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Instrument design and optimization of interferometric reflectance imaging sensors for in vitro diagnostics

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INSTRUMENT DESIGN AND OPTIMIZATION OF
INTERFEROMETRIC REFLECTANCE IMAGING SENSORS
FOR IN VITRO DIAGNOSTICS

by

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I dedicate this to my loving parents, Cheryl and Paul, my competitive siblings Michelle and Anthony, and my grandparents for all their affection and support.

A special dedication to my late great grandma, Emma Elizabeth Shively (1907–2013), and to my late grandpa, Paul White (1940–2013), whose support and laughter will never be forgotten.
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INSTRUMENT DESIGN AND OPTIMIZATION OF INTERFEROMETRIC REFLECTANCE IMAGING SENSORS FOR IN VITRO DIAGNOSTICS

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ABSTRACT

In the field of drug discovery and disease diagnostics, protein microarrays have generated much enthusiasm for their high-throughput monitoring of biomarkers; however, this technology has yet to translate from research laboratories to commercialization. The hindrance is the considerable uncertainty and skepticism regarding data obtained. The disparity in results from different laboratories performing identical tests is attributed to a lack of assay quality control. Unlike DNA microarrays, protein microarrays have a higher level of bioreceptor immobilization variability and non-specific binding because of the more complex molecular structure and broader physiochemical properties. Traditional assay detection modalities, such as fluorescence microscopy and surface plasmon resonance, are unable to overcome both of these sources of variation.

This dissertation describes the hardware and software design and biological validation of three complementary platforms that overcome bioreceptor variability and non-specific binding for diagnostics. In order to quantify the bioreceptor quality, a label-free, non-destructive, low cost, and high-throughput interferometric sensor has been developed as a quality control tool. The quality control tool was combined with a wide-field vi
fluorescence imaging system to improve fluorescence experimental repeatability. Lastly, a novel high-throughput and label-free platform for quality control and specific protein microarray detection is described. This platform overcomes the additional complexities and time required with labeled assays by discriminating between specific and non-specific detection by including sizing of individual binding events.

Protein microarrays may one day emerge as routine clinical laboratory tests; however, it is important that the proper quality control procedures are in place to minimize erroneous results. These platforms provide reliable and repeatable protein microarray measurements for new advancements in disease diagnostics with the potential for drug discovery.
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LIST OF ABBREVIATIONS

AFM  Atomic force microscopy
ASR  Angular spectrum representation
BOE  Buffered oxide etchant 6:1
BSA  Bovine serum albumin
CaFE Calibrated fluorescence enhancement
CaSP Calibrated single particle
CLC  Co-located calibration
CMOS Complementary metal-oxide semiconductor
DAQ  Data Acquisition Device
DI   Deionized water
ELISA Enzyme-linked immunosorbent assay
FBS  Fetal Bovine Serum
FET  Field-effect transistor
FOV  Field-of-view
FWC  Full well capacity
GUI  Graphical User Interface
HSA  Human serum albumin
HMDS Hexamethyldisilazane
IgE  Immunoglobulin E
IgG  Immunoglobulin G
IRIS Interferometric reflectance imaging sensor
LED  Light emitting diode
LOD  Limit of detection
NA   Numerical Aperture
ONC  On-chip calibration
PBS  Phosphate buffered saline
PBST PBS with Tween 20
PD   Photodetector
PFU  plaque-forming units

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<table>
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<th>Abbreviation</th>
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<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
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<tr>
<td>POC</td>
<td>Point-of-care</td>
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<td>PSF</td>
<td>Point spread function</td>
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<tr>
<td>RAM</td>
<td>Random access memory</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>RP</td>
<td>Reference particle</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SIFT</td>
<td>Scale invariant feature transform</td>
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<tr>
<td>SIMD</td>
<td>Single instruction, multiple data</td>
</tr>
<tr>
<td>SiO&lt;sub&gt;2&lt;/sub&gt;/Si</td>
<td>Silicon dioxide on silicon</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>SP-IRIS</td>
<td>Single particle interferometric reflectance imaging sensor</td>
</tr>
<tr>
<td>SRIB</td>
<td>Spectral reflectance imaging biosensor</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate</td>
</tr>
<tr>
<td>SSE</td>
<td>Sum of squared errors</td>
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<tr>
<td>TE</td>
<td>Transverse electric</td>
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<tr>
<td>TM</td>
<td>Transverse magnetic</td>
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<tr>
<td>Tris</td>
<td>Trisminomethane</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polysorbate 20</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>wtVSV</td>
<td>Wild-type Vesicular Stomatitis Virus</td>
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Chapter 1

INTRODUCTION & BACKGROUND

This dissertation describes the design and characterization of three instruments (the interferometric reflectance imaging sensor (IRIS), the calibrated fluorescence enhancement (CaFE), and the single particle interferometric reflectance imaging sensor (SP-IRIS)) for high-throughput, sensitive, and repeatable protein microarray assay detection and quality control. These platforms are compact, low-cost, and non-destructive quality control tools [1], [2]. Moreover, the platforms discussed reduce operational complexity and user-variability through automation. The user-friendly nature of these tools has facilitated the distribution to our collaborative laboratories at the Istituto di Chimica del Riconoscimento Molecolare, (Milano, Italy) for assay development and the Microbiology Dept., (Boston University, Boston, MA) for viral hemorrhagic fever and allergy diagnostics [3]–[6].

Chapter 1 will give a general description of biosensors and a detailed discussion on the current and emerging technologies in multiplexed protein detection. In Chapter 2, the underlying principles of IRIS are described along with design considerations and the development of the 2nd generation IRIS instrument. The design of the two CaFE chips and the CaFE instrument are discussed in Chapter 3. Chapter 4 discusses the rationale for SP-IRIS, the theory behind nanoparticle sizing for specific detection, and the development of an automated, standalone prototype. Finally, Chapter 5 contains comments for further research and conclusive remarks for further developing these technologies.
1.1 Introduction

The complex binding relationships of biological molecules drives the operation of living organisms. System irregularities, such as disease symptoms, occur when the balance of the molecules is interrupted through the introduction of foreign particles or perturbation in functionality or concentration of biomarkers. Although several diseases are detectable through profiling DNA biomarkers, DNA does not directly indicate cellular activity. Measuring the relative or absolute protein concentration offers a more complete understanding of the disease pathogenesis for diagnosis and treatment [7], [8].

Although many microarray detection platforms have been demonstrated for massively multiplexed (tens of thousands of ligands) and high sensitivity measurements (picogram/mL to nanogram/mL concentrations) in pure solutions spiked with protein biomarkers, their practicality in clinical protein samples like whole blood or sputum has been hindered because of low repeatability and high noise floors [9], [10]. The repeatability is affected by the physicochemical variation between proteins that induces fluctuation in the immobilized probe density [11], [12]. A high noise floor arises from false signal due to non-specific binding in clinical samples, where other biomolecules are present in concentrations a billion times greater than the target biomarkers [13]. Current detection methods are able to overcome probe immobilization variation or non-specific binding but not both.

To address probe immobilization variability, the IRIS platform has been developed using optical interferometry and spectroscopy to quantify mass accumulation on a silicon dioxide on silicon (SiO₂/Si) chip. The 1st generation IRIS design provided rapid label-free
measurements of thousands of proteins simultaneously with an intrinsic mass detection limit of 4 pg/mm² [14]. Refining this design, the 2nd generation platform improves the ease-of-use, the footprint, the robustness, and the cost ($30K to $10K) without sacrificing throughput or sensitivity [1]. The new IRIS design implements light-emitting diodes (LEDs) as the illumination source, a new SiO₂/Si design to account for the LEDs’ spectral range, a novel IRIS analytical model for the LEDs’ linewidth, and an on-chip intensity monitoring region to eliminate the need for an external photodetector (PD) [15].

The CaFE platform integrates IRIS to quantify probe immobilization with wide-field fluorescence imaging for discrimination between specific and non-specific detection. This novel microarray technique has been shown, using separate IRIS and fluorescence instruments, to correct for inter-experimental error from disparity in probe density [2]. For this technique, two SiO₂/Si chips have been designed: 1) a chip with two SiO₂ thicknesses optimized for fluorescence enhancement and IRIS measurements for on-chip calibration (ONC) and 2) a single SiO₂ design for same spot fluorescence and IRIS measurements at reduced performance for co-located calibration (CLC) [2]. The ONC chip design calibrates the fluorescence signal with the immobilization density of nearby probes. This design addresses inter-chip variation. For further calibration accuracy, the CLC chip design can be used for same spot calibration to account for inter- and intra-probe variation. Lastly, an integrated IRIS and wide-field fluorescence platform has been designed for all-inclusive CaFE measurements [16]. The optical path design has been designed for sensitive fluorescence detection, a novel IRIS angular spectrum representation (ASR) analytical model has been developed for high numerical aperture
IRIS imaging, and the platform has shown equivalent performance to the separate IRIS and fluorescence readers.

The final platform, SP-IRIS, overcomes the drawbacks of labeling (e.g. labels can affect protein functionality [17], labeled assays require more time and reagents, and the labeled reagents must be stored in a dark, refrigerated environment) with a label-free single nanoparticle method for non-specific binding filtering. Coupling this platform with IRIS provides a completely label-free approach to quantifying probe immobilization and specific detection. The SP-IRIS technique detects single nanoparticles on an interferometric surface using a CCD camera. By analyzing the strength of individual nanoparticles, the nanoparticle size can be determined for non-specific size filtering. The drawback to SP-IRIS is the nanoparticle signal is tightly confined in the focusing axis. In order to remove user focusing error, a standalone, portable, and automated SP-IRIS platform has been designed. A custom graphical user interface (GUI) synchronizes the stage, camera, and LED control for automated data acquisition and processing. With this software in place, the user interaction is confined to loading the chip, defining the microarray geometry, and clicking run.

1.2 Biological Sensors

Known as molecular diagnostics, the observation of events on a molecular level has opened avenues for new and more effective medical treatments [18]. In order to detect and quantify molecular interactions, scientists and engineers have developed biosensors for numerous applications. At a basic level, biosensors consist of a bioreceptor and a transduction device.
Bioreceptors are molecules, such as proteins, enzymes, antibodies, and nucleic acids, capable of recognizing other molecules through specific interactions. These molecules are designed to interact with a specific target molecule, similar to a lock and key. For example, our bodies operate on a series of metabolic processes. In order to fuel our bodies, we eat food. This food is broken down into small molecules through steps called catabolism. The anabolism process then uses these small molecules to make proteins and nucleic acids for our body. These reactions are able to occur because of enzymes, a type of bioreceptor. Biosensors exploit the recognition behavior of bioreceptors to specifically identify target molecules.

Once the bioreceptor captures the target molecules, the binding event must be detected. The detection method depends on the transduction type. A transduction device measures the physical or chemical change due to target-receptor binding event. As illustrated in Figure 1.1, the transduction method can be mechanical, electrical, or optical in nature.

![Figure 1.1: Biosensor schematic. The bioreceptors recognize their specific target molecule. When the target is present, they bind to the bioreceptors producing a physical or chemical change which is changed into a measurable signal.](image)

Biosensors are separated into several general categories based on (1) if the
bioreceptors must be affixed to a surface or are floating in-solution, (2) whether the target molecules are detected directly (label-free) or through secondary molecules (labeled), and (3) the transduction method for detection. This research will focus on detection modalities that take advantage of fixing the bioreceptors to a surface for multiplexed applications.

1.3 Detection Formats

Today’s biosensors have progressed in prevalence as well as sophistication and complexity. According to a market study released by GlobalData, the blood glucose monitoring device market in 2010 was valued at $8.9B world-wide with a compound annual growth rate of 5.2% since 2003 [19]. Although the USA has 40% of this market, much of the growth going forward will stem from expanding to emerging markets who cannot afford the current technology [20]. Therefore, not only do emerging medical technologies need to continue to improve performance, but costs and ease-of-use must also be considered for the emerging markets.

1.3.1 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISA) are the most dominant format for high-throughput, singleplex assays used today. These assays detect antibodies or antigens by non-specifically immobilizing the patient sample inside a well of a microtiter plate with 96-, 384-, or 1536-wells via adsorption or specifically using a bioreceptor on the surface. (A 96-well plate is shown in Figure 1.2a.) After the target molecule is captured, a secondary bioreceptor is added to each well to “sandwich” the target. Finally, a general
bioreceptor with a linked enzyme is attached. The enzyme reacts with a chromogenic substrate\(^1\) to induce a color change in the chromogenic substrate for detection. Figure 1.2b shows a schematic of this process.

![Figure 1.2: Process flow of an ELISA assay. (a) Schematic of a 96-well microtiter plate. Each well runs singleplex (1 target/well) detection. The multiple-well configuration enables a higher throughput and multiplexing. (b) In each well, the probes are adhered to the detection surface for specific capture of the target (step 1). Step 2 shows the target molecule is captured by the probe bioreceptor. In step 3, a secondary bioreceptor is bound to the target molecule to act as an adapter between the target molecule and the detection bioreceptor. Step 4 is the attachment of a tagged bioreceptor to the secondary bioreceptor. Step 5 shows the enzyme interacting with the substrate to induce a color change in the substrate for detection. Step 1 is skipped for protocols that non-specifically adsorb all molecules to the surface.]

At first glance, the use of a secondary bioreceptor and an enzyme-linked bioreceptor may seem redundant. If the indicator molecule is bound directly to the secondary bioreceptor instead of a tertiary bioreceptor the number of steps, the amount of reagents, and the assay time would be reduced. However, there are thousands of biomarkers detected using ELISAs and each biomarker has a corresponding probe receptor and secondary bioreceptor. The task of chemically linking an enzyme to each secondary bioreceptor while maintaining functionality is complicated and laborious. Instead, a tertiary bioreceptor, which is designed to recognize multiple secondary bioreceptors, is linked to the enzyme.

\(^1\) Molecules which undergoes a color change in the presence of an enzyme
1.3.2 Microarray-based Biosensors

The progressive demand for high-throughput and multiplexed detection of biomarkers has sped up the development of a large variety of biosensors [8]. A particularly promising high-throughput and multiplexed biosensor format is known as microarrays. Introduced in the early 1980s [21], [22], microarrays have grown in popularity by providing multiple, parallel protein and nucleic acid measurements with the same sample volume. Similar to ELISAs, microarrays can employ primary, secondary, and tertiary bioreceptors and a solid surface to bind and recognize target molecules. Unlike ELISA, microarrays do not physically separate the bioreceptors in wells, they are not limited to antigens and antibodies, and they are not limited to enzymatic reactions for detection. The probes are adhered to a solid surface (usually a glass slide) in a 2D grid (typically 100 μm diameter bioreceptor spots with a 200 μm center-to-center pitch), see Figure 1.3. This configuration allows a single sample volume to be tested for tens of thousands of target molecules concurrently.
Figure 1.3: Illustrative sketch of a microarray. Each disk represents a cluster of probes adhered to the surface. The multiple colors mimic the use of different bioreceptors to specifically capture target molecules 1, 2, and 3. The co-poly(DMA-NAS-MAPS) surface chemistry used in our experiments has shown bioreceptor densities of about $2 \times 10^{11}$ molecules/mm$^2$ [23], [24]. The exact bioreceptor density can be measured with IRIS.

1.4 Current State of Biosensors

Molecular biosensors have been designed to measure electrical [25], [26], mechanical [9], [27], and optical [28]–[32] fluctuations. Since the 1980s, numerous biosensors have been developed and characterized in these fields, shown in Figure 1.4 and Figure 1.5. These biosensors can be broken into two main groups: a) labeled detection and b) label-free detection. Understanding the strength of each approach is essential to developing molecular diagnostics.
1.4.1 Labeled Versus Label-Free Detection

Biosensors of every variety have been developed for labeled and label-free detection. Whether electrical, mechanical, or optical in nature, the biosensor is reading a chemical or physical signal in the local environment. The transduction signal can come from either the target molecule directly (label-free) or an indicator molecule (labeled).

Labeled microarrays are the first clinically adopted biosensors. These microarrays
follow the same procedure as an ELISA. The key advantage of this approach is signal specificity. Unless the secondary bioreceptor binds to the target molecule and then the tertiary bioreceptor binds to the secondary bioreceptor, a signal will not be generated. The redundancy lessens the chance of signal generation from non-specific binding (unintended attachment of non-target molecules). Consequently, the drawback to this added specificity is assay complexity (bioreceptors must be selected to prevent crosstalk, especially when multiplexing) and assay time (multiple incubation steps). In most tests, each incubation step requires 1 hour, but this time depends on the binding affinity, molecule concentration, molecular diffusion rate, and detection sensitivity. Therefore, in general, labeled detection is not applicable to time critical situations or where assay complexity is an issue.

Label-free detection is a simpler assay that does not require additional incubation steps to label the target molecules. Observation of target binding is directly measured through an electrical, mechanical, or optical material property. In buffer solutions, label-free techniques have shown competitive sensitivities in a reduced number of steps by eliminating the secondary and tertiary bioreceptors [14]; however, removing the additional binding steps sacrifices selectivity [33]. The discrimination between specific and non-specific binding is no longer improved by the binding affinities of the secondary and tertiary bioreceptors. This lack of specificity becomes apparent in a complex solution, such as serum or whole blood, where non-specific binding, not the detection technique, limits the sensitivity [34], [35]. To compete with labeled detection in clinically-relevant solutions, label-free detection techniques must either implement
complex sample purification procedures [36] or detect and discriminate binding label-free, discussed in Chapter 4.

