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Nonlinear wave-mixing spectroscopic methods for bioanalytical and biophysical applications with sensitive detection at the single cell level

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Nonlinear Wave-Mixing Spectroscopic Methods for Bioanalytical and Biophysical Applications with Sensitive Detection at the Single Cell Level

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Chemistry

by

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2006
This Dissertation of Adrian Ashley Atherton is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego
San Diego State University
2006
DEDICATION

To my family with all my love
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PUBLICATIONS


5. **Atherton, A. A.,** Lopez, M., Tong, W. G., “Nonlinear Laser Spectroscopy for Cytokine Detection by a Antibody-Based Protein Array” (manuscript to be submitted)

6. **Atherton, A. A.,** Osher, O., Tong, W. G., “Laser Wave-Mixing Spectroscopy for in vitro biomaterial-induced Cytokine Expression and Detection on Antibody-Based Protein Microarrays” (manuscript to be submitted)


9. Lopez, M., **Atherton, A. A.,** Tong, W. G., “Ultrasensitive Detection of Proteins and Antibodies By Laser Wave Mixing Using a Chromophore Label”, (manuscript to be submitted)

**FIELD OF STUDY**

**Major Field:** Analytical Chemistry

Studies in Nonlinear Wave-mixing Spectroscopy

Professor William G. Tong, Chair
Nonlinear laser wave-mixing spectroscopy is presented as an absorption-based detection method for single cells and cellular components. Compared to other laser-based techniques, wave mixing offers several advantages including excellent detection sensitivity, high spatial resolution and small probe volumes suitable for detection of biomolecules. The analyte travels through the laser probe volume created by two focused input beams. Laser-induced dynamic gratings are generated in the presence of absorbing analytes. The resulting wave-mixing signal beams are coherent laser-like beams, and hence, they can be collected conveniently and efficiently.
Laser wave mixing is demonstrated as a sensitive detection method for proteins. An optical absorption shift is monitored upon chromophore protein complexation. Ultrasensitive detection limits of \(4.7 \times 10^{-19} \text{ M}\) (i.e., \(2.4 \times 10^{-22} \text{ mol}\)) and \(9.3 \times 10^{-14} \text{ M}\) (i.e., \(3.7 \times 10^{-17} \text{ mol}\)) are determined BSA and HPV antibodies, respectively.

Wave mixing is used to investigate and detect heavy metals entrapped in a sol-gel optical sensor. Preliminary detection limits of \(1.7 \times 10^{-14} \text{ M}\) (0.2 ppq) and \(1.15 \times 10^{-9} \text{ M}\) (59 ppt) are determined for Fe(II) and Cr(VI) ions, respectively. Further development of this sol-gel immobilization technique may lead to sol-gel biosensors for proteins.

Nonlinear wave mixing offers detection of biospecific interactions on antibody or DNA microarrays. Microarray spots have specific cytokines or oligonucleotide sequences that bind to their corresponding target molecules. The wave-mixing laser probe volume traverses microarray spots for sensitive mapping and profiling of biospecific interactions with intra-spot spatial resolution. A preliminary detection limit of \(6.4 \times 10^{-13} \text{ M}\) or 0.01 pg/mL is determined for cytokines.

For the first time to our knowledge, cellular components within a single cell are detected and imaged by nonlinear wave-mixing spectroscopy. Wave mixing profiles and images intact cells and detects cellular proteins that are lysed and separated by capillary electrophoresis. A preliminary mass detection limit of 10 fg/mL is determined for proteins.
1

INTRODUCTION

1.1 Detection at the Single-Cell Level

1.1.1 Motivation

Spectroscopy is the study of the interactions of radiation with matter. Nonlinear laser spectroscopy is an absorption-based optical method used to detect cellular components in cells as well as to sense biomolecular interactions. Results obtained using spectroscopy at both the single-cell level and the single-molecule level has a high potential for contributing to early disease detection, diagnosis, and treatment. The purpose of this work is to determine if the technique of nonlinear laser spectroscopy at the nano-liter level can be applied to biomolecules to assist in early detection of diseases.

Some diseases involve irregularities in the genetic code of cells resulting in altered expressions of gene products, RNA and protein.¹ Biomolecules, like proteins, are used in understanding cell development and function in response to these irregularities. Some irregularities in cells that are infected by a disease that only constitute a minor fraction of the total population of cells cannot effectively be detected by population-based techniques.² Because these biological materials are in extremely limited amounts, detecting them is a challenge. Hence, nano-level detection methods play a crucial role in disease detection. The nano scale is defined as the creation of functional materials, devices and systems through control of matter at the scale of 1 to 100 nanometers, a context compatible with use in the human body.¹
Exploitation of novel properties and phenomena at this scale is needed to enhance early disease detection, providing direct measurements of protein expressions, both at the single-cell and the single-molecule levels. Currently, many diseases cannot be detected at their earliest stages, in part because detection methods are not directed at the cellular changes.\(^1\) Thus, identifying and understanding protein expression levels within a single cell will require very high detection sensitivity levels.

Different levels of expressed proteins are characterized as biomarkers. The severity of the progression of cancer can be detected through these biomarkers. For example, the outcome of the detection may be used to determine which cancer patient is at a higher risk of recurrence of the disease.\(^3\) This work will show how the development of highly sensitive nonlinear laser-based detection methods will play a major role in the early detection of diseases, like cancer.

1.1.2 Detection Methods

The study of diseases at the cellular level has created a need for high resolution, high throughput, and ultrasensitive detection methods. Conventional spectroscopic methods such as emission spectroscopy and atomic absorption spectroscopy are widely used in analytical chemistry, but they offer poor sensitivity and spectral resolution because of their use of conventional light sources or the way the light signal is measured. The introduction of lasers has dramatically improved spectroscopic studies due to the unique properties and characteristics of lasers. Some of the most popular methods today have adapted lasers as an excitation source, and hence, significantly enhanced sensitivity and specificity levels.
1.1.3 Lasers

Lasers (light amplification by stimulated emission of radiation) have advanced since the first solid-state ruby laser was demonstrated in 1960. Today, lasers are commercially available as compact inexpensive sources of radiation in a wide range of wavelengths. Lasers can be tightly focused and interfaced to miniaturized devices such as microfluidic chips and microarrays that can host small biomolecules. Numerous properties of lasers, including high spatial resolution, coherence length, directionality, power density, spectral profile, monochromaticity and wavelength tunability, contribute immensely to the field of analytical chemistry, and more importantly, to spectroscopic studies of single cells and biomolecular interactions. Laser-induced fluorescence spectroscopy and microscopy, thermal lens spectroscopy, surface plasma resonance, Raman scattering and Rayleigh scattering are some of the most popular methods that use lasers effectively as an excitation source.

1.2 Nonlinear Spectroscopy

High power density levels available from laser sources make it possible to observe many optical phenomena that do not vary linearly with light wave intensity. When laser light interacts with an analyte medium, it induces a redistribution of electrons, thereby creating polarization. When using low light density levels, polarization induced upon the sample medium is linearly proportional to the electric field of the light wave. When using high light density levels, the analyte no longer maintains the linear dependence of polarization on the electric field of the light wave, thereby creating a nonlinear dependence. Studying how nonlinear optical phenomena
affect molecular transmission in biological samples can provide alternative information as compared to that available from conventional spectroscopic methods.

1.2.1 Historic Review of Nonlinear Wave Mixing

In 1972, Boris Zel’dovich and colleagues first observed optical phase conjugation using stimulated Brillouin backscatter (SBS) experiments in methane gas. They found light passing through the same medium twice produced minimal light distortion. They performed an experiment in which the output of a ruby laser beam was intentionally distorted by passing it through a frosty glass slide. The beam was then directed into a cell containing methane gas that produced a backward-traveling beam of light. This reverse propagating beam of light traveled back through the frosty glass and emerged, virtually undistorted. The SBS interaction generated a well defined “predistorted” beam with a wavefront that exactly canceled the distortions produced by the frosted glass. The generated backward beam is referred to as a “time reversed” replica of the incident beam and the SBS interaction volume is an example of a phase-conjugate mirror (PCM). This surprising new process, analogous to holography, had an inherent problem with the initial phase conjugation setup. A slight frequency shift was observed.

In 1976, Amnon Yariv extended the optical phase conjugation process by proposing three-wave mixing in an attempt to eliminate the frequency shift (i.e. loss of the pictorial information). Unfortunately, because of inherent restrictions on light source requirements and nonlinear phase-matching conditions that could not be satisfied, this method was not pursued.
In 1977, Amnon Yariv and David Pepper proposed degenerate four-wave mixing (DFWM) as a solution to eliminate the aforementioned flaws of three-wave mixing.\textsuperscript{30} Yariv and Pepper demonstrated a coupled-wave treatment of degenerate four-wave mixing. The geometry involves two counter-propagating pump beams of the same frequency, $\omega$, that illuminate a medium with a third order nonlinear susceptibility, $\chi_3$. They showed that a third probe beam of the same frequency interacts with the pump beams and the medium to produce a phase-conjugated beam propagating in the opposite direction. Similarly, near the same time, Robert Hellwarth introduced this same technique.\textsuperscript{31} Building on this concept, Hellwarth pointed out that phase matching is ensured for all angles (multimode counter-propagating pump wave), making this technique even more attractive for applications requiring wide fields of view.

In 1979, Hellwarth demonstrated optical phase conjugation in liquid-phase samples of carbon disulfide.\textsuperscript{32} The generated phase conjugate beam was produced from a thermally generated refractive index grating.

1.2.1 Degenerate Four-Wave Mixing

In this work degenerate four-wave mixing (DFWM) is a nonlinear spectroscopic technique that offers excellent detection sensitivity levels for biomolecules. In a typical optical setup, one laser source is split into two input beams and then mixed inside the analyte to generate two degenerate signal beams. The DFWM signal has a quadratic dependence on analyte absorption and refractive-index change based on temperature change and it has a cubic dependence on laser power. Despite this, one can use laser power levels that are orders of magnitude lower than those for conventional laser
methods. These nonlinear dependences allow sensitive detection of both non-fluorescing and fluorescing analytes. The signal is a coherent laser-like beam providing nearly 100% collection efficiency over a dark background. Furthermore, laser wave mixing has been demonstrated to offer high spectral resolution and detection sensitivity levels for a wide range of sample types (solid, liquid and gas) and for various applications.\textsuperscript{11-19}

For gas-phase samples, DFWM offers sensitive detection of isotopes and hyperfine structures. Isotopes are widely used for many applications including detection of trace amounts of environmental pollutants, geological dating and homeland security applications. These gas-phase samples can be atomized in different atomizers such as graphite furnace, flames, dc discharge plasmas and inductively coupled plasmas (ICP). Laser wave mixing allows measurement of isotope and hyperfine profiles with minimum chemical interference problems.

Table 1.1 summarizes some of the isotope analyses in the gas phase. Advantages include excellent detection of trace amounts of stable isotopes, negligible Doppler and Lorentzian broadenings and high spectral resolution.\textsuperscript{17-23} Laser wave mixing can also measure hyperfine structures, the spectroscopic fingerprints of isotopes. Conventional atomic absorption methods lack the spectral resolution needed to resolve hyperfine structures, resulting in ambiguous isotope measurements.\textsuperscript{24-25}

This work focuses solely on the application of laser wave mixing for liquid-phase analytes. They have a broader absorption profile that grant the option of using a variety of excitation sources. Laser wave mixing can be interfaced to several analytical tools, such as microchips and microarrays, for the analysis of many analytes including
proteins, chiral samples and inorganic compounds. Excellent sensitivity levels can be achieved even if the excitation wavelength is away from the maximum absorption wavelength. Table 1.2 summarizes previous and current DFWM works for liquid-phase samples.

1.3 Dissertation Outline

The following chapters in this dissertation present DFWM as a sensitive detection method for cellular components at the single-cell level and as a sensor for specific biomolecular interactions on miniaturized platforms. Chapter 2 introduces the basic DFWM theory and its advantages. Chapter 3 briefly describes a general forward wave-mixing experimental setup with key parameters used to enhance signal detection. In Chapter 4, laser wave mixing is presented as a sensitive detector for proteins and cancerous antibodies using a chromophore label that does not fluoresce, but still offers equal or better detection sensitivity levels as compared to those of fluorescence-based methods. Sol-gel sensors for the detection of Fe (II) and Cr (VI) ions are presented in Chapter 5. Wave mixing sensitively detects the spectrophotometric shift of metal ions that bind to chelating ligands entrapped inside sol-gel pores. Development of the sol-gel technology can lead to protein entrapment, yielding biosensors that are suitable for wave-mixing detection. Chapters 6, 7 and 8 introduce laser wave-mixing spectroscopy as an effective method that offers intra-spot resolution and biospecific detection on microarray platforms. Specifically, Chapter 6 demonstrates wave-mixing detection for oligonucleotides hybridized to a DNA microarray. Chapter 7 focuses on sensitive
Table 1.1 Summary of analytical results obtained from gas-phase samples using laser wave-mixing spectroscopy and different atomizers.

<table>
<thead>
<tr>
<th>Lasers and Atomizers</th>
<th>Gas-Phase Samples</th>
<th>Concentration Detection Limit (g/mL)</th>
<th>Mass Detection Limit (g)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar ion laser pumped ring dye laser DC cathode plasma</td>
<td>Na</td>
<td>6 $\times$ 10$^{-8}$ g</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Ar ion laser pumped ring dye laser Air-acetylene flame</td>
<td>Na</td>
<td>2 $\times$ 10$^{-10}$ g/mL</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>Pulsed excimer laser pumped dye laser DC cathode plasma</td>
<td>Ca</td>
<td>3.2 $\times$ 10$^{-7}$ g</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Ar ion laser pumped ring dye laser Air-acetylene flame</td>
<td>Ba</td>
<td>5.0 $\times$ 10$^{-8}$ g/mL</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Pulsed excimer laser pumped dye laser Air-acetylene flame</td>
<td>Li</td>
<td>2.5 $\times$ 10$^{-9}$ g/mL</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Laser Diode Graphite furnace</td>
<td>Rb</td>
<td>7.0 $\times$ 10$^{-19}$ g</td>
<td></td>
<td>17</td>
</tr>
</tbody>
</table>
Table 1.2 Summary of analytical results obtained from liquid-phase samples using laser wave-mixing spectroscopy.

<table>
<thead>
<tr>
<th>Lasers and Analytical Tools</th>
<th>Liquid-Phase Samples</th>
<th>Concentration Detection Limit (M)</th>
<th>Mass Detection Limit (g) or (mol)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar ion laser Cell</td>
<td>Eosin B</td>
<td>2.1 x 10^{-11} M</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Ar ion laser Capillary Cell</td>
<td>Cobalt (II)</td>
<td>2.2 x 10^{-5} A.U.</td>
<td>2.6 x 10^{-10} g</td>
<td>20</td>
</tr>
<tr>
<td>Ar ion laser</td>
<td>Eosin B</td>
<td>7.1 x 10^{-9} M</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Ar ion laser CD Sample cell</td>
<td>(+)Co(en)$_3^{2-}$</td>
<td></td>
<td>6.8 x 10^{-13} g</td>
<td>12</td>
</tr>
<tr>
<td>Diode laser</td>
<td>Rhodamine 800</td>
<td>7.7 x 10^{-8} M</td>
<td>1.2 x 10^{-17} mol</td>
<td>8</td>
</tr>
<tr>
<td>Ar ion laser CE system</td>
<td>Dabsylglycine</td>
<td>8.5 x 10^{-8} M</td>
<td>1.3 x 10^{-17} mol</td>
<td>18</td>
</tr>
<tr>
<td>Ar ion laser HPLC system</td>
<td>Glycine</td>
<td></td>
<td>7.8 x 10^{-13} mol</td>
<td>14</td>
</tr>
<tr>
<td>He-Ne laser Capillary cell</td>
<td>BSA</td>
<td>3.4 x 10^{-19} M</td>
<td>1.7 x 10^{-17} mol</td>
<td>This work</td>
</tr>
<tr>
<td>Ar ion Laser Sol-gel sensor</td>
<td>Fe(II)</td>
<td>1.7 x 10^{-14} M</td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>Ar ion laser DNA microarray</td>
<td>DNA</td>
<td>8.7 x 10^{-16} M</td>
<td>3.0 x 10^{-24} mol</td>
<td>This work</td>
</tr>
<tr>
<td>Ar ion laser Protein microarray</td>
<td>Cytokines</td>
<td>6.4 x 10^{-15} M</td>
<td>6.0 x 10^{-21} mol</td>
<td>This work</td>
</tr>
<tr>
<td>Ar ion laser CE system</td>
<td>Cellular proteins</td>
<td>3.5 x 10^{-16} M 6.6 fg/mL</td>
<td></td>
<td>This work</td>
</tr>
</tbody>
</table>
detection of cytokines and Chapter 8 demonstrates an in vitro study for evaluating the immunological impact of biomaterials on macrophage cells. Temporal profile of cytokine expression from the cells interrogating the biomaterial is sensitively detected by laser wave-mixing spectroscopy. Chapter 9 describes an in depth demonstration of DFWM spectroscopy at the single-cell level. Cellular components within a single cell are injected, lysed and labeled inside a capillary. Capillary electrophoresis separates cellular proteins. Chapter 10 describes single-cell detection and imaging of cellular components within intact cells. Cells are sandwiched between glass slides and a hologram-like wave-mixing signal of labeled analytes within a cell is generated by intrinsic nonlinear wave-mixing properties. Varying concentration levels of absorbing analytes within the cell are mapped using wave-mixing imaging. Chapter 11 presents conclusions for this work.

1.4 References


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2

THEORY

2.1 Nonlinear Laser Wave-Mixing Spectroscopy

Nonlinear laser wave-mixing spectroscopy involves the mixing of two input laser beams inside an absorbing medium. Initially, two focused laser beams cross at a small angle to create a pattern of light intensity modulations known as an interference pattern. Inside the medium, analytes absorb light energy in response to the interference pattern, yielding laser-induced gratings. Gratings are only present when both laser beams are present. The signal is generated and measured by detecting photons from the probe beam that diffract off the gratings at a defined angle away from the incident laser beams. Figure 2.1 is a pictorial representation of a portion of photons from an incident probe beam diffracting off laser-induced gratings and, effectively, creating two signal beams. The generated signals are coherent beams that are easily detected over a dark background. To completely understand the advantages of this method, it is essential to investigate the mechanisms that give rise to the laser-induced gratings as a result of the light interference pattern.

2.1.1 Interference Pattern

As stated above, a laser-induced grating begins with the formation of a light interference pattern. Two coherent beams from one laser source cross to form an
Figure 2.1 Photons from probe laser beam diffract off laser-induced gratings to create two signal beams.
interference pattern resulting from constructive and destructive wave interactions. The interference pattern is dependent on the angle the beams cross, the wavelength, the laser intensity, and the specific polarizations of each laser beam. Figure 2.2 shows a two dimensional picture of an interference pattern formed by two Gaussian input beams of the same polarization. The polarized fields add and cancel each other, forming dark and light regions as shown in Figure 2.2. This contrast in light intensity may be described by the equation \( \beta = \frac{2\sqrt{I_1 I_2}}{I_o} \) \[ 2.1 \]
where the variable \( \beta \) represents the light pattern contrast and it is dependent on laser intensities \( I_1 \) and \( I_2 \), and \( I_o \) is the sum of \( I_1 \) and \( I_2 \) (\( I_o = I_1 + I_2 \)). The resulting fringe spacing, \( \Lambda \), otherwise known as spatial periods of the interference pattern, is expressed by the following Bragg equation \( \Lambda = \frac{\lambda}{2\sin(\frac{\theta}{2})} \) \[ 2.2 \]
The spatial periods are dependent on the wavelength, \( \lambda \), and the beam crossing angle, \( \theta \). The overall intensity, \( I(x) \), of the interference pattern is expressed by \( I(x) = I_o \left[ 1 + \beta \cos\left(\frac{2\pi x}{\Lambda}\right)\right] \) \[ 2.3 \]
where \( x \) is the spatial coordinate. Subsequently, the absorbing medium placed inside the interference region mimics the spatial modulation of the interference pattern, thus, generating the laser-induced gratings.

### 2.2 Laser-Induced Grating Formation
There are several mechanisms that give rise to the formation of the laser-induced gratings. For absorbing liquid samples, thermal gratings contribute most significantly. A thermal grating is generated by the absorption of photons in the analyte, and subsequently, from thermalization due to the relaxation of excited states of the analyte. Excited analytes release energy in the form of heat, i.e., nonradiative decay, and this leads to large changes in the analyte density and refractive index that produce strong wave-mixing signals. This nonradiative relaxation process produces heat that raises the temperature of the analyte matrix in corresponding regions. The thermal expansion of the analyte matrix changes the refractive index of the matrix resulting in the formation of a thermal grating. The reader is directed to the book by Eichler et al. for a complete discussion of all other mechanisms describing transient laser-induced gratings.

