Label-free cell-substrate adhesion imaging on plasmonic nanocup arrays

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Abstract: Cell adhesion is a crucial biological and biomedical parameter defining cell differentiation, cell migration, cell survival, and state of disease. Because of its importance in cellular function, several tools have been developed in order to monitor cell adhesion in response to various biochemical and mechanical cues. However, there remains a need to monitor cell adhesion and cell-substrate separation with a method that allows real-time measurements on accessible equipment. In this article, we present a method to monitor cell-substrate separation at the single cell level using a plasmonic extraordinary optical transmission substrate, which has a high sensitivity to refractive index changes at the metal-dielectric interface. We show how refractive index changes can be detected using intensity peaks in color channel histograms from RGB images taken of the device surface with a brightfield microscope. This allows mapping of the nonuniform refractive index pattern of a single cell cultured on the plasmonic substrate and therefore high-throughput detection of cell-substrate adhesion with observations in real time.

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OCIS codes: (240.6680) Surface plasmons; (280.1415) Biological sensing and sensors; (100.2960) Image analysis; (170.0180) Microscopy; (170.1530) Cell analysis; (170.3880) Medical and biological imaging.

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1. Introduction

Cell adhesion drives crucial cellular functions including differentiation, cell migration, cell survival, and angiogenesis [1–3]. In addition, changes in cell adhesion can define the presence of diseases such as cancer, arthritis, osteoporosis, and atherosclerosis [2]. Tumor cells often exhibit changes in adhesion to an extracellular matrix (ECM) determined by the cell and oncogene type [4, 5]. Therefore, the development of methods to detect and quantify cell-cell and cell-substrate adhesion at the single cell level is important for understanding cell signaling pathways, determining the effects of biochemical treatments, drug discovery research, cancer metastasis studies, and disease diagnosis.
When a cell binds to a surface, transmembrane proteins called integrins cluster into focal adhesion (FA) complexes that serve to connect the cell to the ECM [6, 7]. FA complexes are highly localized and altered by integrin clustering on the nanoscale [3]. The cell membrane is typically separated from the substrate by a distance of approximately 150 nm, but at FA sites this distance shrinks to 10-15 nm. FA complexes vary in size, but typically are on the order of 1-10 µm [8]. Research has shown that chemical and mechanical cues both play crucial roles in determining cellular behavior based on adhesion changes [9–11].

Cell adhesion is currently measured using a variety of biophysical methods. The locations of FA complexes can be directly visualized by fluorescent labeling of adhesion molecules such as integrins [11]. However, this doesn’t allow real-time observation and measurement. Interference reflection microscopy, where partially reflected light is used to create an interference pattern, can also be used for direct visualization of cell-substrate separation, but measurements are difficult to quantify [12, 13]. Cellular adhesion profiles can also be determined by measurements of cellular forces generated at the cell-substrate interface. Polyacrylamide (PA) gel-based traction force microscopy uses PA gels functionalized with fluorescent beads near the gel surface [14, 15]. Forces generated at the FA sites cause the fluorescent beads to displace, which can be quantified down to 1 µm using advanced data processing [16]. However, this method suffers from uncertainties in tracking the bead positions.

Tools have also been developed in order to measure the forces required to physically detach a single cell from a substrate of interest. One method uses an atomic force microscope (AFM) probe where the force is quantified by the probe deflection [17–19]. Another method uses a micropipette to apply suction to a cell observed under a microscope [20]. Approaches like these that can monitor cell-substrate separation at the single cell level provide useful information, but these methods suffer from low throughput and high probability for unintended cell damage. There remains a need for a simple noninvasive method to monitor the adhesion and detachment of single cells from a substrate in response to biochemical or biophysical inputs or in different states of disease. This tool should allow observations and measurements in real time, use easily accessible equipment, and have low time and labor requirements.

The use of plasmonics for detecting cell-substrate adhesion has been greatly underutilized. In one noteworthy study, a SPR microscope was constructed in order to detect and quantify cell-substrate adhesion for primary goldfish glial cells cultured on aluminum coated glass prisms [21]. Quantitative measurements of distances between the cell membrane and the aluminum surface were made by scanning the angle of incidence and taking local measurements of the angle required for surface plasmon polariton (SPP) excitation. In another noteworthy study, a scanning localized surface plasmon microscope was used in order to detect FA complexes in single C2C12 cells [22].

