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Review Article

Immuno-Nanoparticles for Multiplex Protein Imaging in Cells and Tissues

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Abstract Simultaneous visualization of numerous proteins in cells and tissues is fundamental for understanding their diversity and dynamics and to address the heterogeneity of human diseases. Despite the many advantages of multiplex analysis, current methods typically rely upon immunohistochemistry, which can only be used to detect a few proteins. The exceptional optical properties of nanoparticles (NPs) have improved multiplex imaging in cells and tissues. Here, we review recent advances in multiplex imaging-based molecular diagnosis using antibody-conjugated NPs (immuno-NPs). With the aim of furthering development, we anticipate that NP-based multiplex imaging techniques will complement classical immunohistochemistry methods and provide insight into the biological diversity of cells and tissues.

Keywords: Multiplex, Imaging, Antibody, Nanoparticle, Quantum dot, SERS

Introduction

Visualizing proteins and their modifications in cells and tissues is important for understanding their physiological roles at the subcellular level[1]. Protein concentrations within a cell are highly dynamic (diversity in protein abundance within a cell spans >7 orders of magnitude[2]) and altered in response to different environmental cues, which influences the cellular phenotype, metabolic processes, and cell-to-cell variations[3]. Reliable methods for quantifying proteins in cells are being developed with studies focusing on high-quality multicolor cell images, which provide diverse information on expression levels, localization, and interactions of multiple targets (multiplex) without disrupting intact samples. This multiplex technique allows us to understand, classify and differentiate human diseases based on heterogeneous biomarkers compared to single protein-based analysis such as classical immunohistochemistry (IHC). In addition, multiplex imaging enables time-efficient high-throughput analysis, low consumption of biological samples, and high comparative ability with a low error rate.

Affinity-based multiplex protein imaging has long been used in conjunction with fluorophores or colorimetric reagents generated by dye- or enzyme-labeled antibodies, respectively[4], but classical methods are generally time-consuming, show low sensitivity, and have limited multiplicity of fluorophores (because of the low spectral separation of emission with multiple excitation wavelengths) or chromogenic reagents (because of the low high cross-reactivity among enzyme-labeled antibodies). Although smart multiplex immunoassays applicable for *in vitro* diagnostics using Luminex technology[5], immuno-polymerase chain reaction technology[6,7], or interferometric spinning-disk technology[8] have been rapidly developed and some products have been commercialized, these techniques generally require the disruption of intact cells, which may make it difficult to discern molecular signatures, particularly

