ACS SENSORS

pubs.acs.org/acssensors

Perspective

Optical Sensing Strategies for Probing Single-Cell Secretion

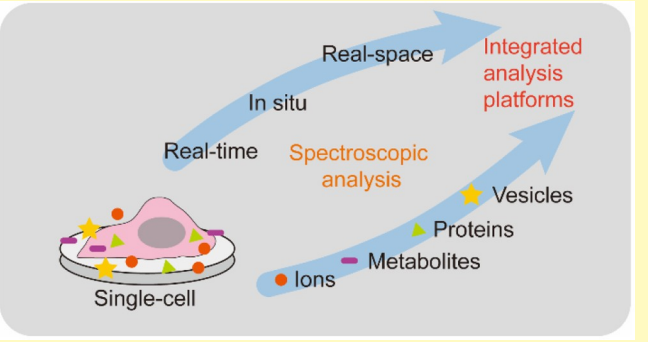
Qingmei Zhong, Xuedong Huang, Rongrong Zhang, Kun Zhang,* and Baohong Liu*

Cite This: *ACS Sens.* 2022, 7, 1779−1790

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Measuring cell secretion events is crucial to understand the fundamental cell biology that underlies cell−cell communication, migration, proliferation, and differentiation. Although strategies targeting cell populations have provided significant information about live cell secretion, they yield ensemble profiles that obscure intrinsic cell-to-cell variations. Innovation in single-cell analysis has made breakthroughs allowing accurate sensing of a wide variety of secretions and their release dynamics with high spatiotemporal resolution. This perspective focuses on the power of single-cell protocols to revolutionize cell-secretion analysis by allowing real-time and real-space measurements on single live cell resolution. We begin by discussing recent progress on single-cell bioanalytical techniques, specifically optical sensing strategies such as fluorescence-, surface plasmon resonance-, and surface-enhanced Raman scattering-based strategies, capable of in situ real-time monitoring of single-cell released ions, metabolites, proteins, and vesicles. Single-cell sensing platforms which allow for high-throughput high-resolution analysis with enough accuracy are highlighted. Furthermore, we discuss remaining challenges that should be addressed to get a more comprehensive understanding of secretion biology. Finally, future opportunities and potential breakthroughs in secretome analysis that will arise as a result of further development of single-cell sensing approaches are discussed.



KEYWORDS: *Single cell, secretion, bioanalysis, optical sensing, fluorescence, surface plasmon resonance, surface-enhanced Raman scattering*

Secretion is a fundamental and ubiquitous cellular process of delivering intracellular species, such as ions, metabolites, and proteins, to the cell exterior in a highly controlled way. These released substances execute a variety of key roles in maintaining normal cellular biology. Abnormal variations in secretion patterns and extracellular concentrations of the secretions can lead to cell dysfunction and diseases.[1−3] For example, neurotransmitters, including some amino acids, biogenic amines, and neuropeptides, released from peripheral and autonomic nerves are well-known signaling molecules in mediating the stimulatory or inhibitory neuronal functions.[4,5] An imbalance of neurotransmitter can provoke various disorders including Alzheimer's disease,[6] Parkinson's disease,[7] schizophrenia,[8] inherited disorders of neurotransmitter metabolism,[9] and malignant tumors.[10] Precise measurement of cell secretion, therefore, is not only crucial to understanding the fundamental cell biology that underlies cell−cell communication, migration, proliferation, and differentiation, but also for discovering new biomarkers and therapy targets.[11,12]

Conventionally, cell secretion is analyzed by ensemble biochemical assays of a large population of cells with relatively low sensitivity, temporal and spatial resolution, and throughput. They are unable to reveal the heterogeneous secretory phenotype and function of individual cells, to quantify extremely trace amounts of released species including ions, molecules, and vesicles, to track the fast, dynamic, and site-specifically occurred secretion events.[13,14] Especially, an increasing number of studies have revealed the pervasive existence of heterogeneity in cellular systems that is commonly manifested in phenotypic differences between cells.[15,16] Cells possessing identical or near-identical genomic DNA sequences can have very different behaviors and biological functions, for example, the exocytotic rate.[17] Ensemble-averaged measurements only reflect the dominant biological features in a cell population, whereas the cell-to-cell variations are usually masked. Furthermore, population-averaged analysis can obscure the presence of rare subpopulations of cells with distinct exocytotic activities, posing challenges for building accurate mechanistic models of secretion related diseases (e.g.,

Received: March 5, 2022
Accepted: June 8, 2022
Published: June 16, 2022

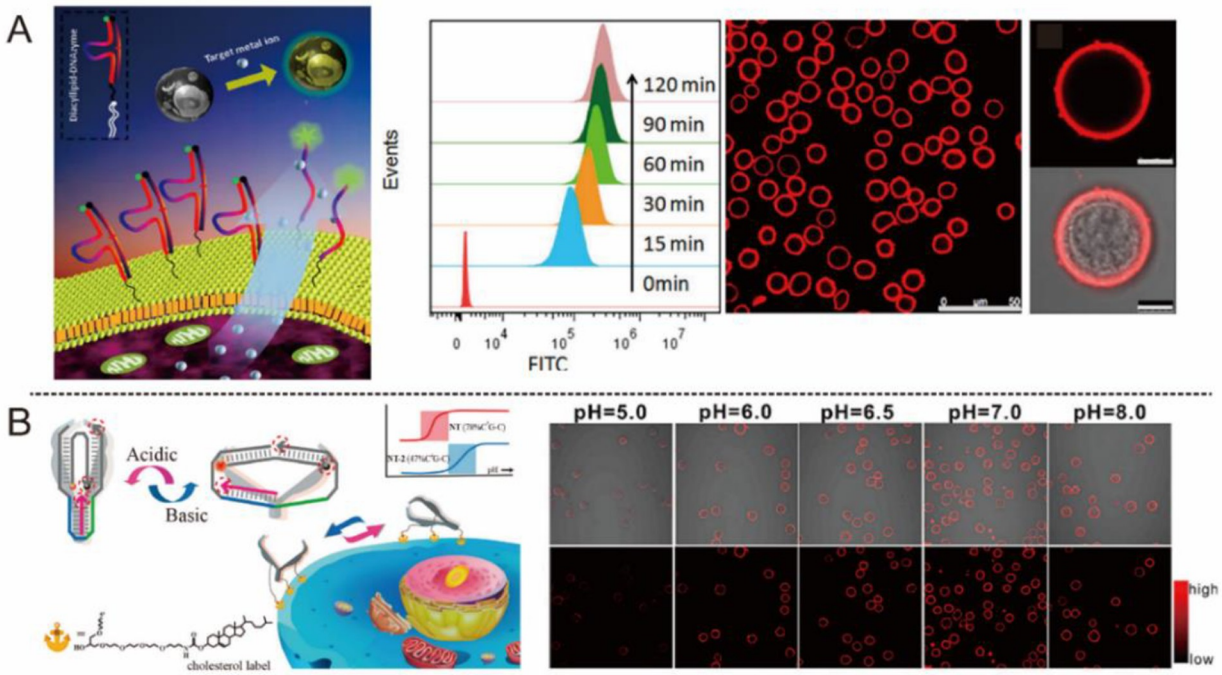


Figure 1. (A) Design of a cell membrane anchored DNAzyme for real-time monitoring of metal ions in the cellular microenvironment. Reprinted with permission from ref 42. Copyright 2014 American Chemical Society. (B) Schematic illustration of a cell surface-anchored DNA nanomachine as reversible and tunable pH sensor. Reprinted with permission from ref 43. Copyright 2018 American Chemical Society.

neurological and metabolic diseases).[18] Given these challenges, it is highly necessary to analyze cellular secretion at the single-cell level.

At present, electrochemical analysis represents one of the mainstream techniques for single-cell secretion analysis and relies on the electrical response of electrodes upon binding to the cell-released molecules.[19−21] The design and preparation of micro/nano electrodes has allowed continuous detection and monitoring of molecular release with high resolution.[22] However, the technical challenge of precisely positioning the probe electrode in the cellular microenvironment may compromise the spatial resolution. The selective detection of compounds with overlapping redox potentials (e.g., dopamine and ascorbic acid) by electrochemical assays is still difficult.[23] In addition, perturbation of cellular states by applying an extra voltage or current to living cells is unknown and needs to be further elucidated.

Optical spectroscopic analysis is attracting increasing attention in the research field of single-cell secretion. Chemical conversion or a physical interaction of the target analytes at a biosensing surface/interface produces the optical signal (e.g., photon emission or frequency shift).[24] Fluorescence imaging and surface plasmon resonance (SPR) imaging microscopies have permitted the in situ monitoring of secretion events with unprecedented sensitivity and resolution, down to the single-molecule level.[25] Although the number of molecules and ions secreted by a single cell to the extracellular space is usually small, the advances in the development of novel fluorescent probes and fluorescence signal-amplification techniques including the enzymatic and nonenzymatic approaches enable accurate detection and quantification of live cell secretions.[26,27] Due to the large extinction coefficient, the plasmonic scattering of a single noble metal nanoparticle can be facilely detected by a commercial microspectroscope and imaged by a standard dark-field microscope. This enables analysis of trace molecules by using a single nanoparticle as the optical biosensor. Similarly, with the rapid progress of bionanophotonics, surface-enhanced Raman scattering (SERS) has emerged as another powerful tool for highly sensitive analysis of cellular secretion with the single-cell resolution.[28,29] The enhancement of Raman signal comes from the excitation of localized surface plasmons with metal nanostructures, especially nano assemblies with tiny interparticle gaps (the so-called SERS hot spots).[30] Compared with fluorescence and SPR-based strategies, SERS has an additional advantage of proving the "spectral fingerprint" of the analyte molecules which contains a wealth of information about the molecular composition, and thus enabling label-free detection and identification of the target molecules released from living cells.[31] The aim of this Perspective is to detail important technological advances toward real-time, in situ, and high-resolution investigation of single-cell secretion. Specifically, we will focus on advances of fluorescence, SPR, and SERS assays for the qualitative and quantitative analysis of different species released by single living cells which include inorganic ions, small molecules, cytokines, and vesicles. We will discuss the pros and cons of each method and finally provide our version of the future of the single-cell secretion analysis.

