

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}

^a*Department of Biomedical Engineering
University of Texas at Austin
Austin, TX 78712*

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

Electronic Supporting Information

Experimental Section

Chemicals: From Sigma Aldrich (St. Louis, MO): Silver Nitrate (99.9999% Trace Metals Basis, 204390), Sodium Citrate Dihydrate (W302600), L-Ascorbic Acid (A5960), Hydrogen Peroxide (30% 216763), Sodium Borohydride (480886), 2-Propanol (I9516), (3-mercaptopropyl)trimethoxysilane (175617), Custom Oligonucleotides (tufB: 5'-[Thi-C6]CGTGCGATTGACAAGCCGTTCC, DXS: 5'-[Thi-C6]ATCGCTGAACGCTACGCTGGTCGATAT)), PCR Primers: DXS (Forward: 5'-CGAGAAACTGGCGATCCTTA, Reverse: 5'-CTTCATCAAGCGGTTTCACA), tufB (Forward: 5'-TGGGAAGCGAAAATCCTG, Reverse: 5'-CAGTACAGGTAGACTTCTG), Poly-L-Lysine (0.1% w/v Aqueous Solution, P8920), SybrGreen Dye (10000X, S9430), Bovine Serum Albumin (2mg/mL), Glycerol (99.9%, G5516), LB Broth Powder (L3022).

From ThermoFisher (Waltham, MA): AmpliTaq Gold PCR Master Mix (4390939), UltraPure Agarose (16500500), 10X BlueJuice Bromo-phenol Blue Tracking Dye (10816015), OneShot Stbl3 Chemically Competent *Escherichia coli* (C737303), pUC19 Plasmid (SD0061).

Bacterial Culture: Stbl3 *Escherichia coli* (ThermoFisher, Waltham, MA), a variant of the HB101 strain, were transformed using the pUC19 plasmid (ThermoFisher) for selection by ampicillin. Briefly, 1µL of concentrated pUC19 plasmid was injected into a prepared hot-shot vial of E.coli, and mixed by gently turning the vial back and forth. The E.coli were allowed to incubate at room temperature with the plasmid for 30 minutes before injecting 4µL into a prepared vial of 2mL LB Broth with 100µg/mL ampicillin. The vial was then incubated at 37°C in a Model 10-140 Incubator (Quincy Lab, Chicago, IL) for two days to allow the ampicillin resistant bacteria to

grow. The bacteria were then centrifuged for 1 minute at 2000g to form a pellet. The pellet was resuspended in 100 μ L of LB Broth, and then mixed with 100 μ L of 50% LB Broth, 50% glycerol in a screw-cap vial for storage at -20°C.

DNA Isolation: DNA solutions were prepared from Stbl3 E.coli transformed with the pUC19 plasmid (Sigma Aldrich, St. Louis, MO). Bacteria were grown in an incubator at 37°C for two days in LB Broth containing 100 μ M ampicillin prior to harvesting. The resulting bacteria were lysed and DNA was isolated using a DNEasy Blood and Tissue Kit (Qiagen, Hilden, Germany) for solid phase extraction and purification of DNA. DNA samples were then analysed using a NanoDrop (ThermoFisher) to determine concentration and to verify purity.

Agarose Gel Electrophoresis: Agarose gels were prepared using UltraPure Agarose (Invitrogen, Carlsbad, CA). Briefly, 1g of agarose was added to 50mL of 0.5x Tris-Acetate-EDTA buffer and heated using a microwave oven until the agarose powder fully dissolved into solution. The resulting 2% agarose solution was then poured into a gel casting mold. The gel was allowed to cool until it had firmly set, and then was stored in 0.5x TAE buffer until needed for use. In a typical electrophoretic experiment, samples were incubated in 1X SybrGreen for 30 minutes to allow the dye to intercalate into any DNA present in the sample. Then, 2 μ L of bromophenol blue tracking dye (10X BlueJuice, Invitrogen) was added to 20 μ L sample aliquots just prior to loading into the wells of the agarose gel. Agarose gel electrophoresis was then performed at 100V on a JouleBox electrophoresis system (Stratagene, San Diego, CA) with the anode at the base of the gel until the tracking dye neared the end of the gel. The gels were photographed using either a BioRad transmission UV plate reader or an UltraLum UV Transilluminator.

