# Digital detection of biomarkers for high-sensitivity diagnostics at low-cost

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## ABSTRACT

We have demonstrated Interferometric Reflectance Imaging Sensor (IRIS) with the ability to detect single nanoscale particles. By extending single-particle IRIS to in-liquid dynamic imaging, we demonstrated real-time digital detection of individual viral pathogens as well as single molecules labeled with Au nanoparticles. With this technique we demonstrate real-time simultaneous detection of multiple targets in a single sample, as well as quantitative dynamic detection of individual biomolecular interactions for reaction kinetics measurements. This approach promises to simplify and reduce the cost of rapid diagnostics.

**Keywords:** Optical biosensors, interferometry, digital detection.

## 1. INTRODUCTION

Due to the lack of molecular analysis devices, clinical diagnosis relied mostly on medical history and physical examination until about a hundred years ago. However, there are many diseases that exhibit indistinguishable symptoms, making direct diagnosis based on clinical presentation difficult. Clinical diagnostics date back thousands of years. Although the microbial cause of infectious diseases was understood over the last few hundred years, observable properties of bodily fluids were utilized in diagnostics much earlier. Doctors have always sought complementary validation and rudimentary clinical tests such as the color and odor of urine were utilized in diagnostics. In modern medicine, in vitro tests are indispensible components of clinical practice with the sensitivity of standard immunoassays measuring protein biomarkers at picomolar concentrations [1]. This level of sensitivity is sufficient for the diagnosis of infectious diseases when clear symptoms are present, however, it falls short – perhaps by a factor of many thousands – for the detection of proteins that are important in cancer [2], neurological disorders [3], and the early stages of infection [4]. Devastating epidemics have exposed the limitations of current technologies and emphasized the importance of continuing innovation and refinement of in vitro diagnostics – especially at an affordable cost.

Synergistic collaboration of medicine with engineering has been crucial for the advances in disease diagnostics. Often most sophisticated diagnostic tools are available first in the wealthy populations and only after years of engineering and manufacturing improvements they become available at a manageable cost. Our efforts in molecular diagnostics aim at making the most sensitive and specific technology available at a low-cost from the very beginning. Perhaps one of the most exciting recent technological developments in biomarker analysis is single-molecule counting or digital detection, an approach that provides resolution and sensitivity beyond the reach of ensemble measurements [5, 6]. Digital detection not only provides very high sensitivity, but also has the potential of making the most advanced disease diagnostic tools available at low cost. This is precisely our technological development goal. One can draw an analogy from the transition of audio recording industry to digital media. Prior to digital recording, the sound quality from vinyl analog media depended on the sophistication of the player accurately reproducing the precise analog signal. An expensive (high fidelity) reading instrument was required to accurately transduce the analog recording to electrical signal. Following the digital recording of audio on compact discs, sound quality no longer depended on the reader because it is easier to measure the presence or absence of signal than to detect the absolute amount. Reading a binary recording (1s and 0s) or detection of single particles, when possible, is easier than precise measurement of the ensemble quantities [7].

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## 2. INTERFEROMETRIC REFLECTANCE IMAGING FOR SINGLE PARTICLE DETECTION

We have demonstrated an optical imaging technique termed Single-Particle Interferometric Reflectance Imaging Sensor (SP-IRIS) with the ability to detect single nanoscale particles [8]. The technology is based on interference of light from an optically transparent multi-layer dielectric structure. The specular reflection of incident light from the dielectric structure provides the reference field that interferes with the scattered light from the nanoparticles on the surface. We have adapted a thermally-grown oxide thin film on top of a Si substrate to provide the interference enhancement. The interference of light reflected from the sensor surface is modified by the presence of particles producing a distinct signal that reveals the size of the particle that is not otherwise visible under a conventional microscope. Using this simple platform, we have demonstrated label-free identification of various viruses in multiplexed format in complex samples. Size discrimination of the imaged virions allows for rejection of non-specifically bound particles to achieve a limit-of-detection competitive with the state-of-the-art laboratory technologies. We have demonstrated the simultaneous detection of Ebola and Marburg VSV pseudotypes in serum or whole blood [9]. SP-IRIS has also shown promising results for detection of protein [10] and DNA molecules labeled with small Au nanoparticles – showing attomolar sensitivity and meeting the requirements for most *in vitro* tests.