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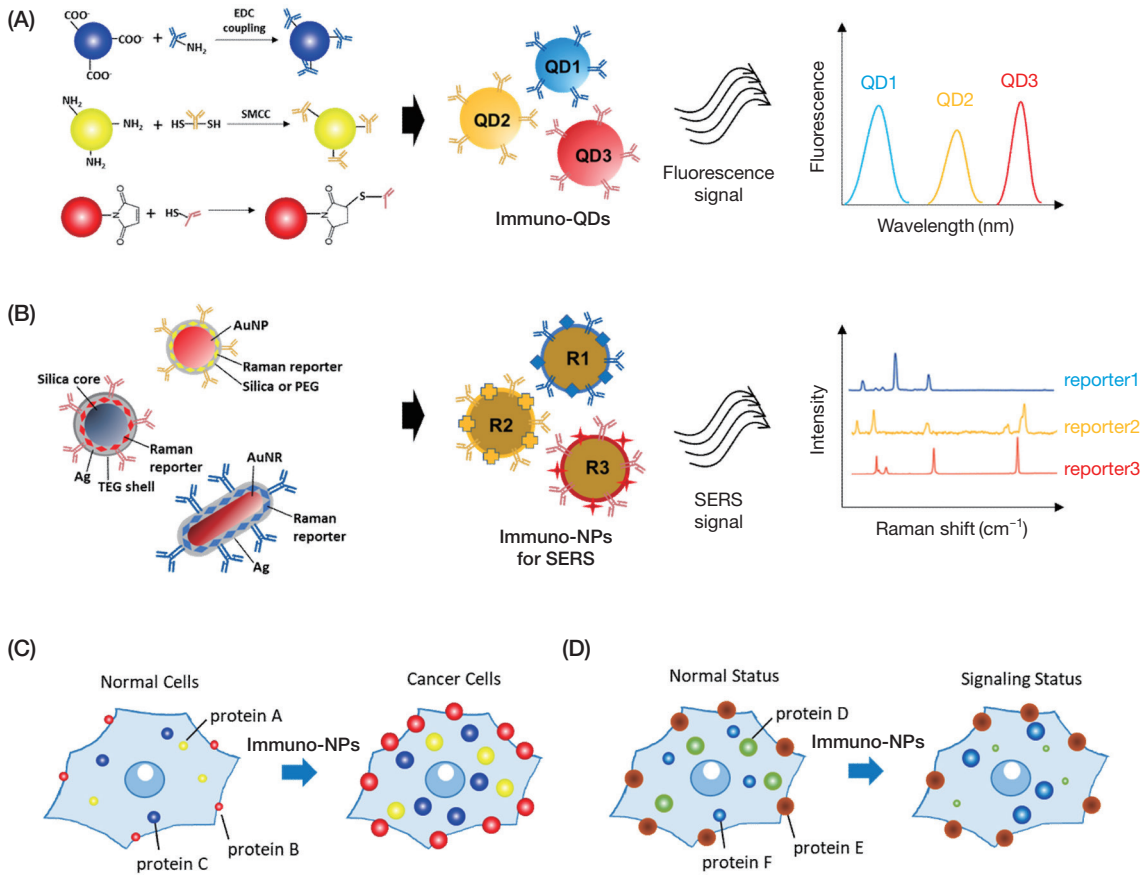

Figure 1. Schematic representation of multiplex imaging using immune-NPs. Antibody conjugation and simultaneous analysis of (A) immuno-QDs or (B) SERS immuno-NPs for multiplex imaging. Application of multiplex imaging: (C) targeting cancer cells or (D) fingerprinting intracellular signaling.

in heterogeneous cell mixtures. Therefore, multiplex protein imaging in intact cells and tissues is required to study intracellular dynamics and to observe protein heterogeneity at the single-cell level.

Over the past few decades, nanoparticles have extensively been used as imaging probes in biochemical and disease-diagnostic studies, because of their distinct physiochemical properties[9,10]. Particularly, quantum dots (QDs) and surface-enhanced Raman scattering (SERS) nanoparticles (NPs) as imaging carriers can be used for simultaneous identification of a broad range of biomolecules in cells[11-14]. Beyond classical IHC which generally targets a few of proteins using a dye-conjugated antibody within a cell, IHC based on antibody-laden nanoparticles (termed immuno-nanoparticles) makes it possible to obtain multiple color images with high spatial resolution, long-term observation, and lower photobleaching effect.

Here, we review recent advances in immuno-nanoparticles and their applications for multiplex imaging in cells to overcome existing limitations. Microbeads or nanoparticles for use in *in vitro* multiplex analysis of various analytes have been described in many review papers[15-20]. To avoid redundancy, we focused on cell and tissue-level imaging based on fluorescent QD- and SERS-enabled multiplex imaging of proteins. We also discuss improvements compared to traditional methods and issues to be solved in the future.

Immuno-Nanoparticles (Immuno-NPs) for Multiplex Imaging

As illustrated in Figure 1, we categorized multiplex protein imaging approaches using immune-NPs as QD-based fluorescence imaging (Figure 1A) and NP-based SERS imaging in cells and tissues (Figure 1B). Through distinct optical signatures and antibody-derived multiple affinity via different conjugation methods, QDs and SERS NPs enable identification of target proteins (membrane, nuclear, or cytosolic proteins) simultane-

ously, leading to a rapid molecular diagnosis of human diseases or specific signaling with high sensitivity and reliability (Figure 1C and 1D). The multiplex capability of such immune-NPs is generally greater in terms of readout time, sample consumption, long-term monitoring, and spatial resolution compared to classical methods such as western blotting and IHC.

