Supporting Information

PCR-free, Multiplexed Expression Profiling of microRNAs using Silicon Photonic Microring Resonators

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Experimental Details

Materials

UltraPure DEPC-treated water (Life Technologies) was used for all experiments. A 10X PBS buffer (pH 7.4) was diluted to 1X and used to reconstitute all nucleic acid samples. A high stringency hybridization buffer consisting of 30% formamide, 0.2% sodium dodecyl sulfate, 4× saline−sodium phosphate−ethylenediaminetetraacetic acid (EDTA) buffer (SSPE, USB Corp.), and 3× Denhardt's solution (Invitrogen) was used for all nucleic acid hybridization steps. A PBS running buffer (pH 7.4) was reconstituted with 0.05% Tween20 and was used to dilute all protein containing steps. The silane (3-aminopropyl)triethoxysilane (APTES) was purchased from Fisher. All custom synthesized nucleic acid sequences were obtained from Integrated DNA Technologies (IDT; Coralville, Iowa). Nucleic acid sequences are included in Table S1. miRNA RT-PCR kits used for RT-qPCR profiling were purchased from Life Technologies, and target specific assay IDs are listed in Table S2. Drycoat Assay Stabilizer solution was purchased from Virusys Corporation and used as received. Streptavidin-conjugated horseradish peroxidase (SA-HRP), one-step 4-chloro-1-napthol solution, and all other reagents were purchased from ThermoFisher and used as received.

Instrumentation

Sensor chips and read-out instrumentation were obtained from Genalyte, Inc. (San Diego, CA). Sensor chips were fabricated at a silicon foundry on 8 in. silicon-on-insulator wafers using deep UV photolithography and dry etch methods, spin-coated with a fluoropolymer cladding layer, and diced into individual 4 × 6 mm chips, each having an array of 132 individually addressable microrings. The fluoropolymer cladding is selectively removed from 128 of the rings, leaving these exposed to the solution and responsive to binding events. The four occluded rings serve as control elements for subtracting thermal drift. Chips were fitted with a laser etched Mylar gasket, which defines flow chambers when sandwiched with a Teflon lid, and loaded into the readout instrumentation. All experiments were performed with automated fluidic handling using the recipes summarized in Table S3.

Resonant wavelengths for each microring were determined by coupling a tunable laser source (centered at 1560 nm) into an adjacent linear waveguide via on-chip grating couplers. The laser output was then swept through an appropriate spectral window and the light intensity at the distal end of the linear waveguide was used to determine the resonance wavelength. This process was then serially repeated for each ring in the array, and the resultant shifts in resonance as a function of time were recorded.

The resonance condition that is supported by the microring resonators is governed by the following equation:

$$m\lambda = 2\pi r n_{eff}$$

where m equals a nonzero integer, λ is the wavelength of propagating light, r is the microring radius, and n_{eff} is the effective refractive index of the local microring environment. Boundary conditions of light propagating in linear waveguides via total internal reflectance result in an evanescent field extending into a region very close to the ring surface. Interactions between the evanescent field and the local

environment cause a change in the resonant wavelength, which is then monitored by the optical scanning instrumentation. Therefore, the binding of higher refractive index biomolecules and accompanying displacement of water results in a resonance shift to longer wavelengths: a positive shift that is listed in units of Δ picometers (Δpm).

Sensor surface functionalization with capture probes:

Prior to covalent modification of capture probes, sensor chips were cleaned in a piranha solution (3:1 H_2SO_4/30% H_2O_2) for 35 seconds. (Caution: Piranha solutions are extremely dangerous and react explosively with organics.) Following a 2 min rinse in acetone, chips were incubated in APTES (5% in acetone) for 4 minutes. After rinsing sensor chips in acetone (2 min) followed by IPA (2 min), a bis(sulfosuccinimidyl)suberate solution (25 µL, 2.85 mg/mL in acetic acid), an amino-to-amine crosslinker, was pipetted onto the sensor surface and left to incubate for 3 minutes. Chips were dried with N_2, and then small aliquots (300 nL) of an aminated DNA capture probe specific to an individual miRNA target were deposited onto the microring surface so that the solution covers a specific set of microrings. The chips were left in a humidity chamber (1 hour). Then, they were rinsed in Drycoat Assay Stabilizer solution and stored at 4°C until use.

