Analyzing DNA-protein interactions on a chip

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Abstract: Gene expression is regulated by multi-protein complexes binding to short non-coding regions of genomic DNA, called *cis*-regulatory elements. A long-term goal of genomics is to identify and annotate all essential elements of the genome. Most coding elements of the genome have been identified and annotated; however, most regulatory elements have not. This chapter describes a method for the high throughput identification of DNA-protein interactions to identify *cis*-regulatory elements.

Introduction

DNA protein interactions control gene expression

An important aspect of the analysis of genomic DNA is not only identifying its structure or sequence, but also its function. If its function is to encode sequences of amino acids, decoding it is trivial. The human genome encodes at least two important functions. The first, DNA sequence that encodes protein sequence, has now been almost completely identified. The second, DNA sequence that binds specific proteins, is almost completely unknown. In eukaryotes, the DNA sequence that binds specific proteins is known as *cis*-regulatory elements. Multi-protein complexes bind to these short, specific DNA sequences regulating the transcription process.

The human genome contains vast amounts of *cis*-regulatory elements, most of which have not yet been identified. These regulatory sequences are responsible for directing spatial and temporal patterns of gene expression, which affect metabolic requirements and developmental programs[1]. *Cis*-regulatory elements are generally less than 20 base pairs and usually located in the promoter region of the regulated gene. The regulatory elements can be located upstream or downstream from the transcription start site.

The simplest binding model involves a molecule (a particular region in a single protein tertiary structure, P) that can bind to a specific binding site (a specific sequence of DNA, D) and form a resulting complex, C, which can enhance or repress expression.

$$P + D \rightarrow C$$

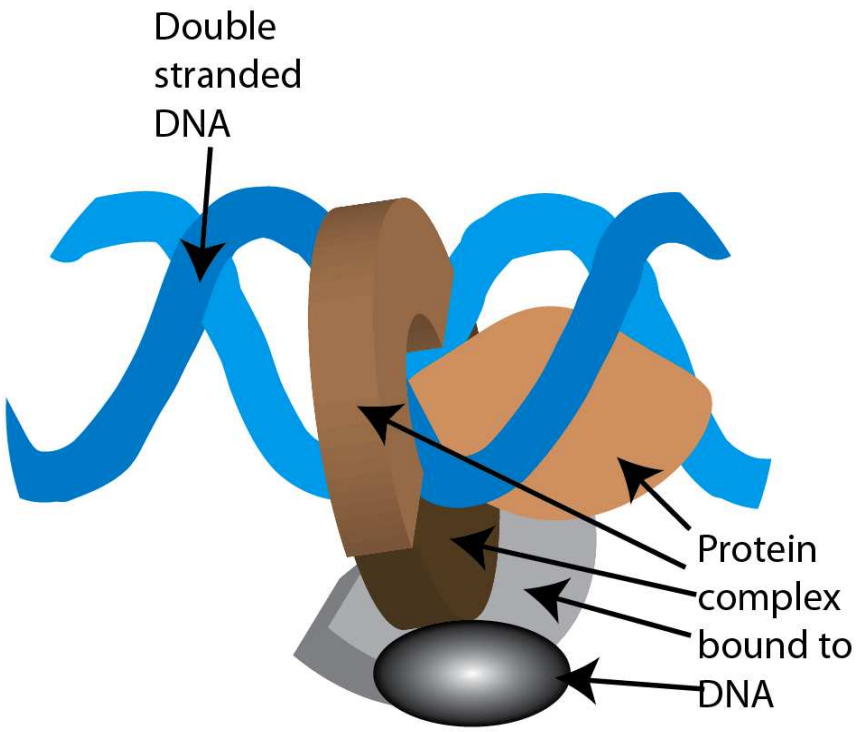

Figure 1 This schematic diagram depicts a multi protein complex binding to a short, specific sequence of genomic DNA known as a *cis* regulatory element. This binding enhances or represses gene expression. Identifying these *cis*-regulatory elements is a challenge, since the identity of the proteins in the complex are unknown.

Their interactions with the targets range from the highly specific (P binds a single site) to the nonspecific (P binds most sites)[2]. Gene expression may be regulated through a combination of multi-protein complexes binding to several distinct elements of the promoter, and cooperative interactions, as shown in Figure 1. A comprehensive identification of the location and relative strength of all these *cis*-regulatory elements on every gene is key to understanding the composition and function of the regulation networks that carry out the essential processes of living organisms[3].