Multiplex Protein Imaging Using Fluorescent Immuno-QDs in Cells and Tissues

Since breakthrough studies in QD-based *in vivo* imaging were reported[21,22], QD-based multiplex imaging at the subcellular level has been developed to overcome the limitations of classical western blotting and IHC. As reported previously[12,23], QDs are semiconductor nanocrystals with size-dependent optical properties and have been extensively exploited in biological applications over the past several decades. A strong advantage of QDs in multiplex imaging is that their different colors can be simultaneously generated from a single excitation wavelength without bleed-through effects, in contrast to traditional fluorophores that require multiple imaging. Compared to organic dyes and fluorescent proteins, these nanoparticles also have superb properties, including large molar extinction coefficients, high quantum yields, less photobleaching, and broad excitation and narrow emission bandwidths[24,25]. Most importantly, because it is simple to introduce functional groups on the surface of QDs, antibodies can be easily conjugated to QD via cross-linkers, enabling control of antibody valency or avidity[26-28]. Here, we describe the significant achievements of using immuno-QDs in multiplex imaging.

An initial report of QD-based imaging was published by the Quantum Dot Corporation[29]. They demonstrated the ability to simultaneously detect the cancer biomarker HER2 and nuclear antigens (NA). Double-labeling of two biomarkers was performed using mouse anti-HER2 and human anti-nuclear antigen antibodies, followed by green QD535-conjugated to anti-mouse IgG (for HER2) and biotinylated anti-human IgG and red QD630-streptavidin (for NA) in fixed SK-BR-3 cells. In addition to membrane and nuclear biomarkers, they attempted to stain microtubules or actin filaments using a biotinylated secondary antibody (or biotinylated reagent) and QD-streptavidin in fixed mouse 3T3 fibroblast cells, and demonstrated that QDs were effective for two-color fluorescence labeling of distinct cellular components. Despite these successful results, a protocol for the surface conjugation method and an optimal buffer for QDs are needed to generalize this strategy.

A breakthrough in QD-based multiplex cell imaging was made by Gao and O'Regan groups in 2007[30]. They reported QD-based quantitative and simultaneous profiling of five biomarkers in breast cancer cells and clinical tissue specimens. QD525, QD565, QD605, QD655, and QD705 were directly conjugated to primary antibodies against HER2, ER, PR, EGFR, and mTOR, respectively, followed by confocal imaging analysis in two fixed breast cell lines (MCF-7 and BT474) and tumor biopsy samples. Figure 2 shows the QD image-based expression profile of three representative biomarkers (ER, PR, and HER2) in three breast cancer cell lines, which showed a good agreement with the results of western blotting. Due to the discernible signatures of several biomarkers, the expression profile of proteins by QD-based spectroscopy enabled breast cell lines and tumor biopsy samples to be discriminated based on each fluorescence emission peak. Most importantly, QD-based analysis was more accurate, quantitative, and detectable at low protein concentrations compared to traditional IHC using the same set of breast cancer cell lines. This is primarily because of the remarkable photostability of QDs, leading to extended exposure to excitation lights and reliable reconstruction of high-resolution 3D projection. Furthermore, because most cancer tumors are highly heterogeneous, this QD-based molecular profiling technology is very effective at the single-cell level and can be generally applied to clinical samples using a classical fixation protocol. For more general usability, the preparation for immuno-QDs (antibody-conjugated QDs via active ester maleimide-mediated amine and sulfhydryl coupling) must be simplified to facilitate the determination of molecular fingerprints in clinical samples. This multiplex concept was further validated by Song *et al*. using hypermulticolor single cell imaging cytometry by immune-QDs[31]. When simultaneous monitoring and quantitative estimation of four targeted receptors (EGFR1, HER2, ER and PR) was carried out by QD-based multiplex imaging cytometry, breast cancer cell lines (MCF-7, SK-BR-3, JIMT-1, and HCC-70) were quantitatively classified into each subtype based on the expression profile of four receptors. Importantly, this method verified the remarkable heterogeneity of primary cells in biopsied tissues from breast cancer patients, indicating the great potential for cancer prognosis and classification of tumor heterogeneity. For clinical assays, Wang's group reported QD-based IHF imaging for multiplexing cancer biomarker detection on formalin-fixed and paraffin-embedded (FFPE) tissues[32]. When QDs labeled with secondary antibody were used to image elongation factor 1α (EF1α, housekeeping protein) and survivin (target protein expressed