APPROACHES TO SINGLE CELL

Ions. Ions play an important role in the regulation of cell function and enzyme catalysis.[32−34] Although the concentration of these ions is usually low under normal conditions, it may increase by several orders of magnitude under some pathological conditions.[35] With the rapid progress of highly bright and specific fluorescent probes, fluorescence-based strategies have been extensively explored for analyzing and/or imaging ions secreted from single living cells.[36−39] Because of the low abundance of specific ions released from a single cell, isolation and enrichment of the target ions in a confined

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

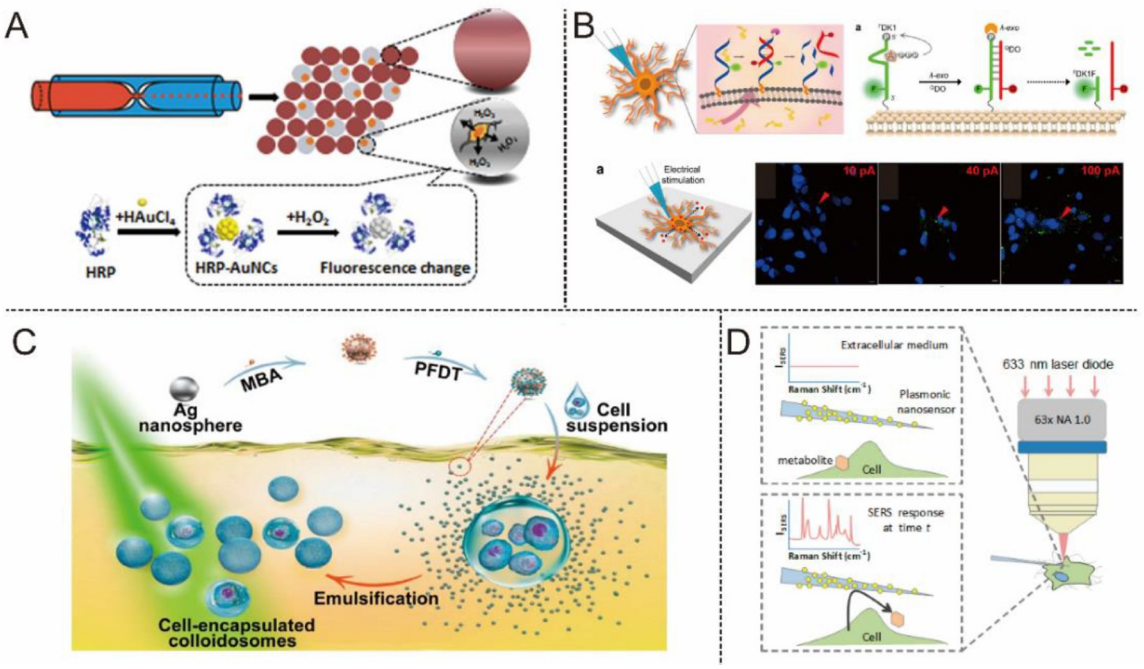


Figure 2. (A) Schematic illustration of detecting hydrogen peroxide in the single-cell encapsulated droplets in combination with HRP-AuNCs. Reprinted with permission from ref 63. Copyright 2018 American Chemical Society. (B) Schematic illustration of a cell-surface-anchored ^{F}DK1 sensor for fluorescence imaging of extracellular ATP. Reprinted with permission from ref 64. Copyright 2021 American Chemical Society. (C) Schematic illustrating the formation of a plasmonic colloidosome-based single cell detector. Reprinted with permission from ref 70. Copyright 2019 American Chemical Society. (D) Representation of the concept of a SERS nanosensor to monitor cellular secretion. Reprinted with permission from ref 73. Copyright 2016 American Chemical Society.

space is necessary to improve the fluorescence intensity and signal-to-noise ratio. The encapsulation of individual living cells in uniform, picoliter (pL) microfluidic droplets has already shown huge promise in elevating the local concentration of the analyte ions due to the largely decreased solution volume.[40] By combining single-cell droplet microfluidics with fluorescence cytometry, proton secretion from single circulation tumor cells was evaluated in a high-throughput (1 kHz) manner, which was a remarkable achievement.[41] Considering the rapid diffusion and dilution of released ions in the bulk extracellular medium, fluorescent probes capable of anchoring at the cell membrane have been designed using dye-labeled DNA oligonucleotides and genetically encoded fluorescent proteins as recognition and signal reporters.[42−44] A representative cell-surface anchored fluorescent biosensor used a diacyllipid-DNAzyme conjugate consisting of three parts: the DNAzyme sequence at the 3′-end region, a diacyllipid tail at the 5′-end region, and a PEG linker located between the two regions. The DNAzyme and the substate strand were labeled with a fluorescent dye and a quencher, respectively. Target ions were detected based on the enzymatic reaction-induced structural changes of the membrane-anchored sensors in the presence of target ions (see Figure 1A).[42] In the absence of target ions, fluorescence was quenched due to the close proximity between the fluorescent dye and the quencher. In the presence of target ions, the DNAzyme was activated to cleave the substrate strand into two fragments which dissociated from the DNAzyme strand due to a decrease of the hybridization stability. This resulted in separation of the quencher from the fluorophore and, in turn, the fluorescence restoration at the cell surface.[38] By adjusting the DNA sequence and the lipid unit, this fluorescence switching approach can be extended to other ions such as H^+ and K^+.[43,45,46] Recently, Jiang et al. reported a dynamic pH-responsive DNA nanodevice which is anchored on the cell surface via multiple cholesterol anchors. This nanodevice could experience a rapid reversible triplex−duplex conformation change accompanied by the off−on fluorescence switching, thus possessing an ability for dynamic detection and imaging of H^+ excretion at the single-cell level (Figure 1B).[43] Despite this progress, the problem of photobleaching and phototoxicity needs to be considered in live fluorescence imaging.

SERS has a sensitivity comparable to that of fluorescence, but is less influenced by photobleaching. A simple yet efficient approach for SERS analysis of H^+ excretion from living cells was developed by directly culturing cells on the SERS substrates premodified with pH-sensitive Raman probes such as 4-mercaptopyridine and 4-mercaptobenzoic acid.[47−49] The detection was based on the deprotonation and protonation of the probe molecules at different pH conditions, which resulted in a detectable change of their SERS spectra.[47−49] To avoid the effect of ion diffusion and dilution in the extracellular space, the strategy of tethering probes on the cell surface was also feasible for SERS analysis. For example, a cell membrane-anchored SERS biosensor was designed for measuring extracellular pH at micrometer-scale resolution.[50] To achieve that, the outer membrane surface of the living cell was first biotinylated via the NHS-ester amine reaction. Gold nanoparticles cofunctionalized with streptavidin and 4-mercaptobenzoic acid were then conjugated onto the cell surface by streptavidin−biotin interaction.[50] The SERS sensor was able to probe a highly localized variation of pH induced by H^+ release which was particularly upregulated in tumor cells.[50] Despite this progress, robust and site-specific labeling of the cell membrane with metal nanoparticles is experimentally challenging that involves multistep biochemical operation and can

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

Table 1. Optical Strategies for Analysis of Metabolites Secreted from Single Cells

analytical modality	probes/tools	targets	pros and cons	refs
Fluorescence	Microdroplet and Au nanoclusters Photoactivatable DNAs NIR fluorescence carbon nanotube arrays Genetically encoded fluorescent proteins	H_2O_2, ATP, dopamine, serotonin, lactate	**Pros** • High throughput • High spatiotemporal resolution (100 ms; 60 μm) • High sensitivity (beyond 10^{-12} M) **Cons** • Probes needed • Simultaneous multitarget analysis is difficult	61,62 64−69
SERS	Au/Ag-based plasmonic nanotips	H^+, ROS, pyruvate, lactate, ATP, ADP, glucose, glutamine, urea	**Pros** • Direct label-free analysis is allowed • Good temporal resolution (500 ms-1 s) • High sensitivity (down to a single molecule) **Cons** • Low throughput (one tip one cell)	70−74
SPR	Au nanorods-based plasmonic hydrogel	H_2O_2	**Pros** • Label-free analysis is allowed • Easy-to-use **Cons** • Single-target analysis	75

cause cellular damage. Another strategy for SERS sensing of cellular ions utilizes a SERS-active nanopipette.[51−53] By using a three-dimensional micromanipulation system which has long been employed in patch-clamp electrophysiology, the plasmonic nanopipette could be positioned precisely in close proximity to the outer cell membrane and thus was able to measure metabolically induced changes in the extracellular pH of individual cancer cells by real-time recording of the SERS signature on the nanopipette.[51] Although effective, the assay throughput of this approach is low; only one cell can be measured at a time. Furthermore, there is still a lack of enough Raman probes to target different ions. New probes could be synthesized, but screening effective dual-function molecules capable of specifically recognizing target ions and stably binding to the SERS substrate is time- and labor-intensive.[54,55]

Metabolites. Metabolites secreted by single cells are important factors for cells to respond to microenvironmental stress.[56−62] Single-cell-secreted H_2O_2 has been measured by coupling microfluidic droplet biosensors and fluorescent Au nanoclusters (Figure 2A).[63] In the presence of H_2O_2, redox reactions on the metal nanoclusters took place, which led to fluorescence quenching.[63] The method allowed sensitive quantification of H_2O_2 with a detection limit of 1.0 nM. However, change of cell status from the adherent state to the suspended state in microdroplet may affect the intrinsic secretion profile of cells. Similar to the analysis of secreted ions, the diffusion of metabolites in bulk extracellular space would cause a deviation between the measured value and the real value of the secretion activity. To address this problem, a cell-surface-anchored self-phosphorylating DNAzyme was designed for fluorescence imaging of ATP secreted from single cells. The presence of ATP resulted in a structural change of the DNA sequence accompanying a restoration of the prequenched fluorescence. The DNAzyme exhibited sub-second response kinetics, high specificity, and micromolar sensitivity (Figure 2B).[64] Such a strategy can be further extended to other metabolites such as dopamine and lactates.[65,66] A drawback of these strategies is that they use fluorophores with optical radiation in the visible spectral region which can subject to influence of the intrinsic emission of cellular components. Recently, single-wall carbon nanotubes emitting near-infrared fluorescence have been used as optical probes for spatiotemporal imaging of neurotransmitter secretion from a single living cell.[67−69] Although promising, this sensing strategy is limited by the availability of metabolites that are capable of binding carbon nanotubes and affecting their luminescence.