2.2.1 Induced Thermal Grating

It is necessary to note that the interference pattern is not what produces the wave-mixing signal beam. It is the analyte, in response to the illuminated spatial fringes formed by the interference pattern that creates the signal beams. Optical excitation, through electronic transitions of molecules inside the regions of constructive interference, initially creates a spatial distribution of excited states, i.e., an amplitude grating. The excited-state molecules do not remain in the excited state for long and quickly decay nonradiatively. The period of the induced thermal grating is determined by the corresponding fringe spacing (Λ), established by the interference pattern. The thermal gratings, and consequently, the refractive-index changes in the analyte produce the wave-mixing signal.
Figure 2.2 Interference pattern formed by two intersecting input laser beams.
2.2.2 Time Scales of Grating Creation and Decay

The details of the creation of the thermal grating and its decay are based on manifold parameters. Under simplified conditions, the relaxation of the sample medium is a relatively fast process, i.e., thermal relaxation occurs typically on the order of microseconds. Table 2.1 lists various time scales of competing processes that release the energy absorbed within the thermal grating.\(^6\)

Grating decay is partially caused by thermal diffusion across the grating. Thermal diffusivity and mass transportation of the medium is related to the decay rate of the grating period. This is on the order of milliseconds and attributed to mass transport of the analytes. The use of a smaller angle \(\theta\) between the two grating-forming beams can extend the grating lifetime since the grating period becomes wider and thermal relaxation within the grating takes longer.

2.2.3 Wave-Mixing Signal

The thermal grating formed in an analyte from laser excitation can be read by the diffraction of photons from a probe laser beam. Depending on the type of four-wave mixing geometry employed in the experiment, the probe laser beam can be either one of the excitation beams producing the grating or a separate laser beam. In either scenario, two detectable wave-mixing signal beams are produced.
Table 2.1 Time scales of competing processes that release energy absorbed within a thermal grating.

<table>
<thead>
<tr>
<th>Physical Process</th>
<th>Time Constant / Type</th>
<th>Relative Time (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal grating decay ((\tau_r))</td>
<td>Rayleigh</td>
<td>(10^3 - 10^4)</td>
</tr>
<tr>
<td>Effective solute thermalization ((R))</td>
<td>Thermalization</td>
<td>0.5 - 15</td>
</tr>
<tr>
<td>Thermalization non-instantaneous and non-local solute-solvent energy transfer ((1/\tau_c))</td>
<td>Energy transfer</td>
<td>0.3 - 10</td>
</tr>
<tr>
<td>Radiative relaxation ((\tau_f))</td>
<td>Fluorescence</td>
<td>1 - 6</td>
</tr>
</tbody>
</table>
This dissertation will only focus on the forward-scattering wave-mixing optical geometry. As shown in Figure 2.3, two incident pump laser beams, arbitrarily denoted as \( E_{\text{pump}1} \) and \( E_{\text{pump}2} \), intersect at an angle \( \theta \) inside an absorbing medium. The wave-mixing process inside the absorbing medium creates the thermal grating. In this forward-scattering geometry, \( E_{\text{pump}1} \) and \( E_{\text{pump}2} \) are also the probe beams that diffract a portion of photons, yielding signal beams, \( E_{\text{signal}1} \) and \( E_{\text{signal}2} \), respectively. Diffraction of a probe beam is viewed as the simultaneous interactions of three input beams (i.e., \( E_{\text{pump}1}, E_{\text{pump}2} \) and \( E_{\text{probe}1} \)) through the nonlinear polarization of the sample, thereby, generating the fourth signal beam, \( E_{\text{signal}1} \). The situation is reversed for the creation of signal beam \( E_{\text{signal}2} \). The process is called degenerate four-wave mixing (DFWM) if the frequencies (wavelengths) of the three incident beams and the diffracted signal beam are the same. Under Bragg conditions and in order for predictable signal propagation and effective energy transfer to occur, both energy and momentum conservation must be satisfied. The conservation of energy requires that\(^1-4\),

\[
\omega_{\text{signal}_1} = \omega_{\text{pump}_1} + \omega_{\text{probe}_1} - \omega_{\text{pump}_2}
\]

where \( \omega \) is the frequency followed by the subscripts (signal, pump, and probe beams) with their arbitrary number assignment. Since, all incident laser beams have the same frequency, the generated signal beam will also have the same frequency, \( \omega_{\text{signal}} \) (hence, the term degenerate). Furthermore, the conservation of momentum requires that\(^1-4\)

\[
k_{\text{signal}_1} = k_{\text{pump}_1} + k_{\text{probe}_1} - k_{\text{pump}_2}
\]

where \( k_{\text{signal}_1} \), the signal wave vector, indicates the most effective propagation direction for the degenerate four-wave mixing signal. Figure 2.3 also shows the phase matching...
diagrams for the formation of the individual signal beams when both pump beams also
serve to probe the grating. The signal beams, $E_{\text{signal1}}$ and $E_{\text{signal2}}$, have wave vectors $k_{\text{pump1}}$ and $k_{\text{pump2}}$, respectively that form vectors $k_{\text{signal1}}$ and $k_{\text{signal2}}$, respectively. Since these vectors are not completely parallel to one another, there is a minimal tolerated mismatch denoted as $\Delta k$.

2.2.4 Diffraction Characteristics

Diffraction primarily results from changes in the refractive index (phase), but the absorption coefficient of the analyte matrix (amplitude) may also contribute to the signal strength. Consequently, the thermal grating acts on both the amplitude and the phase of the probe beam by modulating the electric field and causing a portion of photons to diffract. Subsequently, the probe beam diffracts from the grating at a specific angle according to the following equation:\textsuperscript{1,2}

$$\theta_{\text{signal}} = \sin^{-1}\left(\frac{\lambda}{2\Lambda}\right) = \theta$$

where $\theta_{\text{signal}}$ is the Bragg angle of the first-order diffracted signal beam, defined relative to the bisector of the two excitation beams. At the Bragg angle, the diffraction efficiency for weakly absorbing analytes is described as\textsuperscript{1,2}

$$\eta = \left(\frac{\pi L \Delta n}{\lambda}\right)^2 + \left(L \Delta \alpha/4\right)^2$$
Figure 2.3 Forward-scattering optical geometry with phase matching diagrams for each signal beam.
where \( L \) is the grating thickness, \( \Delta n \) is the peak-to-null difference of the refractive index, and \( \Delta \alpha \) is the change in absorption coefficient. Furthermore, the diffracted signal is angularly resolved from the two incident laser beams, and any further deviation from the Bragg angle results in a rapid decrease in diffraction efficiency.\(^1\)

### 2.2.5 Wave-Mixing Signal Intensity

A majority of the diffracted signal intensity depends on changes in the density of the medium with respect to changes in temperature. This relationship of signal intensities and other experimental parameters can be described as\(^2,3,9\)

\[
I_{\text{signal}1} \approx C \left( \frac{b}{8\pi} \right)^2 I_1^2 I_2 \frac{\lambda^2}{\sin^4 \left( \frac{\theta}{2} \right)} \left( \frac{dn}{dT} \right)^2 \frac{\alpha^2 \phi_{th}^2}{\kappa^2}
\]

\[
I_{\text{signal}2} \approx C \left( \frac{b}{8\pi} \right)^2 I_1 I_2^2 \frac{\lambda^2}{\sin^4 \left( \frac{\theta}{2} \right)} \left( \frac{dn}{dT} \right)^2 \frac{\alpha^2 \phi_{th}^2}{\kappa^2}
\]

where \( I_{\text{signal}1} \) and \( I_{\text{signal}2} \) correspond to the signal intensities, \( \frac{dn}{dT} \) is the change in the refractive index with respect to temperature, \( \alpha \) is the absorption coefficient, \( \kappa \) is the thermal conductivity, \( b \) is the path length of the laser beam cross section, and \( \phi_{th} \) is the thermal yield of the sample. Since, it is desirable to collect only one signal beam, a beam splitter is used to distribute laser intensity at a 70:30 (T/R) ratio, and hence, concentrating the signal output in one direction only. Thus, only one equation shown above, \( I_{\text{signal}1} \) or \( I_{\text{signal}2} \), is used to describe the wave-mixing signal intensity for this work.

Some of the most important and unique characteristic properties of the DFWM signal are described in the above equation. The diffracted signal beam intensity has a
significant nonlinear dependence. Diffraction efficiency grows in proportion to the square of absorption coefficient and temperature gradient of refractive index.

2.2.6 Wave-Mixing Advantages

Laser-induced grating spectroscopy is a highly sensitive way for detecting analytes based on optical absorption. The diffracted probe beam has nonlinear dependencies that contribute to sensitive detection of weakly absorbing analytes. Light absorption outside the interference pattern does not contribute to the diffraction of the wave-mixing signal. The signal is collected by a photodetector that is not directly illuminated by the incident beams (a zero background method). In addition, this technique takes advantage of the unique spatial and temporal coherence properties of laser radiation in producing a signal beam with all the same coherent properties, and therefore, allowing virtually 100% optical signal collection efficiency.

2.3 Degenerate Four-Wave Mixing Holography

DFWM can be considered a real-time dynamic holography process involving recording and reconstruction of interference patterns as illustrated in Figure 2.4. The physical properties of the sample are encoded within the interference pattern called the hologram and reconstructed by illuminating the hologram with the reference wave, or as in DFWM, the diffraction of a probe beam. While holography is a sequential two-step process of recording and reconstruction, these steps occur simultaneously in DFWM while involving four photons. Holograms of absorbing nonlinear mediums are produced and read by the signal diffraction determined by the interference-induced
thermal grating. For example; any concentration variation within the nonlinear medium will be encoded in the interference pattern. A probe laser beam will virtually read the interference pattern and map the concentration variation onto the signal beam profile, producing an image.
Figure 2.4 (A) Hologram recording process. (B) Hologram reading or reconstruction process.
2.4 References


EXPERIMENTAL APPARATUS AND INSTRUMENTATION

3.1 Forward Degenerate Four-Wave Mixing Optical Geometry

Forward degenerate four-wave mixing (F-DFWM) is the most commonly used optical arrangement for liquid-phase analytes. A diagram of this forward-scattering geometry is presented in Figure 3.1. Laser light from a tunable laser, operating at a specific wavelength and laser power, is used as the analyte excitation source. The laser beam is initially split into two excitation input beams with different intensities using a 70/30 R/T beam splitter. The two input beams propagate in the forward direction traveling the same optical path length in a box-like formation. The laser beams are focused and mixed with a focusing lens. Absorbing analytes in this region are excited in the form of the interference patterns, and thus, creating laser-induced gratings. Portions of the photons from the two input beams diffract off thermal gratings to form our laser wave-mixing signals. The input beam with the higher laser intensity produces the stronger signal beam, which is collected by a simple photodiode detector (ThorLabs, Inc., Model PDA55). A mechanical chopper (Stanford Research Systems, Model SR 541) operating at 200 Hz and a lock-in amplifier (Stanford Research Systems, Model SR 810 DSP) are used to enhance the S/N. An analog-to-digital converter digitizes and stores the wave-mixing signal on a personal computer. Beam blockers and pinholes are strategically placed within the optical setup to further minimize background optical noise levels.
A major advantage of this nonlinear optical technique is that the signal beam emerges in a predictable direction that is spatially distinguished from those of the two input beams as shown in Figure 3.2. The wave-mixing signal is a collimated coherent laser-like beam that allows highly efficient optical signal collection against a dark background. Unlike other laser-based methods, the ability to collect nearly 100% of our signal gives us an edge on detection sensitivity. Furthermore, optical alignment is fairly simple since the signal beam is visible to the naked eye and it can be aligned directly into the detector without any guesswork.

The focusing length of the focusing lens (approximately 10 cm) primarily defines the wave-mixing region and the resulting laser probe volume. The focused laser spot size is in the micrometer range, yielding nanoliter-level analyte probe volumes. Even though probe volumes are extremely small, the photon density available from the wave-mixing region is high, resulting in strong signal intensity levels. Nonlinear DFWM is an absorption-based method, but it yields strong signal levels even when using short optical absorption path lengths (i.e., mm). Small probe volumes allow for easy interfacing to miniaturized devices and sample cells.

3.2 Laser Sources

One may use any tunable or pulsed laser source in a DFWM experimental setup as long as the electric field from the laser couples with the sample of interest. Figure 3.3 shows a general laser design consisting of a lasing material and two mirrors (one partially reflecting) serving as the resonator. The lasing material is pumped optically, chemically or by an electrical discharge, to create population inversion. The photons
Figure 3.1 Forward-scattering degenerate four-wave mixing (F-DFWM) experimental setup.
Figure 3.2 Signal beams diffract off thermal gratings at a predictable propagation direction that is spatially distinguished from those of the two incident laser beams. The center-to-center distance of the input beams is denoted by d. Generated signal is the same distance, d, from the two incident laser beams.
bounce back and forth along the direction of the two mirrors. This oscillation continues until the photons have built sufficient amplitude to transmit through the partially reflecting output mirror, creating the laser output beam.

### 3.2.1 Argon Ion Laser

The experiments in Chapters 5 to 10 use a continuous-wave argon-ion laser (Coherent, Inc., Model Innova 90-6). This laser has several different output wavelengths, but the two strongest lines, 514.5 nm (green) and 488.0 nm (blue), are selected for our experimental setups. The output beam yields a TEM\(_{00}\) laser mode. The field distribution is Gaussian for both the electric and magnetic field normal to the resonator axis.\(^2,3\)

### 3.2.2 Helium-Neon Laser

Chapter 4 describes a protein detection method using a helium-neon laser, (Uniphase, Model 1125P). This gas laser produces visible radiation at 632.8 nm (red) and the maximum output power is 5 mW.

### 3.3 Sample Cells

Sample cells were mounted on a 3-D translational stage (Newport, Model 461) for precise XYZ translational movements. Precise placement of the sample cell within the wave-mixing probe volume greatly increases signal intensity. Additional motorized
Figure 3.3 General laser resonator design. Photons propagate back and forth between two mirrors until photons have built sufficient amplitude to escape the partially reflecting output mirror.
actuators (Zaber Technologies, Model ZLA-28) is interfaced to the translational stage for smooth controlled movements. A computer program is used to control the Zaber actuator for micrometer movements along one axis of the translational stage for more precise sample placements. This actuator is used in the work described in Chapter 6, 7 and 8.

3.3.1 Capillary Cells

Flexible fused silica capillary tubes (Polymicro Technologies, Inc.) are used in many experimental setups for sample handling and detection. The capillaries are made with an outer standard polyimide coating around a synthetic fused silica capillary. A transparent optical window is created by burning off the outer coating and exposing the inner capillary to the input laser beams. A 150 µm i.d. capillary is used in the protein detection setup described in Chapter 4. A 50 µm i.d. capillary is used for the single cell analysis described in Chapter 9. For initial studies and alignment protocols, a peristaltic pump (Rainin, Inc., Model Rabbit-Plus) is used to flow alignment dye solutions and samples through the capillary.

3.3.2 Microarrays

Microarrays are glass slides with thousands of reaction spots that allow high throughput analyses. Multiple target molecules (DNA or protein) are attached for rapid detection. Microarray slides are generally 22 mm x 76 mm in dimensions. The experiments discussed in Chapter 6, 7 and 8 use DNA and protein arrays. DNA
microarrays include gene expression profiling and protein arrays are used for the measurement of protein abundances.

DNA microarrays (TeleChem International, Inc., Checkit Chips) have oligonucleotides, synthetic strands of genes, printed on the glass surface. TeleChem’s print-head technology prints two identical 10 x 10 grids of 100 different oligonucleotides. Each spot is approximately 200 to 300 µm in diameter and the distance is 430 µm (center to center).

Protein array chips are purchased from Neupro Technology Co. (NEU001). These protein arrays are spotted with cytokine antibodies, IL-2, TNF-α and IFN-γ. The protein arrays have six identical 5 x 6 grids for a total of 30 spots per grid. Each spot is approximately 200 µm in diameter and the distance is 300 µm (center to center).

### 3.3.3 Sol-Gel Sensors

Sol-gel hosts have been used to entrap analytes of interest non-covalently within a silica matrix. This silica matrix is formed by the hydrolysis, polymerization and aging of the precursor sol, Tetramethylorthosilicate (TMOS). Analytes are mixed with the sol-gel solution to form a transparent glass-like sensor. Sol-gel sensors are then sandwiched between two glass slides for detection purposes. The experiment in Chapter 5 describes this sol-gel technology.
3.3.4 Capillary Electrophoresis

A custom-built capillary electrophoresis (CE) system is used in the work described in Chapter 9. Negatively charged particles move through the capillary from the anode to the cathode when an electric field is applied. This CE system is custom assembled from commercially available components for fast, high-resolution protein separation. A 40 cm fused-silica capillary (50 µm i.d., 360 µm o.d.) is mounted on a translational stage for easy optical alignment. An optical window (4 mm wide) is created 5 cm from the cathode end of the capillary. Nickel-coated cadmium electrodes are mounted and placed in the CE reservoirs. A 30 kV dc power supply (Glassman High Voltage Co., Model PS/MJ30P0400-11) is used for CE separation, as shown in Figure 3.4. The small capillary diameter allows small sample sizes and low reagent consumption. It has already been demonstrated that DFWM can accommodate extremely small detection probe volumes within CE capillaries with excellent sensitivity.4

3.3.5 Charge-Coupled Device (CCD)

A CCD camera (Sony XCD-SX910CR) is used as a high-resolution imaging device in the work described in Chapter 10. This color CCD camera has an incorporated external trigger function for capturing fast or still images in low-light environments. A computer program provided by the manufacturer is used to operate the CCD camera.
Figure 3.4 Custom-built capillary electrophoresis system.
3.4 References


4

NONLINEAR WAVE-MIXING SPECTROSCOPY FOR DETECTION OF PROTEINS AND ANTIBODIES USING A CHROMOPHORE LABEL

4.1 Abstract

Nonlinear wave-mixing spectroscopy is presented as a sensitive optical method for the detection of proteins and antibodies labeled with a chromophore, Coomassie Brilliant Blue. A low-power He-Ne laser is focused within a 150 µm capillary cell for the detection of Coomassie Brilliant Blue complexes. The wave-mixing signal is detected after 10 minutes of room-temperature incubation time for the antibody complex and 18 minutes for the protein complex. Preliminary concentration detection limits of $3.4 \times 10^{-19}$ M and $6.4 \times 10^{-14}$ M (S/N 2) are determined for bovine serum albumin and human Papillomavirus antibody, respectively. Based on the small laser probe volume used (i.e., overlap volume of the two input beams), preliminary mass detection limits of $1.7 \times 10^{-22}$ mol and $2.6 \times 10^{-17}$ mol are determined for bovine serum albumin and human Papillomavirus antibody, respectively. All solutions were prepared in aqueous buffer without the addition of organic modifiers.

4.2 Introduction

The detection of proteins and antibodies for the study of disease markers has created a need for an ultrasensitive detection method, based on optical absorption. Biomolecules are produced from diseases in the body at trace amounts well in advance
of any external symptoms. Early disease diagnosis by a reliable detection method of these marker compounds at low concentration levels can help treatment and prevention. The American Cancer Society estimates that there will be about 10,520 new cases of invasive cervical cancer in the United States. About 3,900 women will die from this disease.\(^1\) When found and treated early, cervical cancer can often be cured. The most important risk factor for cervical cancer is infection with HPV (human Papillomavirus). Papillomaviruses are a group of more than 70 types of viruses that are species-specific and with tumors that contain variable amounts of infectious virus. "High risk" HPV types include antibody HPV-16 and HPV-18.\(^2,3\)

Currently, a Pap smear is the established way to test for cervical cancer. Although, these results are quick, they are sometimes ambiguous due to the lack of sensitivity. Other diagnostic tests for infections HPV require sample amplification steps. Amplification steps are necessary when starting material is limited and detection needs to be more sensitive. Sample amplification steps are not only more involved, but may create problems such as cross contamination providing false results.\(^4,5\) For this reason, it is important to develop a reliable method that will not only provide excellent sensitivity but avoid additional sample preparation steps.

Coomassie Brilliant Blue (CBB) was first introduced as a method to determine proteins.\(^6\) CBB allows measurement of proteins and polypeptides with molecular weight greater than 3000-5000 Daltons, depending on the charged groups that bind to the dye.\(^7\) The CBB dye does not bind to small molecular weight molecules, so little interference should be expected.\(^8\) CBB exists in cationic, neutral and anionic forms. It is the anionic form that complexes with the proteins or antibodies. Protonation of the
dye structure, upon binding to protein, occurs with the arginine amino acids rather than primary amino groups. Other basic (His, Lys) and aromatic residues (Try, Tyr, and Phe) give slight binding responses. The dye-protein binding behavior is attributed to Van der Waals forces and hydrophobic interactions. The Bradford assay is popular because it is rapid, relatively inexpensive, and specific for protein and antibodies. There are some known interferences created by compounds like bases, detergents and others that affect the reaction equilibrium between the three dye forms. Many known interferences in the samples can be compensated for by adding the interference agent to the control blank, thus still allowing accurate determination of proteins.

CBB is widely used as a marker for protein position in electrophoretic gels. The most common way CBB is used to detect proteins is by measuring absorbance increases with an UV-Vis spectrophotometer. Although this instrumentation offers the advantage of being very simple to operate, it is not very sensitive, and typical results obtained are on the order of micrograms or mg/L of protein. Other protein detection methods use fluorescent dyes, instead of chromophores, to bind with proteins. Fluorescence-based detection methods offer better detection sensitivity levels as compared to absorption based methods; however they require fluorophores, and some dyes give high background levels. Mass spectrometry is also used, but it is not as cost effective.

In this chapter, we describe a simple yet extremely sensitive method of detecting antibodies and proteins labeled with CBB. Wave mixing is an absorption-based method that has been proven to be an excellent detection technique for both liquid and gas-phase analytes and has already been demonstrated as a suitable method for the detection of biomolecules. Wave mixing-based detection of marker proteins and antibodies
(BSA and HPV) offers numerous advantages including shorter analysis time, less consumption of sample due to small probe volume, detection sensitivity in the low attomole range, and a broader range of useable labels (both fluorophores and chromophores).

