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Subcellular Resolution Mapping of Endogenous Cytokine Secretion by Nano-Plasmonic-Resonator Sensor Array

Sheng Wang[†], Sadao Ota[†], Bin Guo[†], Jongeun Ryu[‡], Christopher Rhodes[†], Yi Xiong[†], Sheraz Kalim[§], Li Zeng[†], Yong Chen[†], Michael A. Teitell[§], and Xiang Zhang^{*,†,||}

[†]NSF Nanoscale Science and Engineering Center (NSEC), 3112 Etcheverry Hall, University of California, Berkeley, CA 94720

[‡]Department of Mechanical and Aerospace Engineering, University of California, Los Angeles, CA 90095

[§]Department of Pathology and Laboratory Medicine, University of California, Los Angeles, CA 90095

^{||}Materials Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720

Abstract

Local extracellular signaling is central for cellular interactions and organizations. We report a novel sensing technique to interrogate extracellular signaling at the subcellular level. We developed an in-situ immunoassay based on giant optical enhancement of a tunable-nano-plasmonic-resonator (TNPR) array fabricated by nano-imprint lithography (NIL). Our nano-plasmonic device significantly increases the signal-to-noise ratio to enable the first time sub-micron resolution quantitative mapping of endogenous cytokine secretion. Our study shows a markedly high local interleukin-2 (IL-2) concentration within the immediate vicinity of the cell which finally validates a decades-old hypothesis on autocrine physiological concentration and spatial range. This general sensing technique can be applied for a broad range of cellular communication studies to improve our understanding of subcellular signaling and function.

Keywords

Biosensing; plasmonic resonator; in-situ immunoassay; cytokine; T cell; nano-imprint lithography (NIL)

Extracellular signaling controls how cells communicate, differentiate, and organize to form unique biological systems with extraordinary physiological functions. Signaling molecules transfer information not only via their chemical compositions, but also through their spatiotemporal distributions. Cells could confine intended information within a local area by controlling the spatial profile of signaling molecules or through regulated spatial variations in target cell response thresholds. Therefore, to decode cellular communications at the subcellular level, a high spatial resolution signaling molecule sensing platform is required. For instance, autocrine signaling, which regulates a cell through self-secreted factors feeding back on that cell, requires spatiotemporal control over secreted signaling molecules to

*Corresponding authors, xiang@berkeley.edu .

Supporting Information: Experimental details and supplementary figures S1-S3 with corresponding figure legends. This material is available free of charge via the Internet at <http://pubs.acs.org>.

regulate local essential physiological and pathological processes¹⁻⁴. For example, an interleukin-2 (IL-2) autocrine loop is essential for T lymphocyte expansions and also related to cancerogenesis^{1, 3, 4}. While our understanding of autocrine processes is limited by traditional biosensing techniques⁵, it has been hypothesized that autocrine loops should be highly localized processes, suggesting unproven spatial constraints on cellular secretions to regulate autocrine signaling responses⁵⁻⁷. Theoretical analyses^{5,6} and indirect evidence obtained by microphysiometer and ligand-binding assays⁷ predict that the physiologically effective local concentration of cytokines in the proximity of target cells should be several orders of magnitude higher than the global concentrations measured in the serum. However, direct quantification has thus far not been possible due to the limitations of current biosensing techniques to probe spatially-confined signaling processes⁵.

