Single-Cell Analysis

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# Probing Low-Copy-Number Proteins in a Single Living Cell

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***Abstract:*** *Single-cell analysis techniques are essential for understanding the microheterogeneity and functions of cells. Low-copy-number proteins play important roles in cell functioning, but their measurement in single cells remains challenging. Herein, we report an approach, called plasmonic immunosandwich assay (PISA), for probing low-copy-number proteins in single cells. This approach combined in vivo immunoaffinity extraction and plasmon-enhanced Raman scattering (PERS). Target proteins were specifically extracted from the cells by microprobes modified with monoclonal antibody or molecularly-imprinted polymer (MIP), followed by labeling with Raman-active nanotags. The PERS detection, with Raman intensity enhanced by 9 orders of magnitude, provided ultrasensitive detection at the single-molecule level. Using this approach, we found that alkaline phosphatase and survivin were expressed in distinct levels in cancer and normal cells, and that extended culture passage resulted in reduced expression of survivin. We further developed acupuncture needle-based PISA for probing low-copy-number proteins in living bodies.*

Single-cell analysis techniques are essential for unveiling the microheterogeneity and behaviors of cells.[1] As essential constituents of cells, proteins play vital roles in cellular behaviors and functions. Particularly, low-copy-number proteins (present at fewer than 1000 molecules per cell) play important roles in cellular functions, such as signalling and the regulation of gene expression.[2] Although a large variety of approaches have been developed for the analysis of protein expression in single cells, such as capillary electrophoresis (CE),[3] mass spectrometry,[3c,4] flow cytometry,[5] microfluidics,[2b,6] single-molecule imaging,[7] and single-cell Western blotting,[8] the low sensitivity frequently limits these techniques to highly expressed proteins. Owing to its ultrahigh sensitivity, laser-induced fluorescence (LIF) played a central role in existing approaches for the detection of low-copy-number proteins.[2b,3a,6,7] Coupled with CE[3a] or microchip electrophoresis,[2b] LIF permitted the detection of multiple proteins within single cells from the $10^{-21}$ mol level to the single-molecule level. While integrated with a microfluidic device[6] or microscope,[7] LIF imaging allowed for real-time monitoring of protein expression in single cells with single-molecule sensitivity. However, these techniques required lysing the cells or introducing a fluorescent reporter(s) into cells, either killing cells or perturbing the normal composition of the cells. Comparable to LIF, surface-enhanced Raman scattering (SERS) is a powerful technique that can provide single-molecule sensitivity.[9] Due to its intrinsic advantages over LIF, such as lack of photobleaching or self-quenching and being less susceptible to sample and experimental environments, SERS is particularly appealing in the field of bioanalytics,[9c,10] and its potential in single-cell analysis has been demonstrated.[11] However, applications of SERS in the probing of low-copy-number proteins in single cells have not been reported to date.

Herein, we present a new approach called plasmonic immunosandwich assay (PISA) for measuring the expression of low-copy-number proteins in single cells. This approach relies on the combination of in vivo immunoaffinity extraction and ultrasensitive plasmonic detection. A monoclonal antibody and a synthetic antibody mimic produced by a state-of-the-art molecular imprinting technique[12] were used for the immunoaffinity extraction, which ensured that adequate target protein molecules within the cell are specifically extracted onto the microprobe. Meanwhile, a simple but effective alternative of SERS, plasmon-enhanced Raman scattering (PERS), was developed to provide ultrasensitive detection at the single-molecule level. Almost all of the reports on single-molecule SERS rely on clusters or aggregates formed by individual silver or gold nanoparticles,[9c] whereas existing PERS detection usually requires a scanning tunneling microscopic tip[13] or near-field cavities,[14] which are unsuitable for in vivo extraction. In this study, ultrahigh sensitivity was achieved through forming surface plasmon on gold-based extraction microprobes to further enhance the SERS signal of silver-based Raman nanotags attached on the microprobes. The principle and procedure of PISA is illustrated in Scheme 1. A gold-based immunoaffinity microprobe is inserted into a cell of interest through a micromanipulator. After extraction, the microprobe is taken out, washed to remove unwanted species and incubated with silver-based Raman-active nanoparticles (NPs) with immunoaffinity or boronate affinity for a glycoprotein target. Thus, sandwich-like immunocomplexes form on the extraction probe. After washing off excessive Raman nanotags, the microprobe is subjected to Raman detection. As the extraction can be finished within a short period, damage to the cell is limited and the cell can still be alive after extraction. Upon irradiation with a laser beam, surface plasmon on the gold-based extraction microprobe is generated, which further enhances the SERS signal of the silver-based nanotags and thereby enables the detection of low-copy-number proteins.