In the wave-mixing optical setup, two input beams cross inside the analyte, generating an interference pattern, and subsequently, a thermal grating. The signal beam has a square dependence on analyte concentration and a cubic dependence on laser power as shown in Equation 4.1

\[
I_s \approx C f^2 I_1 I_2 \left( \frac{\lambda^2}{\sin^4 \frac{\theta}{2}} \right) Q \left( \frac{\alpha^2}{\kappa^2} \right)
\]

where \(I_1\) and \(I_2\) represent signal intensities from the probe and pump beams, respectively. The analyte fluorescence quantum efficiency, \(f\), is proportional to the square of sample path length, \(b\), divided by \(8\pi\), i.e., \((b/8\pi)^2\), and the solvent parameter \(Q\) is related to refractive-index change based on temperature change, i.e., \((\text{dn/dT})^2\). The above equation shows that the wave-mixing signal has a quadratic dependence on concentration, absorption path length and temperature gradient of the refractive index. These dependencies, along with small probe volumes, translate into a very sensitive detection technique for measuring smaller changes in analyte properties as compared to conventional absorption or fluorescence methods. Using only a 5-mW He-Ne laser, preliminary concentration detection limits of \(3.4 \times 10^{-19} \text{ M}\) and \(6.4 \times 10^{-14} \text{ M} (\text{S/N} 2)\) are determined for BSA protein and HPV antibody, respectively. Based on the small laser probe volume used (i.e., overlapping volume of the two input beams), preliminary mass
detection limits of $1.7 \times 10^{-22}$ mol and $2.6 \times 10^{-17}$ mol (S/N 2) are determined for BSA protein and HPV antibody, respectively.

4.3 Experimental

4.3.1 Wave-Mixing Setup

The wave-mixing optical setup is shown in Figure 4.1. A He-Ne laser (Uniphase, Model 1125P) operating at 632.8 nm with a laser power of 5 mW is used as the excitation light source. The output of the laser beam is split by a 70/30 (R/T) beam splitter to form two input excitation beams. The reflected beam from the beam splitter, E1, has the higher laser intensity, and therefore, is used as the forward pump/probe beam. The transmitted beam, E2, is used only as the pump beam. A mechanical chopper is used to modulate the amplitude of the pump beam at 200 Hz. The chopper control is interfaced to a lock-in amplifier (Stanford Research Systems, Model SR810 DSP), which is interfaced to a computer for data acquisition and display. The two input excitation beams travel equal distances before they are focused at the capillary detector cell, creating a small laser probe volume. Two signal beams, E3 and E4, are generated and they propagate in the forward direction. These signal beams resemble the two input excitation beams, E1 and E2. The signal beam, E3, is directed into a photodiode detector (Thorlabs, Inc., Model PD55) after passing through an iris and a focusing lens.
Figure 4.1 Experimental setup for the detection of proteins and antibodies labeled with a chromophore, CBB.
The wave-mixing detector cell is a 150 µm inner-diameter capillary cell (Polymicro Technologies, Inc.) interfaced to a peristaltic pump (Rainin Instrument Co., Model Rabbit) to keep the analyte solution flowing at a rate of 2.5 mL/min. The probe beam E1 intersects the pump beam inside the cell at a very small angle. An alignment dye solution, $1 \times 10^{-3}$ M Nile Blue (Aldrich, Cat. No. 37,008-8), is first injected into the system. The resulting wave-mixing signal beam is visible to the naked eye and it is used to optimize the optical setup. The optical alignment remains stable during each experiment. To further verify alignment stability, Nile Blue is reintroduced into the capillary cell after all protein solutions have been analyzed.

### 4.3.2 Absorption

A UV-visible spectrophotometer (Hewlett Packard, Model 8452A) is used for UV-visible absorption measurements in disposable polystyrene cuvettes with a 1 cm path length. Absorption spectrum is blanked against the buffer system used.

### 4.3.3 Analyte Solutions

The CBB dye reagent is obtained and prepared according to the manufacturer’s instructions (Bio-Rad Laboratories, Cat. No. 500-0006) to a concentration of 0.1 mg/mL. Tris HCl buffer solution (0.50 M, pH 4) is prepared in deionized doubly distilled water and filtered (Whatman 44 ashless filter). Mouse anti-human Papillomavirus type 16 is obtained from US Biological (Cat. No. P3105-15). All protein stock solutions are prepared by dissolving a weighed amount of protein in pH 4 Tris HCl buffer solutions. The stock protein solution is then serially diluted down to
working concentrations. All solutions are made fresh each day. Different mixing ratios for CBB to protein or antibody are used along with different incubation times. Unless otherwise noted, 200 µL of CBB are mixed with 800 µL of BSA protein or buffer and incubated for 20 minutes. For time-dependence studies, a specific amount of CBB dye reagent is pipetted into a 2 mL plastic vial containing the protein solution, mixed by vortexing, and incubated at room temperature before each measurement. The following ratios are used: 75 µL of CBB are mixed with 400 µL of HPV at a 3.6 x 10⁻⁹ M concentration, and 25 µL of CBB are mixed with 800 µL of BSA at a 1.15 x 10⁻¹² M concentration.

### 4.4 Results and Discussion

The anionic form of CBB binds with protein or antibody in low pH buffer. Wave mixing monitors the absorption shift of the chromophoric dye CBB. The dye normally has an absorption maximum at around 465 nm, and upon binding to a specific protein or antibody, the absorption maximum shifts to approximately 590 nm. It is this hypsochromic shift that is monitored and related to the amount of protein or antibody bound to CBB. Figure 4.2 shows the absorption spectra of CBB with and without protein. CBB has an absorption maximum at 465 nm, and upon binding to a specific protein or antibody, the absorption maximum shifts to 590 nm. Both solutions have the CBB dye at the same concentration as the stock solution. The chromophore alone has a significant absorption peak at the same wavelength as the new protein-complexation peak. The use of appropriate CBB-protein ratios effectively minimizes this background level.
Figure 4.2 Optical absorption shift of CBB bound to protein, 2.4 x 10^{-7} M BSA. CBB solution has only buffer, no added protein. The concentration of the CBB remains the same.
Figure 4.3 shows the results of CBB optical absorption profiles at different protein concentration levels using a UV-visible spectrophotometer. The magnitude of the absorption maximum shift at higher wavelengths is a function of the protein concentration. As shown in Figure 4.3, protein concentration levels below $1 \times 10^{-7}$ M are difficult to detect with a conventional spectrophotometer. Hence, to monitor smaller amounts of protein, a more sensitive method is needed. Wave mixing is orders of magnitude more sensitive and can measure protein absorption shifts at very low concentration levels that are suitable for many biomedical samples.

Traditionally the reaction has been monitored at 595 nm with a UV-visible spectrophotometer. We choose to monitor the absorption shift of the CBB protein or antibody reactions using a compact low-cost 632-nm He-Ne laser in our wave-mixing setup. Future studies can be made effectively using various solid-state diodes. For our studies, the low-power He-Ne laser produces a strong coherent wave-mixing signal beam.

Several procedures are used prior to sample analysis in order to enhance the wave-mixing signal. First, the glass capillary cell is securely mounted on a translational stage. Any movement of the capillary can create fluctuations of the thermal grating that generates the signal beam. The capillary is threaded through a mount attached to an XYZ translational stage. The two input beams intersect directly within the center of the capillary channel. The XYZ stage allows convenient optical alignment to enhance grating sharpness and minimize light scattering. An alignment dye solution is flowed through the capillary to produce a visible wave-mixing signal beam, which offers convenient optical alignment and detection as compared to other laser-based methods.
Figure 4.3 Conventional UV-VIS absorption profiles of CBB in buffer with increasing concentration levels of BSA protein: (1) only buffer and CBB, (2) 2.4 x 10^{-7} M, (3) 6.1 x 10^{-7} M, (4) 1.5 x 10^{-6} M, (5) 5.1 x 10^{-6} M and (6) 3.2 x 10^{-5} M.
The alignment-dye signal is collected before and after each experiment to assure alignment stability of the setup. The capillary cell is rinsed thoroughly with an acid solution, a base and then a buffer solution to remove any residues from previous analyte runs.

A peristaltic pump is used to flow analytes through the glass capillary at different flow rates. As expected, slower flow rates above the static mode (i.e., no flow) yield higher signal intensity since analyte solution within the laser probe volume is refreshed. Very fast flow rates may attenuate the wave-mixing signal due to the grating washout effect. An optimal flow rate for this study is determined to be 2.5 mL/min. Although there is an optimum flow rate, the wave-mixing setup yields excellent signal levels over a wide range of commonly used flow rates for many applications.

The CBB-protein complexation process is important for the detection sensitivity studies. Figure 4.4 illustrates the dependence of (a) CBB-BSA and (b) CBB-HPV signal versus incubation time. Figure 4.4 (a) shows that the CBB-BSA signal has a maximum at approximately 18 minutes and Figure 4.4 (b) shows that the CBB-HPV complex signal has a maximum increase after 10 minutes. All trend lines are based on the best-fit analysis. The CBB-HPV complex reaches the maximum signal intensity level at a faster binding rate. This is most likely due to the availability of more docking sites on HPV, resulting in faster binding with CBB. In both studies, the signal decrease is due to aggregation and this effect has been observed before for this type of assay. Wave mixing has the capability to sensitively measure CBB-protein binding almost immediately, and therefore, long incubation times are not required. This is
Figure 4.4 Wave-mixing signal increases with incubation time of CBB binding with protein. (A) Maximum signal observed at 18 minutes for CBB-BSA (B) Maximum signal observed at 10 minutes for CBB-HPV.
especially advantageous when performing multiple assays to obtain higher analyte throughput.

The wave-mixing signal detection sensitivity is also dependent on the CBB-protein ratio. The amount of total CBB plays an important role in the binding reaction and the subsequent selection of an appropriate detection method. Furthermore, enough protein must be present to bind to the CBB so that an absorption shift can be observed. If there is an excess amount of reagent present, it may increase the background signal.

Figure 4.5 demonstrates the total amount of CBB and the effect it has on the wave-mixing signal with time when the protein volume and concentration are held constant at 25 µL of 1 x 10^{-12} M BSA. Figure 4.5 (a) has 15 µL of CBB and (b) has 25 µL of CBB. As expected, a small addition in the amount of CBB changes the intensity of the wave-mixing signal and incubation time. Signal is generated faster in the presence of more CBB molecules. After 15 minutes of incubation, the absorption signal decreases due to the precipitation of CBB-protein complex. Optimal ratios for sensitive detection are determined to be 1:20 for CBB-BSA (i.e., 25 µL CBB to 500 µL BSA), and 1:5.3 for CBB-HPV (i.e., 75 µL CBB to 400 µL HPV).

Figure 4.6 shows wave-mixing signal dependence on increasing protein concentration levels. In this study, the optimal CBB-BSA ratio mentioned above is used. Although the amount of CBB is held constant, the protein, at higher concentration levels, binds more effectively, resulting in an increase in the wave-mixing signal. The analyte wave-mixing signal is also compared with a buffer solution without
Figure 4.5 Wave-mixing signal varies with different levels of CBB and a constant protein concentration as expected. CBB levels affect signal intensity and incubation time. (A) 15 µL of CBB has maximum signal after 20 minutes of incubation, (B) 25 µL of CBB has maximum signal after 15 minutes of incubation.
Figure 4.6 Wave-mixing signal increases with increasing protein concentration levels. Optimal CBB-protein binding ratios are applied.
any protein present. It is apparent that the analyte signal is strong and reproducible, and it is well above the buffer background level.

The wave-mixing detection sensitivity is verified by running a series of BSA analytes ranging in concentration from $1 \times 10^{-19}$ M to $3 \times 10^{-16}$ M using the optimal CBB-BSA binding ratio. Fig. 4.7 shows the wave-mixing signal of BSA at the $4.7 \times 10^{-19}$ M concentration level. The CBB-BSA wave-mixing signal is collected in three signal intervals. The wave-mixing signal is also compared to a “blank” solution, which consists of the buffer and the CBB reagent at the same concentration levels as those in the analyte. Although the blank solution does not contain any protein, a small background signal is observed since CBB itself absorbs slightly at the wavelength used as shown in Fig. 4.7. A preliminary concentration detection limit of $3.4 \times 10^{-19}$ M, $2.3 \times 10^{-17}$ g/mL or 23 attogram/mL (S/N 2) is determined for BSA. Using 500 µL for sample volume and 68 kDa for BSA, a preliminary mass detection limit of $1.7 \times 10^{-22}$ mol or $1.2 \times 10^{-17}$ g (S/N 2) is determined for BSA. This corresponds to about 100 molecules of BSA protein present at any one time inside the small laser probe volume.

The wave-mixing detection sensitivity is also verified for HPV by running a series of HPV analytes ranging in concentration from $1 \times 10^{-15}$ M to $1 \times 10^{-8}$ M in a similar manner using the optimal CBB-HPV binding ratio. Fig. 4.8 shows the wave-mixing signal at the $9.3 \times 10^{-14}$ M HPV concentration level. A preliminary concentration detection limit of $6.4 \times 10^{-14}$ M or $9.6 \times 10^{-12}$ g/mL (S/N 2) is determined for HPV. Using 400 µL for sample volume and 150 kDa for HPV, a preliminary mass detection limit of $2.6 \times 10^{-17}$ mol or $3.8 \times 10^{-12}$ g (S/N 2) is determined for HPV.
Figure 4.7 Sensitive wave-mixing detection of CBB-BSA.
Figure 4.8 Sensitive wave-mixing detection of CBB-HPV.
Table 4.1 compares the detection sensitivity levels of wave mixing and those of other currently available methods for protein detection. Laser wave mixing offers excellent detection sensitivity and yet it is applicable to both chromophore and fluorophore labels.

4.5 Conclusion

We have demonstrated wave mixing as a sensitive detection method for proteins and antibodies. The advantages of wave mixing include better detection sensitivity, more rapid analysis, less amount of reagents used, lower cost and less environmental waste. Another key feature of this study is that the samples are analyzed in pure buffer without the addition of an organic modifier to enhance the signal. For biological analyses, it is desirable to keep everything at physiological conditions as much as possible. Hence, wave mixing is an attractive detection method for protein studies. Future plans include enhancement of CBB-protein detection selectivity by using specific coupling to popular antibody separation and selection systems including ELISA assays.
Table 4.1 Comparison of currently available techniques for the detection of proteins.

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<th>Technique</th>
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<th>Mass Detection Limit (g or mol)</th>
<th>Concentration Detection Limit (M or g/mL)</th>
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<td>Fluorescein</td>
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<td>Isoelectric focusing</td>
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<td>SPYRO Ruby</td>
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<tr>
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<td>7 x 10^{-8} g/mL</td>
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<tr>
<td>Mass spectrometry</td>
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<td>Epitope-tagged</td>
<td>0.8 x 10^{-15} mol to 5.5 x 10^{-15} mol</td>
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<tr>
<td>Laser wave mixing</td>
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<td>Coomassie Brilliant Blue</td>
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<td></td>
<td>HPV antibody</td>
<td></td>
<td>BSA: 1.7 x 10^{-22} mol, 1.2 x 10^{-17} g</td>
<td>BSA: 3.4 x 10^{-19} M, 2.3 x 10^{-17} g/mL.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>HPV: 2.6 x 10^{-17} mol, 3.8 x 10^{-12} g</td>
<td>HPV: 6.4 x 10^{-14} M, 9.6 x 10^{-12} g/mL</td>
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4.6 References


SOL-GEL OPTICAL DETECTION FOR METAL POLLUTANTS BY NONLINEAR WAVE-MIXING SPECTROSCOPY

5.1 Abstract

Sol-gel sensors are presented as optically transparent monoliths that are effective for sensitive detection of potential environmental contaminants including Cr (VI) and Fe (II) ions. Organic reagents, 1,10-phenanthroline and 1,5-diphenylcarbazide (DPC), are non-covalently entrapped within sol-gel pores and cast into 1 mm thick cassettes. Sensors are dipped into aqueous metal ion solutions forming Cr (VI) 1,5-diphenylcarbazide and Fe (II) 1,10-phenathroline complexes. These compounds are sensitively detected based on their optical properties within the sol-gel medium. The wavelength shift is monitored by laser wave-mixing spectroscopy using a 514 nm argon-ion laser. Laser wave mixing offers excellent detection sensitivity for metal ions entrapped within the sol-gel matrix. Quadratic dependence of the wave-mixing signal on analyte concentration allows more sensitive monitoring of smaller changes in analyte properties, and hence, it is especially effective for sensor applications. Preliminary detection limits of 115 ppt for Cr (VI) and 1.7 ppq for Fe (II) are determined. The combined advantages of sol-gels and wave-mixing detection allow this absorption-based optical technique to offer many potential applications including portable field sensors for environmental contaminants.
5.2 Introduction

Reliable analyses of metals are important for the detection of environmental contaminants. Some transition metals are known for their health and ecological challenges and problems. In recent years, there has been considerable interest in developing portable and sensitive detection methods for these environmental pollutants. Since they are dangerous even in trace amounts, more sensitive detection methods are needed for transition metal remediation.

Monitoring metal pollutants in the U.S. is primarily driven by legislation enacted by the U.S. Congress, such as the Toxic Substances Control Act (TSCA) and the Clean Water Act. Under the TSCA, the Toxics Release Inventory (TRI) requires industries to report the amount of listed compounds released into the environment. For instance, chromium is used in several commercial applications including refractory electroplating cleaning agents in the metal finishing industry, fungicides, pigments in paint, and power plants. In 1998 alone, 1,954 facilities released 30,366,148 pounds of chromium. The enormous abundance of chromium is alarming, and more importantly, during certain processes, chromium can be released into the atmosphere, soils and water supplies, making the chromium ion a significant environmental contaminant.

Extremely toxic chromium and chromium-containing compounds pose potential risks to humans if not detected and monitored at extremely low concentration levels. For instance, Cr (VI), a known carcinogen, damages DNA and causes lung cancer, gastrointestinal disorders, dermatitis and ulceration of skin. According to the national TRI, the Environmental Protection Agency (EPA) classifies chromium as a
“de minimis” carcinogen, i.e., the minimum amount of the chemical set by OSHA is considered to be carcinogenic (1 mg/m³ TWA).

The maximum contaminant level (MCL) for chromium has been set at 0.1 ppm and the detection limit of the analytical procedure (DLAP) is 0.09 ng, which is the lowest level that will give commercial detectors a response significantly different from the blank.⁵ Current analytical methods use ion chromatography and UV-Vis spectrometry. Cr (VI) is first separated from potential interferences using post-column derivations of Cr (VI) 1,5-diphenylcarbazide complex and detected by a UV-visible detector at 540 nm. Unfortunately, conventional optical absorption methods offer inherently poor detection sensitivity levels.

Similarly, iron ions and iron-containing compounds should also be monitored in our environment due to the diverse important roles they play in many biological processes. Iron, in both oxidations states of Fe (II) and Fe (III), is the fourth most abundant element in the Earth’s crust. In general, iron plays essential roles for plant, animals and humans with functions in photosynthesis, and it serves as an active centre of a wide range of proteins and a limiting nutrient for phytoplankton growth.⁶ The concentration of iron ions and the factors governing the insertion of the correct metal ion into metalloproteins have been studied for various applications.⁷ Therefore, effective monitoring of Cr (VI) and Fe (II) is very important for a wide range of applications including environmental and biological studies.

The ideal detector for these hazardous metals should be sensitive, portable and rugged for field use. The sol-gel technology promises these desirable features for sensitive detection of Cr (VI) and Fe (II). Recent advances in polymeric support for
chemical sensors contributed to significant enhancement of sol-gel technology. Sol-gel provides the way to obtain porous silicate glass matrices in which organic reagents may be easily entrapped. The sol-gel process of hydrolysis, condensation and polymerization occur at room temperature. The properties of the final material may be modified in order to entrap a wide range of analytes. Proper use of appropriate precursors, catalysts and drying additives controls the final outcome of these sol-gel sensors. Important parameters and characteristics that can be customized for entrapment and detection purposes include hydrophobicity, thickness, reactivity and stability of the glass material. Another modification is porosity of the sol-gel matrix, which ranges from 2 to 200 nm. The size of the sol-gel pore governs successful chemical entrapment.8 Entrapped chemicals remain exposed to the surrounding environment and may interact with water-soluble species, which can penetrate through the pores.9

Sol-gels have many attractive properties for the application of chemical sensing. Chemical and thermal stability, chemical inertness, high purity and homogeneity are some of the promising properties that make sol-gels attractive as sensors in comparison to other hosts. Other sensors containing immobilized reagents for detection of metal ions have some limitations. For instance, chromophoric groups often require participation of more than one ligand and covalently bonded ligands have only a limited degree of freedom to reorient on the glass surface.9 Unlike these other methods, sol-gel provides a non-covalent entrapment of chelating agents between several microenvironments within the sol-gel derived material. This feature preserves the full
integrity of the chelating agents. Toxic soluble metal species can then penetrate through the pores of the sensor and chelate with their appropriate ligand.

Multi-photon laser wave mixing is presented as a sensitive absorption detection method for sol-gel-based chemical sensors. This sensitive optical absorption method is especially suitable for sol-gel sensors since it offers excellent detection sensitivity while probing a very small analyte volume, i.e., the microenvironment of a sol-gel pore. Small beam overlap volumes resulting from crossing of the two input beams allow convenient sensing of spectrophotometric properties of the metal ion species when bound to the chelating agent. In this paper, we present a sol-gel chemical sensor coupled with a wave-mixing detection system that offers excellent detection limits for metal ions.