Construction of Microfluidic Chip: Lexan polycarbonate sheet (0.12", SABIC) was cut to 40x55mm and channel structures were milled to a depth of 0.2mm and width of 2mm using a HAAS-TM2P CNC Milling Center (Haas Automation, Oxnard, CA) with a 0.12 carbide 2 flute endmill following tool paths generated by GibbsCAM (3D Systems, Moorpark, CA) from a 3D CAD sketch prepared with AutoDesk Inventor (Autodesk, San Rafael, CA). Ramps to DNA hybridization zone at 0mm depth were prepared by holding the chip at a 2.5° angle for milling. Following patterning of microfluidic channels, the chip was flipped and a 12.3mm circle was milled to a depth of 2.84mm for placement of a piezoelectric buzzer. Holes for inlets and outlets were drilled using a drill press with a 0.03" diameter bit.

After preparation of microfluidic structures on the polycarbonate substrate, the piezoelectric buzzer was put into place for the pumping system. An AB1290B-LW100-R

piezoelectric buzzer on a 12.3mm diameter brass deflection disc (PUI Audio, Dayton, OH) was coated with 100um 467MP dry film adhesive (3M, Maplewood, MI), followed by 0.016mm aluminum sheet (Alcoa, New York City, NY). The buzzer was then sealed into place on the chip with NOA68 UV-Cured Adhesive (Norland Adhesives, Cranbury, NJ). Then, sections of 0.0625"OD, 0.03"ID PEEK tubing (IDEX, Lake Forest, IL) for inputs were sealed into place with NOA68 UV-cured adhesive.

The chip was incubated in 1mg/mL bovine serum albumin (Sigma Aldrich, St. Louis, MO) for 1 hour to improve PCR compatibility, after which it was dried under a jet of nitrogen, rinsed in diH₂O and dried again under a jet of nitrogen. A 100 µm layer of 467MP dry film adhesive pre-cut with channel structures was then adhered to the polycarbonate chip and used to bond 36 gauge aluminum sheet (St. Louis Crafts, St. Louis, MO) and a 24x40 glass cover slip coated with a nanoprism monolayer. The gap between the aluminum and glass substrates was sealed with a 1mm wide strip 467MP dry film adhesive and 1mm wide cut glass, followed by NOA68 UV-cured adhesive to prevent leaks.

PCR Thermocycling System: PCR thermocycling was accomplished through the use of 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). Current flow from the DC power source was regulated using an L6203 Power Relay (ST Microelectronics, Geneva, Switzerland) controlled through digital output from an Arduino Uno microcontroller (Arduino) utilizing custom PID software. The circuit diagram of the temperature control system is shown in Fig. S1. Temperature sensing was accomplished using a PPG102A6 1kΩ platinum resistance temperature detector (RTD) with +/-0.06% tolerance (US Sensor, Orange, CA). The RTD was connected in a wheatstone bridge layout with 1kΩ and 1.3kΩ resistors. Each resistor was tested for actual resistance value using a BK Precision 2703C multimeter (B&K Precision, Yorba Linda, CA) to determine parameters for a temperature equation. The RTD was held in place directly adjacent to the TEC surface such that it would contact the aluminum underside of the chip. Temperature calibration was then performed using phase transition testing.

Following the 35 cycles, a final 10s extension period at 72°C was performed. During PCR experiments with LSPRi measurements, one image set was taken every 3 minutes.