1.4.2 Transduction Types

**Electrical Biosensors.** Electrical biosensors operate on the concept of measuring change in conductance to detect molecular binding [25], [26], [37]. The two common types of readout modalities are label-free with field-effect transistors (FETs) for charged target molecules [26], [38] or labeled detection using interdigitated electrodes – an addressable comb-like electrode structure – and an enzymatic reaction [25], [37]. In both cases, a sensing region is functionalized with the primary bioreceptor, as shown in Figure 1.6. When the target molecule binds to the sensing region, the local conductance is changed directly or through an enzymatic reaction with a substrate that produces a chemical product.

![Diagram of a basic electrical biosensor](image)

Figure 1.6: Diagram of a basic electrical biosensor. Binding of the target protein induces a change in the local conductance directly or through an enzymatic reaction. Reprinted from [39].
Nanowire sensors have shown real-time measurement of femtomolar to nanomolar protein concentrations using carbon or silicon nanowires [8]. For example, traditional FET configurations have shown the capability of Dengue fever serotyping with peptide nucleic acids on silicon nanowires [40]. Silicon nanowires have also been applied to DNA and protein detection with attomolar to nanomolar sensitivity [41]. Sarkar et al. suggested a new FET scheme, termed impact-ionization FET, can increase sensitivity an additional four orders of magnitude [26].

Scaling-up of fabrication to high-volume manufacturing is the major limitation of this technology. Current top-down and bottom-up nanowire fabrication methods require the nanowires and electrodes to be fabricated separately then assembled. This fabrication process has low device yield and is incompatible with large-scale semiconductor device processes [36], [42], [43]. Recently, Shalev et al. addressed the manufacturing issue with the introduction of an electrostatically-formed nanowire. Their approach to nanowire sensing demonstrated a 10 pg/mL (~340 fM) detection limit of cardiac troponin on a sensor fabricated using standard complementary metal-oxide semiconductor (CMOS) practices, including thermal diffusion doping, low-pressure chemical vapor deposition, plasma-enhanced chemical vapor deposition, and sputtering [44].

Nanowire sensors have shown great promise as real-time, portable measurement tools for the label-free detection of charged molecules. As innovations in the fabrication procedures and sensor design continue, the non-standard fabrication processes that decrease device yield and performance can be replaced, enabling high-density arrays. The sensitivity of nanowires, however, shows the same limitation as many other label-free
technologies – the specificity of detection relies solely on the probe affinity to the target molecule. For clinical applications, sample purification steps will be required to minimize non-specific binding for practical use of nanowires.

**Nano-mechanical Biosensors.** With advancements in nanofabrication techniques, mechanical transducers with deformable features are used for mass sensing. Nano-mechanical type biosensors rely on two types of changes when molecules bind to immobilized bioreceptors: physical displacement and resonance frequency shifts. Nano-mechanical systems function with nanometer-sized mechanically resonant structures, like graphene sheets, free-standing cantilevers, doubly clamped beams, or membranes, [27].

In the case of nanocantilever biosensors, the stress-induced on the surface from binding causes a bending of the cantilever arm and shifts the resonance frequency proportionally [45], as shown in Figure 1.7. This approach to mass sensing has demonstrated 1 ng/mL detection of cyanovirin-N, a carbohydrate-protein [46] and single cell growth with femtogram sensitivity [47]. Although nano-mechanical biosensors are theorized to detect zeptogram mass changes – the mass of a single protein – with double-wall carbon nanowires [48] or even yoctogram mass changes with graphene sheets [49], experimentally there has not been a demonstration beyond 1 ng/mL in biological experiments. As noted by Arlett et al., the detector performance of these sensors is limited in biological samples by non-specific binding effects and not the intrinsic biosensor performance [13].
Figure 1.7: Operation modes of nano-mechanical biosensors. As biomolecules bind to the cantilever arm, the stress applied to the surface induces a bending. (a) Static mode measures the arm-displacement to infer the amount of stress applied, which correlates to mass. (b) Dynamic mode monitors the resonant frequency and the corresponding shifts as mass attaches. Reprinted from [27]

**Optical Biosensors.** As shown in Figure 1.4, research on optical transduction methods exceeds all other techniques. The popularity of optical devices comes from the diversity of properties available for detection. Fluorescence sensors [50], high-Q optical resonators [51], [52], photonic crystal sensors [53], [54], and plasmonic sensors [31], [55] are just a few of the optical methods employed in biological experiments.
Fluorescence assays are the industry standard for clinical DNA microarrays [56], [57]. This detection method is a labeled technology that relies on a secondary bioreceptor linked to a fluorophore to achieve a high sensitivity and selectivity. Fluorophores are chemical compounds that absorb photons and re-emit lower energy photons. The Stokes shift\(^2\) between the absorption and emission photons enables spectral filtering of non-signal wavelengths for zeroing the background. Despite spectral filtering, the background is not truly eliminated. Background signal is still detected from fluorophores non-

\(^2\) Spectral shift between the absorbed photon and emitted photon
specifically bound and autofluorescent molecules\textsuperscript{3}. In order to increase the fidelity of measurements, research has concentrated on enhancing sensitivity through time-gate detection \cite{58}, total internal reflection fluorescence \cite{59}, Förster resonance energy transfer \cite{60}, incorporating interferometric surfaces for a higher photon collection efficiency \cite{61}, incorporating photonic crystal structures for a higher photon emission and collection efficiency \cite{28}, \cite{53}, and/or physically separating the fluorophores to prevent cross-talk in multiplexed conditions \cite{50}.

Although many techniques have been developed to enhance fluorescence detection, fluorescence imaging is not easily applicable to multiplexed protein assays. As mentioned in Section 1.3.1, labeled assays are a multi-stage process with multiple bioreceptors. For each target protein in the multiplexed assay, the primary and secondary bioreceptors must be selected to prevent cross-reactivity. The direct binding of the secondary or tertiary bioreceptors to the primary bioreceptors will result in false signals. Also, additional complexity is introduced in labeling the bioreceptors with a fluorophore without altering the molecular functionality \cite{17}. As an alternative, research has progressed on the development of multiplexed protein biosensors that do not require labeling of the bioreceptor.

Label-free detection modalities directly measure the target molecule binding to the probes. Previously, the label-free detection methods were discussed using local conductance shifts (nanowire biosensors), and mass changes (nanocantilever biosensors). In optical biosensors, the transduction method is in reflection from thin-film interference

\textsuperscript{3} Materials that naturally emit photons when illuminated
local refractive index changes on resonant structures (i.e. cavities, toroid, and spheres) and local refractive index changes in surface plasmon resonance. These sensors compared to other label-free technologies have shown competitive sensitivities (e.g. an intrinsic sensitivity of a single particle and femtomolar detection of BSA) in both single-point and imaging modalities, a higher level of throughput (1000s of measurements simultaneously), multiplexing capabilities, and potential for specific and non-specific discrimination.

In conclusion, the current state-of-the-art optical biosensors are divided between labeled and label-free detection modalities. Whether mechanical, electrical, or optical, these techniques have not achieved their intrinsic detection limit. The limiting noise for these biosensors is either non-specific binding or probe immobilization density. Labeled detection is able to overcome non-specific binding with additional bioreceptors and labeling procedures; however, since a measurable signal is not generated until the label is present no information is provided about the probe immobilization density. Label-free methods are able to simplify the assay by forgoing any additional bioreceptors and incubation steps, but at the cost of specificity. This dissertation presents multiple approaches to surpass the limitation of labeled and label-free techniques through a hybrid label-free/label detection method and a novel label-free method capable of discriminating between specific and non-specific binding with nanoparticle sizing.
Chapter 2

PRINCIPLES OF THE INTERFEROMETRIC REFLECTANCE IMAGING SENSOR

The incorporation of protein microarrays in clinical diagnostics has been limited by a lack of accurate and precise biosensors for clinical samples and quality control tools. The IRIS platform has been designed to provide rapid, multiplexed, and sensitive probe and biomarker quantification in an easy-to-use and low-cost system. The drawbacks of other detection technologies is their intricate design of sensors and instrumentation that prevents direct translation to commercialization [68], [69]. The IRIS technique has been developed with practically of commercial and clinical scalability in mind. This chapter will discuss the underlying principles of IRIS, layout considerations for an IRIS platform, the 1st and 2nd generation designs, and validation procedures.

2.1 Basic IRIS Principles

IRIS is a spectroscopic imaging technique for measuring biomass accumulation. Utilizing optical interference from a SiO$_2$/Si chip, this technique is able to calculate the optical path difference induced by the SiO$_2$ layer. By imaging the spectral response of select wavelengths, minute amounts of mass binding to the surface can be quantified in a high-throughput format.

The reflection and transmission of light at a single dielectric interface were first described by Augustin-Jean Fresnel in 1827. The Fresnel equations explain the behavior of transverse electric (TE) waves, s-polarized, and transverse magnetic (TM) waves, p-polarized, incident to a planar dielectric interface. The ratio of the incident and reflected
fields, called the Fresnel coefficients, \( r_s \) and \( r_p \), shown in Equations 2.1 and 2.2, are derived by imposing continuity at the interface [70].

\[
\begin{align*}
  r_s (\lambda, \theta_i) &= \frac{n_1(\lambda) \cos \theta_i - n_2(\lambda) \cos \theta_t}{n_1(\lambda) \cos \theta_i + n_2(\lambda) \cos \theta_t}, \\
  r_p (\lambda, \theta_i) &= \frac{n_1(\lambda) \cos \theta_i - n_2(\lambda) \cos \theta_t}{n_1(\lambda) \cos \theta_i + n_2(\lambda) \cos \theta_t},
\end{align*}
\]

(2.1)  

(2.2)

\[
R_p (\lambda, \theta_i) = |r_p (\lambda, \theta_i)|^2,
\]

(2.3)

\[
R_s (\lambda, \theta_i) = |r_s (\lambda, \theta_i)|^2.
\]

(2.4)

Where \( \theta_i \) and \( \theta_t \) are the incident and transmitted angles, \( n_1 \) and \( n_2 \) are the indices of refraction of the two materials, and \( R_s \) and \( R_p \) are the total reflected intensities. For unpolarized light, the total reflected intensity is an average of the polarizations,

\[
R(\lambda, \theta_i) = \frac{1}{2} (R_s (\lambda, \theta_i) + R_p (\lambda, \theta_i)).
\]

(2.5)

For the special case of near-normal incident, the Fresnel coefficients can be further simplified using a small-angle approximation, called the paraxial approximation. As seen in Equation 2.6 and 2.7, applying this approximation to the Fresnel equations, 

\[
\sin \theta_i = \theta_i = 0,
\]

removes the polarization-dependent behavior of the reflection.

\[
\begin{align*}
  r_s (\lambda, \theta_i = 0) &= r_p (\lambda, \theta_i = 0) = \frac{n_1(\lambda) - n_2(\lambda)}{n_1(\lambda) + n_2(\lambda)}, \\
  R_s (\lambda) &= R_p (\lambda) = \left(\frac{n_1(\lambda) - n_2(\lambda)}{n_1(\lambda) + n_2(\lambda)}\right)^2,
\end{align*}
\]

(2.6)  

(2.7)

In the case of a SiO2/Si chip, the reflection model includes the optical resonance from
the SiO₂ cavity. As illustrated in Figure 2.1, the incident light to the Air-SiO₂-Si stack contains two main reflections, the reflection from the Air-SiO₂ interface and the aggregate reflection from the SiO₂/Si interface. These two reflections interfere producing the measured far-field signal. Note the light that is transmitted into the Si layer is not modeled as it will be absorbed.

![Reflection model of an Air-SiO₂-Si configuration](image)

Figure 2.1: Reflection model of an Air-SiO₂-Si configuration

The close-form equation of the 3-layer chip reflection, shown in Equation 2.8, is derived using geometric optics [70]. This model has the assumptions of near-normal incidence and a narrow linewidth illumination source.

\[
R(\lambda) = \frac{r_1^2 + r_2^2 + 2r_1 r_2 \cos(2k_z d)}{1 + r_1^2 r_2^2 + 2r_1 r_2 \cos(2k_z d)},
\]

(2.8)

\[
r_1(\lambda) = \frac{n_1(\lambda) - n_2(\lambda)}{n_1(\lambda) + n_2(\lambda)},
\]

(2.9)

\[
r_2(\lambda) = \frac{n_2(\lambda) - n_3(\lambda)}{n_2(\lambda) + n_3(\lambda)},
\]

(2.10)

\[
k_z(\lambda) = \frac{2m_2(\lambda)}{\lambda},
\]

(2.11)

Where \( r_1 \) and \( r_2 \) are the Fresnel reflection coefficients of the Air-SiO₂ interface and
SiO2-Si interface, $d$ is the thickness of SiO2 layer, and $k_z$ is the wavenumber in SiO2 layer perpendicular to the interface.

IRIS utilizes the Air-SiO2-Si analytical model and acquired spectral data to extract the SiO2 layer thickness and the surface bound density of adsorbed biomass. In the analytical model, all the variables are well defined except the SiO2 thickness, $d$. Using an error minimization algorithm, such as the Levenberg-Marquardt algorithm, the SiO2 layer thickness and the spectral data are determined for each pixel in the image. As biomass accumulates on the surface, it induces an additional phase delay in the optical signal. Treating the amassing of biomass on the SiO2 layer as a growth or increase in the SiO2 thickness with a constant refractive index, the IRIS method is able to measure mass buildup with 4 pg/mm² sensitivity [14]. To quantify the amount of mass bound to the surface, a conversion factor from effective SiO2 thickness growth to biomass density has been experimentally established [71].

2.2 Instrument Design

The key components in designing an IRIS are the chip structure, optical path, camera selection, illumination source, and software interface. These items affect the system sensitivity, footprint, throughput, and cost.

2.2.1 Chip Design & Illumination Source Selection

The IRIS platform spectrally monitors biomass binding to a surface through spectral illumination of a chip. The structural design of the chip (material and selected thickness) drives the affordability, sensitivity, and robustness of the IRIS platform. Material
selection was made with compatibility to high-volume manufacturing processes, existing surface chemistries, model complexity, and chemical inertness. Moreover, the spectral signature and the resulting phase shifts from biomass binding must be predictable for sampling by the illumination source.

The sample design for IRIS has been selected to be a SiO₂/Si design in order to produce a distinctive, predictable spectral signature. SiO₂/Si wafers are a standard configuration in the integrated circuits industry for CMOS technology. The wide-availability and demand from the semiconductor market have driven down fabrication costs, established fabrication protocols, and high-volume manufacturing. The SiO₂ layer has a similar index of refraction to the biomolecules enabling the 4-layer system to be modeled as a 3-layer system with a conversion factor, 1 nm of SiO₂ for 1.28 ng/mm² of bound BSA [71]. Simplifying the analytical model complexity in this way reduces the processing time by 3-fold (0.035 s to 0.01 s per calculation). The final advantage of SiO₂/Si chips is this configuration is compatible with common glass chemistries for surface functionalization [72].

With the chip configuration, the SiO₂ layer thickness must be engineered to optimize sensitivity and dynamic range of the IRIS platform. This thickness of the SiO₂ layer is also designed based upon with the spectral range and linewidth of the illumination source. The thickness of the SiO₂ layer sets the frequency of oscillation in the reflectance curve. The illumination source must be able to accurately sample the reflectance and detect the phase shifts from binding for sensitive mass measurements.

The light source of the IRIS platform provides selective and robust multi-spectral
illumination of the chip surface. Wavelength selectivity has the following criteria to ensure the reflectance signal is properly sampled over a large range of SiO₂ thicknesses.

1) The sampled spectral range includes at least half of a reflectance period.
2) The spectral sampling rate satisfies the Nyquist rate
3) At least 3 spectral wavelengths are sampled

Not adhering to these criteria will result in a higher intrinsic noise floor from the diminished accuracy and precision. Spectrally sampling less than half a reflectance curve period diminishes the operational range of the platform. Undersampling the reflectance curve at the Nyquist rate leads to a SiO₂ thickness dependent error. When fabricating a SiO₂/Si wafer, the standard protocol is the SiO₂ thickness will be within 5% of the target thickness. The smaller operational range resulting from not following these criteria, places a tighter constraint on the manufacturing process that will greatly affect the fabrication costs and the quality control procedures.

Aside from these criteria, the selection of the source is flexible. To a certain extent, design choices made in the light source can be accounted for in the SiO₂ layer design. For example, a light source with a fine spectral resolution would be paired with a thicker SiO₂ layer (faster varying reflectance curve) to satisfy the wavelength selectivity requirements. Additionally, the linewidth of the source must be narrow to resolve the oscillations in the reflectance. Looser spectral resolution requires a broad spectral range as the SiO₂ layer thickness would be set accordingly; however, the gentleness of the reflectance curve would relax the linewidth requirements of the light source. The tradeoffs between the SiO₂ layer thickness and the illumination source linewidth, spectral range, and spectral...
resolution is pivotal when designing an IRIS platform.