As a result, the development of general and efficient assays of DNA-protein interactions has been extensively studied[4]. However, the ability to identify and predict functions of the *cis*-regulatory elements is limited. Many different approaches to this challenging problem have been tried.

Approaches to identify *cis*-regulatory elements

In general, the approaches developed to identify and predict functions of *cis*-regulatory elements fall into two classes: experimental and computational. Experimental approaches focus on the identification of regulatory elements for individual genes. Computational approaches focus on scanning large data sets to identify *cis*-regulatory elements.

Experimental strategies for identification of cis-regulatory elements

The search for *cis*-regulatory sequences generally involves various trial-and-error strategies. Traditional experimental approaches to identify regulatory elements for individual genes include: (1) Generation of deletion constructs to determine the minimal sequences necessary for transcription in cell-culture-based systems; (2) Gel shift assays, in which DNA molecules binding to proteins migrate more slowly in the gel relative to the samples with no protein[5]; to determine the sequences that bind various regulatory proteins (3) DNA footprinting assays to identify a region of DNA protected from digestion by DNAse I by a bound protein (usually a transcription factor)[6]. These methods generally require labeling with a fluorescent or radioactive tag. The procedures are complicated and time-consuming.

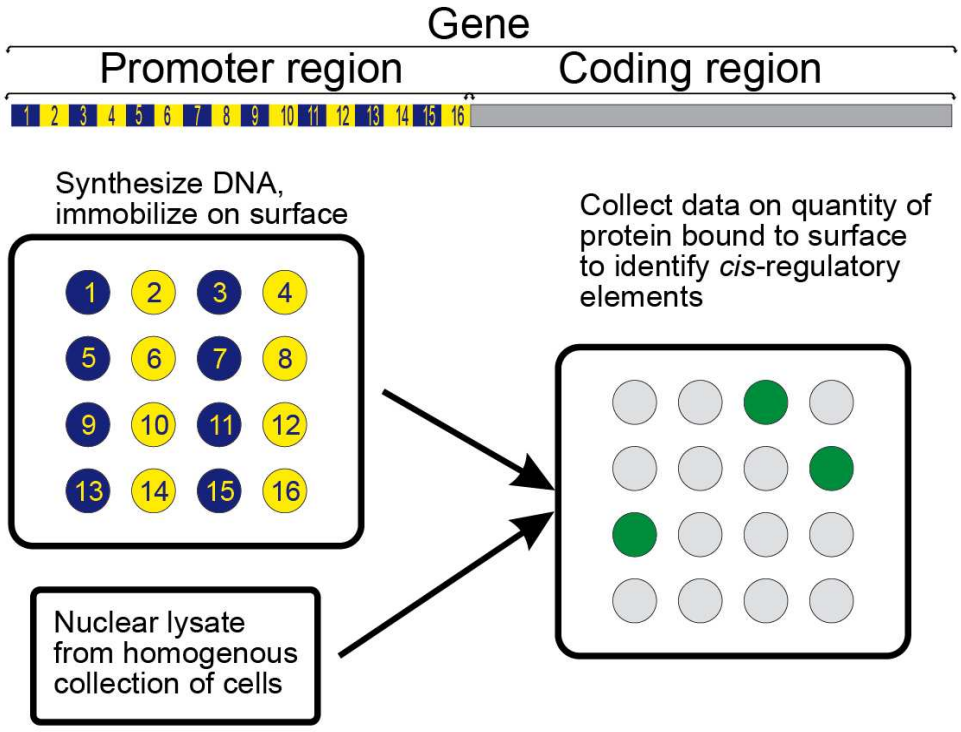

Figure 2. A schematic diagram showing a strategy for identifying *cis*-regulatory elements. The promoter region of a gene is split into small overlapping subsections, each about 20-40 bp long. These DNAs are synthesized and immobilized onto physically distinct areas of a surface. Nuclear lysate from a homogeneous collection of cells is incubated on the surface. Simultaneously, the amount of protein bound to the surface is monitored. Surface areas exhibiting high levels of bound protein will indicate regions of the promoter where protein complexes bind. These are *cis*-regulatory elements.