To gain a maximum plasmonic enhancement, we first optimized the combination of extraction microprobe and

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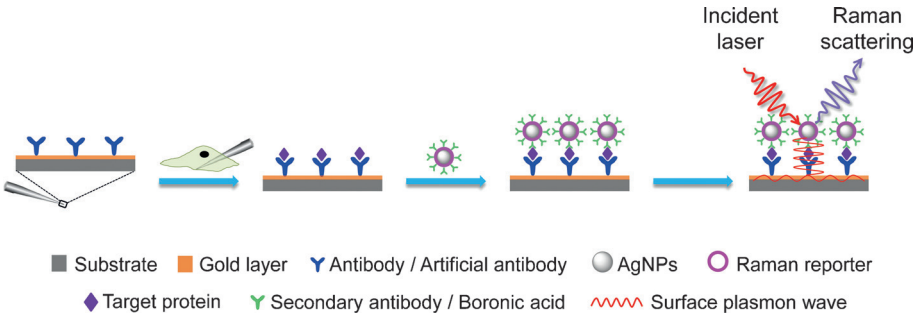


***Scheme 1.*** Overview of probing protein expression in a single cell by plasmonic immunosandwich assay.

detection nanotag that were made of different materials. Figure 1 a shows that the best combination was gold microprobe and silver nanoparticles (AgNPs), which generated 41.3-times higher signal compared to the combination of glass microprobe and gold nanoparticles (conventional SERS mode). Because gold is expensive, gold thin-layer-coated glass was used as an alternative, which generated a slightly lower signal intensity (37.6-times higher compared to the conventional SERS mode). Extraction probes were then fabricated through tapering borosilicate glass capillaries into tiny tips of 1–2 μm diameter followed by chemical plating with a thin layer of gold and functionalization with the antibody or artificial antibody. Figure 1 b shows a representative microprobe. The core size of AgNPs for the preparation of detection nanotags was approximately 50 × 70 nm (Figure S3). Alkaline phosphatase (ALP) and survivin, two potential cancer biomarkers,[15] were used as target proteins in this study. Two Raman reporters, 4-mercaptophenylbronic acid (MPBA) and 4-aminothiophenol (PATP), were used for the detection of ALP and survivin, respectively. Figures 1 c and 1 d show transmission electron microscopic (TEM) images of these Raman-active AgNPs. Figure 1 e displays their Raman spectra while Figure 1 f shows immunosandwich complexes formed on an extraction probe. These nanotags exhibited different characteristic Raman spectra. Characteristic peaks at 1072 and 1435 $cm^{-1}$ were employed for the detection of ALP and survivin, respectively. Under PERS mode, the signal enhancement factor was measured to be $1.8 \times 10^9$ and $2.5 \times 10^9$ for MPBA and PATP, respectively (Figure S5). According to the estimation by Kneipp,[9c] such an enhancement factor is high enough to provide single-molecule sensitivity.

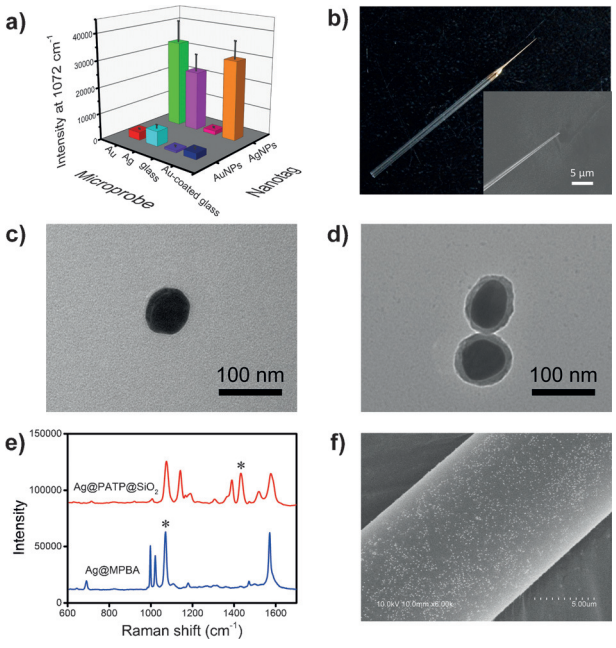


***Figure 1.*** a) Dependence of Raman intensity on the combination of extraction microprobe and nanotag. Error bars represent standard deviations. b) Photo and SEM images of an extraction microprobe. c, d) TEM images of MPBA-immobilized and PATP-encapsulated AgNPs, respectively. e) Raman spectra of the Raman nanotags (peaks marked with * were used for PISA). f) SEM image showing immunosandwiches formed on an extraction microprobe after extracting target protein from a single cell and being labeled with Raman nanotags.

