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Surface Plasmon Resonance Microscopy: From Single-Molecule Sensing to Single-Cell Imaging

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Abstract

Surface plasmon resonance microscopy (SPRM) is a versatile platform for chemical and biological sensing and imaging. Great progress in exploring its applications, ranging from single-molecule sensing to single-cell imaging, has been made. In this Minireview, we introduce the principles and instrumentation of SPRM. We also summarize the broad and exciting applications of SPRM to the analysis of single entities. Finally, we discuss the challenges and limitations associated with SPRM and potential solutions.

Graphical Abstract

Surface plasmon resonance microscopy has emerged as a versatile platform for single-molecule sensing and single-cell imaging with high spatiotemporal resolution. This Minireview highlights the recent advances of SPRM in the analysis of single entities. Future challenges and their limitations as well as potential research directions are discussed.

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Conflict of interest

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Keywords

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1. Introduction

Advanced optical imaging techniques have revolutionized studies exploring micro- to nanoscale objects with high temporal and spatial resolution.^[1] Tracking and imaging targets with intrinsic features in real time is important in chemistry, life science and physics to elucidate the underlying mechanism and has been a long-term goal of the whole imaging community.^[2] To maintain the native state of the targeted objective, surface plasmon resonance microscopy (SPRM) has emerged as a versatile sensing platform because of its capability of imaging single entities ranging from several nanometer to micrometer scales.^[3]

SPRM was derived from surface plasmon resonance (SPR) spectroscopy, which has been widely used in studying biomolecular binding kinetics.^[4] SPR refers to the electromagnetic wave-induced collective oscillations of free electrons at a metal-dielectric interface. These electron oscillations generate surface plasmon polaritons (SPPs) propagating along the interface and create an evanescent wave that decays exponentially in the vicinity of the interface. As a result, the reflected light intensity decreases significantly at a specific incident angle (resonance angle) due to the transformation of radiant energy into surface

plasmons. Subtle refractive index differences near the metal-dielectric interface cause remarkable changes in the resonance angle. Thus, SPR is a popular surface analysis approach when species adsorb or bind to the surface or to any material coated onto the surface. This label-free optical technique has been commercialized and extensively applied in fundamental biological research, clinical diagnosis and drug discovery.^[5]

Although powerful, conventional SPR spectroscopy lacks spatial resolution or imaging capability, hindering its broader applications. When a digital camera is employed for image acquisition, acquisition of two-dimensional images (termed SPR imaging) is feasible, endowing this technique with visualization capability for high-throughput analyses.^[6] SPR imaging has since become a robust analytical tool for multiplexed microarray detection of biomolecular binding events, especially for *in vitro* drug screening.^[7]

However, the prism-based SPR imaging system has limited spatial resolution. Upgrading the prism with a high numerical aperture (NA) objective provides SPRM an unprecedented detection capability with high spatial resolution and without image distortion.^[3a] Using this capability, SPRM has been extensively adopted to sense single DNA molecules, *in situ* quantify the binding kinetics of membrane proteins, detect conformational changes in cytochrome c, monitor organelle dynamics inside cells and characterize the cellular response to external stimuli.

Furthermore, the density of oscillating electrons can be tuned by applying an alternating current (ac) potential to the sensing surface, thus creating plasmonic-electrochemical impedance microscopy (P-EIM).^[8] Owing to its sensitivity to surface charge density, P-EIM is capable of optically imaging impedance and mapping subcellular electrical polarizability and conductivity, revealing detailed information about cellular structure and ionic distribution. Recently, burgeoning developments in P-EIM have been reported, including the detection of charged molecules, binding kinetics of small molecules, and phosphorylation and the study of the electrical activities and intracellular processes of live cells (e.g., apoptosis, electroporation, calcium signaling).

In this Minireview, we will start with an overview of the principles and instrumentation of SPRM. We then discuss the latest progress in biomolecular sensing and imaging and single-cell analysis. Finally, we address the current challenges and present our perspective of SPRM in single-molecule/single-cell analysis. Aspects related to applications of conventional SPR spectroscopy to the study of biomolecules and their interactions are not within the scope of this review.

