Spectroscopy-Assisted Label-free Molecular Analysis of Live Cell Surface with Vertically Aligned Plasmonic Nanopillars

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1. Introduction

The exterior cell surface is rich in molecular markers that play key roles in a broad range of cellular functions, from adhesion to pathogen recognition. Subtle modifications to the plasma membrane protein expression can drive dramatic changes in macroscopic phenomena, and result in difficult-to-treat diseases. For instance, cell surface composition changes in terms of lipid bilayer, cell surface glycoprotein, glycolipids, and morphology are strong indicators of carcinogenesis, and hence are promising targets for cancer detection.[1]

Advances in live cell imaging have yielded stunning insights into how cell surface behavior is regulated at the onset and during advancement of disease. In particular, the recent development of super-resolution imaging techniques has broken the diffraction limit enabling the visualization of cellular features with unprecedented detail in 3D.[2] This has revealed, e.g., the co-localization of protein pairs contradicting previous reports based on low-resolution imaging[3] while diffusion properties of membrane proteins have also been mapped to high resolution.[4] Yet, fluorescent labeling may perturb the function of a molecule, and limits the capacity of discovery as it is only applicable to known species. While the bulk of attention has been focused on exploiting fluorescent probes for surface-selective molecular sensing such as in total internal reflection fluorescence microscopy, surface plasmons provide an intriguing
complementary route for such interrogation of biological samples in unperturbed natural conditions.

Surface-enhanced Raman spectroscopy (SERS), in particular, has emerged as an attractive label-free alternative owing to its molecular sensitivity and selectivity\(^8\) as well as an innate immunity to photobleaching.\(^6\) SERS leverages the excitation of localized modes of surface plasmon polaritons at the plasmonic nanoparticle surface. Any microscopy involving this confined light field has a spatial resolution comparable to the size of the confined field, which is the same as the size of the nanostructure.\(^7\) A notable embodiment of this phenomenon is tip-enhanced Raman spectroscopy, which has been used to study numerous nanomaterials.\(^8\) Additionally, plasmonic imaging is extremely advantageous in terms of optical throughput. While spontaneous Raman spectra offer rich label-free information and unequivocal detection without quenching concerns, the probability of the Raman process is much smaller than that of fluorescence owing to the second-order optical process involved. Field enhancement is, thus, of key significance in Raman scattering and has transformed SERS into a potent analytical tool by virtue of its near single-molecule sensitivity. Importantly, SERS allows selective probing of the composition of the surface of a cell since the enhancement is only perceived by the molecular constituents in the close vicinity (\(\sim 10\) nm) of the nanostructured plasmonic substrate. SERS could, therefore, potentially enable a spectroscopic identification of tumor cells based on cell-surface-specific molecular features.

Work over the last decade has demonstrated that SERS assays can exceed the detection limits of conventional immunosassays and fluorescent approaches, providing a potential opportunity to spectroscopically quantitate multiple biomarker concentrations.\(^9\) The varied possibilities of such a versatile, ultrasensitive approach have spurred the innovation and development of a wide array of new plasmonic substrates for biosensing.\(^10\) Specific to cell surface sensing, label-free approaches as well as SERS nanotags have been proposed for profiling the membrane composition.\(^11\) While these are important developments, the nondeterministic nature of the aggregate formation (and the inherent difficulty of reproducing the same pattern) impedes localization of the SERS signals and, hence, of spatial profiling of the membrane. Furthermore, the colloidal entities are prone to cellular internalization thus making selective surface probing challenging. Hence, the development of an SERS platform that offers reproducible, sensitive, localized detection of cell surface markers, while also eliminating the need for complex sample preparation, remains highly desirable.