Here we introduce a new method for detecting cell-substrate adhesion based on the excitation of surface plasmons on an extraordinary optical transmission (EOT) nanocup array substrate. EOT refractive index (RI) sensors are known to operate based on a combination of SPR and localized surface plasmon resonance (LSPR) [23, 24]. The fields are evanescent with decay lengths on the order of 200 nm or less, which gives a high sensitivity to the RI directly at the metal-dielectric interface. As a result of this property, SPR sensors theoretically have a vertical resolution that is sub-nanometer [26]. In EOT sensors, an increase in RI is measured using the red shift of the transmission peak in the visible light range [25]. In our previous work, we have reported on the use of colorimetry to detect these RI changes on an EOT plasmonic device [26, 27]. The decay length of this EOT plasmonic sensor has been previously determined to be 193 ± 10 nm [28].

Using the advantage of colorimetry, we show that an increase in RI can be detected and quantified by shifts in peak intensity values for the normalized red and green channel histograms from RGB images taken of the device surface. The highest sensitivity occurs for
the difference between the green and red channels because the spectral red shift occurring due to an increase in the RI corresponds to an increase in the red intensity and decrease in the green intensity of the RGB image. This work demonstrates that EOT sensors have an untapped potential to serve as low-cost imaging sensors of samples with uniform and nonuniform RI profiles based only on images taken with a brightfield microscope. We apply this method to image spatially varying adhesion of 3T3 cells cultured directly onto the EOT substrate. Furthermore, using the decay length of the EOT sensor, we are able to determine a cell-substrate separation distance from the experimental data. This work can fill the missing gap for high-throughput, high-accessibility quantitative cell adhesion mapping of live cells in real time.

2. Materials and methods

2.1 Nanoplasmonic device fabrication

The nanocup array plasmonic device is fabricated using a replica molding process described in our previous work [30]. Briefly, we begin with a 4-inch diameter quartz mold patterned with tapered nanopillar arrays, which is fabricated by laser interference lithography. UV-curable polymer (NOA-61) is cast over the mold followed by a PET sheet backing. Once the polymer evenly spreads over the mold, it is cured for 60 s by exposure to UV light. Following curing, the PET sheet with the nanostructured polymer is carefully peeled off of the mold. In order to make the devices plasmonically active, 90 nm of gold (Au) with a 9 nm titanium (Ti) adhesion layer is deposited on the substrate by e-beam deposition. The resulting nanocup arrays have a periodicity of 350 nm, cup diameter of 180 nm, and cup height of 500 nm.

2.2 Cell culture and plating

3T3 fibroblast cells were maintained in Dulbecco’s Modified Eagle’s Medium without phenol red (Life Technologies) supplemented with 10% bovine calf serum (Life Technologies) and 1% Penicillin-Streptomycin solution (Mediatech). The cells were grown and maintained in 75 cm² flasks at 37°C in a humidified atmosphere of 5% CO₂. The cells were subcultured when they reached 75% confluence. Before plating the cells onto the plasmonic substrate, the nanocup array was cleaned in IPA, water, and IPA followed by drying in N₂. The device was then immersed in 70% ethanol for sterilization followed by drying in N₂. The substrate was then placed at the bottom of a 25 mm glass culture plate and the cells were directly plated onto the plasmonic device at a concentration of 1x10⁴ cells/mL. The cells were allowed to grow on the substrate for 48 hours in order to ensure sufficient attachment. Before cell imaging, the cell media was removed and replaced with phosphate-buffered saline (PBS) warmed to 37°C. A glass coverslip was placed over the cells plated on the device and the images were immediately taken. In addition to the stagnant images, we also monitored the cell adhesion profile over time when the cell was put under osmotic stress. In order to create an environment of osmotic stress, excess NaCl was added to the PBS to a final concentration of 500 mM. In this case, PDMS spacers were placed between the coverslip and the cells in order to suppress cell detachment during the imaging.