Recently, our group reported a SERS detection of lactic acid secreted from individual single cells by using 4-MBA functionalized plasmonic colloidosomes as biosensing units (Figure 2C).[70] This method demonstrates high sensitivity and reliability by ratiometric sensing, but colloidosomes prepared by manual emulsification show size variation and may cause signal fluctuation. Furthermore, the lack of automation and biodegradability may limit the practical sensing applications. To address these issues, a renewable nanotip manipulated by the optical fiber system was constructed to identify reactive oxygen species (ROS) from single living cells by SERS.[71] The 4-mercaptophenol-modified Ag nanotip was used for SERS sensing of the intra- and extracellular ROS.[71] To build upon this reversible SERS analysis, the nanopipette contributes to surmounting clearance-based problems.[71] It should be noted that these Raman probe-based indirect measurements are still limited in multiplexing. Using a plasmonic nanosensor (so-called SERS optophysiology) prepared by coating borosilicate nanopipettes with gold nanoparticles, multiple metabolites, such as pyruvate, lactate, ATP, and urea were detected simultaneously in a label-free and real-time manner. However, a chemometric sorting algorithm or more advanced artificial neural network algorithm is required to identify the spectra (Figure 2D).[72,73] Another multiplexing strategy is the development of a SERS-microfluidic droplet platform to realize the label-free simultaneous analysis of metabolites at the single-cell level. By analyzing the intrinsic SERS fingerprints of pyruvate, ATP, and lactate, the metabolic heterogeneity of single cancer cells has been evaluated, which represents a large advance

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

Table 2. Optical Strategies for Analysis of Proteins Secreted from Single Cells

analytical modality	underlying principle	targets	pros and cons	ref
Fluorescence	• Sandwich immunoassay in microchambers • Fluorescence switch of cell surface-anchored aptamers/peptides • Signal-on imaging in microfluidic droplets	TNF-a, IL-6, IL-10, IFN-γ, protease	Pros • High throughput • In situ real-time analysis is allowed Cons • Probes needed • Challenging in multiplexed analysis	80−83
SERS	• Microdroplet-SERS immunoassay	VEGF, IL-8	Pros • High sensitivity (1.0 fg/mL) • Multiplexed analysis (1.0 fg/mL) Cons • Weak stability of large SERS nanotags	84
SPR	• Immunobinding induced change of SPR frequency/intensity	TGF-β, IgG, Thrombopoietin, IL-2, IL-6, IL-8, VEGF	Pros • Label-free analysis is allowed • Single-molecule resolution • In situ real-time analysis is allowed Cons • Challenging in multiplexed analysis	85−92

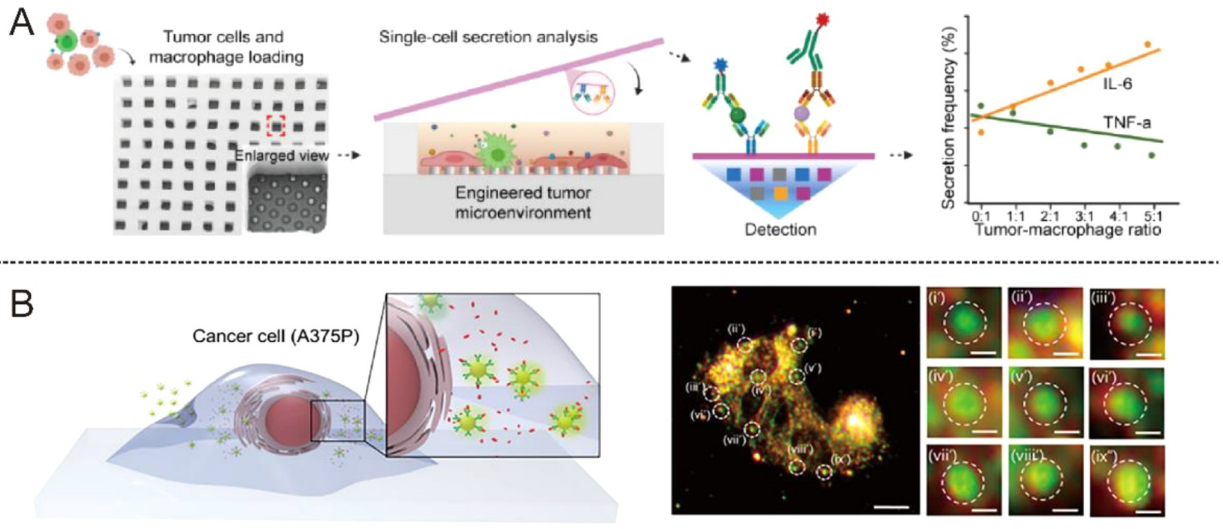


Figure 3. (A) Engineering and characterization of single-cell secretion analysis platform. Reprinted with permission from ref 82. Copyright 2021 American Chemical Society. (B) Immunoplasmonic approach for transforming growth factor-β. Reprinted with permission from ref 85. Copyright 2021 American Chemical Society.

toward real-time single-cell secretion analysis.[74] In addition, an SPR strategy has recently been developed for tracking the release of metabolites from single cells. although the analytes were limited to the highly reactive molecules such as hydrogen peroxide.[75] The pros and cons of each sensing modality are summarized in Table 1.

Proteins. The cell-released proteins, such as cytokines and proteases, play critical roles in regulating physiology and homeostasis in the change process of the extracellular environment.[76−81] Optical spectroscopies based on fluorescence, SERS, and SPR have been exploited in the protein secretion analysis (Table 2). A fluorescent immunoassay-based single cell analysis platform has been developed for investigating the differential modulation effect in cytokine secretions by the tumor microenvironment (Figure 3A).[82] However, as other classical immunoassays, this method faces a problem for which multistep washing and incubation are needed; thus it is not compatible with real-time analysis. Recently, Figdor et al. developed a membrane anchored aptamer sensor combined with a droplet microfluidic platform to detect cytokine secretion.[83] Based on the hydrophobic interaction between the cholesterol tail and the cell phospholipid layer, the cholesterol-linked aptamer probe could be stably anchored on the cell surface. After binding to secreted target cytokines, fluorescence emission of the aptamer probe would be lightened up. By using a microfluidic chip system, aptamer-modified T cells can be individually encapsulated into droplets, which enabled high-throughput

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

Table 3. Optical Strategies for Analysis of Cell-Secreted Vesicles and Their Cargos

strategy	target	LOD	real-time analysis	assay throughput	multiplexed analysis	ref
Antibody barcode-based fluorescent immunoassay	EV surface protein	/	No	>1000 samples	Yes	99
Fluorescence imaging of cellular membrane deformation during vesicle release	Vesicle	/	Yes	Single sample	No	100
Fluorescence imaging of vesicle fusion with cellular membrane	Exosome	/	Yes	Single sample	No	101
Ag staining-based immunoassay	EV surface protein	~10^5 vesicles/μL	No	>1000 samples	No	102
Metal-enhanced fluorescence detection after Immunomagnetic isolation	Exosomal miRNA124	/	No	Single sample	No	104
Hybridization induced fluorescent lighting	Exosomal miRNA21	45.4 fM	No	>45 samples	No	105
Catalytic hairpin assembly driven fluorescence amplification with spatially confined DNA nanocubes	Exosomal miRNA21	9.8 × 10^7 particles/mL	No	Single sample	No	106
Hybridization induced decline of SERS emission	Exosomal let-7a	5.3 aM	No	Single sample	No	111