Here, we report a new platform, entitled nano-pillar-based Raman optical detection (nano-PROD), for selectively interrogating the molecular composition of cell membranes through fabrication of a plasmonic substrate tailored for supporting live cells. In particular, we employ vertical nanopillars to localize the surface plasmons to nanoscale regions by restricting the propagation of light along the substrate. Our work also builds on efforts to isolate and harness the specific microenvironmental cues of 3D culture that have resulted in a variety of micropatterned substrates.\(^12\) The nano-PROD presents such a patterned substrate where the nanoscale topographic features can be tailored to influence the positioning of the plasma membrane, surface receptors, and structures associated with them. The metal plasmonic nanoparticle-coated vertical nanopillar array is fabricated for growing and culturing cells, and to perform SERS analysis at the single-cell level. Our design is premised on the nanofocusing ability of the periodic nanopillar arrangement, its ability to sustain the cells in near-physiological conditions as well as the consequent elimination of the cellular internalization of the plasmonic nanoparticles. The nano-PROD platform is fabricated using a recently developed ink-based solution processing technique,\(^13\) which provides uniform and reproducible SERS enhancement. Structured arrays of nanopillars with well-defined diameter, length, and pitch parameters have received considerable attention owing to their tunable reflectance, transmittance, and absorption properties.\(^14\) While an optimal combination of these parameters can lead to desirable optical attributes, achieving precise control over fabrication of such nanostructures, particularly over large scales, remains challenging. Most fabrication approaches, such as e-beam lithography, are difficult to implement over such length scales due to the complexity and cost of the involved processes. This method permits facile, inexpensive production of substrates with unique analytical resolution that simultaneously provides analytical space proportional to the dimension of a single cell and also sufficient nanoparticle coverage to enable longitudinal monitoring of numerous cells on a single platform. The sensing area provides a stable platform for the cells to attach while preserving their vitality and functions. Wrapping of the cell membrane around the pillars ensures tight attachment to the substrate and prevents any drift under the experimental conditions.\(^15\)

We demonstrate the capability of the nano-PROD platform for label-free sensitive molecular detection of cell surface markers by employing prostate cancer cells that overexpress prostate specific membrane antigen (PSMA), a type II membrane glycoprotein (Figure 1). Because of its differential expression on the surface of prostatic neoplasias, particularly in castration-resistant, advanced, and metastatic disease,\(^16\) detection of PSMA presents an important challenge in itself as well as a useful model for other clinically relevant cell surface markers. Our results show the ability of nano-PROD to differentiate between phenotypically similar prostate cancer cells that differ only in PSMA expression while highlighting its nonscopic detection volume that enables spectroscopic probing of a single proteomic domain. PC3 cells were transduced to overexpress PSMA,\(^17\) and the nano-PROD platform could differentiate these cells from the phenotypically similar PC3 Flu cells which lacked the membrane-localized PSMA protein. Furthermore, only a few Raman peaks (specifically the amide modes) can be used for rapid analysis of the cell membrane proteomics. Efficient application of chemometric algorithms to the pool of spectra obtained from either cell lines led to higher reproducibility and differentiation ability with a specificity of 100%. Our findings underscore the promise of nano-PROD for discerning the molecular pathology of cancers as well as uncovering chemo-mechanical coupling at the nanoscale.

2. Results and Discussion

2.1. Fabrication and Characterization of Nano-PROD

Here, we leveraged a solution-based nanomanufacturing technique, which minimizes production time, to fabricate large-area
silicon nanopillar array coated with nanoscale layer of plasmonic silver (Ag) (Figure 2A). We built on the precursor solution-derived fabrication strategy that has been used for large-area roll-to-roll solar cell fabrication,[18] and tailored it to match the attributes required to exploit surface plasmons while sustaining cell viability. Our nano-PROD fabrication method permits precise control over diameter (700 nm), pitch (2000 nm, center to center), and height while also affording significant coverage over wide areas (2 mm × 2 mm, >250 000 nanopillars)—thereby eliminating the need for complex epitaxial and/or lithographic processes.

