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A Guide to Quantitative Biomarker Assay Development using Whispering Gallery Mode Biosensors

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Abstract

Whispering gallery mode (WGM) sensors are a class of powerful analytical techniques defined by the measurement of changes in the local refractive index at or near the sensor surface. When functionalized with target-specific capture agents, analyte binding can be measured with very low limits of detection. There are many geometric manifestations of WGM sensors, with chip integrated silicon photonic devices having been first commercialized on account of robust, wafer-scale device fabrication, facile optical interrogation, and amenability to the creation of multiplexed sensor arrays. Using these arrays, a number of biomolecular targets have been detected in both label-free and label-enhanced assay formats. For example, sub-picomolar detection limits for multiple cytokines were achieved using an enzymatically enhanced sandwich immunoassay that showed high analyte specificity suitable for detection in complex, clinical matrices. This protocol describes a generalizable approach for the development of quantitative, multiplexed immunoassays using silicon photonic microrings as an example WGM platform.

Keywords

whispering gallery mode sensors; immunoassay; biomarker; protein detection; multiplex; quantitative analysis

Introduction

Whispering gallery mode (WGM) sensors are promising for a range of (bio)chemical detection applications, and several recent reviews have described overall progress in this field (Fan and White, 2011; Foreman et al., 2015; Luchansky and Bailey, 2012; Wade and Bailey, 2016). Rather than survey recent progress and specific advances in sensor geometry or analytical performance, this article describes a generalizable protocol for the development of quantitative, multiplexed biomarker assays for robust analysis of biomarkers from within complex, biologically relevant samples (serum, plasma, whole blood, etc.). Although alternative WGM sensors with different geometries and materials systems are viable platforms for detection, this protocol is written from the perspective of using arrays of chip-integrated silicon photonic microring resonators (Iqbal et al., 2010)—a platform that has been commercialized by Genalyte, Inc. Furthermore, this protocol focuses on enzymatically enhanced sandwich immunoassays (Kindt et al., 2013; Valera et al., 2016), which have been found by our lab to provide the highest specificity and lowest limits of detection for protein

detection in complex matrices; however, many of the general steps are based upon or closely related to earlier examples of label-enhanced (Luchansky and Bailey, 2011; Valera et al., 2015), or label-free protein biomarker detection (Washburn et al., 2009; Washburn et al., 2010). Similar approaches on the same detection platform using different capture agents have been also been applied to the detection of viruses (McClellan et al., 2012) and nucleic acid targets (Kindt and Bailey, 2012; Qavi and Bailey, 2010; Qavi et al., 2011a; Qavi et al., 2011b; Scheler et al., 2012).

Like other WGM devices, microring resonators support optical modes that are sensitive to changes in the local refractive index at or near the sensor surface. By monitoring changes in the optical properties of resonators functionalized with target-specific capture agents, often through shifts in the spectral position of resonances, biomolecules can be detected with high sensitivity (Figure 1) (Wade and Bailey, 2016). The general immunoassay components include a capture antibody, sample/target protein standard, biotinylated secondary tracer antibody, streptavidin-horseradish peroxidase (SA-HRP) conjugate, and chloronaphthol solution (4-CN) for enzymatic signal enhancement (Figure 2). As with all immunoassays, performance is fundamentally related to attributes of the capture agents, including the affinity, avidity, specificity, and kinetics of analyte binding and unbinding. For silicon photonic sensors, antibodies can conveniently be covalently coupled to the sensor surface using standard bioconjugation methods. This chemistry can also be adapted to sensor functionalization with nucleic acids, including amine-terminated single-stranded DNA. After binding of the analyte (from the sample or standard-containing solution), the biotinylated secondary tracer antibody recognizes the bound antigen and provides increased specificity for the target protein—particularly for detection in complex matrices where non-specific binding of highly abundant matrix proteins can obscure target binding to the capture antibody. Binding of the SA-HRP conjugate to the tracer antibody provides an opportunity to tremendously enhance the resonance wavelength shift by the catalytic decomposition of 4-chloro-1-naphthol, which leads to the deposition of an insoluble product onto the sensor surface. The enzymatic signal enhancement is not necessary but provides a notable improvement in the limit of detection (Kindt et al., 2013; Luchansky and Bailey, 2011). Beyond standard sensor functionalization, considerable effort is devoted towards optimizing assay parameters, including the volume, concentration, rate and duration of delivery, and other assay reagent considerations. Once optimized, the assay can be rigorously calibrated, often using protein standard solutions, and then used for robust and reliable analyte quantitation.

