Visualizing and Calculating Tip–Substrate Distance in Nanoscale Scanning Electrochemical Microscopy Using 3-Dimensional Super-Resolution Optical Imaging

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Supporting Information

ABSTRACT: We report a strategy for the optical determination of tip–substrate distance in nanoscale scanning electrochemical microscopy (SECM) using three-dimensional super-resolution fluorescence imaging. A phase mask is placed in the emission path of our dual SECM/optical microscope, generating a double helix point spread function at the image plane, which allows us to measure the height of emitting objects relative to the focus of the microscope. By exciting both a fluorogenic reaction at the nanoscale electrode tip as well as fluorescent nanoparticles at the substrate, we are able to calculate the tip–substrate distance as the tip approaches the surface with precision better than 25 nm. Attachment of a fluorescent particle to the insulating sheath of the SECM tip extends this technique to nonfluorogenic electrochemical reactions. Correlated electrochemical and optical determination of tip–substrate distance yielded excellent agreement between the two techniques. Not only does super-resolution imaging offer a secondary feedback mechanism for measuring the tip–sample gap during SECM experiments, it also enables facile tip alignment and a strategy for accounting for electrode tilt relative to the substrate.

Nanoscale scanning electrochemical microscopy (SECM) is evolving as a powerful tool to obtain electrochemical images and investigate electron-transfer processes with nanoscale resolution.1–3 Using nanometer-sized electrodes as scanning probe tips, it is possible to image the topography of electrochemically active nano-objects,4–5 probe electrocatalytic reactions at single nanoparticles,6,7 detect short-lived reaction intermediates,8,9 and interrogate biological cells.10,11 In a feedback-mode SECM experiment, a voltage is applied to an electroactive tip immersed in a solution of a redox-active mediator in order to generate a current, which is then measured as the tip approaches either an electrochemically inert or active substrate. The current at the tip either decreases or increases as the tip approaches the substrate due to the hindered diffusion of the redox mediator by the inert substrate (negative feedback) or the regeneration of the redox mediator at the active substrate (positive feedback), respectively.1 By fitting the current versus distance approach curve, it is possible to use measured currents to extract information about the tip–substrate gap in subsequent experiments. A feedback mode experiment utilizes very fast mass transport and can provide high axial spatial resolution, which has been extensively used for topographic imaging1–7 and determining rapid electron-transfer kinetics.12–15

While the steady-state tip current strongly depends on the tip–substrate distance, obtaining accurate spatial information along this axial dimension is essential to interpret feedback mode data. Typically, the minimum electrochemically measurable tip–sample gap is limited by the size of the electrode and the thickness of the protective sheath (usually glass) that surrounds the electrode, making it difficult to measure nanoscale tip–sample gaps. Moreover, to extract the separation distance between the tip and the substrate, approach curves for a diffusion-controlled process are fit to a theoretical model, which depends on the geometries of the tip electrode and substrate.1 Difficulties in characterizing the nano electrode geometry, compensating for thermal drift,16 and achieving perfect alignment of the nanotip to the substrate17 often cause considerable uncertainties in the distance determination using an electrochemical approach curve. Further, in an actual experiment, the measured current will depend on both the tip–sample gap as well as any electrochemical activity at the sample itself. Thus, if the sample drifts over time, it is extremely difficult to know whether the change in the measured current is due to drift in the tip–substrate gap or real changes in the sample gap.

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sample gap, changes in the electrochemical activity of the sample, or some combination of the two. To overcome these limitations, hybrid techniques using tip position modulation, shear force, atomic force, and intermittent contact SECM have been developed to facilitate tip positioning and distance control by providing a secondary distance-dependent feedback signal. In these hybrid techniques, either the tip has to be modified in its shape, requiring extensive fabrication efforts, or is oscillated in solution, which perturbs the native environment of the system. Moreover, several of these techniques have limited dynamic range in determining the tip–sample gap due to the tip–substrate interaction that is necessary to achieve this secondary feedback.