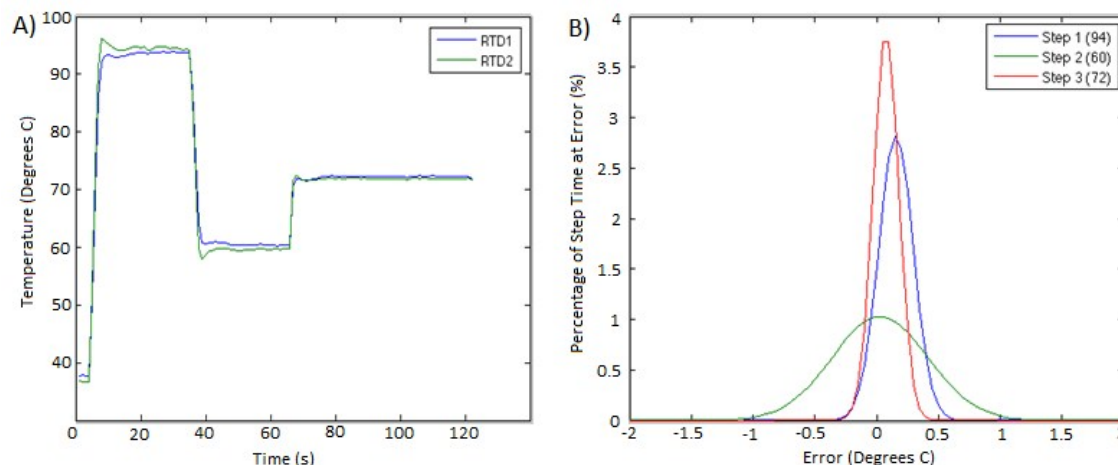


Figure S2. A) Measured temperature at two points on the chip surface over time during a PCR cycle with TopTaq protocol. RTD1 measurements were used for control of the PID heating system. B) Histogram of thermal stability at each step as measured over the course of 40 cycles. Error was consistently within 0.5 degrees of target temperature, with a slight positive bias (~0.1C) at the denaturing step.

Piezoelectric Pumping: Piezoelectric pumping was accomplished based on design parameters developed by Forster et al¹ and Izzo et al². Pumping apparatus consisted of a 10mm diameter pumping chamber with a depth of 300um directly beneath an AB1290B-LW100-R piezoelectric buzzer on a 12.3mm diameter brass deflection disc (PUI Audio, Dayton, OH). This pumping chamber was connected to the flow path by no-moving-parts (NMP) valves. Two valve designs were tested for use in the PCR chip – diffuser NMP (D-NMP) and recirculating NMP (R-NMP). The parameters for each are shown in Fig. S3. Pumping flow was controlled by an HP 33120A signal generator (Hewlett Packard, Palo Alto, CA) connected to a Model 7500 voltage amplifier (Krohn-Hite, Brockton, MA) set to 10X voltage amplification.

The two designs were compared directly in a PCR chip configuration for flow rate characterization. A series of marks were etched into the chip at 1mm intervals, and the chip was filled with diH₂O containing 0.1% w/w 45um diameter silicon beads. The marked area of the chip was placed under a Zeiss microscope equipped with Hamamatsu C2400 CCD camera (Hamamatsu, Naka-ku, Hamamatsu, Japan) and videos of bead flow were taken at input frequencies of the piezoelectric buzzer ranging from 60 to 160Hz at 70V (Fig. S4A). For both

1. F. K. Forster, R. L. Bardell, M. A. Fromowitz, N. R. Sharma and A. Blanchard, *ASME-PUBLICATIONS-FED*, 1995, 234, 39-44.
2. I. Izzo, D. Accoto, A. Menciassi, L. Schmitt and P. Dario, *Sensors and Actuators A: Physical*, 2007, 133, 128-140.

valve designs, maximum flow rates occurred at 110Hz. This frequency was then used to characterize flow rate at voltage input ranging from 30 to 100V (Fig. S4B-C). The D-NMP valve resulted in significantly higher flow rates, and was used in further experiments in this paper. Following characterization, pumping parameters of 40V at 110Hz were chosen to achieve an in-channel flow rate of 45 $\mu\text{L}/\text{min}$.