2. Principles and Instrumentation of SPRM

2.1. SPRM

SPR occurs at the metal/dielectric interface only when the wave vector of the incident light matches that of the surface plasmons. This wave vector matching can be achieved in the Kretschmann configuration in the total internal reflection geometry.^[9] The coupling of surface electromagnetic waves to oscillating free electrons of a metallic surface results in

SPPs propagating along the interface, creating an evanescent wave that decays exponentially in the vicinity of the interface.^[10]

In SPRM, the optical system is comprised of a laser source for SPPs excitation, an inverted microscope and a CCD camera for image acquisition. A beam of *p*-polarized monochromatic light is focused into the back-focal plane of a high-NA oil-emersion objective to illuminate a gold-coated coverslip in total internal reflection mode (Figure 1a). The light beam focused by the high-NA objective can be used for exciting SPPs. When the incident angle of the light beam is modulated, a sharp decrease in reflectivity can be monitored by a camera recording the reflected beam through a tube lens. The angle at the reflectivity minimum is referred to as resonance angle or SPR angle. The resonance angle (θ_R) is given by^[11]

$$\sin(\theta_R) = \sqrt{\frac{\epsilon_1 \epsilon_m}{(\epsilon_1 + \epsilon_m) \epsilon_2}} \quad (1)$$

where ϵ_1 is the dielectric constant of the buffer solution, ϵ_2 is the dielectric constant of the objective, and ϵ_m is the real part of the dielectric constant of the metal film. The resonance angle is extremely sensitive to subtle refractive index variations adjacent to the interface (~300 nm). Traditional SPR biosensors detect the shift in the resonance angle by measuring the intensity change in the reflected light at a fixed incident angle close to the SPR angle.

The above model describes the basic principle of SPR biosensors with an approximation of a uniform surface. However, the classical model is not suitable for describing the observed images from individual small objects (e.g., molecules, nanoparticles, and viruses). Typically, a parabolic-shaped diffraction pattern appears when an object is placed in the vicinity of the metal surface. This behavior can be explained by the intrinsic interferential detection scheme from SPRM. At the prism/metal interface, the incident light is partially reflected (E_r) and partially absorbed to excite surface plasmon wave (E_{sp}) (Figure 1b). This propagating wave is scattered by the object on the surface, creating a scattering field (E_s), which can be expressed as βE_{sp} , where β is the scattering strength. The reflected light intensity detected in the SPR image (I_{SPR}) is the superposition of the reflected field, the evanescent field and the scattering field and is given by^[12]

$$I_{SPR} = |E_r + E_{sp} + E_s|^2 = |E_{sp} + E_r|^2 + 2\text{Re}\{E_s(E_{sp}^* + E_r^*)\} + |E_s|^2 \quad (2)$$

Notably, the first term in Equation 2 generates a uniform SPR background, and the pure scattering contribution from the third term is typically negligible for small particles. The second term yields a distinct parabolic pattern of an object. The characteristic parabolic pattern of 200 nm polystyrene nanoparticles is clearly shown in Figure 1c.^[13]

2.2. Plasmonic-based Electrochemical Impedance Microscopy

Conventional electrochemical impedance spectroscopy (EIS) electrically characterizes the surface impedance by monitoring current changes with driving potentials. In contrast, by connecting a gold sensing surface serving as a working electrode, P-EIM converts the

electrical signal into an optical readout, which is finally imaged with a camera. Thus, P-EIM provides local impedance information that could not previously be obtained using EIS. (Figure 1d). The external field changes the electron density and induces a dielectric constant change in the metal surface (ϵ_m) according to the Drude model^[8a]

$$\epsilon_m(f) = 1 - \frac{n_e e^2}{\epsilon_0 m_e 4\pi^2 f^2} \quad (3)$$

where n_e , e , and m_e are the electron density, charge, and mass, respectively; f is the surface plasmon frequency; and ϵ_0 is the vacuum permittivity (8.85×10^{-12} F/m). The change in the dielectric constant ultimately affects the resonance angle θ_{SPR} and is detected in P-EIM.

To image impedance, a small sinusoidal potential modulation is applied to the surface. The surface charge density (σ) oscillates in response to the potential modulation (V), which induces a modulation in the SPR signal (θ). θ is proportional to σ , given by $\sigma = a \theta$, where a is a coefficient that can be calculated theoretically or calibrated experimentally. The local impedance (Z) density can be calculated by the ac component of the SPR signal (θ), given by

$$Z^{-1}(\omega) = \frac{j\omega a \Delta\theta}{\Delta V} \quad (4)$$

where $j=(-1)^{1/2}$ and ω is the angular frequency of the potential modulation. The results generated by P-EIM are consistent with those generated by EIS while also providing local impedance information.^[14] It exhibits superior sensitivity to small molecules, as it is sensitive to surface charge instead of mass.

