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Imaging of Protein Secretion from a Single Cell Using Plasmonic Substrates

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Abstract

Detecting, imaging, and monitoring cell function on a single cell basis is very important in the field of immunology research where many molecules are secreted from cells in response to external stimuli including immunization. Here we introduce substrates with plasmonic nanoparticles and fluorescence microscopy as promising imaging methods for studies on molecular processes controlling cell behavior, particularly secretion of cytokines. We developed unique composition of silver and silica layers of plasmonic nanostructures which resulted in fluorescence enhancement of more than 200-fold for ensemble of molecules in the immunoassay. For the proof of concept demonstration, we used primary mouse macrophages and imaged tumor necrosis alpha (TNF- α) secretion after stimulation of the cells with lipopolysaccharide (LPS). We demonstrate that metal-enhanced fluorescence assay provides imaging capability detection of cytokine secretion from a single cell without extensive biochemical procedures as required with standard methods. In addition it is demonstrated that cell viability can be controlled during secretion.

Keywords

Plasmonic nanostructures; Silver nanoparticles; Metal-enhanced Fluorescence; Microphage cells; Cell assays; Tumor necrosis factor alpha

1 Introduction

Development of nanoprobes for plasmon and fluorescence-based sensing and imaging is a very active research field. The unique optical properties of noble metals such as Au, Pt, and Ag, and others like Al and Cu display negative real and small positive imaginary dielectric constants which results in surface plasmon resonance over the UV-NIR wavelengths. These optical properties have been widely exploited in construction of surface plasmon resonance (SPR)-based sensors using thin layers of Au or Ag and fabrication of metallic nanoparticles and nanostructures that highly enhance Raman scattering and fluorescence. There are several recent review articles that describe the optical properties of single metal nanoparticles and single nanoparticle clusters [1, 2], application to surface enhanced Raman spectroscopy (SERS) [3], and metal-enhanced fluorescence (MEF) [4–8]. While fluorescent probes are dominant for cellular imaging, there are only a few reports on application of plasmonic materials for enhanced fluorescence cellular imaging, excluding quantum dots.

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Gold nanoparticles conjugated to fluorescently-labeled proteins were used to study their cellular uptake with flow cytometry and confocal microscopy [9]. Plasmonic-fluorescent nanoparticles consisting of an Ag/Au core, polymeric dye-doped shell [10], and a reverse configuration with silica-dye encapsulated silver shell [11] were applied for cellular imaging. In another configuration, dye-protein conjugates were attached to Ag nanoparticles [12] and successfully used for imaging of cell surfaces. It was shown that typically, plasmon-fluorophore probes display substantially stronger emission, shorter lifetime, and improved photostability compared to the fluorophore itself [4–8, 6–12]. There are also approaches that explore the enhancement of electromagnetic field without metallic nanostructures for fluorescence enhancement such as one-dimensional photonic crystals [13–15].

The use of planar plasmonic substrates for enhanced cellular fluorescence imaging has not been sufficiently explored. The main reason is that the distance between the metal coated surface and the intracellularly located fluorophore, is in most cases, larger than the distance where efficient enhanced electromagnetic field occurs. Therefore, the intracellular fluorescent probes are practically unaffected by the enhanced substrate surface fields. An attempt to use silver coated substrates with a thin dielectric layer resulted in 4-fold enhancement of cellular imaging [16] which was due to the constructive interferences and improved collection efficiency. One opportunity for a partial signal improvement is that the signal from cell surface bound fluorescent probes because of possibility of placement of probe in the proximity to the plasmonic surface. Another unexplored opportunity for cellular imaging using planar substrates is in the development of single cell assays. Recently it was shown that a combination of grating-coupled surface plasmon resonance (GCSPR) and surface plasmon-coupled emission (SPCE) enables the detection and characterization of the function of antigen-specific T cell subsets [17]. The microspot regions with immobilized antibodies as the cell capture ligands mixed with an anti-cytokine antibody permitted the detection of secreted cytokines and made possible to differentiation between T help 1 (TH1) and TH2 inducing conditions.