2.2.2 Optical Path

Accurate IRIS measurements require uniform, near-normal illumination of the entire image. As the angle of illumination deviates from normal, $\theta_i=0$, the error in IRIS measurements rises as the analytical model is no longer valid, see Figure 2.2. Additional inaccuracy in the IRIS measurements can be presented through non-uniform illumination as the signal-to-noise ratio (SNR) of each image is limited by shot-noise [73].

Two prominent optical designs for uniform illumination are Nelsonian illumination and Köhler illumination. Both of these optical designs are illustrated in Figure 2.3.

Nelsonian illumination, also called critical illumination, forms an image of the light source on the sample using a condenser lens. The result is a bright, but not always even, illumination. Any structure and misalignment in the source will cause gradients or hotspots of intensity. Coherent sources with a uniform emission, such as lasers and laser diodes, are the most suitable.
Köhler illumination provides uniform illumination for non-uniform sources, such as arc lamps and LEDs. Developed in 1983, August Köhler proved an incoherent light source can project an even intensity on a sample by including additional optics and adjustable apertures. This optical configuration controls the angular properties of the illumination and projects the Fourier plane onto the sample surface.

![Optical configuration diagram](image)

**Figure 2.3**: Optical configuration. (a) Nelsonian illumination projects the sample image onto the sample. This minimizes the number of lenses but suffers from source artifacts at the sample plane. A diffuser can be used for slight improvement. (b) Köhler illumination includes additional optics to project the Fourier plane of the illumination onto the sample plane. This approach offers even intensity with no filament artifacts.

### 2.2.3 Camera Selection

The camera of an IRIS instrument is the driving force behind throughput, sensitivity, measurement time. Camera selection relies on the several characteristics: full well capacity (FWC), frame rate, readout noise, and sensor size.

IRIS is an imaging technique where each pixel measures the effective SiO$_2$ thickness of the corresponding surface area. Thus, the throughput of the system is directly linked to
the camera's sensor size. It is important to note, however, that a larger sensor is not always the answer to throughput. The more pixels within each image proportionally increases the image's processing time. As the objects of interest, the protein microarray spots, are typically 100 μm diameter circles, it is recommended the pixel resolution (pixel size divided by magnification) should be set to approximately 10 μm x 10 μm to maximize the field-of-view (FOV).

Other considerations for camera selection are the FWC, camera noise, and frame rate. The accuracy and acquisition time of the IRIS technique is driven by the SNR of each image. Reliable IRIS measurements require the SNR of the process spectral data be 400, as shown by Figure 2.4. Each acquired image contains shot noise, dark noise, and readout noise.

\[ \sigma_{\text{noise}}^2 = \sigma_{\text{shot}}^2 + \sigma_{\text{readout}}^2 + \sigma_{\text{dark}}^2 \]  

(2.12)

Where \( \sigma_{\text{noise}}^2 \) is the total variance in the image and \( \sigma_{\text{shot}}^2, \sigma_{\text{readout}}^2, \) and \( \sigma_{\text{dark}}^2 \) are the variances due to shot noise, readout noise, and dark current noise, respectively.

![Figure 2.4: Image quality effect on IRIS error](image)

The fundamental variation in the emission of photons from a light source is called
shot noise. The other types of noise are the thermal generation of electrons in each pixel over time (dark noise), and the frame readout electronic (readout noise). Dark and readout noise are set characteristics of each camera, but shot noise is a function of the amount of photons collected, \( N \).

\[
\sigma_{\text{shot}} = \sqrt{N}
\]  

(2.13)

For short exposure times and a single frame acquired, shot noise is the dominate noise in the image. A higher SNR can be achieved through a larger FWC and/or averaging multiple frames, \( M \). Averaging more frames offers the ability to achieve a higher SNR at the expense of acquisition time.

\[
\text{SNR}_M = \frac{M \ast \text{FWC}}{\sigma_{\text{noise}}(M)} = \sqrt{M \ast \text{FWC}}
\]  

(2.14)

In conclusion, the ideal IRIS camera has a large sensor size, a deep FWC, and a fast frame rate. In order to image a large number of spots in every image, the sensor size should be as large as possible, but keep in mind the number of pixels affects the processing time. To streamline image processing as much as possible, the target pixel resolution has been set at 10 \( \mu \text{m} \times 10 \mu \text{m} \). This resolution gives a 10x10 sampling of each microarray spot. Finally, each acquired images requires a SNR of 400 for reliability. Since IRIS operates in a shot-noise limited regime, the FWC of the sensor determines the noise level in each image. Further improvement to the image SNR can be made by averaging frames but at the cost of speed.

2.3 **IRIS 1st Generation Design**

The first generation IRIS design, designated the spectral reflectance imaging
biosensor (SRIB), acquires an image of 1000s of biomarkers every 30 seconds [14]. This platform utilizes an external cavity tunable laser to spectrally illuminate the SiO₂/Si chip, see Figure 2.5. The illumination path for uniform intensity is a Nelsonian optical design. Rotating ground glass units are included in the illumination path to remove speckle artifacts from the laser coherence. An external PD monitors the incident intensity for fluctuations. A 5 μm SiO₂ on Si chip is used to measure phase shifts.

Figure 2.5: IRIS tunable laser system. (a) Schematic of the system. A tunable laser is coupled into an optical fiber. The fiber is split with half of the light going to a PD. The other half of the light is randomized by rotating ground glass before illuminating the sample. The reflected light is imaged onto a CCD camera. The external cavity tunable laser (TLB6300, New Focus, San Jose, CA) provides the spectral illumination for measurements. This source has a spectral range of 20 nm at 1 nm increments from 765 nm to 785 nm. The linewidth is 1 nm. Coupled with a 5 μm SiO₂/Si chip, this source samples a half period of the reflectance curve, see Figure 2.6. To remove spatial coherence artifacts, such as speckle, from the image, see Figure 2.7, rotating ground glass discs are implemented.
Figure 2.6: Spectral reflectance curve of a 5 μm SiO₂ on Si chip. The vertical lines indicate the external cavity tunable laser emission wavelengths. A half period is sampled for accurate fitting. Each wafer costs over $200 from SVMI Inc. (Santa Clara, CA, USA).

Figure 2.7: Laser imaging artifacts. In order to disrupt the artifacts, the illumination beam is passed through two ground-glass disks, where at least one is rotating. Reprinted from the supporting materials [14].

In measuring phase, the magnitude of the incident field needs to be precisely measured. Before every experiment, a mirror image is acquired for each wavelength to establish the baseline intensity and to correct for spatial fluctuations. Then, an external PD is utilized to monitor temporal fluctuations during data acquisition. Before processing, the data is normalized by both the mirror image and PD measurements.

The 1st generation IRIS system successfully demonstrated proof-of-concept experiments; however, in order to commercialize this platform, the component costs, system robustness, and instrumentation complexity need to be addressed. The
components that have been selected for redesign include the external cavity tunable laser, rotating ground glass, and external PD. External cavity tunable lasers are expensive sources ($20k) that require periodic alignment. The limited spectral range requires the SiO₂ layer on the sensor chip to be 5 μm increasing manufacturing costs to $200 per wafer (25 chips per wafer). The associated power and control electronics are bulky and make up the majority of the system's footprint. The coherence artifacts generated from imaging with a narrow linewidth source are removed with rotating ground-glass; any moving pieces in instrumentation become a potential point-of-failure, thus the need to eliminate this component. The external PD increases the system complexity and presents an additional point-of-failure mostly resulting from potential misalignment. Eliminating these components from the design would reduce the overall system cost, remove multiple points of failure, and decrease the footprint to one comparable to an inkjet printer.

2.4 IRIS 2nd Generation Design

The second-generation interferometric biosensor, IRIS, implements design changes to reduce the size, cost, and complexity without sacrificing sensitivity. The goal of this platform was to replace the external cavity tunable laser, eliminate the need for rotating ground glass, and remove the external PD. The new platform employs on-chip intensity monitoring [15], a multi-wavelength LED illumination source [1], and a new analytical model, see Figure 2.8. These improvements have the potential to transform the $30k larger first generation IRIS system to a $10k portable platform, see Figure 2.9.
2.4.1 On-chip Intensity Monitoring

Accurately measuring the spectral reflectivity requires precise knowledge of the incident intensity. Typically, the incident beam is divided with one fraction proceeding to the sensor surface and the other to an external PD for monitoring [63], [74], [75]. This
approach complicates the optical design and decreases the optical efficiency.

To simplify the optical layout, an on-chip intensity monitoring technique was developed [15]. The implementation of an on-chip intensity monitoring supersedes using an external PD by integrating a surface into each image with a predictable and robust signal, see Figure 2.10. The trade-off is the negligible loss of area in the image.

In the case of the SiO$_2$/Si chips, the predictable signal comes from bare silicon surface. Figure 2.10b illustrates the concept. By patterning the surface using photolithography then wet-etching with buffered-oxide etchant 6:1 (BOE), the surface is patterned to expose silicon, as shown in Figure 2.11a. Acting like a mirror, the exposed silicon reflects incident light in a predictable way, relative to the incident intensity.

![Figure 2.10: Types of intensity monitoring. (a) The beam is split with a fraction proceeding to the chip for sensing and a fraction going to an external PD for monitoring (IRIS first generation). (b) and (c) show proposed methods of on-chip monitoring. By monitoring an unperturbed region, the signal measured by the camera can be used for normalization. Reprinted from [15].](image)

The main concern of using an on-chip reference region is maintaining signal fidelity.
The on-chip reference region interacts with all the microarray solutions. For the on-chip reference region to provide an accurate intensity measurement, the spectral shift should not be observed in the reflection when non-specific binding occurs. Simulations of different thickness SiO₂ layers (0 nm, 5 nm, and 15 nm) on the reference region, to emulate 0 ng/mm², 5 ng/mm², and 15 ng/mm² of biomolecular density, show less than a 1% shift in the reflection, shown Figure 2.11c.

To assess the performance of the on-chip intensity monitoring region, an end-point experiment was conducted on a human serum albumin (HSA) assay. Consecutive measurements of a 0.5 mg/mL HSA array were acquired without moving the chip. The 19 consecutive measurements were repeated while varying the number of images averaged. During image acquisition, the intensity fluctuations were recorded both by the reference region in the image and an external PD. The performance of no intensity monitoring, monitoring by PD, and monitoring by reference region were compared by calculating the optical thickness of the HSA spots for each normalization approach.
After processing each image, the average density for 15 protein spots were found for each monitoring method by subtracting the average of the local background from the spot average. The process was completed for each of the 19 scans. Figure 2.12 shows the standard deviation of these scans while the number of frames averaged was increased.

The number of frames averaged was steadily increased for a greater SNR. As discussed by Özcumur [73], the dominating noise for measurements is shot-noise. Therefore, averaging images (acquiring more photons) will increase the image SNR. In these experiments, if the temporal intensity fluctuations are not properly normalized, the error in IRIS measurements will be dominated by noise resulting from temporal intensity fluctuation, leading to a higher noise floor.

As expected, the standard deviation for each approach improved with diminishing returns as more frames are averaged. At higher frames averaged, PD and self-reference show superior performance over no reference and comparable noise levels to each other. The conclusion can be drawn that the temporal intensity noise is corrected comparatively whether the reference region or the PD in end-point experiments is used.

![Figure 2.12: Performance of the intensity monitoring methods. As more frames are averaged, the noise floor for each spot improves. The PD and reference region show comparable performance to each other and superior performance to the no reference case. Reprinted from [15].](image)
The dynamic protein detection experiment was performed using immobilized bovine serum albumin (BSA), rabbit Immunoglobulin G (IgG), and mouse IgG, as shown in Figure 2.13a. The chips were secured within a custom flow cell with a 500 μL capacity. IRIS measurements were taken continuously at 1 min intervals. Fifty frames were averaged for each image. Each solution was driven over the chip surface using a peristaltic pump at 400 μL/min rate for 15 min/solution. The chamber was first filled with 1x phosphate buffered saline (PBS) followed by anti-mouse IgG at 10 μg/mL, another 1x PBS wash, and then anti-rabbit IgG at 10 μg/mL. The PBS washes remove unbound material and flush the flow cell for the next incubation. For analysis, every data set has the SiO_2 thickness extracted for the three monitoring techniques.

Analyzing the resulting curves shows both noise normalization tactics perform better than no normalization. As shown in Figure 2.13b, the no reference approach has more noise than self-reference and PD. Examination of the anti-mouse incubation at minutes 31-40, see Figure 2.13c, shows the reference region noise reduction was even greater than...
the external PD. In this case, the PD does not account for the buffer effects, such as adsorption and scattering, resulting in greater performance from the on-chip reference.

In summary, both end-point and dynamic monitoring show the on-chip reference provides accurate monitoring of the incident intensity. For end-point experiments, whether the intensity monitoring is conducted by an external PD or on-chip region, a similar noise level can be attained. However, real-time binding analyses will see lower noise levels with on-chip reference regions for high-sensitivity measurements.

2.4.2 LED Illumination

The first generation IRIS platform utilized an external cavity tunable laser to spectrally illuminate the surface over a 20 nm range. This type of illumination source is expensive, requires a stable environment, and necessitates rotating ground glass discs to overcome speckle artifacts. In order to decrease the platform’s complexity, fragility, and component prices, the external cavity tunable laser was exchanged for discrete LED devices.

LEDs are semiconductor light sources formed from direct band gap p-n junctions. These sources are mechanically robust, spatially incoherent, and low-cost. For multispectral illumination, multiple LEDs can be mounted adjacently on the same LED package, see Figure 2.14. This “Design-Your-Own” configuration allows custom selection of the wavelengths and each LED can be individually powered or all illuminated simultaneously. For the IRIS platform, the wavelengths selected are 455 nm (blue), 518 nm (green), 598 nm (yellow), and 635 nm (red).
Incorporating the LEDs in the IRIS system also involves redesigning the optical illumination path and the SiO₂/Si chip configuration. While the illumination source is changing, the same parameters described in Section 2.2.1, still govern the basis for the IRIS model; however, integration of an LED introduces modifications that invalidate assumptions in the original optical model. In a Nelsonian illumination configuration, the structure of the LED surface will be projected onto the sample resulting in illumination non-uniformity. Moreover, the LEDs are not directly on the optical axis, thus the spatial deviation from the optical axis will create intensity gradients in the image. Lastly, the broad spectral linewidth of LEDs invalidates the IRIS analytical model, which assumes a narrow illumination linewidth. Not accounting for the broad spectral linewidth introduces error.

To alleviate intensity non-uniformity issues, the optical path has been reconfigured into Köhler illumination with a diffuser. The Köhler illumination optical design provides constant intensity across the imaged plane for incoherent, non-uniform emission sources. Including a diffuser between the collection lens and the LEDs introduces a spatial mixing to reduce effects from the co-located LEDs. As shown in Figure 2.15, implementing this optical configuration provides speckle-free illumination with less than 3% variation in the image intensity.
Figure 2.15: Illumination quality of LEDs with a Köhler optical configuration. (a) An IRIS image of a microarray. The inside square is the SiO$_2$ with spotted protein. The border is the reference region for on-chip intensity monitoring. Note there is no speckle. (b) The intensity histograms from imaging a mirror for each LED. The enactment of a diffuser and Köhler illumination gives each LED an even intensity profile (<3% standard deviation for each LED). The x-axis shows the pixel fill percentage (pixel count normalized by camera bit depth).

In order to ensure the LEDs are able to sense the phase shifts from biomass accumulation, the SiO$_2$ layer thickness must be engineered to optimize sensitivity. For the external cavity tunable laser, a 5 µm SiO$_2$ layer set the reflectivity oscillations at a frequency conducive to imaging with a 1 nm linewidth source over a 20 nm spectral range, as shown in Figure 2.6. For the LEDs, the 5 µm design will not suffice. These sources have a broader spectral range and linewidth, as shown in Figure 2.16a. Thinning the SiO$_2$ layer to 500 nm decreases the frequency of oscillation for spectral sampling, see Figure 2.16b.
Figure 2.16: SiO$_2$/Si reflectivity curves. (a) Reflectivity curve of a 5 $\mu$m thick SiO$_2$ with the LEDs' linewidth overlaid. (b) Reflectivity curve of a 500 nm thick SiO$_2$ with the LEDs' linewidth overlaid.

The final aspect to account for is the linewidth of each LED. Discussed in 2.1, the current IRIS analytical model assumes a narrow linewidth illumination. Current commercial LEDs have a 10 nm – 30 nm spectral linewidth that can be approximated by a Gaussian. As seen in Figure 2.17, the reflectance between a 1 nm linewidth and a 10 nm or 30 nm linewidth is easily noticeable for a 500 nm SiO$_2$/Si chip. Therefore, ignoring the spectral linewidth of a LED will lead to erroneous IRIS data.

