Surface plasmon resonance imaging of cell-substrate contacts with radially polarized beams

K.J. Moh¹, X.-C. Yuan²*, J. Bu², S. W. Zhu³, and Bruce Z. Gao⁴

¹School of Electrical & Electronic Engineering, Nanyang Technological University, Nanyang Avenue, 639798, Singapore
²Institute of Modern Optics, Key Laboratory of Optoelectronic Information Science & Technology, Ministry of Education of China, Nankai University, Tianjin, 300071, China
³Tianjin Union Medicine Centre, Tianjin 300121, China
⁴Department of Bioengineering and Center for Optical Materials Science and Engineering Technologies, Clemson University, 501-5 Rhodes Research Center, Clemson, South Carolina 29634, USA

Abstract
We demonstrate the proof-of-concept for surface plasmon resonance sensing and imaging via a virtual probe at the cell-substrate interface of a biological cell in aqueous media. The technique is based on the optical excitation by focused radially polarized beams of localized surface plasmons, which forms a virtual probe on the metal substrate. The intensity distribution at the back focal plane of the objective lens enables quantitative measurements to be made of the cell-substrate contact. The acquired data is then visualized in the form of a local refractive index map.

1. Introduction
Surface plasmons (SP) have generated a considerable amount of interest because they are highly sensitive to perturbations in the environment, making SPs useful for a host of optical sensing and imaging applications [1,2]. However, the long propagation length of the SP in general can impose limitations on the lateral resolution of an experiment based on the Kretschmann-Raether (KR) configuration [2]. An elegant technique of improving the resolution to within the order of the diffraction limit was proposed by Kano et al who focused a laser beam with a high numerical aperture (NA) objective [3–6]. This focused beam technique enables a spatial localization of the SP on planar metal surfaces without resorting to specialized structured metallic surfaces. Somekh et al combined the technique with a scanning heterodyne interferometer and obtained improved contrast and resolution when the lens was slightly defocused [7,8].

These studies have involved simple test structures on a metal-air interface but have yet to apply the technique for practical in-vivo imaging and sensing of biological samples in an aqueous medium. A recent related study showed that the spatial localization of the SP takes the form of an evanescent Bessel beam, with a full width half maximum (FWHM) peak of 0.343 λ on the dielectric-metal interface when radial polarization (RP) is focused via a high NA lens [9]. This central peak can be used to non-invasively probe the structure and examine the dynamics around the contact regions of cells. These cell-substrate contact...
regions are of significant interest in cell biology with important areas of focus such as understanding the mechanisms for cell motility and the structural organization of cell surface receptors in response to external signals [10]. An SP microscopy technique based on the KR method was utilized by Giebel et al to study these contact localities because the cells disrupted the evanescent field associated with the SP excitation; however, their SP experimental images possessed low lateral resolution and contrast [11]. Recently a Köhler illuminated, high NA objective coupled widefield SP microscope was also used to image the cell surface interface in a fluid medium at improved contrast and resolution [12]. In addition to the imaging aspect, another key to understanding cell behavior is the collection of quantitative data (the sensing aspect) at these surfaces.

This paper demonstrates the application of a virtual near-field probe formed by the optical excitation of localized SP using RP beams for the practical observation and quantitative sensing of cell-substrate contacts. The concept of a purely evanescent pattern as a virtual or immaterial probe is of interest in scanning microscopy [13,14], in this work the probe improves the lateral resolution compared to the standard KR configuration for SP imaging and returns measurements in the form of a refractive index (RI) map of the cell-substrate contacts.

2. System configuration and experimental procedure

Figure 1 shows the optical configuration through the high NA objective lens and at the sample plane for our experiment. A standard glass cover slip of 170 μm thickness and refractive index of ng = 1.515 was coated with 50nm of gold by electron beam evaporation. The sample to be investigated is placed on this metal layer. The gap between the total internal reflection fluorescence (TIRF) microscope objective (Nikon 100x NA 1.49) and the cover slip was filled with an index matching immersion fluid. The finite NA value of the objective lens imposes a limit (~79.57°) to the range of angles available for angular modulation. For a given set of conditions, the angle which corresponds to the SP excitation/resonance condition lies in between the maximum angle of the lens and the critical angle for total internal reflection as seen in Fig. 1. The reader is directed to the seminal work of Kano et al for details and a deeper theoretical understanding of producing SPs by a focused laser beam [3–6]. Here the technique is in essence the Kretschmann and Raether optical configuration for SP excitation with rotational symmetry about the optical axis of the lens when the laser is an RP beam. These unique beams exhibit axially symmetric polarizations over their cross sections and thus the entire beam is p-polarized with respect to the interface as shown in Fig. 1. The virtual probe is formed at the vicinity of the focus on the side of the metal layer facing the sample.