2.3. Spatial and Temporal Resolution, Selectivity, and Detection Limit

Owing to the propagating SPPs, the spatial resolution of SPRM differs in two directions, and each can provide specific information. The utilization of a high-NA objective ensures that the lateral resolution of SPRM is diffraction-limited in the transverse direction. The longitudinal resolution is restricted by the propagation length of SPPs extending over several micrometers, which is observed as a V-shaped parabolic tail of a small object described previously. This characteristic feature has been used for the sensitive detection of small particles that are below the diffraction limit in size.

To solve the anisotropic resolutions in different directions, multiple methods have been proposed. One approach is image reconstruction. Since the parabolic tail is considered a point spread function of SPRM,^[15] a deconvolution algorithm can be applied to the image for high-resolution reconstruction.^[16] In addition, a manual frequency shift in k-space has been applied as a decoding method for resolving the interference terms in eq. (2).^[17] In this way, single exosomes have been visualized and analyzed after image reconstruction.^[18] Alternatively, a high-spatial-resolution SPR image has been achieved by multidirectional or azimuthal rotation illumination, resulting in an order of magnitude enhancement in resolution compared to that of conventional SPRM.^[19]

In addition to the lateral (xy-direction) imaging capability, the SPR imaging technique is ultrasensitive in the vertical direction (z-direction). Since the evanescent field decays rapidly into the media, the SPR intensity is an exponential function of the z distance between the surface and object. With the measured decay constant of the SPRM system, the plasmonic imaging intensity change could be translated into z-axis displacement with a localization accuracy of ~5 nm in the z-direction.^[20] Moreover, by repeatedly driving the nanoparticles to oscillate vertically, a more precise accuracy of ~0.1 nm can be achieved.^[21] The sensitivity of displacement in the z-direction enables SPRM to be a powerful tool for investigating the interfacial dynamics of single entities.

Notably, SPRM is sensitive to different types of scatterer on a surface.^[22] Theoretical and experimental studies have established relationships among the pattern, particle size and refractive index by quantifying the scattering patterns of nanoparticles. Metallic oxide and metal nanoparticles with different radii can be distinguished without any calibration.

The temporal resolution of SPRM is largely limited by the speed of the imager. It can be fundamentally enhanced by utilizing an ultrafast camera together with a high-power light source. A commercially available CMOS camera with a temporal resolution of 10 μ s has been applied to explore electrical activities in single neurons.^[23] In another example, nanosecond temporal resolution was achieved when an ultrafast photodiode along with microelectrodes was adopted to monitor conformational changes in redox molecules.^[24]

3. Biomolecular Imaging

3.1. Single DNA Molecule Imaging

The imaging capability and sensitivity of SPRM make single-molecule detection possible. Although fluorescence imaging has demonstrated its capability in single-molecule detection, photobleaching and blinking interfere with its practical applications, and fluorescence tags may also alter the native state of molecules. Tao and coworkers have demonstrated a differential SPR (DSPR) technique for imaging and measuring the length of single DNA molecules (Figure 2a-d).^[16] Technically, two images were captured at two positions by translating the sample laterally between the two positions. By subtracting one image from the other, a differential image was acquired to remove background noise. A high-contrast image with minimized scattering patterns was acquired via an image deconvolution algorithm. The average length of single stretched DNA molecules was determined to be approximately 14.6 μ m from the deconvolved images. Its label-free and quantitative nature make SPRM an attractive imaging platform for single DNA molecule analysis.