5.3 Experimental

Figure 5.1 shows the forward-scattering wave-mixing optical setup. An argon-ion laser (Coherent, Inc., Model Innova 90-6) operating at 514 nm with a laser power of 4.5 mW is used as the excitation light source. The output of the laser beam is split by a 70/30 (R/T) beam splitter to form two input excitation beams. The higher intensity beam acts as both the pump and probe beam. Two coherent signal beams are created by the wave-mixing thermal gratings. The more intense signal beam is collected by the photodiode detector (Thorlabs, Inc., Model PD55). A mechanical chopper modulates the amplitude of the pump beam at 200 Hz. A lock-in amplifier (Stanford Research
Figure 5.1 Experimental setup for the detection of sol-gel sensors.
Systems, Model SR810 DSP) enhances the S/N and the signal is digitized and stored on a personal computer.

All chemicals and reagents are purchased from Sigma Aldrich unless otherwise specified. Solutions are prepared with double distilled (DDI) water at room temperature. A solution of $1 \times 10^{-3}$ M erythrosin B in DDI water is prepared for preliminary sol-gel sensor experiments. These initial studies help determine optimal wave-mixing parameters such as laser power and optical alignment. Individual stock solutions of 1.4 M hydroxylamine ($H_3NOHCl$), 1.2 M sodium acetate ($NaC_2H_3O_2$) and 0.01 M 1,10-phenanthroline are prepared with DDI water.

A $3.5 \times 10^{-4}$ M ferrous ammonium sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2*6H_2O$) stock solution is prepared and diluted to a final concentration of $4 \times 10^{-14}$ M. A 0.01M 1,10-phenanthroline ($C_{12}H_8N_2$) solution is prepared with hydroxylamine ($H_3NOHCl$) and sodium acetate ($NaC_2H_3O_2$). The hydroxylamine solution is used as a reducing agent to prevent oxidation of ferrous iron to ferric iron, and sodium acetate is used to adjust pH to a value of 4 (conducive for complexation).

The Cr (VI) stock solution is prepared by dissolving 0.1014 g of potassium chromate in DDI water. A few drops of concentrated sulfuric acid are initially added to help dissolve potassium chromate. The $3.4 \times 10^{-4}$ M Cr (VI) ion stock solution is diluted with DDI water to a concentration of $2 \times 10^{-9}$ M. A separate solution of 0.01 M 1,5-diphenylcarbazide (DPC) is prepared with acetone and 0.18 M $H_2SO_4$.

The sol-gel solution is prepared by combining 7.6 mL of tetramethyl orthosilicate (TMOS), 2 mL of DDI water and 200 µL of 0.04 M $HCl$ in a glass vial. The sol-gel solution is sonicated for 15 min. to create a clear viscous solution. Equal
amounts of the analyte solution and the sol-gel solution are mixed and cast inside 1 mm thick plastic cassettes. The cassettes are then covered with Parafilm and set aside until the sol-gel is formed.

Several monolith sensors are prepared by entrapping (doping) with a chelating agent. Sensors only have the chelating ligand entrapped in the sol-gel matrix. A semi-viscous sol-gel solution is added in equal proportions to the solutions of chelating agents, 1,10-phenanthroline and DPC. For example, the iron sol-gel sensors are created by mixing 3 mL of sol-gel solution and 3 mL of 1,10-phenanthroline solution. Similarly, chromium sol-gel sensors are prepared by mixing 3 mL of sol-gel solution and 3 mL of DPC solution. Iron and chromium mixtures are then separately cast in 1 mm thick cassettes for the aging processes.

The aging process and duration depend on the doping agent and the chemistry employed. For instance, the iron sol-gel sensors are formed in one day and chromium ion sensors in four days. Each sensor shrinks approximately 30% during the aging process. They are then easily removed from the casting cassette and cut into 1 cm (L) x 1 cm (H) x 0.7 cm (W) monoliths. Sensors are finally sandwiched between glass slides and mounted on a translational stage for wave-mixing optical detection.

5.4 Results and Discussion

Erythrosin B doped sol-gel monoliths are used as a tool for the study of added precursors, aging time and detection optimization. They appear pink, confirming proper dye entrapment and minimum leaching. The monoliths absorb at 514 nm and Figure 5.2 shows the wave-mixing signal of a doped erythrosin B sol-gel monolith as compared to that of a sensor prepared with only the sol-gel solution. Minimum
absorption is observed for the blank sol-gel as compared to the erythrosine B doped sol-gel sensor. Three wave-mixing signal peaks are collected to verify signal strength, signal reproducibility and noise levels. Laser alignment is optimized using the erythrosin B sol-gel. It is apparent that a strong wave-mixing signal is observed from the microenvironments of the sol-gel sensor with minimum background noise levels. The laser wave-mixing signal strength can be described as:\textsuperscript{11-17}

\[ I_s \propto C I_1 I_2^2 \frac{\lambda_e^2}{\sin^2 \theta} \left( \frac{\partial n}{\partial T} \right)^2 \left( \frac{\alpha^2 \eta^2}{\kappa^2} \right) \] (1)

Where \( C \) is a product of constants, \( I_1 \) and \( I_2 \) are the two pump beam intensities, \( \lambda_e \) is the excitation wavelength, \( \frac{\partial n}{\partial T} \) is the refractive-index gradient based on temperature, \( \kappa \) is the thermal conductivity of the solvent, \( \eta \) is the efficiency of conversion of photon energy into heat, \( \alpha \) depends on concentration and molar absorptivity, \( \theta \) is the angle between the two pump beams, and \( C \) is a constant.\textsuperscript{11-17} The signal has a quadratic dependence on both the analyte concentration and the change in index of refraction with respect to temperature. The signal also has a cubic dependence on laser power. Figure 5.3 verifies the cubic power dependence using the erythrosin B doped sol-gel sensor with a slope of 2.973 and a \( R^2 \) value of 0.9164.
Figure 5.2 Wave-mixing signal for erythrosin B doped sol-gel sensor as compared to that of a blank buffer sensor (buffer 0.05 M Tris HCL).
Figure 5.3 Cubic power dependence of wave-mixing signal for $1 \times 10^{-5}$ M erythrosin B doped sol-gel sensor.
Figure 5.4 shows a conventional UV-visible spectrum of an iron sol-gel sensor with minimal optical absorption from 400 to 600 nm, as expected. This profile is compared to a sol-gel sensor with 3.5 x 10^{-3} M of Fe (II) ions. Chelating Fe (II) with 1,10-phenanthroline causes a wavelength shift that is conveniently detected by the 514 nm argon-ion laser. Metal-ligand complexation is visually observed when an aliquot of 3.5 x 10^{-3} M (Fe(NH₄)₂(SO₄)₂*6H₂O) is dropped onto the sensor. Free Fe (II) ions bind to the chelating agent 1,10-phenanthroline and turn the sol-gel sensor pink.

5.4.1 Iron Wave-Mixing Signal

Figure 5.5 shows the wave-mixing signal obtained from the Fe (II) ion sol-gel with approximately 100 µL of 4 x 10^{-14} M or 2.2 parts per quadrillion (ppq) Fe (II) ions. The wave-mixing signal is collected 20 minutes after sample introduction to allow sufficient time for the Fe (II) ions to penetrate the pores of the sol-gel sensor. A preliminary concentration detection limit of 1.7 x 10^{-14} M or 1.7 ppq is determined for Fe (II) (S/N 2). Based on the analyte probe volume of approximately 5 nL used in the optical setup, a preliminary mass detection limit of 0.17 zepto mole (S/N 2) is determined for Fe (II).

Figure 5.6 demonstrates homogeneity of the sol-gel sensors. Several regions of the iron sol-gel sensor are probed in order to confirm homogeneity. Three wave-mixing signal peaks are averaged over five different regions of a sol-gel sensor. A similar study confirms analyte homogeneity for the iron sol-gel sensor that contains 4 x 10^{-14} M Fe (II) ions.
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Figure 5.5 Sensitive wave-mixing detection of Fe (II) 1,10-phenanthroline sol-gel sensor.
Figure 5.6 Sol-gel homogeneity study for five different probe areas on a 0.01 M 1,10-Phenanthroline sol-gel sensor doped with 100 µL of $8 \times 10^{-14}$ M Fe (II). Five different spots on the sensor are detected, averaged and compared to a “blank” 0.01 M 1,10-Phenanthroline sol-gel sensor.
Figure 5.7 shows the amount of time needed to achieve Fe (II) complexation, and hence, the maximum wave-mixing signal intensity. The signal intensity remains constant after 20 minutes. Since the wave-mixing probe volume is very small, it can detect Fe (II) ions even after only 5 minutes of sample incubation. In summary, iron sol-gel sensors are capable of entrapping Fe (II) ions in solution at extremely low concentration levels and then detected conveniently by laser wave-mixing spectroscopy.

5.4.2 Chromium Wave-Mixing Signal

The chelating agent DPC for Cr (VI) has similar properties as those of 1,10-phenanthroline. There is no optical absorption for DPC until complexation with Cr (VI) ions. Figure 5.8 shows optical absorption of DPC solution (1) at different concentrations of Cr (VI) 1,5-diphenylcarbazide complex in solution, (2) at 2 x 10^-6 M, (3) at 4 x 10^-7 M, (4) at 9 x 10^-6 M, and (5) at 2 x 10^-5 M. The maximum absorption wavelength for Cr (VI) 1,5-diphenylcarbazide complex solution is 540 nm. The lowest concentration of Cr (VI) ion detected by our conventional UV-visible spectrophotometer is 9 x 10^-7 M. There is a minimal difference between the DPC (blank) solution and the metal-ligand complex when using this conventional UV-visible spectrometer.
Figure 5.7 Wave-mixing signal dependence on incubation time for 100 µL of 3 x 10^{-4} M Fe (II) added to 0.01M 1,10-Phenanthroline sol-gel sensor.
Figure 5.8 Conventional UV-visible absorption profile of (1) 0.01 M DPC compared to different concentrations of Cr (VI), (2) $2 \times 10^{-6}$ M, (3) $4 \times 10^{-6}$ M, (4) $4 \times 10^{-6}$ M, and (5) $2 \times 10^{-5}$ M.
Figure 5.9 shows sensitive wave-mixing detection of Cr (VI) ions in a sol-gel sensor, with significant advantages over conventional absorption detection methods. Three reproducible wave-mixing peaks are collected with a minimum background level for 100 µL of 2 x 10^{-9} M Cr (VI) ions, after 20 minutes of incubation time. A preliminary concentration detection limit of 1.15 x 10^{-9} M or 115 ppt is determined for Cr (VI) ions (S/N 2). Based on the analyte probe volume used, a preliminary mass detection limit of 10 atto mole is determined for Cr (VI). The wave-mixing detection limit far exceeds those required by the EPA, i.e., 9.6 x 10^{-7} M or 0.1 ppm for Cr (VI).

5.5 Conclusions

Laser wave-mixing spectroscopy is demonstrated as a sensitive absorption-based detection method for environmental pollutants using sol-gel sensors. Doped sol-gel sensors allow fast and efficient entrapment of toxic metal ions. Sol-gel sensors coupled with laser wave mixing can easily detect small shifts and changes in optical properties of specific metal ligand complexes. As shown in Table 5.1, wave mixing offers excellent detection sensitivity levels as compared to those of other detection methods for Cr (VI) and Fe (II) ions.\textsuperscript{16-21} The chelating ligands used in this study are not specific to one metal, and hence, one could use a chip-based separation method or species-specific ligand doped sol-gels for more complex real-world samples.
Figure 5.9 Sensitive wave-mixing signal detection of Cr (VI) DPC sol-gel sensor.
Table 5.1 Comparison of currently available techniques for the detection of metal ions.

<table>
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<th>Technique</th>
<th>Metal Analyte</th>
<th>Mass Detection Limit</th>
<th>Concentration Detection Limit</th>
<th>Ref.</th>
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5.6 References


SENSITIVE WAVE-MIXING DETECTION OF DNA MICROARRAY SPOTS HYBRIDIZED WITH CY3

6.1 Abstract

Sensitive laser wave-mixing spectroscopy is used as a detection method for reading DNA hybridization events on a microarray. The DNA microarray consists of 70-mers oligonucleotide targets that are hybridized with a probe solution containing Cy3-labeled random 9-mers at concentrations ranging from 10 µM to 1 fM. After hybridization, the DNA microarray is scanned with an argon ion laser operating at 515 nm in a laser wave-mixing setup. Intra-spot resolution is obtained by mounting the array on a translational stage and focusing laser beams down to a diameter of 32 µm. An actuator conveniently scans the stationary microarray producing backward and forward scans of DNA spots and rows. Multiple scans determine accuracy and reproducibility of the wave-mixing signal. Microarray marker spots are sensitively detected and compared to oligonucleotide sample spots. Preliminary concentration detection limits of 7.4 x 10^{-16} M and 8.7 x 10^{-16} M (S/N 2) are determined for the marker spot and the oligonucleotide spot, respectively. Preliminary mass detection limits range from 2 to 8 fluorophores detected inside the laser probe volume.
6.2 Introduction

DNA microarray technology is immensely popular due to its widespread use in the advancement and understanding of diseases that may be correlated to gene expression. A microarray is an ordered array of microscopic elements on a planar substrate that allows the specific binding of genes or gene products. Microarrays are used to analyze thousands of multiple reactions at the same time with great speed and precision. The enormous capacity of microarrays makes it possible to analyze the entire human genome in one single experiment. Because gene expression can be correlated with function, microarrays can be used to provide information on human disease, aging, drug, mental illness, diet, and many other clinical matters that otherwise would be difficult and tedious to study. The capabilities of microarrays are opening new venues for testing, which are taking research to a new era of genetic screening and diagnostics. These exciting new advancements in microarray technology might also lead to the development of personalized medicine, a new field called Pharmacogenomics.

One of the earliest applications of DNA microarray technology to clinical problems was the study of tumors. DNA microarray analysis of a patient’s tumor might provide information that could improve their clinical treatment by profiling tumors and clarifying their complex biochemical pathways. Identifying single-gene products that are expressed in tumor cells, but not in normal tissue, can be used as tumor markers. In addition, microarrays have been used to analyze changes in gene expression in several other diseases including muscular dystrophy, Alzheimer’s disease, schizophrenia and HIV infection.
The platforms used for the microarrays are usually membranes or glass chips containing target molecules, specifically synthetic oligonucleotides (single stranded short nucleotide molecules arranged in a chain) or cDNA. The first microarray experiments were developed and performed in the early 1990s with cDNA. These microarrays consisted of DNA printed to the glass substrate and hybridized with cDNA, nucleic acid molecules derived from mRNA. Both target molecules are hybridized with a probe solution containing the complimentary half of the oligonucleotide or DNA and labeled with a fluorophore. For target preparation, mRNA from cells or tissue is extracted, converted to DNA and labeled. The labeled DNA is then hybridized to the elements on the array surface and detected. These hybridization reactions between target and probe molecules of DNA occur by hydrogen bond formation between the bases of complimentary nucleic acid sequences. Other competing methods of analysis require up to several micrograms of total RNA. The estimated recovery of RNA is approximately 10 micrograms per million cells. Therefore, it is very important to develop methods that require less RNA input or a great enhancement of detection sensitivity. The field of microarrays holds great promise for this area since the amount of sample required is very small even for the study of multiple reactions.

A sensitive detection method will help the advancement of this technology. Currently, fluorescence is the most commonly used method for the analysis of microarrays. Each spot fluoresces with an intensity that is proportional to the activity of the expressed gene that is spotted on the array. Time-resolved fluorescence measurement with a CCD camera offers some advantages over conventional fluorescence detection. Although sensitive, fluorescence-based methods have inherent
limitations. Analytes must fluoresce or they must be labeled with fluorophores, and the amount of sample required is still relatively high. In addition, background fluorescence levels are high from the glass, plastic substrate or the chemicals used in microarray processing.

Other methods for detection of microarrays include resonance light scattering (RLS). This method uses gold RLS particles coated with anti-biotin antibodies to bind to biotin-labeled targets. RLS particle labels are colloidal metals that scatter a light with specific color and intensity when illuminated by white light. The characteristics of the scattered light are determined by the metal particle size, shape and composition. RLS offers enhanced detection sensitivity over optical absorption methods by 3 or more orders of magnitude. However, several extra labeling steps complicate the detection procedure. Another disadvantage is that more steric effects are present due to the relatively large size of the RLS particle (40-120 nm in diameter).

Laser wave mixing is presented as a sensitive optical method for microarray analysis. This technique has been demonstrated and reported by our group for various applications. For these applications, wave mixing offers small probe volumes, intra-spot reproducibility, effective scanning of smaller DNA spots, better detection sensitivity, shorter analysis time, and higher spatial resolution suitable for higher density arrays. Wave mixing is especially effective in detecting smaller amounts of genetic material per spot, and hence, it can eliminate time-consuming PCR steps. In this chapter, we present wave mixing as an ultrasensitive optical absorption method for the detection of DNA molecules. Since analytes only need to absorb light and fluorescence is not required, both chromophores and fluorophores can be used as labels.
Preliminary concentration detection limits of $7.4 \times 10^{-16}$ M and $8.7 \times 10^{-16}$ M (S/N 2) are determined for the Cy-3 marker spot and the oligonucleotide spot, respectively.

6.3 Experimental

6.3.1 Microarrays

Figure 6.1 shows a schematic drawing of the DNA microarray slide, purchased from TeleChem International, Inc., (CheckIt Chips, Sunnyvale, CA). Slides are used as is. Each microarray chip (25 mm x 76 mm) is printed with 200 oligonucleotide targets in 200-300 µm spots, and configured as two identical 10 x 10 subgrids spaced 4.5 mm apart. Each spot is about 430 µm from the neighboring spots (center to center) as shown in Figure 6.1. The individual oligonucleotide targets consist of 70-mers from human, mouse and E.coli, which are printed onto a superamine substrate surface. The superamine surface contains primary amine groups on a glass slide that carry a positive charge at neutral pH. This charge permits the formation of ionic bonds with the negatively charged DNA phosphate backbone. A covalent bond is formed between the DNA and the surface with the use of ultraviolet light or heat. In addition, marker spots are placed on each corner of the grids. Marker spots are used to validate the observed signal.
Figure 6.1 Diagram of a DNA microarray slide.
6.3.2 Hybridization

The hybridization protocols outlined by the manufacturer are followed with some minor modifications. The microarray chip is processed to remove the unbound target sequences prior to hybridization. The chip is subjected to a 2 min. wash at room temperature in 2X saline sodium citrate (SSC) buffer in 0.1% sarkosyl solution. The chip is then washed for 2 min. in 2X SSC solution. The SSC buffer is made from sodium citrate (Fisher Scientific, NJ, USA), sodium chloride (VWR, PA, USA) and in-house double distilled water. The 0.1% sarkosyl (sodium lauroyl sarcosinate) solution is made from molecular biology grade sarkosyl solution (Fisher Scientific, NJ, USA). The microarray is then treated for 2 min. at 100 °C in distilled water and cooled to room temperature. Finally, the microarray is treated for 2 min. in ice-cold 100% ethanol and dried by slight shaking. A 10 µL drop of probe solution is added to the microarray and a cover slip is gently placed on top to distribute the solution on the entire grid. Microarray chips are hybridized in a hybridization chamber for approximately 3 hours at room temperature. The hybridization chamber consists of an empty pipette tip container with a damp paper towel at the bottom to prevent the microarray from drying out during the incubation period. After the hybridization step, the microarray is washed with the SSC buffers and dried by gentle shaking.

6.3.3 Probe Solution

Figure 6.2 illustrates the hybridization reaction and detection of the probe solution before scanning. The probe solution consists of a universal random 9-mer labeled with Cy3 label on the 5’ end, which will hybridize to all the spots in the
microarray. The 50 µL of 5x (50 µM) universal probe solution is included in the microarray kit along with a 1.0 mL of 1.25x superhyb hybridization solution. The stock probe solution is diluted to the desired concentration (i.e., serial dilutions using double distilled deionized water). The serial dilution is performed first by mixing 2 µL of the 50 µM probe stock solution with 8 µL of the 1.25x superhyb buffer. Then, 80 µL of the 1.25x superhyb buffer solution is mixed with 20 µL of water to prepare a buffer concentration of 1x. Next, the 10 µM probe solution is diluted 10x with the 1x superhyb buffer to yield a probe concentration of 1 µM. This 10x dilution is repeated until the final probe concentration of 1fM is obtained as shown below.

Serial dilutions:

\[
\begin{align*}
(1.0 \text{ µl})(10 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=1 \text{ µM probe} \\
(1.0 \text{ µl})(1 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-1} \text{ µM probe} \\
(1.0 \text{ µl})(0.1 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-2} \text{ µM probe} \\
(1.0 \text{ µl})(0.01 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-3} \text{ µM probe} = 1 \text{ nM} \\
(1.0 \text{ µl})(0.001 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-4} \text{ µM probe} \\
(1.0 \text{ µl})(0.0001 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-5} \text{ µM probe} \\
(1.0 \text{ µl})(0.00001 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-6} \text{ µM probe} = 1 \text{ pM} \\
(1.0 \text{ µl})(0.00001 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-7} \text{ µM probe} \\
(1.0 \text{ µl})(0.000001 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-8} \text{ µM probe} \\
(1.0 \text{ µl})(0.000001 \text{ µM probe}) &= (10 \text{ µl})(x) \quad x=10^{-9} \text{ µM probe} = 1 \text{ fM}
\end{align*}
\]

All solutions are prepared fresh each day, starting with the stock probe solution included in the kit. The pipette used for the serial dilution is a 2 µL P-2 Pipetman from Rainin Instruments (1.5% accuracy +/- 0.03 µL).