This sandwich immunoassay protocol is generally applicable for a range of protein biomarkers that can be specifically targeted using commercial or custom-generated antibody (or aptamer) capture agents. Furthermore, these approaches are amenable to single- or multiplexed protein detecting assays, with multiplexed assays facilitated by microspotting different capture agents at discrete spatial locations across the sensor array substrate (Figure 3). Assay optimization and operation are further facilitated by automated liquid handling, which significantly mitigates the reproducibility issues that can plague manual plate- and bead-based assay formats. Finally, considerations that reduce required sample input and assay time-to-result are also important when considering translational application to the analysis of complex clinical samples.

Strategic planning

Selection of the biomarker targets and the capture agents requires careful consideration. Once functionalized with a specific capture agent, the performance of the sensor is significantly dependent on the unique binding properties of the capture agent (e.g., affinity, avidity, and specificity). This is critical to remember when comparing limits of detection as a performance metric of sensors and similar assays. That is to say that limits of detection for specific assays are often more a result of higher affinity reagents than the performance of the technology itself—this is particularly important to note when critically evaluating new platforms that invoke irregularly high affinity interactions (e.g., biotin-avidin) for proof-of-principle reports. Furthermore, the stability of capture agents can dictate long-term storage and other assay conditions. The native matrix in which a biomarker needs to be detected is also an important consideration, particularly if the complexity will require sample dilution. Mitigating matrix effects (i.e., non-specific interactions) through dilution is common, but can be non-ideal if the biomarker is present at low abundance. Early consideration of the target, primary and tracer capture agents, and potential for matrix effects can significantly reduce optimization time and assay failure.

Finally, the development of a quantitative, multiplexed assay is rarely a straightforward process. The most robust assays require rigorous optimization regardless of the sensing platform. Moreover, care must be given to ensure assay reproducibility over time and perhaps even across multiple lots of reagents, which can have profound effects on performance. Therefore, it may be necessary to iteratively repeat the basic protocols presented herein to achieve robust and reliable performance for the entire protein panel.

The protocols in this article present a general method for the development and implementation of a multiplexed quantitative protein detection assay using WGM sensors. Three basic protocols are described. First, the WGM sensor is functionalized with capture probes selective for the protein targets. Second, the assay performance is optimized and characterized in terms of cross-reactivity, non-specific binding conditions, and optimal conditions. Third, the sensor array is calibrated for quantitative response in a matrix comparable to experimental conditions. As a specific example to illustrate these steps, an amplified sandwich immunoassay is described here.

Basic protocol 1: Functionalization of the sensor surface with antibody capture probes

Sensor fabrication is highly dependent upon the WGM sensor geometry and materials system utilized; however, in all instances, the initial post-fabrication step involves the functionalization of the sensor surface with a target-specific capture agent(s) for an analyte(s) of interest. A common method to achieve this, which is compatible with silicon photonic sensor arrays, includes chemical modification with aminopropyltriethoxysilane (APTES), followed by reaction with a homobifunctional cross-linker, such as bisulfosuccinimidyl suberate (BS3), which can subsequently react with lysine residues and N-terminal amines on capture antibodies (Figure 4). Other bifunctional crosslinkers could be used with minimal impact on sensitivity, since the evanescent field is far longer than the

length of chemical linkers. This chemical modification strategy and cross-linking process is applicable to many suitable WGM resonator materials systems and was selected on account of its robustness and generality. Other attachment schemes may be suitable provided they are compatible with the experimental conditions. With BS3, this protocol can also be used for the functionalization of other amine-containing capture agents, including commercially available amine-terminated nucleic acids. This protocol is also amenable to sensor functionalization using robotic microarrayers, and in this way can be used to batch fabricate many identical single- or multiplexed detection arrays.

Materials

- 3-Aminopropyltriethoxysilane (1% solution in acetone)
- Acetone
- Isopropanol
- Distilled water or Milli-Q purified water
- Acetic Acid (2 mM in distilled water)
- Bissulfosuccinimidyl suberate (5 mM in 2 mM acetic acid—conveniently available in 2 mg No Weigh Format from Thermo Scientific)
- PBS buffer with BSA (10 mM with 0.5% BSA)
- Glycerol
- Capture antibody stock solution (at least 0.25 mg/ml, <1% sodium azide)
- StartingBlock (PBS) buffer (Thermo Scientific)
- DryCoat assay stabilizer (Virusys, #AG066-1)
- WGM sensor/sensor array (Commercially available as 128-microring sensor arrays from Genalyte Inc.)
- 20 mL scintillation vials
- Tweezers
- Stereoscope
- Clean room cloth

Silanizing the sensor surface

- 1 Clean the sensors using appropriate organic solvents, such as acetone or isopropyl alcohol. *When handling the sensor, use clean tweezers and be careful to avoid damaging the device.*

Be sure to give the array a final rinse in clean solvent before proceeding to the next step.
- 2 Silanize chips in 1% APTES solution in acetone. Allow the chip to soak with mild agitation for 4 min.