2.3 Imaging and image analysis

Brightfield images were taken with an Olympus BX51 Upright Fluorescence Microscope operating in transmission mode using a 50X objective and halogen light source. Transmission spectra were also taken using the halogen light source from the Olympus BX51 Microscope with a portable spectrometer (Ocean Optics). The transmission spectra from the sample were normalized with the light source spectrum in order to obtain the final transmission data. Multispectral images were taken using a multispectral imaging setup consisting of a halogen light source, monochromator, and microscope with a 10X objective. The multispectral imaging was controlled by a custom LabVIEW program. The frame rate for the brightfield
images was 0.275 s and the frame rate for the multispectral images was 0.8 s. These frame rates were chosen in order to maximize the signal without reaching saturation. These frame rates can be reduced to observe processes occurring on a faster timescale. In order to maintain the same signal-to-noise ratio for a shorter frame rate, the light source power can be increased or a lower noise camera with a higher sensitivity can be used. Overall, the ability for measurements to be carried out in real time will be limited by the frame rate.

All image analysis was carried out using MATLAB (Mathworks) and the MATLAB Image Processing Toolbox. The RGB image was split into the three color channels. Each image was normalized by taking each channel’s intensity at each pixel and dividing by the total RGB intensity for that pixel. For example, the first pixel of the red color channel image is equal to: pixel1\_red, normalized = pixel1\_red / (pixel1\_red + pixel1\_green + pixel1\_blue). The result is an image in the range of 0 to 1 for each color channel that gives an accurate representation of the color contribution to the image. This procedure removes the effect of intensity variations in the three color channels caused by the light source or the specimen. For example, overall increases or decreases to the intensity in the RGB image due to transmission variations from cell structures will be corrected for in the color channel images during the normalization step. The difference image is obtained by subtracting the red color channel image from the green color channel image (green-red). The built-in MATLAB colormaps (parula, hot) were used for cell adhesion map visualization where the colorbar scale was set based on the measured calibration curves. Nonuniform illumination correction and contrast enhancement of RGB cell images were done by filtering with morphological opening of the image using a disk-shaped structural element with a radius of 20 pixels before splitting the image into the three color channels. The images taken with the multispectral system were directly subtracted and enhanced by contrast stretching in the range of 0 to 1.

2.4 Simulation study

A 3D finite element method (FEM) simulation study of the plasmonic nanocup device was done using the COMSOL Multiphysics RF Module. A single nanocup with a period of 350 nm, cup height of 500 nm, top cup diameter of 180 nm, and bottom cup diameter of 160 nm was modeled. A 9 nm Ti layer and a 90 nm Au layer were placed on the bottom and top of the cup to make the device plasmonically active. The polymer substrate RI was set to 1.56, the superstrate RI was set to 1.33, and the wavelength dependent RIs of Ti and Au were interpolated from the experimental data reported by Johnson and Christy [29, 30]. The plane wave source propagated in the + z direction at normal incidence with polarization along x. The total simulation region was 350 nm x 350 nm x 2000 nm. Periodic boundary conditions were imposed along x and y and nonreflecting boundary conditions were imposed along the z axis by the use of periodic ports for transmission measurements.

3. Operating principle

A schematic of a single cell cultured on the Au nanocup array is shown in Fig. 1(a) and a top-down scanning electron microscope (SEM) image of the plasmonic device is shown in Fig. 1(b) to show the surface on the microscale. The operating principle for our cell adhesion visualization method is based on the evanescent fields created by surface plasmon generation combined with the local changes in RI that will occur at the cell-substrate interface depending on the adhesion pattern. At FA complexes, the cell membrane is 10-15 nm away from the device surface, which will result in a larger local change in the resonance condition compared to the rest of the cell. EOT nanohole arrays exhibit peaks because of LSPR and SPR with the SPP-Bloch wave (SPP-BW) typically having a high sensitivity. The wavelength required for SPP-BW ($\lambda_{SPP-BW}$) excitation can be predicted based on theoretical momentum matching at the interface, which gives $\lambda_{SPP-BW} = \frac{p}{\sqrt{l^2 + j^2 \sqrt{\varepsilon_m \varepsilon_d}}}$ where $p$ is the periodicity of the
square lattice nanohole array, \( i \) and \( j \) are integers defining the scattering order, \( \varepsilon_m \) is the dielectric constant of the metal, and \( \varepsilon_d \) is the dielectric constant in the sensing region [31]. The generated SPP-BW results in an evanescent electromagnetic wave at the metal-dielectric interface and therefore the sensitivity exponentially decays away from the interface with the highest sensitivity directly at the device surface.