The buffer background noise is determined by hybridizing a microarray slide with only buffer and without any probe solution. The slide is defined as the “blank”
Figure 6.2 DNA microarray hybridization reaction steps
A blank wave-mixing signal is monitored for the blank glass surface between the microarray spots to ensure that there is no hybridization (i.e., no target).

### 6.3.4 Forward-Scattering Wave-Mixing Geometry

Microarrays are scanned using a forward-scattering wave-mixing setup and detected by Cy3 absorption at 535 nm. An argon-ion laser (Coherent, Inc., Model Innova 90-6) is used to excite Cy3 at 515 nm using a single 10-cm focal length lens. As shown in Figure 6.3, the argon-ion laser beam is split by a 70/30 (R/T) beam splitter and later focused and mixed on the microarray. The mixed laser pump beams create a thermal grating inside the array spot producing the signal beam. The signal is then sent through a spatial filter and detected by a photodiode detector (ThorLabs, Inc., Model PDA55, Newton, NJ, USA). An optical chopper (Stanford Research Systems, Model SR541, Sunnyvale, CA, USA) is used to modulate the weaker pump beam. The reference signal from the chopper is sent to a lock-in amplifier (Stanford Research Systems, Model SR810 DSP, Sunnyvale, CA, USA) and the signal is sent to a desktop computer for analysis. The laser power ranges from 2 to 4 mW during the microarray scanning process. The microarray is mechanically scanned in 20 µm increments using a motorized actuator (Zaber Technologies, Model ZLA-28, Vancouver, BC, Canada) controlled by a personal computer. Glass background noise is determined by scanning the glass surface between the microarray spots and the buffer background noise is determined by scanning the buffer slide. These background noise levels are compared to that from a probe hybridized spot.
Figure 6.3 Experimental setup for the detection of oligonucleotides on a DNA microarray.
6.4 Results

6.4.1 Wave-Mixing Signal

In a nonlinear wave-mixing setup, two input laser beams, I₁ and I₂, create an interference pattern inside an absorbing analyte, i.e., a thermal grating, which in turn diffracts incoming photons to create two signal beams, I₃ and I₄. These laser-like coherent signal beams have exactly predictable propagation directions, and thus, signal detection is very easy and efficient. The wave-mixing signal intensity, I₄, can be described as:

\[ I₄ = C \left( \frac{b}{8\pi} \right)^2 I₁^2 I₂ \frac{\lambda^2}{\sin^4(\theta/2)} \left( \frac{dn}{dT} \right)^2 \frac{\alpha^2}{\kappa^2} \]

where \( \lambda \) represents the laser wavelength, \( \theta \) is the angle between the incoming beams, \( \frac{dn}{dT} \) is the change in the index of refraction of the solvent with respect to temperature, \( \alpha \) is the absorption coefficient, \( \kappa \) is the thermal conductivity, and \( b \) is the path length of the laser beam cross section. Eq. 6.1 shows that the signal has a cubic dependence on laser power and a quadratic dependence on the concentration of the analyte. These nonlinear dependencies inherently make wave mixing very sensitive especially for monitoring small changes in microarray spots.

6.4.2 Wave-Mixing Spot Size

The spot size of the focused beams is calculated using the following approximation formula:

where \( \lambda \) represents the laser wavelength, \( \theta \) is the angle between the incoming beams, \( \frac{dn}{dT} \) is the change in the index of refraction of the solvent with respect to temperature, \( \alpha \) is the absorption coefficient, \( \kappa \) is the thermal conductivity, and \( b \) is the path length of the laser beam cross section. Eq. 6.1 shows that the signal has a cubic dependence on laser power and a quadratic dependence on the concentration of the analyte. These nonlinear dependencies inherently make wave mixing very sensitive especially for monitoring small changes in microarray spots.
\[ \omega'_o \approx \left( \frac{\lambda}{\pi \omega_o} \right) f \]  

where \( f \) is the focal length of the lens, \( \omega_o \) is the laser beam radius before the lens, \( \lambda \) is the laser wavelength, and \( \omega'_o \) is the beam radius at the focal point. Using the laser wavelength of 515 nm, the focused spot diameter of the laser is found to be 29 \( \mu \)m. In the actual experiment, the sample is not exactly placed at the 10 cm focal point of the lens. The reason for this is that the maximum wave-mixing signal is obtained 2 mm off the focus point. This maximization of the signal is done while visually monitoring the signal intensity on the computer while manually adjusting the sample in the x-direction of the focusing lens. This off-focus spot size can be calculated by using the Gaussian beam formula\(^{18}\):

\[ W^2(z) = W_o^2 \left[ 1 + \left( \frac{\lambda z}{\pi W_o^2} \right)^2 \right] \]

where \( W(z) \) corresponds to the calculated laser width at the new focal point, \( W_o \) is the width at lens focal point, \( \lambda \) is the absorbed wavelength of the laser, and \( z \) is the distance from the focal point. The estimated spot size diameter is then calculated to be 32 \( \mu \)m.

### 6.4.3 Grating Period

Using a 1.1-degree angle and the 32 \( \mu \)m focused beam diameter, the grating period is calculated to be 13 \( \mu \)m (i.e., the distance between the constructive and destructive interference bands). The following equation is used to calculate the grating period: \(^{19}\)
where $\Lambda$ corresponds to the grating period, $\lambda$ corresponds to the laser wavelength, and $\theta$ is the angle between the two laser beams. Signal can be obtained from two or more grating periods in the probe volume\textsuperscript{19}. This calculation yields approximately 3 grating periods inside our probe volume.

### 6.4.4 Resolution Studies

The laser wave-mixing probe volume is much smaller than the DNA microarray spot of 200-300 $\mu$m, which allows intra-spot scanning resolution. Hence, each microarray spot hybridized with a 10 $\mu$L probe solution can be scanned, detected and signal strengths compared. The first spot scanned is a marker spot, Spot 1, which is prelabeled with Cy3 single-stranded control oligonucleotides. The wave-mixing probe is used to scan Spot 1 several times in the forward direction. Figure 6.4 shows scanning profiles yielding intra-spot resolution for a microarray, Spot 1, using a laser power level of 4 mW. Two distinct peaks are observed across this marker spot, which is likely due to the uneven printing process of the target oligonucleotides.

It is known that not all spots on the microarray have the same shape or size.\textsuperscript{20} Various spot shapes observed on microarrays include circular, elliptical and non-convex types. If one assumes that all the spots have the same shape, e.g., circular, some error
Figure 6.4 Spot 1 on the DNA microarray hybridized with 10 µM of probe solution. Multiple scans of Spot 1 in the forward direction.
will be introduced in the data analysis. An image quantification software package can be employed to reduce these types of errors by segmenting the spots and assigning more accurate areas for the signal. One could also reduce errors caused by spot non-uniformity by monitoring the background level and the spot signal at each image pixel. Compared to these complex and time-consuming methods, wave mixing offers a faster and simpler way of determining the signal and blank spots in only one scan.

In comparison, Spot 109 is scanned in both the forward and backward directions producing a single reproducible peak across the scan. Spot 109 is an oligonucleotide spot hybridized with the complimentary labeled 9-mer strands. Figure 6.5 shows the average scanning profiles for Spot 109 with the (A) backward scanning direction and (B) forward scanning direction. Reproducible peak heights indicate that the wave-mixing signal strengths are reproducible and that the computer driven actuator movements are accurate. Small discrepancies between the scan-profile can be attributed to small mechanical lags in the actuator movement in different drive directions, as indicated by actuator manufacturer specifications. The scan profiles are more reproducible when the actuator is driven in the same direction. The actuator scans the microarray spots at 20 μm increments in order to collect 10 data points across the entire spot size of 200 μm. This method produces intra-spot resolution, and if desired, can be enhanced by focusing the laser beams more tightly and by using smaller actuator movements (as small as 0.1 μm).
Figure 6.5 Spot 109 on the DNA microarray hybridized with 10 μM of probe solution. Multiple scans of Spot 109 in both the (A) forward scanning and (B) backward scanning directions.
Conventional microarray scanners take an average or blend of incremental signals to obtain spot profiles. As scan resolution approaches the spot diameter, the spots appear grainy and pixilated, and quantification of the microarray signal is adversely affected. Our wave mixing-based microarray detection offers some advantages since the spot signals are strong and reproducible, and the background noise is negligible. As mentioned above, multiple scans are not needed and the intra-spot resolution is high. As a result, wave mixing allows detection of smaller microarray spots, and hence, the effective and reliable use of higher density arrays, which in turn allows more efficient use of smaller amounts of DNA or probe molecules.

6.4.5 Reproducibility Studies

Rapid automated scans of ten spots demonstrate method reproducibility. The microarray slide is hybridized with 10 μM of probe solution. Figure 6.6 shows six 5-second scans of ten spots, Spots 10 to 100. The computer driven actuator is programmed to scan the microarray with 350 μm increments. Data is normalized against the marker spot, Spot 100. The marker spot yields the highest signal intensity. Spots 10 to 90 have differences in sequence composition of the 70-mer oligonucleotide targets, and they yield varying signal intensity levels as compared to the marker spot, as expected. Figure 6.6 shows that our rapid automated scans of microarray rows yield reproducible scan profiles with good mechanical stability.
Figure 6.6 Scan of rows 10 to 100 on the DNA microarray hybridized with 10 μM probe solution.
6.4.6 Sensitivity Hybridization Studies

To determine the sensitivity of our method, hybridizations with different concentrations of probe solutions are measured. Furthermore, when working with low femtomolar-level probe hybridized arrays, one might expect that not all of the target molecules on the microarray spot will hybridize. Taking advantage of the intra-spot resolution capability, individual spots can be mapped and the effective area where the probe solution binds to the target molecules can be determined. Figure 6.7 shows the wave-mixing signal collected from 3 spots, (A) 1, (B) 109, and (C) 160, as they are mapped along the forward direction of the actuator movement. The effective hybridized area measured ranges from 60 µM to 120 µM for these spots. Multiple scans are collected to ensure reproducibility. As one can expect, the probe does not bind perfectly over the entire spot area, resulting in reduced hybridized areas in some spots. Although the array manufacturer indicates a 300 µm target size, only 120 µm of the spot has effective hybridization, and hence, measurable wave-mixing signal when using a probe concentration of 10 µM. One can assume that the effective hybridized array size is even smaller when lower probe concentration levels are used.

6.4.7 Detection Sensitivity

The wave-mixing detection sensitivity is tested using hybridizations with different probe concentrations ranging from 1 fM to 10 µM. The wave-mixing signal of Spot 100, the marker, is compared to that of Spot 190, an oligonucleotide target spot. Figure 6.8 (A) shows the wave-mixing signal for Spot 100 hybridized with 1 fM probe
Figure 6.7 Effective hybridized area of Spots 109, 1 and 160. Forward scanning direction.
Figure 6.8 Sensitive wave-mixing detection of (A) Spot 100 and (B) Spot 190 of the DNA microarray hybridized with 1 fM probe solution.
solution. To determine the background signal, a microarray slide hybridized with buffer containing no probe is analyzed. This blank slide is prepared using the same procedure as the other slides but without the Cy-3 probe. The wave-mixing signal levels from Spot 100 on the blank slide and Spot 100 on the 1 fM probe slide are collected in multiple scans with good reproducibility. As expected, the slide with the 1 fM probe solution shows drastically higher signal intensity as compared to the clean baseline level collected from the blank slide. A preliminary concentration detection limit of \(7.4 \times 10^{-16}\) M is determined for the Cy-3 probe solution (S/N 2).

Figure 6.8 (B) also shows the wave-mixing signal for DNA sample Spot 190 hybridized with 1 fM probe solution. The signal of Spot 190 is compared with that of the same spot on the blank slide. To ensure that the glass surface between Spot 190 and Spot 200 is clean without any baseline signal, it is scanned and the results compared. A preliminary concentration detection limit of \(8.7 \times 10^{-16}\) M (S/N 2) is determined for regular oligonucleotide Spot 109 using a laser power of 2 mW. Comparing the signal for Spot 100 in Figure 6.8 (A) and that for (B) Spot 109, it is apparent that the marker Spot 100 yields a higher wave-mixing intensity level, as expected.

A probe volume of 10 \(\mu\)L is used to cover 200 DNA spots on a microarray slide. Based on a probe-to-target ratio of 1:1, coverage density is estimated to be \(5 \times 10^{-8}\) L/spot. There is an estimated 30 fluorophores per spot when multiplying the coverage density by the number of fluorophores per liter used. Based on the effective hybridized spot diameter of 60 \(\mu\)m, the microarray spot area of 2827 \(\mu\)m\(^2\), laser probe diameter of 32 \(\mu\)m, and a laser probe area of 804 \(\mu\)m\(^2\), we estimate that there are approximately 9
fluorophores in the laser probe area. Using similar calculations for a microarray spot diameter of 120 µm (i.e., the upper range of measured hybridized area), we estimate that only 2 fluorophores are present in the laser probe area. Therefore, depending on the effective hybridized area, the range of fluorophores present in our laser probe area is 2 to 9.

For regular oligonucleotide spots, e.g., Spot 109, the concentration detection limit is determined to be $8.7 \times 10^{-16} \text{ M}$, as mentioned above. This corresponds to a mass detection limit of 2 to 8 fluorophores or 0.003 to 0.01 zeptomole for an effective hybridization range of 120 to 60 µM. For the Cy-3 marker spots, e.g., Spot 100, the concentration detection limit is determined to be $7.4 \times 10^{-16} \text{ M}$, as mentioned above. This corresponds to a mass detection limit of 2 to 6 fluorophores or 0.003 to 0.01 zeptomole for an effective hybridization range of 120 to 60 µM. As mentioned above, our effective hybridization area range of 60 to 120 µM is estimated using a relatively high concentration level (µM), and this range is expected to be smaller when using lower probe concentration levels. Hence, the corresponding mass detection limits could be even better than those mentioned above.

Table 6.1 compares the sensitivity level of wave mixing with those of other sensitive methods. In the near-IR time-resolved fluorescence method, a probe concentration of 100 nM is used to obtain a detection limit of 0.38 molecules/µm² (S/N 2). Sensitivity is improved by a factor of 10 if time-gated detection is used for the same slide. The detection limit for the probe remains high. Resonance light scattering (RLS) method, shown in Table 6.1, uses gold RLS particles coated with anti-biotin
antibodies to bind to the biotin labeled targets, a more stringent labeling protocol. Wave mixing offers faster analysis in a simple setup. Even when using a conservative estimate of a mass detection limit value of 8 fluorophores for the regular oligonucleotide spot (0.0093 fluorophores per µm² at S/N 2), wave mixing offers favorable results as compared to other methods.

6.5 Conclusion

DNA microarray technology combined with laser wave mixing offers many advantages for sensitive detection of gene expressions. High throughput analyses for multiple reactions are detected with excellent sensitivity. Detectable oligonucleotides concentration of 1 fM demonstrates that laser wave mixing is effective at detecting smaller amounts of genetic material per microarray spot. Small laser probe volumes provide intra-spot resolution that may be used for higher density arrays for faster and less expensive analyses. Since it is based on absorption, wave mixing can be used to detect both fluorescing and non-fluorescing molecules, providing a larger choice of array labels. Future work includes the analysis of smaller diameter spot DNA microarrays and the use of chromophore-labeled probes.
Table 6.1 Comparison of currently available techniques for the detection of DNA using DNA microarrays.

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<tr>
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<th>Concentration Detection Limit</th>
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6.6 References


LASER WAVE-MIXING DETECTION OF ANTIBODY-BASED PROTEIN MICROARRAYS

7.1 Abstract

Sensitive laser wave-mixing detection of cytokines is presented using ELISA-based reactions performed on a microarray surface. Microarray slides contain 12 identical grids. Each grid consists of 3 different antibodies, along with a positive and negative control, spotted six times for a total of 30 spots per grid. Cytokine antibodies, IL-2, TNF-alpha and IFN-gamma, are immobilized on the microarray surface and hybridized with their corresponding antigens. Alexa-labeled streptavidin binds to the biotin-labeled antibodies. An argon-ion laser operating at 514 nm excites the dye and scans the array using a motorized actuator. Tightly focusing the input beams within a spot, and scanning the spots incrementally allow intra-spot spatial resolution. Three-dimensional images of entire rows and spots are obtained. Positive and negative control spots are compared to antibody spots for verification and detection of true wave-mixing signal. Excellent selectivity is obtained with some cross-hybridization between the antigens. Preliminary detection limits of 0.01 pg/mL and 0.05 pg/mL (S/N 2) are obtained for IL-2 and IFN-gamma, respectively.
7.2 Introduction

Protein microarrays allow convenient and fast measurement of cytokine activities and abundances. These microarrays provide information on proteomic functions and interactions within cellular networks as well as integrated views of disease mechanisms for the discovery of new disease markers.\(^1\) Microarrays allow convenient, fast, multiple protein analyses at minimal cost and sample volume. They allow studies of numerous samples and interactions between proteins, low molecular weight compounds, DNA and peptides. They are the new diagnostic tool for proteomics and many other fields that deliver thousands of parameters in a single experiment.\(^2\) This sensitive and high-throughput analytical tool is very useful for studying complex cytokine pathways.

Cytokines, glycoproteins grouped into separate families according to structural and functional similarities, play crucial roles in cell-to-cell signaling.\(^3\) They have very important roles including regulation of inflammatory responses and direct interference with invading pathogens. Cytokines affect neighboring cells even in relatively small concentrations due to their high affinity for ligands. Cytokines are potent molecules that are active at picomolar to femtomolar concentration levels.\(^4\) Key cytokines that carry out the most important functions are IL-2 (interleukin), IFNs (interferons), and TNF (tumor necrosis factor). Macrophages produce TNF-alpha in response to bacteria and other pathogens. TNF has anti-tumor properties giving it the ability to modify certain cell activity, such as uptake for tumor destruction or reversal of tumor growth. A wide range of cells, when under attack from viruses and other non-self pathogenic
antigens, produces IFNs. The IFN-gamma and IL-2 antigens act on the growth, differentiation and activation of T, B and other cells, regulating all phases of immunity and inflammatory responses. Imbalances in the production of cytokines, particularly those affecting immuno regulations, can have profound effects, and they are implicated in numerous disease states.

These new types of protein and antibody arrays are similar to cDNA microarrays. Although, cDNA microarrays yield the sequence information of each protein, they offer very little information about their localization, structure, modifications, interactions, activities and ultimately their functions as well as abundances. Studies show a clear disparity between the relative expression levels of mRNA and their corresponding proteins. Antibodies and proteins are chemically and structurally different, requiring some modifications to the existing cDNA microarray technology.

The protein microarray format used in this study is a sandwich protein detection array, which is capable of recognizing target proteins in biological samples found in low concentrations, such as cytokines. Specific cytokines are immobilized in a predetermined position on a solid glass support, retaining their capabilities of recognizing and capturing their unlabeled antigens. A cocktail antibody solution then captures these antigens of interest. The cocktail antibody solution is a biotinylated antibody that is incubated with streptavidin-conjugated Alexa dye for detection purposes. These particular sandwich arrays take advantage of the proven utility of enzyme-linked immunosorbent assay (ELISA), and due to the small size of the arrays, they offer minimum consumption of analytes and reagents.
Current detection methods for proteins include MALDI mass spectrometry after separation by 2-D gel electrophoresis, laser-based fluorescence and surface plasmon resonance (SPR). Each technique is widely used but has some inherent limitations when used for microarrays. For instance, the amount of captured protein may not be sufficient to be detected in MALDI MS and excessive sample handling steps can lead to erroneous protein peaks due to sample contamination. Although SPR is a sensitive method, it has yet to be shown that this technique can be adapted to the scale already employed in conventional microarray technology. Fluorescence spectroscopy is the most widely used detection method for antibody-based arrays. Recent advances in the field of low-level antibody detection include the development of stronger fluorophores. Although these techniques can be quite powerful, greater sensitivity and specificity are often required, particularly when working with limited amounts of analytes and low antibody density levels. Amplification steps, such as rolling circle DNA, have been studied, but they require multiple sample preparation steps. Protein arrays have been used with radioactive labeling or horseradish peroxidase-substrate systems, but they are not compatible with currently available detection systems.

Wave mixing is a very sensitive method for the detection of biomolecules. This nonlinear optical technique has the ability to detect and measure biospecific interactions on an antibody-based microarray platform. The wave-mixing signal is a coherent collimated beam, and hence, signal collection efficiency is virtually 100%. In this chapter, wave mixing is demonstrated as a sensitive absorption-based detection method for protein profiling of three biologically relevant cytokines on an antibody microarray. A preliminary concentration detection limit of 0.01 pg/mL or $3 \times 10^{-15}$ M is
obtained for IL-2 and 0.05 pg/mL for IFN-gamma. A preliminary mass detection limit of 1 femtogram or $6 \times 10^{-20}$ mole (S/N 2) is obtained for IL-2 and IFN-gamma.

7.3 Experimental

7.3.1 Microarray Fabrication

Figure 7.1 shows a diagram of the microarray slide purchased as part of a human cytokine test kit (Neupro Technology, NEU001). The microarrays are spotted 6 times per grid with IL-2, TNF-alpha and IFN-gamma antibodies, and two control spots. The positive control spot contains Biotin label Mouse polyAb and the negative control spot has PBS-T. The antibodies are immobilized on the slide by aldehyde condensation. The microarray chip measures 25 mm x 76 mm. Spot diameters are 200 µm and the spot-to-spot distance is 300 µm. The array surface has a total of 12 identical grids. Each grid is incubated with different concentrations and types of antigens. Roman numerals are assigned to each grid for identification purposes.