To address the aforementioned challenges, we have integrated SECM with three-dimensional (3-D) super-resolution optical microscopy in order to use optical feedback to measure tip–sample gaps with a dynamic range of ~2 μm and, in many cases, resolution better than 25 nm. In 3-D super-resolution imaging, an optic such as a cylindrical lens or spatial light modulator is inserted into the emission path of an optical microscope to induce a distortion on the point spread function (PSF) of an emitter. This distortion depends strongly on the axial position of the probe relative to the focus of the microscope. Here, we use the 3-D super-resolution approach described by Moerner and co-workers, in which they generated a double helix PSF (DH-PSF) to measure the axial position of a fluorescent emitter with precision better than 25 nm. To use this technique for measuring tip–substrate gaps in SECM, we introduce two emission sources, one at the substrate surface and the other at the SECM tip, which produce different emission patterns based on their relative axial positions. By fitting the DH-PSF of the two emitters, we are able to calculate the distance between the tip and the substrate, independent of the electrochemical activity of the system and with no dependence on the geometry of the nanoelectrode. For our initial experiments, we used a fluorogenic resorufin redox reaction occurring at the nanoelectrode to monitor the axial position of the tip, while later experiments extended this technique to nonfluorescent redox reactions by attaching a fluorescent particle to the protective sheath of the SECM tip. Using this 3-D super-resolution approach, we demonstrate excellent agreement between the optical and electrochemical approach curve data, indicating that optical feedback provides a robust strategy for measuring tip–sample gaps with large dynamic range and excellent spatial resolution.

### EXPERIMENTAL SECTION

#### Chemicals and Materials

Ferrocenemethanol (FcMeOH, 97%), resorufin (dye content 95%, \( \lambda_{\text{emission}} = 585 \) nm), fluorescent nanodiamonds (800 NV/particle, 100 nm average particle size, \( \lambda_{\text{emission}} = 690 \) nm), potassium chloride (BioXtra, \( \geq 99.0\% \)), sodium hydroxide (BioXtra, \( \geq 98\% \)), D-(+)-glucose (99.5%, GC), and (3-aminopropyl)triethoxysilane (APTES, \( \geq 98\% \)) were purchased from Sigma-Aldrich. Fluorescent sky blue nanospheres (220 nm, \( \lambda_{\text{emission}} = 610 \) and 660 nm) were purchased from Spherotech Inc. Ethyl alcohol (200 proof) was purchased from Pharmco-Aaper. Glass coverslips (25 × 25 mm, 0.17 mm thickness) and glass slides were purchased from Fisher Scientific. Quartz capillaries (1 mm outer diameter, 0.3 mm inner diameter and 7.5 cm in length) were purchased from Sutter instruments. A 25 μm diameter Pt wire was purchased from Goodfellow. Polishing materials were purchased from Ted Pella Inc. All solutions were prepared with nanopure water (18 MΩ cm, arium pro, Sartorius).

#### Electrochemical Measurements

All electrochemical measurements were performed using a CH750E potentiostat (CH Instruments). A Pt wire and an Ag/AgCl (1 M KCl; CH Instruments) were used as a counter and reference electrode throughout the experiment. A Pt disk electrode with the diameter of 230 nm (ratio of the insulator sheath radius to the Pt disk electrode radius, RG: 40) and 510 nm (RG: 20) were fabricated using a laser puller and polished (see Supporting Information for detailed procedure). The fabricated electrodes were characterized electrochemically by performing cyclic voltammetry with 1 mM FcMeOH in 0.1 M KCl (Figure S-1) and the radius of the electrode was calculated from the diffusion-limited steady state equation (Supporting Information).

Negative feedback electrochemical approach curves were obtained using 1 mM FcMeOH in 0.1 M KCl in a homemade spectroelectrochemical cell with a glass coverslip as the substrate and performed on top of an inverted optical microscope (see below). The tip electrode was positioned ~20 μm above the substrate using a stepper motor (Microdrive, Mad City Labs Inc.). The process is continuously monitored with the optical microscope in a bright-field geometry. A piezo controller (Thorlabs) is employed for further approach of the tip towards the substrate with a step size of 40 nm. The current from the bipotentiostat is acquired using a data acquisition card (PCI-6221, National Instruments) and synchronized with the piezo controller using home written LabView code (National Instruments).

