

Preparation of DNA for Multiplex Experiments

DNA samples were prepared by separately performing PCR on an Eppendorf MasterCycler commercial PCR system targeting *tufB* and *DXS* genes with *E. coli* DNA input. The samples were separated using gel electrophoresis on a **2% agarose gel run on a joulebox electrophoresis system (stratagene, san diego, ca) at 100v for 20 minutes**. The bands corresponding to the two genes were cut out of the gels, melted with guanidine hydrochloride and separately isolated using a DNEasy solid-phase extraction kit (Qiagen, Hilden, Germany).

PCR Reagents

The AmpliTaq Gold PCR protocol (see Supporting Information) was utilized for all experiments. PCR solutions consisted of 50% v/v AmpliTaq Gold PCR Master Mix (ThermoFisher), 10% v/v sense primer (5µM), 10% v/v anti-sense primer (5µM) and 30% DNA solution prepared from bacterial lysate to reach final concentrations ranging from 5 to

5000 fg/ μ L. All solutions were prepared fresh just prior to thermocycling. Primers were used targeting *E. coli* *tufB* and *DXS* genes, selected as single allele genes consistently present in the *E. coli* variant used in these experiments. Thermocycling was accomplished using a four minute hot-start period at 95°C followed by 35 three-step cycles of 5s at 96°C, 5s at 60°C at 10s at 72°C. Please, refer to supporting information for DNA primer sequences.

On-chip PCR

In a typical experiment, the PCR chip was loaded with a small quantity of PCR-grade mineral oil (SigmaAldrich, St. Louis, MO) to coat channel edges and reduce interfacial air bubbles. Then 200uL of prepared PCR solution was loaded into the chip. The thermocycling zone of the chip was then placed on the TEC with a thin layer of thermal paste (TX-4, Tuniq); the sensor zone was positioned outside of the TEC and in line with the illumination beam from the LSPRi system. A baseline spectral sweep from 560 to 640nm was performed as described above prior to activating the piezoelectric pumping and thermocycling control. Experiments were allowed to run for 35 cycles, or approximately 30 minutes. Spectral LSPR imaging was carried out every three minutes during the PCR thermocycling for continuous monitoring of DNA hybridization events.

Results and Discussion

First, fluorescent gel electrophoresis was used to validate effective on-chip pcr amplification separately from lspri detection. to this end nanoprisms in the sensor zone were functionalized with dna probes specific for the *E. coli* *tufB* gene, while primers for the *dxs* gene were used for pcr amplification; this mismatch was used to prevent dna loss due to hybridization in the sensor zone during on-chip thermocycling. the full pcr/lspri chip was loaded with 200ul of pcr mixture with the final dna concentration of 5pg/ μ l and thirty-five thermocycles were carried out using the tec thermocycler (fig. 5a). after on-chip pcr, the sample was removed from the chip, stained for dna, loaded into a 2% agarose gel and was run on a joulebox electrophoresis system (stratagene, san diego, ca) at 100v for 20 minutes. the resulting gel was then imaged on an ultralum uv transilluminator (fig. 5b). fluorescent bands were observed for samples that had undergone on-chip thermocycling, indicating successful dna amplification during flow pcr.

Following verification of on-chip PCR, we validated our localized surface plasmon resonance imaging (LSPRi) system. To do this, spectral measurements were performed on nanoprism monolayers under different dielectric solvent conditions and were compared with a commercial system - PARISS hyperspectral imaging device (LightForm, Inc.) attached to a Leica optical microscope.³⁴ Three different solvents were used, chosen for their large difference in dielectric constants, – air ($n=1.0$), diH₂O ($n=1.333$) and glycerol ($n=1.472$) (Fig. 6). For LSPRi measurements, nanoprism monolayers were placed in the path of the collimated output beam (legend B in Fig. 4) and a spectral sweep was performed as described above. A clean glass cover slip was used to measure the spectral throughput of the imaging system (Fig. 6A); this blank spectrum was used for normalization of the intensity data to obtain transmittance spectra under each solvent condition (Fig. 6B). The transmittance spectra showed an expected strong red shift associated with an increase in a

dielectric constant of a surrounding media around silver nanoprisms in the detection zone. Furthermore, LSPRi spectra showed a very good correlation with the commercial hyperspectral imaging device (Fig. 6, C–D). Using the observed shifts in plasmonic peaks under different solvents, the sensitivity of the system was determined to be $S_n \approx 130\text{nm/Refractive Index Unit (RIU)}$.³⁵