To overcome the drawback of conventional e-beam evaporation of silver in a vacuum chamber leading to shadowing effects with 3D nanostructures and subsequent formation of coating only on selected portions, we have employed a different strategy to ensure more homogenous coating of the pillars with an organic Ag precursor ink, which was mixed with isopropanol to cause dewetting of the film. Once heated to a particular temperature for a relatively short time duration (∼15 min), this precursor transforms into a sheet of closely intertwined Ag nanoparticles (∼10 nm in size), thus imparting plasmonic attributes (see Figure S1A, Supporting Information). Figure 2B shows the energy dispersive X-ray analysis (EDAX) spectra of the silver nanoparticle-coated silicon nanopillars showing the presence of elemental Ag, which can be attributed to the Ag nanoparticles

Figure 1. A) Cartoon of cells adherent on the nano-PROD platform constituted by silver nanoparticle-coated silicon nanopillars. B) Schematic of acquiring SERS signal from the cell on the nano-PROD. C) Basic construct of the cell membrane consisting of a lipid bilayer and other organelles. Here, the prostate cancer biomarker, prostate specific membrane antigen (PSMA) is shown localized on the surface of the plasma membrane.

Figure 2. A) 1–6 represents the steps of self-assembly of polystyrene spheres on the wafer, reactive ion etching (RIE), silver deposition, wet etching using HF and H₂O₂, silver nanoink deposition, and annealing to form final plasmonic nanoparticle-coated nanopillar array. B) EDAX spectrum of the silver nanoparticle-coated silicon nanopillars showing the presence of elemental Ag. C) Schematic of the cell membrane and nuclear deformation facilitated by the nano-PROD platform. This geometry ensures substantial contact of the plasma membrane with the surface of the plasmonic nanopillars without the penetration of the cell. D) False color FESEM image of cell positioned on the nano-PROD. The white arrows indicate the points of attachment of the cell to the nanopillar substrate. E) Confocal fluorescence image of cells grown on silicon nanopillars. Fluorescence images constructed from tdTomato signals of the transfected MDA-MB-435 cell reveal the nature of cellular deformation. Three Z-sections are shown (red dashed line), beginning from the basal surface of the cell.
coating. It is worth noting that the amount of ink dilution with isopropanol gives rise to different coating morphologies from continuous sheet to nanohole arrangements thus affording a high degree of plasmonic tunability. An important feature of such Ag-ink-coated substrates for real sample measurements is that they are reusable, as shown in one of our previous publications, following sequential washes with conc. HCl and liquor ammonia.

To quantitatively characterize the substrate reproducibility, we calculated the relative standard deviation of the prominent peak at 1574 cm\(^{-1}\) with 50 SERS spectra (10\(^{-5}\) m 4-nitrothiophenol) from different locations on nano-PROD substrates (Figure S2, Supporting Information). From the recorded spectral profiles of 4-nitrothiophenol, we compute the relative standard deviation of the SERS intensity to be less than 13\%, which underscores the excellent reproducibility of the nano-PROD substrate.

### 2.2. Imaging Cell Morphology on Nano-PROD

For greater SERS signal strength from cell membrane, it is imperative that the cell wraps tightly around the nanopillars, thus exposing to the biomarkers to the plasmonic near field (Figure 2C). This mode of cellular probing is in contrast to the use of free-standing nanoparticles for SERS studies wherein the nanoparticles are generally internalized by the cells by endocytosis, and it becomes difficult to clearly demarcate the modes arising from the plasma membrane from the cytosolic features.\(^{(20)}\) Figure 2D shows that the cell is anchored on the nanopillar arrays—as evidenced by its unchanged position even after several washes—during the field-emission scanning electron microscope (FESEM) sample preparation.

Our choice of the size and spacing of the nanopillars hinged on the following functional requirements: the pillars should not penetrate the cell membrane; there should be tight junctions with the pillars; and the cell should externally engulf the pillars. Prior studies on cell adhesion and proliferation on nanopillar substrates informed our design.\(^{(21)}\) The diameter, height, and the spacings of the nanopillars play a major role in determining the interaction between the cell and the nanopillars. The cell membrane is around 5–10 nm thick, and nanopillars with smaller dimensions can penetrate the cells to enter the cytoplasm. Such platforms have been employed for intracellular delivery of molecules and also capture of target molecules.\(^{(22)}\) On the other hand, pillars larger than 500 nm and with spacing of more than a micron is suitable for engulfment by cells, whereas smaller spacing results in suspension of the cells on the surface of the nanopillar arrays. A diameter greater than 500 nm and with spacing of more than a micron were selected to address the aforementioned functional requirements.