#### 3-D Super-Resolution Imaging

Optical measurements were performed on an inverted microscope (Olympus IX-73) with an Olympus 60× oil immersion objective (Olympus PlanoApo N) in a widefield epi-illumination geometry (Figure 1). The objective was attached to a piezo stage (Piezosystem jena), allowing its vertical position (and thus the focal plane of the imaging system) to be adjusted in 50 nm steps. A 532 nm laser excitation (Spectra-Physics, 532–50-CDRH) was introduced through the objective to excite fluorescence from the sample, which was then collected by the same objective and...
Figure 2. (A) DH-PSF images of a nanodiamond surface marker when it is below, in, and above the focal plane. (B) Optical calibration curve relating the angle between the two lobes in the DH-PSF images and the distance between the surface marker and the focus of the microscope \((d_s)\). (C) DH-PSF images of the resorufin fluorescence at the SECM tip when it is below, in and above the focal plane. The insulting sheath of the SECM tip can also be observed in these images. Insets show zoomed-in images of the lobes at the electrode. (D) Optical calibration curve of the resorufin fluorescence at the Pt nanoelectrode relating the angle between the two lobes in the DH-PSF images and the distance between the electrode and the focus of the microscope \((d_e)\).

RESULTS AND DISCUSSION

The first step of these experiments is to measure calibration curves, in which we relate the emission pattern induced by the double helix phase mask to the distance of the emitter from the focus of the microscope. Figure 2A shows representative emission patterns from a single nanodiamond surface marker as the focus of the microscope is changed relative to the substrate. When the surface marker is in focus, it exhibits two lobes horizontal to each other; we define this angle as 0°. When the surface marker is below or above the focus of the microscope, the angle between the two lobes rotates, as shown. By fitting each lobe to a two-dimensional Gaussian function, we are able to extract the angle between the two lobes (Figure S-4). To create the calibration curve shown in Figure 2B, we move the focus of the microscope by 50 nm steps roughly 1 \(\mu \text{m}\) above and below the substrate surface and calculate the angle between the two lobes. These data then allow us to calculate the distance between the surface marker and focal plane \((d_s)\) based on the angle between the lobes.

Similarly, a calibration curve was obtained for the electrode using resorufin fluorescence at the electrode tip as shown in Figure 2C,D. The focal plane of the microscope was set roughly 3–5 \(\mu \text{m}\) above the substrate, and the electrode was held close to this focal plane while an oxidation potential of +0.4 V was applied to generate fluorescent resorufin (Figure S-2). When the electrode is at the focal plane of the microscope, the two lobes associated with resorufin emission appear roughly horizontal, and they rotate as the electrode is moved in steps of 50 nm roughly 1 \(\mu \text{m}\) above and below the focal plane (Figure 2C). We create the calibration curve shown in Figure 2D by stepping the position of the electrode relative to the fixed focal plane of the microscope. The calibration curve allows us to calculate the distance between the electrode and the focal plane \((d_e)\) based on the angle between the two lobes. We point out that the two lobes are not as clearly resolved in the images in Figure 2C.
comparison to the surface marker images from Figure 2A. This is most likely due to diffusion of the resoruﬁn probe from the electrode surface, which causes broadening in the resulting diffraction-limited spot. To reduce this diffusion-based effect, we work in a solution of glucose in NaOH, which chemically reduces the resoruﬁn produced at the electrode to nonﬂuorescent dihydroresoruﬁn, thereby mitigating the impact of diffusion on our images. Further, we also use electrodes for these measurements that have diameters at or below the diffraction limit of light, such that we observe clean double helix point spread function images associated with the resoruﬁn emission. This electrode size restriction is a limitation of monitoring ﬂuorogenic reactions at the electrode surface, which will be addressed in more detail below.

Using the two calibration curves, we can now determine the tip–substrate distance as the height of the electrode is changed relative to the substrate. Figure 3 shows a series of images where both the emission from the resoruﬁn at the tip (yellow circle) and the emission of a surface marker at the substrate (white circle) are observed. Note that the two emitters are spatially separated such that the two images do not overlap; otherwise, we would be unable to determine their individual angles and therefore axial position. The focus of the microscope is set between the substrate and the initial position of the electrode, as indicated by the angle between the lobes of the resoruﬁn distribution (Figure S-5), as indicated in each of the panels of Figure 3.