Preparation and addition of miRNA target to sensor surface:

Target miRNA solutions were first reverse transcribed according to the manufacturer's protocol (TaqMan microRNA Reverse Transcription Kit, Life Technologies) using the following thermal profile: 16°C (30 min), 42°C for (30 min), and 85°C (5 min). Following reverse transcription, the solution was incubated in equal volume alkaline hydrolysis buffer (50 mM Na_2CO_3, 1 mM EDTA, pH 8.5) at 95°C for 30 min. An aliquot of this solution was then diluted 100 fold in hybridization buffer (4 µL sample aliquot in 396 µL hyb) and to that a biotinylated DNA probe (0.4 µL, 200 µM) that is complimentary to the "stem" region of the stem loop primer is added. This solution is incubated at 72°C for 10 min. After incubation, the solution is allowed to cool back to room temperature and loaded into a 96 well plate for subsequent analysis on chip.

For on chip analysis, the diluted miRNA target sequences were flowed at 15 µL/min for 14 min. For total RNA analysis, the solution was allowed to hybridize for 25 minutes. A sensor baseline was established before and after hybridization by flowing hybridization buffer (30 µL/min) for 3 min and 1 min respectively.

Horseradish peroxidase enzymatic amplification:

Following miRNA hybridization, a PBST (0.05% Tween20) solution is flowed across the surface (30 µL/min) for 5 minutes to establish a new baseline resulting from the running buffer change. Afterwards, a solution of SA-HRP (4 µg/mL) is introduced (30 µL/min, 3 min) and binds to the biotinylated compliment that is bound on the sensor surface. This is followed by another PBST rinse (30 µL/min, 2 min) to prepare the surface for 4-CN precipitation. 4-CN is then introduced to the sensor (30 µL/min) for 9 min, and a final buffer rinse (30 µL/min) is conducted for 3 min to establish the net sensor response before and after 4-CN amplification. It is important to note that the surface can be regenerated on the microring sensor chips without a decrease in performance.

*Complete outline of automated fluidic handling shown in Table S3.

Total RNA samples:

Both brain (Lot No. 1307018) and lung (Lot No. 1410019) total RNA samples isolated from patient tissue were obtained commercially (Life Technologies) and stored at -80°C until further use. Samples were thawed on ice for approximately 2 hours prior to use, and a 1 µg and 100 ng input amount was used for microring and RT-qPCR analysis respectively.

Data analysis:

Data analysis was performed using Origin pro 9.0. All data was corrected for temperature drift, bulk refractive index shifts and differential sensor response by subtracting the response of control rings functionalized with a non-complementary capture probe from the active rings. To calculate the initial slope of DNA binding, we used a modified 1:1 Langmuir Binding Isotherm, as described by:

$$S(t) = A(1 - e^{-B(t-t_0)})$$

To determine the initial slope of the binding response, the first derivative of the previous equation was evaluated at $t = t_0$, yielding:

$$\frac{dS}{dt} = AB$$

The average of the initial slopes was taken over a number of sensors for each concentration. As a general rule, the first 5 minutes of collected data was used to obtain the fit. For low concentrations where the modified Langmuir Binding Isotherm could not effectively fit the sensor trace, a linear fit was used to approximate the initial slope.