In order to confirm the vertical sensitivity of our device, we built a simplified 3D-FEM model of a cell near the surface of an EOT sensor. A single period of the plasmonic nanocup was modeled with periodic boundary conditions in \( x \) and \( y \). The superstrate was set to be a dielectric with a RI of 1.33. Figure 1(c) shows the simulated transmission spectra when the superstrate RI is increased to 1.38 and 1.41. A red shift occurs as expected. The sensitivity of the simulated device is 130 nm/RIU, which is significantly less than the expected sensitivity of the EOT plasmonic device utilized in this study. The higher sensitivity of the experimental device is primarily because of the presence of Au nanoparticles that form along the cup sidewalls due to the directional e-beam Au deposition, which were not included in the 3D-FEM model.

In order to model a cell membrane, a second object was added to the superstrate with a RI of 1.46 and set to be separated from the device surface by a distance \( d \) of 15 nm or 150 nm. The length and width of the object was 350 nm such that it filled the entire simulation space above the height determined by the \( d \) value. The RI inside the cup and at the device surface was set to 1.33. The transmission spectra and electromagnetic near field distributions for the three cases (water only, \( d = 15 \) nm, and \( d = 150 \) nm) were then compared as shown in Fig. 1(d)-1(e). The \( d = 15 \) nm case results in a red shift of 7 nm while the \( d = 150 \) nm case results in no detectable red shift. A change to the amplitude \( |E|\), the \( E_z \) component, and the \( H_y \) component of the electromagnetic field also occurs for the \( d = 15 \) nm case. The near fields were also visualized in order to confirm that the main peak in the transmission spectrum corresponds to the SPP-BW as is shown in the \( E_z \) and \( H_y \) field components in Fig. 1(e). Stronger field intensity was observed with smaller displacement of the cell from the device surface. These results show that the EOT nanocup sensor has a sensitivity to vertical displacement from the device surface, as expected.
4. Experimental results

The experimental transmission spectra of the nanocup array are shown in Fig. 2(a) when the superstrate is changed from air to PBS to PBS with a cultured cell. Figures 2(b)-2(c) show the normalized histograms for the red, green, blue, and difference (green-red) color channels for air and PBS, respectively, based on RGB images taken of the device surface. In the transmission spectra, there is a clear red shift as the superstrate RI increases from air (RI = 1) to PBS (RI = 1.33). The spectral properties of this plasmonic nanocup device have been previously studied [26, 28, 32]. In PBS, the longer wavelength peak corresponds to the SPP-BW while the shorter wavelength peak is primarily due to LSPR resonances, such as at the nanocup rim. In air, the two peaks overlap to form a single peak. The red shift of the LSPR peak with increasing RI is minimal compared to the SPP-BW peak and therefore the focus of our analysis is on the longer wavelength SPP-BW peak. In the color channel histograms, the result is an increase in the peak intensity value for the red channel, a decrease in the peak intensity value for the green channel, and a decrease in the peak intensity value for the difference channel. In order to characterize the spectral and color channel sensitivities of the nanocup array, glycerol solutions with increasing concentrations (0-60%) were added to the device surface. The spectra and RGB images were taken with a brightfield microscope and 50X objective. The red shift with increasing RI is shown in Fig. 2(d). There is a linear relationship with a device sensitivity of 230 nm/RIU. Figures 2(e)-2(h) show the normalized color channel histograms for the red, green, blue, and difference channels, respectively, with increasing RI. The results are summarized in the plot shown in Fig. 2(i). The relationship between the color channel peak intensity (I) and RI is also linear. The red color channel has a calibration equation of $I = 0.37(RI) - 0.13$ with $R^2 = 0.82$, the green color channel has a calibration equation of $I = -0.38(RI) + 0.98$ with $R^2 = 0.96$, the blue color channel has a calibration equation of $I = 0.02(RI) + 0.15$ with $R^2 = 0.48$, and the difference (green-red) color channel has a calibration equation of $I = -0.79(RI) + 1.18$ with $R^2 = 0.91$. Based on these results, the red, green, and difference color channel histograms can all be used for detecting RI changes with the difference channel having the highest sensitivity.
The RGB images of cells cultured on the plasmonic nanocup array before and after filtering are shown in Fig. 3(a)-3(b). The red color channel image is shown in Fig. 3(c), the green color channel image is shown in Fig. 3(d), and the difference color channel image is shown in Fig. 3(e). In the red color channel image, locations of greater RI show higher intensity visualized in the parula MATLAB colormap as more yellow and less blue. In the green and difference color channel images, locations of greater RI show lower intensity visualized in the same colormap as more blue and less yellow. Filtering results in a decrease in background variation making the locations of high adhesion easier to visualize. These results show that the number of cells that can monitored simultaneously using this method is limited only by the microscope field of view. However, in this work we focus on single cell measurements.
Greater details of the RI variations at the cell-substrate interface can be seen more clearly by looking at single cells as shown in Fig. 4. Figures 4(a)-4(b) show the RGB image before and after filtering. Figures 4(c)-4(e) show the red, green, and difference channel images. These image types are repeated for another single cell and shown below in Fig. 4(f)-4(j). The difference color channel images (Fig. 4(e) and 4(j)) both contain a second colorbar, which corresponds to the cell-substrate separation distance. The cell-substrate separation distance was calculated based on the method outlined by L. S. Jung et al. [33]. Briefly, a three-layer structure was assumed to be present above the device surface. The first is a PBS layer of thickness equal to the cell-substrate separation distance \( d \) with a RI value of \( \text{RI}_{\text{PBS}} = 1.33 \). The second layer is a cell membrane with a thickness of 10 nm and a RI of \( \text{RI}_m = 1.46 \) and the third layer is the cell body with a RI value of \( \text{RI}_c = 1.37 \). RI values and the cell membrane thickness were taken from relevant literature [34, 35]. The sensor response \( R \), defined here as \( \Delta I \), is given in Eq. (1):