The APTES solution should be prepared fresh from stock solution stored in a desiccator under nitrogen. APTES will have a short shelf life if not stored correctly.

This is easily performed in a 20 mL scintillation vial.

- 3 Rinse chip for 2 min in acetone and then isopropanol with mild agitation.
Both steps should take place in separate 20 mL scintillation vials.
- 4 Rinse the chip(s) in deionized water and dry under N₂ gas stream.

Bioconjugation with BS3 cross-linker

- 5 Prepare 5 mM BS3 cross-linker solution in 2 mM acetic acid solution.

The BS3 solution should be prepared fresh and exposed to the sensor within 1 h. The NHS-ester moiety readily hydrolyzes and becomes non-reactive so fresh reagents and timely use is critical. If using 2 mg No Weigh Format (above in Materials) dissolve an entire aliquot into 700 mL of 2 mM acetic acid solution. The preparation of this reagent with regard to pH and concentration is consistent with the commercial recommendations. The use of acetic acid and the length of the reaction was empirically determined for Genalyte sensors.
- 6 Place chip into a well plate and apply ~20 µL of BS3 solution onto the chip taking care to cover the entire sensor surface.

When functionalizing multiple chips, complete this step with each chip in an individual well of a 24 well plate. Carefully add the solution on the chip surface and avoid contact with the plate or adding too much liquid.
- 7 Allow the BS3 coated chip to sit for 3 min. Remove liquid with N₂ gas stream.

If the liquid was not contained to the surface of the chip, carefully move the chip to gas stream. Chips may stick to the surface of the plate.

Spotting capture probes on chip surface

- 8 Prepare 20–50 µL of >100 µg/ml capture agent solution by diluting in 10 mM PBS and 5% glycerol.

The capture concentration varies depending on optimal response. The glycerol minimizes capture agent drying before complete conjugation. When considering capture agents, always additionally include an off-target control capture, such as an isotype control antibody compatible with the detection assay. This should be included in every assay as a measure of non-specific binding.
- 9 To create a multiplexed sensor array with resolution greater than that achievable by eye, place chip under stereoscope. Deposit capture target in 5% glycerol onto

specific regions of the sensor array by carefully spotting liquid onto distinct sensor array clusters.

A 2.5 μL pipet set to 0.2–0.5 μL dispense volume works well. Spot slowly. To spot multiple capture probes, be sure to avoid cross contamination of the capture agent spots by carefully spotting small and non-overlapping volumes.

10 Transfer spotted chips to humidity chamber for one hour.

A humidity chamber can be easily constructed by adding a thin strip of damp clean room cloth over the well plate containing sensors and closing the lid. This will decrease the likelihood of spot evaporation before complete surface functionalization.

11 Coat chips in DryCoat assay stabilization reagent by pipetting several drops over the surface and place in desiccator at 4 C until ready to use.

To pre-block the sensors, use DryCoat with BSA to avoid non-specific protein binding. This can also be achieved by blocking during the experiment with StartingBlock buffer. After blocking, sensors are immediately ready to use and do not require DryCoat or 4 C storage prior to use. This step is necessary only for stable storage of functionalized sensors.

Basic protocol 2: Assessing optimal conditions, cross-reactivity, and matrix effects for whispering gallery mode (WGM) assay optimization

Traditional immunoassays require iterative development to assess the optimal concentration and protocol for each target analyte. Similarly, WGM immunoassays require this development as well as the evaluation of potential non-specific interactions between multiple targets. First, it is critical to determine the minimum concentration that will yield the maximum signal for each component of the assay. By minimizing reagent use, the assay becomes more cost-efficient and has minimal non-specific binding. A second component of assay optimization is the development of the assay protocol. In this example, the assay protocol, also known as the recipe, is optimized in terms of the flow rate and volume of each reagent (Table 1).

Significant variation exists in the fluid delivery approaches used in different WGM sensor configurations. Examples of fluid delivery for the Genalyte M1 and M24 platforms is shown in Figure 5, along with an example of an optical configuration to monitor a microsphere resonator in a polymeric chamber as described by the Vollmer group (Baaske et al., 2014). While specific details may not be generalizable between platforms/configurations, the focus on sensor care, consistent fluid flow, and their impact on data quality is shared.

Our usual approach has been to semi-optimize single-target assays and before incorporating reagents for multiple targets. With multiplexed assays, the potential for cross-reactivity is evaluated for each component (primary capture and tracer antibodies). The final step of

assay optimization consists of evaluating the effects of a complex matrix on non-specific binding and maximum signal. The sensors have shown robust measurements in different biological environments (e.g., serum, plasma, etc.), but the assay performance should be evaluated, optimized, and calibrated in the matrix most closely resembling the format of the final samples to be analyzed. This protocol was optimized for the development of a robust multiplexed immunoassay in complex matrix using the Genalyte silicon photonic platform. Steps that are specific to the Genalyte, Inc. platform are indicated with an asterisk (*).