The overall experimental setup shown in Fig. 2 is based on an inverted microscope configuration with a tunable Ti:sapphire femtosecond (FS) laser producing pulses of ~200 fs at 778 nm. While a conventional continuous wave laser can be used, we built the system around a short-pulse laser because it allows nonlinear phenomenon to be studied with the technique in the future. Furthermore working with this class of lasers has additional practical benefits; near infrared wavelengths enables the SP resonance angle to be within the limit imposed by the finite NA of the lens (for a sample in water), its low temporal coherence produced clearer CCD images free from interference fringes and hence a better signal-to-noise ratio (which aided in the data collection process described below). We applied a relatively simple technique comprising of a micro-fabricated spiral phase plate and an azimuthal-type linear analyzer to generate RP light, interested readers are directed to references 15 and 16 for details of the method. The RP beam is a solution of Maxwell’s equations that exhibit an axially symmetric polarization over its cross section and possesses several unique properties suited for various applications [17–19].

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The panels in the left hand column of Fig. 3 shows the typical images of the reflected beam captured on the CCD camera when the metal is exposed to air and with a homogenous glucose solution of known RI. The test solution was prepared by dissolving glucose in deionized (DI) water and its RI was measured with a refractometer (Kyoto Electronics, model: RA-130, accuracy of ±0.0005). The inner dark ring, indicated by the arrow in Fig. 2, corresponds to the angle providing the SP resonance condition and enlarges when the sample is changed from air to glucose (RI of 1.378). The regions that yielded no useful data within the raw images (portions beyond the lens NA and below the SP resonance angle for air) were masked out and the remaining regions processed with a combination of filtering techniques (e.g., Nth order and low pass filters). The resulting images then showed a set of clearly defined annular rings with clear contrast to the background as seen in the panels contained in the right hand column of Fig. 3. A shape recognition program with an algorithm capable of finding a best fit circle was then applied to this set of images in order to extract the value of the radius which is normalized to the edge of the field of view captured on CCD.

The normalized radius is converted into the corresponding SP resonance angle by calibrating with two known samples. Consequently, the experimentally obtained angle data can be further processed into the local effective refractive index at the point of focus using the equations relating the momentum \( k_x \) of the incident beam to the SP \( k_{sp} \) [6]. Table 1 shows a set of calibration values and subsequent verification of an experimentally measured result with a RI reading (1.3275 ±0.0005) obtained by the commercial refractometer. The mean value of the measured refractive index was 1.3293 with standard deviation of 0.0004 (to 4 decimal places) from 50 readings of a uniform sample of DI water. The deviation in the two RI values is expected as the refractometer measured the samples at a wavelength of 589.6nm while the incident and SP wavelength is in the infra-red. However the experimentally obtained result is still within the expected value for water (~1.33) and the system in its current configuration provides the same order of accuracy (to 4 decimal places) as the commercial refractometer. Therefore a complete 2 dimensional (2D) effective refractive index map of a sample can be acquired by scanning the virtual SP probe within the area of interest. The system modules (i.e. scanning, image processing, shape recognition and determination of the index values) described here are fully automated in real time with the aid of Labview.

3. Refractive index maps

A suspension of micro-particles with a mean diameter of 0.99 \( \mu \)m was dispersed on the gold substrate. They were allowed to dry in order to enhance the adhesion to the surface before deionized water was added as a buffer medium. Fig. 4 shows the vertical and horizontal profiles of one such particle imaged with the technique. The insert in Fig. 4 is the scanned 2D refractive index map. The image shows the particle having dimensions corresponding to the expected size of the particle (~1 \( \mu \)m at FWHM). Since surface plasmons cannot resolve features below their propagation length in the typical KR configuration, it implies a resolution of 1 \( \mu \)m or better for our current setup.

