Enhancing Label-Free Molecular Recognition Studies with Meru Biotechnologies' Free Solution Analysis (FSA)

Summary

Understanding and measuring molecular binding remains a critical component of biochemical research, drug discovery, and diagnostics development. Despite enormous emphasis on collecting biologically relevant binding affinity data, many existing methods struggle to sensitively measure these interactions through chemical manipulation of one or more binding partner. While other more complex methods improve on this point, none of these accomplish this task without significant method development, cost, and data analysis. To meet this need, Meru Biotechnologies' **Free Solution Analysis** (FSA) builds on the strengths of existing label-free methods, while eliminating their individual challenges. Here, we discuss the existing state-of-the-art approaches, FSA's enabling technology, and case studies that demonstrate the improvements it offers in a variety of fields.

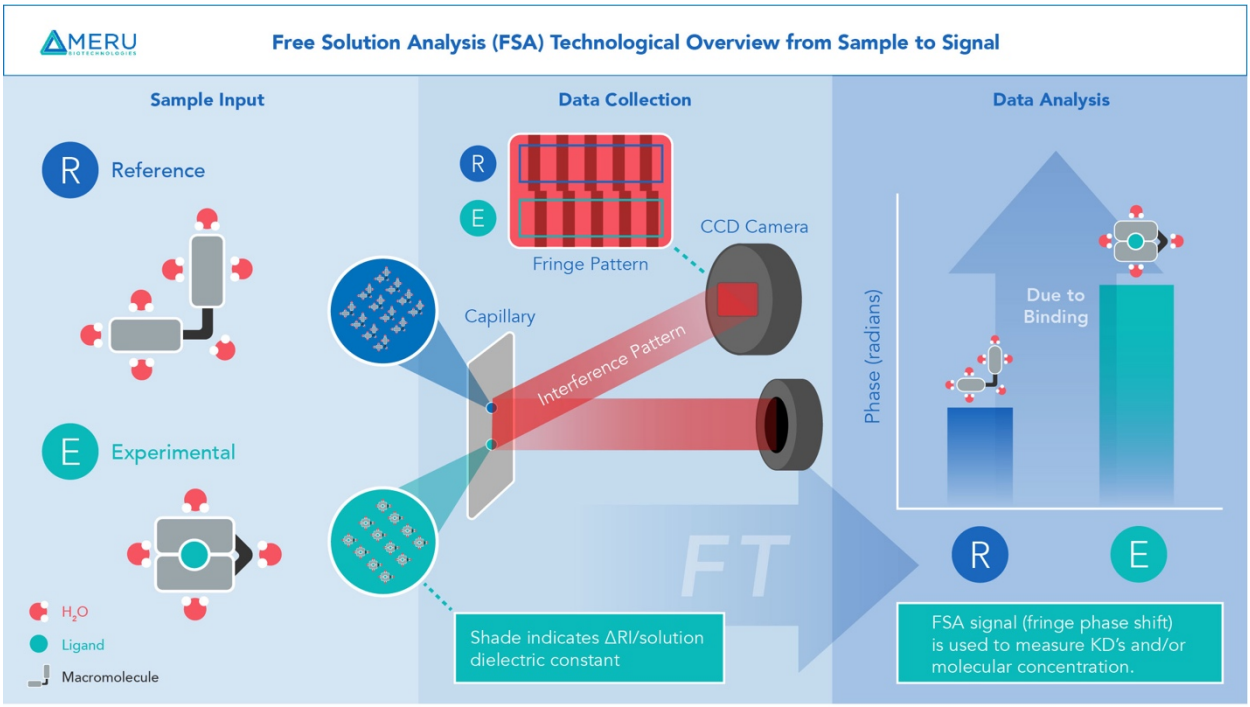

Figure 1: FSA Schematic. *Sample Input:* Two samples in the same solution matrix are loaded into a capillary with the Experimental sample including the binding pair and the Reference sample containing one binding partner. Binding induced solvation, conformational, and electronic structure changes alter solution Refractive Index (RI) and dielectric constant. *Data Collection:* A compensated interferometer detects solution changes through fringe pattern phase shift collected using a CCD camera. *Data Analysis:* Using a Fourier Transform (FT), image data from CCD camera is analyzed to construct FSA signal graphs. Through variations of molecular concentration or calibration curves construction, FSA signal measures dissociation constants (KD) or molecular concentration, respectively.