Surface plasmon resonance-based assay to identify cis-regulatory elements

With a purely computational approach, uncertainty remains as to whether a predicted *cis*-regulatory element actually possesses the expected function [7, 8]. With a purely experimental method, it is difficult to predict *cis*-regulatory elements on a large scale. The union of experimental and computational strategies is a new trend in deciphering *cis*-

regulatory codes [9]. The most successful of these approaches to date appear to be those that rely on gene expression profiles from DNA microarrays [10, 11].

However, these approaches are all indirect methods. A direct approach, which identifies DNA elements that bind to nuclear protein, is better, because this approach is more likely to provide an accurate measurement. Many different assays exist for measuring DNA/protein binding, but the identification of *cis*-regulatory elements has specific constraints:

(1) The assay needs to work with raw nuclear extract rather than an isolated and purified protein.

(2) The assay should allow parallelization or use other approaches to enable high throughput measurements.

A surface plasmon resonance based assay satisfies these requirements. In the surface plasmon resonance assay, the first biomolecule, DNA, is immobilized onto a surface, and a change in signal occurs if a second molecule or molecular complex binds to the first as shown in Figure 2. A surface plasmon resonance-based assay offers several advantages over other biomolecular binding assays: the assay can monitor binding rapidly, in real time, and without any labels; and information about specificity, affinity, and kinetics can be extracted from a surface plasmon resonance analysis.

Surface plasmon resonance-based assays

The physical principle known as surface plasmon resonance can be used to detect interactions of biomolecules. For instance, surface plasmon resonance has been successfully used to detect interactions between biomolecules with applications in various fields such as drug discovery [12], cell signaling[13], immunoassays[14, 15] and virology [16].

Surface plasmon resonance signals are proportional to the refractive index close to the sensor surface, and are related to the amount of bound macromolecules [17]. The main advantage of surface plasmon resonance over the conventional assays of molecular recognition, such as the enzyme-linked immunosorbent assay (ELISA), is its ability to provide continuous real-time monitoring and its ability to work without chemical labels. The specificity and kinetics of the interaction of the biomolecules can be extracted from a surface plasmon resonance analysis.

Several commercial surface plasmon resonance instruments are currently available [18]. The first and best known is the BIAcore biosensor made by BIAcore AB (Uppsala, Sweden) [19-21]. However, its size and cost (more than $100,000) limit its use. In the late 1990's Texas Instruments developed the Spreeta, an integrated electro-optical sensor package[18, 22, 23]. Spreeta utilizes surface plasmon resonance to measure the refractive index (RI) of liquid to monitor precisely the change in its composition. It can be adapted to detect DNA-protein interactions and obtain kinetic rate constants. This sensor is cost-effective (a single Spreeta sensor package costs only $20) and portable.

Using surface plasmon resonance to measure DNA-protein interaction

The physical principle of surface plasmon resonance

Surface plasmon resonance is based on a unique optical phenomenon [24]. The underlying physical principle is that at an interface between two transparent media (e.g. glass and water) with different refractive indices, the light coming from the side of higher refractive index is partly reflected and partly refracted.

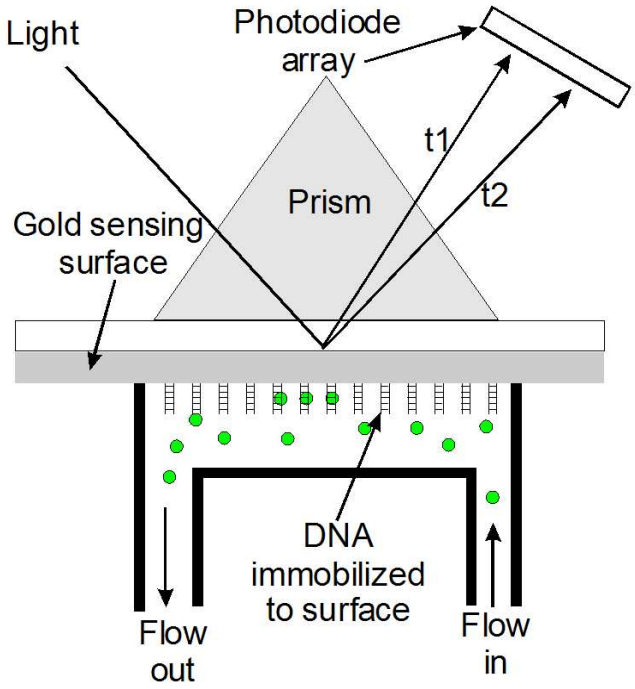

Figure 3. A schematic describing surface plasmon resonance. Light is aimed through a prism and reflects off the surface to a photodiode array. The line projected at time =t1 corresponds to the situation before binding of protein to the DNA on the surface and the line projected at time =t2 corresponds to the position of resonance after binding. The binding causes a shift in the angle of minimum reflection, where the resonance condition is satisfied.