Materials

- Running buffer (10 mM PBS, 0.5% BSA, Detergents such as Tween20 are optional)
- Standard protein solutions (1–100 µg/ml stock diluted in running buffer or matrix solutions)
- Biotinylated tracer antibodies (diluted to 0.5–4 µg/ml in running buffer)
- Streptavidin-horseradish peroxidase conjugate (diluted to 1–6 µg/ml in running buffer)
- Low pH Glycine solution (10 mM in Milli-Q water, pH 2.2)
- Concentrated matrix (e.g., plasma or serum, aliquoted and stored frozen)
- 1-step chloronaphthol solution (4-CN, Thermo Scientific, containing 4-chloro-1-naphthol and proprietary peroxide-containing buffer)
- Vortex mixer
- *96-well plates (400 µL maximum volume)
- *Pre-cut Piercable Films for Robotics (X-Pierce, Excel Scientific)
- *Cartridge assembly (Genalyte Inc.)
- *Teflon sipper tubes (0.01" ID Teflon tubing)
- *Precision torque wrench and screwdriver (1/16")
- *Maverick optical scanning instrumentation (Genalyte Inc.)

Preparation of reagents for signal optimization

- 1 Prepare 400 µL of standard protein solution in running buffer (10 mM PBS, 0.5% BSA). Gently vortex each solution prior to use.

This should be a maximum concentration recommended by the product guide for an immunoassay. If this is unavailable, it is recommended to start with a concentration between 10–100 ng/ml. However, the saturation concentration can be highly variable and should be iteratively tested.
- 2 Prepare 400 µL of biotinylated tracer antibody in running buffer. Gently vortex each solution prior to use.

The optimal concentration of the secondary antibody will need to be determined empirically, but start by consulting the product guide. If this is unavailable, it is recommended to start with 1–4 µg/ml. Aim to achieve the maximum signal response with the minimum antibody concentration for that signal.

- 3 Prepare 400 µL of streptavidin-horseradish peroxidase solution in running buffer. Gently vortex the solution prior to use.

This concentration should be comparable to the concentration of the biotinylated tracer antibody. Optimize this concentration simultaneously with the secondary antibody concentration. Minimize light exposure throughout solution preparation and during the experiment.

- 4 *Transfer all reagents to a 96 well plate including 1-step chloronaphthol solution (4-CN) as the final step in the assay. Cover with a Pre-Cut Pierceable Film.

The plate cover must be pre-cut for the sipper tube to access the reagents. This is achieved using the Pre-Cut Pierceable plate covers for 96 well plates. The 4-CN incubation should be followed by a short buffer rinse to remove excess reagent. Include enough running buffer for this step.

Mounting WGM sensor in fluidic housing, pre-assay checks, and running the assay

- 5 Assemble capture agent-functionalized WGM sensor into suitable cell for fluidic delivery.

- 6 *Load a pre-functionalized chip into a base cartridge holder.

Use tweezers and carefully align the chip with fluidic gasket and assemble cartridge using screwdriver and torque wrench. Tighten the screws as evenly as possible applying gentle pressure to the whole cartridge top to maintain even stress on the chip and gasket. Attach Teflon tubing to cartridge. Check flow before attaching to fluid delivery system.

- 7 Load the assembled sensor into the optical mounting system.

*Load the assay protocol into the software and register the chip. Chip registration performs a quick scan of each sensor and the readout determines which sensors on the chip are viable for the experiment. If numerous sensors fail to register or the entire chip fails to register, this may indicate the chip alignment is off or the chip is damaged.

- 8 Before starting the assay, check the flow quality across the sensor by performing a pre-scan buffer rinse step for approximately 5 minutes.

Watch the fluid lines for visual confirmation of flow and reduction in bubbles. At this point, no data is generated (“pre-scan”), but bubbles may be seen in the tubing. Watching the progress of a bubble flowing in

the tubing lines is a good indication of fluid flow. It is critical that the flow is established before scanning to avoid poor reagent interactions with the sensor. If there are many bubbles present in the fluid lines or the lines are dry, manually purge the lines with buffer or water via syringe.

- 9 Deliver reagents sequentially to the pre-functionalized sensor surface.

