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Multiplexed Detection and Label-Free Quantitation of microRNAs using Arrays of Silicon Photonic Microring Resonators**

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MicroRNAs (miRNAs) are short (19 to 24 nucleotides), single-stranded, non-protein-coding RNAs that are powerful transcriptional and post-transcriptional regulators of gene expression. Unlike small interfering RNAs (siRNAs), miRNAs are genomically encoded and play key roles in a range of normal cellular processes, including proliferation, apoptosis, and development.[1-4] Not surprisingly, miRNAs have also been implicated in a number of diseases, including cancer,[5-8] neurodegenerative disorders,[9-11] and diabetes,[12-14] and represent promising biomarker candidates for informative diagnostics. Despite their increasingly well-understood importance in gene regulation, the development of sensitive analytical techniques for the quantitation of multiple miRNAs has lagged behind. Furthermore, current methodologies for miRNA expression analysis are not applicable to a clinical setting where sample sizes are limited and assay cost and time-to-result is of tremendous importance.

In contrast to most nucleic acid analysis technologies that advantageously utilize the polymerase chain reaction (PCR) to increase the amount of the target sequence, miRNAs are not easily amplified on account of their small size, which prohibits standard primer hybridization.[15] Although creative approaches that enable reverse transcriptase-PCR amplification have been developed,[16-18] many conventional miRNA analyses are prone to sequence-biased amplification or hindered by the need for large amounts of sample. The most widely reported miRNA analysis technique, Northern blotting, requires substantial amounts of starting material, is extremely laborious, and is not amenable to large-scale multiplexing.[19] Recently, a number of new miRNA analysis methods have been reported that feature high sensitivity, but often at the expense of assay simplicity and scalability, multiplexing capability, or rapid analysis time.[20-27]

In this paper, we report a label-free, direct hybridization assay enabling the simultaneous detection of multiple different miRNAs from a single sample using commercially fabricated

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and modularly multiplexable arrays of silicon photonic microring resonators. Using complementary single-stranded DNA capture probes, we are able to rapidly (10 min) quantitate down to ~ 150 fmol of miRNA and show the ability to discriminate between single nucleotide polymorphisms within the biologically important let-7 family of miRNAs. We also demonstrate the applicability of this platform for quantitative, multiplexed expression profiling by determining the concentration of four miRNAs from within a clinically-relevant sample size of a cell line model of glioblastoma with minimal sample preparation.

Microring resonators are a promising class of refractive index-sensitive devices that have recently been applied to monitoring chemical reactions and biomolecular binding events. [28-36] Light coupled via an adjacent linear waveguide is strongly localized around the circumference of the microring under conditions of optical resonance, as defined by the cavity geometry and the surrounding refractive index environment. Given a defined microring structure, the resonance wavelength is sensitive to changes in the local refractive index, in this case the hybridization of miRNAs to complementary ssDNAs on the surface, as illustrated in Figure 1a. Monitoring the shift in resonance wavelength after exposure to the sample of interest allows determination of the solution-phase analyte concentration.

We have previously described the use of silicon-on-insulator (SOI) microring resonators for the sensitive detection of proteins.[28-30] A wavelength-tuneable laser centered at 1560 nm is coupled into on-chip waveguides that interrogate the microrings and determine resonance wavelengths. The sensor chips, each containing 32 individually-addressable 30 μm diameter microrings, are coated with a fluoropolymer cladding layer that is selectively removed over the active sensing elements using reactive ion etching. Figure 1b shows a small portion of the sensor array, and the inset highlights a single microring and its adjacent linear interrogation waveguide.

The first step in modifying sensors to detect particular miRNAs is to covalently modify the native oxide-coated surface of the silicon microrings with single-stranded DNAs complementary to the target(s) of interest. After appropriate derivitization, the shifts in resonance wavelength accompanying hybridization of miRNA to the microrings can be followed in real time, as shown in Figure 2. At $t = 15$ min, a 2 μM solution of miR-24-1 is flowed over the sensors and its hybridization to complementarily functionalized microrings elicits a shift of ~ 40 pm in the resonance wavelength. Returning to PBS buffer at $t = 45$ min gives an immediate increase in resonance peak shift on account of differences in bulk solution refractive index. The opposite shift (a negative change in bulk refractive index) occurs for the injection of miRNA solution, but is largely counteracted by the hybridization of miRNA.