Visualization of protein secretion from a single cell could be exciting new tool for cell biology studies. One such field where this technique could significantly facilitate on-going research is cytokine biology. Cytokines are small, secreted regulatory proteins that exhibit a wide range of biological activities. Secreted cytokines induce a magnitude of biological effects including stimulation or inhibition of cell proliferation, cytotoxicity/apoptosis, antimicrobial activity, differentiation, and regulation of expression of intracellular, secreted, and surface proteins [18]. Most cytokines are circulated at very low levels under normal conditions (less than 10 pg/mL or ~ 0.5–5 pM) [19]. The detection of cytokines is hampered by their biological properties, e.g., local secretion, rapid uptake and utilization, and short half-life. These properties have led to the development of several techniques commonly used to measure cytokines, such as the enzyme-linked immunosorbent assays (ELISA) [19–22], intracellular cytokine cytometry (ICC) [22–24], enzyme-linked immunospot (ELISPOT) [25–27], and fluorescence-based FLUOROSPOT [28–29]. Most of the cytokine assays developed over the past decade are based on specific anti-cytokine antibodies using a variety of reporter systems including enzymes and fluorescent tags. ELISA is mostly used to measure cytokines in liquid samples including cell supernatants. The disadvantage of ELISA for cellular applications is that although it measures the total amount of analyte very sensitively, does not provide information on the amount produced by a single cell and therefore does not represent correctly the cellular secretion in a heterogeneous cell population. The presence of cytokine binding proteins in serum might reduce the cytokine levels in biological fluids, making results obtained by ELISA less representative [20–21]. Intracellular cytokine cytometry (ICC) or flow cytometry is currently mostly used for measurements of cell surface molecules and intracellular levels of cytokines. Monoclonal

antibodies labeled with fluorophores bind to the cell surface or intracellular antigens and the populations of cells based on expression of the markers are determined. There are several advantages of flow cytometry: single cell measurement, multiple biomarker detection, and cell populations can be sorted for subsequent analysis. There are also several limitations: expensive equipment and trained personnel are required, inability to measure secreted proteins from live cells, and difficulties in performing analysis with samples of limited cell number. Synthesized cytokines can also be identified by combining cell surface and cytoplasmic staining in flow cytometry. However, for many cytokines this requires *in vitro* stimulation of the isolated cells [22], usually involving the addition of inhibitors of intracellular transport (such as brefeldin A or monensin), which can limit the viability of the cells [23]. Furthermore, flow cytometry detects synthesized but not necessarily secreted cytokines. Therefore, detection of cytokines before their secretion might not be a reliable reflection of the released effector cytokine [24].

ELISPOT assays allow the detection of well-defined spots that clearly represent numbers of cytokine-secreting cells present in the sample. Their sensitivities permit the *ex vivo* identification of cells actively secreting cytokines and can detect a single active cell out of a million, provided that the releases close to 100 cytokine molecules per second [30]. There are several limitations of ELISPOT assays such as limited quantitative information on the amount of secreted markers (only threshold of detection), difficult multiplexing (even double cytokines is difficult), and a multi-step protocol makes ELISPOT difficult to use [26–27]. In principle, the FLUOROSPOT assay follows the ELISPOT procedure in that the capture antibody is immobilized on the membrane, cells are added and incubated, secreted cytokines are captured and detected using biotinylated secondary antibodies followed by streptavidin-conjugated dye [28]. It was also demonstrated that dual color FLUOROSPOT allowed significantly easier discrimination between single and double cytokine secreting cells than ELISPOT [28–29]. The main drawback of FLUOROSPOT is insufficient fluorescence signal using typical organic dyes and the requirement for biochemical amplification [28].