### 2.1 Operation Principles and Optical System

Detection of small nanoparticles using optical microscopy methods presents unique challenges. Most significantly, natural nanoparticles of interest, such as virions, are much smaller than the wavelength of light in the visible spectrum and thus beyond the resolution ability of optical microscopes. Since we can approximate the nanoparticles as point scatterers, the contrast of the particles depends very strongly on the size of the particle, proportional to the scattering cross-section:

$$I_{det} \propto \sigma_{sca} \propto \left|\frac{\epsilon_p - \epsilon_m}{\epsilon_p + 2\epsilon_m}\right|^2 R^6 \qquad (1)$$

The scattering cross section also depends strongly on the dielectric index of the nanoparticle $\varepsilon_p$ and of the medium $\varepsilon_m$. For small nanoparticles, the scattered field is vanishingly small compared to background noise and thus particles such as viruses cannot be observed under conventional microscopes without labels. Detection in liquid environment is even more challenging due to reduced dielectric contrast of the particle with respect to the surrounding medium.

The basic concept of SP-IRIS is shown in Figure 1. To detect nanoparticles, SP-IRIS shines light from visible LED sources onto the sensor surface, which consists of a silicon dioxide layer on a silicon substrate**.**

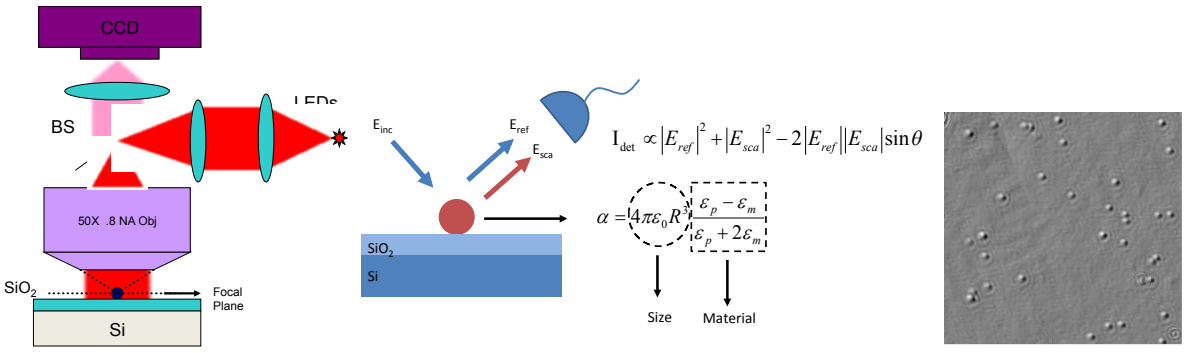



Figure 1. SP-IRIS is a simple optical microscopy system. A visible LED provides illumination and bright field reflection image is captured on a CCD camera **(left)**. The key to improved visibility of nanoparticles on the SP-IRIS system is mixing of the scattered light with reference field reflected from the Si surface **(middle)**. The interference signal can be orders of magnitude larger than the scattered intensity (middle term) for small low-index nanoparticles. Oxide thickness is chosen to get largest cross-term for a particular color (wavelength) of illumination resulting in enhanced visibility of surface bound nanoparticles (viruses). Detected particles appear as diffraction limited dots on the image and the contrast of the dots can be correlated to their sizes. Image shows 100nm polystyrene particles in an SP-IRIS image **(right)**.