One challenge with using the optical feedback approach we have described thus far is that it requires a ﬂuorogenic redox probe to deﬁne the electrode surface, which limits the applicability of the technique. Moreover, because we use glucose to quickly oxidize the resoruﬁn produced at the SECM tip back to dihydroresoruﬁn, the currents that we measure as we approach the electrode to the surface do not follow the expected negative feedback proﬁle, making it diﬃcult to compare our optical data to electrochemical data. To overcome these limitations and improve the generality of this technique, we attached a ﬂuorescent nanodiamond to the nonconducting glass sheath of our SECM tip, which provides an optical readout of the tip position while allowing us to probe any electrochemical reaction of interest at the electrode (see Supporting Information for details about nanodiamond attachment). We will refer to the nanodiamonds attached to the sheath of the SECM tip as electrode markers, for clarity.

For correlated 3-D super-resolution optical and electrochemical experiments, we immersed the nanodiamond-functionalyzed electrode (here, a 510 nm diameter Pt electrode) in 1 mM FcMeOH in 0.1 M KCl and applied +0.4 V versus Ag/AgCl (1 M KCl). The focal plane of the microscope was set several nanometers above the substrate (as deﬁned by the surface markers) and the nanodiamond-functionalyzed tip was allowed to approach the substrate in steps of 40 nm using the piezo controller. The current was continuously monitored and when the electrode reached ~1 μm above the focal plane, we started to observe emission corresponding to the electrode markers (see video in the Supporting Information). Figure 4A shows four representative optical images, indicating the surface marker (white circle) and the brightest electrode marker (yellow circle). The lobes associated with the electrode marker rotate as the electrode approaches the surface (Figure 4A, panels 1–3) while the lobes associated with surface marker remain at a consistent angle. Using calibration curves for this system, we calculate the distance of both the electrode and the surface marker relative to the focal point (Figure 4B). As expected, $d_e$ is constant as the electrode approaches, while $d_s$ changes smoothly as the electrode moves through the focal plane. The summation of $d_e$ and $d_s$ results in the tip–substrate distance ($1.24, 0.8$, and $0.27 \mu m$, respectively, for Figure 4A, panels 1–3). Once the tip hits the substrate, the lobes in the PSF of both the electrode and the surface markers rotate (Figure 4A, panel 4), resulting in changes in both $d_e$ and $d_s$ along with significant error in their determination (Figure 4B).

Next, we compared the results of the optical experiment to the electrochemical data that was obtained simultaneously. The normalized current versus distance approach curve is shown in Figure 5A. As expected, as the tip approaches the insulating substrate, the tip current gradually decreases, and then abruptly levels off, indicating that the glass sheath of the tip touched the substrate surface. The electrochemical data was ﬁt to negative feedback theory, “using the tip radius and RG ratio determined from the steady-state voltammogram and the optical microscope.

Figure 3. 3-D super-resolution optical images to determine the tip–substrate distance for various positions of the tip relative to the substrate. The emission in the white circle is from a surface marker at the substrate and the emission in the yellow circle is due to resoruﬁn ﬂuorescence at the electrode. The calculated tip–substrate distance at various position are (A) 1.92, (B) 1.51, (C) 0.92, (D), 0.74, (E) 0.50, (F) 0.36, (G) 0.27, and (H) 0.15 μm. Scale bars: 3 μm.
The piezodisplacement of zero separation distance \( z_0 \) was used as an adjustable parameter. While the curvature of the distance-current curve essentially depends on the tip radius (\( a \)) and RG, the \( z_0 \) value could be confidently determined from the fitting. The tip-substrate separation distance of each data point was evaluated from the tip current and compared with optical results (see Supporting Information).

Figure 5. (A) Experimental electrochemical approach curve (symbols) fitted to the theory for pure negative feedback (blue solid line) with RG = 20. The distance is normalized by tip radius, \( a = 255 \) nm and the tip current is normalized by \( i_\infty = 75 \) pA. (B) Tip-substrate separation distances vs piezo displacement determined from the optical measurement (blue squares), SECM approach curve fit (black solid line), and tip currents (red triangle). Note that the piezo displacement increases relative to its starting position as the tip gets closer to the substrate surface.