Nanosensing techniques offer new opportunities for in situ high resolution extracellular sensing⁸, for example, redox signaling molecule H_2O_2 can be mapped in subcellular resolution with carbon nanotube array sensors⁹. Modern molecular and cellular biology research calls for a general nanosensing tool for many kinds of extracellular signaling molecules detection with subcellular resolution. Plasmonic nanostructures provide a novel approach to concentrating electromagnetic energy at the nanoscale, and they can significantly enhance biosensing signals, such as for Surface Enhanced Raman Scattering (SERS) measurements¹⁰. Many novel nanostructures have been explored for ultrasensitive biosensor applications¹¹⁻¹⁵ with one powerful structure being a tunable-nano-plasmonic-resonator (TNPR)^{16, 17}. TNPRs are multilayered nano-plasmonic structures that consist of feature sizes in the range of 100 nm that are assembled from alternating noble metal and dielectric films^{16, 17}. TNPRs provide unique advantages, such as extraordinary field enhancements, tunable resonance frequencies, and compatibility with a top-down mass production process which enables the fabrication of large numbers of uniform nano-plasmonic structures with controllable resonance spectra and electromagnetic field enhancements. Plasmon-enhanced fluorescence is regarded as an emerging tool in biosensing^{18, 19}, while it has not yet been fully explored in biosensing applications due to contrasting effects on signal enhancement and quenching^{20, 21}. Mechanistic studies have shown that in order to achieve maximal fluorescence enhancement there is an optimized range of distances between a fluorophore and the device metal surface which, fortunately, falls within the length scale of antibody-antigen-antibody sandwich structures, such as those used in fluorescent immunoassay²⁰. Furthermore, a large number of uniform biosensing nanostructures can be fabricated by nano-imprint lithography (NIL)^{22, 23}. The NIL mass fabrication method produces high quality TNPRs at a low cost, which potentially offers a unique opportunity for high resolution mapping of signaling molecule secretion. Thus, the combination of NIL^{22, 23} for large scale TNPR fabrication and the theoretical ability for TNPR enhancement of in situ immunoassays suggests a possible method to significantly enhance a fluorescent detection signal along with markedly improving the signal-to-noise ratio for accurate biosensing. The signal amplification of the conventional enzyme-linked immunosorbent assay (ELISA) relies on the catalyzation of the enzyme, the diffusive nature of the reporter molecules limits its applications in high resolution sensing. Conventional fluorescent immunoassay is based on spatial integration of individual signals. Due to the weak signal strength, the signal-to-noise ratio drops drastically in subcellular scale. On the other hand, the averaging of the signals over larger area sacrifices the spatial resolution and hampers its ability to sense the local extracellular secretion. There were efforts in signaling molecule sensing at the single cell level attempting to overcome this significant limitation²⁴⁻²⁶. However, in situ biosensing techniques, such as elispot²⁵, cannot achieve subcellular spatial resolution because of poor signal-to-noise ratios or blurry staining from diffusive reporters at the subcellular scale. The significant optical enhancement of TNPRs is crucial to remove the requirement for the large area spatial integration thus makes it possible for subcellular resolution biosensing. Here, we report a novel technique, termed a TNPR-

enhanced in situ immunoassay, which we successfully demonstrate quantifies and maps endogenous cytokine secretion from individual cells at subcellular resolution. Our study is the first direct quantification that confirms the theoretical decades-old hypothesis⁶ that IL-2, which can function as an autocrine signaling molecule, has a significantly elevated local target cell concentration.

The experimental platform for the TNPR-enhanced in situ immunoassay for subcellular resolution mapping of cytokine secretion is shown schematically in figure 1. A TNPR array 100 nm in diameter with a 500 nm periodicity was fabricated by NIL (Supplementary Fig. S1) and was coated with poly-D-lysine and an anti-IL-2 antibody. Jurkat T cells were added to the anti-IL-2 functionalized TNPR surface, imaged by scanning electron microscopy (SEM, Fig. 1 inset) to verify cell orientation on the array, and followed by co-stimulation with a phorbol ester, phorbol myristal acetate (PMA), and a calcium ionophore, ionomycin. A portion of the large amount of IL-2 secreted by Jurkat T cells using this activation method²⁷ was captured by IL-2 antibodies immobilized on the device surface. This was followed by the addition of a fluorescence-conjugated anti-IL-2 antibody to detect the captured IL-2 on the TNPR surface. Formation of the antibody-antigen-antibody sandwich complex brings the fluorophore into the optimal distance for TNPR-dependent signal enhancement. Nonspecifically bound fluorescence-conjugated antibody will be too close to the TNPR surface and therefore experience less signal enhancement due to a dominant quenching effect. Also, there will be no signal enhancement when nonspecific binding occurs in the empty areas of the device surface between TNPRs. Therefore, the anticipated signal-to-noise ratio should be strongly improved as the TNPRs will specifically enhance signal detection from antibody-antigen-antibody sandwich complexes over nonspecific binding interactions. Following a thorough rinse to remove the Jurkat T cells, the profile of secreted IL-2 will be mapped at submicron resolution because of the enhanced signal strength and improved signal-to-noise ratio.