Next, the combined PCR/LSPRi chip was tested for detection of DNA for input concentrations ranging from 5 to 5000fg/ μL . Before each experiment, the PCR chip was disassembled and thoroughly cleaned by treatment with DNase, sonication in 70% ethanol for one hour, and heating to 70°C for 30 min to remove any residual enzymes and other chemicals from prior reactions. Then, the chip was reassembled with a freshly prepared sensor zone consisting of a glass coverslip coated with nanoprisms functionalized with DNA probes specific for a sample DNA. The chip was filled with PCR solutions containing either 0, 5, 50, 500 or 5000 fg/ μL of DNA isolated from Stbl3 *E. coli*. For each sample, 35 PCR thermocycles were carried out under continuous flow conditions with the sample flowing through and interacting with DNA probes in the sensor zone. LSPRi measurements were taken every three minutes to monitor DNA hybridization using spectral changes in the absorbance of nanoprism array in the sensor zone. The spectral measurement at each time point consisted of a series of 250ms acquisitions for each of 40 wavelength points in the 560–640nm wavelength range. The collected data were used to generate difference maps for each wavelength point ($I_{i,j}^\lambda$) by calculating the difference at each pixel ($I_{i,j}^c$) from the corresponding baseline image ($I_{i,j}^o$) normalized by the baseline value according the

equation, $I_{i,j}^\lambda = \frac{|I_{i,j}^o - I_{i,j}^c|}{I_{i,j}^o}$. The difference maps were then summed across the measured spectrum to obtain an integrated difference map representing spectral changes as a

percentage of the original spectrum, $I_{i,j}^f = \sum_{\lambda} I_{i,j}^\lambda / n_{\lambda} = \sum_{\lambda} \frac{|I_{i,j}^{o,\lambda} - I_{i,j}^{c,\lambda}|}{I_{i,j}^{o,\lambda}} / 40$, which was displayed using a heat map; in this equation, $I_{i,j}^\lambda$ represents each pixel in the calculated difference map for a given wavelength, λ ; $I_{i,j}^{c,\lambda}$ represents each pixel in the raw image at one wavelength; $I_{i,j}^o$ represents each pixel in the baseline image at one wavelength; $I_{i,j}^f$ represents the final calculated difference map across all wavelengths, and n_{λ} represents the number of wavelength bands measured.

Figure 7A shows an example series of output images from the 5fg/ μL PCR experiment demonstrating clear increase in signal over the course of thermocycling, indicating successful detection of DNA hybridization, as compared to a negative control with 0fg/ μL of sample DNA added (No Template control, Figure 7B). Figure 8 shows histograms of calculated spectral difference for all pixels in the region of interest for both 5fg/ μL and negative control experiments. The mean pixel value continuously increased over time for the 5fg/ μL sample while the distribution of the negative sample remained unchanged. The percentage of pixels in the hybridization zone (red outlines in Figure 7) with values exceeding a noise threshold increases with time from 3.0% at 9minutes to 46.3%, 62.7% and

74.1% at 15, 21 and 30 minutes, respectively (Figure 8). The noise threshold was calculated as three standard deviations above the mean pixel value for the negative control experiment. It is interesting to note that while average spectral difference in the ROI for 5fg/ μ L sample was 0.18% at 30 minutes, the maximum spectral change reached 0.45% with many pixels at 0.3–0.35%. Substantial pixel-to-pixel variance that is evident from Figures 7 and 8 is likely due to fluctuations in DNA hybridization efficiency that could be due to differences in DNA probe density in the sensor zone as well as steric hindrance of DNA hybridization. This observation indicates the need for further improvement of the functionalized nanoprism sensor array that was outside of the scope of this feasibility study.