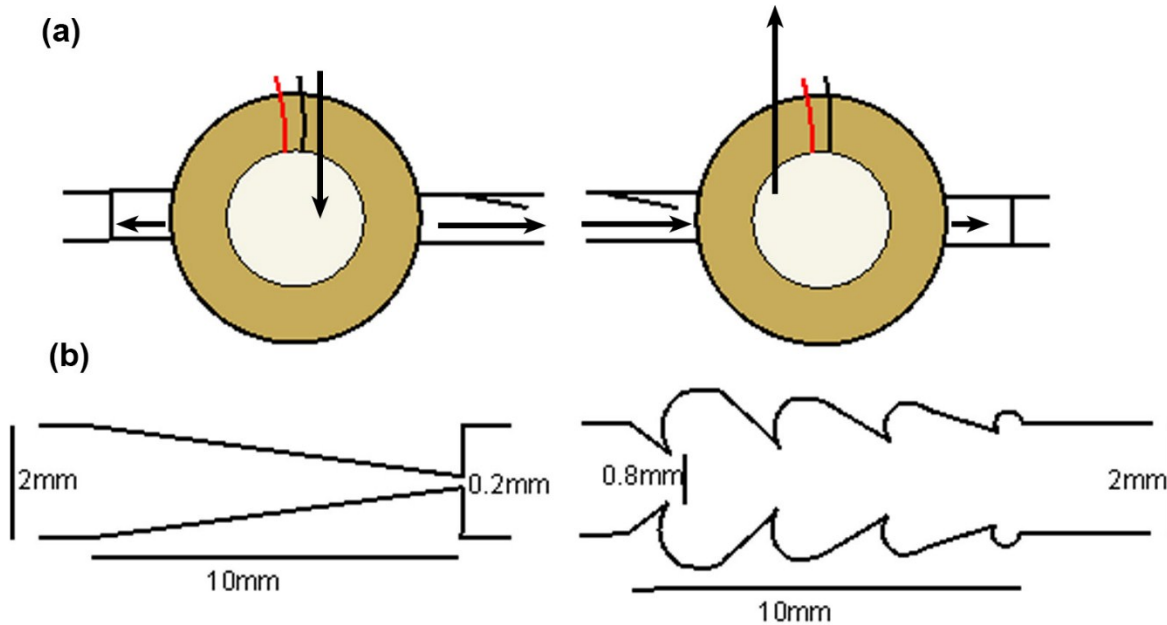


Figure S3. A) Operational principle diagram of piezoelectric pump. No-moving part (NMP) valves used to rectify flow created from deflection of the piezoelectric buzzer. Flow enters the chamber when the buzzer deflects upwards, and out when it deflects downwards. The NMP valves each limit flow in one of these directions, leading to a net unidirectional flow through the pump. B) Fluidic NMP valve designs: diffusion (left) and recirculating (right). Two identical copies of the same valve design were connected to 2mm wide channel structure on one side and 10mm diameter pumping chamber on the other to create unidirectional flow. All valves were fabricated with a depth of 300 μm .