Figure 2.17: Calculated reflectance of a 500 nm SiO$_2$/Si chip for a 1 nm linewidth source and (a) a 10 nm Gaussian linewidth source or (b) a 30 nm Gaussian linewidth source. LEDs typically have a non-symmetric Gaussian-like profile with a linewidth ranging from 10 nm to 30 nm. These calculations show the linewidth of the LEDs do significantly alter the reflectance.
To correct the linewidth inaccuracy in the analytical model, the IRIS model must be more sophisticated. It is necessary to integrate the reflectance over the spectral linewidth for each LED.

\[
R = \int \left| \frac{n_1 + r_2 e^{-j2k_1d}}{1 + r_1 r_2 e^{-j2k_1d}} \right|^2 d\lambda
\]  

(2.15)

From an analytical viewpoint this is a near-trivial modification; however, the computational complexity for the new model is significantly increased. The SRIB model was calculated once for each wavelength. This new method must calculate the reflectance for the discrete steps of the LED’s spectral linewidth and then execute a Riemann sum:

\[
R = \sum_{n=0}^{n_{1\lambda}} \beta_n \left| \frac{n_{1,n} + r_{2,n} e^{-j2k_{1,n}d}}{1 + r_{1,n} r_{2,n} e^{-j2k_{1,n}d}} \right|^2
\]  

(2.16)

Where \( \beta_n \) is the spectral weight for \( n_{1\lambda} \) samples of the LED spectral linewidth. The computation required for this model is directly proportional to the sampling of the LED linewidth. Currently, the linewidth is sampled at over 330 discrete points for each LED.

While the device itself is more suitable for low-cost portable implementations, the processing necessary to compensate for the design changes greatly limits the utility. As shown in Table 2.1, a single computer requires in excess of 4 hours processing for a 2 megapixel image. One laboratory-based solution is to divide the image across a large computing grid for parallel processing. This is not appropriate for most applications. Noticing each pixel in the image is independent from its neighbors but undergoes the same arithmetic, a graphics card algorithm has been written to parallelize the processing on a single computer [76], discussed further in Appendix D.
Table 2.1: IRIS processing time for multiple hardware methods.

<table>
<thead>
<tr>
<th>Hardware Type</th>
<th>Single CPU</th>
<th>Grid of i7-920 CPUs</th>
<th>Graphics Card GTX 285</th>
<th>Graphics Card GTX 680</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processing time (2MP)</td>
<td>15785.0 sec</td>
<td>322.2 sec</td>
<td>174.0 sec</td>
<td>39.5 sec</td>
</tr>
</tbody>
</table>

2.4.3 IRIS Chip Fabrication

The reference patterns required for on-chip referencing are fabricated using photolithography patterning, processing, and wet-etching. SiO₂/Si wafers with 500 nm of thermally grown SiO₂ are purchased from Silicon Valley Microelectronics (Santa Clara, CA). The surface is cleaned of organic residue by sonicating in acetone for 10 minutes and plasma ashing in an oxygen environment at 300 sccm and 500 W for 10 minutes. For patterning, hexamethyldisilazane (HMDS) and Shipley S1813 positive resist are spun onto the surface at 2000 rpm for 30 seconds followed by a soft bake at 90 °C for 3 minutes. Next, the chip is exposed to UV light for 30 seconds at 15 mW in the SUSS MA6 Mask Aligner and a custom designed photolithography mask. The exposed wafer is developed for 45 seconds in Micro-Dev resist developer diluted 1:1 with deionized (DI) water, and hard baked for 5 minutes at 120 °C.

Once the pattern has been transferred from the mask to the photoresist on the wafer, the surface must be etched. BOE selectivity etches SiO₂ over Si at a rate of 77 nm/min. To expose the underlying Si layer, the wafers are submerged in BOE for 7 minutes, utilizing the Si layer as a natural etch stop. Over etching cannot occur since BOE does not attack the silicon surface.

With the pattern transferred to the chip surface, the photoresist is stripped from the
surface following the same procedure to strip organics (sonication in acetone for 10
minutes and oxygen plasma ash at 300 sccm and 500 W for 10 minutes). A new layer of
S1813 photoresist is spun on then hard baked. This layer will protect the wafer surface
during the dicing process. The covered wafer is diced into 15 mm x 15 mm chips using a
Disco dicing saw. Finally, the protective photoresist layer is stripped using the organic
removal procedure previously described. Figure 2.18 shows a single chip with the IRIS
pattern.

![Figure 2.18: A 15 mm x 15 mm IRIS chip with 500 nm of SiO₂. The chip is divided into four SiO₂
quadrants (purple) to allow a reference region (gray) to be captured in every image. The coloring of the
SiO₂ is a function of the layer thickness.](image)

2.5 Instrument Validation Procedure

The 2nd generation IRIS design decreases the complexity and cost enabling potential
for the commercialization of the technology. Each platform constructed must have an
instrumentation performance validation process. This section details the standard
calibration protocol for ensuring the performance of IRIS platforms. The standard
procedure will tune the intensity of the LEDs, measure the LEDs’ linewidth, construct the
spectral weight function for the IRIS model, and validate performance with artificial non-
biological samples.

As discussed in Section 2.2.2, the quality of illumination (uniformity and strength)
affects the signal accuracy. The target uniformity and strength for each LED is a standard
deviation less than 5% at 60% FWC. To guarantee a high SNR image, the LED intensities are adjusted to 60% of the camera’s FWC. This procedure aligns the intensity of each LED using a custom PCB board. This PCB board has been designed to provide each LED a constant current, see Figure 2.19. To begin, a bare Si chip (a mirror) is placed on the sample platform and brought into focus. Using the histogram function, the intensity profiles of each LED are aligned by tuning the potentiometer on the circuit board. Since the yellow LED has the lowest efficiency, it is recommended the yellow LED current be maximized and used as the baseline for the other LEDs. If the width of the intensity profile exceeds 5% standard deviation, there is a misalignment in the system. System alignment is performed by leveling the illumination path, sample platform, and imaging path. Fine adjustment can be completed with feedback from the image gradients.

Figure 2.19: IRIS LED Current PCB. The circuit can be powered by a USB connection, barrel power connector, a 2-pin connector, or the data acquisition (DAQ) device’s 6-pin connector. Each LED is interfaced with a TL4242 LED driver to provide a constant current up to 500 mA. A potentiometer is coupled to the reference pin of the TL4242 for tuning the current. The LEDs are individually toggled on/off using a push button, dip switch, or DAQ device.
With the system aligned and the LEDs tuned, the LED profile must be captured, normalized, and integrated into the IRIS processing software. This is done by aligning the input fiber of a spectrometer (USB 2000, Ocean Optics, Florida, USA) to the sample plane and bringing it into focus. First, the dark spectrum is acquired and then each LED is sequentially illuminated and the spectral profile captured. For a high SNR, the exposure time is adjusted to fill the detector to 70% of the FWC and average at least 10 measurements. The composite profile is saved to a text file. The text file is loaded into MATLAB and the integral of the profile is normalized to 1. Finally, this profile is saved into a mat-file ([instrument name].mat) using the correct variable names (wavelength, step_wavelength, and weight). The spectral mat-file can now be integrated into the IRIS processing software, MGrid. Note, when implementing new instruments, the GRID_initGrid.m and fitdata.m files must be updated.

After implementing the new system’s linewidth profile in the IRIS processing software, the calculation of SiO₂ thickness must be verified. First, a calibration sample should be fabricated. Starting with a 500 nm SiO₂/Si chip, etch patterns into the surface to emulate biomass binding. Next, measure the absolute SiO₂ thickness for each depth with ellipsometry and/or atomic force microscopy (AFM). Finally, acquire IRIS images and compare. (Figure 2.20 shows the analysis method for fitted IRIS data.) For the first 2nd generation IRIS instrument, a 0.06% and 4.97% percent error was observed when comparing results from ellipsometry and AFM, respectively. The discrepancy between the AFM and IRIS measurements comes from substrate bowing in the AFM results [77], see Figure 2.21.
Figure 2.20: Fitted IRIS Image Processing. The average of the spot (green circle) is subtracted from the average of the local background (red ring) to determine the effective SiO\textsubscript{2} thickness of the biomass. The thickness due to the biomass is then converted into density using the quantification factor.

Figure 2.21: AFM measurement of the calibration sample. (a) AFM image. (b) Profile of step. The red arrows indicate the two points analyzed to determine the vertical distance. The points analyzed were selected to minimize effects from AFM induced bowing artifacts [77]. The percent error between the IRIS (8.644 nm with a 19 pm standard deviation) and the AFM results is 4.97%.

2.5.1 Quantifying Surface Mass Density

The principle of IRIS detection is the quantification of bio-mass accumulation on the surface. The IRIS method relates the bound density to a growth (or increase) in the SiO\textsubscript{2} layer thickness. Since SiO\textsubscript{2} and the biomass do not have identical refractive indices, the phase delay induced by mass accumulation does not have a 1-to-1 relationship to the growth in SiO\textsubscript{2} thickness measured by IRIS. Özkumur et al. demonstrated the 1\textsuperscript{st} generation IRIS instrument had a BSA conversion factor of 1.21 ng/mm\textsuperscript{2} for 1 nm of
SiO₂ [71]. These experiments have been repeated for the 2\textsuperscript{nd} generation IRIS platform.

Determining the response of the system to the adsorption of mass to the surface requires precisely spotting a known quantity of mass onto the surface. Described in detail in Appendix A, the response of the system to 25 pg to 400 pg of mass on the surface was quantified using purified BSA (Sigma, St. Louis, MO). As shown in Figure 2.22, the effective SiO₂ thickness from the spotted mass has been extracted for the 3 x 6 array of BSA. The measured IRIS signal has been scaled by the analyzed area to account for the variable spot size. From the inverse slope of the linear fit, a relationship between the surface density and measured optical thickness is calculated to be 1.3 ng/mm\(^2\) for 1 nm of SiO₂ thickness [1]. This calibration factor is 8% higher than the previous value. The difference between factors is attributed to user variation in the experimental preparation process, such as inaccuracies in the preparation of the known protein solutions.
To conclude, the 2nd generation IRIS platform builds upon the 1st generation design to decrease the system component costs and complexity, while increasing robustness. The external PD has been substituted with an on-chip reference region. Experiments validated that the on-chip reference region achieves a similar noise floor to the PD. The external cavity tunable laser has been exchanged with a 4 LED source. This substitution removed the speckle artifacts from the spatial coherence of the laser and the need for rotating
ground glass. In order to implement the LEDs, the SiO₂/Si chip has been thinned from 5 μm to 500 nm – reducing the cost per wafer 10 fold – and the IRIS analytical model has been modified to include linewidth. Quantification experiments have been conducted to determine the LED IRIS optical thickness to mass density conversion factor (1.3 ng/mm²). Finally, a standard calibration procedure has been established for system alignment and validation.
Chapter 3

MULTI-MODAL MICROARRAY DETECTION

Although the IRIS platform has been proven to be quantifiable and sensitive in protein detection, the technique cannot discriminate specific from non-specific binding in clinical samples. Fluorescence imaging, a labeled detection technique, is not susceptible to this problem, because it uses additional bioreceptors to increase signal specificity. The drawback to labeling, however, is the loss of information about the microarray quality. For complex molecules, like proteins, the density of immobilized probe varies resulting in noisy fluorescence data from experiment to experiment. In order to strengthen both techniques, a hybrid label-free/label detection method has been developed. This approach takes advantage of the quantification of IRIS and specificity of fluorescence imaging for sensitive and robust clinical microarray assays.

This chapter discusses the fusion of IRIS with fluorescence imaging for dual-modality detection. This new method, termed CaFE, facilitates rapid microarray development [4] and achieves inter-experiment linearity by relating the fluorescence response to the surface-bound probe density. Two SiO₂/Si designs aimed at broadband and selective fluorescence enhancement are described. Finally, the practically of the CaFE technique is proven with the design of an integrated dual-modality microscope.

3.1 Calibrated Fluorescence Enhancement SiO₂/Si Chips

In order to provide IRIS and wide-field fluorescence measurements, a common chip design has been implemented [78]. The standard chip design for fluorescence imaging is
a glass slide. Attempts to replace the standard glass slide have led to enhancement techniques with photonic crystals [53], [79], plasmonic metal layers [80], [81], nanoparticles [82], [83], and dielectric spacer configurations [84]–[87]. However, the glass slide is still the standard for its low-cost, low auto-fluorescence, design simplicity, and compatibility with established surface chemistries. The SiO$_2$/Si chips used by IRIS are able to compete with glass slides on all these metrics with the added benefit of enhancing the fluorescence signal beyond glass [61].

Two SiO$_2$/Si design have been pursued for IRIS and fluorescence imaging. The ONC chip design aims to yield emission enhancement for maximum coverage of wavelengths while performing label-free sensing on a separate spot on the same chip. This chip design relies on spotting repeatability within a chip in order to allow for optimized label-free and broadband fluorescence sensing on different SiO$_2$ regions. The CLC chip design remedies potential issues with intra-chip array spotting consistency by performing a same spot analysis. This approach consists of a single SiO$_2$ thickness for IRIS analysis and selective enhancement of Cy3 and Cy5 fluorophores. These configurations facilitate the use of IRIS and fluorescence imaging modalities to calibrate for inter-chip and intra-chip variations.

3.1.1 Chip Design

Selection of the SiO$_2$ thickness for the ONC and CLC designs were carried out using the dipole emission model for an interferometric chip [2], [88], [89]. In Figure 3.1a, the calculated fluorescence signal at 0.7 NA for a 100 nm and 320 nm SiO$_2$ layer is compared with glass. The 100 nm SiO$_2$ design offers the broadband enhancement and
will be coupled with 500 nm SiO₂ regions for the ONC design. The 320 nm design provides both selective enhancement of both Cy3 and Cy5 and IRIS measurements. In Figure 3.1b, the NA dependence of emission enhancement of Cy3 is plotted for the two optimized designs. As expected, the 320 nm SiO₂ layer provides a smaller enhancement to the signal, but both designs provide higher fluorescence signal than glass.

The 320 nm SiO₂ design must also be able to provide accurate label-free IRIS measurements for same spot imaging. As shown in Figure 3.2a, the reflectance curve satisfies the design criteria laid out in Section 2.2.1. IRIS Monte Carlo simulations, Figure 3.2b, confirm the 320 nm chip will provide similar performance to the IRIS chip.

Figure 3.1: Labeled evaluation of CaFE chip designs. (a) The fluorescence signal profile of the two SiO₂/Si designs with respect to glass. Notice the 100 nm SiO₂ design increases the signal across the visible while 320 nm SiO₂ selectively enhances Cy3 and Cy5. (b) The calculated fluorescence enhancement for the 2 designs with respect to glass.
Figure 3.2: Label-free evaluation of CaFE chip designs. (a) Reflectance curve for 500 nm, 320 nm, and 100 nm SiO$_2$/Si chips. According to the chip design criteria discussed in Section 2.2.1, 320 nm is expected to have more accurate IRIS measurements than 100 nm. (b) The absolute error in SiO$_2$ thickness calculations for the LED IRIS platform. As expected, the noise levels in 100 nm are twice as great as 320 nm and 500 nm.

3.1.2 Fabrication Procedure

The CaFE chip comes in two designs. The ONC design has two SiO$_2$ areas: (1) a 500 nm thick SiO$_2$ region dedicated to IRIS quantification and (2) a 100 nm thick SiO$_2$ region to provide enhanced fluorescence. The CLC design contains a single SiO$_2$ region at 320 nm thick for both measurements. Starting with a 320 nm SiO$_2$/Si wafer, fabrication of the CLC design follows the procedure outlined in Section 2.4.3. To fabricate an ONC wafer the following procedure is outlined below.

Starting with an IRIS patterned wafer – see Section 2.4.3 for fabrication details – select SiO$_2$ islands must be etched to 100 nm for fluorescence enhancement. Photolithography is used to transfer the mask pattern to the chip surface for selective wet etching. Acetone and oxygen plasma-ash are used to clean the surface of organic residue. HMDS and Shipley S1818 positive resist are spun onto the surface. The patterned is transferred to the photoresist with contact photolithography. Etching the exposed SiO$_2$
from 500 nm to 100 nm is performed with BOE 6:1 diluted 1:40 with DI water to achieve an etch rate of 4 nm/min. The ellipsometer (VASE ellipsometer, J.A. Woollam Inc., Lincoln, NE), is used to verify the SiO₂ thickness. Finally, the wafer is cleaned and prepped for dicing.

In Figure 3.3 the ONC chip, applied to allergen microarrays, is illustrated. The chip is 15 mm x 15 mm and divided into four quarters (Figure 3.3a), three regions have been etched to 100 nm thick SiO₂ and one region has a 500 nm thick SiO₂. Each quarter of the chip was spotted with the same protein array within the same spotting session. After binding proteins and blocking unreacted sites, the quarter of the chip exhibiting the 500 nm SiO₂ layer was imaged using IRIS. The immobilized density for each condition is quantified; the morphology and consistency of spots are also verified by visual inspection. The chip was then incubated first with serum samples containing allergen specific immunoglobulin E (IgE) and then with fluorescent anti-IgE antibody. Next, the 100 nm SiO₂ quadrants were analyzed in a fluorescence scanner for quantification of fluorescent signals. Figure 3.3b shows the composite image obtained, for the same allergen microarray, before the serum incubation (label free detection of immobilized allergens) and after incubations with an allergic patient's serum and fluorescent secondary antibody.
Figure 3.3: Fluorescence/IRIS chip design. The SiO₂/Si chip is a 15 mm x 15 mm square divided into four quadrants (a). Quadrants 1, 2, and 3 exhibit a SiO₂ layer of 100 nm for fluorescence and quadrant 4 is a SiO₂ thickness of 500 nm. Each quarter of the chip is spotted with the same protein array within the same spotting session. The quarter of the chip exhibiting the 500 nm SiO₂ layer is imaged by IRIS. The other three quarters are analyzed by a fluorescence scanner. (b) A composite image obtained, for the same allergen microarray, before the serum incubation (label free detection of immobilized allergens) and after incubations with an allergic patient’s serum and fluorescent secondary antibody.