For a detailed observation of the cellular engulfment around the nanopillars, we used confocal fluorescence microscopy to record stacks of 2D images (Z-sections) that reveal the cell–array interaction. In particular, tdTomato-transfected MDA-MB-435 cancer cells that were cultured on the nanopillar array were imaged to elucidate the topology of the cell on the nano-PROD substrate (Figure 2E). In the recorded confocal scan, the cell does not appear in the first Z section (Figure 2E, first panel). When subsequent sections further along the imaging axis are acquired, the precise position of the interface between the pillars and the cell is revealed (via the appearance of the black dots), which in turn informs the shape of the deformed cell (Figure 2E, second panel). As the imaging section moves further, the black dots representative of the cell-nanopillar contact disappear (Figure 2E, third panel).

#### 2.3. Finite Difference Time Domain (FDTD) Simulations

The nanopillar architecture provides a unique prospect for the observation of light focusing phenomenon that gives rise to additional plasmonic enhancement. To understand the plasmonic response of the nano-PROD, we have performed 3D FDTD simulations. The near-field intensity (|\( \mathbf{E} \)|\(^2\)) can be obtained from these calculations and can be directly correlated with the SERS enhancement G (\( \propto |\mathbf{E}|^2 \)).\(^{(23)}\) We reasoned that the presence of cell on the nanopillar arrays will alter the light scattering behavior, and will give rise to a differential distribution of the plasmonic near-field. Therefore, these parameters (namely, the presence and absence of cell and the Ag layer) were modeled individually to study their effects on the near field intensities.

Figure 3 shows the various models with simulation parameters obtained directly from our FESEM measurements. The enhancement in the Raman signals comes from the molecules which are in close association with the plasmonic nanoparticle surface, and is the combined effect of the local increase in light intensity of the illuminated light and also the enhancement in the strength of the Raman dipole-emission transition at a slightly longer wavelength. Hence, the signal-enhancing electromagnetic field distribution around the plasmonic nanostructure is critical, and it has been found to diminish as a factor of distance (\( \propto 1/r^3 \)) from the surface.\(^{(24)}\) In case of analyzing a cellular system, this decay in the field intensity would enable the detection of the biochemical ingredients of the plasma membrane selectively as it spans several nanometers, encompassing the field of influence (the “hotspots”). The hotspot distribution is visible in the magnified regions around a single nanopillar (Figure 3).

It is worth noting that the silver coating of nanoscale roughness over the Si nanopillars can trap light efficiently with the refractive index contrast at the air/metal/Si interface increased as compared with that at the air/Si interface. This also leads to a stronger Fabry–Perot resonance leading to larger field intensities.\(^{(25)}\) Such large field confinement cannot be seen in case of nanostructures on flat Si substrates, as the geometric resonance and the antireflection properties of the underlying nanostructures drive the electric field confinement at the metal–semiconductor interface.\(^{(25)}\) Our results show that the occurrence of higher field confinement is more prominent when the antireflectivity of the underlying substrate is increased, which is introduced by the presence of a live cell, whose specific arrangement of refractive indices with respect to the pillars, the silver, and the surrounding medium produces a nanofocusing effect, i.e., multiple internal reflections within the nanopillar cavity itself. Specifically comparing the scenarios with (Figure 3C) and without the cell (Figure 3B), we note that the cell-Ag-Si pillar arrangement leads to significant amplification of the E-field enhancement (approximately six times) as evident from the near-field intensities obtained from the FDTD simulations.
calculations. These simulation results can be linked to the characteristics of the nanopillar substrate for understanding and tailoring the plasmonic behavior of the nano-PROD platform.