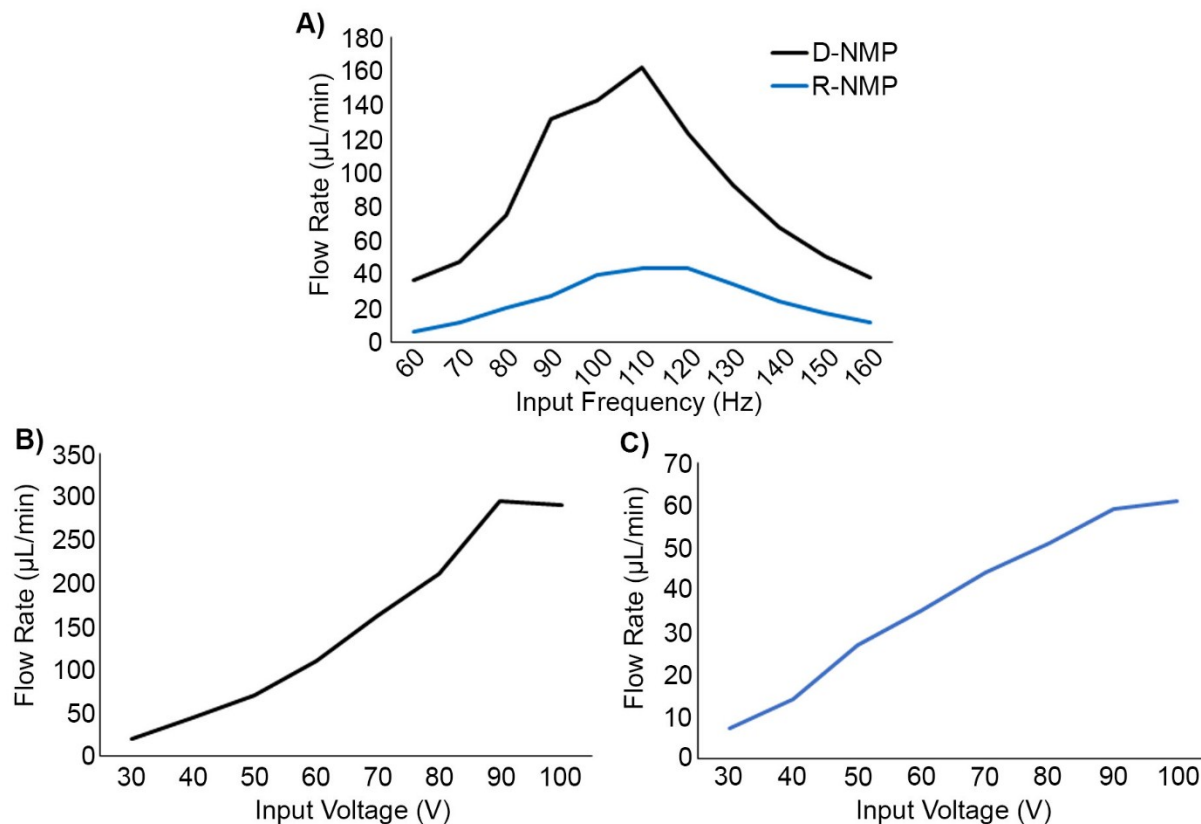


Figure S4. A) Flow rate generated from piezoelectric pump at 70V for diffusion (D-NMP) and recirculating (R-NMP) no-moving part valve designs at frequencies from 60 to 160Hz. Maximum flow rates occurred at 110Hz for both valve designs, resulting in a peak of 162μL/min for the diffusion valve, and 44μL/min for the recirculating valve. B) Pumping flow rate for D-NMP valve as a function of voltage at constant frequency of 110Hz. Maximum attained flow rate was 300μL/min that was more than sufficient for the required in-loop flows. C) Pumping flow rate for R-NMP valve as a function of voltage at constant frequency of 110 Hz. Flow rate appeared to plateau at a maximum of approximately 60μL/min.

Synthesis of silver nanoprisms: All reagent solutions were prepared fresh because sodium borohydride and L-ascorbic acid are reducing agents and thus might lose reduction potential over time. In a typical experiment, a solution consisting of 39.3mL diH₂O, 2mL Trisodium Citrate (TSC) (75mM), 256μL Hydrogen Peroxide (H₂O₂) (0.6%), 186μL Silver Nitrate (AgNO₃) (10mM) was prepared. Under vigorous stirring, 192μL of Sodium Borohydride (NaBH₄) (100mM) was rapidly added to initiate reduction, and the solution immediately changed to a pale yellow color. After about five minutes, the color shifted to a golden yellow. After this color shift, the colloidal seed solution was stored overnight at room temperature to allow the NaBH₄ to complete its

reduction. Following this aging period, 2.1mL of the seed stock were added to a clean 8mL vial and stirred vigorously. Two hundred μL of L-Ascorbic Acid (5mM) were added to this mixture, followed by dropwise addition of AgNO_3 (10mM, typically 100 μL) until silver nanoprisms with desired optical properties were formed that was tested by color changes and was confirmed by UV-Vis spectrophotometry.