Recipes can vary based upon the specifics of an individual assay. The initial running buffer rinse steps may be used to establish the baseline and confirm fluid flow. If there are significant bubbles or limited flow, there will be significant deviation in the sensor response in the first 1–2 minutes. If this occurs, stop the assay and re-purge the device. Once the recipe proceeds to the analyte step, the assay should not be restarted. Running buffer rinse steps typically range from 1–5 minutes, Sample from 5–20 minutes, and remaining reagents from 5–20 minutes in sequential steps. Flow rates can range from 10–40 $\mu\text{L}/\text{min}$. A final rinse of the fluid lines should be 10–30 minutes of water. For biological samples, include a 10% bleach rinse (~10 min) prior to the water rinse. For a sandwich immunoassay with enzymatic signal amplification an example recipe is shown in Table 1.

* Prepare assay recipe by defining each step of the assay, duration, and flow rate in a CSV file. All sensor rings must be assigned as a probe sensor or control sensor.

- 10 *Start recipe and optical monitoring of resonance wavelength shift. Once the recipe is completed, rinse fluid lines completely.
- 11 Examine results and iteratively adjust reagent concentrations, flow rates, and flow durations to optimize the magnitude of sensor response for a given analyte concentration.

Assessing cross-reactivity for multiplexed assays

- 12 Once multiple, individual sandwich assays are independently optimized, multiple capture antibodies can be spotted at distinct spatial locations across the array.
- 13 Separately prepare the analyte and secondary antibody reagents for each target.

The analytes and secondary antibodies will be introduced to the chip one at a time.
- 14 Transfer the reagents, buffer, and a 10 mM glycine rinse to a 96 well plate or other fluid containment system.

This cross-reactivity assay requires that each analyte and secondary antibody is introduced one at a time. Between each target introduction, the low pH glycine rinse acts to disrupt the analyte-capture interaction to regenerate the surface. Assessment of cross-reactivity is performed

by sequentially flowing buffer, analyte, buffer, tracer antibody, buffer, glycine, buffer, repeat.

- 15** Sequentially deliver each standard protein and tracer antibody across the array one-at-a-time.

Each analyte and secondary antibody should elicit a response only at the sensor functionalized with the target-specific capture agent. The low pH glycine rinse will disrupt the binding between the capture agent and target protein causing the target and tracer to be removed from the sensor between every analyte-specific sequence of the recipe.

- 16** Review resonant wavelength shifts for each individual assay across the entire multiplexed capture antibody array and check for cross-reactive responses.

A cross-reactive response is determined as a signal response from a sensor functionalized with a capture with the addition of an unrelated target protein and secondary tracer. If the off-target response is observed for only one step and disappears with a buffer rinse than it may be weakly interacting and removed with a longer buffer rinse step during the actual assay. If cross-reactive responses are observed, try a lower analyte concentration that more closely mimics concentrations expected in the sample matrix. Sometimes cross-reactivity is observed at artificially high concentrations used for initial assay trials, but this can be irrelevant for assays performed at lower concentrations, such as those seen in real biological matrices.

Assessing matrix effects

- 17** Using the optimized concentrations, prepare 400 μ L analyte solutions containing each analyte at a concentration at or near saturation. The solutions should be prepared with varying concentrations of desired sample matrix (serum, plasma, etc.) ranging from 100% serum to 100% running buffer.

By spiking solutions with a high concentration of analyte, a small amount of natively present analyte will not significantly change the sensor response. This will provide a direct observation of the matrix effects. However, when assessing the possible interactions of a complex matrix with the assay, review the matrix composition. For example, commercially available, pooled human serum or plasma may contain detectable levels of the target analyte, which can contribute a specific background signal during matrix evaluation.

- 18** Complete steps 5–10 of this protocol using an optimized assay recipe. Review the data for any non-specific binding and changes in the magnitude of the signal. Perform some experiments to explore matrix effects at lower concentrations more similar to what would be expected in the native biological matrix.

Use these data to assess necessary dilutions for future calibration and sample treatment. If the signal is greatly affected at a certain percentage

of complex matrix then the calibration and samples should be appropriately diluted to mitigate signal loss.

Basic protocol 3: Calibrating the optimized multiplexed assay in complex matrix for analyte quantitation

For high quality, quantitative measurements, assay calibration is critical. Described below are the necessary steps to calibrate the response of a fully optimized multiplexed immunoassay. There are two main approaches to preparing calibrant solutions: serial dilution and random concentration. Serial dilutions are favored because it minimizes pipetting errors and involves a more linear standard preparation process. However, the concentration profiles of standard solutions generally do not represent the complex matrix of a real world sample, which can lead to a masked effect related to high or low protein concentration. Random concentration solutions may represent more realistic solution concentrations, but require that each solution is prepared individually. This preparation is more tedious to mitigate potential dilution and pipetting errors. Together, the two approaches can be used to complement each other in fully characterizing assays.

Using the data collected from the calibration experiment, a calibration curve can be constructed and critical metrics, including limit of detection (LOD) and limit of quantitation (LOQ), can be determined. Using this information, the assay is now ready for experimental sample analysis. Example data and the resulting calibration curve with delineated LOD and LOQ are shown in Figure 6.