3.2 Microarray Calibration Proof-of-Concept

Detection of allergen-specific IgE molecules necessitates the use of secondary antibodies to distinguish them from the large amount of physiologic allergen-specific IgG molecules that bind to the probe but are not indicative of allergic sensitization. The additional quantification challenge imposed by the large variability in immobilized probe density makes in-vitro allergy diagnostics a perfect candidate for the demonstration of CaFE technology [2].

The experiments conducted in this section are performed using 2 separate instruments, the LED IRIS platform and a fluorescence reader (GenePix 4000B, Molecular Devices LLC, Sunnyvale, CA, USA).

3.2.1 Assay Preparation

To demonstrate the universal application of the CaFE method, we have performed the quantification and correlation of captured IgG and β-lactoglobulin probe to fluorescence
signal of Cy3-labeled-secondary antibody. For these experiments, ONC chips are used for optimized fluorescence and IRIS sensitivity. IgG and β-lactoglobulin proteins were selected for their well-established spotting protocols and reliable spotting morphologies.

**IgG Procedure.** As a proof of concept, 20 replicates IgG of varying concentrations (0.015, 0.03, 0.063, 0.125, 0.25, and 1 mg/mL) were spotted onto 2 chips. After overnight humid chamber incubation, the chips were washed with 50 mM ethanolamine in TRIS/HCl 1 M pH 9 for 1 h, rinsed with water, dried with a stream of Argon gas, and then measured using IRIS. They were then incubated with 100 μL of specific labeled antibody in incubation buffer (Tris/HCl 0.05 M pH 7.6, NaCl 0.15 M, Tween 20 0.02%) with 1% w/v BSA for 1 h at 1 μg/mL. Another IRIS measurement was taken after washing with PBS for 10 min, rinsing with water, and drying with Argon.

Fluorescence evaluation was performed by a fluorescence scanner using 40% photomultiplier tube (PMT) gain and 33% laser power for maximum fluorescence value without saturation. Mean fluorescence intensity and standard error from all 20 spots is depicted.

**β-Lactoglobulin Procedure.** To model a sandwich assay similar to the allergen immunoassay and to demonstrate the versatility of the CaFE method, 20 replicates of β-lactoglobulin of varying concentrations (0.015, 0.03, 0.063, 0.125, 0.25, and 1 mg/mL) were spotted onto three chips. After overnight humid chamber incubation, the chips were washed with 50 mM ethanolamine in TRIS/HCl 1 M pH 9 for 1 h, washed with water, dried with a stream of argon gas, and then measured using IRIS. Then, the chips were incubated with 100 μL of specific antibody in incubation buffer with 1% w/v BSA, for
2hr at 10 ng/mL. Next, the chips were washed with washing buffer (Tris/HCl 0.05 M pH 9, NaCl 0.25 M, Tween20 0.05%) for 10 min, rinsed with water, and dried with Argon gas. Following drying, the chips were incubated with 100 μL of the solution of the specific labeled secondary antibody 1 μg/mL in incubation buffer for 1 h. Finally, the chips were washed with PBS for 10 min, rinsed with water, dried with Argon, and measured with IRIS.

Fluorescence evaluation was performed using 90% PMT gain and 90% laser power for maximum fluorescence value just below saturation. Mean fluorescence intensity and standard error were plotted for the 20 replicate spots.

**Allergen Procedure.** To evaluate CaFE as a clinical diagnostic platform, two major allergens, peanut (Ara h1) and timothy grass (Phl p1), were spotted in replicates of three at four concentrations (0.25, 0.5, 0.75, and 1.0 mg/mL) on two ONC chips. In addition, PBS and IgG were spotted as negative and positive control parameters. After overnight humid chamber incubation, the chips were washed with 50 mM ethanolamine in TRIS/HCl 1 M pH 9 for 1 h, washed with water, dried with a stream of Argon gas, and then measured using IRIS. The chips were then incubated with 100 μL of patient serum with documented allergy to peanut (specific IgE 19.40 kU/L, Phadia ImmunoCAP) and timothy grass (positive allergen skin prick test) in incubation buffer with 1% w/v BSA for 2 h at 10 μg/mL (Subject recruitment was approved by the Boston University Medical Campus Institutional Review Board, ProtocolH-29428.). Another wash was applied with washing buffer for 10 min, followed by a rinse with DI water and drying with Argon gas. After incubating with 1 ng/mL anti-IgE labeled with Cy3, the chips were washed with
PBS (10 min), rinsed with DI water, dried with Argon, and measured with IRIS.

Scanning for fluorescence evaluation was performed with 90% PMT gain and laser power to maximize the signal-to-noise ratio without pixel saturation.

3.2.2 Results and Discussion

_IgG and β-Lactoglobulin Calibration._ Images of both label-free measurements (Figure 3.4a) and fluorescence measurements (Figure 3.4b) show a gradient that correlates with varying concentration of immobilized rabbit IgG and captured Cy3-anti-rabbit IgG. The fluorescence enhancement of the 100 nm SiO₂ islands is noticeable when compared to the 500 nm SiO₂ island. Similar effects are seen in the β-lactoglobulin array but are not shown.

Theoretically, each chip should bind the same probe density on the surface. IRIS measurements show that both IgG and β-lactoglobulin exhibit large immobilization variation between chips resulting in varied fluorescence measurements. Quadrant-to-quadrant variation on chip was also present in the IgG data. After applying the CaFE method, see Figure 3.4c, d, each protein demonstrates a calibrated, strong linear response (\(R^2 > 90\%\)) between the fluorescence signal and probe density despite the chip-to-chip variability. The slopes of the calibrated curves differ because each protein has different finite binding capacities. Overall, these results suggest that the CaFE method will be an effective and versatile platform to quantify and correlate the probe density to the fluorescence signal. Going one step further, inter-grid variation could be addressed by implementing the CLC chip design, which would assess each spot individually.
Figure 3.4: CaFE CLC chip concept. (a) Label-free IRIS image of varying concentrations of rabbit IgG on the 500 nm island of CLC chip. (b) Fluorescence image of spotting array on ONC chips for varying concentrations of captured Cy3-anti-rabbit IgG. (c) CaFE measurement for rabbit IgG and (d) β-lactoglobulin. Error bars represent the standard deviation of 20 spots. For the IgG calibration of Chip 3, two quadrants (Q1 and Q2) are analyzed to show on chip variability. Results show the CaFE method yields calibrated, linear responses between experiments for a variety of proteins.

**Allergen Assay Calibration.** The performance of the two chips, defined by the fluorescence signal from secondary IgE, varies between the allergens because different levels of IgE exist in serum for each particular allergen. When analyzing the fluorescence signal of anti-IgE in the fashion of a typical ELISA and microarray assays, measurement variation of allergen-specific IgE of the same allergen between chips is seen, particularly for Phl p1 allergen (see Figure 3.5a). The degree of chip-to-chip variation between allergens most likely occurs because of the physiochemical properties of the allergens.
themselves (i.e., affinity to immobilize to the surface) and technical variation (i.e., spotting). While only slight chip-to-chip fluorescence variations are seen for Ara h1 allergen ($R^2$ of 0.88), a significant chip-to-chip fluorescence variation is seen for Phl p1 allergen ($R^2$ of 0.24), despite the same conditions, reagents, and serum samples used in this single experiment. On the basis of the fluorescence data alone, it is unknown as to why Phl p1 allergen chips yield different fluorescence responses.

To account for this deviation, the CaFE method is applied to collected fluorescence and IRIS data. As a result, a calibrated, linear response between allergen-specific IgE and amount of allergen immobilized on surface emerges (see Figure 3.5b). In both the Ara h1 and Phl p1 examples, the $R^2$ value increases to at least 90%, demonstrating the higher degree of correlation between fluorescence signal and immobilization density compared to spotting concentration. Although the CaFE method only slightly improves upon Ara h1 allergen data, the effects of including label-free IRIS measurement are dramatically seen in the Phl p1 data. Most importantly, use of the CaFE method clarifies that higher immobilization density of Phl p1 allergen on chip 2 results in higher fluorescence signal, indicating that any variation in immobilization density will affect the amount of IgE captured. In accordance with the literature, this data supports that high variation in allergen immobilization microarrays is a concerning issue [90]. The strong linear correlation between fluorescence and immobilization density demonstrates the value of the CaFE method as an opportunity for calibrated quantitative assessment of serum allergen-specific IgE and should improve accuracy in predicting clinical reactivity in susceptible individuals.
Figure 3.5: Calibration of the fluorescence response. (a) The standard "calibration" of secondary antibody for diagnosis of allergy is measured as the degree of fluorescence as a function of spotting concentration is shown for Ara h 1 peanut allergen (left) and Phl p 1 timothy grass allergen (right) for two chips. (b) Using CaFE, results show calibrated, linear responses for allergy testing analysis compared to traditional "semiquantifiable" analysis in part a.

3.2.3 Conclusions

Relying on the consistent immobilization of probes for every assay inherently introduces error. As shown in Figure 3.5, the spotted concentration is neither indicative of the bound density nor the resulting fluorescence signal. The disagreement between the final fluorescence signals comes from the immobilized density that varies by protein and spotting run. Correlating the fluorescence signal to the immobilized probe density regains linearity despite chip-to-chip variance. The application of the ONC chip design has been demonstrated with an IgG, β-lactoglobulin, and allergen assay. Further improvement to account for grid-to-grid variance is shown to be possible through the utilization of the
3.3 Integrated Instrument for Calibrated Fluorescence Protein Microarrays

The CaFE method has shown using a label-free in conjunction with a labeled method is able to increase reproducibility between fluorescence microarrays by correlating the fluorescence signal to the surface-bound probe density [2]. The initial CaFE experiments utilized an IRIS instrument to measure the probe immobilization and a separate fluorescence reader to measure the fluorescence signal from fluorophores linked to secondary bioreceptors. If these instruments can be integrated, the labor and instrumentation costs associated with this method of calibration can be reduced. This section details the hardware and software design for a combined apparatus [16]. Ultimately, this work can lead to a modular attachment for commercial wide-field fluorescence imagers.

3.3.1 Optical Design

The first step in design an integrated platform is analyzing the design requirements and points of synergy between the modalities. From an optical design standpoint, IRIS and wide-field fluorescence imaging have several points of symmetry and two dissimilarities. Both platforms are top-illuminated, also known as reflection-mode, microscopes. For reliable data collection, these instruments require uniform and stable illumination of the sample across the FOV. The caveats requiring attention are fluorescence imaging necessitates spectral filters and a high NA collection.

Fluorescence imaging optimizes sensitivity by maximizing the illumination intensity
at the fluorophore plane and then spectrally filters all non-signal wavelengths in the imaging path to nullify background signals. To achieve spectral isolation, an excitation filter, a dichroic mirror, and an emission filter are built into the optical path. Figure 3.6 illustrates the ideal spectral design of the fluorescence filters. The excitation filter, typically a short-pass filter, is placed in the illumination path to eliminate any overlap between the illumination spectrum and the detection spectrum. The emission filter, typically a long-pass or band-pass, filter is in the imaging path to block signal from the illumination and external sources. For additional spectral isolation and greater illumination/imaging efficiency, fluorescence imaging utilizes a long-pass dichroic mirror instead of a beamsplitter.

![Figure 3.6: Transmission spectra of Select Cy3 Filters. The absorption spectrum of Cy3 is from 500-550 nm. The emission spectrum extends from 550 - 600 nm. The emission and excitation filters were selected to spectrally isolate the illumination and fluorescence signals for filtering. The dichroic mirror reflects the excitation wavelengths and transmits the emission wavelengths for high excitation intensity on the sample and high efficiency of the emission path for the fluorescence signal but not the excitation signal.](image)

Taking into account the requirements of the modalities, the hybrid instrument has been designed as shown in Figure 3.7. IRIS illumination is provided by the 4-LED package (ACL01-MC-RGYB-E08-C01-L-0000, Excelitas Technologies, Waltham, MA, USA). A single high-powered green LED (M530L2, Thorlabs, Newton, NJ, USA) with a
short-pass filter for fluorescence imaging of Cy3 (FF01-529/24-25, Semrock Inc, Rochester NY, USA) is included for fluorescence excitation. These sources are combined using a 50:50 non-polarizing beamsplitter before entering the illumination optics. Since the light sources are LEDs, the illumination paths have been merged into a common Köhler configuration for even intensity across the FOV. In order to maintain a large FOV while providing a high NA collection, the imaging path consists of a 5x 0.5NA objective (1-MVX200, Olympus, Center Valley, PA, USA) coupled with the corresponding tube lens (3-MVX200, Olympus, Center Valley, PA, USA) and a 0.63X magnification adapter (MVX-210, Olympus, Center Valley, PA, USA). The resulting FOV is 2.5 mm x 3.4 mm with a 1” format camera sensor (Retiga 2000R, QImaging, Surrey, BC, CA). For efficient switching of modalities, the custom 52 mm x 58 mm dichroic mirror (Di01-R532-52x58, Semrock Inc, Rochester, NY) and emission filter (FF01-575/15-25, Semrock Inc, Rochester, NY) for Cy3 are designed for quick removal and swapping with the 50:50 non-polarizing beamsplitter required for IRIS imaging.

Figure 3.7: CaFE instrument design. (a) Schematic of the optical layout. For fluorescence, the second beamsplitter is swapped for a dichroic mirror and the emission filter is inserted. (b) Model of instrument with key components labeled. (c) Image of the constructed instrument.
3.3.2 High NA Reflectance Model

The objective selected for sensitive fluorescence measurements has a high NA. As discussed in Section 2.4.2, the 2nd generation IRIS analytical model accounts for the linewidth of light sources, but the paraxial approximation is still applied to simplify calculations. Adjusting the analytical model appropriately leads to an ASR analytical model. Additional complexity can be immediately seen as the reflectance of TE, \( R_s \), and TM, \( R_p \), must now be calculated independently, see Equations 3.1 and 3.2. Since the LEDs are unpolarized, the contribution from each polarization can be considered equal, see Equation 3.3.

\[
R_s = \left| \frac{r_{s,12}(\lambda, \theta) + r_{s,23}(\lambda, \theta)e^{-j2k_\lambda(\lambda, \theta)d}}{1 + r_{s,12}(\lambda, \theta)r_{s,23}(\lambda, \theta)e^{-j2k_\lambda(\lambda, \theta)d}} \right|^2, \tag{3.1}
\]

\[
R_p = \left| \frac{r_{p,12}(\lambda, \theta) + r_{p,23}(\lambda, \theta)e^{-j2k_\lambda(\lambda, \theta)d}}{1 + r_{p,12}(\lambda, \theta)r_{p,23}(\lambda, \theta)e^{-j2k_\lambda(\lambda, \theta)d}} \right|^2, \tag{3.2}
\]

\[
R = \int \int \frac{R_s(\lambda, \theta) + R_p(\lambda, \theta)}{2} d\lambda d\theta, \tag{3.3}
\]

The Fresnel coefficients and the wavenumbers for an angular illumination are:

\[
r_{s,12}(\lambda, \theta) = \frac{n_2 \cos \theta_2 - n_1 \cos \theta_1}{n_2 \cos \theta_2 + n_1 \cos \theta_1}, \tag{3.4}
\]

\[
r_{s,23}(\lambda, \theta) = \frac{n_3 \cos \theta_3 - n_2 \cos \theta_2}{n_3 \cos \theta_3 + n_2 \cos \theta_2}, \tag{3.5}
\]

\[
r_{p,12}(\lambda, \theta) = \frac{n_2 \cos \theta_1 - n_1 \cos \theta_2}{n_2 \cos \theta_1 + n_1 \cos \theta_2}, \tag{3.6}
\]
\[ r_{p,23}(\lambda, \theta) = \frac{n_1 \cos \theta_2 - n_2 \cos \theta_3}{n_1 \cos \theta_2 + n_2 \cos \theta_3}, \quad (3.7) \]

\[ k_2(\lambda, \theta) = \frac{2\pi n_2}{\lambda} \cos \theta_2, \quad (3.8) \]

Where \( \theta_1, \theta_2, \) and \( \theta_3 \) are the propagation angles in air, SiO\(_2\), and Si, respectively. These angles can be calculated using Snell’s Law.

The new analytical model greatly complicates the computational process. For the proposed instrument, the processing time will be greatly affected by the implementation of the ASR model. Determining the NA where the error from the paraxial approximation model exceeds tolerance governs if the ASR model or paraxial approximation model should be used. Monte Carlo simulations are conducted to estimate the IRIS error as a function of NA. As IRIS measurements relate the difference between the spot thickness (SiO\(_2\) + biomass) and background thickness (SiO\(_2\)) to extract the amount of mass bound, Monte Carlo simulations were conducted to emulate a 1 nm SiO\(_2\) spot. For an increasing NA, the reflectance values of a 500 nm SiO\(_2\) layer and a 501 nm SiO\(_2\) layer were generated, noise approximating the system noise (shot noise, readout noise, and dark current noise) was added, and then the noisy data was fitted with the ASR and paraxial approximation models. The metric of evaluation selected is the percent error for the 1nm SiO\(_2\) growth.