One additional advantage of our 3-D super-resolution optical feedback approach is that we are able to calculate the tilt of the electrode. In traditional SECM experiments, electrode tilt cannot be measured directly and is instead inferred when an approach curve deviates significantly from ideal feedback behavior. Using the optical approach, if there are multiple markers on the SECM tip, we can measure the vertical distance between them: if the electrode is tilted, the emission from electrode markers will have different angles between the lobes in the DH-PSF images, indicating that the markers are in different axial planes (Figure S-7), whereas if the electrode is flat, the electrode markers will appear in the same axial plane (Figure S-8). We have demonstrated the ability to measure tilt angles as large as 22° and as small as 1.8° using this technique, allowing us to have excellent control over tilt. Moreover, because we can place multiple markers on both the substrate and the tip, we are able to monitor both tilts simultaneously, providing us with the ability to ensure that the two electrochemical components are well-aligned relative to each other.

\section*{CONCLUSION}

In summary, we have developed a nano SECM combined with a 3-D super-resolution optical microscope to determine tip-substrate distance optically with axial resolution better than 25 nm. The optical approach curve data agrees with simultaneously obtained electrochemical approach data, providing a secondary feedback mechanism for SECM tip-sample gap measurements.

Figure 4. (A) Optical images in panels 1–4 represent four different positions of the electrode, corresponding to the labeled points in panel (B). The white and yellow circles correspond to emission from the surface and electrode markers, respectively. The optically calculated tip-substrate distances are indicated in panels 1–3. The angle between the lobes of the marker changes when the electrode hits the surface (panel 4). Scale bars: 5 \( \mu \)m. (B) Calculated distances (angles) of a surface marker (red triangles) and an electrode marker (blue circles) from the focal plane as a function of piezo positions. The green dotted line indicates the position of the focal plane and the red dashed line represents the position at which the tip hits the substrate. The distances between the focal plane and surface marker as well as the focal plane and electrode marker are shown as \( d_s \) and \( d_e \), respectively. The calculated distances (angles) of the surface and tip electrode markers fluctuate after the electrode hits the surface. Note that the piezo displacement increases relative to its starting position as the tip gets closer to the substrate surface.
in an experiment. Moreover, our approach allows us to measure the tilt of both an electrode and a surface without the risk of electrode damage.

Despite the success of these initial experiments, there are some improvements that can be made to yield even better axial resolution. For example, our DH-PSF phase mask is optimized for a 665 nm emitter, which is red of the emission of both the resorufin and the nanodiamonds. Thus, using a wavelength-optimized phase mask (or a spatial light modulator which allows dynamic phase mask control) would improve the quality of the optical images and yield even better resolution. Second, we have tested two different fitting algorithms to extract the angle between the two lobes in the DH-PSF: fitting to two separate two-dimensional Gaussians (Figure S-4) and using radial symmetry fitting. While the former yielded better precision in the fits, the latter is significantly faster and would be easier to implement in a real-time SECM experiment. Thus, finding an algorithm that can determine the angle between the two lobes of the different PSFs and make drift corrections in real time remains a future improvement to this experiment. Finally, minimizing vibration of the tip is a significant experimental challenge that further limits our resolution. For some of our experiments, we obtain precision better than ~25 nm (e.g., Figure 2), but in other cases, we observe small vibrations in the tip that limit the resolution to ~50 nm (Figures 4 and 5). Further improvements to the tip holder and introducing improved vibration isolation will help address these issues and allow even better axial precision. However, even with these experimental challenges, we believe this initial demonstration of correlated 3-D super-resolution and SECM offers a promising avenue towards improved performance as we push towards even more nanoresolved electrochemical imaging.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b04073.

Attachment of fluorescent particles as surface markers to the substrate, nanoelectrode fabrication, fluorogenic reaction of resorufin occurring at Pt nanoelectrode, attaching fluorescent nanodiamond to the nonconductive sheath of the nanoelectrode, calculation of angle between the double-helix lobes, optical determination of tip–substrate distance, deviation in the position of fluorescent surface marker when electrode hits the substrate and calculating the tilt of the nanoelectrode (PDF). Video shows the emission from surface and electrode markers, in which the electrode is approaching towards the substrate (AVI).

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