The results from the DNA concentration series experiment were used to determine the limit of detection of the PCR/LSPRi chip. Figure 9A shows the average measured spectral change in the sensor zone over the course of thermocycling for various starting concentrations of DNA. For DNA concentrations 50 fg/ μ L and higher, the maximum spectral change was observed within 10–15 minutes, or in less than 20 cycles. A consistent increase in spectral changes over time was still observed at the concentration of 5fg/ μ L indicating that while amplification occurred and could be measured, the signal did not reach saturation. The signal growth at this concentration was also linear, rather than exponential, indicating that the concentration of 5fg/ μ L is at the limit of detection of the system, which corresponds to a quantity of DNA equivalent to ~60 copies of the target gene in a 200 μ L of a PCR input sample³⁶, or approximately 300 bacteria per mL for a target with one copy per cell. This compares favourably with the theoretical lower detection limit of PCR, which has been estimated to be between 10 and 300 copies of the target gene, depending on reaction conditions^{37, 38}. Figure 9B shows the average spectral change as a function of input DNA concentration after 35 thermocycles, indicating a plateau at an average spectral change of ~0.4%. It is interesting to note that the real-time nature of the results allows for sigmoidal regression analysis to measure the slope and timeframe of amplification, which can theoretically be used for determining the initial concentration of starting DNA following the development of a PCR standard curve.

Following demonstration of single-target PCR, the feasibility of multiplex PCR utilizing the recirculating PCR/LSPRi chip was evaluated. A sensor zone was prepared using two different DNA probe containing droplets placed on two separate regions of interest – one targeting the *tufB* and the other the *DXS* genes. A full PCR/LSPRi chip was assembled with the multiplex sensor zone. PCR experiments for the multiplex chip were then performed by preparing PCR solutions with different combinations of the two target genes: negative for both, and positive/negative for each gene. Concentrations of both sense and antisense primers were held at 200nM for each target, with 500fg/ μ L concentrations of positive input DNA to ensure complete amplification. Figure 10 shows pseudocolor difference map images for the multiplex PCR experiment. As expected no signal was detected in the case of negative *tufB*/negative *DXS* combination (–*tufB*/–*DXS*) (Fig. 10A). The +*tufB*/–*DXS* experiment resulted in a strong plasmonic shift co-localized with the region containing *tufB* probes with practically no changes observed in the adjacent region with *DXS* probes (Fig. 10B), and the opposite result was obtained for the –*tufB*/+*DXS* sample (Fig. 10C). This strong specificity of detection shows feasibility of a multiplex detection of genetic targets on the recirculating PCR/LSPRi chip.

The observed differences in two-target gene experiments were quantified using histograms of calculated spectral difference for all pixels in the region of interest (figure 11). In the *tufb*+/*dxs*- experiment, average spectral difference was found to be 0.48% for the *tufb* region and 0.04% for the *dxs* region. As expected, this pattern was reversed in the *tufb*-/*dxs*+ experiment where average spectral difference was 0.09% for the *tufb* region and 0.55% for the *dxs* region. Analysis of the relative number of pixels above noise threshold showed 92% and 28% above the threshold respectively for *tufb*+/*dxs*- case, and 33% and 99% above the threshold respectively for *tufb*-/*dxs*+ experiments. This analysis demonstrates specificity of the proposed method in detection of two target genes.

Conclusions

A microfluidic PCR chip utilizing piezoelectrically-pumped recirculating flow and an LSPR based detection method was constructed and validated, demonstrating a limit of detection of 5 fg/ μ L of DNA from *E. coli*, or ~300 bacteria/mL which is comparable to or better than most conventional PCR systems. Detection of target markers was achieved consistently within 15 minutes, allowing for a rapid diagnosis. Furthermore, feasibility of on-chip multiplex detection of DNA hybridization was demonstrated that could be significantly increased by the use of standard microcontact printing for creating arrays of DNA probes in the sensor zone, which has been demonstrated for successful deposition of DNA probes in spot sizes below 20 μ m³⁹. Analysis of data presented in this paper indicates a possibility of achieving the minimum effective detection spot size of *ca.* 70 μ m with improved DNA probe deposition methods. This estimate was carried out using data obtained at the saturated signal. First, a square area in the ROI center with visually uniformly distributed signal was selected. The selected area was divided into four quadrants and the mean signal value for each quadrant was compared with the mean value of the overall area using t-test. Then, each quadrant was further divided into four quadrants if the means were statistically the same. The process was continued until the mean signal of one of the quadrants was statistically different from the mean of the whole spot resulting in the 70 μ m spot size. Based on this value, it would be theoretically possible to construct an array of up to 800 hybridization spots assuming 70 μ m spacing between adjacent spots – meaning that the system would be limited by the complexity of optimizing the PCR reaction for balanced amplification output and primer conditions rather than by multiplex detection.