In more detail, the Monte Carlo simulations: (1) generate the reflectivity of a SiO\(_2\)/Si chip for a given NA, LED spectral profile, and the two SiO\(_2\) thicknesses. The LEDs are assumed to have Gaussian spectra with bandwidths approximating the VHL ACULEDs from Excelitas technologies. (2) The system noise (shot noise, readout noise, and dark
noise) is calculated for each simulation and added. Note, the quantity of shot noise added is randomized for each LED and follows a Poisson distribution. (3) The analytical model (ASR or paraxial approximation) is applied to the noisy data using the Levenberg-Marquardt algorithm. The result yields the calculated optical thickness for the noisy data. (4) The two optical thicknesses are subtracted to determine the relative SiO₂ thickness change. (5) The percent error between the simulated and the actual SiO₂ thickness change is determined.

As shown in Figure 3.8, the paraxial approximation model becomes erroneous at higher NA while the ASR model stays consistent. Therefore, to accommodate high NA systems, such as sensitive fluorescence imaging platforms, the more complex ASR model should be utilized for accurate and precise measurement of the probe density.

![Graphs showing error vs. NA for different models](image)

Figure 3.8: Monte Carlo simulations of IRIS accuracy for a 1 nm thickness change (~1 ng/mm² of protein) while increasing NA. (a) The paraxial approximation model becomes more erroneous at higher NA leading to a model breakdown. (b) However, the ASR model shows a significantly reduced and constant error. The vertical error bars are ±1 standard deviation for N=100.

### 3.3.3 Validation Assay Procedure

Instrument validation is performed using a β-lactoglobulin assay [16]. A direct label-
free comparison between the IRIS platform and the CaFE reader is completed to ensure an accurate measurement of probe immobilization with the ASR model. In addition, the calibration capabilities of the CaFE platform are compared to the IRIS and GenePix 4000B systems through an anti-β-lactoglobulin/β-lactoglobulin fluorescence sandwich assay using Cy3-label-secondary antibodies. The anti-β-lactoglobulin/β-lactoglobulin system was selected for its well-established spotting protocols and reliable spotting morphologies.

A traditional sandwich assay using an anti-β-lactoglobulin/β-lactoglobulin system is conducted to model a variety of different antigen/ligand interactions. Ten replicates of anti-β-lactoglobulin of varying concentrations (0.015, 0.03, 0.063, 0.125, 0.25, and 1 mg/ml) are spotted onto 4 ONC chips and 4 CLC chips. After overnight humid chamber incubation, all slides are washed 3 times with 1X PBS with Tween 20 (PBST), 3 times with 1X PBS, and 2 times with DI water for 4 minutes each, washed with water, and dried with a stream of argon gas. At this point, the immobilized probe density is measured using the IRIS platform and the label-free modality of the calibrated fluorescence reader. The slides are blocked with 50 mM ethanolamine in TRIS/HCl 1 M pH 9 for 45 minutes and incubated with 10 µg/mL of β-lactoglobulin in PBS for 2 hours. Next, the slides are washed with 1X PBST for 10 minutes, rinsed with water, dried with Argon gas, and incubated with 1 µg/mL of Cy3 labeled secondary antibody against β-lactoglobulin in 1% weight/volume BSA in PBS for 1 hour. Finally, the slides are washed with PBS for 10 minutes, rinsed with water, and dried with Argon before the fluorescence signal is measured with the GenePix 4000B scanner and the calibrated fluorescence
Fluorescence evaluation with the GenePix4000B was performed using 90% PMT gain and 90% laser power for the maximum fluorescence signal without saturation. Fluorescence evaluation with the CaFE reader was performed using a 60 second exposure time at maximum LED power. The mean fluorescence intensity of each concentration was normalized by the maximum detected fluorescence signal for each calibration approach and then plotted against the corresponding immobilized surface probe density.

### 3.3.4 Results and Discussion

The CaFE instrument integrates the IRIS technique and fluorescence imaging to calibrate the fluorescence response to the probe immobilization of a microarray. Figure 3.9 shows the label-free accuracy of the integrated reader against the IRIS platform. The integrated platform results agree with the IRIS system for both chip configurations. Thus, the conclusion can be drawn that the ASR model for high NA imaging offers equal accuracy and sensitivity to the IRIS platform.
Figure 3.9: Comparison of the IRIS platform and the calibrated fluorescence reader's label-free modality. Diminishing concentrations of probe were spotted onto 4 slides for ONC and 4 slides for CLC. The resultant average and standard deviation of the 10 replicate spots at each spotted concentration on both calibration slide types are shown here for both instruments. An $R^2$ value of $>0.995$ in both cases exemplifies the validity of the ASR model for high NA IRIS measurements. Error bars are ±1 standard deviation for $N=10$.

With the new high NA model authenticated, the calibration performance of the ONC and CLC designs can be evaluated for the integrated platform. Figure 3.10a shows a correlation of 0.95 for the ONC and 0.93 for CLC. These results illustrate the CaFE platform's capability to calibrate the microarray accurately. Comparing the calibration response of the integrated system to the optimized IRIS and GenePix 4000B combination shows almost identical normalized responses (Figure 3.10b). The near-complete overlap of the integrated platform calibration and the IRIS|GenePix calibration proves the integrated system is able to operate with comparable IRIS and fluorescence sensitivities for accurate assay calibration.
Figure 3.10: Validation of the integrated system calibration. (a) The fluorescence signal shows a highly linear relationship to the measured probe density for both the ONC and CLC configurations on the calibrated fluorescence reader. (b) Comparing the integrated reader response to the IRIS and GenePix 4000B instruments shows a close relationship in calibration response. In both plots, the fluorescence is normalized by the maximum measured signal.

3.4 Conclusion

The performance of a microarray can be affected by the array geometry, probe density, spot morphology, background signal, probe specificity, and detection sensitivity. Through careful design of the array layout, surface chemistry, antibody selection, and the detection modality, most of these sources of variation are controlled [91]–[93]. Probe immobilization, however, inherently fluctuates because of the complex and delicate relationship between protein biochemistry and immobilization/spotting conditions.

An integrated CaFE reader has been designed and validated to correct for variation in the probe immobilization. This reader incorporates IRIS and wide-field fluorescence imaging measurements to calibrate the fluorescence response of the assay to the probe density. The ASR analytical model for high NA IRIS measurements has been validated through direct comparison of the integrated reader and IRIS system. A sandwich assay was conducted by spotting anti-β-lactoglobulin, incubating β-lactoglobulin, and then incubating a Cy3-labeled-secondary antibody against β-lactoglobulin to verify the
fluorescence sensitivity and linearity of the calibrated response. The resulting calibrated curve shows a strong correlation among all the chips. Additionally, the CaFE reader showed equal calibration performance to the IRIS and GenePix 4000B readers. These experiments verify both the label-free and fluorescence detection capabilities as well as illustrate the capability to calibrate an assay with a single, integrated platform.
Chapter 4

DIGITAL MICROARRAY DETECTION

Clinical protein microarray applications have been hindered by repeatability and sensitivity issues due to bioreceptor immobilization variability and non-specific molecular binding. The CaFE technique prevailed over these problems with a hybrid label and label-free approach [2]. In the CaFE technique, wide-field fluorescence detection was utilized to specifically detect the biomarkers. Although labeled techniques have a distinct advantage over label-free techniques in detection specificity, designing the labels to not affect the protein's functionality and to minimize cross-reactivity is cumbersome for a multiplexed assay. Furthermore, labeled assays require additional incubation steps that increase the assay time. The development of a label-free modality capable of specific molecular detection is essential for highly multiplexed and/or rapid clinical assays.

This chapter will detail the theory and design of a single particle detection platform, called SP-IRIS, capable of discriminating between specific and non-specific binding. Utilizing interferometry and a high resolution microscope, the SP-IRIS method images the diffraction pattern of biological nanoparticles down to 60 nm in diameter directly [67]. Analysis of the imaged nanoparticle response has been shown to yield information about the particle size that can then be used to identify specific binding [66], [67], [94]. This digital detection of nanoparticles coupled with sizing verification has shown to dramatically improve the sensor's detection capabilities in clinical samples [6]. To eliminate user error and disseminate this technology to clinical laboratories for further
testing, an automated SP-IRIS detection platform has been built [95].

4.1 SP-IRIS Detection Principle

The detection of single biological particles, like whole viruses, using light scattering is challenging. Biological materials, whether cellular or a molecular, have a small scattering cross-section when they are in the presence of scattered light because of the small refractive index differences when compared to the surrounding medium. As described by Mie [96], the electric field scattered from a nanoparticle in free-space, \( E_s \), is proportional to the induced dipole moment, \( p \), that can be described by the polarizability of the particle, \( \alpha \), and the incident electric field, \( E_i \) [97].

\[
E_s \propto p = \alpha E_i, \tag{4.1}
\]

For particles much smaller than the wavelength of light, the scattered signal can be approximated by Rayleigh scattering theory. In this case, the polarizability scales with a cubic dependence on the radius, \( r \).

\[
\alpha = 4\pi\varepsilon_0 r^3 \frac{\varepsilon_p - \varepsilon_m}{\varepsilon_p + 2\varepsilon_m}, \tag{4.2}
\]

Where \( \varepsilon_p \), \( \varepsilon_m \), and \( \varepsilon_m \) are the permittivity of the particle, surrounding medium, and vacuum, respectively. This cubic dependence indicates that techniques that measure the scattering intensity of the nanoparticle have a signal that scales with the particle’s radius to the sixth power, see Equation 4.3. (In other words, the scattered intensity from a 1 \( \mu \)m bacterium is 1 million times greater than a 100 nm viral particle.) This rapidly diminishing response for biological nanoparticles is consumed by the system noise.
SP-IRIS utilizes a common path interferometer to image and discriminate single nanoparticles. Introducing a spacer layer and mirror below the particle introduces an interference effect in the far-field signal, see Equation 4.4. The resulting detected intensity is described by the addition of the scattering and reflected field, $E_r$. In this configuration, the far-field intensity approximately scales to the radius cubed for enhanced visibility of nanoparticles, see Figure 4.1a. The enhanced nanoparticle contrast enables the detection of particles down to 60 nm; see Figure 4.1b, in a large FOV (240 μm x 180 μm). Additionally, since the signal has an approximate cubic dependence on the radius, nanoparticle can be classified by size [67], [98].

$$I_{det} = |E_s + E_r|^2 = |E_s|^2 + |E_r|^2 + 2|E_r||E_s|\cos\theta \propto r^3$$  \hspace{1cm} (4.4)

Where $\theta$ is the relative phase between the reflected and scattered fields. In this case, $|E_s|^2$ is equivalent to the previous case and will scale to the sixth power, $|E_r|^2$ contributes constant background intensity and the interference term forms the dominant optical response of the nanoparticle.
A common path interferometer is selected over double path configurations that are common to holography for robustness when using LEDs. For instance, when performing interferometry with a Michelson interferometer, the incident beam is split into two arms, a detection and reference arm, as shown in Figure 4.2. Both arms propagate an equal distance, $2L$, but the detector arm is passed through an unknown sample. Upon recombining, the beams interfere and the resulting intensity on the detector is proportional to the phase delay induced on the detection arm by the unknown sample. Any vibration on one or both of the arms will result in a spatial displacement of the beam. For a spatially coherent source, like a laser or an incoherent source passed through a pinhole, the spatial displacement does not affect the interference of the beams; however, spatially incoherent sources, like LEDs, will have an unpredictable response. Therefore, the SP-IRIS technique employs a common path interferometer for a greater level of stability.
Figure 4.2: Michelson Interferometer. The input beam (source) is split into 2 arms. The split beams travel equal distance, reflect off a mirror, and recombine onto a detector. However, one arm is lagged by an unknown sample. The resulting interferometric signal is a function of the induced phase delay.

The SP-IRIS optical path is a Köhler illumination path and a 4-f imaging system, see Figure 4.3a. The nanoparticles are detected by interfering the scattering from the nanoparticle (arm 1) with the reflection off the buried SiO₂/Si interface (arm 2), see Figure 4.3b. The sub-diffraction interference signal is relayed to the imager and produces an airy function, see Figure 4.3c and d. The contrast of these particles, peak value divided by the local background, is then extracted to determine the particle size.

Figure 4.3: SP-IRIS Detection Concept. (a) Optical schematic of the SP-IRIS platform. (b) The scattering from a nanoparticle is interfered with the reflection from a buried interface. (c) A cropped image showing the detection of 150 nm diameter polystyrene beads for 635 nm illumination. (d) Response of a particle from (c). This image is modified from [67].
4.2 Automated Single Particle Platform

The difficulty in using this technique is focusing. The imaged particle response is dependent on the relative phase between the reflections, the permittivity of the particle and surrounding environment, the incident wavelength, the collection NA, and the imaged focal plane. All of these parameters except the imaged focal plane are known and controlled. The phase difference is set by the SiO$_2$ layer thickness. The particle permittivity and surrounding environment permittivity are specific to the assay. The instrument design sets the incident wavelength and collection NA. Only the focal plane is a user-defined parameter. As shown in Figure 4.4, the range of defocus values where the nanoparticles (100 nm) are visible over the background is small (± 0.5 μm) and rapidly varying. For accurate and repeatable data, the user must be able to adjust the focus with an accuracy of ± 50 nm.

![Figure 4.4: Defocus profile of a 100 nm nanoparticle on a 100 nm SiO$_2$/Si chip. The background signal (horizontal red line) limits the visibility of the nanoparticle to a ± 500 nm range. Additionally, the peak contrast extends over only a 100 nm range. These constraints complicate the data acquisition process.](image)

For this technology to move forward as a diagnostic platform, the dependence on the user's focusing skill must be removed. To this end, an automated and easy-to-use SP-
IRIS platform has been designed, constructed, and tested, see Figure 4.5. This standalone platform interfaces automated XYZ stages and a custom GUI with the SP-IRIS optical path to allow for automated acquisition and processing. Two complimentary focusing algorithms are implemented in tandem to achieve the ± 50 nm accuracy for robust nanoparticle detection. For validation, the detection capabilities of this automated prototype are compared against the manual benchtop system on a wild-type vesicular stomatitis virus (wtVSV) assay.

![Figure 4.5: SP-IRIS prototype. (a) Image of the prototype. (b) Patterned chip loaded into the sample holder. The center square region is for spotting. (c) Image of an antibody spot on the prototype.](image)

4.2.1 Hardware Design

In the following discussion, the components and materials of individual design elements (the sensor chip, the optics, the electronics, and the mechanics) are given. For each design component, the specific part numbers are provided. These components were selected to meet or exceed design requirements.

**Sensor Chip Design.** For SiO2/Si chips, a thickness of 100 nm has been selected to maximize the phase angle term [94]. Silicon chips with thin layers of SiO2, fabricated by SVMII, Inc. (Santa Clara, CA, USA), were designed to be used with a reusable loading holder Figure 4.6. Fiduciary patterns enable the use of an automatic spot detection
algorithm, in addition to providing contrast for autofocusing algorithms. The dimensions of the chips are $10 \text{ mm} \times 10 \text{ mm} \times 0.5 \text{ mm}$, with a $2.3 \text{ mm} \times 2.3 \text{ mm}$ active center region for spotting capture probes. Small, periodic etched squares ($15 \mu\text{m} \times 15 \mu\text{m}$ every $143 \mu\text{m}$) in the capture probe area ensure that regions of high contrast are present in the field-of-view for coarse focusing, independent of the precise location of each capture probe spot.

Figure 4.6: Floor plan of an SP-IRIS chip. The active region in the center is used for biosensing. Additional fiducial marks outside of the active region enable repeatable robotic spotting.

**Optical Design.** The SP-IRIS technique is based on a top-illumination microscope scheme. The key parameters in the optical path design are the illumination wavelength, uniformity, NA, magnification, and camera pixel size. These parameters determine the accuracy of sizing, throughput, and minimum detection size of the system.

In imaging systems, the uniformity of illumination is vital to obtain comparable performance across the sensor surface. For the SP-IRIS technique, improper illumination will affect the detection and sizing capabilities. In particular, because sizing is calculated via contrast of the central peak to near-neighbor background pixels, non-uniformity of the illumination will induce sizing ambiguity. As discussed in Section 2.2.2, for LEDs and filament sources, uniform illumination is achieved through Kohler illumination.

Adjusting the NA offers a tradeoff between nanoparticle contrast and usable FOV.
The image of the nanoparticle is calculated from a forward model that is derived using the angular spectrum representation, where the nanoparticles are modeled as dipoles to determine the expected far-field signal of the virus [99]. To ensure reliable detection of the virus, an NA offering a SNR of at least 3 over the background noise was selected. Figure 4.7 shows the contrast response of a 100 nm virus (equivalent to H1N1) and the peak of the response as a function of NA.