To confirm the hybridization, we introduced a solution containing RNase H, an enzyme that selectively cleaves DNA:RNA heteroduplexes, at $t = 60$ min. The rapid increase in resonance wavelength corresponds to a bulk refractive index change, but the enzymatic activity of RNase H dissociating the duplex quickly leads to a decrease in relative peak shift. Control experiments without hybridized miRNA or with DNA:DNA duplexes show a stepped response that reflects only the bulk index change to and from the RNase H-containing solution, but without the net decrease corresponding to heteroduplex cleavage. Returning the microring to RNase H buffer and then PBS buffer confirms the hybridization of miRNAs to the ssDNA capture strands and also demonstrates that the sensor surfaces can be regenerated. Utilizing this RNase H protocol, we have found that sensors can reproducibly respond to miRNA hybridization after more than twenty regeneration cycles.

Exposure of microrings to different solutions of miR-24-1 varying from 2 μ M to 1.95 nM reveals a concentration-dependent response, as shown in Figure 3a. Rather than utilize the absolute wavelength shift, which saturates as miRNAs hybridize to all of the available ssDNA capture probes, we determine the rate at which the resonance peak changes immediately after target introduction and use the initial slope response for quantitation. Advantages of this approach include generation of a linear sensor calibration curve and greatly reduced assay time ($\sim$ 10 min), which is significantly faster than waiting for the system to establish binding equilibrium, a concentration-dependent period that can take many hours. Figure 3b shows the linear relationship between the initial slope of sensor response, determined via fitting of the real time resonance wavelength shift data, and the concentration of miR-24-1.

A significant challenge for all nucleic acid analyses that is particularly important for miRNAs is the ability to distinguish single base differences in sequence. Therefore, we developed an isothermal method of distinguishing single base differences between two members of the biologically important let-7 family of miRNAs by performing hybridizations in the presence of formamide, which is a chaotropic agent that competes for hydrogen bonding sites. Under normal hybridization conditions (no formamide) the miRNA isoforms let-7b and let-7c, which differ only by a single base change at position 17, both bind to the non-specific DNA capture probe designed to be perfectly complementary to the other sequence (Figure S6). However, when hybridization is performed in a 50% (v/v) formamide solution, the single base difference is easily distinguished.

A key advantage of the microring resonator sensing platform is its potential for high-level multiplexing. SOI microring resonators are fabricated using scalable semiconductor-processing techniques that enable a large number of sensors to be incorporated and individually interrogated on the same chip. Utilizing microarray spotting or other patterning methodologies, each ring can be functionalized with a unique capture agents (cDNAs, antibodies, etc.), allowing many different biomolecules to be simultaneously quantitated.

To demonstrate the multiplexing capability of our platform, we constructed a four-component array by differentially functionalizing microrings on the same chip with unique ssDNAs complementary to four dissimilar miRNAs. Figure 4 shows the real time shift in resonance wavelength for 4 sets of microrings, each functionalized with a different ssDNA, during the sequential introduction of miR-133b, miR-21, miR-24-1, and let-7c. Sequence-specific responses are observed at appropriate microrings only when the complementary miRNA solution is exposed to the sensor array. Small changes in resonance wavelengths arising from differences in bulk refractive index are observed at time points where solutions are switched, but in each case the sequence-specific response is clearly discernable above baseline.

Furthermore, we simultaneously determined the expression levels of the same four miRNAs extracted from U87 MG cells, an established model for grade IV gliomas, including glioblastoma and astrocytoma.[37,38] The entire small RNA content from 5×10^7 U87 cells was extracted using a commercial purification kit and flowed over a sensor surface with microrings functionalized with ssDNA capture probes complementary to the target miRNAs. Each microring was individually calibrated to account for differences in signal response between target miRNAs (Figure S9). The initial slope of sensor response upon addition of the U87 small RNA sample was measured and the concentration of each target miRNA in solution determined (miR-21: 18.9 ± 3 nM, miR-24-1: 3.3 ± 0.2 nM, miR-133b: 60 ± 20 nM, let-7c: 4 ± 3 nM).

Given the drive towards even smaller sample sizes, future work with this platform will focus heavily on improvements in sensitivity. One method for improving might include the incorporation of higher affinity oligo capture probes, such as locked nucleic acids (LNA) or peptide nucleic acids (PNA). Previous studies have shown that both classes of synthetic oligos increase the specificity as well as sensitivity of miRNA assays.[26,39] Another approach might include the implementation of sequence-independent, secondary amplification techniques to increase the total mass bound to our sensor surfaces. Two candidate methods include the RNA-primed array-based Klenow enzyme assay (RAKE) or Poly(A) polymerase enzymatic amplification, both of which utilize enzymes to specifically add nucleotides to the 3' end of miRNAs hybridized to the sensor surface, after which additional amplification steps can be included to further boost the amount of bound mass. [21,40]