2.4. Experimental Determination of Cell Viability on Vertical Nanopillar Array

Since the proposed platform is composed of inorganic materials, e.g., noble metals and silicon, its biocompatibility needs to be ascertained for cellular imaging applications. To address this concern, we performed in vitro toxicity studies with the silver-coated nanopillar platform. In vitro studies were done using ISO protocols and 3T3 fibroblasts stably expressing green fluorescent protein (GFP). For the direct contact test, the cells were grown on plane silicon wafers and on our platform. After 24 and 48 h of incubation, the media was removed from the wells and the 3T3 cells were rinsed with phosphate buffered saline (PBS) and then stained with Ethidium Homodimer-1 (EthD-1) to identify dead cells in the population. We observed no difference in cell viability between the positive controls and our platform, while the methanol fixation control caused increased cell death. The findings, shown in Figure 4, confirmed the biocompatibility of our platform.

2.5. SERS Study of the Cells Grown on Nano-PROD Platform

The SERS performance of the nano-PROD was first evaluated in the absence of the prostate cancer cells (Figure S1B, Supporting Information). Next, SERS spectra of the prostate cancer cell lines were recorded; the mean spectral profile corresponding to the PSMA-deficient PC3-flu and PSMA overexpressing PC3-PIP prostate cancer cells are shown in Figure 5A. The spectral profiles, which are exclusively indicative of the cell membrane constituents owing to the surface-selective nature of the plasmonic enhancement, exhibit common spectral features as well as interesting differences. Tentative assignments of the observed peaks are summarized in Table S1 in the Supporting Information. Prominent SERS bands common to both flu and PIP cells were observed at 670, 880, 1124, 1264, 1356, 1398, 1509, 1552, and 1607 cm⁻¹. The features seen here can be attributed to

![Figure 3](image-url)  
**Figure 3.** The focusing effect of the cells on the incoming laser beam causes further enhancement of electric field distribution. Panels (A)–(C) show different electric field intensity distributions of instances where A) the Ag layer and the cell are absent, B) only the cell is absent, and C) the cell is present. The normalized field intensities are obtained through FDTD calculations.

![Figure 4](image-url)  
**Figure 4.** Direct contact cytotoxicity test results obtained with the nano-PROD platform. A1,A2) GFP-expressing 3T3 fibroblast cells were grown on the nano-PROD platform, stained with EthD-1 and imaged after 24 h. B1,B2) Imaged after 48 h. C1,C2) 3T3 fibroblast cells were grown on plain Si, stained with EthD-1 and imaged after 24 h. D1,D2) Negative controls in which the cultured 3T3 cells on plain Si were fixed in methanol (note the significant increase in the number of dead cells in D2). Scale bar: 100 µm.
the different vibrational modes of proteins, lipids, and modes from other molecular components of the cell membrane. The SERS modes arise from the plasma membrane, which has a fluidic nature and consists of a mosaic of globular proteins within the phospholipid bilayer. The protein-lipid interactions form the basis of several important cellular functions and house several important biomarkers that are involved in recognition, signaling, and other downstream processes. The phospholipids are also zwitterionic and include components such as phosphatidylcholine. The Raman modes of these groups can be seen in SERS spectra of the PC3 cells. The C-N vibrational mode of the membrane phospholipid head can be seen at 720 cm$^{-1}$. This region also overlaps with the symmetric stretch vibration of choline group, N$^+$\(\text{CH}_3\)_3 and the C-C-N symmetric stretching in phosphatidylcholine. The phospholipid is arranged as an interrupted bilayer with a small portion of lipid having intimate association with integral proteins. This heterogeneity in composition as well as thickness of the membrane phospholipid head can be seen at 720 cm$^{-1}$.