Currently, microfluidic PCR systems come in a wide variety of forms, most of which utilize one of three main approaches: arrays of microchambers⁴⁰, continuous flow paths passing over multiple temperature zones⁴¹, and bulk PCR reactors⁴². While the system described in this paper is under the category of bulk reactors, it carries notable advantages in the use of a recirculating design that can allow qPCR with a hybridization array and that integrates well with continuous flow sample preparation. The primary advantages of our system, however, are the capability for multiplexing and real-time qPCR. While microfluidic systems currently exist for multiplexing (in-line hybridization arrays⁴³, electrochemical detection⁴⁴), and qPCR (fluorescent detection in chamber reactors⁴⁵), the design proposed herein combines both capabilities while maintaining bulk PCR levels of sensitivity – something previously only attainable by microchamber arrays. However, microchamber arrays require splitting of samples that reduces reliability in detection of lower concentration targets.³⁸

Other systems either cannot manage massive multiplexing due to reliance on fluorescent signals in a single chamber, or cannot be used for qPCR, as the PCR melt cycle interrupts DNA hybridization to target probes⁴⁶ – a problem solved by the use of a recirculating pump to keep sample flow alternating between thermocycling and a separate detection zone without allowing the temperature to drop enough to initiate primer-dimer formation.

Throughput of our system as presented is comparable to microchamber arrays and is more limited than continuous flow systems. However, the recirculating nature of the chip can allow adaptation to continuous flow, improving throughput to the same level as the latter that is ultimately limited by the time-in-system required for PCR amplification (typically >15 minutes). In terms of reagent consumption, our system is comparable to other microfluidic PCR systems since PCR reagent usage is dependent on sample volume that is comparable in different systems. However, our system does not require labels or fluorescent probe reagents. With regard to simplicity and cost, the use of a DMD provides combination of flexibility and cost-effectiveness in LSPR imaging. Further, the electronic circuitry is less complex than would be required for electrochemical detection⁴⁴, and no microelectrodes or moving parts are necessary as in case of other in-channel pumping systems^{47, 48}. Regarding sensitivity, our system is advantageous over most other microfluidic systems as it accomplishes down to ~300 bacteria/mL detection in real time, which is comparable to the most optimized PCR reactions. The use of bulk recirculating PCR allows for higher sensitivity compared to arrays of microchambers as discussed above. Additionally, the label-free nature of LSPRi as compared to standard fluorescent detection allows for the possibility of simultaneous real-time analysis of multiple target genes. The looped design of the device makes it ideal for addition to a continuous flow sample preparation system, such as for isolation of bacteria from a blood sample. This work serves as a proof-of-concept for the recirculating PCR/ LSPRi chip with low-cost LSPR imaging as a novel detection set-up for microfluidic PCR systems. This chip design showed both high sensitivity and specificity, and utility for real-time qPCR measurements that can provide data on initial pathogen concentration in samples for clinicians following the development of a PCR standard curve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by NIH grant R01 CA103830. Machining was provided by Mitchell R. Hawkins at the Radiation Physics CABIR Machine Shop at MD Anderson.

Notes and references

1. C. Centers for Disease and Prevention. *Annals of Emergency Medicine*. 2011; 58:447–450. [PubMed: 22018400]
2. Blot S, Vandewoude K, Hoste E, Colardyn F. *Critical Care*. 2001; 5:1–2.
3. Alonso-Echanove J, Edwards JR, Richards MJ, Brennan P, Venezia RA, Keen J, Ashline V, Kirkland K, Chou E, Hupert M, Veeder AV, Speas J, Kaye J, Sharma K, Martin A, Moroz VD, Gaynes RP. *Infection Control & Hospital Epidemiology*. 2003; 24:916–925. [PubMed: 14700407]