Molecular imprinting has been an important technology to create synthetic mimics of antibodies.[16] Compared with antibodies, molecularly-imprinted polymers (MIPs) are easy to prepare and more stable in a sample environment and storage. Recently, by using boronic acids as ligands to bind the sugar moieties of glycoproteins, we have synthesized high-performance MIPs that permits the specific recognition of glycoproteins from complex real samples such as human serum.[10b,12] Herein, ALP-imprinted MIP was prepared as an antibody mimic for the extraction of the glycoprotein ALP, while a monoclonal antibody was used to extract survivin. The imprinting efficiency and imprinting factor, two critical parameters describing the performance of the molecular imprinting approach and the obtained MIPs, were measured to be 28.8 % and 10.5, respectively (see the Supporting Information), which are excellent for molecular imprinting. The binding isotherms are shown in Figure S7. As compared with a non-imprinted extraction microprobe, the ALP-imprinted microprobe exhibited significant affinity toward the target protein. The binding isotherms obeyed a typical logarithmic function; the signal intensity increased linearly with the logarithm of the protein concentration within a certain range. The linear range spanned 4- and 5-orders of magnitude for ALP and survivin, respectively. The dissociation constant ($K_d$) was measured to be 6.3 nM for ALP-imprinted microprobe and 0.05 nM for the anti-survivin antibody-immobilized microprobe. Such excellent affinity ensured that low-copy-number proteins could be efficiently extracted from single cells. The selectivity of the extraction probes was examined, and the cross-reactivity is shown in Figure S8. The microprobes exhibited excellent specificity. Considering the logarithmic signal intensity/concentration-dependence, the cross-reactivities were very limited, even in the presence of high-abundance interferents. Compared with the antibody, the molecularly imprinted extraction probe exhibited better storage stability (Figure S9). The limit of detection (LOD) of the approach was measured to be 6.0 ×

$10^{-11}$ M for ALP and $3.0 \times 10^{-13}$ M for survivin (S/N > 3), which matches well with the expression levels in single cells. We applied the PISA approach to detect ALP and survivin in a volume ranging from 10 to 40 pL, which is comparable to the volume of mammalian cells. Figure S10 indicates that the PISA approach allowed for detecting 360 ALP molecules and 7 survivin molecules (S/N = 5).

The spatial distribution of ALP in a single human cervical cancer (HeLa) cell was examined (Figure 2a). As compared to the relatively even distribution in the control experiments shown in Figure S12, Figure 2b indicates that ALP was not evenly distributed within the cell, being less abundant in the region close to the cell membrane but relatively highly expressed within the central region. Considering protein diffusion during the in vivo extraction (3 min), the observed distribution might not be exactly the one within the cell; however, it implies that uneven distribution should be taken into account for signal readout. In later experiments, the probes were inserted into the central region of the cells with the tip close to the far-end cell membrane, and signals read from the central region of the probes were averaged. The copy numbers of survivin in normal hepatic cells (L-02) and hepatoma carcinoma cells (HepG-2) were measured by PISA assay of single cells and cell lysates, respectively (Supporting Information). As shown in Figure 2c, no survivin was found in both the single L-02 cells and the L-02 cell lysates. This is in agreement with the fact that healthy cells barely express survivin.[15b] In contrast, significant survivin expression was observed in HepG-2 cells through the PISA assays of both single-cells and cell lysates. However, the average copy number/cell value measured with single cells ($83 \pm 56$) was lower than that with the cell lysates ($1000 \pm 165$). This is reasonable because the single-cell analysis extracted only free target protein within the cells, whereas in cell lysates, many if not all of the originally bound target protein molecules could become free. Because L-02 cells do not express survivin, survivin-spiked L-02 single-cells were used to test single-molecule detection within single-cells. As shown in Figure 2d, 6 survivin molecules spiked in single L-02 cells were reproducibly detectable (S/N = 4), which validates the detection of single-molecules in single-cells using PISA. The approach was then applied to probe ALP and survivin in single cells of a variety of cancer cell lines, with standard solution and normal cells for comparison (Figures 2e,f and S16–S18). When normal cells (L-02) were analyzed, the signal intensity for ALP and survivin was comparable to the noise level, indicating that ALP and survivin were either unexpressed or limitedly expressed (Figure S18). In contrast, when HepG-2, HeLa, and human mammary cancer (MCF-7) cells were analyzed, both ALP and survivin were expressed and their expression level varied from cell to cell (Figures 2e,f and S18). The run-to-run variations for standard solutions and cell lysates were low, with RSD ranging from 8.4 to 14.3 % and 6.0 to 13.8 %, respectively (Figure S14). In comparison, the cell-to-cell RSD values for ALP and survivin in cancer cells were much higher (24.5 to 83.3 %), which can be largely attributed to cell microheterogeneity. Through the calibration curves shown in Figure S7, the signal intensities in Figures 2e and f can be roughly converted into protein concentrations (Figure S19).