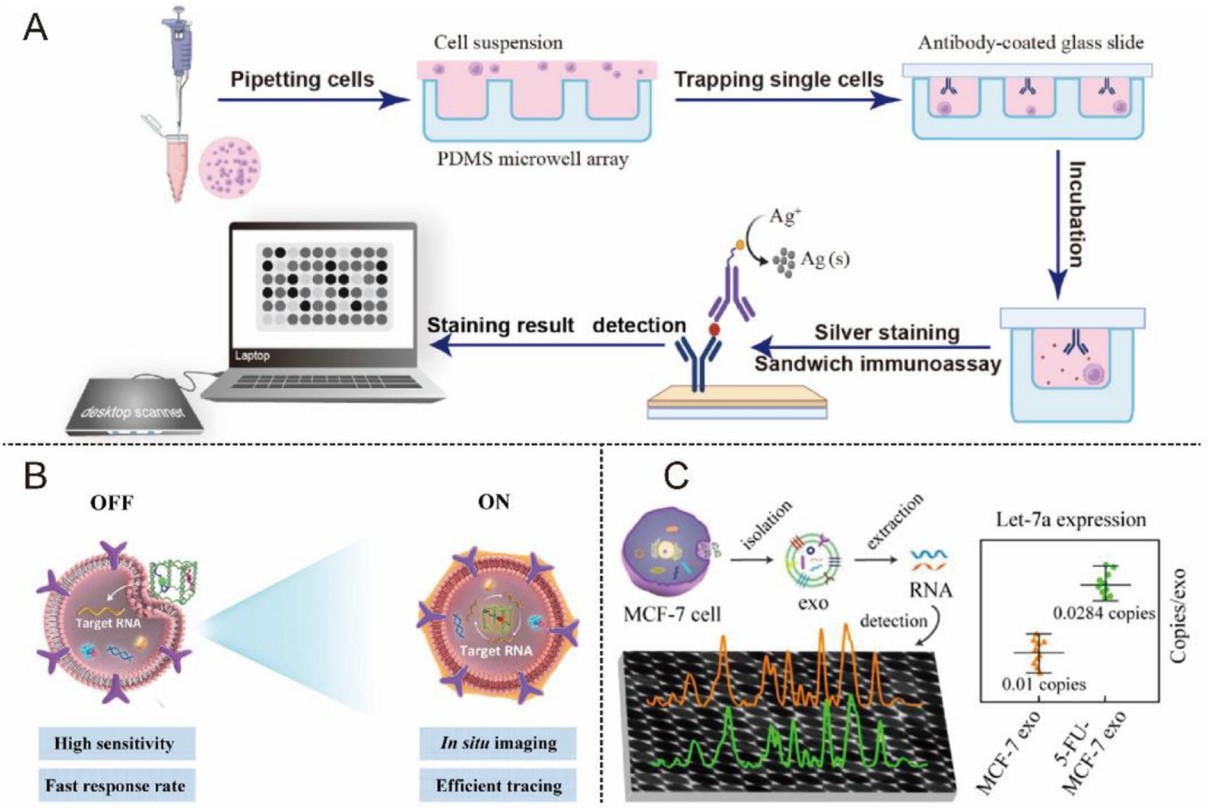


Figure 4. (A) Workflow illustration of trapping of single cells and using gold nanoparticle-enhanced silver staining to enable detection with the desktop scanner for the single-cell EV secretion assay. Reprinted with permission from ref 102. Copyright 2021 American Chemical Society. (B) In situ monitoring of exosomal miRNAs with double-accelerated DNA cascade amplifier nanocubes. Reprinted with permission from ref 106. Copyright 2022 American Chemical Society. (C) Schematic illustration of the SERS sensing exosomal microRNAs. Reprinted with permission from ref 111. Copyright 2021 American Chemical Society.

real-time detection of cytokine secretion at a single cell level, although simultaneous multiprotein quantification is still challenging.[83] To circumvent this issue, a SERS immunoassay combined with droplet microfluids has been developed. The narrow vibrational spectra allowed simultaneous SERS detection of the vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) released from single living cells in monodispersed droplets.[84] A significant limitation of this method is that complex SERS nanotags are needed for signal transformation. The insufficient stability of metallic particles in biological samples such as the extracellular medium may decrease the detection accuracy.

The protein secretion has also been studied by SPR-based strategies which allow label-free, real-time detection of molecular interactions occurring on or near a noble metal surface by monitoring changes in refractive index induced by molecular binding.[85−92] For example, Choi et al. reported an immunoplasmonic approach for transforming growth factor-β (TGF-β) (Figure 3B).[85] The method used antibody-conjugated single gold nanoparticles as optical detection probes.

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

Adsorption of the secreted TGF-β on surface of the gold nanoparticle induced an apparent shift in the plasmonic scattering peak of the particle due to variation of the local refractive index.[85] The method was capable of detecting TGF-β with picomolar sensitivity and monitoring the extracellular TGF-β level secreted from cells under stress conditions.[85] In another work, two antibody-modified gold nanorods, which showed distinct SPR peaks, were used to create a plasmonic microdroplet platform for label-free, real-time, high-throughput, and multiplexed monitoring of the secretion of IL-8 and VEGF from single cancer cells.[93] Besides spectral analysis, SPR imaging has also been employed for real-time monitoring of protein secretion from single cells.[94−96] These advanced methodologies not only expand the assay throughput but also bring an improvement for high spatiotemporal measurement. The major drawback of SPR strategies lies in the weak ability for multiplexing due to the limited resolution of spectrometers for the SPR peaks of metal nanostructures.

Vesicles. Extracellular vesicles (EVs) are important intercellular mediators regulating intercellular communications and transferring biological information molecules like cytosolic proteins, lipids, and nucleic acids.[97,98] Various optical spectroscopic strategies have been proposed for EV quantification and functional analysis (Table 3). An antibody barcode-based fluorescence approach has been developed for high-throughput, multiplexed profiling of single-cell EV secretion. The approach used nanoliter microwell arrays for isolating and enriching EVs released from single cells and spatially resolved antibody barcode for fluorescence detection of EV surface proteins. Single-cell heterogeneity underlying EV secretion within a phenotypically similar cell population was revealed.[99] For a deep understanding of the biosynthesis and secretion of EVs, in situ real-time analysis is urgently needed.[100] Recently, Verweij et al. described a total internal reflection fluorescence microscopy that enabled imaging of vesicles based on tetraspanin reporters.[101] The approach allowed real-time measurement of exosomes released from a single live event and the identification of subcellular sites of fusion events.[101] However, these fluorescent methods mainly rely on expensive or unwieldy instruments, which hinder the practical application. On the bases of gold nanoparticle-enhanced silver staining and the Poisson distribution statistics, a home-use scanner platform was developed.[102] After seeding single cells in microchamber arrays, gold nanoparticle-labeled antibodies were used to execute the sandwich immunoassay, which enabled further generation of a plasmonic silver coating with intense light scattering and reflection (Figure 4A).[102] The method was used to analyze the different phenotypes and the number of extracellular vesicles secreted from the single cell, and has the characteristics of high-throughput and no cell counting.[102]

Extracellular vesicles encapsulate a variety of substances such as microRNAs (miRNA) and proteins which play a pivotal role in the disease progression and have received wide attention as next-generation biomarkers.[103] However, the low abundance and protection of lipid bilayer make it challenging to sense these substances. Several signal amplification-based fluorescent strategies, such as metal-enhanced fluorescence, hybridization chain reaction, and confined space acceleration of hybridization, have been designed to quantify EV miRNAs.[104−106] Li et al. developed a double-accelerated DNA nanocube, which combines the spatial confinement effect and the catalytic hairpin assembly reaction to achieve fast fluorescence signal amplification, for in situ monitoring of miRNA21 in exosomes released from cancer cells (Figure 4B).[106] Furthermore, with the continuous progress in fabrication of novel SERS substrates via colloid synthesis and interfacial self-assembly,[107−109] SERS has emerged as a powerful analytical tool for analysis of EV miRNAs.[110,111] By self-assembling nanoparticles at the oil−water interface, a close-packed and ordered Au octahedra array was developed. The Au nanoarray was used to construct a SERS sensor for exosomal miRNA let-7a, which exhibited broad linearity ranging from 10 aM to 10 nM and could discriminate the target from other miRNA sequences with single-base resolution (Figure 4C).[111] It should be noted that most existing methods, either fluorescence-based techniques or SERS-based ones, are not compatible with a single-cell EV miRNA analysis due to their inability to capture EVs and label the EV miRNAs in situ. However, recently it was reported that the rigid DNA nanostructures and liposome-based vesicle fusion techniques could be used to effectively deliver optical probes to into the EVs.[112,113] The integration of these novel technologies with single-cell microfluids such as microchamber arrays may enable highly-throughput analysis of EV miRNAs released from single cells.

CONCLUSIONS AND OUTLOOK

New strategies for accurate detection and monitoring of single-cell secretion are critical to enable deeper understanding of the heterogeneous cell states between normal and pathological conditions. This knowledge will boost the development of precision medicine by the discovery of meaningful screening and diagnostic biomarkers and therapeutic targets, thereby allowing clinicians to tailor treatment according to the specific characteristics of each patient. They will be help to improve the outcome of patients with malignant diseases, both acutely and in the long term, by providing earlier medical intervention, precise management, and prognosis.

Compared to population-based methodologies, such as liquid chromatography−tandem mass spectrometry (LC-MS/MS) or immunoassays (e.g., ELISA in multiwell plates), single-cell techniques exhibit remarkable resolution. In general, LC-MS/MS methods require several hundred microliters of samples (>10^5 cells),[114] whereas microscopic optical strategies have achieved nanoscale subcell resolution.[50,115] Moreover, cells are lysed in LC-MS/MS assays, and are thus incapable of real-time and in situ recording of secretion events of live cells. Although advanced immunoassays such as digital ELISA has an improved sensitivity at the single-cell level, they use a sandwich-based approach, hence involve multistep operations, and are not applicable for measuring secretion events at a high spatial−temporal resolution.

Despite great progress in optical spectroscopic analysis of single-cell secretion over recent decades, formidable challenges remain to be addressed. First, new principles and methods for improving the signal-to-noise ratio (SNR), and hence the sensitivity and temporal resolution during the in situ observation of the secretion dynamics must be established.[116] As detailed in this Perspective, current efforts to meet this challenge have largely focused on amplifying the signal generated by target analytes as well as on new insights into elimination of the nonspecific background noise. As SNR is related to both the signal and noise levels, preventing nonspecific binding would not only enable a sensitivity and resolution enhancement but also be helpful to avoid measurement bias. Second, as secretion events occur locally on the live

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

cell surface, methods with unprecedented spatial resolution must be developed. Recent advances in live-cell super-resolution imaging may facilitate observation of secretion with nanoscale resolution.[117] Moreover, to continuously observe molecular efflux from the local cell surface for extended periods of time, facile methods for site-specific engineering of the outer cell membrane with rationally designed probes should be further developed. To this end, in addition to classical bioconjugation techniques (e.g., biotin–streptavidin coupling, *N*-hydroxysuccinimide (NHS) ester–primary amine reaction),[118] biorthogonal click chemistry is playing an increasingly prominent role because of the high selectivity and biocompatibility.[119] In addition, since oligonucleotide sequences can be facilely edited and labeled with other chemical/biological moieties (e.g., cholesterol), noncovalent anchoring of oligonucleotide-based optical probes such as aptamers at the cell surface would offer new access to single-cell secretion measurements. It should be noted that, in comparison to antibodies, currently available aptamers, especially the ones targeting specific proteins or small molecules, are still relatively insufficient. We expect that with the continuous development of aptamer discovery techniques (e.g., machine learning guided discovery),[120] this challenge can be effectively addressed. Furthermore, the fluorescence- and SPR-based techniques to date are only capable of detection and imaging of very few targets (≤3 kinds of targets) simultaneously due to spectral overlap. To truly enable high-throughput molecular profiling of single-cell secretion by these techniques, integrated platforms that combine optical spectroscopic analysis with other techniques such as spatial and spectral encoding, microfluidic chips, MS, or flow cytometry should be further studied.[121−125] For SERS analysis, although the molecular narrow band spectra theoretically permit multiplexed monitoring of cellular species, accurately extracting and quantifying the intensities of the characteristic SERS peaks of the target molecules remain a challenge due to the heterogeneous distribution of SERS hot spots on the enhancing matrix. In this regard, the continuous progress in molecular manipulation by DNA origami and host-gest chemistry may facilitate the construction of highly reproducible SERS substrates and the precise placement of target analytes in the hot spots.[126−129]

AUTHOR INFORMATION

Corresponding Authors

Baohong Liu − *Department of Chemistry, Shanghai Stomatological Hospital, State Key Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai 200433, China*; orcid.org/0000-0002-0660-8610; Email: bhliu@fudan.edu.cn

Kun Zhang − *Shanghai Institute for Pediatric Research, Shanghai Key Laboratory of Pediatric Gastroenterology and Nutrition, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China*; Email: kunzhang@shsmu.edu.cn

Authors

Qingmei Zhong − *Department of Chemistry, Shanghai Stomatological Hospital, State Key Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai 200433, China*

Xuedong Huang − *Department of Chemistry, Shanghai Stomatological Hospital, State Key Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai 200433, China*

Rongrong Zhang − *Department of Chemistry, Shanghai Stomatological Hospital, State Key Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai 200433, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssensors.2c00474

Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of the National Natural Science Foundation of China (21934001 and 21974088), and the Science and Technology Commission of Shanghai Municipality (21ZR1481300).