\[
\Delta I = m(\text{RI}_{\text{eff}} - \text{RI}_c)
\]

where \( \text{RI}_{\text{eff}} \) is the effective RI of the three-layer structure above the EOT device surface and \( m \) is the bulk sensitivity from the color channel calibration data. The effective RI should take into consideration that the generated electromagnetic field for a plasmonic sensor is evanescent with a decay length \( l \) such that \( \text{RI}_{\text{eff}} \) is given by Eq. (2):

\[
\text{RI}_{\text{eff}} = \frac{2}{l} \int_0^l \text{RI}(z)e^{-2z/l} \, dz
\]

where \( \text{RI}(z) \) is the RI value at a given height \( z \) above the device surface. The integral in Eq. (2) can be solved under the assumption that the RI is equal to \( \text{RI}_{\text{PBS}} \) from \( z = 0 \) to \( z = d \), equal to \( \text{RI}_m \) from \( z = d \) to \( z = d + 10 \), and equal to \( \text{RI}_c \) from \( z = d + 10 \) to \( z = \infty \). It is important to note that the analysis given here assumes that the cell thickness is large enough that it can be approximated as extending out to infinity. Equation (2) was then solved to obtain \( \text{RI}_{\text{eff}} \). This expression was then plugged into Eq. (1) and the equation was rearranged such that \( d \) was solved for in terms of the other known parameter. The accuracy of this method depends on the accuracy of the assumed RI values and cell membrane thickness in addition to the accuracy of \( l \), which was previously experimentally determined for this plasmonic sensor to be \( 193 \pm 10 \) nm [28].
In order to confirm our method, control sample images of single cells were also taken on a glass slide and on an Au thin film (9 nm Ti with 90 nm Au) deposited on a flat polymer sheet and the same image processing method was applied. The images are essentially blank with no cell information. These results suggest that the plasmonic nanocup array substrate is critical for enabling the sensing of cell adhesion. Thus, the method and analysis presented here apply specifically for mapping the RI variations of the cells cultured on the plasmonic nanocup array substrate.