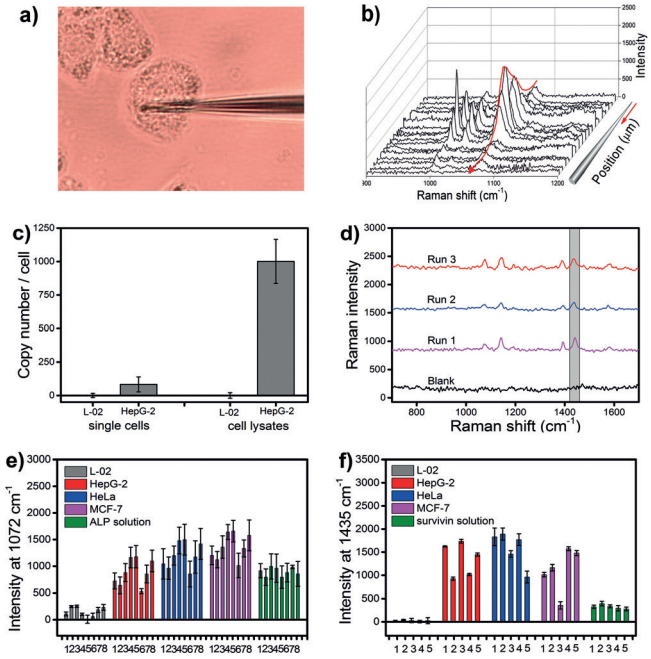


***Figure 2.*** a) An image showing an extraction microprobe inserted into a single cell. b) Spatial distribution of ALP within a single HeLa cell. c) Comparison of the copy numbers of survivin measured through PISA of single cells and cell lysates. Error bars represent standard deviations for 3 parallel measurements. d) Raman spectra showing the detectability of 6 survivin molecules within single L-02 cells (S/N = 4, each cell was injected with 100 fL of $1.0 \times 10^{-10}$ M survivin solution). e) Raman intensity of ALP in individual cells of different cell lines and ALP solutions (1.5 U L$^{-1}$, 5 μL). f) Raman intensity of survivin in individual cells of different cell lines and survivin solutions (10 pg mL$^{-1}$, 5 μL). Error bars represent standard deviations for signals from 10 spots on a single extraction microprobe.

We further investigated on survivin expression in cells along cell culture passage. We found that over culture passage of HeLa cells resulted in reduced survivin expression (Figure S20). This finding might provide a warning that over culture passage should be avoided to maintain the cells' original state.

To facilitate wider applications, we expanded the extraction probes to acupuncture needles, which allow for probing compounds facilely and minimally invasively from living bodies. ALP and survivin were found significantly expressed within HepG-2, HeLa, and MCF-7, and human gastric cancer cell (MGC-803)-bearing mice but minimally expressed within the normal cell (L-02)-bearing mice (Figures S21 and S22). Even at the beginning of cell inoculation, trace survivin within the mice was detectable (Figure S23).

In summary, we have established an approach for measuring low-copy-number proteins in single cells and living bodies. The PISA approach was simple and straightforward. If a portable Raman spectrograph is used, the acupuncture needle version of PISA can permit point-of-care and on-site assay. In addition to ALP and survivin, target proteins can be expanded to other protein biomarkers.

  

Additionally, the approach can be modified to detect other species, such as microRNAs. Therefore, this approach could be a promising tool for multiple important applications, such as cancer diagnosis, cancer prognosis, and cell quality control.

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