Nanoprism Adhesion to Glass Substrates: Glass cover slips were coated by nanoprisms using a two-step process. First, 24x40mm borosilicate glass cover slips (VWR, Radnor, PA) were cleaned using aqua regia and carefully washed with diH_2O prior to incubation in a solution of 0.01% Poly-L-Lysine (PLL) for 10 minutes. The PLL physisorbed to the glass substrate, creating an amine-modified surface. The cover slips were then blown dry under a jet of nitrogen, rinsed in diH_2O and incubated for 20 minutes in a colloidal nanoprism solution with OD >3. Following this incubation period, the cover slips were removed from the nanoparticle solution, rinsed in diH_2O and dried under a jet of nitrogen. This technique resulted in reliable formation of consistent nanoprism monolayers.

Nanoprism monolayer stability was tested under operational conditions of the PCR/LSPR chip. As shown in Figure S5, silver nanoprism monolayers maintained integrity under heating to 85 $^{\circ}\text{C}$ for 30 minutes in a microchannel filled with deionized water (Fig. S5A) as well as under flow created by one hour of piezoelectric pumping using the same conditions as would be used during on-chip PCR (Fig. S5B).

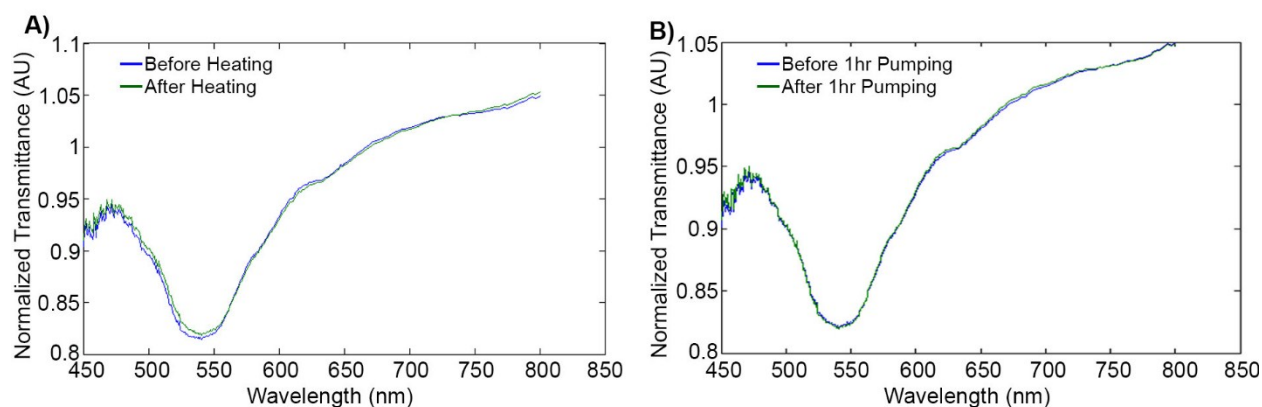


Figure S5. A) Heating stability test: transmittance spectra before and after heating at 85 $^{\circ}\text{C}$ for 30 minutes in a flow channel. B) Flow stability test: transmittance spectra before and after one hour under piezoelectric pumping at 70V and 110Hz. Neither test showed any significant spectra changes, indicating stability under PCR reaction conditions on the chip.

Nanoprism Functionalization: Thiol-linked probes targeted to the DXS and *tufB* genes for *Escherichia coli* were obtained from Sigma Aldrich, (*tufB*: 5'-[Thi-C6]CGTGCGATTGACAAGCCGTTCC, DXS: 5'-[Thi-C6]ATCGCTGAACGCTACGCTGGTCGATAT). A 10 μ L water droplet containing DNA probes at 35 μ M concentration was incubated on the detection zone of a nanoprism coated coverslip for one hour. The coverslip was then washed with diH₂O and dried under a nitrogen jet.