The emergence of miRNAs as important regulators of gene expression and as valuable disease biomarkers places an impetus on developing next-generation detection methodologies. Particularly valuable will be those that can operate under the sample size limitations and time-to-result requirements of clinical analyses. Furthermore, multiplexed analyses in which a significant fraction of the “miRNA-ome,” predicted to be comprised of ~1000 miRNAs for humans,[41] can be simultaneously analyzed will prove exceedingly important in deciphering the complex regulatory action of these molecules. In pursuit of these needs, we have developed a new platform for the sensitive, sequence-specific, and label-free quantitation of miRNAs using direct hybridization to arrays of ssDNA-functionalized silicon photonic microring resonators. We demonstrate the ability to quantitate the expression level of multiple miRNAs from clinically relevant sample volumes within a 10-minute data acquisition time using a pre-calibrated sensor array. Future efforts will be directed towards improving sensor limits of detection as well as increasing levels of multiplexing by interfacing microring resonator arrays with microarray spotting technologies for rapid encoding of many unique sensing elements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Lee RC, Feinbaum RL, Ambros V. *Cell*. 1993; 75:843. [PubMed: 8252621]
2. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom N, Kelnar K, Ovcharenko D, Wilson M, Wang XW, Shelton J, Shingara J, Chin L, Brown D, Slack FJ. *Cancer Res*. 2007; 67:7713. [PubMed: 17699775]
3. Jovanovic M, Hengartner MO. *Oncogene*. 2006; 25:6176. [PubMed: 17028597]
4. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RHA. *Science*. 2005; 309:310. [PubMed: 15919954]
5. Nelson, Peter T.; Wang, WangXia; Rajeev, Bernard W. *Brain Pathol*. 2008; 18:130. [PubMed: 18226108]
6. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebet BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. *Nature*. 2005; 435:834. [PubMed: 15944708]
7. Bartels CL, Tsongalis GJ. *Clin Chem*. 2009; 55:623. [PubMed: 19246618]
8. Calin GA, Croce CM. *Nature Rev Cancer*. 2006; 6:857. [PubMed: 17060945]
9. Hebert SS, De Strooper B. *Science*. 2007; 317:1179. [PubMed: 17761871]
10. Perkins DO, Jeffries CD, Jarskog LF, Thomson JM, Woods K, Newman MA, Parker JS, Jin JP, Hammond SM. *Genome Biol*. 2007; 8:R27. [PubMed: 17326821]