In this approach the dielectric layered structure acts as an optical antenna optimizing the elastic scattering characteristics of nanoparticles for sensitive detection and analysis. Then, the intensity of the detector depends on both scattered and reference (specular reflection) field intensities, which interfere according to their phase difference given by path length difference between the scattered light from the nanoparticle and the reference light can be expressed as:

$$I_{det} \propto \left|E_{ref} + E_{sca}\right|^2$$
$$I_{det} \propto \left|E_{ref}\right|^2 + |E_{sca}|^2 + 2\left|E_{ref}\right||E_{sca}|cos\theta \quad (2)$$

The interference signal in Eqn (2) can be orders of magnitude larger than the scattered intensity (middle term) for small low-index nanoparticles. Oxide thickness is chosen to get largest interference contrast for a particular color (wavelength) of illumination resulting in enhanced visibility of surface bound nanoparticles. While viruses are natural nanoparticles and SP-IRIS can detect those without labels, for much smaller particles such as individual protein and nucleic acid molecules additional nanoparticle labels are required. The advantage of SP-IRIS over other labelled detection techniques for molecular detection lies in its ability to detect small and non-fluorescent nanoparticle labels. Thus, the resulting detection modality is digital allowing for counting of individual biomolecular interactions. We have described the two modalities of SP-IRIS in detail in earlier publications [8,9,11] and recently we have described the detailed physical modeling of detection and characterization of nanoparticles on SP-IRIS platform [12].

### 2.2 In-liquid detection

As discussed above, reduced dielectric contrast when the dielectric nanoparticles (such as viruses) are surrounded by liquid presents a significant challenge in detection. Compared to dry measurements, the contrast of viral particles is reduced by a factor of about 3 when they are imaged in a liquid such as buffer, serum, or plasma. Despite the reduced contrast and additional optical imaging challenges in a microfluidic cartridge, it is an essential requirement for applications in infectious disease diagnostics. It would be desirable to have the assay and the target blood sample confined in an enclosed cartridge. Recently, we have overcome the challenges of in-liquid detection and demonstrated dynamic detection of viruses in undiluted fetal bovine serum [13].

## 3. RESULTS AND DISCUSSION

Earlier, we have demonstrated identification of virus particles on washed and dried sensor chips capture from complex samples for replication-competent wild-type vesicular stomatitis virus (VSV), defective VSV, and Ebola- and Marburg-pseudotyped VSV with high sensitivity and specificity. Size discrimination of the imaged nano-particles (virions) facilitates elimination of nonspecifically bound particles to achieve a limit-of-detection (LOD) of $5x10^3$ PFU/mL (< 10 atto-molar of viable virus) for the Ebola and Marburg VSV pseudotypes [9].

For "digital" detection of individual protein and nucleic acid molecules, SP-IRIS utilizes Au nanoparticles as labels. Since we can detect Au nanoparticles as small as 20nm, which is only about twice the hydrodynamic diameter of an antibody, single molecule counting can be achieved without significantly altering the affinity of molecular interaction. Using this approach, we have shown detection of protein biomarker, β-lactoglobulin, in unprocessed serum and human whole blood with a detection limit of 60 aM and 500 aM, respectively [10].

Recently, the ability to image, detect and identify viruses in a liquid environment as they are captured on the sensor surface led to real-time detection. In this modality, we can distinguish individual binding events temporally and spatially. Time resolution requirement is closely related to frequency of binding events and a high temporal resolution is not needed for low-concentration samples that produce infrequent binding events. Thus, for a detection platform aimed at low-concentration samples a 30 s time resolution was considered sufficient.

To test this modality, a simple microfluidic flow cell with an optical window was used to allow imaging of the virus capture during sample incubation. External fluidic connections on the chip allowed fluid to be driven across the chip sensor surface by a syringe pump before exiting to a waste reservoir. Figure 2 presents a summary of the concept illustrating multiplexed detection of viruses and molecular biomarkers. Through integration with microfluidics, SP-IRIS may be implemented for critical point-of-care applications such as detection of biomarkers from unprocessed blood for early diagnosis or sample-to-answer molecular diagnosis in resource-poor rural settings for infectious disease control and containment during an outbreak.