The chosen probe concentration was utilized as the minimal concentration required to maximize shift in the absorption spectra of the nanoprism – past an asymptotic point, further addition of probes did not result in an appreciable spectral shift, and thus no further improvement in functionalization of nanoprisms. To determine this concentration, 10 μ L droplets containing DNA probes for the *E. coli* DXS gene at concentrations ranging from 17.5 μ M to 70 μ M were placed on the surface of a nanoprism coated cover slip and were incubated for one hour. Then, the cover slip was washed, dried under a jet of nitrogen, and transmittance spectra from each functionalized region were taken using hyperspectral imaging, along with a reference zone that had been incubated with diH₂O.

Hyperspectral Imaging: Hyperspectral imaging of silver nanoprism monolayers was carried out using a PARISS hyperspectral imaging device (LightForm Inc.) installed on Leica DM6000M microscope (Leica, Wetzlar, Germany). The microscope was used in brightfield transmitted light mode with a broad halogen lamp illumination and 20x objective. Prior to taking hyperspectral measurements, a reference lamp spectrum was recorded by placing an uncoated glass slide on the microscope stage. Once the lamp spectrum was acquired, a hyperspectral image of a nanoparticle coated slide was recorded. After image acquisition, resulting data were analyzed in MatLab using custom functions. Lamp spectra were used to normalize sample spectra to obtain 2D maps of transmittance spectra. These maps were then averaged to obtain the average transmittance spectra of nanoprism monolayers in the sensor zone in the 450 - 800nm wavelength range.

LSPR Imaging Optical System: Design of the optical imaging system was predicated on low-cost methods for 2D spectroscopic imaging. All optical components were purchased from ThorLabs (Newton, NJ) except as noted. A 5W Cree XP-G2 white LED (RapidLED, Burlingame, CA) was used for illumination, powered with a B&K Precision 1651 24V DC power source (B&K Precision, Yorba Linda, CA). Light from the LED passed through a 1mm pinhole prior to collimation through an 18mm PCX lens (f=20mm) and 12mm asphere (EFL=8mm). The collimated beam passed through a 0.3mm slit aperture prior to a 600 groove/mm diffraction

grating. The diffracted beam was re-collimated at a 12° angle from the original beam using an 18mm PCX lens ($f=25\text{mm}$), and then incident on a DLP3000 digital micromirror device (DMD) (Texas Instruments, Dallas, TX). The DMD was set at a 50° angle from the horizontal perpendicular to the beam at a distance such that only the spectrum from 560-640nm region was incident on its surface. A lens pair, 25mm PCX ($f=125\text{mm}$) and 25mm PCX ($f=50\text{mm}$), was used in the path of the reflected beam to collimate and focus it onto a 1mm diameter multimode optical fiber ($\text{NA}=0.05$). The optical fiber was terminated with an SMA connector attached to an F280SMA 3.3mm diameter fiber optic collimator. After passing through the nanoprism sensor zone of the PCR chip, the light was detected by a QICam 12-bit Retiga EXi FireWire camera (QImaging, Surrey, BC, Canada). See Table 1 for a complete list of optical components used.

Table 2. Optical Components in LSPR Imaging System

Component	Source	Cost
5W Cree XP-G2 White LED	RapidLED	\$4.00
DLP LightCrafter Evaluation Kit which includes DLP3000 Digital Micromirror Device Chip that was used to simplify DMD control (optional component).	Texas Instruments	\$603.70
DLP3000 Digital Micromirror Device + DLPC300 Digital Controller (DLPC300 can be also used to control DMD)	Texas Instruments	\$173.19
1mm Pinhole Aperture	ThorLabs	\$40.50
18mm PCX Lens ($f=20\text{mm}$)	ThorLabs	\$32.00
12mm Asphere Lens ($\text{EFL}=8\text{mm}$)	ThorLabs	\$83.25
Adjustable Mechanical Slit Aperture	ThorLabs	\$253.00
25mm Diffraction Grating (600 grooves/mm)	ThorLabs	\$107.00
18mm PCX Lens ($f=25\text{mm}$)	ThorLabs	\$31.25
1" PCX Lens ($f=125\text{mm}$)	ThorLabs	\$31.00
25mm PCX Lens ($f=50\text{mm}$)	ThorLabs	\$32.50
1mm Diameter Multimode Optical Fiber ($\text{NA}=0.05$)	ThorLabs	\$111.00
F280SMA 3.3mm Fiber Optic Collimator	ThorLabs	\$148.00
Total		\$1477.20

