Surface Plasmon Resonance Imaging Using a High Numerical Aperture Microscope Objective

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We designed, constructed, and tested a surface plasmon resonance (SPR) microscope using a high numerical aperture objective from a commercially available inverted optical microscope. Such a configuration, combined with various methods to shorten the surface plasmon propagation length, achieves diffraction-limited spatial resolution in the transverse direction and near-diffraction-limited resolution in the longitudinal direction. A virtue of the objective-type SPR imaging is that we achieve distortion-free angle-resolved SPR imaging, allowing the angle-dependent reflectivity of the sample to be examined on a pixel-by-pixel basis, thus offering high-resolution information about surface properties.

The surface plasmon resonance (SPR) phenomenon occurs when the external light energy resonantly induces the free electrons of the metal to oscillate at a metal/dielectric interface. As a result, the radiant energy is absorbed by the metal at a certain incident angle, and the metal displays a reflectivity minimum whose angular position is extremely sensitive to the index of refraction of the dielectric medium in contact with the metal surface. Most SPR measurements are based on the Kretschmann–Raether configuration, i.e., a prism is used to couple the light into the metal film. When scanning the incident angle beyond the critical angle where total internal reflection (TIR) happens, a dip of reflectivity is monitored. Using the prism configuration, it is also possible to achieve two-dimensional (2D) imaging, which is called surface plasmon resonance imaging (SPRI). Since its invention in 1988, SPR has become a powerful tool to image subtle interfacial features by means of refractive index contrast. The high sensitivity of SPR has made it a good tool to read biomolecular binding events on, for example, DNA or protein microarrays, in a label-free fashion.

Unfortunately, the physical constraint of the prism limits the numerical aperture (NA) and magnification of an imaging system, thus providing poor spatial resolution. For example, due to diffraction, the imaging resolution of a lens with an NA of 0.1 is ~3 μm at a wavelength of 633 nm. The prism also distorts SPR images and causes the images to move when the incident angle is scanned. In order to correct the SPR image and improve its quality, compensating imaging optics are used, such as a double-prism geometry, a cylindrical prism geometry, or a tilting CCD camera. In fact, the cylindrical prism geometry introduced by Shumaker-Parry and Campbell has claimed to be able to do angle-resolved SPRI of a biological microarray when the feature size is large (~200 μm) and the lateral resolution is not critical.

We describe here an alternative approach using a high NA microscope objective to launch the SPR. This configuration is similar to the widely used through-the-objective configuration in total internal reflection fluorescence (TIRF) microscopy, which solves the image distortion and movement problem. The use of a high NA and high magnification imaging system ensures that the resolution of the imaging optics is diffraction-limited (~300 nm). In fact, this diffraction limit defines the lateral resolution of SPR (perpendicular to the incident plane of light). However, at this high optical resolution, the longitudinal resolution (parallel to the incident plane of light) becomes limited by the propagation length of the surface plasmon, which is several micrometers in typical applications. There is a special case where Smolyaninov et al. have used 2D SPR optics to magnify nanostructures on a metal surface, although its applications are limited to features on the metal film that create strong scattering of the surface plasmon.

Two other groups have tried to solve the longitudinal resolution problem using a localized surface plasmon polariton excited from a wide range of azimuthal angles, but these approaches require a slow x–y scanning process or compromise image contrast owing to the existence of s-polarized excitation and multiple incident angles arising from the width of the mask.

There exist much simpler solutions to improve the longitudinal resolution, by increasing the damping of SP waves in the metal film, thus reducing the propagation length of the surface plasmon, using a lossy metal or a lossy wavelength of light. We will show...
objective, $f$ is the focal length of the objective, and $\theta$ is the incident angle. Although it is possible to use the absolute value of $d$ to derive the incident angle, the calculation is usually not accurate because of imperfect alignment and aberrations in the objective. Therefore, we calibrate the incident angle with respect to the relative displacement from the critical angle or a known SPR minimum angle:

$$d - d_0 = f (\sin \theta - \sin \theta_0)$$  \hspace{1cm} (2)

where $d_0$ and $\theta_0$ are the reference offset and the reference angle, respectively.

**Imaging Substrates.** Microscope cover glasses (VWR) are cleaned by sonicating in 1 M KOH and then in water. Gold slides are prepared by sequentially depositing a ~2-nm Cr layer and a ~50-nm gold layer at a rate of 0.1 nm/s and a pressure of $1 \times 10^{-6}$ Mbar using an Edwards 306 thermal evaporator. Copper slides are prepared by depositing a ~2-nm Cr layer and a ~30-nm copper layer at a rate of 0.1 nm/s at a pressure of $9 \times 10^{-7}$ Mbar on a Veeco evaporation station. Patterned poly(dimethylsiloxane) (PDMS) stamps are fabricated using standard soft lithography techniques.