REFERENCES

(1) Esteve, P.; Rueda-Carrasco, J.; Mateo, M. I.; Martin-Bermejo, M. J.; Draffin, J.; Pereyra, G.; Sandonís, Á.; Crespo, I.; Moreno, I.; Aso, E.; Garcia-Esparcia, P.; Gomez-Tortosa, E.; Rábano, A.; Fortea, J.; Alcolea, D.; Lleo, A.; Heneka, M. T.; Valpuesta, J. M.; Esteban, J. A.; Ferrer, I.; Domínguez, M.; Bovolenta, P. Elevated levels of secreted-frizzled-related-protein 1 contribute to Alzheimer's disease pathogenesis. *Nat. Neurosci.* **2019**, *22*, 1258−1268.

(2) Rai, S.; Hansen, N.; Hao-Shen, H.; Dirnhofer, S.; Tata, N. R.; Skoda, R. C. IL-1β secreted from mutant cells carrying JAK2-V617F favors early clonal expansion and promotes MPN disease initiation and progression. *Blood* **2019**, *134*, 307.

(3) Hurley, P. J.; Marchionni, L.; Simons, B. W.; Ross, A. E.; Peskoe, S. B.; Miller, R. M.; Erho, N.; Vergara, I. A.; Ghadessi, M.; Huang, Z.; Gurel, B.; Park, B. H.; Davicioni, E.; Jenkins, R. B.; Platz, E. A.; Berman, D. M.; Schaeffer, E. M. Secreted protein, acidic and rich in cysteine-like 1 (SPARCL1) is down regulated in aggressive prostate cancers and is prognostic for poor clinical outcome. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 14977−14982.

(4) Hodo, T. W.; de Aquino, M. T. P.; Shimamoto, A.; Shanker, A. Critical neurotransmitters in the neuroimmune network. *Front. Immunol.* **2020**, *11*, 1869.

(5) Mittal, R.; Debs, L. H.; Patel, A. P.; Nguyen, D.; Patel, K.; O'Connor, G.; Grati, M.; Mittal, J.; Yan, D.; Eshraghi, A. A.; Deo, S. K.; Daunert, S.; Liu, X. Z. J. Neurotransmitters: the critical modulators regulating gut-brain axis. *Cell. Physiol.* **2017**, *232*, 2359−2372.

(6) Nam, E.; Nam, G.; Lim, M. H. Synaptic copper, amyloid-β, and neurotransmitters in Alzheimer's disease. *Biochemistry* **2020**, *59*, 15−17.

(7) Factor, S. A.; McDonald, W. M.; Goldstein, F. C. The role of neurotransmitters in the development of Parkinson's disease-related psychosis. *Eur. J. Neurol.* **2017**, *24*, 1244−1254.

(8) Uno, Y.; Coyle, J. T. Glutamate hypothesis in schizophrenia. *Psychiatry Clin. Neurosci.* **2019**, *73*, 204−215.

(9) Ng, J.; Papandreou, A.; Heales, S. J.; Kurian, M. A. Monoamine neurotransmitter disorders-clinical advances and future perspectives. *Nat. Rev. Neurol.* **2015**, *11*, 567−584.

(10) Jiang, S. H.; Hu, L. P.; Wang, X.; Li, J.; Zhang, Z. J. Neurotransmitters: emerging targets in cancer. *Oncogene* **2020**, *39*, 503−515.

(11) Obenauf, A. C.; Zou, Y.; Ji, A. L.; Vanharanta, S.; Shu, W.; Shi, H.; Kong, X.; Bosenberg, M. C.; Wiesner, T.; Rosen, N.; Lo, R. S.; Massagué, J. Therapy-induced tumour secretomes promote resistance and tumour progression. *Nature* **2015**, *520*, 368−372.