Here we describe a modified FLUOROSPOT approach in which the fluorescence signal is optically amplified by a planar plasmonic substrate. Our detection method exploits the interaction of excitation light and excited fluorophores with metallic nanostructures that cause the significant amplification of fluorescence and simultaneous decrease in fluorescence lifetime. We have previously reported a simple method of nanofabrication of plasmonic substrates that provide more than 200-fold fluorescence enhancement in a typical surface immunoassay configuration [31–32]. Because fluorescence enhancement caused by plasmonic structures is frequently called as metal-enhanced fluorescence (MEF), we term our single cell assay method the MEFspot. This single step method directly detects the local concentration of analyte with sensitivity and resolution sufficient for cell imaging and significantly simplified protocol compared to FLUOROSPOT or ELISPOT and enables real-time monitoring of protein secretion from a single cell.

2 Methods

2.1 Substrate Fabrication

Glass slides (from VWR) were cleaned with “piranha solution” (35% $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$, 1:3) overnight, rinsed with distilled deionized water and dried with nitrogen before performing vacuum deposition steps. Metallic and dielectric layers were deposited using magnetron sputtering (Hummer 6.6 RF from Anatech USA). First, a 5 nm TiO_2 layer was deposited on glass for adhesion of a 100 nm-thick Ag layer followed by a 40 nm-thick silicon dioxide layer and a final thin (about 13 nm-thick) layer of Ag. After deposition of the metallic/dielectric layers, the substrate was thermally annealed in air at 180° C for 30 min in order to

convert outer Ag film into nanoparticles. Detailed experimental evaluations and supportive numerical calculations regarding multi layered substrates were recently reported [31–32].

2.2 Substrate Surface Activation

On the surface of the MEF substrate, a microwell array was attached with dimensions of 5 mm diameter and 1.5 mm depth that accommodated about 30 μ L solutions in each well. Array of wells allowed for preparing bioactive surfaces and subsequent plating, activation, incubation, and imaging of cells. The sensing MEF substrate surface was prepared by immobilization of capture monoclonal antibody (tumor necrosis factor alpha (TNF- α): MTNFAI and interferon gamma (IFN- γ): M700a from Thermo Scientific) by direct physical adsorption. The phosphate buffer solution (PBS, pH 7.4) of 50 μ g/mL of antibody was incubated for 2 h, followed with a blocking solution of 1% bovine serum albumin (BSA) in phosphate buffer saline (PBS) at room temperature for 1 h. For cellular application, substrates were sterilized by flushing with culture medium and exposing wells to UV light for 1 h.

2.3 Cells and culture conditions

Primary microphages were obtained by peritoneal lavage from mouse strain C57BL/6J 4 days after intraperitoneally injection of 3 ml thioglycollate broth (Remel). Cells were maintained in RPMI 1640 medium (RPMI: Roswell Park Memorial Institute) supplemented with 2% fetal bovine serum (FBS) at 37 °C in 5% CO₂ atmosphere. Cells (30,000 per well) were placed on the MEF substrates and allowed to attach to the surface overnight. Separate wells were used for detection of TNF- α and IFN- γ . To induce cytokine production, cells were stimulated by lipopolysaccharide (LPS, 100 ng/ml) and incubated for up to 16 h. Before fluorescence imaging, half of the culture medium was supplemented with AF-647-labeled detection antibody at a final concentration of 1 μ g/ml. To detect cell viability, Calcein AM was added to a final concentration of 25 ng/ml.

2.4 MEF assay procedure

Initial testing of the assay sensitivity was performed using recombinant cytokines. In this case two spots were activated for detection of TNF- α and IFN- γ within individual wells to accommodate simultaneous detection of both cytokines. Assay performance was tested using a mixture of cytokines in a concentration range from 5 pg/ml to 1000 ng/ml, followed by detection with a cocktail of secondary-matched detection monoclonal antibodies. Detection antibodies (TNF- α : M3TNFABI and IFN- γ : M701 from Thermo Scientific) were dye-labeled (APEX Alexa Fluor 647 Labeling Kit A10475 from Invitrogen) and used at a concentration of 1 μ g/ml each. Following incubation for 1 h at room temperature, the array of wells was imaged.