All materials in this step are identical to those described in the previous two basic protocols.

Preparing multiplexed calibration solutions

- 1 Prepare 400 μ L multiplexed analyte solutions in the complex matrix, varying the concentration from zero to the pre-determined maximum (signal saturation). Include a minimum concentration near or just below the expected limit of detection for each analyte for full characterization.

The matrix composition and concentration range should be pre-determined from the optimization process (Basic Protocol 2). The solution preparation can be completed via serial dilution or by preparing each analyte solution with random concentrations of each analyte. Both calibration approaches are recommended to fully characterize the signal response.

Use fresh (or recently thawed) recombinant protein standards and keep all samples and matrix on ice or at 4 C throughout preparation.

- 2 Prepare multiplexed biotinylated tracer antibody solutions for the entire calibration curve analysis. The antibodies should be diluted in running buffer.

Keep tracer solution on ice or at 4 C throughout preparation and use. Prepare solutions fresh for the experiment unless otherwise tested.

- 3 Prepare the streptavidin-HRP solution for the entire calibration curve.
Keep solution on ice or at 4 C throughout use and limit exposure to light. The concentration of this solution may need to increase to compensate for the increased concentration of total detected analyte. If the maximum signal is lower than expected, increase the concentration until comparable maximum signal from the optimization is achieved.
- 4 Prepare sensor and solutions for assays.
Leave the prepared solutions on ice or at 4 C for as long as possible before their use in the assay.

Collecting and pre-analyzing data

- 5 Analyze all calibration samples including a blank (no analyte, only matrix) using the steps outlined in Basic Protocol 2, steps 5–10.
- 6 Once the data collection is complete, check that all data has been saved and itemize by target.
*Data will be output in a folder organized by sensor number.
- 7 Import the data into any data processing program that suits the following analysis steps.
Most analysis programs or statistical programming languages would provide the necessary tools for data processing.

Data analysis

- 8 If baseline controls were used then subtract the signal from this response.
*On-chip thermal controls may be used to correct for thermal fluctuations.
- 9 Plot the sensor response as a function of time.
- 10 Average the data of redundantly spotted sensors (technical replicates).
*Redundantly spotted sensors in the array can be considered as technical replicates, allowing for data averaging.
- 11 Using the averaged data, calculate the net shift during the enzymatic amplification step for the assay readout.
Calculate this by choosing a consistent time point during the amplification step and subtracting that signal from the signal of a time point prior to the amplification (i.e., buffer step prior to amplification). Use the same time points for the analysis of all samples.
- 12 Plot the data for each target analyte as sensor response (in pm) versus time and fit to the logistic function:

$$f(x) = \frac{A_1 - A_2}{1 + \frac{x^p}{x_0^p}} + A_2$$

where A_1 is the initial value (pm), A_2 is the final value (pm), x is the analyte concentration (pM), x_0 is the center value (inflection point, pM), and p is the power parameter affecting the slope of the linear portion of the fit surrounding the inflection point.

Using this data, the limit of detection and limit of quantitation can be determined for each target. Both metrics are necessary for quantitative sample analysis.

Reagents and Solutions

Milli-Q water, or equivalently pure water, is used in all recipes and protocol steps. All buffers are filtered with sterilized, low protein binding disposable filters. All protein and antibody solutions should be prepared fresh and maintained at as high of concentrations as possible for storage. Long-term storage at diluted concentrations should be thoroughly tested. Consideration must also be given to the presence of carrier proteins or antimicrobial compounds within commercially available antibody and protein standard solutions.

COMMENTARY

Background Information

Whispering gallery mode biosensors—Whispering gallery mode (WGM) resonators function by detecting changes in the local refractive index (Wade and Bailey, 2016). Figure 1 shows the operating principles for microring resonators wherein light is coupled onto the chip via a grating coupler and propagates down the linear waveguide via total internal reflectance (Iqbal et al., 2010). For other WGM sensor geometries, light is commonly coupled in via an extruded fiber optic or prism. The waveguide is directly adjacent to a circular microring, and light couples into the microring when the following resonance condition is met:

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

where λ is the wavelength of light, r is the microring radius, m is an integer, and n_{eff} is the effective refractive index sampled by the microcavity. Resonances are measured by monitoring dips in transmittance through the waveguide past the resonator as the probe wavelength is swept through a suitable spectral range (Iqbal et al., 2010).

WGM immunoassays are an alternative to the traditional plate- or more recent bead-based protein assays. The gold standard plate-based enzyme-linked immunosorbent assays (ELISAs) are labor intensive and typically single-plex. Most bead-based assays have multiplexing capabilities, but numerous manual steps can introduce significant error. When

validating WGM-based immunoassays, parallel analysis using ELISA or other existing immunoassay platform is strongly encouraged to robustly validate and correlate the results. For multiplexed assays this is extremely cumbersome; however, side-by-side validation of all targets across a subset of biological samples can instill confidence in the approach.