(12) Robinson, J. L.; Feizi, A.; Uhlén, M.; Nielsen, J. A systematic investigation of the malignant functions and diagnostic potential of the cancer secretome. *Cell Rep.* **2019**, *26*, 2622−2635.
(13) Fathi, M.; Joseph, R.; Adolacion, J. R. T.; Martinez-Paniagua, M.; An, X.; Gabrusiewicz, K.; Mani, S. A.; Varadarajan, N. Single-cell cloning of breast cancer cells secreting specific subsets of extracellular vesicles. *Cancers* **2021**, *13*, 4397.
(14) Shirasaki, Y.; Yamagishi, M.; Suzuki, N.; Izawa, K.; Nakahara, A.; Mizuno, J.; Shoji, S.; Heike, T.; Harada, Y.; Nishikomori, R.; Ohara, O. Real-time single-cell imaging of protein secretion. *Sci. Rep.* **2015**, *4*, 4736.
(15) Zomer, A.; van Rheenen, J. Implications of extracellular vesicle transfer on cellular heterogeneity in cancer: what are the potential clinical ramifications? *Cancer Res.* **2016**, *76*, 2071−2075.
(16) Hu, Z.; Sun, R.; Curtis, C. A population genetics perspective on the determinants of intra-tumor heterogeneity. *Biochim. Biophys. Acta, Rev. Cancer* **2017**, *1867*, 109−126.
(17) Moqtaderi, Z.; Geisberg, J. V.; Struhl, K. Extensive structural differences of closely related 3′ mRNA isoforms: links to Pab1 binding and mRNA stability. *Mol. Cell* **2018**, *72*, 849−861.
(18) Huang, Y.; Cai, D.; Chen, P. Micro- and nanotechnologies for study of cell secretion. *Anal. Chem.* **2011**, *83*, 4393−4406.
(19) Wang, K.; Xiao, T.; Yue, Q.; Wu, F.; Yu, P.; Mao, L. Selective amperometric recording of endogenous ascorbate secretion from a single rat adrenal chromaffin cell with pretreated carbon fiber microelectrodes. *Anal. Chem.* **2017**, *89*, 9502−9507.
(20) Zhang, J.; Zhou, J.; Pan, R.; Jiang, D.; Burgess, J. D.; Chen, H.-Y. New frontiers and challenges for single-cell electrochemical analysis. *ACS Sens.* **2018**, *3*, 242−250.
(21) Larsson, A.; Majdi, S.; Oleinick, A.; Svir, I.; Dunevall, J.; Amatore, C.; Ewing, A. G. Intracellular electrochemical nanomeasurements reveal that exocytosis of molecules at living neurons is subquantal and complex. *Angew. Chem., Int. Ed.* **2020**, *59*, 6711−6714.
(22) White, K. A.; Kim, B. N. Quantifying neurotransmitter secretion at single-vesicle resolution using high-density complementary metal-oxide-semiconductor electrode array. *Nat. Commun.* **2021**, *12*, 431.
(23) Ferapontova, E. E. Electrochemical analysis of dopamine: perspectives of specific in vivo detection. *Electrochim. Acta* **2017**, *245*, 664−671.
(24) Li, B.; Liu, Y.; Liu, Y.; Tian, T.; Yang, B.; Huang, X.; Liu, J.; Liu, B. Construction of dual-color probes with target-triggered signal amplification for in situ single-molecule imaging of microRNA. *ACS Nano* **2020**, *14*, 8116−8125.
(25) Zhang, H.; Huang, X.; Liu, J.; Liu, B. Simultaneous and ultrasensitive detection of multiple microRNAs by single-molecule fluorescence imaging. *Chem. Sci.* **2020**, *11*, 3812−3819.
(26) Gui, R.; Jin, H.; Bu, X.; Fu, Y.; Wang, Z.; Liu, Q. Recent advances in dual-emission ratiometric fluorescence probes for chemo/biosensing and bioimaging of biomarkers. *Coord. Chem. Rev.* **2019**, *383*, 82−103.
(27) Zhou, H.; Liu, J.; Xu, J.-J.; Zhang, S.-S.; Chen, H.-Y. Optical nano-biosensing interface via nucleic acid amplification strategy: construction and application. *Chem. Soc. Rev.* **2018**, *47*, 1996−2019.
(28) Li, X. X.; Duan, X. Y.; Yang, P.; Li, L.; Tang, B. Accurate in situ monitoring of mitochondrial H_2O_2 by robust SERS nanoprobes with a Au-Se interface. *Anal. Chem.* **2021**, *93*, 4059−4065.
(29) Zheng, X.-S.; Zong, C.; Wang, X.; Ren, B. Cell-penetrating peptide conjugated SERS nanosensor for in situ intracellular pH imaging of single living cells during cell cycle. *Anal. Chem.* **2019**, *91*, 8383−8389.
(30) Bell, S. E. J.; Charron, G.; Cortes, E.; Kneipp, J.; Chapelle, M. L.; Langer, J.; Prochazka, M.; Tran, V.; Schlucker, S. Towards reliable and quantitative surface-enhanced raman scattering (SERS): from key parameters to good analytical practice. *Angew. Chem., Int. Ed.* **2020**, *59*, 5454−5462.
(31) Zong, C.; Xu, M. X.; Xu, L. J.; Wei, T.; Ma, X.; Zheng, X. S.; Hu, R.; Ren, B. Surface-enhanced Raman spectroscopy for bioanalysis: reliability and challenges. *Chem. Rev.* **2018**, *118*, 4946−4980.
(32) Mbatia, H. W.; Burdette, S. C. Photochemical tools for studying metal ion signaling and homeostasis. *Biochemistry* **2012**, *51*, 7212−7224.
(33) Ackerman, C. M.; Lee, S.; Chang, C. J. Analytical methods for imaging metals in biology: from transition metal metabolism to transition metal signaling. *Anal. Chem.* **2017**, *89*, 22−41.
(34) Trebak, M.; Kinet, J. P. Calcium signaling in T cells. *Nat. Rev. Immunol.* **2019**, *19*, 154−169.
(35) Lelievre, P.; Sancey, L.; Coll, J. L.; Deniaud, A.; Busser, B. The multifaceted roles of copper in cancer: a trace metal element with dysregulated metabolism, but also a target or a bullet for therapy. *Cancers* **2020**, *12*, 3594.
(36) Gee, K. R.; Zhou, Z.-L.; Qian, W.-J.; Kennedy, R. Detection and imaging of zinc secretion from pancreatic β-cells using a new fluorescent zinc indicator. *J. Am. Chem. Soc.* **2002**, *124*, 776−778.
(37) Chen, M.; Zhang, S.; Xing, Y.; Li, X.; He, Y.; Wang, Y.; Oberholzer, J.; Ai, H. Genetically encoded, photostable indicators to image dynamic Zn^{2+} secretion of pancreatic islets. *Anal. Chem.* **2019**, *91*, 12212−12219.
(38) Zhang, J.; Cheng, F.; Li, J.; Zhu, J. J.; Lu, Y. Fluorescent nanoprobes for sensing and imaging of metal ions: recent advances and future perspectives. *Nano Today* **2016**, *11*, 309−329.
(39) Wu, D.; Chen, L.; Lee, W.; Ko, G.; Yin, J.; Yoon, J. Recent progress in the development of organic dye based near-infrared fluorescence probes for metal ions. *Coord. Chem. Rev.* **2018**, *354*, 74−97.
(40) Joensson, H. N.; Andersson Svahn, H. Droplet microfluidics: a tool for single-cell analysis. *Angew. Chem., Int. Ed.* **2012**, *51*, 12176−12192.
(41) Del Ben, F.; Turetta, M.; Celetti, G.; Piruska, A.; Bulfoni, M.; Cesselli, D.; Huck, W. T. S.; Scoles, G. A method for detecting circulating tumor cells based on the measurement of single-cell metabolism in droplet-based microfluidics. *Angew. Chem., Int. Ed.* **2016**, *128*, 8723−8726.
(42) Qiu, L.; Zhang, T.; Jiang, J.; Wu, C.; Zhu, G.; You, M.; Chen, X.; Zhang, L.; Cui, C.; Yu, R.; Tan, W. Cell membrane-anchored biosensors for real-time monitoring of the cellular microenvironment. *J. Am. Chem. Soc.* **2014**, *136*, 13090−13093.
(43) Liu, L.; Dou, C. X.; Liu, J. W.; Wang, X. N.; Ying, Z. M.; Jiang, J. H. Cell surface-anchored DNA nanomachine for dynamically tunable sensing and imaging of extracellular pH. *Anal. Chem.* **2018**, *90*, 11198−11202.
(44) Burgstaller, S.; Bischof, H.; Rauter, T.; Schmidt, T.; Schindl, R.; Patz, S.; Groschup, B.; Filser, S.; van den Boom, L.; Sasse, P.; Lukowski, R.; Plesnila, N.; Graier, W. F.; Malli, R. Immobilization of recombinant fluorescent biosensors permits imaging of extracellular ion signals. *ACS Sens.* **2021**, *6*, 3994−4000.
(45) Zeng, S.; Liu, D.; Li, C.; Yu, F.; Fan, L.; Li, C.; Huang, Y.; Nie, Z.; Yao, S. Cell-surface-anchored ratiometric DNA tweezer for real-time monitoring of extracellular and apoplastic pH. *Anal. Chem.* **2018**, *90*, 13459−13466.
(46) Liu, J.; Li, W.; Li, R.; Yin, X.; He, S.; Hu, J.; Ruan, S. Programmable DNA framework sensors for in situ cell-surface pH analysis. *Anal. Chem.* **2021**, *93*, 12170−12174.
(47) Sun, F.; Zhang, P.; Bai, T.; Galvan, D. D.; Hung, H.-C.; Zhou, N.; Jiang, S.; Yu, Q. Functionalized plasmonic nanostructure arrays for direct and accurate mapping extracellular pH of living cells in complex media using SERS. *Biosens. Bioelectron.* **2015**, *73*, 202−207.
(48) Xu, M.; Ma, X.; Wei, T.; Lu, Z.-X.; Ren, B. In situ imaging of live-cell extracellular pH during cell apoptosis with surface-enhanced Raman spectroscopy. *Anal. Chem.* **2018**, *90*, 13922−13928.
(49) Skinner, W. h.; Chung, M.; Mitchell, S.; Akidil, A.; Fabre, K.; Fabre, K.; Goodwin, R.; Stokes, A. A.; Radacsi, N.; Campbell, C. J. A SERS-active electrospun polymer mesh for spatially localized pH measurements of the cellular microenvironment. *Anal. Chem.* **2021**, *93*, 13844−13851.