Two different biological samples were used in this work to demonstrate SP sensing and imaging at the cell-substrate interface of a biological cell in aqueous media. For the first example, we scrapped the squamous epithelial cells from the basal mucosa of a donor. The cheek cells were gently spread and allowed to adhere on the gold substrate before adding a surrounding medium of water. Figure 5(a) shows a cheek cell observed under epi-illumination conditions, while Fig. 5(b) and 5(c) shows the obtained refractive index map of the same cell scanned by the virtual SPR probe. The general outline of the cell’s index profile (Fig. 5(b)) is seen to be consistent with the overall shape perceived in Fig. 5(a). In
the scanned image, the detected data range from the index of the surrounding aqueous medium (~1.33) to approximately 1.35 within the cell. Since the SPR probe is affected by variations in the cell cytoplasm close to the membrane or to subtle deviations in the cell-substrate distance, the index map reveals details about the cell physiology and correlates to the cell’s footprint. Therefore, we believe that direct refractive index measurements using this technique may aid in studies of cell growth, division, aspects of membrane transport or as an indicator of cell health.

Human 3T3 fibroblast cells which form the extracellular structural framework in tissues where used in the second example. A thin glass coverslip with a 50nm thick gold layer was first coated with Poly-L-lysine (PLL), a pipette tip was then used to slowly seed the 3T3 fibroblast cell suspension on to the coverslip. The cells were left overnight to form attachments to the surface, subsequently the sample was washed with phosphate buffer saline (PBS) before fixing with 4% paraformaldehyde. The cell-coverslip was kept in a dilute PBS for scanning microscopy. Fig. 6(a) shows the image of the fibroblast cells observed under a light microscope while Fig. 6 (b) is the refractive index map for the group of cells. The areas of higher refractive index indicates the localities that are in direct contact with the surface and regions of greater density, which we believe reflect where the microfilaments in the cytoskeleton have contracted to support the rest of the cell. Furthermore it seen in the map, that the portions (A,B,C in Fig. 6 (b)) with the highest refractive index and hence greatest cytoplasm density correspond to the locations of the cell nucleus (A,B,C in Fig. 6 (a)). However it should be noted that the refractive index map in general does not provide a direct point to point correspondence with the optical image of the biological sample. Instead the SP virtual probe returns quantifiable data on changes in the cytoplasm or to subtle deviations in the cell-substrate distance which may not be optically visible or differentiated. Therefore a potential use is in the study of extracellular matrix (ECM) mediated cell signaling where the interactions between cells and the ECM play an important role in cellular responses to their microenvironments. These interactions involve coupling between integrins on the cell surface and their corresponding ligands in the ECM which activates a series of pathways that initiate ECM mediated cell differentiation, growth, migration, or apoptosis. Thus a critical step towards the complete understanding of these molecular regulations is to directly image the cell-ECM interface at nanometric resolutions.

4. Conclusion

In summary, we show the proof-of-concept for SPR sensing and imaging via a virtual probe at the cell-substrate interface of a biological cell in aqueous media with improved resolution over the traditional KR configuration. Further research is currently being directed to use the technique for multiphoton microscopy and its potential to increase nonlinear optical effects at interfaces. This may be relevant for studies in the visualization of reversible bound fluorescent ligands at membrane receptors or studying the interaction of many types of molecular structures with biological membranes.

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Fig. 1.
Optical configuration through the high NA objective lens and at sample plane. There is full rotational symmetry about the optic axis.
The setup is based on an inverted microscope configuration. Optical elements include a linear polarizer (P), quarter-waveplate ($\frac{\lambda}{4}$), azimuth-type analyzer (AA) and spiral phase plate (SPP) for generating radially polarized light. The polarization Rotator (PR) consists of two half-wave plates. The half-wave plate ($\frac{\lambda}{2}$) allows the power to be varied without adjusting the femtosecond laser (FS).
Fig. 3.
Image captured on the CCD camera when the metal is exposed to air and with a homogenous glucose solution of known refractive index. The panels on the right column show the processed images after digital filtering.
Fig. 4.
Acquired refractive index map of 0.99 μm particle in a 5×5 μm² area, suggesting a 1 μm or better resolution for the system.
Fig. 5.
Color online. (a) shows a cheek cell observed under epi-illumination conditions, (b) and (c) shows the obtained refractive index map of the same cell scanned by the virtual SP probe.
Fig. 6.
Color online. (a) 3T3 fibroblast cells, (b) obtained refractive index map of the same group of cells scanned by the virtual SP probe.
**Table 1**

System calibration and accuracy

| Sample    | Normalized radius | Reference values | From experiment |
|-----------|-------------------|------------------|-----------------|
| Air ♦     | 0.6927            | 1.0003*          | -               |
| Glucose ♦ | 0.9509            | 1.3455 ± 0.0005† | -               |
| DI water  | 0.9323            | 1.3275 ± 0.0005† | 1.3293 (±0.0004) |

♦ Calibration points,
* From [20] to 4 decimal places,
† Measured with refractometer.