A numeric analysis and characterization of the TNPR-enhanced IL-2 immunoassay was performed. As we reported previously^{16, 17}, the TNPR structure is 100 nm in diameter and consists of two 20 nm gold layers with an intervening 5 nm silicon dioxide thin film (Fig. 2a). The TNPR extinction spectrum shows that the resonance peak is ~670 nm (Fig. 2a) with the strongest electric field enhancement located close to the TNPR edge around the polarization direction of the incident light (Fig. 2b). The light intensity enhancement factor reached two to three orders of magnitude at this edge (Fig. 2b). Because optimized fluorescence enhancement occurs at ~10 nm from a gold surface^{20, 21}, the TNPR functions robustly in an IL-2 sandwich immunoassay based upon the proximal geometry of this antibody-antigen-antibody complex. Notably, there is still significant field enhancement 10 nm away from the gold TNPR surface (Fig. 2b). Spectrum matching is also essential for plasmon-enhanced fluorescence. Thus, we chose APC (allophycocyanin) conjugated anti-IL-2 as the detection antibody, which has a matched overlap spectrum with the designed TNPR. To determine the extent of TNPR enhancement, we assessed a purified IL-2 solution without cells using an APC conjugated anti-IL-2 antibody for detection (Fig. 2c). These clearly resolved individual TNPRs in the fluorescent image (Fig. 2c) were not only indicating the enhancement, but also showing the submicron spatial resolution as high as 500 nm of the pitch size. The normalized fluorescence signal was enhanced 117-fold in the TNPR area versus the area of the platform that lacked TNPRs. Factors contributing to signal enhancement and noise reduction include the local plasmon field and the gold TNPR surface, which has a higher affinity for antibody binding.

Next, a cytokine secretion experiment was performed. Jurkat T cells were cultured on top of an anti-IL-2 antibody-functionalized TNPR array. After overnight stimulation, APC conjugated anti-IL-2 antibody was added followed by washing to remove the cells and

unbound antibody. A high resolution map of the IL-2 secretion profile from individual Jurkat T cells was generated (Fig. 3a). Results showed the highest concentration of IL-2 in the immediate vicinity of the cell, close to the center of the profile pattern from each cell, which also produced a fast signal decay moving radially from this region into a low signal intensity background. As the Jurkat T cells were nonpolarized, it was not surprising that the secretion pattern was round and symmetrically decayed into the background. Control experiments showed that stimulation with PMA and ionomycin was necessary to induce IL-2 secretion (Supplementary Fig. S2) and that the enhancement from TNPR was essential to achieving enough sensitivity for high resolution sensing this secretion (Supplementary Fig. S3). The secretion data were quantified using an IL-2 calibration curve (Fig. 3b). Our results (Fig. 3c) showed that the local concentration of IL-2 at the cell membrane was on the order of hundreds of $\mu\text{g/ml}$ to mg/ml which fell into the right range covered by the calibration curve. By contrast, for a cell density of 10^6 cells/ml after stimulation the global IL-2 concentration was in the ng/ml range.