Imaging of cytokine secretion by stimulated microphage cells were performed similarly as for the assay with recombinant cytokines. To maintain the cell culture conditions, samples were kept in the CO₂ incubator during the incubation periods and between biochemical and imaging steps. Experiment included wells with no stimulated cells as the controls. Imaging was performed at the defined time of stimulation.

2.5 Spectroscopic Measurements

Fluorescence imaging was performed using a fluorescence lifetime imaging microscope (FLIM) system (Alba 5 from ISS). Olympus microscope (model IX71S), objective 10 $\times$ 0.3 NA (UPlan Olympus) and diode laser 473 and 640 nm (LASOS) were used for cell imaging. Lower magnification objective (4 $\times$ NA, Olympus UPlan) was used for imaging of double spots using recombinant cytokines to allow larger imaging area when using galvo-controlled

scanning mirrors. Images were acquired using following setting: image size of 256×256 pixels, scan speed 1 ms/pixel with two overlapping scans. A multiband dichroic filter (470/635 nm), long pass filter above 650 nm (for AF647) and band pass filter of 514/30 nm (for Calcein AM) were used to separate the fluorescence from laser lights. Fluorescence was detected using two channels; one with an avalanche photodiode (SPCM-AQR-14, Perkin Elmer) for AF647 and second channel with photomultiplier (H7422-40, Hamamatsu). FLIM data were acquired using A320 FastFLIM and analyzed with Vista software (both from ISS). FastFLIM was calibrated using the known lifetime of the Cy5 in water of 1.1 ns

3 Results and Discussion

3.1 MEF allows for sensitive immunoassays

Before experimentation with cells, we tested the performance of MEF substrates using recombinant cytokines. The active spots of about 500 μm in diameter were generated by spotting capture antibodies on the MEF substrate using a handheld spotter. After incubation for 1 h, the remaining surface was blocked with 5% BSA solution. This activation procedure was also applied for cellular studies. Next, spots were exposed to a mixture of recombinant TNF- α and IFN- γ at various concentrations. After 1 hr incubation, the mixture of detection antibodies labeled with Alexa Fluor 647 was added in concentrations of 1 $\mu\text{g}/\text{ml}$ each. Measurements of intensity and lifetime (phase shift) images were performed without washing. Figure 1 shows the selected images and calibration curves generated based on the average values of intensity over the duplicate spots area. Phase shifts are shown in Figure 2 at 80 MHz modulation frequency. The trend in phase shift responses are opposite to the intensity as is expected for MEF-based sensing. As more probe bound to the surface, the intensity increased while the lifetime decreased (lower phase value). Experimental data were fit to the four parameter logistic function, which are shown as lines in Figures 1 and 2 using Origin Pro 7.0 software. MEF-based TNF- α and IFN- γ assays perform similarly and clearly show that the concentrations of 10 pg/ml or lower can be determined. For example, the midpoints of intensity responses are 4.2 and 4.0 ng/ml for TNF- α and IFN- γ , respectively. The midpoints for phase responses are lower than for intensities, 0.56 and 0.86 ng/ml for TNF- α and IFN- γ , respectively. The higher sensitivity observed from phase shift measurements are related to different weighing of signals with short lifetime (bound probe) and longer lifetime (free probe) [33]. This sensitivity is comparable with ELISA method which is widely used in studies of cellular secretion of cytokines. In ELISA method, the supernatant is typically collected and the average cytokine concentration is determined, or as in ELISPOT, enumeration of spots is used. A MEFspot approach is significantly simpler because there is no requirement for washing out cells thus an average cytokine concentration in the supernatant can be determined directly by the readout of intensity and/or lifetime (in areas between cells) and comparison with calibration curves. In case of low number of active cells, the spots can be enumerated and potentially cytokine concentration per cell can be estimated.