RT-qPCR

For RT-PCR experiments, total RNA (100 ng) was used. Reverse transcription was performed using TaqMan microRNA Reverse Transcription Kit (Life Technologies) and miRNA-specific stem loop primers provided in the kit (see Table S2 for miRNA specific Assay IDs). Reverse Transcription was carried out on a BioRad T100 Thermal cycler at 16°C (30 min), 42°C for (30 min), and 85°C (5 min). RT products were then subjected to quantitative PCR in triplicate using PCR primers from the same miRNA Assay Kit. The reaction was performed at 95°C (10 min), followed by 40 two-step cycles of (1) 95°C for 15 s and (2) 60°C for 1 min. All quantitative PCR work was done using an Applied Biosystems 7900HT Fast Real-Time PCR System. All procedures and reactions were carried out according to the protocols provided by the manufacturer. Levels of miRNAs were normalized to miR-26a, and fold change was calculated using the 2^(-ΔΔCt) method.

Table S1. Summary of nucleic acid sequences.

	Sequence
hsa miRNA-26a	UUC AAG UAA UCC AGG AUA GGC U
hsa miRNA-106a	AAA AGU GCU UAC AGU GCA GGU AG
hsa miRNA-21	UAG CUU AUC AGA CUG AUG UUG A
hsa miRNA-222	AGC UAC AUC UGG CUA CUG GGU CUC
hsa miRNA-335	UCA AGA GCA AUA ACG AAA AAU GU
hsa miRNA-219	UGA UUG UCC AAA CGC AAU UCU
hsa miRNA-29a	UAG CAC CAU CUG AAA UCG GUU A
Conserved region of stem loop primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTT GGA TAC GAC … … miRNA specific overhang
miR-26a SLP Overhang	AGCCTATCC
miR-106a SLP Overhang	CTACCTGCA
miR-21 SLP Overhang	TCAACATCAG
miR-222 SLP Overhang	GAGACCCAG
miR-335 SLP Overhang	ACATTTTTCG
miR-219 SLP Overhang	AGAATTGC
miR-29a SLP Overhang	TAACCGATTT
miR-26a Capture Probe	TTC AAG TAA TCC AGG ATA GGC TGT
miR-106a Capture Probe	AAA AGT GCT TAC AGT GCA GGT AGG
miR-21 Capture Probe	TAG CTT ATC AGA CTG ATG TTG AGT
miR-222 Capture Probe	AGC TAC ATC TGG CTA CTG GGT C
miR-335 Capture Probe	TC AAG AGC AAT AAC GAA AAA TGT GT
miR-219 Capture Probe	TGA TTG TCC AAA CGC AAT TCT GT
miR-29a Capture Probe	TAG CAC CAT CTG AAA TCG GT
Control Capture Probe	/5AmMC12/CTACAAGTGCCTTCACTGCAGT
Stem Loop Primer Compliment	5’-biotinTEG-ATACCTCGGACCCTGCACT-3’

Table S2. RT-qPCR assay IDs for miRNA targets (sequences not provided by vendor).

Target	Assay ID
miR-26a	000405
miR-106a	002169
miR-21	000397
miR-222	000525
miR-335	000546
miR-219	000522
miR-29a	002112

Table S3. Details on fluid flow conditions used in the assay.

Step	Flow Rate (µL/min)	Duration (min)
Hybridization Buffer	30	3
RT Product	15	14*
Hybridization Buffer	30	1
0.05% PBST	30	5
SA-HRP (4 µg/mL)	30	3
0.05% PBST	30	2
4-CN	30	9
0.05% PBST	30	3

*For total RNA profiling, the duration was increased to 25 minutes, which improved hybridization specificity.

Table S4. The brain and lung RNA samples used in Figure S3 were subjected to RT-qPCR analysis. Each sample was reverse transcribed (100 ng input) and subjected to PCR in triplicate. Outlined in the table are the average C(t) values and the standard deviation of the measurements for individual miRNA targets.