Above a certain critical angle of incidence, total internal reflection is obtained without any refraction across the interface. In this situation, the electromagnetic field component penetrates a short (hundreds of nanometers) distance into a medium of a lower refractive index, generating an exponentially attenuated evanescent wave. If the interface between the media is coated with a thin layer of metal (e.g. gold), then waves, called surface plasmons, form from the oscillation of mobile electrons at the surface of the thin metal layer. The mechanism of surface plasmon resonance is related to the resonance energy transfer between the evanescent wave and the surface plasmon. If the light is monochromatic and p-polarized (meaning the electric field of the light wave lies in the same plane as the incident wave and the surface normal), and the wave vector of the incident light matches the wavelength of the surface plasmons, then surface plasmon resonance occurs.

Some of the energy from the incident light is transferred to the electron density waves in the metallic film, and therefore the intensity of the reflected light is reduced. The specific incident angle at which this occurs is called the surface plasmon resonance angle. This

surface plasmon resonance angle can be measured by observing the intensity of the reflected light at a range of different angles and then identifying the angle at which the intensity is minimized.

The surface plasmon resonance angle is affected by the amount and type of materials adsorbed onto the thin metal film. A satisfactory linear relationship exists between this angle and the mass concentration of biochemically relevant molecules such as proteins, sugars and DNA adsorbed to the surface. The angle can be monitored in real time. Therefore, it is possible to determine the analyte and ligand association and dissociation rate, and simultaneously detect interactions between unmodified proteins and directly measure kinetic parameters of the interaction. The surface plasmon resonance angle is also a function of the refractive index adjacent to the sensor surface and is often reported in units of refractive index.

Although surface plasmon resonance-based assays are typically performed one assay at a time, work is progressing on making these measurements amenable to parallelization. One approach is to immobilize different elements in physically distinct locations on a surface, and then to acquire a surface plasmon resonance image of the surface. This approach is feasible; several different surface plasmon resonance imagers have been developed[25-27]. The primary limitation to this approach is sensitivity.

Surface plasmon resonance imagers usually work fundamentally different from the Kretschmann geometry shown in Figure 3. Imagers generally measure changes in signal through changes in optical intensity. Most other surface plasmon resonance instruments use the Kretschmann geometry, which measures a change in angle. It is easier to measure a very small change in angle as compared to a small change in intensity. Hence, the Kretschmann geometry is significantly more sensitive than most current surface plasmon resonance imagers are. A challenge, then, is to design surface plasmon resonance imagers with sensitivity close to the Kretschmann geometry.

Tests using a Spreeta biosensor

To test this concept in a low throughput experiment, we used the Spreeta biosensor made by Texas Instruments to do real-time measurement of the interaction between protein and DNA. Spreeta was designed for applications in diagnostics, food/beverage quality and safety[19]. As compared with other instruments, Spreeta's main advantages are low cost and compact format. Figure 4 is a sketch of the Spreeta biosensor.