The cell adhesion profile for a single 3T3 cell was also monitored over time when the cell was placed in an environment of osmotic stress (500 mM NaCl) that is expected to cause the cell to shrink. The difference color channel images are shown for time point 1 (0 minutes), time point 2 (30 minutes), time point 3 (60 minutes), and time point 4 (90 minutes) in Fig. 5(a)-5(d) using the hot colormap. The changes in cell adhesion are better visualized by a close up image of the cell body for the four different time points as shown in Fig. 5(e)-5(h). The maximum intensity value is also decreased from 0.225 to 0.15 to increase the visualized contrast in the image. In this case, the higher RI is given by the lower intensity and visualized as more red. Locations of decreasing adhesion with respect to time are highlighted in Fig. 5(e)-5(h) by black arrows. In addition, second colorbars in Fig. 5(e)-5(h) indicate the cell-substrate separation distance, calculated as described previously. Because of the intensity cutoff at 0.15, all d values that are 160 nm or greater are set to the maximum value. By limiting the maximum intensity value and therefore increasing the contrast, locations of high adhesion are more clearly identified.
In addition to brightfield microscope RGB images, grayscale images taken using a multispectral imaging system also can be used for plasmonic RI mapping and cell adhesion imaging [36]. In this case, an image is taken at a single wavelength at a time and two images taken at different wavelengths are subtracted to obtain the difference image. Therefore, the same plasmonic cell adhesion mapping method is applied as for the brightfield images, but with a controlled input. For almost all applications, the brightfield image acquisition with color channel analysis is preferred because of the reduced acquisition times and instrumentation requirements. However, with the controlled input of the multispectral imaging system, the plasmon resonance wavelength at each pixel can be known with a higher precision, which may be beneficial for certain applications. The schematic of the multispectral imaging system used for this study is shown in Fig. 6(a). A white light source is sent through a monochromator where a grating is used to select the wavelength of interest. The nanocup array is then illuminated with this light and imaged with a 10X objective lens and camera. The grayscale image taken at $\lambda = 625$ nm is shown in Fig. 6(b). Figures 6(c)-6(h) show the difference images, $I(\lambda_1)-I(\lambda_2)$, between $\lambda_2 = 625$ nm and $\lambda_1 = 450$, 475, 500, 525, 550, and 575 nm, respectively. The contrast and detail in the images increase as $\lambda_1$ leaves the blue spectral range and goes further into the green closer to the spectral peak of the plasmonic nanocup array. Figure 6(i)-6(k) show a close up image of single cells cultured on the plasmonic device for the difference image where $\lambda_1 = 575$ nm.
5. Discussion and conclusion

In this work, we have presented experimental results which show that RI changes can be detected and quantified by detecting shifts in the peak intensity values for green and red channel histograms with the difference channel (green-red) showing the highest sensitivity. This result has many important consequences. It demonstrates that RI changes can be detected and quantified using an EOT plasmonic substrate and brightfield microscope images therefore enabling high-throughput low-cost RI mapping of uniform and nonuniform samples alike. We demonstrated how this method allows for a new way to carry out cell adhesion mapping of single cells using brightfield microscope images taken of living cells cultured on plasmonic nanocup arrays.

This work presents a proof-of-concept and there are still important biological details to consider. For example, the 3T3 cells in this work were cultured on the plasmonic nanocup arrays directly without any surface treatment. Coating the substrates with an ECM protein such as collagen or fibronectin is common in cell adhesion studies and is an important step in our future work to ensure relevant adhesion profiles. Our device can easily be applied to cell adhesion mapping with different surface treatments as long as the calibration curves are done with the device including the same surface treatment. The timescale limitations of this technique may also require further study depending on the application. For the brightfield
image acquisition, a frame rate of 0.275 s was chosen in order to maximize the signal without reaching saturation. This is sufficient for real time monitoring of important processes such as cell migration, differentiation, and detachment. However, if a biological process on a faster timescale needs to be studied, then the frame rate can be reduced, but the signal-to-noise ratio will also be reduced. Modifications that will improve the signal-to-noise ratio include increasing the light source power or using a detector with a higher sensitivity. The plasmonic device can also be further optimized if a faster frame rate is required. For example, the Au thickness can be reduced to increase the transmitted intensity.

The tool presented in this work enables mapping cell-substrate separation in real time where the cell can be exposed to a wide variety of biochemical and biophysical stimuli. Therefore, it fills an important gap in the set of available tools for cell adhesion studies.

**Funding**

Funding for this work was provided by the U.S. Department of Energy (grant number DE-FG02-07ER46471). The authors would like to thank the Linda Su-Nan Chang Sah Doctoral Fellowship for funding L.P.H.

**Acknowledgments**

This work was carried out in part in the Micro and Nanotechnology Laboratory and the Frederick Seitz Materials Research Laboratory Central Research Facilities at the University of Illinois at Urbana-Champaign.