Key Points

By applying FSA, researchers were able to:

- Collect quantitative affinity values in a wide variety of biological systems
- Publish high-quality research reports in high-impact journals
- Make *in vitro* molecular recognition studies more biologically relevant
- Lower analytic limits of quantification (LOQs), compared to "gold-standard" techniques
- Quantify important low abundance chemical and biological markers from clinical samples to improve diagnosis and toxicology
- Reduce sample and cost requirements for sample analysis

The Challenge of Measuring Native Biomolecular Interactions

Advances in biochemical and biomedical science in recent decades are in large part due to the introduction of new technologies that have opened the doors to a detailed understanding of nanoscale interactions. While identifying and classifying individual molecular contributions to biological function is of critical importance, the ability to measure the interplay between these many contributors to generate an understanding of the system around which life revolves is often more impactful. Through considerations of submicroscopic interactions, we can begin to understand how chemicals behave on human-relevant scales and utilize this information for our collective benefit.

For this reason, foundational biotechnology research, drug pipelines, and diagnostics development all rely on the ability to meaningfully measure molecular recognition of biomacromolecules. However, a persistent challenge for traditional methods remains: Assessing the interaction quantitatively, without perturbing its natural state. Doing so successfully has two immediate benefits. First, it provides an accurate reflection of the native interaction and reduction of assay development time. Along these lines, many existing and commonly used methods also rely on the chemical addition of a label/tag (i.e., fluorescent, enzymatic, radioactive, etc.), non-native binding conditions, and/or unnatural surface immobilization. In each case, recognition nativity is sacrificed to enable or enhance detection, and chemically modified biomolecules must be carefully assessed to ensure that the modification doesn't disproportionately skew experimental results.

Label-Free Methods

The use of molecular recognition methods without chemical labels represent an important step towards better understanding the natural state of biomolecular recognition. Some popular label-free approaches have become widespread in research communities. In recent years, the

use of label-free, solution-based methods, such as **Nuclear Magnetic Resonance** (NMR), **Isothermal Calorimetry** (ITC), and **Mass Spectroscopy** (MS), have grown dramatically. Respectively, they detect binding through energy emission, temperature, and mass. While useful, these methods come with significant drawbacks that can limit their application. Both NMR and ITC lack the sensitivity found in fluorescently labeled systems and often require significant sample quantities.[1] While MS-based methods offer significant sensitivity and can measure multiple analytes simultaneously, its instrumentation is costly, and the data collection/output are often complicated and difficult to deconvolute.[2]

Beyond NMR, ITC, and MS, refractive index (RI), which is a measure of the speed of light as it passes through a specific material, has also been employed to detect binding. As a result, optical analytical methods that detect changes in RI and/or the related solution dielectric constant have become important label-free alternatives to measure molecular recognition.

The most commonly used label-free optical method is Surface Plasmon Resonance (SPR), which has become a primary method for determining the thermodynamic and kinetic properties of binding interactions.[3] While SPR does not require chemical labeling, it requires one of the binding partners to be immobilized on a metal surface. When molecules adsorb or bind to the metal detection surface in SPR, the instrument measures film surface thickness through resulting RI changes that alter the angle of light required to generate surface plasmon wave resonance. Generally, SPR can only measure the interactions between the probe (on the surface) and the target in solution in simple matrices (i.e., containing buffer and a single purified biomolecule).[4] Furthermore, extremely strong affinity interactions (K_D< 1 nM) and those involving small molecules (<180Da) can be very challenging to access using SPR.[3]

Bio-Layer Interferometry (BLI) is another common RI-based method that detects changes in optical thickness of a membrane biolayer. Unlike SPR, interferometry approaches measure changes in RI through superimposing the nascent light wave with a counterpart wave that has passed through a sample of interest. Superimposing these two light waves results in an interference pattern that can indicate changes in RI that result from changes in biolayer optical thickness. Similar to SPR, BLI also relies on surface immobilization of one binding partner and quantifies recognition through the detection of mass added to the surface through binding. Importantly, since mass must be added to the membrane biolayer to detect binding, entrained membrane recognition, where both partners are embedded in the membrane, is not possible through BLI.

While these label-free methods have opened many new avenues of research and fortified our understanding of molecular interactions, they each have shortcomings that can impact their use in medical research and diagnostics. Considering existing label-free methods and their limitations together, the ideal biomolecular recognition analysis technique eliminates *a priori* chemical modification (labels/immobilization), maintains high sensitivity, requires low sample amounts, generates reproducible outcomes, provides quantitative assessments under realistic biological conditions, and allows rapid data collection and analysis.

Meru Biotechnologies' FSA

Meru Biotechnologies' FSA combines the strengths of existing label-free approaches in a straightforward analytical platform, powered by advancements in **Compensated Interferometry,** which uses a single laser to measure experimental and reference samples in the same microfluidic channel simultaneously (Figure 1).[5] FSA's unique signal transduction mechanism, which detects changes in molecular conformation and hydration that in turn alter its dielectric constant and RI (Fig. 1, Sample Input), enables sensitivity that rivals methods that use fluorescence and eliminates the need for surface immobilization strategies needed in SPR and BLI.[1,6] Furthermore, FSA is pair agnostic, does not require mass addition, and can be performed in both complex matrices and entrained membrane receptors. These features collectively add to the biological relevance of FSA data output.