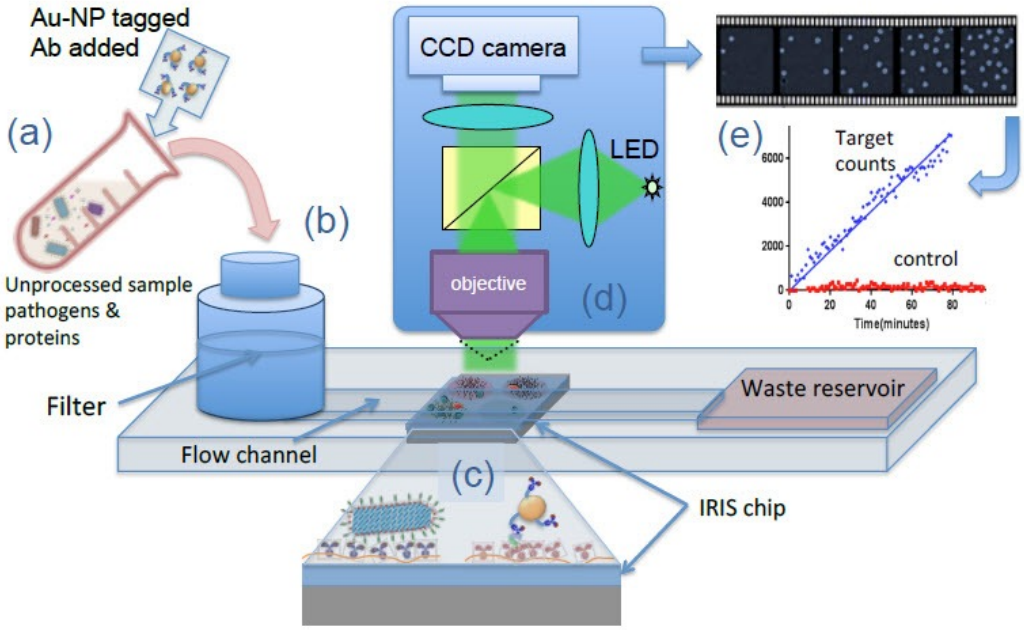
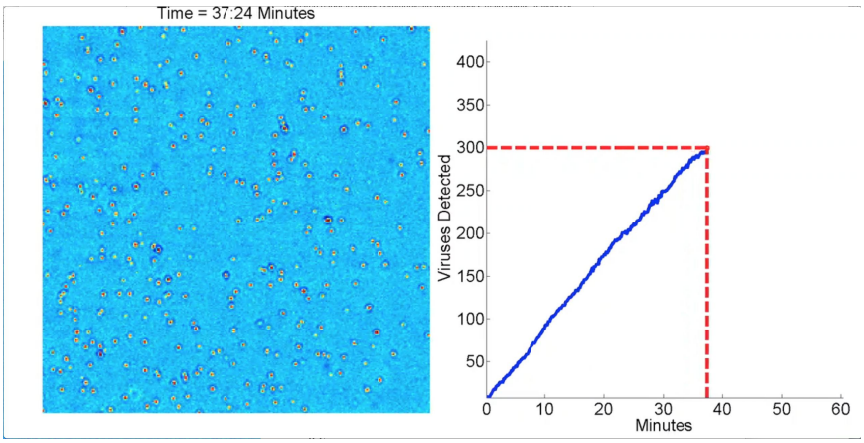



Figure 2. Concept of SP-IRIS for a diagnostic assay. (a) An unprocessed sample (E.g., serum or whole blood) suspected of containing the target pathogens and molecular biomarkers is mixed with nanoparticle conjugated secondary antibodies. (b) Sample is introduced into a microfluidic cartridge to facilitate incubation with the sensor chip (c) that has a multiplexed array of capture probes. After incubation with the target solution, SP-IRIS instrument (d) captures images allowing for direct visualization of pathogens and nanoparticle labeled individual molecules (e) that yields visualization of individual nanoparticles and quantitative counts of multiplexed targets.