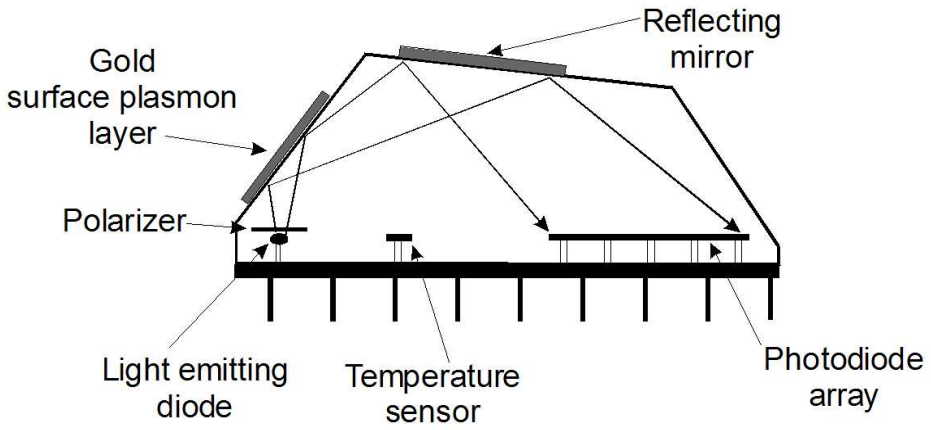

Figure 4 Sketch of Spreeta biosensor. The Spreeta sensor incorporates a LED, polarizer, thermistor, and two silicon photodiode arrays, all mounted on a printed circuit board. Components are encapsulated with a clear, optical epoxy. A surface plasmon layer (the sensing surface) and reflecting mirror surface are coated on the epoxy. The photodiode array measures intensity of light reflected at different angles. The integrated temperature sensor is necessary since the refractive index varies significantly with temperature, for water it varies by about 1 part in 10,000 per degree C.

A Spreeta biosensor occupies a few square centimeters. It consists of a light-emitting diode (LED), a sensing surface, and a light detector, which are integrated into a compact electro-optical package. Electrical connections are made to the sensor *via* pins at the bottom of the device.

When the liquid contacts the gold surface plasmon layer and the appropriate signals are applied to the pins, the sensor generates an output that corresponds to the refractive index of the liquid or the mass adsorbed on the surface. The output of the Spreeta sensor is a series of analog voltages, one per clock pulse, from which the refractive index of the liquid is derived when the voltages are digitized and processed.

Tests using a Spreeta biosensor (the miniaturized surface plasmon resonance system) were performed to evaluate the feasibility of the concept presented in Figure 1. A Spreeta evaluation module (EVM), which consists of a Spreeta biosensor, a flow cell with temperature compensation, and an electronic PC interface control along with comprehensive software, was used. The Spreeta biosensor is made by Texas Instruments, and the other three parts are made by Nomadics.

Experimental set up

A schematic diagram of the Spreeta is shown in Figure 4. All of the components are immobilized in an optically transparent material. Near infrared light generated from a light-emitting diode (LED) passes through a polarizer, reflects off the back of the gold sensing surface, and is then directed onto a linear array of silicon photodiodes. Each detection pixel corresponds to a narrow range of incident angles. The signals arising from the reflected light are monitored to determine the minimum signal intensity versus scattering angle, which occurs at the surface plasmon resonance angle.

In the sample handling and flow system of Spreeta, solutions are imported to a flow cell that is attached to the front of the sensor and then flow across the gold sensing surface. A rectangular channel cut into a rubber gasket defines the volume of the cell. The volume of the flow cell we used is 8 µl. The flow cell has an internal thermistor that is used for temperature compensation. Figure 5 shows the mechanism of how the flow cell works in the Spreeta evaluation module (EVM).

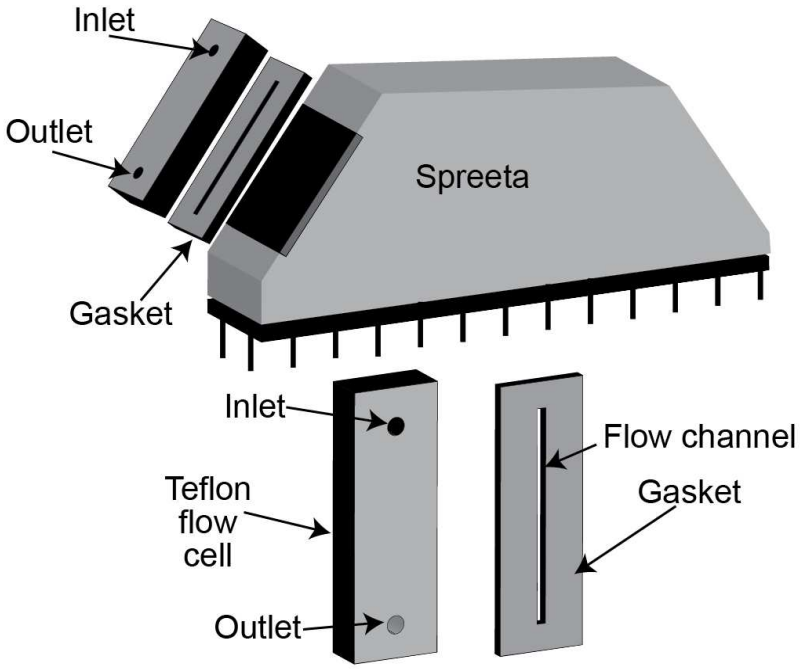

Figure 5. This drawing is a sketch of the fluidics in the Spreeta Evaluation Modules (EVM) made by Nomadics. A Teflon flow cell with two holes drilled through it is clamped onto a gasket to form a flow channel across the sensing surface of the Spreeta.