3.2. Small Molecule Binding Kinetics Detection

SPR is a detection strategy that has found extensive applications in the field of biomolecular interactions. The sensitivity of conventional SPR is determined by refractive index contrast, which is related to the molecular mass. Thus, small-molecule detection remains a challenge owing to the extremely low refractive index alterations when small molecules bind to a surface. To enhance the signal of biomolecular binding events, two strategies have been proposed. The first is signal amplification. Au nanoparticles (Au NPs) have been extensively

employed to enhance the sensitivity of SPR-based biosensors and the role of Au NPs is to amplify the signals of small-molecule detection. The introduction of functionalized Au NPs could cause a pseudo-increase in the mass of the analyte. This mass change ultimately provides a significant increase in the magnitude of refractive index changes and accordingly amplifies the signal. For example, Halpern et al. used DNA-functionalized Au NPs for the real-time detection of DNA hybridization kinetics (Figure 2e).^[13] Au NPs are generally linked with thiolated oligonucleotides, and their hybridization to a complementary surface sequence can result in an approximately 1000-fold increase in the magnitude of the refractive index change.

The other approach to obtain increased SPR signals for small-molecule detection is the P-EIM technique. As previously stated, P-EIM measures the interfacial capacitance, which does not scale with the mass of the molecules, providing superior sensitivity compared with that of traditional SPRM. For example, the capability of P-EIM for sensing small molecules, namely, ethylenediamine and ethanolamine, chemically linked to a dextran-coated gold film was studied.^[25] The charge status of both the substrate and the small molecules was regulated by adjusting the solution pH because their terminal groups could be protonated/deprotonated depending on the solution pH value. The phase component of the P-EIM signals represented the charge status of the small molecules, and any differences from the phase component of the substrate could be detected and distinguished by obvious phase contrast (Figure 2f). Following the demonstration of its capability for small-molecule identification via charge differences, the P-EIM technique has also been applied to evaluate the binding kinetics and affinity between small-molecule drugs (imatinib) and their target proteins (kinases Abl1) in a protein microarray.^[26] Upon injecting an imatinib solution, the SPR signals rapidly increased, while the simultaneously recorded impedance signals exhibited no response to nonspecific absorption, resulting in a single-exponential increase in admittance. For label-free molecular binding assays, nonspecific adsorption is one of the most challenging issues. Unlike conventional SPR signals, the impedance signal is independent of nonspecific binding arising from electrostatic interactions but sensitive to the intrinsic kinetic information about specific molecular interactions.

4. Single Virus and Bacteria Imaging

4.1. Single Virus Detection and Mass Measurement

Optical visualization and detection of viruses remain challenging due to their small size and low dielectric contrast. Compared with non-imaging techniques (dynamic light scattering et al.), SPRM can explicitly resolve individual viruses to enable multiplexed and high-throughput virus analysis. The size and mass of multiple individual viruses could be measured at the same time. Wang et al. has validated the imaging, detection, and size and mass measurements of single viruses with SPRM.^[3c] Individual viruses were imaged as characteristic parabolic patterns due to the scattering of SPPs (Figure 3a-c). The SPR intensity of a nanoparticle has been proved to be proportional to the volume of the nanoparticle. To obtain the size and mass of the influenza A virus, silica nanoparticles of a known size were used as calibration standard. The refractive index of influenza based on its protein and lipid contents is approximately 1.48, close to that of the silica nanoparticles with

a refractive index of 1.46. Based on the linear intensity-volume curve, volumetric information about the influenza virus could be extracted. The diameter of the virus was determined to be 109 ± 13 nm; therefore, a mass of 0.80 ± 0.35 fg was obtained using the known density of H1N1 influenza A virus.

4.2. Single-Bacterium Motion Classification and Binding Measurement

Micromotion is a signature of life.^[27] Considerable efforts have been devoted to detecting this phenomenon in bacteria and to correlating metabolic activity with micromotion using atomic force microscope (AFM) cantilevers.^[27-28] This approach utilizes the sensitivity of nanomechanical oscillators to transduce small fluctuations related to the micromotion of bacterial cells. However, the cantilever detects the overall signals of hundreds of bacterial cells on the cantilever, lacking the capability to resolve the micromotion of a single bacterial cell. The z-direction-sensitive feature of SPRM enabled the analysis of microbial vibration with nanometer precision. Recently, Syal et al. tracked the motion of individual bacterial cells and correlated the nanomotion with their metabolic activity.^[29] In this work, *Escherichia coli* O157:H7 cells were attached to a sensor surface by antibody coupling. The large fluctuations (in the z-direction) were revealed by translating the plasmonic intensity changes of single bacterial cells into vertical displacement (Figure 3d and e). When the bacteria were exposed to high concentrations of an antibiotic, the nanomotion decreased substantially. In spite of the substantial variability among different cells, the antibiotic-triggered decrease in bacterial nanomotion was a robust and statistically evident phenomenon, which could potentially be used for an antimicrobial susceptibility test (AST).