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The proteins associated with the plasma membrane can be peripheral or integral, with the peripheral ones exposed to the solvent on the outer side. The integral proteins are also globular proteins with ionic residues, and the extent to which they are embedded is dependent on the size and nature of the molecule. Due to this distribution of globular membrane proteins, which can reach up to 40% of the total membrane protein content, we observe that the SERS features representative of aromatic amino acids like Phe, Tyr, Trp, and His as well as those of amide modes, which are indicative of the secondary structural elements of the proteins. Notably, PC3-PIP cells preferentially expresses PSMA, a membrane protein and a key prostate cancer biomarker. This protein, also known as glutamate carboxypeptidase I or folate hydrolase, is a 750 residue glycoprotein and has a transmembrane existence. The PSMA protein is constituted predominantly by $\alpha$-helical domains, which are evident from the amide I feature of the SERS spectra that correspond to $\alpha$-helix (Figure 5A). The deconvoluted amide I peak shows the existence of a major resolvable peak at 1658 cm$^{-1}$ which indicates the one-to-one correspondence with the structure of the PSMA protein (Figure 5B). PSMA is a considerably bulky protein with dimensions more than 10 nm in the long axis as evident from the crystal structure (Protein Data Base (PDB) ID: 1Z8L). Thus, we reason that a part of the protein will be exposed to the surface free to interact with the surface of the plasmonic nanopillars. Interestingly, the amide I intensities are greater in case of PIP cells, which express PSMA. Even in the absence of PSMA (in case of PC3-flu cells), we observe a weak amide I band (Figure 5B-ii), which can also be resolved to obtain various secondary structure components corresponding to the other structural and functional proteins intrinsic to the plasma membrane. The differential median and spread of the amide I intensity indicate the intrinsic variations in composition and heterogeneity in the fluidic cell membrane for the two cell types (Figure 5C). Specifically, the intensity values are significantly lower for the PC3-flu cells than for the PC3 PIP cells providing direct evidence that PSMA (whose crystal structure is shown in Figure 5D with clear demarcation of alpha-helical domains) contributes to the total protein content of the plasma membrane significantly. To further verify the differences observed through the peak analysis, we used partial least squares discriminant analysis (PLS-DA) to classify the spectra belonging to the two types of phenotypically similar cells. The developed PLS-DA models offer a classification sensitivity and specificity of 96.7% and 100% on leave-many-out cross-validation analysis. This singular ability to quantify the differential concentrations of proteins directly in the cell membrane opens up the possibility of applications in diverse fields from cancer diagnoses to forensics.
3. Conclusions

A broad range of cellular functions from adhesion to pathogen recognition depends specifically on events in the cell membrane. While important, such activity is often challenging to access with existing techniques due to limitations in resolution, sensitivity, or labeling requirements. Here, we report a vertical nanopillar-based platform, termed nano-PROD, which provides a portal for extended monitoring of the cell surface milieu by enhancing intrinsic molecular signatures. The culturing of epithelial cells on the patterned surface yields an apical-basal recognition that accurately reflects their physiological condition in vivo. SERS provides a powerful probe for profiling the surface of the live cells without necessitating use of exogenous contrast agents while the vertical nanopillars serve to localize the surface plasmons to nanoscale regions. Our experimental results highlight the utility of the platform in probing specific cell surface markers, and demonstrate a proof of concept through the accurate identification of phenotypically identical prostate cancer cells, differing only in PSMA expression. Immediate possibilities for the nano-PROD abound; these include uncovering new markers for recognition of cell types, and performing stain-free pathology assessment in smears. A striking near-term application is in differential diagnosis of clinical effusion samples by offering quantitative and orthogonal analysis of cells in such samples. Further development of the nano-PROD for identification of biophysical and molecular markers for cellular phenotype recognition can provide a fresh route to reproducible diagnoses of malignant transformations without requiring human interpretation. Due to the programmability of the platform especially its nanoscale topological features, we anticipate that the nano-PROD will also offer a new lens for exploring mechanochemical phenomena such as the role of local mechanical environment in spatial organization of surface receptors and its functional consequence on the downstream responses.