7.3.2 Hybridization

The antibody attached to the microarray glass surface binds to a specific antigen. A biotin-conjugated anti-cytokine antibody cocktail is introduced to bind to the antibody-antigen complex (i.e., ELISA hybridization reaction). An Alexa 514 streptavidin conjugate (Molecular Probes, S32353) is introduced to bind to the biotin, and it is the molecule the laser excites and detects.
Figure 7.1 Experimental setup for the detection of cytokines on a protein microarray.
The ELISA hybridization process outlined by the kit manufacturer is followed with some minor modifications. Each microarray slide is treated with 1x blocking buffer for 30 min. with gentle agitation. All agitations are manually performed. After washing with 1x wash buffer II, 20 µL of antigen are added. Incubation with the antigen is performed for 60 min. followed by washing with buffer I and buffer II. Then, 12 µL of 1x biotin-conjugated anti-cytokine antibody cocktail is added to each well and incubated for 30 min. After washing with buffer I and II, 20 µL of 1x streptavidin Alexa 514 conjugate is added and incubated for 30 min. A final wash with both buffers is done, followed by drying.

7.3.3 Solutions

IL-2, recombinant, human (US Biological, I7663-26), TNF-alpha, recombinant, human (US Biological, T9160-10), and IFN-gamma, recombinant (US Biological, I7662-16A) are the antigen solutions used in this study. IFN-gamma and TNF-alpha are reconstituted in 1x PBS (US Biological, D9820) to 1 mg/mL and 0.5 mg/mL stock concentrations, respectively. IL-2 is diluted in 100 mM acetic acid to a concentration of 0.5 mg/mL. The antigens are further diluted in 1x PBS buffer by serial dilution to the desired concentration for hybridization. All solutions are made fresh each day, starting from the stock solutions. Alexa Fluor 514 conjugate is diluted in 1x PBS buffer to a stock concentration of 1 mg/mL. This stock is further diluted 100x in PBS to a working concentration of 0.01 mg/mL that is used to hybridize to the antibody array.
7.3.4 Wave-Mixing Setup

Antibody microarrays are scanned using a forward-scattering wave-mixing setup as shown in Figure 7.2. A 3 mW argon-ion laser beam (Coherent, Inc., Model Innova 90-6) is used to excite the dye at 515 nm. The laser beam is split by a 70/30 R/T beam splitter and later focused by a 10-cm focusing lens on the microarray. As the two pump beams focus on the microarray spot, an interference grating is formed. Sample molecules absorb light energy in the form of the interference pattern, producing thermal gratings that create the wave-mixing signal. The signal is then sent through a spatial filter and then detected by a photodiode detector (ThorLabs, Inc., Model PDA55). An optical chopper (Stanford Research Systems, Model SR541) is used to modulate the weaker pump beam. The reference signal from the chopper is sent to a lock-in amplifier (Stanford Research Systems, Model SR810 DSP) and the signal is digitized and collected by a desktop computer.

7.3.5 Scanning

The microarray is mechanically moved in 25 µm increments by a motorized actuator (Zaber Technologies, Model ZLA-28) controlled by a computer. Glass background noise is determined by scanning the glass surface between the spots. For the selectivity studies, the array is scanned with individual optimization for each spot. The wave-mixing signal is collected, digitized and saved on the computer for further imaging and data analysis.
Figure 7.2 Diagram of a protein microarray. Each microarray is divided into 12 identical grids with 30 spots on each grid.
7.4 Results and Discussion

7.4.1 Wave-Mixing Parameters

More sensitive methods are needed for detection of small amounts of samples hybridized to the microarray spots. Wave mixing offers inherently small analyte probe volumes that can easily be coupled to microarray spots while yielding excellent detection sensitivity. The wave-mixing signal beam has a predictable propagation direction, and thus, signal detection is very easy and efficient. The wave-mixing signal intensity, $I_3$, can be described as:  \[ I_3 = C \left( \frac{b}{8\pi} \right)^2 I_2 I_1 \frac{\lambda^2}{\sin^4(\theta/2)} \left( \frac{dn}{dT} \right)^2 \frac{\alpha^2}{\kappa^2} \]  where $\lambda$ represents the laser wavelength, $\theta$ is the angle between the incoming beams, $dn/dT$ is the change in the index of refraction of the solvent with respect to temperature, $\alpha$ is the absorption coefficient, $\kappa$ is the thermal conductivity, and $b$ is the path length of the laser beam cross section. Eq. 1 shows that the signal has a cubic dependence on laser power and a quadratic dependence on the concentration of the analyte as well as a quadratic dependence on temperature gradient of refractive index. These nonlinear dependencies inherently make wave mixing very sensitive, especially for monitoring small changes in microarray spots.

Some solvents can be used to enhance the wave-mixing signal. Figure 7.3 shows the effect that ethanol addition has on the signal. The wave-mixing signal is collected from a microarray spot as a drop of ethanol is added. Initially the signal intensity decreases dramatically, and after a few moments, the signal intensity increases.
Figure 7.3 Addition of solvent (ethanol) enhances laser wave-mixing signal due to inherent wave-mixing dependences.
After more time, signal strength starts to decrease. Although Figure 7.3 may look complicated, the explanation is simple. The addition of ethanol increases the index of refraction of the medium, and as shown in Eq. 1, it in turn increasing signal intensity. After a few minutes, signal starts to decrease due to ethanol evaporation and the decrease in the index of refraction. This observation is reproduced several times and it confirms the wave-mixing signal. It is believed that the molecules forming the ELISA sandwich act like solvent molecules around the Alexa dye, significantly contributing in the wave-mixing signal.

Studies are performed to show the cubic dependence of the wave-mixing signal on laser power. The wave-mixing signal from a microarray spot hybridized with 200 pg/mL of IL-2 is detected as the laser power is increased. Figure 7.4 shows the expected result with a slope of 2.8 and a $R^2$ value of 0.98. At higher power levels, power saturation is observed as expected especially since the amount of sample present in the probe volume is very small.

### 7.4.2 Selectivity

Each array row is spotted with 3 different antibodies that will only bind a specific antigen. Positive and negative control spots are also added to verify the success of the assay. Ideally, when only introducing one antigen, e.g. IL-2, no cross reactivity should occur and only signal from the IL-2 spot and the positive spot should be observed. This is not always the case and cross reactivity is observed in some of our
Figure 7.4 Cubic power dependence study for cytokines. Power saturation is observed since detected concentration level of cytokine is extremely low.
arrays. Cross reactivity affects our detection limits due to the loss of target antigen to neighboring spots.

Different antigens are individually hybridized on different microarray grids to test for binding selectivity. It is observed that cross reactivity does occur mostly between two different antibodies or spots. Figure 7.5 shows an assay where 100 pg/mL of TNF-alpha is hybridized to one of the grids on the microarray. A peak is seen for TNF-alpha, but also another peak of equal intensity is observed for IL-2 due to cross hybridization. No signal is observed for IFN-gamma since no cross hybridization is present. A bigger signal is observed for the positive control spot and no signal is observed for the negative spot, indicating that the array works properly. If every spot had signal, then selectivity could not be claimed and random hybridization would have occurred. This is not the case here, and selectivity, although limited, is observed.

Figure 7.6 shows an assay where 10 pg/mL of IFN-gamma is hybridized to an array grid. The IFN-gamma shows a signal peak, but cross reactivity with TNF-alpha is observed. There is a signal from the IL-2 spot, indicating possible hybridization. But, it can also be attributed to background since the hybridization concentration of IFN-gamma is low. The positive control spot has a big peak and the negative control spot has no signal, indicating that the assay did work. Figure 7.7 shows an assay where 2000 pg/mL of IL-2 is hybridized to a microarray grid. Since the concentration of IL-2 is so high, the peak is as tall as the positive control peak. The negative and glass spots show some signal also for this same reason. Cross reactivity with IFN-gamma is mostly observed. Some with TNF-alpha is also observed, but it is less than the one with IFN-gamma.
Figure 7.5 Wave-mixing detection of one microarray row incubated with 100 pg/mL TNF-alpha antigen.
Figure 7.6 Wave-mixing detection of one microarray row incubated with 10 pg/mL IFN-gamma antigen.
Figure 7.7 Wave-mixing detection of one microarray row incubated with 2000 pg/mL IL-2 antigen.
Cross reactivity can occur when one antibody has different affinities for many antigens. It can also be a result of the hybridization procedure not performed correctly. The washing steps done after incubation with the antigen may not have been done as rigorously as necessary, leaving some of the mismatched linked antibodies attached on the neighboring spots. This problem could be minimized by using an orbital shaker instead of manual shaking.

### 7.4.3 Mapping

Moving the microarray in two directions by a motorized actuator and a translational stage provides smooth and automated scanning capabilities. Figure 7.8 shows a 3-D wave-mixing signal profile for a positive control spot. The actuator moves in 25 µm increments along the spot. Once the entire spot is scanned in one direction, the actuator returns to the starting position. Then, the array is moved 45 µm by a translational stage in a direction perpendicular to the actuator scan direction. Intra-spot resolution is obtained and distinct topography of the spot is observed. This capability can be used to determine the uniformity of the spotting technique. The figure shows that the spot does not always exhibit the strongest signal at the center.

### 7.4.4 Detection Studies

Figure 7.9 shows the scanning profile of a microarray row hybridized with 0.1 pg/mL of IFN-gamma antigen. A large peak is observed for the positive spot as expected, with nothing observed for the negative control spot or the adjacent glass surface. The glass adjacent to the spots is scanned to determine if anything reacted with
Figure 7.8 3-D wave-mixing signal profile of a single microarray spot showing intra-spot resolution.
Figure 7.9 Sensitive wave-mixing signal detection of protein microarray hybridized with 0.1 pg/mL IFN-gamma antigen.
the glass. The second largest peak corresponds to the IFN-gamma spot, as expected. Some cross reactivity is present with TNF-alpha. Taking IL-2 as the background signal, a preliminary concentration limit of 0.05 pg/mL is obtained for IFN-gamma (S/N 2).

Figure 7.10 shows the row scan of a microarray grid hybridized with 0.01 pg/mL of IL-2. The positive control spot and the IL-2 spot show the strongest wave-mixing signal, as expected. Some cross reactivity is observed, but S/N is greater than 2 when compared to the negative control spot or other antigen spots. Cross reactivity adversely affects the detection limits.

Table 7.1 compares the detection sensitivity of this method to those of other methods for detection of cytokines and other antigens performed on antibody-based microarrays. The wave-mixing method compares favorably with other methods and shows better detection limits. Only one method shows detection limits close to that of wave mixing, however, it required DNA amplification after the ELISA reactions is performed. These extra steps make the overall procedure longer and more complicated. Wave-mixing detection does not require extra reactions or steps.

7.5 Conclusions

Wave mixing spectroscopy offers many advantages as a sensitive detection method for antibody-based microarrays. Inherent nonlinear dependences including quadratic dependence on analyte concentration allow more dramatic monitoring of small changes in analyte properties. Wave mixing allows efficient signal collection since the signal, a coherent laser-like beam, can be measured against a dark background.
Figure 7.10 Sensitive wave-mixing signal detection of protein microarray hybridized with 0.01 pg/mL IL-2 antigen.
Since wave mixing can detect both fluorophores and chromophores, one can choose from a much wider array of labels. The probe volume is very small (nanoliter to picoliter) and it allows excellent spatial resolution for intra-spot probing. Effective 3-D imaging of individual spots is achieved to give precise detail of the distinct topography within spots. This allows a closer look at the spotting efficiency. Selectivity of hybridization is observed, but some problems with cross-hybridization need to be resolved and hybridization conditions optimized. Once these problems are corrected, detection limits can be further enhanced. Future work includes the use of higher density arrays with smaller spot sizes for faster and more cost effective analyses, and the use of expanded probe beams to image the entire grid at once.
Table 7.1 Comparison of currently available techniques for the detection of cytokines using protein microarrays.

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<th>Technique</th>
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<th>Concentration Detection Limit (pg/mL)</th>
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</table>
7.6 References


8

IN VITRO BIOMATERIAL-INDUCED CYTOKINE DETECTION ON ANTIBODY-BASED PROTEIN MICROARRAYS USING LASER WAVE MIXING

8.1 Abstract

A sensitive wave-mixing detection method is presented for proteins based on biomaterial-induced cytokine secretion. Wave mixing monitors small changes in cytokine expressions collected from culture media with 1, 24, 48 and 72 hours of exposure levels to titanium (Ti) particles and lipopolysaccharide (LPS). The supernatants are applied to a protein array via ELISA-based reactions. Each array contains 12 identical grids with six robotically printed rows of capture antibodies, TNF-alpha and IFN-gamma. LPS induces the release of all three cytokines between 1 and 24 hours of treatment, whereas Ti induction of cytokines is prominently expressed after 48 hours. Wave mixing allows simple and sensitive monitoring of changes of cytokine expression induced by Ti particles that resemble in vivo situations near arthroplasties where implant particles make contact with inflammatory cells such as macrophages. Quadratic dependence on analyte concentration allows wave mixing to monitor small dynamic changes of low cytokine concentrations (<10 pg/mL) more dramatically.
8.2 Introduction

Biomaterials used for prosthetic implants and artificial extensions at the bone-joint surface for enhanced joint movement are widely investigated and characterized in order to understand biocompatibility after implantation. With increasing numbers of prostheses being implanted into patients, it is important to understand implant failure mechanisms.\textsuperscript{1,2} Prosthetic implants are normally made from metals or high-density polyethylene biomaterials. Implant failures may be attributed to aseptic loosening. In the absence of infection, the release of wear debris can promote adverse reactions of inflammatory cells. Wear debris of polyethylene, metal or bone cement from implants are formed by many factors including biological response of the bone to stress shielding, micro motion at the bone-prosthesis, bone-cement or cement-prosthesis interface, and biological loosening due to osteolysis caused by unfavorable cellular reactions to debris generated.\textsuperscript{2} Any combination of these factors contribute to the inflammation process.

This chapter studies wear debris that activate cells, such as macrophages, to secrete a variety of cytokines and growth factors. Cytokines are chemical-protein messengers that coordinate inflammatory responses. Cytokines, such as tumor necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma), are secreted during phagocytose, a process in which macrophages engulf and destroy micro-organisms and orchestrate the processes of inflammation and repair at tissue sites that have been subjected to any form of trauma, infection or intrusion by prostheses.\textsuperscript{2} The result is a
gradual loss of bone, known as osteolysis. Hence, bone adjacent to implants contributes to loosening of prostheses.\textsuperscript{3-5}

Temporal profiling of cytokines and growth factors that interrogate biomaterials from implants has the potential to form the basis of a sensitive and predictive assay of implant biocompatibility.\textsuperscript{4-7} In this study, macrophages are exposed to a biomaterial that constitute implant wear debris (e.g., titanium particles), inducing cytokine secretion in the culture medium.\textsuperscript{7-13} A protein microarray, based on enzyme-linked immunosorbent assay (ELISA) and a sensitive wave-mixing detector are used to detect expressed cytokines from macrophage culture media.

Microarrays allow convenient, fast, multiple protein analyses with minimum cost and analyte consumption. Numerous samples and interactions between proteins, low molecular weight compounds, peptides and DNA can be identified and quantified by protein microarrays. They offer many advantages for a wide range of applications including temporal profiling of cytokine expressions.

Interfacing a sensitive wave-mixing detector to protein microarray allows sensitive cytokine-based in vitro studies for biomaterials. Reliable and sensitive detection of small changes in cytokine expressions can be made. Quadratic dependence on analyte concentration and absorption allows wave mixing to detect small changes more dramatically as compared to currently available commercial detectors and scanners.\textsuperscript{13-16}

Wave mixing is based on creating thermal gratings in the analyte medium, resulting from constructive and destructive interferences of two crossing input laser beams. Analyte molecules inside the interference patterns absorb the light energy,
creating a spatial modulation of excited molecules. The absorbed energy is released non-radiatively to surrounding molecules, resulting in a refractive-index modulation (thermal gratings). Incoming photons from the input laser beams diffract off the thermal gratings at specific propagation directions, generating coherent laser-like signal beams. The wave-mixing signal is generated only in the presence of absorbing analytes. Since wave mixing is an unusually sensitive optical absorption method, it can detect both fluorescing and non-fluorescing molecules with detection sensitivity levels comparable or better those of fluorescence-based methods.

### 8.3 Experimental

Figure 8.1 shows the forward-scattering wave-mixing optical setup with an argon ion laser (Coherent, Inc., Model Innova 90-6) operating at 514 nm and 3 mW. The laser beam is split by a 70/30 R/T beam splitter into two input laser beams, a forward pump and a forward pump/probe beam. The two input beams are mixed and focused with a 7.5 cm focusing lens, resulting in a 20 µm diameter probe spot. An interference pattern is formed by the two input beams inside the microarray spot. A thermal grating is formed by absorbing Alexa fluorophores. Only one wave-mixing signal beam, generated from the stronger input laser beam, is collected and detected for cytokine studies.

The protein microarray is mounted on a XYZ translational stage at the focal point of the mixing lens. The protein microarray is then scanned under computer control, from spot to spot, for target antigen detection. The wave-mixing signal beam propagates through the microarray at a 0.5° angle away from incident laser beams, and
Figure 8.1 Wave-mixing experimental setup for the detection of cytokines induced by biomaterial (Ti) on a protein microarray
hence, it can be conveniently filtered and collected with a simple photodetector (ThorLabs, Inc., Model PDA55). An optical chopper (Stanford Research Systems, Model SR541) modulates the weaker pump input beam at 200 Hz. The signal is amplified by a lock-in amplifier (Stanford Research Systems, Model SR810 DSP) and then digitized and stored on a desktop computer.

Protein microarray slides are commercially obtained as a cytokine test kit (Neupro Technology, NEU001). The microarray chip consists of a 25 mm x 76 mm glass slide spotted with immobilized TNF-alpha and IFN-gamma antibodies. Each antibody, including the positive and negative control spots, is spotted 6 times on each grid for a total of 30 spots per grid. Repeated spots are used to compare the presence of antigen when treated with Ti particles and LPS control samples. The positive control spot consists of biotin label mouse polyAb and the negative control spot has PBS-T.

The array spot diameter is 200 µm and the spot-to-spot distance is 300 µm. Each grid is incubated with different concentrations and types of antigens (i.e., supernatant of cell culture) with added biotinylated detection antibody and Alexa conjugated Streptavidin. Quantitative measurements are made by using antigens on some arrays within a slide to generate a standard curve. Microarray hybridization is performed as outlined by the manufacturer.

The protein microarray is mechanically moved in 20 µm incremental steps by a computer controlled actuator (Zaber Technologies, Model ZLA-28). For time dependence studies, the array is scanned manually to allow individual optimization for each spot. Wave-mixing signals from microarray spots are collected and imaged with intra-spot spatial resolution.
8.3.1 RAW Cell Culture.

RAW macrophages are maintained in four 6-well plates (Falcon 353846) at $10^5$ cells/well in 2 mL of a culture medium (i.e., RPMI) containing 0.01% L-glutamine, 90% RPMI 1640 (Sigma) and 10% fetal bovine serum. Cells are grown to an appropriate confluence in an incubator at 37°C and 5% CO$_2$. Each plate represents a different treatment time of 1, 24, 48 and 72 hours. Initially, cultured cells are equilibrated overnight before administering 2 mL of fresh media, 200 µL of phosphate-buffered saline solution (PBS) and sterile Ti particles (8.5 µm in diameter, Sigma) at 50 ng/mL into designated wells. In addition, a 10 ng/mL LPS (Sigma) solution is added to other wells as a positive control.

8.3.2 Standard Solutions.

The antigen solutions used are TNF-alpha recombinant human (US Biological, T9160-10) and IFN-gamma recombinant (US Biological, I7662-16A). IFN-gamma and TNF-alpha are reconstituted in 1x PBS (US Biological, D9820) to prepare 1 mg/mL and 0.5 mg/mL stock solutions, respectively. The antigens are diluted in 1x PBS buffer to desired concentration levels for hybridization. All solutions are made fresh each day, starting from stock solutions. The Alexa conjugate is diluted in 1x PBS buffer to a stock concentration of 1 mg/mL. This stock solution is further diluted 100x in PBS to a working concentration of 0.01 mg/mL and used to hybridize the antibody array. Concentration levels of expressed cytokines are thereby interpolated by optical absorbance values from standard calibration curves.
8.4 Results and Discussion

The Ti particles used in our implant biocompatibility study are 8.5 µm in diameter, a size consistent with phagocytosis and activation of macrophages.\(^5\) The endotoxin, LPS, is used as a positive control for this in vitro study because it induces significant cytokine production. Specifically, LPS stimulates the synthesis of inflammatory mediators such as cytokine TNF-alpha and IFN-gamma. The macrophage cells are exposed in culture to both Ti particles and LPS. Supernatants of different treatment times are added to the protein array to determine, in parallel, the temporal profile of cytokine expression from the cells interrogating the biomaterial.

Figure 8.2 shows a schematic overview of the in vitro study with 50 µL of supernatants added to the protein microarray wells consisting of 6 identical rows of two capture antibodies and two controls. The computer controlled actuator scans a single protein microarray row in 20-micrometer increments, confirming the wave-mixing signal and antigen hybridization. The focused wave-mixing spot size is 20 micrometer in diameter, and hence, a 20-micrometer region of the 200-micrometer array spot is probed in each step along the protein microarray row. These small incremental steps confirm hybridization and selectivity of control spots, cytokine spots and the glass surface areas between the spots.