To confirm the heterogeneity of cells, the interactions of molecules with individual *Escherichia coli* O157:H7 cells were analyzed from a dynamic perspective.^[30] By plotting the SPR intensity versus time, sensorgrams of single bacteria could be fit with a first-order kinetics model. The differential images revealed variations in the SPR intensity among different cells, providing more insights into the kinetics of binding and cell-to-cell variation.

5. Live Cell Imaging

5.1. Visualization of Cellular Morphology

Investigating cell-substrate interactions is crucial for understanding many cellular behaviors, including cell adhesion, growth and subsequent detachment, cell mobility, and cellular responses to the extracellular matrix. Giebel et al. first attempted to apply the prism-based SPR tool for visualizing and quantifying the distance between a goldfish glial cell and a substrate.^[31] The cell-substrate distance is expected to differ across the substrate. Consequently, the resonance angle at different positions varies; therefore, the reflectivity of these positions is different at a fixed incident angle. When optimized with enhanced spatial resolution, SPRM has been used to observe the cell-substrate interface and measure the cleft gap distance.^[32] In this work, the stretched shape of human embryonic kidney 293 cells on Au films was revealed. In addition, the cell-substrate distances were mapped quantitatively, ranging from approximately 40 to 60 nm. SPRM can also be utilized to assess adhesion strength.^[33] An alteration in the extracellular osmotic pressure changed the cell-substrate adhesion strength, inducing a heterogeneous force distribution around the cell membrane.

This effect results in a spatial distribution map of the cell-substrate adhesion strength, which could be acquired based on the plasmonic displacement image (Figure 4a-b).

To image cellular morphology, the sensing range of conventional SPR (cSPR, typically 100-200 nm) could be lengthened by long-range surface plasmon resonance (LRSPR) with a deeper probing distance up to 500-1000 nm. LRSPR was achieved by inducing a buffer layer with refractive index similar to that of the sample. This symmetric structure causes coupling of the plasmon waves on both sides of the metallic layer. Consequently, this coupling enables electromagnetic fields penetrate deeper into the sensing medium compared with cSPR. LRSPR provided remarkably improved sensitivity to the subtle variations in the refractive index in the vicinity of the interface. Recently, cellular micromotion has been measured using LRSPR.^[34] The level of fluctuation for live fibroblast cells was distinct from that of fixed cells. In stark contrast to LRSPR, cSPR was insensitive to the micromotion of cells. Interestingly, rapid vertical (z-direction) cellular membrane fluctuations have also been monitored by employing a broad bandwidth optical analytical method.^[35] The stochastic fluctuations of the plasma membrane of live HEK-293 cells at the nanoscale were detected and found to be closely related to the cytoskeletal structure of the cells.

5.2. Mapping the Binding Kinetics of Membrane Proteins

Membrane proteins, embedded in or attached to the phospholipid bilayer, are crucial for a variety of cellular responses to extracellular stimuli. As therapeutic targets and biomarkers of disease, membrane proteins have been extensively investigated.^[36] However, *in situ* exploration of membrane protein interactions with molecules remains a challenge. Traditional approaches for ex situ membrane protein analysis cannot maintain the native structures and functions of proteins.^[37] The high spatiotemporal resolution and label-free nature of SPRM make it an ideal imaging platform for *in situ* research on the binding kinetics in single live cells.^[3d] The subcellular imaging capability enabled mapping of the local association and dissociation rate constants of single cells, reflecting obvious spatial variability in the binding kinetics of the glycoproteins across the cell surface (Figure 4c-e).

To improve the sensitivity and stability for long-term recording, a temperature control system coupled with a mechanical noise reduction system was implemented, and the light source was also optimized. These strategies accomplished the task of monitoring the binding of drugs to endogenous receptors with low abundance and, more importantly, provided new findings regarding protein-protein interactions. Recently, to explore the origin of drug resistance at the molecular scale, the quantitative analysis of drug-receptor interactions has been reported.^[38] The drug-receptor binding kinetics of Herceptin (an antitumor drug) with its receptor (Her2) in both drug-sensitive and drug-resistant cells were assessed, revealing two different populations of Her2 in the Herceptin-resistant cells. The distribution and interaction kinetics of epidermal growth factor receptor (EGFR) in its native environment could also be quantified.^[39] The heterogeneity of the binding kinetics indicated that the microenvironment of the cellular membranes had an important impact on drug-target interactions.