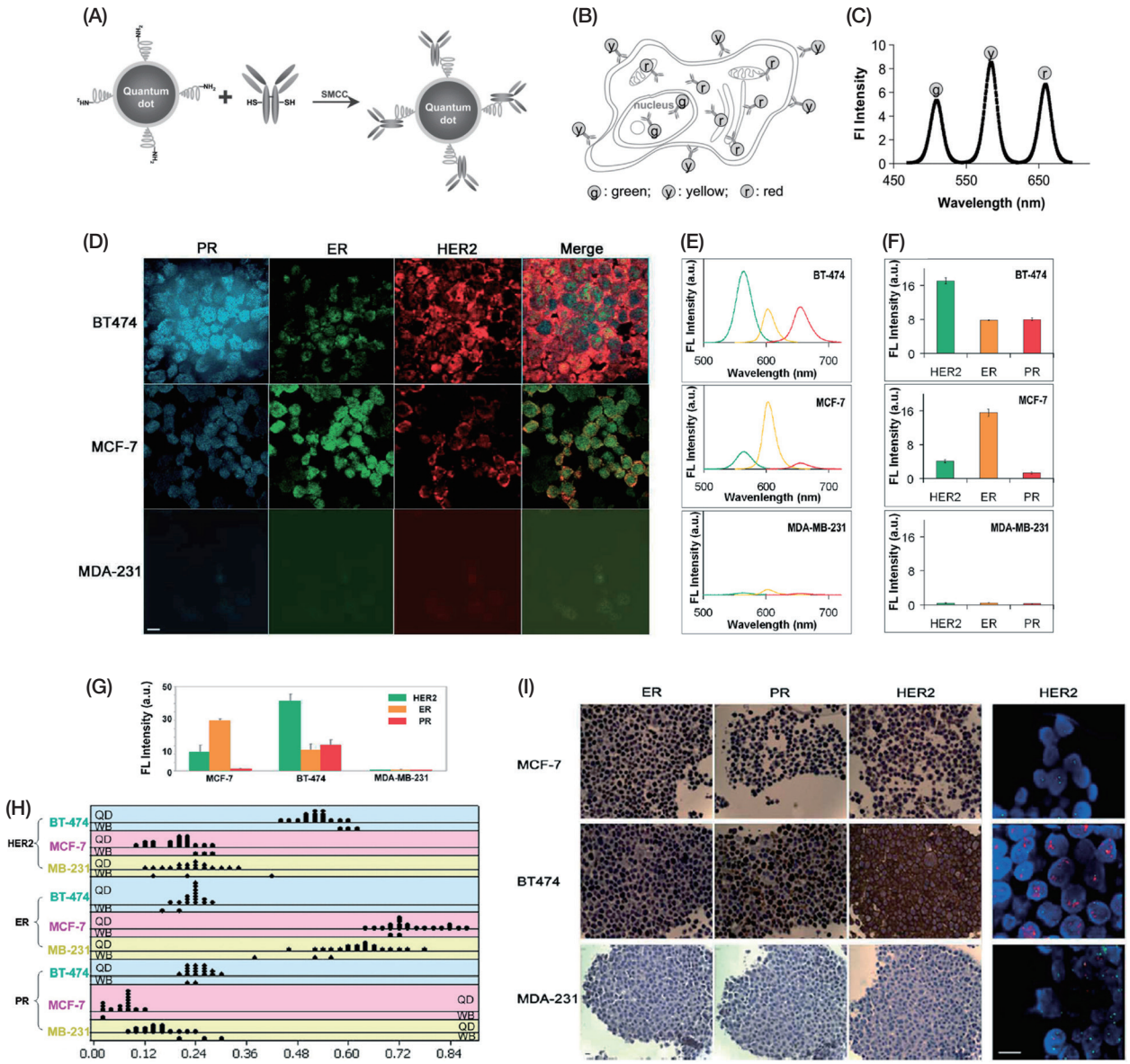

Figure 2. QD-based multiplex imaging of three biomarker proteins in breast cancer cells. (A) Schematic illustration of preparing for antibody-conjugated QDs via SMCC crosslinker. (B) Cell imaging with three QDs targeting PR, ER, and HER2 after fixation. (C) Quantification of biomarker expression using wavelength-resolved spectroscopy. (D) Multiplex image of ER, PR, and HER2 expression in MCF-7, BT-474 and MDA-MB-231 cell lines detected by fluorescence microscopy. (E) Representative spectra of single cell spectroscopy measurement of the protein biomarkers. (F) Average expression levels of HER2, ER and PR determined from the results of spectral analysis of 100 single cells. (G) Western blot of ER, PR, and HER2 in MCF-7, BT-474 and MDA-MB-231 cells. (H) Comparative statistical analysis of QD-based profiling data with western blotting results, showing a significant correlation between the two techniques. (I) Expression of ER, PR and HER2 estimated by IHC in the breast cancer cell lines (left panels) and amplification of the HER2 gene in breast cancer cell lines assessed by FISH (right panel, scale bar is 10 μm). Adapted with permission from the reference[30].

in cancerous cells) in FFPE tissue, a high signal ratio of surviving (QD620) to EF1α (QD530) was observed in cancerous tissue, whereas there was no significant difference in fluorescence between QD620 and QD520 in surrounding tissues, enabling cancer diagnosis with high fidelity using multiple biomarkers. Significantly, QDs were stable for at least 5 months on FFPE tissue, whereas organic dyes were photobleached shortly after exposure to light.