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

(50) Puppulin, L.; Hosogi, S.; Sun, H.; Matsuo, K.; Inui, T.; Kumamoto, Y.; Suzaki, T.; Tanaka, H.; Marunake, Y. Bioconjugation strategy for cell surface labelling with gold nanostructures designed for highly localized pH measurement. *Nat. Commun.* **2018**, *9*, 5278.
(51) Zhao, X.; Campbell, S.; Wallace, G. Q.; Claing, A.; Bazuin, C. G.; Masson, J.-F. Branched Au nanoparticles on nanofibers for surface-enhanced Raman scattering sensing of intracellular pH and extracellular pH gradients. *ACS Sen.* **2020**, *5*, 2155−2167.
(52) Zhao, X.; Campbell, S.; El-Khoury, P. Z.; Jia, Y.; Wallace, G. Q.; Claing, A.; Bazuin, C. G.; Masson, J.-F. Surface-enhanced Raman scattering optophysiology nanofibers for the detection of heavy metals in single breast cancer cells. *ACS Sens.* **2021**, *6*, 1649−1662.
(53) Hanif, S.; Liu, H.; Chen, M.; Muhammad, P.; Zhou, Y.; Cao, J.; Ahmed, S. A.; Xu, J.; Xia, X.; Chen, H.; Wang, K. Organic cyanide decorated SERS active nanopipettes for quantitative detection of hemeproteins and Fe^{3+} in single cells. *Anal. Chem.* **2017**, *89*, 2522−2530.
(54) Liu, J.; Liu, Z.; Wang, W.; Tian, Y. Real-time tracking and sensing of Cu^{+} and Cu^{2+} with a single SERS probe in the live brain: toward understanding why copper ions were increased upon ischemia. *Angew. Chem., Int. Ed.* **2021**, *60*, 21351−21359.
(55) Sreedevi, P.; Nair, J. B.; Preethanuj, P.; Jeeja, B. S.; Suresh, C. H.; Maiti, K. K.; Varma, R. L. Calix[4]arene based redox sensitive molecular probe for SERS guided recognition of labile iron pool in tumor cells. *Anal. Chem.* **2018**, *90*, 7148−7153.
(56) Levy, M.; Blacher, E.; Elinav, E. Microbiome, metabolites and host immunity. *Curr. Opin. Microbiol.* **2017**, *35*, 8−15.
(57) Martin, J. F.; Casqueiro, J.; Liras, P. Secretion systems for secondary metabolites: how producer cells send out messages of intercellular communication. *Curr. Opin. Microbiol.* **2005**, *8*, 282−293.
(58) D'Souza, G.; Shitut, S.; Preussger, D.; Yousif, G.; Waschina, S.; Kost, C. Ecology and evolution of metabolic cross-feeding interactions in bacteria. *Nat. Prod. Rep.* **2018**, *35*, 455−488.
(59) Hirschhaeuser, F.; Sattler, U. G.; Mueller-Klieser, W. Lactate: a metabolic key player in cancer. *Cancer Res.* **2011**, *71*, 6921−6925.
(60) Chiang, J. Y. Bile acid metabolism and signaling. *Compr. Physiol.* **2013**, *3*, 1191−1212.
(61) Duan, Z.; Tan, L.; Duan, R.; Chen, M.; Xia, F.; Huang, F. Photoactivated biosensing process for dictated ATP detection in single living cells. *Anal. Chem.* **2021**, *93*, 11547−11556.
(62) Dinarvand, M.; Neubert, E.; Meyer, D.; Selvaggio, G.; Mann, F. A.; Erpenbeck, L.; Kruss, S. Near-infrared imaging of serotonin release from cells with fluorescent nanosensors. *Nano Lett.* **2019**, *19*, 6604−6611.
(63) Shen, R.; Liu, P.; Zhang, Y.; Yu, Z.; Chen, X.; Zhou, L.; Nie, B.; Żaczek, A.; Chen, J.; Liu, J. Sensitive detection of single-cell secreted H_2O_2 by integrating a microfluidic droplet sensor and Au nanoclusters. *Anal. Chem.* **2018**, *90*, 4478−4484.
(64) Zhao, D.; Chang, D.; Zhang, Q.; Chang, Y.; Liu, B.; Sun, C.; Li, Z.; Dong, C.; Liu, M.; Li, Y. Rapid and specific imaging of extracellular signaling molecule adenosine triphosphate with a self-phosphorylating DNAzyme. *J. Am. Chem. Soc.* **2021**, *143*, 15084−15090.
(65) Zeng, S.; Wang, S.; Xie, X.; Yang, S. H.; Fan, J. H.; Nie, Z.; Huang, Y.; Wang, H. H. Live-cell imaging of neurotransmitter release with a cell-surface-anchored DNA-nanoprism fluorescent sensor. *Anal. Chem.* **2020**, *92*, 15194−15201.
(66) Nasu, Y.; Murphy-Royal, C.; Wen, Y.; Haidey, J. N.; Molina, R. S.; Aggarwal, A.; Zhang, S.; Kamijo, Y.; Paquet, M. E.; Podgorski, K.; Drobizhev, M.; Bains, J. S.; Lemieux, M. J.; Gordon, G. R.; Campbell, R. E. A genetically encoded fluorescent biosensor for extracellular L-lactate. *Nat. Commun.* **2021**, *12*, 7058.
(67) Kruss, S.; Salem, D. P.; Vukovic, L.; Lima, B.; Vander Ende, E.; Boyden, E. S.; Strano, M. S. High-resolution imaging of cellular dopamine efflux using a fluorescent nanosensor array. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 1789−1794.
(68) Yang, S. J.; Del Bonis-O'Donnell, J. T.; Beyene, A. G.; Landry, M. P. Near-infrared catecholamine nanosensors for high spatiotemporal dopamine imaging. *Nat. Protoc.* **2021**, *16*, 3026−3048.
(69) Beyene, A. G.; Delevich, K.; Del Bonis-O'Donnell, J. T.; Piekarski, D. J.; Lin, W. C.; Thomas, A. W.; Yang, S. J.; Kosillo, P.; Yang, D.; Prounis, G. S.; Wilbrecht, L.; Landry, M. P. Imaging striatal dopamine release using a nongenetically encoded near infrared fluorescent catecholamine nanosensor. *Sci. Adv.* **2019**, *5*, No. eaaw3108.
(70) Wang, X.; Ji, J.; Liu, T.; Liu, Y.; Qiao, L.; Liu, B. Plasmonic colloidosome-based single cell detector: a strategy for individual cell secretion sensing. *Anal. Chem.* **2019**, *91*, 2260−2265.
(71) Chen, J.; Wang, J.; Geng, Y.; Yue, J.; Shi, W.; Liang, C.; Xu, W.; Xu, S. Single-cell oxidative stress events revealed by a renewable SERS nanotip. *ACS Sens.* **2021**, *6*, 1663−1670.
(72) Lussier, F.; Missirlis, D.; Spatz, J. P.; Masson, J.-F. Machine-learning-driven surface-enhanced Raman scattering optophysiology reveals multiplexed metabolite gradients near cells. *ACS Nano* **2019**, *13*, 1403−1411.
(73) Lussier, F.; Brulé, T.; Vishwakarma, M.; Das, T.; Spatz, J. P.; Masson, J.-F. Dynamic-SERS optophysiology: a nanosensor for monitoring cell secretion events. *Nano Lett.* **2016**, *16*, 3866−3871.
(74) Sun, D.; Cao, F.; Tian, Y.; Li, A.; Xu, W.; Chen, Q.; Shi, W.; Xu, S. Label-free detection of multiplexed metabolites at single-cell level via a SERS-microfluidic droplet platform. *Anal. Chem.* **2019**, *91*, 15484−15490.
(75) Chen, Y.-C.; Chen, J.-J.; Hsiao, Y.-J.; Xie, X.-Z.; Peng, C.-C.; Tung, Y.-C.; Chen, Y.-F. Plasmonic gel films for time-lapse LSPR detection of hydrogen peroxide secreted from living cells. *Sens. Actuators B Chem.* **2021**, *336*, 129725.
(76) Chen, Z.; Chen, J. J.; Fan, R. Single-cell protein secretion detection and profiling. *Annu. Rev. Anal. Chem.* **2019**, *12*, 431−449.
(77) Barnes, P. J. Targeting cytokines to treat asthma and chronic obstructive pulmonary disease. *Nat. Rev. Immunol.* **2018**, *18*, 454−466.
(78) Kim, J.; Noh, S.; Park, J. A.; Park, S. C.; Park, S. J.; Lee, J. H.; Ahn, J. H.; Lee, T. Recent advances in aptasensor for cytokine detection: a review. *Sensors* **2021**, *21*, 8491.
(79) Wang, Y.; Che, M.; Xin, J.; Zheng, Z.; Li, J.; Zhang, S. The role of IL-1beta and TNF-alpha in intervertebral disc degeneration. *Biomed. Pharmacother.* **2020**, *131*, 110660.
(80) Alexander, A. F.; Kelsey, I.; Forbes, H.; Miller-Jensen, K. Single-cell secretion analysis reveals a dual role for IL-10 in restraining and resolving the TLR4-induced inflammatory response. *Cell Rep.* **2021**, *36*, 109728.
(81) Dhar, M.; Lam, J. N.; Walser, T.; Dubinett, S. M.; Rettig, M. B.; Di Carlo, D. Functional profiling of circulating tumor cells with an integrated vortex capture and single-cell protease activity assay. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 9986−9991.
(82) Li, L.; Shi, W.; Liu, M.; Bai, X.; Sun, Y.; Zhu, X.; Su, H.; Ji, Y.; Zhu, F.; Liu, X.; Luo, Y.; Liu, T.; Lin, B.; Lu, Y. Single-cell secretion analysis in the engineered tumor microenvironment reveals differential modulation of macrophage immune responses. *Anal. Chem.* **2021**, *93*, 4198−4207.
(83) Qiu, L.; Wimmers, F.; Weiden, J.; Heus, H. A.; Tel, J.; Figdor, C. G. A membrane-anchored aptamer sensor for probing IFNγ secretion by single cells. *Chem. Commun.* **2017**, *53*, 8066−8069.
(84) Sun, D.; Cao, F.; Xu, W.; Chen, Q.; Shi, W.; Xu, S. Ultrasensitive and simultaneous detection of two cytokines secreted by single cell in microfluidic droplets via magnetic-field amplified SERS. *Anal. Chem.* **2019**, *91*, 2551−2558.
(85) Park, J.; Lee, S.; Choi, J.; Choi, I. Extra- and Intracellular monitoring of TGF-β using single immunoplasmonic nanoprobes. *ACS Sens.* **2021**, *6*, 1823−1830.
(86) McDonald, M. P.; Gemeinhardt, A.; Konig, K.; Piliarik, M.; Schaffer, S.; Volkl, S.; Aigner, M.; Mackensen, A.; Sandoghdar, V. Visualizing single-cell secretion dynamics with single-protein sensitivity. *Nano Lett.* **2018**, *18*, 513−519.
(87) Juan-Colas, J.; Hitchcock, I. S.; Coles, M.; Johnson, S.; Krauss, T. F. Quantifying single-cell secretion in real time using resonant hyperspectral imaging. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 13204−13209.