Traditional drug discovery usually uses label-based technologies to probe the binding of ligands to their receptors.^[40] A label-free screening platform could simplify the experimental procedures and, more critically, can extract information from proteins in their native state. Therefore, this label-free approach could address the concern regarding whether the labels interfere with the binding kinetics through an easy comparison with label-based methods. For instance, using this imaging modality, Yin et al. revealed that antibody-conjugated Au NPs could significantly affect binding kinetics.^[41]

5.3. Monitoring Intracellular Processes

Studying dynamic intracellular processes is essential for understanding the intrinsic mechanism of modulating the signaling pathways and the transitions between cell cycle stages. Plasmonic imaging techniques have been applied to investigate dynamic intracellular processes, including electroporation and calcium signaling, and intracellular transport. Electroporation is widely used for transformation and transfection in cell biology. After electroporation, the electrical permeability and conductivity of the cell membranes increased, allowing drugs or DNA to be introduced into the cell.^[42] Using the sensitivity of P-EIM to electrical conductivity, the electroporation process was visualized. The SPR intensity in the regions in Figure 5a decreased considerably during this process, indicating local detachment of the cell membrane, and recovered in less than a minute after the large transient change. The P-EIM images (Figure 5b) reveal dramatic changes, showing that electroporation started with a rapid and large increase in the center of the cell that takes dozens of seconds to slowly recover thereafter.

Recently, P-EIM was used to study intracellular calcium signaling for the activation of G-protein-coupled receptors (GPCRs) triggered by histamine in HeLa cells with subcellular and millisecond spatial-temporal resolutions.^[43] In the early stage of histamine-stimulated GPCR activation, the SPR and P-EIM images exhibit highly dynamic responses and heterogeneous behavior in single HeLa cells. Thorough quantitative kinetic analyses of the SPR signals identified the wide variation in the calcium flux activities between individual cells. Furthermore, it was shown that a subpopulation of cells was more active towards agonist stimulation. The P-EIM images revealed the local heterogeneous distribution of transient activities of ion channels during agonist-triggered calcium flux.

For mass-based intracellular dynamic processes, SPRM could be employed due to its high detection sensitivity to changes in mass density. Recently, Yang et al. has studied single organelle dynamics vertically with 5 nm accuracy in a native cell state.^[20] The dynamic motion process of two organelles, marked with arrows in Figure 5c, along the neurite structure are clearly shown in the time-lapsed snapshots of SPR images. Notably, SPRM could track the movement of intracellular organelles vertically (z-direction) in addition to laterally (xy-direction). Combining this z-position with the x-y position information, the 3D structure of the microtubule was extracted from the SPR images. Furthermore, typical Brownian motion trajectories of exosomes on a PEG-coated surface were visualized with high spatial resolution through image-processing optimization.

6. Developments of New Methods and Instruments

6.1. Coupling with a Patch Clamp

SPRM is based on an inverted optical microscope configuration, which is easily compatible with other techniques, allowing the development of new methods and capabilities. The patch clamp technique is a powerful electrophysiological tool that can record single-channel or whole-cell currents flowing across biological membranes via ion channels. The patch clamp technique has been integrated with SPRM to enable multifunctional recording of the same sample and demonstrated the imaging of the electrical activity of single cells with sub-millisecond temporal resolution (Figure 6a).^[23] The patch clamp technique can change the membrane potentials and synchronize the electrical recording with the fast camera recording, which enabled us to extract the optical signals from tens of thousands of plasmonic images. For example, once the action potential was initiated on a neuron, ions flowed in and out of the neuron through the ion channels. A local transient electric field was thus formed, inducing a change in surface charge density on the metal film, which could be imaged using SPRM. The transient P-EIM intensity was similar to the spike of electrically recorded action potential, demonstrating reliable P-EIM imaging of action potential in single mammalian neurons. Figure 6b shows several snapshots at different stages of action potential, covering initial depolarization, spontaneous action potential firing, and action potential dissipation. This integrated platform holds great promise for studying a variety of cellular electrical activities, such as neuronal signaling processes. Similarly, by integration with patch clamp, Yang et al. observed the mechanical movement of cell membrane was coupled with membrane potential changes.^[44]