Our laboratory test bench consists of a computer, a VWR peristaltic pump, and the EVM, which includes a Spreeta biosensor, a Spreeta interface control box that communicates between the host computer and sensor, and an integrated flow cell mounted onto the Spreeta. The interface control module has a 12-bit analog to digital converter. It digitizes the analog signal from each pixel. The whole system has a resolution of about 8 x 10^{-7} refractive index units (RIU). According to the manufacturer, 11 x 10^{-3} RIU is equal to a change in the surface plasmon resonance angle of 1^{o}. Generally, 1 pg/mm^2 of adsorbed protein results in a change of 1x10^{-6} RIU (using Spreeta's measurement units) or a change of 1 RU (using the Biacore's measuring units).

Refractive index is a unitless number, but by convention and for clarity, it is given the dimensionless unit RIU. The resolution and noise level of this system is comparable to other commercially available surface plasmon resonance systems. The analysis software identifies the angle with the least intense reflected light. This angle is converted into RIU, which is recorded every ten seconds.

As compared with traditional time-consuming and costly methods, Spreeta has the advantages of simplicity, low cost, high efficiency, and real-time measurement. There

are several reports on the use of Spreeta to obtain information on biological interactions[17, 28-31].

Measuring DNA-protein interactions using the surface plasmon resonance sensor

The utilization of surface plasmon resonance measurements of DNA/protein binding has been previously published[32, 33]. In principle, the Spreeta should be able to detect transcription factor-DNA binding and other related information. We used the Spreeta to detect transcription factor binding and combine these results with conventional biochemical assays that measure actual transcription rates. In addition, the measurements were performed not only of the interactions between purified protein and immobilized DNA, but also of the detection of the binding between DNA and a multi-protein complex within the raw nuclear lysate.

Surface preparation

The method involves immobilizing a 16-mer biotinylated oligonucleotide on the sensor chip of Spreeta. The general immobilization scheme consists of depositing layers of biotin-BSA, streptavidin, DNA and protein, as shown in Figure 6.

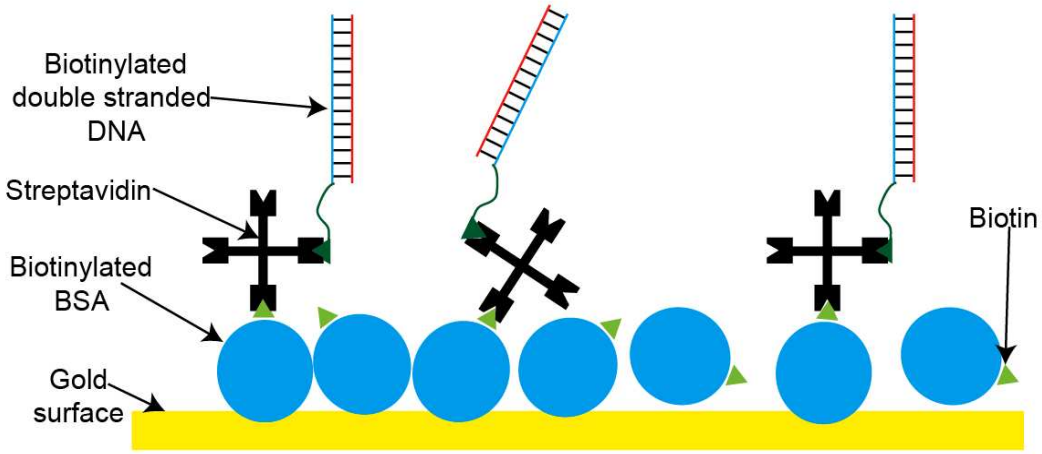

Figure 6. A cartoon schematic of the protein binding layers for the surface preparation chemistry. Biotinylated bovine serum albumin (BSA) binds non-specifically to the gold surface. Streptavidin, which has four biotin binding sites, acts as a linker between the biotinylated BSA and the biotinylated DNA. This prepares the sensor to detect transcription factor binding.