Figure 8.3 shows wave-mixing profiling of the microarray row hybridized with macrophage supernatants exposed to LPS for one hour. As expected, the positive control gives the maximum wave-mixing signal as compared to the antibody spots and the negative control. Selected hybridization is observed for antigens TNF-alpha and IFN-gamma. Jagged peak profiles of each spot are attributed to the printing process of
Figure 8.2 Overview of experimental design on protein microarray.
Figure 8.3 Wave-mixing detection of an array row hybridized with 1 hour of LPS on a protein microarray.
the protein microarray. This protein microarray is also spotted with IL-2 antibody for the detection of IL-2 antigen. IL-2 secretion occurs in T and B cells, and therefore, should not be detected for mouse macrophages. However, some cross-reactivity can occur when one antibody has different affinities for many antigens, and a small wave-mixing signal is still observed for the IL-2 spot on the protein microarray. The background noise levels from the blank glass surface and the negative control is undetectable, offering excellent S/N.

Figure 8.4 shows a three-dimensional profile of an array row hybridized with macrophage supernatants exposed to LPS for one hour. Intra-spot spatial resolution provided by the wave-mixing probe reveals a distinct topography for each spot on the microarray row. The actuator scans each microarray row in the X direction with 20-micrometer increments and a data collection period of 3 sec. per step. Once the array row is scanned, the actuator moves the array 5 micrometers in the Y direction, followed by another set of 20-micrometer incremental steps in the X direction. This zig-zag automated scanning pattern covers different regions within each spot along one microarray row. Each row is scanned 5 times in less than 3 minutes with intra-spot spatial resolution as shown in Figure 8.4.

This detailed scanning and mapping capability also reveals some non-uniformity created by the manufacturer during the array spotting process. Some array spots are observed to exhibit more signal near the center of the spot, and the spotted size is not necessary the size of the spot specified by the manufacturer. Wave mixing offers high intra-spot spatial resolution and even smaller spots on some microarrays could be probed with excellent detection limits.
Figure 8.4 3-D wave-mixing signal profile of a protein microarray row showing intra-spot spatial resolution.
Figure 8.5 shows reproducible results obtained from our wave-mixing scanning detector interfaced to a protein microarray. The computer controlled actuator is programmed to move the microarray in the forward scanning direction along an array row with 20 increments and 80 steps per increment. Figure 8.5 shows (A) forward scanning and (B) backward scanning for a protein microarray row hybridized with LPS for 1 hour. Similar peak characteristics, such as heights and widths, are obtained reproducibly for all array spots when using both scanning directions. Very small variations in signal strengths may be attributed to small mechanical lags in the actuator movement in different drive directions, as indicated by actuator manufacturer specifications. The scan profiles are very reproducible while maintaining intra-spot spatial resolution especially when the actuator is driven in the same direction. Therefore, all 3-D wave-mixing profiling images are obtained from scans detected in one scanning direction.

As shown in Figure 8.4, the positive control has the maximum wave-mixing signal and the negative control the minimum signal, as expected. In this experiment, LPS is expected to induce an immediate response from macrophage cells. Cytokine spots IFN-gamma and TNF-alpha are highly expressed by one hour of LPS stimuli, and they collectively show the highest wave-mixing signal as compared to those for other exposure times.

Figure 8.6 compares the average wave-mixing signals obtained from cytokine expressions corresponding to three duplicate TNF-alpha spots using different experimental condition, i.e., exposure to Ti particles and LPS. In the top plot, wave mixing monitors the production of TNF-alpha in response to the exposure of Ti
Figure 8.5 Reproducible scans in the (A) forward scanning direction and (B) backward scanning direction of a protein microarray row.
Figure 8.6 Sensitive wave-mixing detection of TFN-gamma on a protein microarray hybridized with 1, 24, 48 and 72 hours of exposure to (A) Ti particles and (B) LPS.
Figure 8.7 Sensitive wave-mixing detection of IFN-alpha on a protein microarray hybridized with 1, 24, 48 and 72 hours of exposure to (A) Ti particles and (B) LPS.
particles. This is compared to the controlled studies using LPS, which produces a substantial release of cytokines that decrease with exposure time. The decrease in expression of cytokines to LPS has been recognized and likely contributed to cell death.⁴

Similarly, Figure 8.8 shows the same trend of a gradual increase in the release of cytokine IFN-gamma by the Ti particle-treated cells and a decrease by LPS. Between the two cytokines assayed, TNF-alpha is more prominently expressed while IFN-gamma shows lower expression levels. This is important since different biomaterials could suppress one cytokine while another may be enhanced or unchanged.⁶ The slightly lower detectable levels of IFN-gamma to Ti particles may therefore be attributed to their different immunological roles. IFN-gamma activates the bactericidal mechanisms of macrophages and neutrophils, and TNF-alpha initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection.⁷ In all experiments, positive and negative controls are tested in parallel with the test biomaterials. The dosage of Ti particles and controls can be further optimized to improve the degree of cellular responses.

8.5 Conclusions

Wave mixing-based detection for protein microarrays is demonstrated to be effective for evaluating the immunological impact of biomaterials of macrophage cultures. Protein microarrays offer high throughput analyses and wave-mixing detection offers excellent detection sensitivity levels.
Preliminary concentration detection limits of 0.6 pg/mL and 12 pg/mL are determined for TNF-alpha and IFN-gamma, respectively. Our wave mixing-based spatial and temporal profiling of cytokine expression can be applied to other materials and analyte configurations for in vitro biomaterial testing on pathogenesis of particle diseases and biocompatibility of foreign materials.

8.6 References


9

SINGLE-CELL PROTEIN ANALYSIS BY CAPILLARY ELECTROPHORESIS AND LASER WAVE-MIXING DETECTION

9.1 Abstract

Nonlinear laser wave mixing is presented as a sensitive optical absorption-based detection method for proteins and cellular components within a single cell separated by capillary electrophoresis (CE). Interfacing wave-mixing detection and capillary sodium dodecyl sulfate-Dalton (SDS-DALT) electrophoresis offers many advantages. Laser wave mixing allows sensitive absorption detection even for thin samples including single cells inside a coated 50 µm i.d. capillary. SDS-DALT-CE resolves proteins by their molecular weight at high speed and the analyte requirement is small. When coupled with wave mixing, it allows on-column detection of analytes from individual cells. A single Jurkat cell is visually introduced into the capillary, lysed with SDS, labeled with a chromophore and separated using an 8% Pullulan-sieving matrix. Major proteins, within an individual cell, including β-lactoglobulin, ovalbumin and β-galactosidase are separated and identified by wave mixing using a 488-nm argon ion laser. A preliminary concentration detection limit of 6.6 fg/mL or 0.35 fM and a preliminary mass concentration of 2.2 yocto mole are determined for β-lactoglobulin.
9.2 Introduction

Sensitive and reliable detection techniques are needed for cellular analyses of biological systems at the single-cell level. The living cell, with its amazing integrative abilities of environmental detection, molecular signal amplification, processing, and repertoire of genome-level responses to perturbation, provides the knowledge-building framework for early disease detection, diagnosis and treatment.

Cells can be engineered to become powerful reporters of their own temporal and spatial activities for disease detection.¹ Diseases involve irregularities in the genetic code of cells, resulting in altered expressions of gene products, RNAs and proteins.² Protein analyses are essential in understanding cell development and function in response to these irregularities. Some irregularities in cells that are infected by a disease that only constitute a minor fraction of the total population of cells cannot effectively be detected by population-based detection techniques.³ Since these biological materials are in extremely limited amounts, detecting them is a challenge with currently available techniques.

Conventional single-cell analysis using flow cytometry detects only a few components and provides an average measurement that is extrapolated to the entire cell.⁴ The drawback lies in the assumption that this average measurement is indicative of each individual entity in the population. Hence, a more reliable method is needed to measure individual cell components for the study of irregularities.⁵

A single cell measures a few micrometers to hundreds of micrometers with approximately 18% of the total weight consisting of proteins.⁵ It requires an analytical
method that can separate and detect very small analyte amounts. Capillary electrophoresis uses a narrow-bore fused-silica capillary to separate analytes based on differences in charge density, size and hydrophobicity. It offers high selectivity needed for complex analytes, ease of automation, high throughput and high efficiency. However, conventional CE has some limitations for protein separation including unpredictable sub-micellar mobility rates.

SDS-DALT capillary electrophoresis provides the means to overcome some of these CE separation obstacles by adding sodium dodecyl sulfate to the buffer and a low viscose-sieving matrix to the capillary. These minor additions provide an on-column single-cell lysis chamber, i.e., a separation mechanism with a more reliable estimation for the molecular weight (MW) elution of proteins and a higher separation resolution.

Conventional detection techniques for CE include absorption, fluorescence, electrochemical and refractive index-based detection methods. They are widely popular, but each has certain limitations. For instance, laser-induced fluorescence detection is the most sensitive on-column detection method, but it requires the use of fluorophore labels or chemical derivatization. Many native proteins do not fluoresce sufficiently when excited with light, and fluorescence detectors only detect reasonably fluorescing analytes. Optical absorption-based detectors are popular and widely available with commercial CE systems, however, absorption detection offers inherently poor detection sensitivity and it requires relatively long (e.g., 10 mm) optical absorption path lengths. Some modifications made to enhance analyte path lengths increase detection sensitivity, but they compromise separation efficiency.
In this work, we present laser wave mixing as an absorption-based detection method that offers excellent detection sensitivity for cellular components while requiring a relatively short optical path length, i.e., 1 mm or shorter. The analytes could be in their native form or labeled with chromophores or fluorophores, providing a wider range of applicable analytes and proteins. The wave-mixing signal is created by thermal gratings produced only within an absorbing medium. The wave-mixing signal has a cubic dependence on laser power and a quadratic dependence on analyte concentration, and hence, it requires only a low power laser and it can monitor small changes in chemical properties more reliably.

Wave mixing, coupled with SDS-DALT-CE, offers high CE separation power and excellent absorption-based detection sensitivity for protein analysis within individual cells. SDS-DALT-CE offers efficient separation and improved on-column sample handling and injection of an individual intact cell, guaranteeing 100% cellular content. Wave mixing also offers squared Gaussian peak shapes, inherently enhancing CE peak resolution. This work focuses on enhancements of both separation and detection of proteins within individual intact cells.

9.3 Experimental

Figure 9.1 shows a typical laser wave-mixing optical setup. A 70/30 R/T beam splitter is used to create two input pump laser beams. They propagate in the forward direction traveling equal distances. The two pump beams are mixed and focused using a 7.5 cm focusing lens, producing a spot size approximately 30 µm in diameter. This
Figure 9.1 Experimental setup for the detection of cellular proteins within a single Jurkat cell separated by CE.
small probe size efficiently excites analytes passing through the capillary. Eq. 9.1 is used to estimate the focused spot size:\textsuperscript{14}

\[
2W_o \approx \frac{4f}{\pi D \lambda}
\]

where $W_o$ is the waist radius of the beam when focused, $f$ is the focal length, $D$ is the diameter of the original beam, and $\lambda$ is the wavelength. The two input pump beams cross inside the capillary, creating interference patterns. The laser probe volume is estimated to be approximately 6 nL. The use of low laser power levels (2 -5 mW) allows tight analyte probing without analyte photo degradation or power saturation.

Absorbing analytes passing through the laser probe volume inside the capillary are excited. The excited analytes release their energy nonradiatively in the form of heat to surrounding solvent molecules, creating thermal gratings. Portions of incoming pump photons diffract off the thermal gratings to generate laser-like coherent signal beams. The signal beams propagate in a predictable direction away from those of the pump beams, and hence, it can be collected efficiently with high S/N. An optical chopper (Stanford Research Systems, Model SR540) modulates one of the pump beams at 200 Hz and a lock-in amplifier further enhances the S/N. The wave-mixing signal is detected by a photodiode (ThorLabs, Model PDA 55) and digitized by an ADC, and the electropherograms are displayed on a personal computer.

Each end of the capillary is placed in a 1.5 mL glass vial serving as a CE reservoir. Nickel-coated cadmium electrodes are placed in the CE reservoirs and high voltage is applied using a 30 kV dc power supply (Glassman, Model PS/MJ30P0400-11
30kV) for CE separation. A 488-nm argon-ion laser (Coherent, Inc., Model Innova 90-6) is used as the excitation source.

9.3.1 Single Cell Injection

First, 1 µL aliquots of SDS buffer, QSY35 and a single Jurkat cell suspension are placed in a row on a micro slide. Viewing under a standard inverted phase 10x microscope (Hinkle’s Optical, Inc., Swit Phase Master, Model 765875), the negative end of the capillary (i.e., near the cathode) is elevated and consecutively placed in each aliquot for three seconds. A pressure difference drives the analytes into the capillary and a 3-s injection period results in an injected volume of 6 nL for our 50 µm i.d. capillary. Single cells are visually introduced by pressure injection, followed by another 3-s injection of QSY35 and buffer. This sandwich process helps completely lyse and stain cellular components of single cells. In addition, sandwich-pressure injection efficiently minimizes sample handling, and hence, data bias. Incubation is not required for this QSY35 chromophore label, thus, the capillary end is immediately placed in the CE reservoir. A voltage (12 kV) is applied to the electrodes to move proteins across the capillary.

9.3.2 Chemical Reagents

Aureobasidium Pullulans, acrylamide powder, APS, TEMED, 2-(N-cyclohexylamino) ethanesulphonic acid (CHES), Tris and protein standards, β-galacotsidase and β-lactoglobulin, and trypsin inhibitor are all purchased from Sigma-Aldrich. Protein standards are weighed and dissolved in 0.1 M Tris and 2% SDS
solution at pH 8.6, and diluted to appropriate concentrations. Dissolving the proteins in 2% SDS serves two purposes. SDS denatures and makes proteins negatively charged for separation. QSY 35 chromophore is purchased (Molecular Probes, Eugene, OR) and prepared following the protocol from Molecular Probes. Proteins are labeled with 50 µL of QSY 35 and placed in cubical dialysis chambers (Bel-Art Products) with a dialysis membrane to filter out low molecular fragments from 6000 to 8000 Da. The running buffer for the CE system contains 0.1 M Tris, 0.1 M CHES and 0.1% SDS (pH 8.4). The sieving matrix is prepared by dissolving 8% aueobasidium Pullulans in the running buffer. All sample solutions are stored at 8 °C except for proteins and QSY35, which were stored in –20 °C and thawed at room temperature before each run.

9.3.3 Cell Preparation

Jurkat cells (Cell Culture Bank, Institute of Cytology, Academy of Sciences) are cultured in media consisting of 0.01% L-glutamine, 90% RPM1 1640 (Sigma) and 10% fetal bovine serum. Cells are grown to an appropriate confluence in an incubator at 37 °C and 5% CO₂. Approximately 106 cells are prepared for use by washing cell pellet three times in 1 mL of 1% PBS at pH 7.4, removing all residual substrates from the media culture.

9.3.4 SDS-DALT-CE Wave Mixing

The proteins produced in the Jurkat cell line are analyzed on a custom-built CE wave-mixing system. Separation is performed using a 40 cm bare fused-silica capillary (50 µm i.d., 360 µm o.d., Polymicro Technologies, Inc., Phoenix, AZ). A capillary
window is created by burning and removing the polyimide coating 0.5 cm from the cathodic end (negative electrode) of the capillary. This window is cleaned using tissue papers and ethanol, and the capillary is then mounted on a XYZ translational stage. The capillary is conditioned first with 0.5 M NaOH for 1 hour, rinsed with Barnstead 18.2 MΩ water for 1 hour, rinsed with the MAPS solution for 1 hour, rinsed with DI water, and finally filled with the polymerizing solution containing 4% acrylamide, 0.1% APS and 0.1% TEMED.

9.4 Results and Discussion

Polymerization takes approximately an hour, coating the inner wall by interacting with the silanol groups of the capillary tube. The polymerization step minimizes the availability of silanol groups that might interact with proteins. Furthermore, the acrylamide solution suppresses the electroosmotic flow (EOF) by increasing the capillary surface viscosity. This creates a labyrinth of tunnels and cross-linking for improved protein separation.

Figure 9.2 shows the CE capillary channel coating design. Negatively charged proteins are easily separated by their mass-to-charge ratio as they move from the negative electrode to the positive electrode. The capillary is again rinsed with water to remove any excess polyacrylamide.6

The QSY35 label is an amine reactive chromophore that conjugates to proteins.16 Figure 9.3 shows the absorption profiles of the native protein, ovalbumin and
Figure 9.2 Coating of the 50 µm i.d. capillary wall used for the separation and detection of cellular proteins.
Figure 9.3 Conventional UV-visible absorption profile of 10 µg/mL protein labeled with QSY35 compared to 10 µg/mL protein.
the QSY35-ovalbumin complex. The absorption wavelength maximum of the chromophore protein complex is approximately 460 nm. Since the wave-mixing signal is strong and the optical signal collection is efficient, an argon ion laser operating at 488 nm is more than adequate to excite the QSY35 protein complex. Wave-mixing detection sensitivity is excellent even when the laser excitation wavelength is more than 30 nm away from the maximum absorption wavelength of the analyte.9,10,17

The QSY35 chromophore label has an extinction coefficient of 23,500 cm⁻¹M⁻¹ and no fluorescence quantum yield. Since wave mixing is an absorption-based method, it can detect proteins labeled with fluorophores or chromophores, affording a wider range of useful labels. As shown in Figure 9.4, the molecular structure of QSY35 does not allow fluorescence (NO₂ and other electron withdrawing groups) but QSY35 absorbs light at 488 nm.

Figure 9.5 shows an image of the Jurkat cell with a diameter of approximately 25 micrometers. The 10x inverted phase microscope can illuminate transparent media and capture images of an intact cell traveling through the capillary window. Phase contrast microscopy simulates the phase and intensity distribution at the focal point by a density variation found inside the biological cell. An annular aperture controls the illumination of the object, reducing the intensity of non-diffracted light and giving a quarter-wave phase shift with reference to the diffracted light.18 The resulting image is an interference of the two phases of the diffracted and non-diffracted light beams.

SDS-DALT-CE experiments are initially performed using a mixture of protein standards and an internal reference marker, orange G, which has a molecular weight of 10 kDa. This internal standard does not interact with any of the proteins and it elutes
Figure 9.4 Chemical structure of the QSY35 chromophore label
Figure 9.5 Jurkat cell image flowing through a 50 µm i.d. capillary.
Figure 9.6 Linear correlation between molecular weight and wave mixing-detected protein mobility.

\[ y = 763.27x - 2340.6 \]

\[ R^2 = 0.9772 \]
before the protein analytes. Figure 9.6 verifies linear correlation between molecular weight (MW) and sample mobility \(y = 673.98x - 1959\) and \(R^2 = 0.9911\).

The wave-mixing detector monitors the migration times of orange G, β-lactoglobulin, tris inhibitor, carbonic anhydrase, ovalalbumin and β-glactosidase. Linear correlation of MW and mobility of protein standards allows identification of other proteins found within a cell. Figure 9.6 shows a linear correlation with a wide detectable dynamic range for expressed cellular proteins.

Figure 9.7 shows three reproducible electropherograms of a protein standard, β-lactoglobulin, at 10 µg/mL. Using 3-s injections and an applied voltage of 12 kV, signal peaks are observed with an elution time of approximately 919 sec. Slightly different migration times are observed because analytes were manually injected. Figure 9.7 demonstrates that our custom-built CE wave-mixing system is stable and reliable.

Figure 9.8 shows concentration studies for the same protein standard, β-lactoglobulin, at different concentration levels: (A) 10 mg/mL (diluted 1000x), (B) 10 µg/mL, (C) 10 ng/mL, (D) 10 pg/mL and (E) 10 fg/mL, with minimal background noise levels. Hence, wave mixing offers a wide concentration dynamic range necessary for typical a real-world sample, especially if the average expressed protein in a cell is in the high attomole range. A typical Jurkat cell is 25 µm in diameter and has a volume of about 8 fL (assuming a sphere). Assuming that the Jurkat cell is 10% protein by mass and that the average protein is about 25000 Da, a Jurkat cell would contain a total of about 32 fmol of proteins. If 10,000 proteins are expressed in a single cell, the average
Figure 9.7 Reproducible electropherograms detected by wave mixing for 10 µg/mL protein standard and β-lactoglobulin: (A) Injection 1, (B) Injection 2, and (C) Injection 3.
Figure 9.8 Concentration studies of standard β-lactoglobulin at (A) 10 fg/mL, (B) 10 pg/mL, (C) 10 ng/mL, (D) 10 µg/mL and (E) 10 mg/mL.
protein is present at the attomole range. Thus, highly sensitive detection methods are required for single-cell analyses.

Figure 9.8 also shows well resolved squared Gaussian peaks over a wide concentration range for $\beta$-lactoglobulin with a reproducible CE migration time of approximately 919 sec. for each run. Peak intensity levels decrease with decreasing protein concentrations, as expected.

Figure 9.9 shows ultrasensitive detection of the standard protein, $\beta$-lactoglobulin, with an injected concentration of only 10 fg/mL. A preliminary concentration detection limit of $3.58 \times 10^{-16}$ M is determined for $\beta$-lactoglobulin (S/N 2). Based on the probe volume estimated to be 6 nL, a preliminary mass detection limit of 2.2 yocto mole is determined for $\beta$-lactoglobulin labeled with QSY35. It is important to note that the wave-mixing signal is generated by the absorbing QSY35 chromophore and that $\beta$-lactoglobulin has multiple QSY35 labels per protein and an estimated 15 lysine binding sites per molecule. Figure 9.9 verifies that this absorption-based wave-mixing detection method can detect cellular components at trace concentration levels available in single cells.