6.2. Integration with Microfabrication

Monitoring electron transfer (ET) in molecules is essential for comprehending numerous fundamental chemical and biological processes. A technique with high detection sensitivity and temporal resolution is highly desired to track transient ET processes. Conventional electrochemical measurements usually utilize gold chips with large surfaces, which give rise to a high capacitive charging current and slow response time. Moreover, detection of fast ET kinetics is also plagued by synchronization. Microfabrication of small electrode dimensions can be a perfect means of reducing the charging effect and increasing the response time. Using this strategy, Wang et al. integrated a microelectrode with SPRM and achieved nanosecond temporal resolution.^[24] Thus, the ET in a redox protein (cytochrome c) was measured. The advantage of this integration was the ability to optically detect an electrochemical current with high speed by converting it into a plasmonic signal (Figure 6c). This approach detected both the ET and capacitive charging currents, the latter of which was dramatically suppressed. In addition, the plasmonic signal was insensitive to the electrode area, which enabled a high signal-to-noise ratio when reducing the electrode size. Using this approach, it was found that the ET process occurred at multiple time scales, indicating different conformational changes of cytochrome c. The ultrafast detection capability of SPRM makes it a favorable platform for studying fast electrochemical processes.

6.3. Development of Multifunctional Nano-oscillators

As discussed above, two strategies have been proposed to enhance the signal for small-molecule detection: Au NP-assisted signal amplification and P-EIM. By taking advantage of the basic concepts of the two strategies simultaneously, Shan et al. developed well-organized nano-oscillators for the detection of charges and molecular binding kinetics with high spatiotemporal resolution.^[21b] Each of the nano-oscillators was composed of a gold nanoparticle attached to a gold electrode by means of a flexible molecular tether (Figure 6d). An alternating electric field was applied to the electrode normally. The Au NPs, which could be individually visualized, oscillated owing to electrostatic force, resulting in significant signal amplification. The detection accuracy limit of charges achieved with the nano-oscillators was approximately 0.18 electrons in an aqueous solution. This feature has been employed to detect single microRNA molecules and phosphorylation.^[45] Here, we take phosphorylation as an example to describe the application of a nano-oscillator.^[45b] Phosphorylation is a posttranslational modification of proteins. With the assistance of enzymes (kinases), phosphate groups are specifically bound to proteins. Phosphorylation is involved in nearly all basic cellular processes. Briefly, Au NPs were functionalized with peptides containing an active tyrosine residue. Upon introducing kinase and ATP, the variation in the charge of the Au NPs was monitored by detecting the oscillation amplitude since phosphate group-peptide binding occurred. Similar to routine kinetic analysis, this method can be used to calculate the Michaelis constant and catalytic rate constant. The combination of nano-oscillators with SPRM allowed the simultaneous recording of numerous nano-oscillators and is promising for high-throughput screening of drugs with low molecular weights at the single-molecule level.

We believe that integrating of SPRM with other techniques, such as scanning ion-conductance microscopy (SICM), and atomic force microscopy (AFM), in the future will undoubtedly advance its capability in single-molecule and single-cell analysis.

7. Summary and Outlook

Plasmonic imaging techniques (SPRM and P-EIM) are versatile tools for studying a plethora of biological substances, including single DNA molecules, proteins, bacteria and live cells. Because SPR has the distinctive features of noninvasiveness, a label-free nature and high spatiotemporal resolution, a series of advances in SPR and its applications in biological sensing and imaging have been reported. Although plasmonic imaging techniques have demonstrated impressive feasibility in detecting various biological processes, there are still significant obstacles to overcome to improve their detection limit and expand the scope of their applications. Several challenges can be identified as follows.

The lateral spatial resolution of SPRM or P-EIM in the transverse direction is limited by optical diffraction. However, the parabolic tails of up to several micrometers deteriorate the quality of an image. The optimization of optics by multidirectional or azimuthal rotation illumination and image reconstruction has been proposed to improve the image resolution of SPRM. It is anticipated that the lateral spatial resolution could be further improved without sacrificing sensitivity or temporal resolution by innovations in instrumentation and image processing algorithms.