Previous studies hypothesize that the concentration of IL-2 reduces significantly with distance from a cell^{5, 6, 7} due to diffusion, which suggests that the local IL-2 concentration would be much higher than that measured globally. Indeed, the concentration we measured locally compared to that in the medium was ~ 5 to 6 orders of magnitude different. Since the IL-2 concentration in pre-secretion vesicles is estimated to be tens of mg/ml ⁶ and diffusion at the nano-scale is very fast ($< 1\text{ms}$), it is not immediately clear why the time averaged IL-2 concentration near the cell membrane could reach hundreds of $\mu\text{g/ml}$ to mg/ml . As shown before^{6, 27} both the IL-2 and the α -chain of the IL-2 receptor were largely produced within the same cell after stimulation. As the IL-2 was secreted, the α chain of IL-2 receptor combined with the β and γ chain complex to form high affinity IL-2 receptors ($K_d = 10\text{pM}$) on the cell membrane. A portion of the secreted IL-2 binds to those receptors, which may prevent the rapid escape of IL-2 and maintain the high average IL-2 concentration near cell membrane⁶. A competing factor that could reduce this high local IL-2 concentration would be receptor-mediated endocytosis, but from our measurements this seems to be ineffective at reducing the IL-2 content in the immediate cell vicinity. This spatio-temporal IL-2 secretion profile may have important roles in such extracellular signaling^{5, 6, 7, 28}.

The TNPR technique demonstrated in this report can quantify subcellular cytokine secretion. An important future application is to determine the signaling molecule spatial distribution between polarized cells, such as those during the signaling and communication events between neuronal or immunological synapses^{29,30}. This TNPR technique will also enable the simultaneous detection, spatial mapping, and quantification of multiple, distinct signaling molecules when TNPRs are engineered with multiple resonance peaks for multiple color enhancements. Our technique opens a new avenue in sensing and quantifying subcellular level signaling molecule secretion and could have broad impact for deciphering extracellular signaling and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- (1). Toribio ML, Gutierrez-Ramos JC, Pezzi L, Marcos MAR, Martinez-A C. *Nature*. 1989; 342:82–85. [PubMed: 2812004]
- (2). Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, Dong C. *Nature*. 2007; 448:480–483. [PubMed: 17581589]
- (3). Sporn MB, Roberts AB. *Nature*. 1985; 313:745–747. [PubMed: 3883191]
- (4). Arima N, Daitoku Y, Ohgaki S, Fukumori J, Tanaka H, Yamamoto Y, Fujimoto K, Onoue K. *Blood*. 1986; 68:779–782. [PubMed: 2874849]
- (5). Shvartsman SY, Wiley HS, Deen WM, Lauffenburger DA. *Biophys. J.* 2001; 81:1854–1867. [PubMed: 11566760]
- (6). Kaplan D. *Immunology Today*. 1996; 17:303–304. [PubMed: 8763813]
- (7). Lauffenburger DA, Oehrtman GT, Walker L, Wiley HS. *Proceedings of the National Academy of Sciences*. 1998; 95:15368–15373.
- (8). Sokolov AN, Roberts ME, Bao Z. *Materials Today*. 2009; 12:12–20.
- (9). Jin H, Heller DA, Kalbacova M, Kim J-H, Zhang J, Boghossian AA, Maheshri N, Strano MS. *Nat Nano*. 2010; 5:302–309.
- (10). Nie S, Emory SR. *Science*. 1997; 275:1102–1106. [PubMed: 9027306]
- (11). Prokes SM, Glembocki OJ, Rendell RW, Ancona MG. *Appl. Phys. Lett.* 2007; 90:093105.
- (12). Stewart ME, Anderton CR, Thompson LB, Maria J, Gray SK, Rogers JA, Nuzzo RG. *Chem. Rev.* 2008; 108:494–521. [PubMed: 18229956]
- (13). Wang S, Pile DFP, Sun C, Zhang X. *Nano Letters*. 2007; 7:1076–1080. [PubMed: 17343424]
- (14). Anker JN, Hall WP, Lyandres O, Shah NC, Zhao J, Van Duyne RP. *Nat Mater*. 2008; 7:442–453. [PubMed: 18497851]
- (15). Kabashin AV, Evans P, Pastkovsky S, Hendren W, Wurtz GA, Atkinson R, Pollard R, Podolskiy VA, Zayats AV. *Nat Mater*. 2009; 8:867–871. [PubMed: 19820701]
- (16). Su KH, Wei QH, Zhang X. *Appl. Phys. Lett.* 2006; 88:063118.
- (17). Sun C, Su K-H, Valentine J, Rosa-Bauza YT, Ellman JA, Elboudwarej O, Mukherjee B, Craik CS, Shuman MA, Chen FF, Zhang X. *ACS Nano*. 2010; 4:978–984. [PubMed: 20121209]
- (18). Aslan K, Gryczynski I, Malicka J, Matveeva E, Lakowicz JR, Geddes CD. *Curr. Opin. Biotechnol.* 2005; 16:55–62. [PubMed: 15722016]
- (19). Pompa PP, Martiradonna L, Torre AD, Sala FD, Manna L, De Vittorio M, Calabi F, Cingolani R, Rinaldi R. *Nat Nano*. 2006; 1:126–130.
- (20). Anger P, Bharadwaj P, Novotny L. *Phys. Rev. Lett.* 2006; 96:113002. [PubMed: 16605818]
- (21). Kinkhabwala A, Yu Z, Fan S, Avlasevich Y, Mullen K, Moerner WE. *Nat Photon*. 2009; 3:654–657.
- (22). Chou SY, Krauss PR, Renstrom PJ. *Science*. 1996; 272:85–87.
- (23). Stuart C, Xu Q, Tseng RJ, Yang Y, Hahn HT, Chen Y, Wu W, Williams RS. *J. Vac. Sci. Technol. B*. 2006; 24:539.
- (24). Yee C, Greenberg P. *Nat Rev Cancer*. 2002; 2:409–419. [PubMed: 12189383]
- (25). Lakew M, Nordström I, Czerkinsky C, Quiding-Järbrink M. *Journal of Immunological Methods*. 1997; 203:193–198. [PubMed: 9149813]
- (26). Qian WJ, Aspinwall CA, Battiste MA, Kennedy RT. *Anal. Chem*. 2000; 72:711–717. [PubMed: 10701254]
- (27). Abraham RT, Weiss A. *Nat Rev Immunol*. 2004; 4:301–308. [PubMed: 15057788]
- (28). Andersson J, Abrams J, Björk L, Funa K, Litton M, Agren K, Andersson U. *Immunology*. 1994; 83:16–24. [PubMed: 7821961]
- (29). Howe CL, Mobley WC. *Current Opinion in Neurobiology*. 2005; 15:40–48. [PubMed: 15721743]
- (30). Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair D, Waite J, Sacristán C, Victora G, Zanin-Zhorov A, Dustin ML. *Annu Rev Immunol*. 2010; 28:79–105. [PubMed: 19968559]