Recently, QD-based multiplex imaging in conjunc-

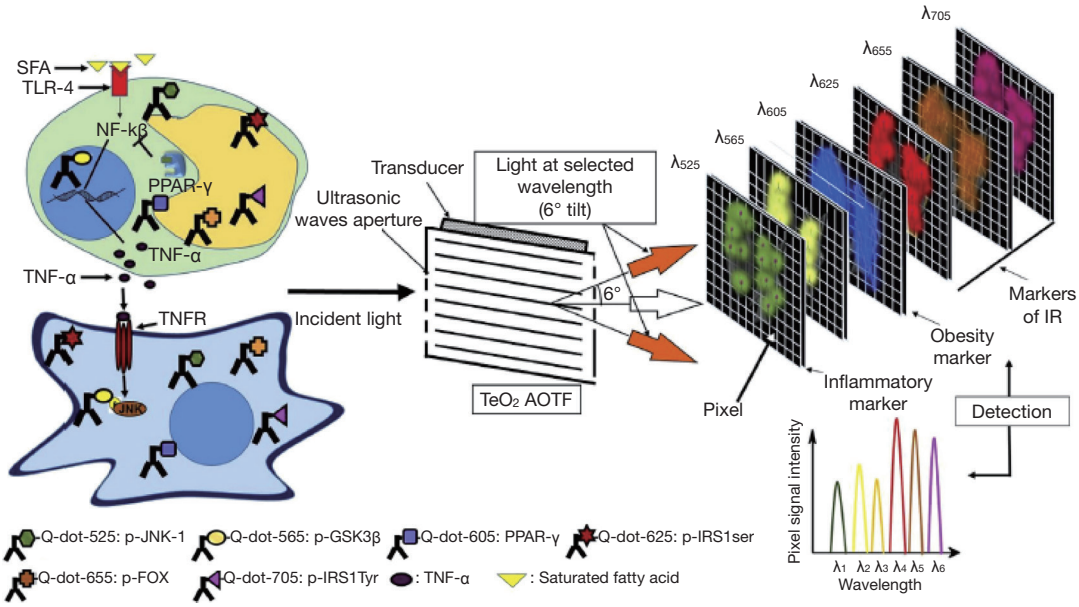

Figure 3. Six-colored multiplex imaging using antibody-conjugated QDs with AOTF in adipocyte and HepG2 cells. QDs with six different colors (QD525, QD565 QD605, QD625, QD655, and QD705) were conjugated to primary antibodies against pJNK-1, p-GSK3β, PPAR-γ, p-IRS1ser, p-FPXO1, and p-IRS1tyr, respectively. Profiling of six signaling molecules that functioned as biomarkers to monitor obesity-induced insulin resistance between adipocyte and HepG2. Adapted with permission from the reference[33].

tion with acousto-optic tunable filters (AOTF) was reported by Song's group to investigate multiple signal molecules using only a small number of fixed cells[33]. As shown in Figure 3, six different signaling proteins including peroxisome proliferator-activated receptor gamma (PPAR-γ), phosphorylated c-Jun N-terminal kinase 1 (p-JNK-1), phosphorylated glycogen synthase kinase 3β (p-GSK3β), phosphorylated insulin receptor substrate-1 at the serine residue (p-IRS1ser), phosphorylated forkhead box protein O1 (p-FOXO1), and phosphorylated insulin receptor substrate-1 at the tyrosine residue (p-IRS1tyr) were simultaneously observed at the single-cell level to monitor obesity-induced insulin resistance between adipocyte and HepG2 cells. To monitor insulin-dependent signaling changes by QD-based imaging, six immuno-QDs were synthesized: QD525-p-JNK-1, QD565-p-GSK3β, QD605-PPAR-γ, QD625-p-IRS1ser, QD655-p-FOX, and QD705-p-IRS1tyr. By utilizing AOTF to effectively avoid the spectral overlap of neighboring emission, QD-based multicolor imaging was achieved in this study. This method suggests that the QD-antibody sensing probe based on AOTF is an excellent tool for multiplex fluorescent imaging analysis. High expression of PPAR-γ (obesity marker) in adipocytes negatively regulates NF-κB-induced TNF-α expression, leading to the secretion of inflammatory cytokines from