Standard protein assay validation—A key step in the development of the protein assay is the validation of the selective detection of a single protein target. This is typically accomplished with a standard protein as a positive control prior to sample analysis. This protein can be obtained commercially or produced in house, and is typically recombinantly expressed or isolated from natural sources (e.g., prostate-specific antigen purified from human plasma or seminal fluid is available from Meridian Life Science). When using antibodies as the capture and tracer agents, consult product information from the vendor regarding recommended pre-screened antibody pairs and compatible protein standards. To further verify the quality and binding characteristics of antibody pairs, validation with ELISA or a related immunoassay platform is advised. However, certain proteins will not have available positive standards. An alternative method to validate an antibody pair is to create or identify reference cell populations with specific expression of the target protein. We did this for a number of phosphorylated protein targets, considering an antibody pair as suitable for a specific target when the signal was significantly above the off-target control response, the assay showed a concentration-dependent response and statistically insignificant signal for negative control experiments, and independent Western blots indicated an appropriate band using the same antibody clone(s) (Wade et al., 2015).

Monitoring all assay steps for a comprehensive view of performance—In the assay described herein, signal quantitation is achieved by utilizing the enzymatically enhanced signal from the tertiary conversion of 4-CN. However, assay optimization is greatly aided by the ability to monitor (in real-time, ideally) the shift in resonance wavelength during each assay step. For example, by separately monitoring primary capture and tracer antibody binding steps a great deal of information can be gleaned, including knowledge of antibody and antigen cross-reactive responses. In one recent example, we were able to utilize primary and tracer binding data to rule out antibody cross-reactivity and reveal that the commercially available protein standard solution, which had been obtained from natural sources, had a minor contaminant that was another biomarker from within our multiplexed assay (Washburn et al., 2016). Real-time analytical capabilities can also provide insight into the kinetics of antigen-antibody interactions, which are important to keep in mind as fast dissociation rates of the capture antibody-antigen complex will complicate detection with the tracer antibody and may lead to a reduction in rinsing times.

Troubleshooting

Low primary capture or tracer antibody binding response—Choosing the antibody pair or any capture target is crucial to the success of the assay. The performance of the assay is dependent on the sensitivity and selectivity of the capture and tracer agents. Focus on pre-validated monoclonal antibody pairs whenever available when selecting antibody pairs. When initially testing an antibody pair, there are a number of reasons for lack of signal. However, if an antibody or protein standard is in question, perform a

secondary validation such as an ELISA. The best side-by-side comparison is an ELISA developed as consistently as possible (i.e. similar buffers, reagent preparation). If this is unsuccessful, replace the antibody pair or protein as needed. Importantly, do not implicitly trust vendor information on antibody compatibility—especially for antibody sandwich pairs. Many times vendors will not describe the targeted epitope, making it difficult to a priori establish whether two separate antibodies will work together. Also, be wary of lot-to-lot variation of antibodies, which can have profound effects on long-term assay stability and reproducibility.

Non-specific binding—Non-specific binding can present as a high signal when the target analyte is not present in the solution and also as high signals for control (off-target) sensors. The primary ways to investigate and mitigate this issue is through thorough optimization and by pre-blocking sensor surfaces. Minimize the concentration of secondary antibodies that may non-specifically bind to the surface or other targets. Complex matrix composition is often responsible for non-specific binding, but be weary of significantly diluting the sample beyond the detection limit. It may also be advantageous to modify the recipe by increasing the length of buffer rinses between steps to clean the surface of weakly bound biomolecules. One of the main advantages of the multi-step sandwich immunoassay described herein is that the quantitative measurement is made in the absence of biological matrix—after tertiary reagent addition and multiple rinses with buffer. In this way, the signal used for quantitation does not contain any non-specific binding response; however, cross-reactive responses from primary capture or tracer antibodies is not corrected for using this (or any other) approach.

Signal Response and Flow—Consistent fluid flow is necessary to deliver reagents to the sensor surface. An issue with fluid flow will manifest as signal drift or a spread in responses for multiplexed assays (i.e., sensor spread, which is when redundant sensors drift apart to give a large deviation in response), or no signal response. For general assays, visually establish fluid flow and lack of air bubbles prior to the introduction of the assay reagents. Inconsistent fluid flow is a common source of error and Table 2 outlines specific flow-related issues and the related solutions.