The streptavidin-biotin coupling is specific due to its extremely high binding affinity, K_a, which is about 10^{13} M [34]. Each streptavidin has four equivalent binding sites for biotins. Streptavidin binds biotin rapidly and the bound complex tolerates a range of temperature and pH conditions. Because of the ease with which a wide variety of molecules can be chemically modified with biotin, the streptavidin-biotin coupling has been extensively used in many different applications in biotechnology [35].

The utilization of surface plasmon resonance enables the real-time monitoring of changes in the refractive index of a thin film close to the sensing surface. The evanescent field created at the surface decays exponentially from the surface and falls to one third of its maximum intensity at approximately 200nm from the surface. In our experiments, the biotinylated BSA layer is about 14.1 nm; streptavidin is about 5.8 nm; and the biotinylated DNA is about 8.0 nm. The total thickness of all the layers for surface preparation is in the range of sensing.

Measurements of Double-Stranded DNA Surface Adsorption

We measured the change in surface plasmon resonance signal when streptavidin and double-stranded DNA were adsorbed to the surface. Based on the usual approximation (a change in refractive index of 1×10^{-6} is the equivalent of 1 pg/mm^2 of adsorbed mass), we calculated the surface density of streptavidin to be $2.6 (\pm 0.3) \times 10^{11}$/cm^2, whereas the double-stranded DNA had a surface density of $1.0 (\pm 0.1) \times 10^{11}$/cm^2. The ratio of double-stranded DNA to streptavidin was measured at 0.38 ($\pm$ 0.06).

Sensitivity of the surface plasmon resonance based sensor

Surface plasmon resonance based sensors rely upon an evanescent electromagnetic field that decays exponentially from the surface. Hence, two factors affect the ultimate limit of detection: the surface attachment chemistry and the optical data collection system. The signal could be enhanced by minimizing the attachment scheme. The surface attachment chemistry should present the capture site as close to the surface as possible. We have used an elaborate, but robust, attachment scheme: gold surface-BSA/biotin-streptavidin-biotin/DNA. This is not optimal and our signal could be increased by using a simpler scheme: attaching thiol/DNA to the gold surface. This, however, presents more problems; the thiol/DNA can form disulfide bonds (dimers) in solution.

The signal could be further enhanced by using a three dimensional attachment scheme. For instance, various investigators have shown the feasibility of coating the gold surface with a thin hydrogel, then attaching biomolecules to attachment sites. This provides enhanced signal as compared to a monolayer.

Surface plasmon resonance array imagers measure changes in signal through changes in optical intensity. Most other surface plasmon resonance instruments use the Kretschmann geometry, which measures a change in angle. A surface plasmon resonance array imager using the Kretschmann geometry would have enhanced sensitivity compared to other imagers. This would also be much slower than other surface plasmon resonance array imagers would. It should have sensitivity equivalent to that which was achieved in these experiments.

Projected sensitivity based on preliminary experiments

To determine the sensitivity of the assay, we measured binding between a known protein, MutS, and an immobilized piece of DNA. This was using the Spreeta, which has an active area of 30 mm^2. We measured a difference of about 0.00005 refractive index units (RIU) that could be attributable to this binding. Using the standard conversion, a change

of 1×10^{-6} RIU ≈ 1pg/mm^2, this corresponds to about 9×10^9 copies of MutS, which is a 90 kDa protein. This was well within our detection limit; we could detect 9×10^8 copies, but not 9×10^7. For comparison, one liter of yeast culture can produce approximately 10^{10} cells. This could be used for 3–5 experiments, and hence each experiment will have nuclear extract from about 2×10^9 cells. If we can detect 9×10^8 copies of a DNA-bound protein complex, we will be able to detect protein complexes that are present at the single copy per cell level.

Surface plasmon resonance is sensitive enough to detect physiologically relevant DNA-protein interactions

Surface plasmon resonance has sufficient sensitivity to accomplish our main goal. We had previously shown that the promoter of the SQSTM1 gene contained a single base pair (out of 1800) that was responsible for 80% of its transcriptional activity in a cancer cell line[36]. First, we quantified the physiological effect of this single base pair. We cloned the promoter and constructed a mutated version that differed by that single key base pair. We inserted these into luciferase vectors, then (in separate experiments) transfected them into NIH/3T3 cells. We measured the luciferase activity, which is a measure of the transcriptional activity of the vectors. We found a significant decrease in the luciferase activity when the key single base pair is mutated, see Figure 7. This established that this single base pair is responsible for a physiological change.