Particle sizing in the SP-IRIS technique is performed by comparing the detected particle peak contrast to the predictive forward model. A potential source of error in this comparison is undersampling of the point spread function (PSF). If the PSF is ill-defined on the camera, the central peak of a nanoparticle’s PSF could be blurred between multiple pixels leading to error in the contrast calculation. To guarantee proper imaging of the PSF, an oversampling rate of at least 3 pixels is recommended. In this case, the diffraction-limit resolution specified by the Rayleigh criterion is 400 nm. Therefore, an effective pixel size of 134 nm x 134 nm should be used. The prototype system meets this requirement with a 50x, 0.8 NA Nikon objective and the Grasshopper 2 GigE CCD camera (Point Grey Research, Inc., BC, Canada) with 2 x 2 binning. The resulting optical parameters are an effective pixel size of 138 nm x 138 nm and a total FOV of 168 μm x 141 μm.
**Figure 4.7:** Theoretical response of a 100 nm virus with a varied NA. The dashed horizontal bar indicates an SNR of three above the background noise. The dotted vertical line indicates the selected NA, 0.8.

**Electrical Design.** For rapid system development, an attempt was made to maximize the use of commercial off-the-shelf subcomponents. This included a Gigabit Wireless Router (AirStation HighPower N450 Gigabit Wireless Router, Buffalo Technology, Austin, TX, USA), an industrial serial RS232 to Ethernet Converter (BF-431, Gridconnect Inc., Naperville, IL, USA), a high-power LED (Golden Dragon LT W5 SM, OSRAM Opto Semiconductors), and a high-speed Gigabit CMOS Camera (GS2-GE-50S5M-C, Point Grey Research, Richmond, BC, Canada).

To integrate all these components together inside the SP-IRIS enclosure some custom electronics are needed to control the LEDs and generate the necessary supply voltages for all subcomponents from the single 24 V DC power supply. To save space, four 64 mm x 64 mm, four-layer printed circuit boards are vertically stacked and connected through a 26 pin cable. Each board serves a single purpose: high efficiency regulation of the necessary 12 and 5 V power supplies, constant current LED driving circuits (TL4242, Texas Instruments, Austin, TX, USA), Ethernet to UART interface, and a microcontroller (MSP430AFE221, Texas Instruments, Austin, TX, USA) to allow TCP/IP commands to
be issued to control the LEDs.

By using a gigabit router for all communications to subcomponents, standard TCP/IP networking protocols were used over 10/100/1000 Mbit Ethernet with category-6 unshielded twisted pair cables. This allows a single interface for communication with components and will enable a seamless transition to high-speed wireless interfaces (802.11n) for control and interfacing. The system uses a peak power of 29 W during power-up and acquisition, and 23–24 W while idle.

**Mechanical Design.** A FOV of 168 μm x 141 μm and a focus dependent particle sizing gives the potential for cross-dataset variability, as small differences in operator technique can result in significant divergence in particle counts. Designing an operator independent protocol is vital for POC devices. The mechanical design of the system solves the issue with rigidity and automation.

The SP-IRIS prototype utilizes a 3-axis orthogonal stack of linear stages (VT-21, MICOS USA, Irvine, CA, USA) to enable the rapid scanning of samples. A removable chip holder secures the sensor in a repeatable location with a spring-loaded latch. The holder is then secured to the stage assembly via a series of alignment pins and magnetic retention features. Fine alignment adjustments are enabled via a pitch/yaw mechanism built into the stage assembly.

All required optics, mechanics, and electronics are incorporated inside a robust and portable housing measuring 17 in x 11 in x 8 in. An aluminum base plate equipped with vibration dampening feet provides a stable mounting platform for all onboard components and doubles as a heat sink for the stage components.
4.2.2 **Software Design**

The user interface of a POC diagnostic instrument must be easy to use. Ideally, an untrained operator with a high school degree or equivalent should be able to use the instrument with a simple set of instructions. Creating a user-friendly interface requires a high level of mechanical and software automation. Applying these criteria to a high-resolution optical technique requires engineering in both image acquisition and image processing. In the analysis of microarray assays, the time to process each spotted condition becomes the determining factor behind measurement time. The following sections detail a user-friendly interface and the novel image processing algorithms to achieve acquisition times less than 1 min per spotted condition.

**Graphical User Interface.** Incorporating a simple, easy-to-use, and readable user interface is pivotal in developing a tool for POC settings. In these environments, the target user cannot be required to have specialized training. With the algorithms discussed in the following sections, the SP-IRIS instrument is able to achieve a 5-click interface, as shown in Figure 4.8. This interface requires user interaction only for loading/unloading and basic array information. Once the user clicks acquire and analysis, the software precedes spot to spot acquiring data. The first spot of the array is located through the use of fiducial marks and subsequent spots are found through the user defined spot pitch. At each spot, the instrument executes the focus algorithms. Once focused, the number of viral particles detected is tallied and recorded in the table. During this time, the operator is available to continue other clinical operations and can return to reload the system once analysis has completed.
Particle Detection. Robust, rapid, and automated particle detection is an enabling feature of an easy-to-use SP-IRIS interface. The detection of the sparse nanoparticle diffraction patterns in the image requires the implementation of several image processing techniques. To start, an important observation is these nanoparticles are well localized and local extrema in the pixel space. A profile of a 100 nm viral nanoparticle is shown in Figure 4.9. Such features are the characteristic of an interest point in computer vision.
Interest point detection is a well-studied subject, and tools from the existing literature have been adapted for robust detection of the nanoparticles. Scale invariant feature transform (SIFT) has been proven to be one of the most robust interest point detection methods and has been used to automate detection in this instrument [100].

![Image of simulated response of a 100 nm spherical viral particle in the SP-IRIS system.](image)

Figure 4.9: Simulated response of a 100 nm spherical viral particle in the SP-IRIS system.

In SP-IRIS images, given the particle type of interest and other experimental variables, the forward model is computed and the expected normalized peak response of the particles of interest is known. For example, if one aims to image 100 nm viral nanoparticles, the expected normalized peak value would be around 1.06. This information is used to eliminate strong interest points that are related to salts and large nonspecifically bound particles. In order to detect strong interest points, the ratio of the maximum to minimum value of a pixel in its local neighborhood is computed and thresholding is used to find regions with high intensity differences. The resulting binary image is named as the anomaly map. Figure 4.10 shows the detection and elimination of anomaly region. Note that for sizable anomalous structures, the pixels toward the center
of the large structures do not have a high intensity difference. In order to overcome this, a morphological close operation is used to fill such gaps in sizable structures.

![Figure 4.10: Anomaly Filtering. (a) Detected anomaly map of nonspecific structures. (b) Using the anomaly map to discard particles within those regions, the nanoparticles of interest (green circles) are accurately identified.](image)

**Closed-Loop Focusing.** The automation of optical devices requires the use of robust autofocusing. For POC devices, a robust, precise, and real-time algorithm is imperative to minimize experimental time and prevent erroneous results. Choosing the correct passive autofocusing algorithm weighs heavily on the imaging modality and the target object [101]. For some applications, the sample alignment conditions are loose. However, in the case of SP-IRIS, focusing is critical as the response of the nanoparticle rapidly peaks. The challenge of a rapid focusing algorithm is compounded by two issues: 1) a narrow detectable window where the nanoparticle contrast is above the background and 2) the optimal focal plane of the nanoparticles does not coincide with the sensor surface. To approach the autofocusing for the SP-IRIS technique in a robust, rapid manner, two complementary algorithms have been implemented.

When the sensor is loaded, the removable chip holder latches into the stage assembly.
Since the nanoparticle detection window overlaps with the sensor surface, the coarse focusing algorithm is executed to bring the imaging plane to the sensor surface by focusing on the 15 μm square fiduciary marks within the FOV. The stages are swept 30 μm acquiring an image every micrometer. A fast Fourier transform is applied to the stack of the images followed by a high-pass filter. The remaining high frequencies are summed. The most in-focus plane corresponds to the maximum sum. With the stages now within 2 μm of the sensor surface focal plane, the algorithm is reiterated over 2 μm with 100 nm steps. The resulting in-focus plane is somewhere within the nanoparticle detection window.

For repeatable sizing, a nanoparticle detection feedback algorithm is executed to locate the nanoparticle peak contrast plane. This algorithm swings from −300 to +300 nm of the current location by 100 nm increments acquiring images. Each image is then processed to detect and size the detected nanoparticles. The optimal focal plane is determined by maximizing the number of particles within the desired particle size range. This algorithm in combination with the coarse focus requires less than 90 seconds, as shown in Figure 4.11.
Figure 4.11: SP-IRIS focusing procedure. The coarse focusing algorithm locates the correct plane using the on-chip reference region. This method is accurate within 1 µm. A nanoparticle feedback algorithm is then executed to maximize the number of particle detected.

These algorithms have shown high repeatability in clean data. Clean data is defined as spots without large debris. Nevertheless, nonspecific binding and salt deposits occur in non-ideal laboratory settings. Anticipating the situations where nonspecific binding will bias the high-pass filter autofocus algorithm to the wrong starting plane, the algorithm also performs shifting window detection. If the optimal plane is determined to be at either extreme of the current window, the focal planes being analyzed will be shifted. The new window of focal planes will be acquired and processed. Shifting the focal plane increases the robustness of the autofocusing but at a cost of time. To prevent runaway situations, the number of iterations is limited to four per spot giving a total analysis range of 2.2 µm to the nanoparticle detection feedback algorithm.

4.2.3 Platform Characterization

Clinical diagnostic tests are rarely performed with simple sample solutions. Typically, biomarker analytes are vastly outnumbered by endogenous proteins and macromolecules
found in unprocessed patient samples. Ensuring that the diagnostic assay can specifically and sensitively detect the target within a complex solution is imperative. The presence of other macromolecules can lead to nonspecific binding that decreases the test confidence. A proof-of-concept experiment showing specific detection of wtVSV spiked in 100% fetal bovine serum (FBS) was performed. To ensure that the constructed prototype operates in a comparable capacity to our laboratory system, this experiment is also used as a benchmark.

An antibody against the wtVSV surface glycoprotein (8G5 monoclonal antibody) and a negative control monoclonal antibody (specific for the Marburg virus glycoprotein) were arrayed onto the sensor that is functionalized with the copoly (DMA-NAS-MAPS) (Lucidant Polymers, Sunnyvale, CA, USA). The array was printed with the sciFlexarray S3 spotter (Scienion AG, Berlin, Germany) and consisted of five replicates of each antibody. After an overnight incubation, the surface was first treated with 50-mM ethanolamine (pH 8), then washed with PBS containing 0.1% tween-20 to remove any remaining unbound material followed by blocking for 1 h in a petri dish with 1% BSA with PBS. Finally, the sensors were thoroughly rinsed in DI water to remove salt. The images of the resulting antibody spots were acquired on both the prototype and laboratory platforms to determine the number of pre-incubation bound particles. Next, the sensor was incubated in another petri dish with undiluted FBS spiked with $5 \times 10^5$ plaque-forming units per milliliter (PFU/mL) of wtVSV for 2 h. Following the incubation, the sensor was washed with PBS and rinsed quickly in DI water. Finally, post-incubation images of the spotted array were acquired. Figure 4.12 shows the specific detection of
100 to 140 nm diameter particles in the post-incubation images of SP-IRIS prototype. wtVSV is rod-like; however, when imaging with non-polarized light, the average polarizability that is equivalent to a spherical particle with a 110 nm diameter is observed [66], [94].

The comparison of the two platforms was conducted via the detected density of the wtVSV particles on both the wtVSV-specific and Marburg-specific antibody spots. Figure 4.13 demonstrates that the prototype is able to detect virus particles trapped on the wtVSV-recognizing antibody with little to no binding on the non-specific spot. An equal density between the platforms illustrates no loss in sensitivity.

Figure 4.12: Specific virus capture and detection. The panels show detection of wtVSV (circled in green) for 0 and $5 \times 10^5$ PFU/ml concentrations for a 2hr incubation in a cropped area of $65 \mu m \times 65 \mu m$. 
4.3 Discussion and Conclusion

The development of a clinical microarray platform necessitates the classification of specific and non-specific binding. Labeled techniques have shown to have a distinct advantage over label-free techniques in specificity, but multiplexed assay design is complicated by making sure the labels do not affect the functionality of the protein and minimizing cross-reactivity. The additional incubation steps that increase the assay time are also undesired.

The SP-IRIS detection technique has been established as a label-free technique with high specificity. This technique is capable of detecting the diffraction pattern of single particles in a large FOV. Through analysis of the diffraction pattern’s contrast, information on the particle’s size can be extracted. With a priori knowledge of the target molecule’s size, the SP-IRIS method can perform specific detection via particle size
filtering. Nevertheless, performing accurate nanoparticle sizing is an operationally complex process as a result of the narrow, rapidly varying nanoparticle response.

Instead of requiring a highly trained operator, a prototype has been designed to automate data acquisition and processing. The automation of the acquisition and processing enables minimally trained or untrained user operation. The custom GUI has a simple 4-button interface and tabular readout for ease of use. This platform has two complementary focusing algorithms to achieve a precise focusing algorithm with 100 nm accuracy. The performance of this automated system was validated against the laboratory platform for wtVSV detection at a clinically relevant level ($5 \times 10^5$ PFU/ml) in undiluted FBS.
Illnesses occur when the balance of biological molecules in living organisms is disrupted by perturbation in functionality or concentration of biomarkers. Although many tools exist for profiling DNA biomarkers, protein diagnostic tools lack sensitivity and/or repeatability for clinical samples. These techniques are hindered by fluctuations in bioreceptor immobilization between protein microarrays and the greater level of non-specific binding.

This dissertation detailed the development of three practical platforms (IRIS, CaFE, and SP-IRIS) for high-throughput, sensitive, and repeatable protein microarray detection. The label-free IRIS platform exploited interferometric spectral reflectance imaging to quantify bioreceptor accumulation on the SiO₂/Si chip surface. The implementation of LEDs, an on-chip intensity monitoring region, and a linewidth analytical model in the 2nd generation IRIS instrument reduced the component costs to under $10k and the footprint to 9 in x 18 in x 4 in while maintaining the capacity to measure 1000s of proteins simultaneously with an intrinsic mass detection limit of 4 pg/mm².

The CaFE platform integrated IRIS with wide-field fluorescence imaging to quantify probe immobilization and discriminate between specific from non-specific binding. Two chip designs, ONC and CLC, have been designed for sensitive IRIS measurements as well as broadband and selective fluorescence enhancement, respectively. An integrated CaFE platform has been designed for all-inclusive measurements. The rationale for integrating the two technologies has been laid out and, since fluorescence requires a high
NA imaging system for sensitive measurements, an ASR analytical model has been developed for high NA IRIS imaging. The CaFE platform’s performance has been verified via a direct comparison with the IRIS and Genepix 4000B readers.

The SP-IRIS methodology offers a label-free method to distinguish between specific and non-specific binding. Labeled assays require careful development to prevent the fluorophore from altering the protein functionality in addition to extra time and reagents. For a simpler and faster assay while maintaining a high level of sensitivity and specificity, the SP-IRIS detects single nanoparticles on an interferometric surface and sizes the nanoparticles for filtering. An automated SP-IRIS platform has been designed to reduce operational complexity and remove user error. A custom GUI which controls the stages, camera, and LED, streamlines the user interaction to loading the chip, defining the microarray geometry, and clicking run.

5.1 Acceleration of SP-IRIS Focusing Algorithm

The SP-IRIS automated prototype is the first step toward a specific label-free technology for reliable assay detection. An advantage of this platform is the specificity arising from the analysis of the nanoparticle signal. Currently, the prototype can acquire and process a spot in under 2 minutes, see Table 5.1. This time was achieved after optimizing each algorithmic step for speed. A 2-minute per condition acquisition and processing time is only practical for assays up to 30 conditions (1 hour acquisition time). For higher throughput arrays, the acquisition time must be quicker. With each individual algorithm optimized for fast acquisition, random access memory (RAM) disk and region of interest (ROI) imaging methods were explored for further improvement.
Table 5.1: SP-IRIS software timeline. The times listed below are the average when the algorithms are optimized for speed. * This time is an estimate. ** Each image is full FOV.

<table>
<thead>
<tr>
<th>Process Description</th>
<th>Automated? (Y/N)</th>
<th>Time to complete (s)</th>
<th>Total time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power on</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>start-up test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Instrument Initialization</td>
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<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Load assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>information</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>User defines assay geometry</td>
<td>N</td>
<td>20*</td>
<td>50</td>
</tr>
<tr>
<td>Load SP-IRIS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chip</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>User places chip on sample holder and loads</td>
<td>N</td>
<td>30*</td>
<td>80</td>
</tr>
<tr>
<td>Focus on chip</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>surface</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locate chip surface</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>Locate spot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>array</td>
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<td></td>
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<tr>
<td>Determines chip lateral offset</td>
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<td>140</td>
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<tr>
<td>Moves to first assay spot</td>
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<td></td>
<td></td>
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<tr>
<td>Spot Focusing</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Coarse &amp; Fine focus</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Repeated for every spot</td>
<td>Y</td>
<td>139 ± 27**</td>
<td>279 ± 27</td>
</tr>
</tbody>
</table>

A RAM disk is a block of RAM that is treated as a disk drive for faster data read/write times. RAM access times, in general, are orders of magnitude faster than hard drive access times. Since the camera images are large, applying this technology may decrease the time required to load the images and process the data. Further experimentation revealed no measurable benefit from RAM disk.