Target	Brain Sample C(t)	Std. Dev.	Lung Sample C(t)	Std. Dev.
miR-26a	21.92	0.23	17.46	0.03
miR-106a	26.33	0.11	19.54	0.04
miR-21	24.78	0.13	17.04	0.05
miR-222	29.00	0.16	23.33	0.03
miR-335	24.78	0.05	20.01	0.03
miR-219	27.56	0.05	34.72	0.28
miR-29a	22.52	0.12	17.74	0.03

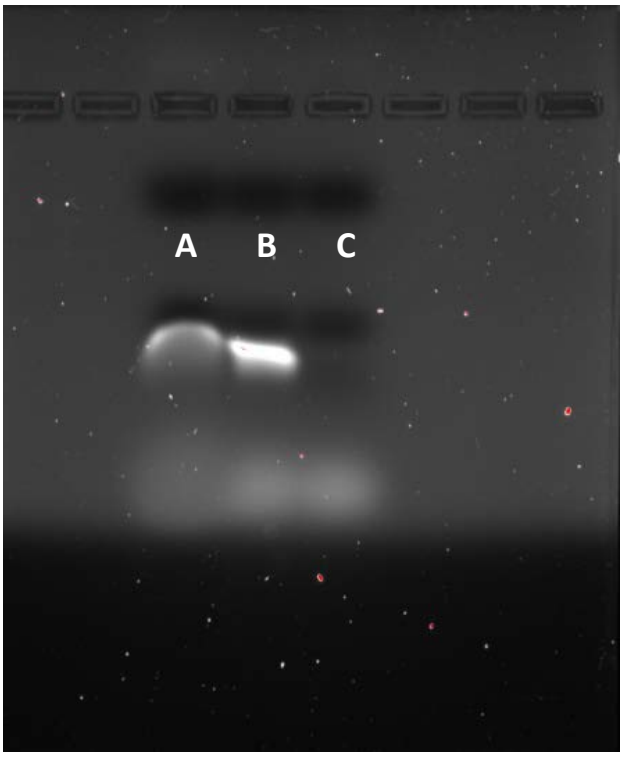

Figure S1. Agarose gel analysis hydrolysis buffer efficiency. Three samples were analyzed on an ethidium bromide stained 2% agarose gel (0.5x TBE running buffer) to assess the efficiency of the alkaline hydrolysis buffer. Lanes A and B show samples that have been reverse transcribed (30 picomole input of miR-26a) and incubated with either alkaline hydrolysis buffer (lane A) or water (lane B). This result confirms that the alkaline hydrolysis buffer successfully hydrolyzes the RNA. The band intensity analysis (ImageJ) shows that the hydrolyzed sample is approximately 50% less intense than the control group.