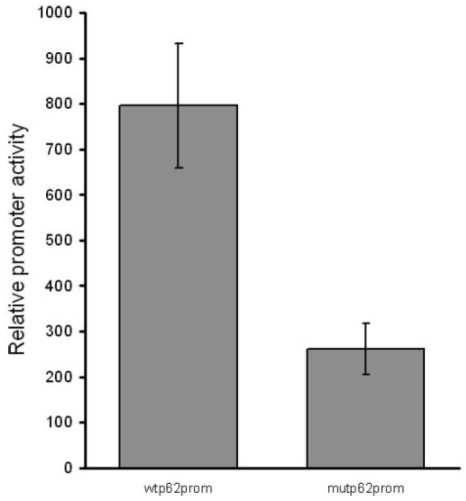

Figure 7. A single base mutation in the SQSTM1 promoter leads to significantly lower promoter activity in NIH/3T3 cells. Two luciferase reporter constructs were made: the first contained the wild-type 1800-bp promoter region from the SQSTM1 gene, the second differed by a single base pair 512 bases before the transcription start site. Each construct was inserted into a luciferase reporter vector and transfected into NIH/3T3 cells, and the luciferase activity was measured.

Next, we measured the amount of nuclear protein that binds to this piece of DNA. We made two short (16 bp) pieces of DNA from the SQSTM1 promoter. These were centered on the key base pair and differed only in the key base pair. Using surface plasmon

resonance, we measured the binding of protein complexes present in raw nuclear lysate to these two pieces of DNA immobilized on the surface of the sensor. We found a significant increase in binding of the modified piece of DNA, see Figure 8.

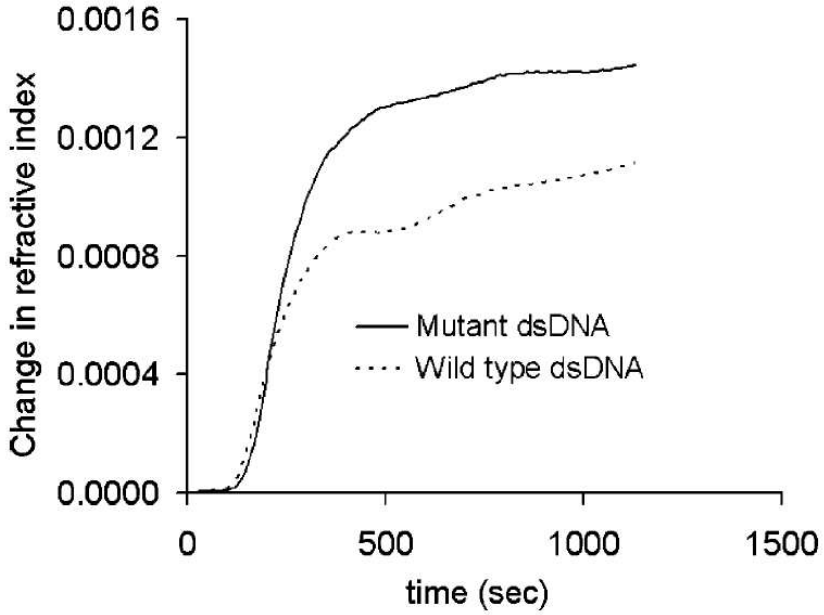

Figure 8. Surface plasmon resonance recordings can detect binding between DNA and proteins present in nuclear lysate. The surface of the sensor chip was coated with a 16-bp sequence of DNA from a region of the SQSTM1 promoter. As a control, a similar sequence containing a single-base-pair mutation was also immobilized in a different experiment. In each case, the surface was exposed to 0.33 mg/mL of nuclear lysate from NIH/3T3 cells. This lysate contains a complex mixture of proteins present within the nucleus. The relative change in refractive index is shown. The mutant and wild type were normalized to the same levels before the introduction or removal of protein. Repeated measurements showed similar behavior

Conclusion

Based upon these experiments we conclude that this surface plasmon resonance-based system was able to identify physiologically relevant *cis*-regulatory elements. Hence, the sensitivity is sufficient to accomplish the main goal of this project. Based on its design, we expect the high throughput version to have similar sensitivity.

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