Four substrates are used for imaging:

1) Unpatterned, metal-coated cover glass. For imaging in water, a PDMS slab with a 1 mm wide × 0.1 mm deep groove is placed on the cover glass to form a flow channel. The SPR curve is first acquired in HEPES-buffered saline (20 mM HEPES, pH 7.5, 100 mM NaCl). Then 1 mg/mL biotinylated bovine serum albumin (BSA; Pierce Biotech) is injected into the channel, incubated for 5 min, followed by buffer washing and injection of 1 mg/mL neutravidin (Pierce Biotech). The SPR curve is scanned again after a second buffer washing.

2) Patterned PDMS stamp directly placed on a metal-coated cover glass. When the incident angle is set to the SPR minimum of air interface, the regions corresponding to the grooves in the PDMS stamp show low reflectivity, whereas the regions in contact with PDMS have high reflectivity because surface plasmons are not excited.

3) Thiol patterns on gold surface created by microcontact printing. A 10 mM octadecanethiol in ethanol solution is applied to the PDMS stamp using a cotton swab. After the ethanol evaporates, the PDMS stamp is placed on a piece of gold-coated cover glass for 1 min, transferring the pattern on the stamp to the self-assembled thiol layer on the gold surface.

4) Poly(tetrafluoroethylene) (PTFE) films made by spin coating a 0.9 wt % solution of Cytop PTFE (CTL-809M) dissolved in CT-SOLV 180 solvent (AGC) on gold-coated cover glass at 2000 rpm should create a PTFE film of ~100 nm. Small pieces of PDMS obstacles are placed on the cover glass during the spin coating to make the PTFE film uneven for imaging purposes.

**Angle-Resolved SPRI.** For angle-resolved SPRI, a series of images are acquired with the position of the excitation laser shifting in 10 nm steps, which correspond to ~0.3° near the critical angle of the glass/gold/air interface using a 100× objective. The SPR minimum angle of each pixel in the image stack is obtained by fitting the dip in reflectance with a quadratic function and calibrated pixel by pixel to the position of SPR minimum on a uniform gold surface. The relationship between the SPR minimum

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RESULTS AND DISCUSSION

Objective-Type SPR. As illustrated in Figure 1A, the objective-type SPR setup uses an immersion objective having a NA that is larger than the index of refraction of the medium. In this way, when the incident light is shifted to the edge of the objective back aperture, it will reach the sample at an angle that is larger than the critical angle. Although this SPR setup has a configuration similar to that of objective-type TIRF microscopy, they differ in the following two aspects.

First, a TIRF microscope uses a dichroic mirror to separate the fluorescence emission from the excitation light. When imaging the coherent reflected laser in SPR, conventional beam splitters create interference patterns in the images because of the reflections at different surfaces (Figure 1B). We solve this problem using a pellicle beam splitter (National Photocolor), which is a linear polarizer that eliminates the vertical stripes caused by interference. We can still observe ring-shaped interference patterns, which is common when using a coherent light source and can be removed in various ways, including the use of s-polarized excitation light as the reference.

Second, because SPR happens at a larger angle than the critical angle, SPIR places more demands on the NA of the objective than TIRF microscopy. The nominal maximum angles of different objectives are compared to the SPR minimum angle with different medium and metal films in Table 1. For example, an objective with a NA of 1.4 is sufficient for TIRF microscopy in water but is unable to cover the SPR minimum angle. To increase the angle coverage, metal films having a small SPR minimum angle (such as aluminum) or special objectives using substrates with high index of refraction (such as an Olympus 1.65 NA objective) can be used.

The angle dependence of the surface reflectivity can be measured by scanning the offset of the incident laser. Figure 2 shows the reflectivity curve of a 50-nm gold film measured in air and in water using 638-nm excitation. In the case of SPR in water, the incident laser is shifted from the back aperture of the 1.45 NA objective before the full dip in reflectance can be revealed. Nevertheless, this limited incident angle range does not affect SPR because it is usually performed at an incident angle smaller than the index of refraction of the medium. In this way, the incident light is shifted to the edge of the objective back aperture, it will reach the sample at an angle that is larger than the critical angle. Although this SPR setup has a configuration similar to that of objective-type TIRF microscopy, they differ in the following two aspects.

Table 1. Maximum Incident Angle, Critical Angle, and SPR Minimum Angle of Popular High NA Objective Systems

<table>
<thead>
<tr>
<th>NA</th>
<th>nsubratea</th>
<th>nominal θa (deg)</th>
<th>θc (deg)</th>
<th>θspp (deg) for 638-nm excitationb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>in air</td>
<td>in water</td>
<td>in air</td>
</tr>
<tr>
<td>Au</td>
<td>Cu</td>
<td>Al</td>
<td>Au</td>
<td>Cu</td>
</tr>
<tr>
<td>1.4/1.45/1.49</td>
<td>1.515</td>
<td>67.5/73.2/79.6</td>
<td>41.3</td>
<td>43.9</td>
</tr>
<tr>
<td>1.69</td>
<td>1.780</td>
<td>68.0</td>
<td>34.2</td>
<td>36.2</td>
</tr>
</tbody>
</table>

a The index of refraction of the immersion medium and the cover glass. Objectives with NA < 1.5 use conventional cover glass, and objectives with higher NA use special cover glasses with high index of refraction.

b The complex permittivity of the metals are −12.30 + 1.298 for Au, −13.30 + 4.13i for Cu, −40.34 + 15.23i for Al, and −12.33 + 20.78i for Cr at λ = 638 nm. Thicknesses of the metal layers are 50 nm for Au (with 2-nm Cr), 30 nm for Cu (with 2-nm Cr) and 15 nm for Al.