Anticipated Results

With manually functionalized arrays, Basic Protocol 1 provides the necessary information to generate consistent multiplexed chips. Basic Protocol 2 provides a general method to rigorously test immunoassays for cross-reactivity, non-specific binding interactions, and matrix effects. This protocol is broadly applicable for the development of clinical assays with numerous WGM sensors. Once a calibration curve with clinically relevant LODs has been obtained, the assay can be used for clinical evaluation of biological fluids.

Time Considerations

Functionalization of the sensor arrays depends on the batch size and number of targets. The chips can be stored at 4 °C or used immediately. The time considerations for Basic Protocol 2 depend on the number of targets and matrix type. Each target will require multiple experiments for optimization and matrix evaluation and an additional experiment for cross-reactivity. Assuming two targets and a 60 min assay time, Basic Protocol 2 requires at least

one full day. Finally, the assay calibration depends on assay length and the number of data points collected. Assuming two targets, 60 min assay, and 8 data points, Basic Protocol 3 requires approximately 6 hours. The overall unit length is highly variable, but an established assay can be completed in well under one hour.

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Figure 1.

Operating principles for microring resonator sensors. (A) A scanning electron microscope (SEM) image shows a 30 μm active microring with an adjacent linear waveguide. (B) Light is coupled onto the chip via a grating coupler and propagates down the linear waveguide via total internal reflectance. Under resonance conditions, light couples into the adjacent microring, resulting in a narrow dip in the transmittance past the microring, which is measured by a photodetector after coupled off-chip by a second grating coupler. (C) Shifts in the resonant wavelength occur due to changes in the refractive index near the surface of the ring. The schematic example depicts a target protein binding to a capture antibody, resulting in a shift of the resonance to a longer wavelength. This figure was adapted with permission from Wade et.al., *ACS Cent. Sci.* **2015**, *1*, 374–382.

Figure 2. Enzymatically-enhanced assay scheme with the signal response for the example system. The assay consists of (1) chip functionalization and protein blocking steps, (2) analyte capture from cell lysate, (3) binding of biotinylated tracer antibody, (4) binding of SA-HRP conjugate to biotinylated tracer, and (5) enzymatic signal enhancement via oxidation of 4CN to the insoluble product, 4-chloronaphthol. This figure was adapted from Wade et.al., *ACS Cent. Sci.* **2015**, *1*, 374–382.

Figure 3.
Schematic representation of the microring arrays on the sensor chip functionalized, in clusters of four microring, with 16 different capture probes in two fluidic channels. The white sensors are on-chip controls to detect thermal drift and fluidic leaks.

Figure 4.
(A) Structure of the silanization agent 3-Aminopropyltriethoxysilane (APTES) and (B) structure of the cross-linking agent Bissulfosuccinimidyl suberate.

Figure 5.

Examples of fluid delivery: Genalyte M1 cartridge assembly for a single chip array (A-B), Genalyte M24 cartridge assembly for 12 arrays with integrated fluidics (C), and an optical configuration to monitor a microsphere resonator in a polymeric chamber as described by the Vollmer group (D). Panels A and B are adapted with permission from *Clinical Biochemistry*, **2016**, 49, 121–126. Panel D is adapted with permission from *Nature Nanotechnology*, **2014**, 9, 933–939.

Figure 6. Example calibration data and the resulting calibration curve for monocyte chemotactic protein 1 (MCP-1) and a control. Each data point in the calibration curve shown corresponds with an average of at least 23 replicates. This figure is being reprinted with permission from Valera, E., Shia, W.W., and Bailey, R.C. 2016. Development and validation of an immunosensor for monocyte chemotactic protein 1 using a silicon photonic microring resonator biosensing platform. Figure adapted with permission from *Clinical Biochemistry*, 2016, 49, 121–126.

Table 1

Example optimized sandwich immunoassay with enzymatic signal amplification. The flow rate of each step is 30 $\mu\text{L}/\text{min}$.

Description	Duration (min)
Running buffer	2
Sample	20
Running buffer	2
Secondary antibody	10
Running buffer	2
SA-HRP conjugate	10
Running buffer	3
4-CN solution	15
Running buffer	7

Table 2

Common problems and solutions to fluid flow related errors. Those problems or solutions marked with an asterisk* are specifically related to the example system. All other problems and solutions are general troubleshooting strategies.

Problem	Solution
Air bubbles	Manually purge the entire flow path with buffer
Seal/fluid junction leak	Adjust connection, reinforce with Teflon tape, or replace connection
Clog in cartridge top* or microfluidic component	Remove component, sonicate if possible, manually purge with multiple solvents, and reassemble
Clog in tubing	Replace tubing (do not force through pumps) or separate from set up and thoroughly purge with multiple solvents
Broken seal between cartridge top and chip*	Remove cartridge top, dry, carefully reassemble*
Reduced or loss of signal after initial troubleshooting	Run a reliable test assay (Instrument calibration)