By detecting individual viruses, this approach represents the ultimate limit of detection sensitivity. To demonstrate the capability of this technique for virus analysis, we showed real-time label-free detection of an Ebola model at100 PFU/mL in less than 30 min directly in serum without the need for additional sample purification or amplification in a low-cost polymer based cartridge [14].

Video 1 illustrates the real-time detection and counting virus particles as they are captured on the SP-IRIS chip. We have developed software for automatically processing the images and counting the particles on an antibody spot.




Video 1. A virus capture experiment running for 60 minutes is shown as a video composed of images acquired at 30 second intervals. Automatic particle identification and counting yields the number of viruses detected on the antibody spot.
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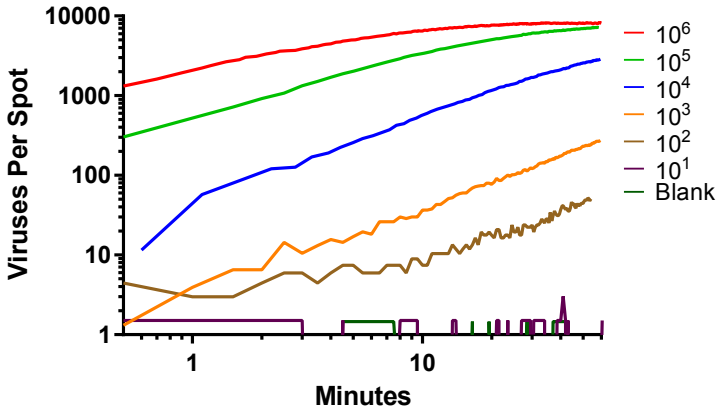


Figure 3. Counting of viruses on an antibody spot for a serial dilution ranging from $1 \times 10^6$ PFU/mL down to a blank sample. The high concentration samples show a very rapid accumulation of viruses followed by a saturation of the sensors. Background binding when incubated with a blank PBS sample is 1-2 viruses per antibody spot. Thus the limit-of-detection is determined by Poisson noise level and it is approximately 10 virus particle counts. A limit of detection of 100 PFU/mL in less than 60 mins has been demonstrated.

## 4. CONCLUSIONS

SP-IRIS offers a robust and low-cost platform for detection of a variety of targets such as proteins, nucleic acids, and whole viruses in a simple assay format and with high sensitivity (at single pathogen or molecule resolution) from complex samples with minimal sample preparation. With in-liquid, real-time detection, we have demonstrated a single-step assay. Basically, a serum sample is introduced to the microfluidic cartridge and captured viruses are counted after an incubation period without a wash step. The system is enclosed providing protection for healthcare providers. The instrument is simple and can be produced at a low-cost. The chips are produced by well-established Si foundry technologies and can be at a negligible cost. The microfluidic cartridges remain as the primary cost concern and we are exploring passive / disposable cartridges.

Another challenge for low-cost point-of-care applications is related to the shelf life of antibody microarrays and the ability to deliver custom chips immediately in case of an epidemic. Recently, we demonstrated feasibility of DNA-directed antibody immobilization for detection of individual viruses on a microarray surface using the SP-IRIS platform [14]. DNA microarrays are easier to prepare than protein microarrays and are highly reproducible. Furthermore, DNA chips can be stored at room temperature for an extended period of time without denaturation. When there is need for viral diagnostics, especially in urgent outbreak situations, these DNA microarrays can be functionalized quickly according to the need thus providing an elegant solution for resource-limited settings.

The digital detection of proteins and nanobioparticles using microarray technologies is poised to impact diagnostics. Perhaps the future of all analytical measurements will be based on single-molecule detection and counting representing the highest achievable signal fidelity. With SP-IRIS platform, we have shown that *digital detection* – perhaps the future of diagnostics – can be implemented at low cost.

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