Figure 1. Schematic of high-resolution mapping of cytokine secretion by a TNPR-enhanced in situ immunoassay

A TNPR array fabricated by NIL was incubated with anti-IL-2 antibody, and after Jurkat T cell plating, stimulation, and removal, a portion of the secreted IL-2 was captured by the antibodies on TNPR. Fluorescence-conjugated antibodies were applied to detect the secreted IL-2 by forming an antibody-antigen (IL-2)-antibody sandwich on the TNPR surface. Insert: SEM image of a Jurkat T cell adherent to a TNPR array (500 nm pitch).

Figure 2. Physical principle, simulation and experimental validation of the TNPR-enhanced immunoassay

(a) Simulation results of TNPR plasmonic resonance spectrum (blue, green and red curves represent extinction, absorption and scattering respectively). (b) Spatial distribution of the optical intensity enhancement at the plane above the top surface of a TNPR (100nm diameter). (c) Fluorescent image showing experimental validation of the TNPR-enhanced immunoassay, with a fluorescence enhancement factor of ~117.

Figure 3. Demonstration of quantitative high spatial resolution mapping of IL-2 secretion from individual Jurkat T cells

(a) Individual Jurkat T cells IL-2 secretion patterns. (b) Calibration curve for increasing concentrations of IL-2 detected with optical signal intensity. (c) Quantitative mapping of Jurkat T cell secretion profiles using information gathered in panels (a) and (b).