4. Experimental Section

Fabrication of Nano-PROD: Silicon wafers of (100) orientation, N-type, resistivity of \(\approx 0.01-0.02 \, \Omega \) were used in the experiments. The wafer was ultrasonicated in acetone and ethanol at room temperature for 10 and 5 min, respectively. The degreased silicon substrate was heated in 80 °C Piranha solution \((4:1, \text{v/v } \text{H}_2\text{SO}_4/\text{H}_2\text{O}_2)\) and RCA solution \((1:15, \text{v/v } \text{NH}_4\text{F}/\text{H}_2\text{O}_2/\text{H}_2\text{O})\) for 30 min each. Subsequently, the silicon substrate was rinsed several times with deionized water. Monolayers of polystyrene spheres (PS) of 2000 nm diameter were coated on the silicon wafer by drop coating with an optimized solution of methanol and Triton X. The spheres (PS) of 2000 nm diameter were coated on the silicon wafer by drop coating with an optimized solution of methanol and Triton X. The PS spheres were etched by reactive ion etching (RIE) at 30 mtorr and 50 W power in oxygen atmosphere. A silver film was deposited using e-beam deposition in a JEE-4X/5B deposition system at a pressure of around 6 \(\times 10^{-6} \, \text{torr} \). Etching at room temperature was achieved by using HF and H$_2$O$_2$. The substrate was subsequently cleaned of the PS spheres by chloroform. The plasmonic silver nanoparticles were deposited on the silicon nanopillars by silver conductive ink (SC-100, 20 wt%, Kunshan Hisense Electronics Co., Ltd., China) spin coating and heating at 130 °C. The samples were characterized by using SEM.

Cell Culture and Sample Preparation: Phenotypically similar prostate cancer cells and human breast cancer cell lines (PC3-PIP & PC3 flu, and MDA-MB-435), and 3T3 cells for cell viability experiments were obtained from ATCC with the authentication from Johns Hopkins Genetic Resource Core Facility. Generation and characterization of MDA-MB-435 engineered to constitutively express a bright red fluorescent protein: tdTomato, to facilitate in vivo and ex vivo tracking, had been previously described.[32] All cell lines were cultured in RPMI-1640 medium, 10% fetal bovine serum and 1% penicillin-streptomycin were added into the medium. The culture condition was 37 °C and 5% CO$_2$ in standard humidified incubators. The prepared substrate was sterilized by immersion in 75% ethanol for 2 h and exposed to a high-dose of UV light for another 2 h to ensure adequate sterilization. Then the sterilized substrate was coated with poly-D-lysine (0.1 mg mL$^{-1}$, Sigma-Aldrich, P6407) solution for 60 min and washed with PBS. After at least 2 h drying, cells PBS suspension and the coated substrate were dispensed in 24-well plates for 30 min before SERS spectral acquisition.

To test the possibility of reusing the nano-PROD platform, the used substrates were immersed in an aqua regia solution for 20 min. This ensures removal of the silver layer along with residual organic contents from the previous cell culture, while keeping the silicon pillars intact. Upon deposition of the silver layer of the same thickness, similar substrate performance was observed.

Raman Microspectroscopy: A custom-built Raman microscope was used to record the spectral profiles from the prostate cancer cell lines. A 514 nm Spectra-Physics laser was used as the excitation source. The laser was focused onto the specimen using a 0.5 NA objective lens (50X, Olympus) that also collected the backscattered signal. The laser spot was about 1250 nm in diameter. The collected signal was then recorded using a T64000 Jobin-Ivon Horiba triple monochromator spectrometer with liquid nitrogen cooled CCD detector. The spectral resolution obtained was 2 cm$^{-1}$. Discrete point sampling was performed and the acquisition time for each spectrum was 10 s and the laser power at the sample was 3 mW. Wavelength calibration was performed prior to spectral acquisition by acquiring spectra from 4-acetamidophenol, a known Raman reporter with well-characterized peak positions. The 550–1800 cm$^{-1}$ fingerprint region was used for the ensuing analysis.

FDTD Calculations: FDTD simulations (Lumerical Solutions Ltd.) were carried out in order to estimate the electromagnetic field strength around the nanoparticle clusters on the tip of the nanopillar. The silver layer was modeled as a film of 100 nm of thickness and a corrugation of 30 nm. Perfectly matched layers were used along the x-axis with a plane wave polarized light of wavelength 532 nm as the source along the y-axis. To get the best resolution while limiting the simulation time, a mesh override region of 3 nm was selected while the simulation time was 100 fs.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.
Data Availability Statement

Research data are not shared.

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