ROI imaging, on the other hand, dropped the focusing 43.8% (79.3 ± 14.3 s). The fine focusing algorithm was the only process to benefit from imaging a smaller FOV because of the CCD camera selected. Typically, with less information to read out per frame, a camera can achieve a faster frame rate. The faster frame rate would have benefited all of the focusing algorithms. However, the selected camera on the prototype, the Point Grey Grasshopper 2, is an exception. Therefore, the benefit from ROI imaging is seen only in image processing during the nanoparticle feedback focusing.
The downside to ROI imaging is the reduction in the number of particles in the FOV for the feedback algorithm. For accurate focusing of the sample, at least 30 nanoparticles must be present in the image. In biological experiments, it is not feasible to assume this kind of coverage on every spot especially since control spots should be clean. As an alternative, reference particles (RPs), 180 nm diameter polystyrene beads, were co-spotted onto the chip surface with the bioreceptors. These particles provide the needed surface coverage for ROI focusing without inhibiting detection, Figure 5.1

![Figure 5.1: RP coverage experiment. Multiple concentrations of RPs were spotted onto the surface to determine the necessary spotting concentration to achieve the required surface coverage. Afterwards, VSV was incubated onto the surface to make certain the RPs do not inhibit the detection of the target molecules.](image)

In conclusion, the first iteration of the SP-IRIS focusing algorithm laid the groundwork for future designs. The current 2-minute per condition acquisition time, although automation, is not sufficient for large assays. Two methods were explored to decrease the acquisition time. The first method, RAM disk, showed no improvement and, thus, eliminated the read/write times of the hard drive as the limiting factor. The second method, ROI imaging, improved the spot focusing time 43.8%. Further investigation into these gains brought to light a flaw in camera selection. The reduction in time came
exclusively from image processing. Image acquisition showed no change contrary to expectations. Therefore, ROI imaging would have provided even greater improvement for a difference camera. Finally, the implementation of RPs to guarantee robust focusing in the abbreviated FOV was discussed. A viral assay was performed to determine the needed spotting concentration, $10^{11}$ particles/mL, and verify the RPs do not affect virus detection.

5.2 Accurate Nanoparticle Sizing

Although the SP-IRIS platform has been demonstrated to perform accurate sizing of polystyrene beads (\(~2\%\) error from the nominal mean report by the manufacturer [67]), further experimentation has shown a sizing dependence on the SiO$_2$ layer thickness, see Figure 5.2. The dependence of the observed nanoparticle contrast on the SiO$_2$ thickness does not affect manual experiments as the user can adjust the data processing algorithm appropriately. However, since the accuracy and robustness of automated focusing relies heavily on nanoparticle sizing, either the SiO$_2$ thickness must be measured then referenced with a multi-dimensional sizing algorithm or sample fabrication must have less than 1\% variation. As a stringent manufacturing process will significantly increase chip fabrication costs, the effect of SiO$_2$ on the particle sizing should be modeled and a multi-dimensional sizing curve should be used.
Figure 5.2: SP-IRIS Nanoparticle Sizing Dependence on SiO$_2$ thickness. Two size populations of polystyrene beads (110 nm and 173 nm diameter) were spotted with 4 replicates onto various SiO$_2$ thicknesses. The average and standard deviation of each replicate spot for both sizes is shown. A SiO$_2$-dependent particle contrast is observed.

5.3 **Calibrated Single Particle Detection Platform**

The design and fabrication of an integrated IRIS and SP-IRIS platform, called calibrated single particle (CaSP), would enable same platform calibration of the particle detection signal. The CaFE platform illustrated the relationship between fluorescence signal and the immobilized probe density. This relationship is not confined to fluorescence detection as a varying probe density affects the binding capacity of the spot [6]. Additionally, as discussed in Section 5.2, the SiO$_2$ thickness must be quantified for accurate nanoparticle sizing.

Many parallels can be drawn between the CaFE and CaSP platforms. The illumination path of both SP-IRIS and IRIS technologies can be combined into a single Köhler illumination. Unlike fluorescence, SP-IRIS does not require a high-powered LED; however, the slight offset of each LED alters the illumination profile at 0.8 NA resulting in perturbation of the nanoparticle signal. Therefore, there must be two illumination sources which are combined using a 50:50 non-polarizing beamsplitter. In addition to
separate light sources, mounting two objectives on a turret is recommended for imaging. At high NA, multiple spectral sources become problematic because of the small depth of focus and chromatic aberration in the objective. However, if a common objective is highly desired, a focusing feedback system with an algorithmic or optical, like the Nikon perfect focus system, could be developed. A preliminary design of the CaSP system is shown in Figure 5.3.

Figure 5.3: CaSP Platform Preliminary Design. A 50:50 beamsplitter couples the two illumination sources into a Köhler illumination system. An objective turret allows for switching between the SP-IRIS high magnification, high NA objective and the IRIS low magnification, low NA objective. A motorized stage is used for focusing and chip translation. The imaging path is a 4f system with objective and tube lens.

5.4 Point-of-care Diagnostics

The efforts in designing compact, low-cost, easy-to-use platforms have opened up new applications for this technology. One particular market of interest is point-of-care
diagnostics. The POC diagnostic device market – devices which operate outside of a laboratory with minimal to no user-training – is one of the fastest growing sectors of the medical industry [102]. The rapid market growth in this sector as compared to traditional diagnostic tests is attributed to the paradigm shift toward targeted early disease detection, therapy, and sample-to-answer automation [103]. For time critical situations, commercially available rapid diagnostic tests take the form of lateral flow immunoassays, which suffer from low sensitivity [104]. The lack of a sensitive and specific rapid test in the primary care setting requires clinical procedures to include a second confirmatory test conducted in a laboratory causing delays in diagnosis, prolonged recovery, and an increased chance of disease transmission [105]. The development of a compact system capable of laboratory quality measurement would represent an invaluable tool in the fight against diseases [106].

The challenges in POC tests are achieving the correct level of operational complexity and safety [107]. Ranging from the hospital staff performing in-patient testing to the lay person doing self-testing, the first task for POC applications is identifying the target environment and user. Once the target tier is known, test-specific criteria must be identified (e.g. platform portability, power consumption, platform costs, operational complexity, disposable costs, disposable storage & shelf-life, assay reagents, and acceptable assay false negative and positive rates).

5.4.1 2nd Generation CaFE Design

The 1st generation CaFE platform outlined the design criteria of a dual IRIS and wide-field fluorescence reader. Further engineering of the platform can follow two diverging
paths: (1) a standalone platform for an automated all-in-one sample-to-answer reader or
(2) a modular unit for existing fluorescence readers.

The development of a standalone platform for complete sample-to-answer measurements would enable POC tests. In POC applications, operational simplicity and operator safety are crucial. The patient sample must be isolated from the user for safety and to prevent contamination. The standalone platform will require the implementation of an automated XYZ platform for focusing and indexing, a microfluidic cartridge, and fluidic controls to automate the assay. Although these items are not trivial, they have been addressed for similar technologies [14], [95].

The modular unit for existing fluorescence readers would escalate the value of this technique to other researchers in the field. The main difference between the CaFE platform and the typical wide-field fluorescence microscope is the light source. Wide-field fluorescence microscopes typically use one of four types of light sources: a xenon arc lamp, a mercury-vapor lamp, a high-power LED, or a supercontinuum source. All of these sources require Köhler illumination or a similar optical path. Therefore, an illumination housing which unifies the fluorescence and the IRIS sources onto the same optical path would allow for the transition from a fluorescence reader to a calibrated fluorescence reader.

5.4.2 SP-IRIS for Point-of-Care Diagnostics

The efforts in designing an automated acquisition and processing instrument have enabled SP-IRIS to approach a new market, POC diagnostics. Although the SP-IRIS prototype provides automated operation, further reduction can be achieved in the power
consumption, footprint, complexity, and cost. The improvements will be seen by incorporating specialized components.

The current prototype is controlled by a laptop, communicates to the component over Ethernet via a router, and uses a scientific CCD camera for acquisition. The footprint of the platform is 17 in x 11 in x 8 in. Instead of having these separate components, a custom embedded system could be designed.

As a proof-of-concept, a manual SP-IRIS instrument has been designed with priority given to cost and footprint, shown in Figure 5.4. The new platform implements a smartphone (Nexus S, Samsung, Seoul, KR) in lieu of a camera and laptop. This redesign decreases the footprint to 15 in x 6 in x 4 in and the cost to under $4k. A custom software application has been written by Konrad Szupinski on the Android 2.3 “Gingerbread” operating system to control the LEDs via the headphone port and the camera.
The detection capability of this platform was tested using a VSV chip and a 20x 0.46NA objective. As shown in Figure 5.5a, the smart platform was able to resolve many diffraction limited particles and salt clusters; however, the image is out-of-focus. Comparing the particle locations between the smart phone system and the benchtop system, Figure 5.5b, many of the diffraction-limited particles are seen to match up. Before conclusive evidence can be obtained on the smart phone system, two design changes are needed. First, the mechanical vibration in this system prevents averaging more than 10 frames, an SNR of approximately 230. As shown in Figure 5.6, an SNR of 400, approximately 30 images for the Nexus S, is needed for accurate particle sizing. The source of the vibration is the smart phone. Anchored to the system by only 4 cage rods,
the smart phone is susceptible to shaking. Second, the smart phone preview function does not have zoom capability preventing accurate manual focusing. Either new software must be written to enable zooming while previewing or an auto-focusing system should be integrated into the platform.

Figure 5.5: Mobile SP-IRIS image. (a) After manually focusing, an image was taken of a VSV chip on the smart phone system. Although the image is out-of-focus preventing verification, this image shows the detection of large salt chucks as well as diffraction-limited particles believed to be virus. (b) An image from the benchtop system of the same spot. Many of the same particles are seen in both images. However, further alignment is testing is needed for conclusive evidence.

Figure 5.6: SNR analysis of SP-IRIS detection. First order monte carlo simulations were performed to estimate the error in the detected nanoparticle contrast signal for a given image SNR. For these simulations, a system with a 2.2 μm square pixel (size of a smart phone camera), a 0.8NA 50x objective, and a 532 nm illumination source imaged a 100 nm polystyrene bead on a 100 nm SiO₂ layer. For accurate sizing, an SNR of at least 400 is recommended.

In conclusion, the results from this system encourage further investigation into
developing a POC diagnostic test based on the SPIRIS method. The proof-of-concept platform demonstrates the viability of utilizing an embedded system for nanoparticle detection, but a direct comparison between the smart phone system and the benchtop platform cannot be conducted until the smart phone system's rigidity and the focusing issues are resolved. Addressing these issues in the next iteration system would enable the detection and sizing nanoparticle on an almost hand-held system.
Appendix A

IRIS QUANTIFICATION EXPERIMENTAL PROCEDURE

Summary. To determine the conversion factor between SiO$_2$ thickness change and mass accumulation, purified BSA suspended in milli-Q water was spotted onto the surface. After allowing the water to evaporate, the signal from a known mass was measured.

Procedure. BSA was purified through dialysis, lyophilized, and re-dissolved in milli-Q water at a concentration of 2 mg/ml. Then, the BSA was serially diluted in milli-Q water using high precision pipettes from Gilson (Middleton, WI). The solutions were spotted using a Scienion SciFlexarrayer S5 piezoelectric arrayer (Berlin, Germany) equipped with the sciDropVOLUME software, a tool to measure the absolute volume of the droplets dispensed by a SciFlexarrayer piezo nozzle. For each spot, a photograph of the droplet in midair is used to calculate the spotted volume. Based on the spot volume and concentration of the BSA solution, 25 pg to 400 pg of BSA was spotted onto the surface. IRIS measurements were made on the sensor's surface after water evaporated leaving behind only the protein on the surface.

Analysis. The fabricated samples were imaged and processed using the IRIS platform. The processed images are analyzed by summing the pixels defining the spot and multiplying the area of analysis. Extracting the slope of the linear curve relating the spotted mass (ng) to the weighted sum of the IRIS thickness (nm$\times$m m$^2$) yields the scaling factor between SiO$_2$ thickness and biomass density.
Appendix B

IRIS IN THE CLASSROOM

**High School Outreach.** Passing knowledge to younger generations is one of the most critical and difficult aspects of science. Working alongside the Boston University Assistant Dean of Outreach and Diversity, educational activities centered around using discrete LEDs to illuminate a SiO2 on Si chip with nanometer deep etched features has been developed. The goal of this activity is to teach students about thin-film interference.

Low-cost IRIS detectors have been developed specifically for these outreach activities, Figure 5.7. By removing the translational stage, reducing the optics to a Nelsonian configuration, and implementing an eye-piece rather than a camera brought the cost below $800 per unit. These changes sacrificed the quantitative ability of the microscope, but observation is the goal of this project. The optical and mechanical parts used are off-the-shelf components and the LEDs are controlled with a custom electronic PCB for manually tuning the brightness.

**Laboratory Module: EC 481.** The Fundamentals of Nanomaterials and Nanotechnology aims to introduce undergraduate students to state-of-the-art optical and electronic techniques that deal with measuring or manipulating matter on the nanometer scale. To aid in the understanding of biological sensors and novel detection modalities, an IRIS laboratory module has been developed to introduce the concept of label-free detection. As shown in Figure 5.8, a stripped down version of the IRIS platform for classroom learning has been developed.
Figure 5.7: IRIS detector for demonstrating thin film interference at a low-cost
Figure 5.8: IRIS module for classrooms. This design implements a lower quality sensor and removes all moving parts to improve the ease-of-use, an important feature for instructional courses.
Appendix C

IRIS Prototype

Advancing the IRIS technology towards a simpler platform and faster computation collimated in the development of a standalone, easy-to-use prototype. A multidisciplinary senior design team ('12) was tasked with developing a standalone, automated IRIS instrument. A Köhler illumination configuration, a 2 in beamsplitter, and a standard 50 mm macro lens attached to a camera are fixed on an aluminum plate for mechanical stability. As shown in Figure 5.9, the end result is a compact instrument (13 in x 7 in x 4 in) with simple GUI and a large FOV (7.8 mm x 5.8 mm which corresponds to a maximum array of >1000 assuming a 200 μm spot pitch). Through the implementation of the graphics card processing algorithm and an automated spot detection algorithm, the acquisition and processing time has been decreased below 2 minutes on a laptop. With the development of a portable instrument capable of rapid measurements, the IRIS platform has reached a state where translation to commercial applications.
Figure 5.9: IRIS standalone prototype. (a) The prototype with the acrylic cover removed. Internally, the optics and electronics are seen. The slide sits on top facing down. (b) A screenshot of the custom software package for instrument control. This software requires few clicks for operation to initiate data acquisition and analysis.
Appendix D

IRIS MODEL FITTING USING CUDA

While the IRIS device has physically become more suitable for low-cost portable applications, the data processing necessary to compensate for the design changes tethers IRIS to a computing grid. In the spirit of moving towards a completely portable and field-deployable system, the time required for data analysis must be addressed.

Data fitting is the process in which the Levenberg-Marquardt algorithm is applied to the collected spectral data to minimize the sum-squared of errors (SSE) on a pixel-by-pixel basis. Since the same algorithm must be applied to each pixel, the fitting of an image benefits from a single instruction, multiple data (SIMD) computing architecture. GPUs have been developed precisely for SIMD situations. Compared to a CPU, which is designed to be flexible and perform sequential operations, GPUs are specialized for simple parallel computation. Written by Dylan Jackson, Boston University M.S. ’12, a CUDA implementation of the fitting process has shown remarkable performance gain, see Table 5.2.
Table 5.2: Performance of IRIS/CaFE fitting algorithms on multiple hardware platforms

<table>
<thead>
<tr>
<th>Hardware Type</th>
<th>Model Type</th>
<th>Convergence Criteria</th>
<th>Average Processing Runtime</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>(1e-8)*SSE</td>
<td>(1e-6)*SSE</td>
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<tr>
<td><strong>GTX 680 (CC 3.0)</strong></td>
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<td>CaFE</td>
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Advisors: Professor Dimitris Korakakis and Professor Jeremy Dawson
Design an optical platform for measuring the waveguide transmission
2007  Summer Research Assistant, Boston University, Boston, MA
Advisor: Professor M. Selim Ünlü
Designed and simulated alternative optical schemes for a biosensor to minimize the footprint and complexity while maintaining performance

Leadership Opportunities
2010–2013  Mentor, ECE and BME multi-disciplinary senior design teams
2012  Officer, Student Association of Graduate Engineers
2012  Co-organizer, CaFE GHECHO Symposium
2011–2012  Instructor, ECE Summer Challenge, Boston University

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Honors and Awards
2009–2013 Smart Lighting Engineering Research Center Fellowship
2013 Center for Integration of Medicine and Innovative Technology (CIMIT) Semi-
finalist in the nation-wide Primary Care Prize competition
2012 Center for Integration of Medicine and Innovative Technology (CIMIT) Semi-
finalist in the nation-wide Primary Care Prize competition
2012 Top 3 in Boston University 15k Business Plan Competition, NeXGen Arrays
2012 Placed 4th in Boston University 50k New Venture Competition, NeXGen Arrays
2011 NSF Accelerating Innovation Research (AIR) Award, Co-author
2010 Boston University Ignition Award, Co-author

Publications
Peer-Reviewed Journals