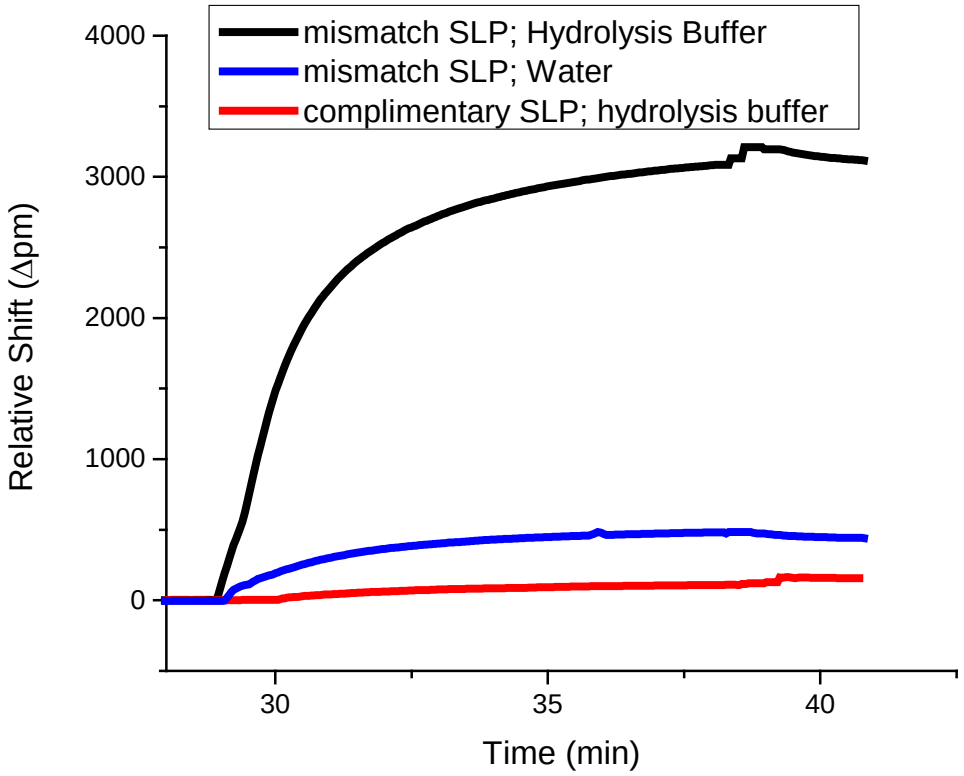

Figure S2. Effect of alkaline hydrolysis buffer and stem loop primer construction on sensor response. A 10 picomole input of miRNA-26a was used and subjected to the RT-HRP assay under three different conditions. The response for experiments that had a perfectly complimentary SLP (T_m = 79.6°C) showed no binding response as the probe was not linearized and able to bind to the sensor surface. Responses were observed when a single nucleotide mismatch was introduced into the SLP design (T_m = 61.8°C); however, the signal was dramatically increased when the miRNA was degraded using hydrolysis buffer. These results show that the stem loop primer choice and miRNA degradation via alkaline hydrolysis are essential steps in the assay design.

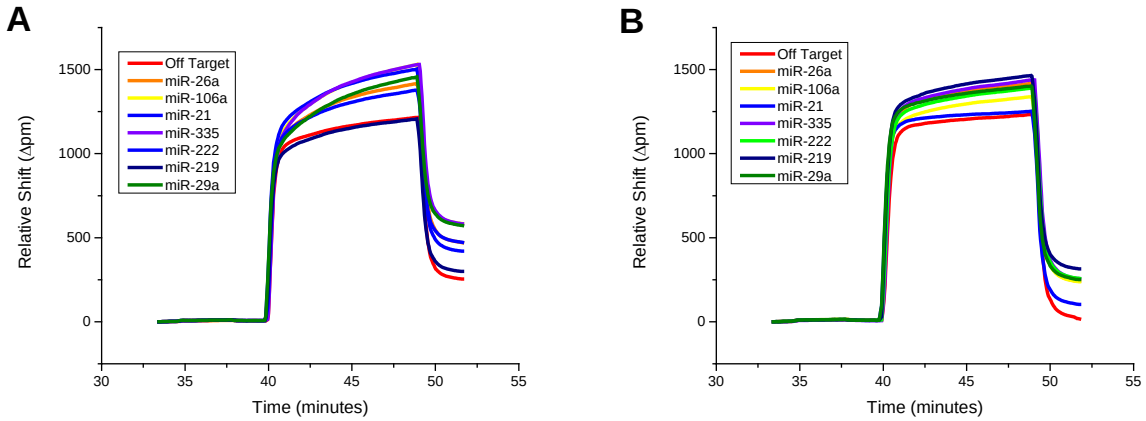

Figure S3. Microring sensor response of total RNA profiling from lung and brain tissue. Microrings previously functionalized with 8 different capture probes (7 complimentary to a specific miRNA target and one off target control) were subjected to the optimized assay. The resulting shifts were quantitated by taking the difference before and after 4-CN oxidation. (A) Binding curves obtained when using an input of 1 µg of total RNA isolated from lung tissue. (B) Binding curves obtained when using an input of 1 µg of total RNA isolated from brain tissue.