Instead of measuring RI changes due to mass adsorption or optical thickness, FSA measures RI changes in solution that are a direct result of molecular binding. When a chemical pair recognize each other, conformational, molecular dipole/electronic structure, and hydration changes occur that collectively impact a solution's RI (Fig. 1, Sample Input).[1] These RI changes are detected through analysis of high-contrast interference fringe patterns that are produced by a laser interacting with a capillary or channel in a chip (Fig.1, Data Collection). The fringes are collected using a charge-coupled device (CCD) array camera. A select region of interest (ROI), composed of ~7 fringes, that exhibits a single spatial frequency is then analyzed using a Fourier Transform (FT). When the RI/dipole changes following binding, positional shifts in this single spatial frequency (phase change) occur that are proportional to the interaction (Fig. 1, Data Analysis). Using FSA, between 10^{-3} and 10^{-7} RI changes can be reliably detected, when compared with a reference sample lacking one binding partner.[1,5] As a result, FSA permits sensitive recognition of a binding event or detection of an analyte with numerous advantages over existing methods. These advantages include lower LOQs than many best-in-class assay systems and opportunities to study native binding for drug discovery, diagnostic endeavors, and clinical applications.

Using FSA to Determine Physiologically Relevant Drug Binding Affinities and Improve *In Vitro In Vivo* Correlation (IVIVC)

In the drug discovery and development pipeline, determining drug affinity and specificity to a target biomolecule is considered an essential stepping-stone towards clinical testing. Often, affinity assessments rely on the determination of molecular dissociation constants (K_D) between drugs and targets, which generally indicate relative binding "tightness". While K_Ds are used to select lead compounds and guide drug dosing in first-in-human clinical trials, they are not always accurate indicators of affinity within complex *in vivo* environments. Nonetheless, they are one of the best indicators available in pre-clinical studies. Therefore, methods that can improve the biological relevance of this measurement, could improve the predictive power of binding assays or K_D determination.

FSA offers significant benefits in this application, as it doesn't require any chemical modification, and it can be used with complex samples. Collectively, these advantages enable FSA to collect more physiologically relevant K_D's that can drive successful pharmacological research, drug development, and clinical study.

As a recent example, FSA was used to study a monoclonal antibody designed to treat inflammatory bowel disease by binding to human MAdCAM, a protein responsible for mediating lymphocyte rolling/adhesion.[4] It was found that the original K_D, collected using SPR, was 30-fold lower than clinically derived values (16.1 pM vs. 528 pM). Significantly underestimating the therapeutic dose due to poorly correlated K_Ds can undermine drug efficacy, safety and progress towards commercial approval. In this example, FSA was used to investigate the source of the discrepancy and to improve the clinical relevance of *in vitro* K_D collection. It was found that assessing this interaction in the context of minimally diluted serum, as opposed to purified buffer systems in SPR, lead to K_Ds that more closely resembled those found in the clinic (596 pM vs 528 pM).

FSA was also used to investigate antibody recognition of tissue homogenates to assess differences in K_D between soluble and membrane-bound MAdCAM. Taken together, FSA's ability to operate in complex solutions means it can account for natural drug target concentrations and densities, which can significantly impact affinity assessment. Eliminating surface immobilization steps also creates opportunities to study the interaction of both soluble and membrane-bound drug targets, which may contribute differently to disease state and drug

efficacy. Both improvements offered by FSA are untenable in SPR, which likely explains the lack of IVIVC.

Highly Sensitive Measurement of a Blood Serum Biomarker to Improve Lung Cancer Diagnosis

Lung cancer is the greatest individual cause of cancer-related deaths in the US.[7] Low-dose chest computed tomography (CT) scans can reduce mortality in high-risk individuals, but they are costly. Blood-based biomarkers have been proposed as a rapid, low-cost complement to CT screening for early detection. Biomarkers would be especially valuable for tumor diagnosis and monitoring tumor recurrence, where early detection and monitoring remains a challenge. However, current biomarker strategies are not sensitive enough to detect a growing tumor within the early stages of tumor growth.[8] In addition to significant sensitivity, biomarker detection assays also depend on rapid workflows and accessible price points.