4. Maki DG, Kluger DM, Crnich CJ. Mayo Clinic Proceedings. 2006; 81:1159–1171. [PubMed: 16970212]
5. Esteve F, Pujol M, Limón E, Saballs M, Argerich MJ, Verdaguer R, Mañez R, Ariza X, Gudiol F. Journal of Hospital Infection. 2007; 67:30–34. [PubMed: 17719682]
6. Yeung SCJ, Escalante CP, Gagel RF. Medical care of cancer patients, Pmhp-usa. 2009
7. Weinstein MP, Reller LB, Murphy JR, Lichtenstein KA. Review of Infectious Diseases. 1983; 5:35–53.
8. Pittet D, Li N, Woolson RF, Wenzel RP. Clinical Infectious Diseases. 1997; 24:1068–1078. [PubMed: 9195059]
9. Pittet D, Tarara D, Wenzel R. JAMA. 1994; 271
10. DiGiovine B, Chenoweth C, Watts C, Higgins M. American Journal of Respiratory and Critical Care Medicine. 1999; 160:976–981. [PubMed: 10471627]
11. Tabah A, Koulenti D, Laupland K, Misset B, Valles J, Bruzzi de Carvalho F, Paiva JA, Çakar N, Ma X, Eggimann P, Antonelli M, Bonten MJM, Csomos A, Krueger WA, Mikstacki A, Lipman J, Depuydt P, Vesin A, Garrouste-Orgeas M, Zahar J-R, Blot S, Carlet J, Brun-Buisson C, Martin C, Rello J, Dimopoulos G, Timsit J-F. Intensive Care Medicine. 2012; 38:1930–1945. [PubMed: 23011531]
12. Micek ST, Lloyd AE, Ritchie DJ, Reichley RM, Fraser VJ, Kollef MH. Antimicrobial Agents and Chemotherapy. 2005; 49:1306–1311. [PubMed: 15793102]
13. Vogelaers D, De Bels D, Forêt F, Cran S, Gilbert E, Schoonheydt K, Blot S. International Journal of Antimicrobial Agents. 2010; 35:375–381. [PubMed: 20122817]
14. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Clinical Infectious Diseases. 1999; 29:239–244. [PubMed: 10476719]
15. Schwalbe RS, Stapleton JT, Gilligan PH. New England Journal of Medicine. 1987; 316:927–931. [PubMed: 3821839]
16. Beekmann SE, Diekema DJ, Chapin KC, Doern GV. Journal of Clinical Microbiology. 2003; 41:3119–3125. [PubMed: 12843051]
17. Tumbarello M, Viale P, Viscoli C, Trecarichi EM, Tumietto F, Marchese A, Spanu T, Ambretti S, Ginocchio F, Cristini F, Losito AR, Tedeschi S, Cauda R, Bassetti M. Clinical Infectious Diseases. 2012; 55:943–950. [PubMed: 22752516]
18. Kollef MH, Fraser VJ. Annals of Internal Medicine. 2001; 134:298–314. [PubMed: 11182841]
19. Espy M, Uhl J, Sloan L, Buckwalter S, Jones M, Vetter E, Yao J, Wengenack N, Rosenblatt J, Cockerill F. Clinical microbiology reviews. 2006; 19:165–256. [PubMed: 16418529]
20. Klein D. Trends in molecular medicine. 2002; 8:257–260. [PubMed: 12067606]
21. Jervøe-Storm PM, Koltzsch M, Falk W, Dörfler A, Jepsen S. Journal of clinical periodontology. 2005; 32:778–783. [PubMed: 15966886]
22. Freeman WM, Robertson DJ, Vrana KE. Biotechniques. 2000; 29:1042–1055. [PubMed: 11084867]
23. Whitesides GM. Nature. 2006; 442:368–373. [PubMed: 16871203]
24. Gong H, Ramalingam N, Chen L, Che J, Wang Q, Wang Y, Yang X, Yap PHE, Neo CH. Biomedical microdevices. 2006; 8:167–176. [PubMed: 16688576]
25. Jiang X, Shao N, Jing W, Tao S, Liu S, Sui G. Talanta. 2014; 122:246–250. [PubMed: 24720991]
26. Anker JN, Hall WP, Lyandres O, Shah NC, Zhao J, Van Duyne RP. Nature materials. 2008; 7:442–453. [PubMed: 18497851]
27. Huang C, Ye J, Wang S, Stakenborg T, Lagae L. Applied Physics Letters. 2012; 100:173114.
28. Sepúlveda B, Angelomé PC, Lechuga LM, Liz-Marzán LM. Nano Today. 2009; 4:244–251.
29. Schneider T, Jahr N, Jatschka J, Csaki A, Stranik O, Fritzsche W. Journal of nanoparticle research. 2013; 15:1–10.
30. Lee HH, Smoot J, McMurray Z, Stahl DA, Yager P. Lab on a Chip. 2006; 6:1163–1170. [PubMed: 16929395]
31. Nabavi M. Microfluidics and Nanofluidics. 2009; 7:599–619.