11. Schaefer A, O'Carroll D, Tan CL, Hillman D, Sugimori M, Llinas R, Greengard P. *J Exp Med*. 2007; 204:1553. [PubMed: 17606634]
12. Tang X, Tang G, Özcan S. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*. 2008; 1779:697.
13. Muhonen P, Holthofer H. *Nephrol Dial Transplant*. 2009; 24:1088. [PubMed: 19145005]
14. Walker MD. *Diabetes*. 2008; 57:2567. [PubMed: 18820213]
15. Wark AW, Lee HJ, Corn RM. *Angew Chem*. 2008; 47:644. [PubMed: 17994653]
16. Li J, Yao B, Huang H, Wang Z, Sun C, Fan Y, Chang Q, Li S, Wang X, Xi J. *Anal Chem*. 2009; 81:5446. [PubMed: 19469541]
17. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM. *RNA*. 2005; 11:1737. [PubMed: 16244135]
18. Chen CF, Ridzon DA, Broomer AJ, Zhou ZH, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. *Nucl Acids Res*. 2005; 33
19. Cissell KA, Shrestha S, Deo SK. *Anal Chem*. 2007; 79:4754.
20. Liang RQ, Li W, Li Y, Tan Cy, Li JX, Jin YX, Ruan KC. *Nucl Acids Res*. 2005; 33:e17. [PubMed: 15684409]
21. Fang S, Lee HJ, Wark AW, Corn RM. *J Am Chem Soc*. 2006; 128:14044. [PubMed: 17061884]
22. Jonstrup SP, Koch J, Kjems J. *RNA*. 2006; 12:1747. [PubMed: 16888321]
23. Li J, Schachermeyer S, Wang Y, Yin Y, Zhong W. *Anal Chem*. 2009; 81:9723. [PubMed: 19831385]
24. Cheng YQ, Zhang X, Li ZP, Jiao XX, Wang YC, Zhang YL. *Angew Chem*. 2009; 48:3268. [PubMed: 19219883]
25. Yang H, Hui A, Pampalakis G, Soleymani L, Liu FF, Sargent EH, Kelley SO. *Angew Chem*. 2009; 48:8461. [PubMed: 19810065]
26. Zhang GJ, Chua JH, Chee RE, Agarwal A, Wong SM. *Biosens Bioelect*. 2009; 24:2504.
27. Zhang Y, Li Z, Cheng Y, Lv X. *Chem Comm*. 2009; 22:3172. [PubMed: 19587903]
28. Washburn AL, Gunn LC, Bailey RC. *Anal Chem*. 2009; 81:9499. [PubMed: 19848413]
29. Washburn AL, Luchansky MS, Bowman AL, Bailey RC. *Anal Chem*. 2010; 82:69. [PubMed: 20000326]
30. Luchansky MS, Bailey RC. *Anal Chem*. 2010; 82:1975. [PubMed: 20143780]
31. Iqbal M, Gleeson M, Spaugh B, Tybor F, Gunn WG, Hochberg M, Baehr-Jones T, Bailey RC, Gunn LC. *IEEE J Sel Top Quantum Electron*. 2010 in press.
32. Xu DX, Densmore A, Delage A, Waldron P, McKinnon R, Janz S, Lapointe J, Lopinski G, Mischki T, Post E, Cheben P, Schmid JH. *Optics Exp*. 2008; 16:15137.
33. Chao CY, Fung W, Guo LJ. *IEEE J Sel Top Quantum Electron*. 2006; 12:134.
34. Yalcin A, Popat KC, Aldridge JC, Desai TA, Hryniewicz J, Chbouki N, Little BE, King O, Van V, Chu S, Gill D, Anthes-Washburn M, Unlu MS. *IEEE J Sel Top Quantum Electron*. 2006; 12:148.
35. De Vos K, Girones J, Popelka S, Schacht E, Baets R, Bienstman P. *Biosens Bioelectron*. 2009; 24:2528. [PubMed: 19200711]
36. Ramachandran A, Wang S, Clarke J, Ja SJ, Goad D, Wald L, Flood EM, Knobbe E, Hryniewicz JV, Chu ST, Gill D, Chen W, King O, Little BE. *Biosens Bioelectron*. 2008; 23:939. [PubMed: 17964774]
37. Clark MJ, Homer N, O'Connor BD, Chen ZG, Eskin A, Lee H, Merriman B, Nelson SF. *Plos Genetics*. 2010; 6:e1000832. [PubMed: 20126413]
38. <http://www.attc.org>
39. Valoczi A, Hornyik C, Varga N, Burgyan J, Kauppinen S, Havelda Z. *Nuc Acids Res*. 2004; 32:e175.
40. Nelson PT, Baldwin DA, Searce LM, Oberholtzer JC, Tobias JW, Mourelatos Z. *Nature Methods*. 2004; 1:155. [PubMed: 15782179]
41. Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z. *Nature Genetics*. 2005; 37:766. [PubMed: 15965474]

Figure 1.

(a) Each microring sensor is functionalized with a capture sequence of DNA (black). The sequence-specific hybridization of the target miRNA (red) causes a shift in the wavelength required to achieve optical resonance. (b) Scanning electron micrograph showing six microrings on a sensor array chip. The inset shows a single microring and its corresponding linear access waveguide revealed within an annular opening in the fluoropolymer cladding layer.

Figure 2.

Real time measurement in shift of microring resonance wavelength during the hybridization of 2 μM miR-24-1 to three separate microring resonators. The resulting heteroduplex is subsequently dissociated by the enzyme RNase H, yielding a regenerated sensor surface.

Figure 3.

a) Response of a single microring to the binding of miR-24-1 as a function of concentration (2 μM to 7.8 nM decreasing top-to-bottom via 2-fold dilutions). The dotted lines designate the fitted curves used to calculate the initial slope of the target miRNA binding. The response of only a single ring is shown for clarity. Responses for miRNA concentrations of 3.91 and 1.95 nM are omitted for clarity, but are resolvable from the zero concentration response. *b)* Average response of microring resonators as a function of miR-24-1 concentration. Error bars represent ± 1 standard deviation for at least nine independent measurements at each concentration.

Figure 4.

Sequence-specific detection of four unique miRNAs on a single chip as the miRNA complementary to the ssDNA on the microring is sequentially introduced into the flow chamber. Microrings were functionalized with complementary ssDNAs against (top to bottom) miR-133b, miR-21, miR-24-1, and let-7c. miRNA solutions were all 1 μ M in PBS. Asterisks (*) denote time points where the solution over the sensors was changed to PBS buffer. In some cases, small changes in resonance wavelength are observed due to small differences in bulk solution refractive index. Each set of rings is offset from the baseline wavelength for clarity.