Figure 9.10 compares electropherograms obtained from (A) cellular $\beta$-lactoglobulin protein from a single cell, (B) $\beta$-lactoglobulin protein standard at 10 $\mu$g/mL, and (C) a mixture solution containing multiple protein standards including $\beta$-lactoglobulin at 1$\mu$g/mL. The $\beta$-Lactoglobulin protein from the cell has a migration time of approximately 919 s., matching those in the protein standard and mixture of
Figure 9.9 Ultrasensitive wave-mixing detection of 10 fg/mL standard β-lactoglobulin protein.
Figure 9.10 Reproducible electropherogram detected by wave mixing: (A) 1 µg/mL β-lactoglobulin in a mixture standard protein solution, (B) 10µg/mL β-lactoglobulin, standard protein only, and (C) β-lactoglobulin protein within a single Jurkat cell.
standards. Peak intensities vary slightly from one electropherogram to another due to different protein concentrations used. The higher background level observed for the single-cell electropherogram is possibly due to the presence of other cellular components.

Figure 9.11 shows a similar study for the β-glactosidase protein. It compares electropherograms obtained for β-glactosidase from (A) a single cell, (B) the standard solution, and (C) the mixture standard solution. The single-cell migration time matches favorably with those of the standards at approximately 1577 sec. Again, peak intensities vary due to different protein concentrations used (10 µg/mL for the standard β-glactosidase and 1 µg/mL for the mixture β-glactosidase). In general, electropherograms from different proteins have different peak shapes due to different numbers of binding sites available and different overall analyte concentrations after dialysis.

Figure 9.12 compares cell contents observed from different cells. Four CE peaks for the β-glactosidase protein are detected after four separate injections of Jurkat cells: (A) Cell 1, (B) Cell 2, (C) Cell 3 and (D) Cell 4. All four independent injections of individual cells, using the sandwich lysis method described above, yield four wave-mixing CE peaks with a migration time of approximately 1577 sec. Each peak shows characteristics of the individual cell due to varying growth rate and protein expression, resulting in different peak intensities and widths. Hence, wave mixing allows detection and fingerprinting of proteins within a single cell.
Figure 9.11 Electropherograms detected by wave mixing for (A) 1 µg/mL β-glactosidase in a mixture standard protein solution, (B) 10 µg/mL β-glactosidase, standard protein only, and (C) β-glactosidase protein within a single Jurkat cell.
Figure 9.12 Reproducible electropherograms detected by wave mixing for β-glactosidase within (A) Cell 1, (B) Cell 2, (C) Cell 3, and (D) Cell 4.
Figure 9.13 Reproducible electropherograms detected by wave mixing for $\beta$-glactosidase on (A) Day 1, (B) Day 2, and (C) Day 3.
The custom-built CE wave-mixing detection system is stable, robust and reliable. Figure 9.13 shows reproducibility results for the detection of $\beta$-glactosidase on different days. The 10 µg/mL $\beta$-glactosidase analyte is injected on three different days, labeled (A) Day 1, (B) Day 2 and (C) Day 3. For each day, the migration time is approximately 1573 sec. and the background level is stable. Furthermore, the same capillary can be used repeatedly on different days.

### 9.5 Conclusions

Laser wave mixing offers many advantages as an ultrasensitive detection method for both fluorescing and non-fluorescing analytes. Analytes may be labeled with chromophores, in addition to fluorophores. Ultrasensitive yoctomole-level detection sensitivity is demonstrated using a non-fluorescing label in our SDS-DALT-CE wave-mixing system. The coherent laser-like wave-mixing signal is collected conveniently with virtually 100% collection efficiency against a dark background. The use of small laser probe volumes allows reliable analysis of single cells and other thin samples. The use of low laser power levels offers a compact and simple design for detection and identification of cellular proteins within a single cell.
9.6 References


10

IMAGING OF DISTRIBUTED ABSORBING SPECIES WITHIN A SINGLE CELL BY NONLINEAR LASER WAVE MIXING

10.1 Abstract

Laser wave mixing is presented as a novel imaging technique for chemical species in biological cells and cell surfaces. Images obtained by laser wave mixing can determine spatial distribution of absorbing species that play an important role in cell function and characterization. In this study, antigens on the surface of a single Jurkat cell are stained with an antibody directly conjugated to a fluorescent probe, Cy5. A small amount of absorbing species around the surface of a single cell is sensitively detected in vitro. Concentration variation of light-absorbing species is recorded and mapped by the wave-mixing signal beam, producing an image with high spatial resolution. The wave-mixing image can be recorded and displayed using 66 nM or 0.66 attomole of Cy5. Wave mixing is also applied to label-free cells for the detection of cytochrome C. This non-intrusive technique sensitively detects both fluorescing and non-fluorescing species, and hence, it is suitable for measuring distribution of cytochrome C within a cell in its native form using a 514 nm argon-ion laser. Dynamic wave-mixing holographic images of chemical species are recorded by a CCD camera and compared to wave-mixing signals detected by a photodetector.
10.2 Introduction

Reliable optical imaging of chemical species within and on the surface of a single biological cell offers many advantages, especially as a diagnostic tool in the study of biological mechanisms. These optical methods produce images that reflect visual representation, characterization and quantification of biological events at the cellular level. Samples are within the authentic environment of a living cell rather than cell-free extracts, as used in many conventional methods (e.g., chromatography, Western blot). Current methods do not provide information on the distribution and change of distribution for analytes, in vitro.

Cellular imaging techniques including confocal fluorescence microscopy, laser-induced fluorescence (LIF) and scanning optical microscopy have been developed for studies on single cells. Although these techniques are effective and sensitive, it is necessary to label the analytes within the cells or on cell surfaces. The use of labels often affects the cell or the cell function under study. Wave mixing is an optical absorption-based detection method, and hence, it can capture dynamic holographic images of both stained and non-stained (native) cells.

The wave-mixing signal is produced by thermal gratings created by non-radiative relaxation of absorbing analytes. In an unsaturated and optically thin medium, the wave-mixing signal has a quadratic dependence on analyte concentration and the local concentration of the species responsible for the nonlinearity of the medium. Thus, when the excitation laser wavelength is tuned to an absorption peak, a coherent laser-like signal beam is created.
Preliminary studies indicate that the wave-mixing signal is not strongly affected by scattering of the input laser beams off membranes and particles in cells when monitoring single-cell images. In this work, we report, for the first time to our knowledge, a novel imaging technique for recording the distribution of chemical species within a single cell based on absorption-based laser wave mixing.

10.3 Experimental

Figure 10.1 shows the wave-mixing optical setup used for monitoring 2-D dynamic holographic images. Two input beams are mixed and focused inside an absorbing analyte medium. Portions of input beam photons are diffracted off laser-induced thermal gratings to create a coherent signal beam that carries the image of the absorbing analyte cell. One can profile the concentration variation of absorbing analytes, e.g., cellular components, within and on the surface of a cell, and record the dynamic images on a CCD camera.

A He-Ne laser operating at 633 nm is used for Cy5-based surface antigen detection, and a 514 nm argon-ion laser is used for native cytochrome C detection. Each laser is positioned in separate yet similar forward-scattering wave-mixing experimental setup. Figure 10.2 shows a typical wave-mixing optical geometry. The two input laser beams are focused and mixed by the focusing lens with a focal length of 75 mm. The beam overlap angle is approximately 1 deg. The wave-mixing signal
Figure 10.1 Wave-mixing optical setup used for monitoring 2-D dynamic holographic images.
Figure 10.2 Forward-scattering wave-mixing experimental setup for imaging studies.
intensity profile is monitored by a CCD camera (Sony Corp., Model XCD-SX910CR) and a photodetector. Additional optics, such as a focusing lens, an aperture and a collimating lens, are placed immediately in front of the CCD camera in order to suppress background incoherent light. A mechanical chopper is used to modulate the amplitude of the pump beam at 200 Hz and a lock-in amplifier (Stanford Research Systems, Model SR810 DSP) is used to enhance the S/N. A data acquisition system digitizes and stores the wave-mixing images.

Wave-mixing imaging, a technique analogous to dynamic holography, is first aligned and optimized using a mesh grid suspended in a dye solution (1 x 10^{-4} M erythrosine B in ethanol). The grid displays apparent distributions of absorbing and non-absorbing (opaque) areas. Figure 10.3 shows a two-dimensional wave-mixing image of the dye mesh grid collected by the CCD camera. The wave-mixing imaging system is then applied to image surface antigens on a Jurkat cell. Antigens on the surface of a single cell are stained with an antibody directly conjugated to a fluorescent probe, Cy5. Single cell images recorded from the cell stained with Cy5 promises good detection sensitivity for label-free (native) detection and imaging of the distribution of cytochrome C within a Jurkat cell. One of the key processes for signal transduction in the process of apoptosis is the release of cytochrome C. Therefore, it is important to monitor small changes of cytochrome C distribution during the apoptosis process for a wide range of potential applications in cellular science.3

Jurkat cells (American Type Culture Collection, Manassas, VA) are cultured at 37°C with RPMI 1640 (Mediatech, Herndon, VA) containing 10 mM HEPES, 2 mM L-
glutamine and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) in a 5% CO₂ chamber. Approximately 10⁶ Jurkat cells are washed three times by pelleting at 50 g for 5 minutes and re-suspending the pellet in PBS (pH 7.4) in a microcentrifuge tube.

For T-cell receptor staining, cells are incubated with 10 μg/mL anti-CD3e mAb OKT3 hybridoma (American Type Culture Collection) in PBS at 4 °C for 30 minutes. The cells are then washed by pelleting at 50 g for 5 minutes, re-suspended in PBS and incubated with 10 mg/mL Cy5 conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) at 4°C for 30 minutes. Mito Tracker Green (Molecular Probes, Eugene, OR) is used to stain mitochondria within a cell. For Mito Tracker staining, cells are incubated with 500 nM Mito Tracker Green in PBS at 37°C for 30 minutes. For each type of staining, cells are then pelleted at 50g for 5 minutes and re-suspended in 3.7% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS for 30 minutes at 4°C. Cells are then washed three times with PBS.

10.3.1 Microscopy

Images created by wave mixing are compared with those from a laser scanning confocal microscope (Leica, TCS SP2, Heidelberg, Germany) using a 63X objective and a 675/20 PMT. Cells are placed on a 0.1 % poly-L-lysine (Sigma-Aldrich, St. Louis, MO) coated slide, covered with ProLong mounting media (Molecular Probes), and finally covered with a cover slip.
10.4 Results and Discussion

When the laser wavelength is tuned to a resonance with an allowed molecular transition, a resonant enhancement of the wave-mixing signal occurs. Excited molecules release energy in the form of heat, creating thermal gratings. The signal is a coherent laser-like beam generated by dynamic thermal gratings formed by the interference of the two input beams. Assuming small absorption, i.e., within a cell, the wave-mixing signal \( I_s \) can be described as:\(^4\text{-}^7\)

\[
I_s \approx CI_1^2I_2\frac{\lambda_{ex}^2}{\sin^4\theta}\left(\frac{dn}{dT}\right)^2\left(\frac{\alpha}{\kappa}\right)^2b^2t^2
\]

where \( C \) is a constant, \( I_1 \) and \( I_2 \) are intensities of the pump excitation beams, \( \lambda_{ex} \) is the laser wavelength, \( \theta \) is the angle between the two input beams, \( \frac{dn}{dT} \) is the change of refractive index with temperature change, \( \kappa \) is the solvent thermal conductivity, \( \alpha \) is the analyte absorptivity, \( b \) is the optical path length, and \( t \) is the thermal quantum yield.

Wave mixing has many attractive features including high detection sensitivity, non-destructive detection, non-invasive probing, and useful nonlinear dependences, e.g., quadratic dependence on analyte concentration for more sensitive monitoring of small changes.

Initially, wave-mixing imaging is optimized and confirmed by measuring a 1 x \( 10^{-4} \) M erythrosine B solution within the defined regions of a mesh grid. An argon ion laser excites erythrosine B in the forward-scattering wave-mixing geometry using a 1000 mm focusing lens. The laser probe diameter is approximately 300 \( \mu \)m and it can excite approximately six squares of the mesh grid. Figure 10.3 (A) shows an image of
the mesh grid viewed under an inverted phase 10X microscope and Figure 10.3 (B) shows a wave-mixing image captured by the CCD camera. As expected, only the absorbing regions within the boundaries of the mesh grid produce thermal gratings, which are then imaged and profiled by the wave-mixing signal beam.

Figure 10.4 (A) shows single cell images obtained from fluorescent microscopy and wave-mixing imaging. Cell surface antibodies are directly conjugated to the Cy5 fluorescent probe. Figure 10.4 (B) shows the distribution of surface Cy5 probes (250,000 M⁻¹cm⁻¹ extinction coefficient) excited at 633 nm using a 5 mW laser. The wave-mixing probe beam diameter is adjusted to approximately 40 micrometers using a 75 mm focusing lens in order to cover and excite the entire single cell (approximately 25 micrometer in diameter). Stained cells produce thermal gratings with two or more calculated grating periods, which exhibit a circular shape of light intensity on the order of the cell size. Aberrant and distorted edges displayed in the wave-mixing image may be contributed to thermalization and non-instantaneous and non-local solute-solvent energy transfer.

In addition to the CCD camera, a photodetector simultaneously detects the wave-mixing signal generated from the same Cy5 stained Jurkat cell. Figure 10.5 shows wave-mixing peaks collected in three signal intervals. The wave-mixing signal is also compared to a “blank” solution containing the PBS buffer only. Although the blank solution does not contain any Cy5, a very small background signal is still observed. A preliminary concentration detection limit of 66 nM or 0.66 attomole is
Figure 10.3 Two-dimensional wave-mixing image of the dye mesh grid collected (A) under an inverted phase 10X microscope and (B) CCD-based wave-mixing imaging
Figure 10.4 Single cell images obtained from (A) fluorescent microscopy and (B) wave-mixing imaging. Cell surface antibodies are directly conjugated to the Cy5 fluorescent probe.
Figure 10.5 Wave-mixing signal generated from the same Cy5 stained Jurkat cell compared to a “blank” solution containing the PBS buffer only. Signal collected by a photodetector.
determined for Cy5.

Figure 10.6 shows a wave-mixing image that captures the presence of almost two Cy5 stained Jurkat cells. The excitation probe diameter is approximately 40 µm, and it can accommodate almost two cells. Two distinct circular bright patterns are observed for two Cy5 stained cells.

Figure 10.6 shows the wave-mixing signal collected simultaneously by the photodetector along with the image on the CCD camera. Figure 10.7 shows the wave-mixing signal generated by two stained Jurkat cells. Three reproducible wave-mixing signal levels are collected by blocking the two input laser beams on and off. The wave-mixing signal intensity increases with the increasing amount of Cy5, as shown in Figure 10.5. The enhanced signal peak is compared to those obtained from lower Cy5 concentration levels and a blank.

Cytochrome C is present at the outer parts of a cell since it is only contained in mitochondria and not in the cell center. Cytochrome C does not fluoresce, and hence, it cannot be observed in situ using fluorescence microscopy, unless stained with MitoTracker Green or other dyes.

Figure 10.8 demonstrates label-free native imaging by wave-mixing dynamic holography. Figure 10.8 (A) shows a fluorescent image of a Jurkat cell stained with MitoTracker Green and Figure 10.8 (B) shows a wave-mixing image of native cytochrome C. Wave mixing is a real-time dynamic holographic process that records and reconstructs cytochrome C distribution. Figure 10.9 shows the wave-mixing signal collected simultaneously by the photodetector along with the image on the CCD
Figure 10.6 Wave-mixing image that captures the presence of almost two Cy5 stained Jurkat cells
Figure 10.7 The wave-mixing signal generated by two stained Jurkat cells collected with a photodetector.
Figure 10.8 Cytochrome C imaging by (A) fluorescence of a Jurkat cell stained with Mito Tracker Green and (B) wave-mixing dynamic imaging of native cytochrome C.
Figure 10.9 Wave-mixing signal collected by the photodetector. Signal is reproducible and strong when compared to that from a blank solution (PBS buffer).
camera. The wave-mixing signal is reproducible and strong when compared to that from a blank solution (PBS buffer).

10.5 Conclusions

Laser wave mixing allows sensitive and convenient detection and imaging of chemical species within biological cells. This technique is based on optical absorption, and therefore, can be used for both fluorescing and non-fluorescing samples. Wave mixing has been demonstrated to be effective and promising as a tool for imaging trace amounts of analytes in single biological cells in vitro. The coherent laser-like wave-mixing signal beam carries physical/chemical properties of the analytes encoded within the nonlinear medium, similar to the processes in dynamic holography. Light absorption outside the nonlinear medium does not contribute in generating the wave-mixing signal. Unlike fluorescence methods, virtually 100% of the signal is collected by the photodetector or the CCD camera. Wave-mixing imaging promises a wide range of potential applications including in situ analysis of intact single cells.
10.6 Reference


CONCLUSION

Nonlinear laser wave mixing is demonstrated as a sensitive absorption-based spectroscopic method for the detection of cellular components in single cells and for monitoring biomolecular interactions. Two input laser beams are mixed within a light absorbing medium to generate dynamic laser-induced gratings. One of the input beams diffracts off these gratings to create a coherent laser-like signal beam. Hence, the signal beam can be monitored with virtually 100% collection efficiency and detected conveniently using spatial filters. Cells and cellular components can be analyzed by monitoring the changes in the wave-mixing signal intensity. Wave mixing allows monitoring of fluorescing and non-fluorescing analytes with detection sensitivity levels comparable to or better than those of widely popular fluorescence-based methods. In addition, the wave-mixing signal has a quadratic dependence on analyte concentration, and hence, it is especially suitable for measuring small changes in cells.

In Chapter 4, laser wave mixing is presented as a sensitive detector for proteins and cancerous antibodies using a chromophore label that does not fluoresce, but still offers equal or better detection sensitivity levels as compared to those of fluorescence-based methods. Preliminary detection limits of $3.4 \times 10^{-19}$ M and $6.4 \times 10^{-14}$ M (S/N 2) are determined for bovine serum albumin and human Papillomavirus antibody, respectively. Based on the small laser probe volume used (i.e., overlap volume of the two input beams), preliminary mass detection limits of $1.7 \times 10^{-22}$ mol and $2.6 \times 10^{-17}$
mol are determined for bovine serum albumin and human Papillomavirus antibody, respectively.

In Chapter 5, sol-gel sensors are studied for the detection of environmental contaminants. Metal ions, Fe (II) and Cr (VI), are entrapped within a sol-gel matrix and wave mixing sensitively detects the spectral shift of these metal ions that bind to chelating ligands entrapped inside sol-gel pores. Preliminary detection limits of 115 ppt and 1.7 ppq are determined for Cr (VI) and Fe (II), respectively. Development of this sol-gel technology can lead to protein entrapment, yielding very sensitive biosensors.

Chapters 6, 7 and 8 introduce laser wave-mixing spectroscopy as an effective method that offers intra-spot resolution and biospecific detection on microarray platforms. Specifically, Chapter 6 describes a wave-mixing detection method for reading DNA hybridization events on a microarray. The DNA microarray consists of 70-mer oligonucleotide targets that are hybridized with a probe solution containing Cy3-labeled random 9-mers at concentration levels ranging from 10 µM to 1 fM. In Chapter 7, cytokines are detected using an ELISA-based reaction performed on a protein microarray surface. Cytokine antibodies, IL-2, TNF-alpha and IFN-gamma, are immobilized on the microarray surface and hybridized with their corresponding antigens. Alexa-labeled Streptavidin binds to the biotin-labeled antibodies. Tightly focusing the input beams within a spot and scanning the spots incrementally allow intra-spot spatial resolution. Preliminary detection limits of 0.01 pg/mL (S/N 2) for IL-2 and 0.05 pg/mL for IFN-gamma are determined. Chapter 8 continues with an in vitro study for evaluating the immunological impact of biomaterials on macrophage cells using a
protein microarray. Temporal profiles of cytokine expression from RAW macrophage cells exposed to titanium (Ti) particles (test biomaterial) and lipopolysaccharide (LPS, positive control) are sensitively measured by laser wave-mixing spectroscopy. This study demonstrates a simple yet effective way of monitoring changes of cytokine expression induced by Ti particles resembling in vivo situations near arthroplasties, where implant particles make contact with inflammatory cells.

In Chapter 9, wave-mixing spectroscopy is used to detect cellular components within single cells. Jurkat cells are injected, lysed and labeled inside a capillary for cellular protein separation based on capillary sodium dodecyl sulfate - Dalton (SDS-DALT) electrophoresis. Major proteins within an individual cell, β-lactoglobulin, ovalbumin, and β-galactosidase are separated and identified by laser wave mixing. A preliminary concentration detection limit of 6.6 fg/mL or 0.35 fM is determined for β-lactoglobulin.

In Chapter 10, single-cell detection and imaging of cellular components within intact cells is described. Cells are sandwiched between glass slides and a hologram-like wave-mixing signal of labeled analytes within a cell is generated by intrinsic nonlinear wave mixing. Preliminary studies detect antigens on the surface of a single Jurkat cell that are stained with an antibody directly conjugated to a Cy5 fluorescent probe. Absorbing Cy5 labels around the surface of a single cell are sensitively detected in vitro and an image is mapped based on wave-mixing signal profiling. Preliminary studies also detect native cytochrome C species inside the cell. Signals detected by a
photodetector are simultaneously compared to images collected by laser wave-mixing spectroscopy.