32. Izzo I, Accoto D, Mencias A, Schmitt L, Dario P. *Sensors and Actuators A: Physical*. 2007; 133:128–140.
33. Li N, Zhang Q, Quinlivan S, Goebel J, Gan Y, Yin Y. *ChemPhysChem*. 2012; 13:2526–2530. [PubMed: 22298378]
34. Aaron J, Travis K, Harrison N, Sokolov K. *Nano letters*. 2009; 9:3612–3618. [PubMed: 19645464]
35. Homola J, Koudela I, Yee SS. *Sensors and Actuators B: Chemical*. 1999; 54:16–24.
36. Kohara Y, Akiyama K, Isono K. *Cell*. 1987; 50:495–508. [PubMed: 3038334]
37. Sykes P, Neoh S, Brisco M, Hughes E, Condon J, Morley A. *Biotechniques*. 1992; 13:444–449. [PubMed: 1389177]
38. Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J. *Nucleic acids research*. 1996; 24:3189–3194. [PubMed: 8774899]
39. Thibault C, Le Berre V, Casimirius S, Trévisiol E, François J, Vieu C. *Journal of Nanobiotechnology*. 2005; 3:7. [PubMed: 15992404]
40. Liu HB, Gong HQ, Ramalingam N, Jiang Y, Dai CC, Hui KM. *Journal of Micromechanics and Microengineering*. 2007; 17:2055.
41. Schneegaß I, Bräutigam R, Köhler JM. *Lab on a Chip*. 2001; 1:42–49. [PubMed: 15100888]
42. Ke C, Kelleher AM, Berney H, Sheehan M, Mathewson A. *Sensors and Actuators B: Chemical*. 2007; 120:538–544.
43. Liu Y, Rauch CB, Stevens RL, Lenigk R, Yang J, Rhine DB, Grodzinski P. *Analytical Chemistry*. 2002; 74:3063–3070. [PubMed: 12141665]
44. Barbaro M, Bonfiglio A, Raffo L, Alessandrini A, Facci P, BarakBarak I. *IEEE Electron Device Letters*. 2006; 27:595–597.
45. Zhao Z, Cui Z, Cui D, Xia S. *Sensors and Actuators A: Physical*. 2003; 108:162–167.
46. Jablonski E, Moomaw EW, Tullis RH, Ruth JL. *Nucleic acids research*. 1986; 14:6115–6128. [PubMed: 3748805]
47. Chen CH, Santiago JG. *Journal of Microelectromechanical Systems*. 2002; 11:672–683.
48. Takao H, Ishida M, Sawada K. *Journal of Microelectromechanical Systems*. 2002; 11:421–426.

Figure 1.

A) Block diagram of microfluidic PCR thermocycler with in-flow LSPR imaging detection of DNA hybridization. B) Top – channel structure layout of microfluidic PCR chip. Aluminum substrate **A** was used for contact with Peltier thermocycler; glass substrate **B** - for optical transmission measurements through nanoparticle sensor zone. Bottom – cross section view of chip construction. Channels constructed from milled polycarbonate are adhered to aluminum and glass substrates by etched dry film adhesive. C) Image of assembled microfluidic chip. Letters from “A” to “G” identify the same structures in panels (B) and (C).

Figure 2.

A) Piezoelectric pumping flow rate under varying input frequency conditions with voltage held constant at 70V. Maximal flow rates were found to occur at 110Hz. B) Piezoelectric pumping flow rate under varying input voltage with frequency held constant at 110Hz.

Figure 3.

A) Schematic of silver nanoprism detection zone. Nanoprisms were adhered to a glass substrate through amine binding with adhered Poly-L-Lysine (PLL). Prisms were then functionalized with 5'-Thiol capped DNA probes specific to target sequences. B) Optical extinction spectrum typical of silver nanoprisms with 555nm extinction peak used in the sensor zone. C) Image of the sensor zone with nanoparticle monolayer on a glass substrate in microfluidic PCR chip.

Figure 4.