In order to optimize spectral resolution and light throughput of the imaging system, images were taken of the spectra resulting from passing the light reflected from the diffraction grating through a series of bandpass filters with known spectral characteristics – XF3084535AF45 (535nm center, 45nm FWHM), XF1021550DF30 (550nm center, 30nm FWHM), XF1045560DF15 (560nm center, 15nm FWHM), QMAX/EX620-650/25 (620-650nm band), and XF3059QBEM90168 (580-615nm band) (Horiba, Alvin, TX). The obtained images

were then correlated with the known spectral bands to optimize performance of the imaging system. As shown in Figure S6 (A), there was a significant dependence of system's spectral resolution of the size of the aperture (A in Figure 4). Based on these experiments the aperture size was set to 0.3 mm because below this value the optical flux of the system was deemed too low for achieving sufficient signal-to-noise ratio. A series of measurements through known bandpass filters was used to determine the spatial separation of different wavelengths projected on the DMD from the diffraction grating (Fig. S6, B). Bandpass filters were placed in the light path following the diffraction grating, limiting light incident on the DMD to a specific, known, wavelength range. The reflected light from the DMD was then incident on a Hamamatsu C2400 CCD camera, creating images of the horizontal distribution of reflected light. To create spatial mapping of the DMD, the horizontal resolution of image was mapped to the width of the DMD surface. Given that the horizontal length of the DMD corresponded to frequency of incident light following diffraction, the known wavelength bands from the bandpass filters were used to correlate this spatial mapping to frequency of light. It was determined that the total spectral range was 540 to 680nm (Fig. S6, B).

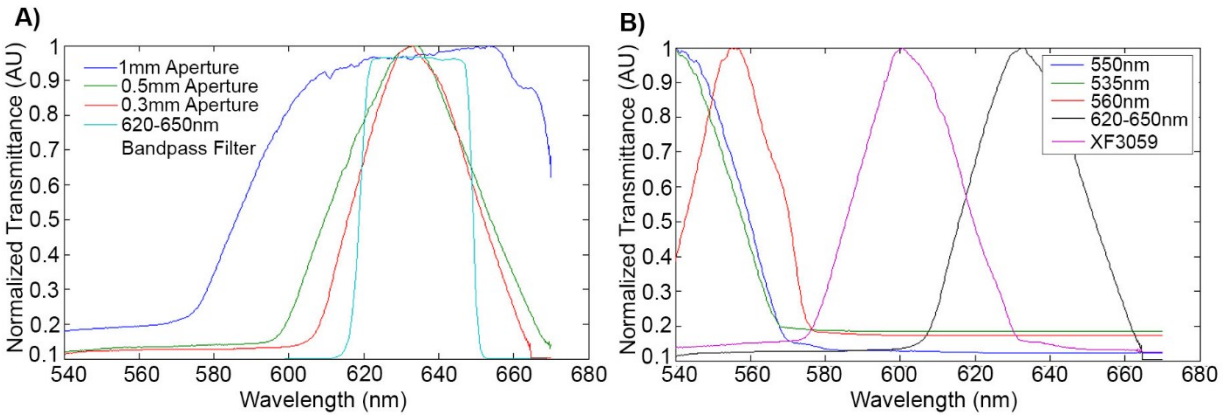


Figure S6. A) Detected transmission through a 620-650nm bandpass filter using 1mm, 0.5mm and 0.3mm aperture sizes as compared to the actual transmission through the filter. The 0.3mm aperture resulted in the full-width half-maximum of 615-652nm that closely matched the actual 620-650nm bandwidth of the filter. B) Measured transmission through a series of bandpass

filters using a 0.3mm aperture. Legend indicates center wavelengths for bandpass filters. XF3059 is a multipass filter with a 580-615nm band in the measured range of wavelengths.