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

(88) Liu, Y. C.; Ansaryan, S.; Li, X.; Arvelo, E. R.; Altug, H. Real-time monitoring of single-cell secretion with a high-throughput nanoplasmonic microarray. *Biosens. Bioelectron.* **2022**, *202*, 113955.
(89) Hassanzadeh-Barforoushi, A.; Warkiani, M. E.; Gallego-Ortega, D.; Liu, G.; Barber, T. Capillary-assisted microfluidic biosensing platform captures single cell secretion dynamics in nanoliter compartments. *Biosens. Bioelectron.* **2020**, *155*, 112113.
(90) Wei, S.-C.; Hsu, M. N.; Chen, C.-H. Plasmonic droplet screen for single-cell secretion analysis. *Biosens. Bioelectron.* **2019**, *144*, 111639.
(91) Zhu, C.; Luo, X.; Espulgar, W. V.; Koyama, S.; Kumanogoh, A.; Saito, M.; Takamatsu, H.; Tamiya, E. Real-time monitoring and detection of single-cell level cytokine secretion using LSPR technology. *Micromachines* **2020**, *11*, 107.
(92) Li, X.; Soler, M.; Szydzik, C.; Khoshmanesh, K.; Schmidt, J.; Coukos, G.; Mitchell, A.; Altug, H. Label-free optofluidic nanobiosensor enables real-time analysis of single-cell cytokine secretion. *Small* **2018**, *14*, 1800698.
(93) Wei, S.-C.; Hsu, M. N.; Chen, C.-H. Plasmonic droplet screen for single-cell secretion analysis. *Biosens. Bioelectron.* **2019**, *144*, 111639.
(94) Milgram, S.; Cortes, S.; Villiers, M.-B.; Marche, P.; Buhot, A.; Livache, T.; Roupioz, Y. On chip real time monitoring of B-cells hybridoma secretion of immunoglobulin. *Biosens. Bioelectron.* **2011**, *26*, 2728−2732.
(95) Stojanovic, I.; van der Velden, T. J.G.; Mulder, H. W.; Schasfoort, R. B.M.; Terstappen, L. W.M.M. Quantification of antibody production of individual hybridoma cells by surface plasmon resonance imaging. *Anal. Biochem.* **2015**, *485*, 112−118.
(96) Qian, J.; Zeng, X.; Gao, Y.; Li, H.; Kumar, S.; Gan, Q.; Cheng, X.; Bartoli, F. J. Intensity-modulated nanoplasmonic interferometric sensor for MMP-9 detection. *Lab Chip.* **2019**, *19*, 1267−1276.
(97) van Niel, G.; D'Angelo, G.; Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell. Biol.* **2018**, *19*, 213−228.
(98) Mathieu, M.; Martin-Jaular, L.; Lavieu, G.; Thery, C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat. Cell Biol.* **2019**, *21*, 9−17.
(99) Ji, Y.; Qi, D.; Li, L.; Su, H.; Li, X.; Luo, Y.; Sun, B.; Zhang, F.; Lin, B.; Liu, T.; Lu, Y. Multiplexed profiling of single-cell extracellular vesicles secretion. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 5979−5984.
(100) Zhang, F.; Guan, Y.; Yang, Y.; Hunt, A.; Wang, S.; Chen, H.-Y.; Tao, N. Optical tracking of nanometer-scale cellular membrane deformation associated with single vesicle release. *ACS Sens.* **2019**, *4*, 2205−2212.
(101) Bebelman, M. P.; Bun, P.; Huveneers, S.; van Niel, G.; Pegtel, D. M.; Verweij, F. J. Real-time imaging of multivesicular body-plasma membrane fusion to quantify exosome release from single cells. *Nat. Protoc.* **2020**, *15*, 102−121.
(102) Zhu, F.; Ji, Y.; Li, L.; Bai, X.; Liu, X.; Luo, Y.; Liu, T.; Lin, B.; Lu, Y. High-throughput single-cell extracellular vesicle secretion analysis on a desktop scanner without cell counting. *Anal. Chem.* **2021**, *93*, 13152−13160.
(103) Makarova, J.; Turchinovich, A.; Shkurnikov, M.; Tonevitsky, A. Extracellular miRNAs and cell-cell communication: problems and prospects. *Trends Biochem. Sci.* **2021**, *46*, 640−651.
(104) Lee, J.-H.; Choi, J.-H.; Chueng, S.-T. D.; Pongkulapa, T.; Yang, L.; Cho, H.-Y.; Choi, J.-W.; Lee, K.-B. Nondestructive characterization of stem cell neurogenesis by a magneto-plasmonic nanomaterial-based exosomal miRNA detection. *ACS Nano* **2019**, *13*, 8793−8803.
(105) Chen, X.; Jia, M.; Liu, L.; Qiu, X.; Zhang, H.; Yu, X.; Gu, W.; Qing, G.; Li, Q.; Hu, X.; Wang, R.; Zhao, X.; Zhang, L.; Wang, X.; Durkan, C.; Wang, N.; Wang, G.; Luo, Y. High-fidelity determination and tracing of small extracellular vesicle cargoes. *Small* **2020**, *16*, 2002800.
(106) Chen, J.; Xie, M.; Shi, M.; Yuan, K.; Wu, Y.; Meng, H.-M.; Qu, L.; Li, Z. Spatial confinement-derived double-accelerated DNA cascade reaction for ultrafast and highly sensitive in situ monitoring of exosomal miRNA and exosome tracing. *Anal. Chem.* **2022**, *94*, 2227−2235.
(107) Ning, C.-F.; Wang, L.; Tian, Y.-F.; Yin, B.-C.; Ye, B.-C. Multiple and sensitive SERS detection of cancer-related exosomes based on gold-silver bimetallic nanotrepangs. *Analyst* **2020**, *145*, 2795−2804.
(108) Zhang, K.; Zhao, J.; Ji, J.; Li, Y.; Liu, B. Quantitative label-free and real-time surface-enhanced Raman scattering monitoring of reaction kinetics using self-assembled bifunctional nanoparticle arrays. *Anal. Chem.* **2015**, *87*, 8702−8708.
(109) Liu, Z.; Li, S.; Xia, X.; Zhu, Z.; Che, L.; Chen, Z. Recent advances in multifunctional graphitic nanocapsules for Raman detection, imaging, and therapy. *Small Methods* **2020**, *4*, 1900440.
(110) Wu, Y.; Zhang, Y.; Zhang, X.; Luo, S.; Yan, X.; Qiu, Y.; Zheng, L.; Li, L. Research advances for exosomal miRNAs detection in biosensing: from the massive study to the individual study. *Biosens. Bioelectron.* **2021**, *177*, 112962.
(111) Kang, T.; Zhu, J.; Luo, X.; Jia, W.; Wu, P.; Cai, C. Controlled self-assembly of a close-packed gold octahedra array for SERS sensing exosomal microRNAs. *Anal. Chem.* **2021**, *93*, 2519−2526.
(112) Zhao, J.; Liu, C.; Li, Y.; Ma, Y.; Deng, J.; Li, L.; Sun, J. Thermophoretic detection of exosomal microRNAs by nanoflares. *J. Am. Chem. Soc.* **2020**, *142*, 4996−5001.
(113) Ning, B.; Huang, Z.; Youngquist, B. M.; Scott, J. W.; Niu, A.; Bojanowski, C. M.; Zwezdaryk, K. J.; Saba, N. S.; Fan, J.; Yin, X.-M.; Cao, J.; Lyon, C. J.; Li, C.-Z.; Roy, C. J.; Hu, T. Y. Liposome-mediated detection of SARS-Cov-2 RNA-positive extracellular vesicles in plasma. *Nat. Nanotechnol.* **2021**, *16*, 1039−1044.
(114) Klünemann, M.; Andrejev, S.; Blasche, S.; Mateus, A.; Phapale, P.; Devendran, S.; Vappiani, J.; Simon, B.; Scott, T. A.; Kafkia, E.; Konstantinidis, D.; Zirngibl, K.; Mastrorilli, E.; Banzhaf, M.; Mackmull, M.-T.; Hövelmann, F.; Nesme, L.; Brochado, A. R.; Maier, L.; Bock, T.; Periwal, V.; Kumar, M.; Kim, Y.; Tramontano, M.; Schultz, C.; Beck, M.; Hennig, J.; Zimmermann, M.; Sévin, D. C.; Cabreiro, F.; Savitski, M. M.; Bork, P.; Typas, A.; Patil, K. R. Bioaccumulation of therapeutic drugs by human gut bacteria. *Nature* **2021**, *597*, 533−538.
(115) Liu, A.; Huang, X.; He, W.; Xue, F.; Yang, Y.; Liu, J.; Chen, L.; Yuan, L.; Xu, P. pHmScarlet is a pH-sensitive red fluorescent protein to monitor exocytosis docking and fusion steps. *Nat. Commun.* **2021**, *12*, 1413.
(116) Labib, M.; Kelley, S. O. Single-cell analysis targeting the proteome. *Nat. Rev. Chem.* **2020**, *4*, 143−158.
(117) Wang, L.; Frei, M. S.; Salim, A.; Johnsson, K. Small-molecule fluorescent probes for live-cell super-resolution microscopy. *J. Am. Chem. Soc.* **2019**, *141*, 2770−2781.
(118) Roy, S.; Cha, J. N.; Goodwin, A. P. Nongenetic bioconjugation strategies for modifying cell membranes and membrane proteins: a review. *Bioconj. Chem.* **2020**, *31*, 2465−2475.
(119) Bird, R. E.; Lemmel, S. A.; Yu, X.; Zhou, Q. A. Bioorthogonal chemistry and its applications. *Bioconj. Chem.* **2021**, *32*, 2457−2479.
(120) Bashir, A.; Yang, Q.; Wang, J.; Hoyer, S.; Chou, W.; McLean, C.; Davis, G.; Gong, Q.; Armstrong, Z.; Jang, J.; Kang, H.; Pawlosky, A.; Scott, A.; Dahl, G. E.; Berndl, M.; Dimon, M.; Ferguson, B. S. Machine learning guided aptamer refinement and discovery. *Nat. Commun.* **2021**, *12*, 2366.
(121) Lu, Y.; Xue, Q.; Eisele, M. R.; Sulistijo, E. S.; Brower, K.; Han, L.; Amir, E.-a. D.; Pe'er, D.; Miller-Jensen, K.; Fan, R. Highly multiplexed profiling of single-cell effector functions reveals deep functional heterogeneity in response to pathogenic ligands. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E607−E615.
(122) Zeming, K. K.; Lu, R.; Woo, K. L.; Sun, G.; Quek, K. Y.; Cheow, L. F.; Chen, C.-H.; Han, J.; Lim, S. L. Multiplexed single-cell leukocyte enzymatic secretion profiling from whole blood reveals patient-specific immune signature. *Anal. Chem.* **2021**, *93*, 4374−4382.

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790

(123) Zhu, H.; Zou, G.; Wang, N.; Zhuang, M.; Xiong, W.; Huang, G. Single-neuron identification of chemical constituents, physiological changes, and metabolism using mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 2586−2591.
(124) Guille-Collignon, M.; Lemaître, F. Overview and outlook of the strategies devoted to electrofluorescence surveys: application to single cell secretion analysis. *TrAC, Trends Anal. Chem.* **2020**, *132*, 116055.
(125) Cho, S. Y.; Gong, X.; Koman, V. B.; Kuehne, M.; Moon, S. J.; Son, M.; Lew, T. T. S.; Gordiichuk, P.; Jin, X.; Sikes, H. D.; Strano, M. S. Cellular lensing and near infrared fluorescent nanosensor arrays to enable chemical efflux cytometry. *Nat. Commun.* **2021**, *12*, 3079.
(126) Zhan, P.; Wen, T.; Wang, Z.; He, Y.; Shi, J.; Wang, T.; Liu, X.; Lu, G.; Ding, B. DNA origami directed assembly of gold bowtie nanoantennas for single-molecule surface-enhanced Raman scattering. *Angew. Chem., Int. Ed.* **2018**, *57*, 2846−2850.
(127) Fang, W.; Jia, S.; Chao, J.; Wang, L.; Duan, X.; Liu, H.; Li, Q.; Zuo, X.; Wang, L.; Wang, L.; Liu, N.; Fan, C. Quantizing single-molecule surface-enhanced Raman scattering with DNA origami metamolecules. *Sci. Adv.* **2019**, *5*, No. eaau4506.
(128) Wang, C.; Tian, L.; Zhu, W.; Wang, S.; Gao, N.; Zhou, K.; Yin, X.; Zhang, W.; Zhao, L.; Li, G. Molecular cage-bridged plasmonic structures with well-defined nanogaps as well as the capability of reversible and selective guest trapping. *Chem. Sci.* **2018**, *9*, 889.
(129) Kim, N. H.; Hwang, W.; Baek, K.; Rohman, M. R.; Kim, J.; Kim, H. W.; Mun, J.; Lee, S. Y.; Yun, G.; Murray, J.; Ha, J. W.; Rho, J.; Moskovits, M.; Kim, K. Smart SERS hot spots: single molecules can be positioned in a plasmonic nanojunction using host-guest chemistry. *J. Am. Chem. Soc.* **2018**, *140*, 4705−4711.

https://doi.org/10.1021/acssensors.2c00474
ACS Sens. 2022, 7, 1779−1790