A) Diagram of optical system for localized surface plasmon resonance imaging (LSPRi) of the PCR/LSPRi chip sensor zone. White light from an LED source **S** was passed through a 1mm pinhole **P** and was collimated using a planoconvex (PCX) lens **L1** and an asphere **L2**. The collimated light was then passed through a 0.3mm aperture **A** and onto a 600 groove/mm diffraction grating **G** and the spectrally separated light was projected via a PCX lens **L3** onto a digital micromirror device **D**. Then, spectral bands selected by **D** were focused through a PCX lens couplet **L4, L5** into an optical fiber (**F**) and delivered to the nanoprism sensor zone **M** through a collimator **B**. The light transmitted through **M** was detected by a CCD camera **C**. B) Picture of the optical assembly that separated white light into a series of predefined spectral bands using DMD device. C) Image of the PCR/LSPRi chip in the optical path. Letter legends in all figure panels correspond to the same optical elements.

Figure 5.

A) A typical thermocycling sequence for PCR/LSPRi chip experiments utilizing AmpliTaq Gold PCR Protocol. B) Zoomed in view of three consecutive thermocycles. Note consistency in temperature cycling. C) Agarose gel electrophoresis results following two PCR runs on fully constructed PCR/LSPRi chip with input DNA concentration of 5pg/μL. Presence of fluorescent bands in the gel indicating presence of DNA confirmed positive amplification. A sample without amplification was used as a negative control.

Figure 6.

A) Raw intensity data collected by the LSPRi system of a blank cover slip and nanoprism monolayers in air, deionized water and glycerol. B) Transmission of nanoprism monolayers in different solvents normalized by throughput of the LSPRi system. C) Corresponding spectral measurements obtained using a commercial hyperspectral imaging device (PARISS, LightForm, Inc.). D) Comparison of commercial hyperspectral and LSPRi system measurements of nanoprism monolayers in air, water and glycerol; note a high level of correlation between the spectra.

Figure 7.

LSPRi spectral measurements over time for (a) 5fg/μL input sample and (b) a negative control (0fg/μL of sample DNA; No Template control). Images are presented at 9, 15, 21 and 30 min. Red circles indicate the region of interest (ROI) – the location of nanoprisms functionalized with DNA probes (the hybridization zone) that was determined by the size of a droplet with DNA probes incubated in the sensor zone. Note clearly increasing signal in the ROI over time in (a). The scale of x-y axis on the images is in millimetres, and the colormap corresponds to the calculated spectral difference (%).

Figure 8.

Histograms of calculated spectral difference for 5fg/μL (red) and negative control (blue) experiments at (a) 9 minutes, (b) 15 minutes, (c) 21 minutes, and (d) 30 minutes. The dashed line indicates the noise threshold, defined as three standard deviations above the mean signal in the negative control. Note steadily increasing signal over time for 5fg/μL experiment.

Figure 9.

A) Average change in transmittance in the ROI for PCR/LSPRi chip limit of detection series. DNA samples at concentrations of 0, 5, 50, 500, and 5000 fg/μL were loaded into the PCR/LSPRi chip and were run for 35 thermocycles. A plateau in the average spectral change in the ROI was observed at 50 fg/μL and above. A positive detection without reaching plateau was observed at 5 fg/μL concentration. B) Average change in transmittance in the ROI for each input concentration. A plateau of ~0.4% average spectral change in the ROI was observed for concentrations of approximately 50 fg/μL and above.

Figure 10.

LSPR imaging results for multiplex PCR tests following 35 thermocycles. A) *tufB*–/*DXS*– input. B) *tufB*+/*DXS*– input. C) *tufB*–/*DXS*+ input. In each case, a graphic indicating expected results (left) and obtained results (right) are presented. The red spheroidal circles outline an approximate area of overlap of the physical location of DNA probes in the sensor zone and the light illumination area. The scale of x-y axis on the images is in millimetres.

Figure 11.

Histograms of calculated spectral difference for ROI regions corresponding to hybridization zones in two-target gene experiments with results for *tufB* hybridization zone shown in blue and for DXS hybridization zone in red: (a) *tufB*⁻/DXS⁻, (b) *tufB*⁺/DXS⁻, (c) *tufB*⁻/DXS⁺. The dashed line indicates the noise threshold calculated as three standard deviations above the mean for negative control experiment. Note significantly increased average signal for target positive experiments compared to target negative.