Figure 2. SPR curves measured by scanning the incident angle. Acquired using a 1.45 NA objective, 638-nm excitation laser, and 50-nm gold-coated cover glass. Reflectivities are calculated by normalizing the reflected laser intensities in the CCD images so that their maximum value matches that in a theoretical SPR curve. (A) Incident angle dependence of reflectivity at glass/gold/air interface, compared with the simulation. (B) Reflectivities at glass/gold/water interface before and after binding biotin-BSA (BB) and neutravidin (NA) to the surface. The arrow indicates the incident angle when the laser beam is moving out of the objective aperture.

Figure 3. Resolution of SPRI. Imaging of a PDMS stamp on a (A) gold film with 638-nm laser, (B) gold film with 532-nm laser, and (C) copper film with 638-nm laser. The incident angle is set at the SPR resonance angle of glass/metal/air interface. Scale bars are 6 μm.
the stage, which may simplify the system design and improve the overall mechanical performance.

When fixing the incident angle at the SPR minimum angle of air interface, images of a PDMS stamp on gold surface can be obtained. Images show the difference between the local SPR minimum angles and that of a glass/gold/air interface. Scale bars are 6 μm. (B) Image of an uneven PTFE film on a cover glass coated with a 50-nm gold film, acquired using 638-nm excitation. The left panel shows the SPR minimum angles in a 45 μm × 25 μm area and the right panel shows the calculated surface topography.

Table 2. Propagation Length and Refractive Index Sensitivity of SPR Excited on Gold and Copper Films at Different Wavelengths

<table>
<thead>
<tr>
<th>metal</th>
<th>wavelength (nm)</th>
<th>optimal thickness (nm)</th>
<th>propagation lengtha (μm) in air</th>
<th>propagation lengtha (μm) in water</th>
<th>rel refractive index sensitivityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>gold</td>
<td>638</td>
<td>50</td>
<td>8.3</td>
<td>3.1</td>
<td>unity</td>
</tr>
<tr>
<td>gold</td>
<td>532</td>
<td>50</td>
<td>0.5</td>
<td>0.2</td>
<td>0.27</td>
</tr>
<tr>
<td>copper</td>
<td>638</td>
<td>30</td>
<td>3.9</td>
<td>1.5</td>
<td>0.40</td>
</tr>
</tbody>
</table>

a The calculation is based on equations in Raether's book.1 The refractive index sensitivity is the calculated slope of reflectivity increment as a function of 1-nm dielectric layer (ε = 2.1) consecutively deposited onto the metal film in air, monitored at a fixed angle slightly smaller than the SPR minimum. The calculated sensitivities in different conditions are normalized to that of gold at 638-nm excitation.

b The refractive index sensitivity is calculated as the slope of reflectivity increment as a function of 1-nm dielectric layer (ε = 2.1) consecutively deposited onto the metal film in air, monitored at a fixed angle slightly smaller than the SPR minimum. The calculated sensitivities in different conditions are normalized to that of gold at 638-nm excitation.

The advantage of objective-type SPR is that the sample and imaging optical paths are fixed when scanning the incident angle, thus allowing even pixel-by-pixel tracking of the reflectivity in the images. In this way, a complete SPR curve can be obtained for each pixel. Figure 4A shows the images of microcontact-printed C18-thiol patterns on a gold surface, which give an SPR minimum angle difference of 0.55 ± 0.10°, obtained by fitting the SPR minimum angles from each pixel when scanning the incident angle. Assuming a refractive index of the SAM of 1.5, this angle shift corresponds to ∼2 nm of thickness variation, which coincides well with the value that has been previously reported.21
way, each pixel generates a SPR curve and the image is constructed using the SPR minimum angle information; therefore, effects of laser intensity variations, such as the inhomogeneous profile and the interferences, are significantly reduced. By referencing using a homogeneous surface, this method also corrects the difference in incident angles at different positions of the image field due to aberrations of the objective lens.

The full SPR curve contains more information than just the reflectivity at a certain incident angle. For example, the position of the SPR minimum angle can be used to determine the thickness of a film on a surface. As a demonstration, we created an uneven PTFE film on gold-coated cover glass. Figure 4B shows that the thickness topography of the PTFE film can be determined with high spatial resolution.

**CONCLUSION**

An objective-type surface plasmon resonance microscope with its distortion-free, angle-resolved imaging capability is expected to extract detailed information on surface properties with nearly diffraction-limited spatial resolution. It is also simple in design because this device employs the widely used objective-type TIR configuration and only requires a small modification of modular elements. Moreover, this configuration opens the possibility for simultaneous detection of SPR signals and fluorescence signals through surface plasmon coupled emission, yielding more insight into surface features and processes. Therefore, we expect that this objective-type SPRI design will find wide applications and become a useful complement to the conventional prism-type SPRI.

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