As described above, electrical activities are always accompanied by mechanical micromotion.^[44,46] It is difficult to distinguish the micromotion response from a P-EIM signal for a complex system. For instance, probing individual electrical activities in cells, such as single action potential events in neurons, remains a challenge for the present capabilities of SPRM, even when integrated with EIS. Further improvement in the detection sensitivity and signal processing algorithms is needed.

In the near future, when these challenges have been addressed by developments in the technical and theoretical aspects, plasmonic imaging techniques will have broader applications and provide further insights for comprehending complex biological and chemical processes.

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Biography

Xiao-Li Zhou received her PhD degree in inorganic chemistry from the University of Science and Technology of China in 2016. Currently, she is a postdoctoral researcher at USTC under the supervision of Prof. Xian-Wei Liu. Her research interests mainly cover the *in situ* optical imaging of electrochemical reactions on nanomaterials.

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Shaopeng Wang received his BS in Biology in 1990 and MS in Biophysics in 1993 from Tsinghua University. He received his PhD in Physical Chemistry from University of Miami with Prof. Roger M. Leblanc in 1999. He was appointed as an associate research professor in Biodesign Center for Bioelectronics and Biosensors at Arizona State University in 2008

and promoted to full research professor in 2015. His research focuses on the development of biosensors and bioinstrumentation for biomedical applications.

Xian-Wei Liu received his PhD in 2011 from University of Science and Technology of China (USTC), where he studied the manipulation and regulation of microbial extracellular electron transfer. After working at the University of Illinois at Urbana-Champaign for one year, he joined the group of Prof. Nongjian Tao at Arizona State University, USA, as a postdoctoral research associate. In 2016, he was appointed as a professor in School of Chemistry and Materials Science at USTC. His group specializes in the optical imaging of interfacial chemistry process.

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Figure 1.

(a) Schematic illustration of SPRM. (b) Particle-scattering model of SPR imaging. (c) SPR difference image of 200 nm polystyrene nanoparticles. (d) Schematic illustration of the P-EIM setup. Copyright 2012, 2014, American Chemical Society. Reproduced with permission from Ref [12, 13, 8a].

Figure 2.

Typical DSPR image (a) and fluorescence image (b) of DNA molecules. (c-d) DSPR image of DNA molecules after (c) and before (d) deconvolution. (e) DSPR image of DNA-functionalized Au NPs binding to a complementary surface. (f) The P-EIM signals from printed small-molecule spots. Copyright 2013, 2014, American Chemical Society. Adapted with permission from Refs. [16, 13, 25].

Figure 3.

(a) SPR images of the influenza A virus and silica nanoparticles. (b) The SPR intensity profiles of selected particles along the X directions. (c) Calibration curve of SPR intensity plotted vs. particle volumes. Copyright 2014, National Academy of Sciences. Adapted with permission from Ref. [3c]. (d) Setup and principle of SPR imaging and tracking of bacterial cells. (e) SPR image of a bacterial cell. Copyright 2016, American Chemical Society. Adapted with permission from Ref. [29].

Figure 4.

(a-b) Mapping cell-substrate interactions with SPRM. Copyright 2012, American Chemical Society. Adapted with permission from Ref. [33]. (c) The distribution of GlcNAc-containing glycoproteins. Mapping of the local association (d) and dissociation (e) rate constants. Copyright 2012, Nature Publishing Group. Adapted with permission from Ref. [3d].

Figure 5.

(a-b) The dynamic electroporation process of a single cell monitored by time-lapsed snapshots of (top) SPR images and (bottom) P-EIM images. Copyright 2011, Nature Publishing Group. Adapted with permission from Ref. [42]. (c) Snapshots of SPR images showing the movement of two intracellular organelles along a neurite structure (denoted by a dashed line). Copyright 2015, Wiley-VCH. Adapted with permission from Ref. [20].

Figure 6.

(a) Setup of plasmonic imaging of action potential in single neurons. (b) Snapshot P-EIM images of action potential at different moments. (c) Optical setup including gold microelectrodes in an electrochemical cell. (d) Schematic illustration of nano-oscillators on a Au electrode. Copyright 2017, Wiley-VCH. Copyright 2017, 2014, American Chemical Society. Adapted with permission from Refs. [23, 24, 21b].