3.2 Detection of time-dependent secretion

A unique capability of the MEFspot is real-time monitoring of secretion. This is due to the fact that the method does not require washing and removal of cells. Time-dependent spots development is shown in Figure 2. Initially, 4 hours post stimulating, a small number of cells responded to the stimulating agent, lipopolysaccharide (LPS) by secreting TNF- α , as indicated by brighter spots (intensity image) and shorter lifetime (lifetime image). With increased incubation time of cells with LPS, the number of activated cells increases (Figure 2c–d) as indicated by the increased number of spots. Also, the amount of secreted cytokine increased in the solution overall as shown with increased intensity and parallel with decreased average lifetime. Time-resolved imaging provides an excellent verification that

spots are formed due to binding of the dye-labeled detection antibody to cytokine captured on the surface. The simultaneous increase in intensity and decrease in lifetime strongly confirms that the spots are created by a sandwich complex of capture Ab1 – TNF- α – AF647-labeled Ab2 on the surface of plasmonic substrate.

One should note that the presented time-course images display different fragments of a well. Real-time monitoring of protein secretion from the same cell population requires building of a chamber that maintains the cell culture conditions as in the CO₂ incubator for an extended time. Nonetheless, the presented results provide strong indication that real-time detection of secretion is feasible.

3.3 Cell secretion and viability

MEFspot does not require washing cells; this unique feature makes possible verification of cell viability simultaneously with detection of protein secretion from a single cell. To demonstrate this we used the Calcein AM, a dye which displays bright fluorescence in live cells. Figure 4 shows that spots formed by secretion of TNF- α correspond to viable cells. This type of imaging allows identification of damaged cells or unspecified artifacts and exclusion of unrelated objects from analysis. In the standard technique such as ELISPOT, the differentiation between live and dead cells is not possible and all spots are counted out.

4 Conclusions

The MEFspot approach enables a real-time monitoring of protein secretion from a single cell. Metal-enhanced fluorescence sufficiently enhances the signal of surface-bound detection probes and provides the opportunity for imaging cellular function on the single cell basis. Increased intensity and reduced lifetime are complementary signals that together allow studying the kinetics of protein secretion simultaneously with controlling cell viability. We believe that MEFspot will be regarded as a complementary detection platform to already established methods such as ELISPOT and flow cytometry, because the new method uses the same assay reagents, requires simplified biochemical procedure and provides the real-time monitoring capability.

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Figure 1. Intensity images of spots exposed to various concentrations of TNF- α and IFN- γ : 1.6, 0.52, and 0.104 ng/ml (from top) and intensity concentration responses. Data (means $\pm$ sd) are from an average intensity of the two duplicate spots. Lines represent the fit of logistic function (OriginPro 7.0).

Figure 2. Phase shift (lifetime) images of spots exposed to various concentrations of TNF- α and IFN- γ : 1.6, 0.52, and 0.104 ng/ml (from top) and phase concentration responses. Phase shift was measured at modulation frequency of 80 MHz. Lines represent the fit of logistic function (OriginPro 7.0).

Figure 3. Time-dependent secretion of TNF- α by macrophage cells upon stimulation with LPS. Formation of the spots by intensity increase (left panel) and by lifetime decrease (right panel) measured after LPS activation: (a) 16 hrs no LPS, (b) 4, (c) 8, (d) 16 hrs. Lifetime images were processed using VistaVision fitting program (ISS) using bi-exponential intensity decay model and displayed as the average lifetime (amplitude weighted).

Figure 4. Simultaneous imaging of cell viability and secretion. (a) Fluorescence image of cells secreting TNF- α detected with AF647-labeled detection antibody (excitation 640 and emission above 650 nm). (b) Fluorescence image of cells stained with Calcein AM (excitation 473 and emission 514/30 nm). (c) Overlay image (note small shift between images due to two different lasers used).