Many studies have sought to study cancer pathogenesis through biomarker identification and detection, but these approaches have struggled to translate to the clinic. Furthermore, no report has demonstrated significant improvement in positive predictive value (PPV) and diagnostic likelihood ratio (DLR) values for lung cancer using an individual lung cancer biomarker.[9]

FSA was applied to quantify CYFRA 21-1, a cytokeratin-19 fragment investigated as a potential lung cancer biomarker candidate, in an attempt to improve IPN diagnosis via serum testing.[9] Previous efforts to harness CYFRA 21-1 as an early-stage blood biomarker in clinical practice was limited by insufficient assay sensitivity. However, performing FSA lead to >6-fold improvement in LOQ, compared to the industry-standard CYFRA 21-1 assay (Cobas ECL). Using a commercial anti-CYFRA antibody, CYFRA 21-1 could be detected in spiked patient serum down to 60 pg/mL, at a rate of ~40 experiments per day using sample volumes less than 1 µL.

Next, the researchers sought to analyze 225 blinded serum samples from patients with suspicious lung lesions. With the improved LOQ of FSA, CYFRA 21-1 was measurable in nearly all samples, whereas standard ELISA assays could not accurately quantify biomarker amounts in nearly 100 samples. When combined with the Mayo model of IPN diagnosis, CYFRA 21-1 testing with FSA provided a strong diagnostic benefit over the Mayo model alone, even with smaller tumors. Increases in PPV (99%) and +DLR (41.4) using FSA highlight the method's potential for early detection of lung cancer. In the notoriously difficult field of early, noninvasive

lung cancer diagnosis, FSA led to improved clinical performance and low concentration biomarker detection.

Rapid Quantification and Detection of Low Abundance Chemicals in Human Samples

A longstanding challenge in diagnostics is detecting low abundance chemicals that, despite their low concentrations, may impact human health or act as early indicators of disease. This can become especially challenging if the sample amount is limited. FSA's sensitivity and low sample requirements make it well suited to address this gap, and its fitness is readily demonstrated in two recent studies in areas of high importance: nerve agent and neonatal opioid exposure.

In both cases, FSA and the compensated interferometry were combined with nucleic acid aptamer probes. Aptamer probes are an excellent fit for FSA as binding to their targets leads to more dramatic conformational and hydration changes compared to proteins, which results in excellent signal-to-noise.

Chemical Nerve Agents

While a number of methods can detect high exposure to chemical nerve agents, low exposure detection remains a challenge. While they may initially produce no detectable symptoms, low-dose chronic exposure to organophosphorus nerve agents (OPNAs) can ultimately lead to peripheral nervous and respiratory system damage as well as depression and cognitive impairment. MS can be used to detect low levels of OPNAs; however, the method is not field deployable or rapid, which is critical for testing real-time exposure to chemical weapons and pollutants. To address this challenge, FSA was used to select specific high-affinity aptamer probes against two different OPNAs (VX and Sarin).[10] This combination of FSA and aptamer probes led to OPNA detection limits 2-to-3 orders of magnitude below MS methods and accurate quantification of nerve agent concentrations from spiked serum samples.

Neonatal Opioid Dependence

Infants are often overlooked victims of the current opioid epidemic. Those born to mothers addicted to opioids can suffer from neonatal abstinence syndrome (NAS). Diagnosing this condition rapidly is critical, but the tests are often difficult to perform at hospitals and clinics with limited resources. Standard testing requires the use of liquid chromatography-MS/MS (LC-MS/MS), which requires >100 µL sample volumes, significant set-up time and operational costs. This limits the application of MS in this setting, especially for premature babies that produce even less urine than full-term neonates.

FSA's minimal sample requirement (~5 µL) and fast data output (~2 hrs) make it a natural fit to meet this important need. FSA and compensated interferometry were used to identify aptamer probes against opioids and major urinary metabolites.[2] Once identified, these probes enabled 5- to 275-fold LOQ improvement relative to LC-MS/MS, at a fraction of the cost. In addition, the improved assay sensitivity creates opportunities to quantify opioids at earlier time points and over longer periods of time, drastically improving the capacity to monitor patient status.

FSA Provides Biologically Relevant Binding Affinities and Improved Sensitivity

The development of our improved compensated interferometer makes FSA a distinct analytical offering that eliminates all of the fundamental limitations of existing standard-practice methods. Without labels and immobilization, binding interactions can be assessed meaningfully in a native environment, regardless of solution complexity, to improve clinical applicability. Enhancing reproducible detection sensitivity allows its users to assess important molecular concentrations beyond the reach of other label-free methods. Finally, vastly reduced sample requirements and straightforward operation make FSA and compensated interferometers ideal for field and resource limited applications.

Though not discussed here, FSA also holds enormous opportunity for other fields, like membrane-ligand,[11] host-pathogen,[12] and carbohydrate recognition studies[1,5] to name a few. The potential of this system is vast, and it continues to expand as science and medicine become more sophisticated. Meru Biotechnologies' FSA can help researchers summit nearly any challenging biomolecular recognition experimentation.

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