adipocytes and inhibition of insulin-signaling in HepG2 cells. When PPAR-γ was expressed after treating adipocytes with palmitic acid and high glucose, sequential phosphorylation of JNK (inflammation marker) and IRS1ser (insulin marker) was induced in HepG2 cells, leading to downstream signaling (e.g. increased p-GSK3β and p-FOXO1 to facilitate the conversion of glucose into glycogen in the liver). When insulin-signaling was prolonged for 8 days with palmitic acid and high glucose, immuno-QD-based imaging showed that insulin treatment to increase p-IRS1tyr, p-GSK3β and p-FOXO1 was not effective on the 8th day, indicating that the TNF-α level was significantly increased in adipose cells alone but not in hepatic cells after long-term administration of palmitic acid and high glucose (i.e. adipocytes may be the major mediators of obesity-induced insulin resistance). Most significantly, because the number of primary cells is low and their culture is difficult, this immune-QD-based multiplex imaging, which can be performed in a small fraction of primary cells, is expected to be very effective for monitoring the pathway of intracellular signaling focused on targeted proteins. In addition to the rapid development of *in vitro* immunoassay technologies, this multiplex imaging system is powerful for tracing protein expression and modification in different cell types.

Multiplex Protein Imaging Using Immuno-NP-based SERS in Cells and Tissues

Compared to the high sensitivity and stability of fluorescent QDs, SERS provides greater sensitivity for molecular analysis. In principal, the signal enhancement can occur in proximity to a nanostructured free-electron metal such as Au, Ag, or Cu, by which surface plasmons create an enhanced electromagnetic (EM) field. Over the past decade, metal nanoparticles (with bare or core-shell structures) have made SERS more powerful and general for clinical diagnostics with high sensitivity[34]. The use of SERS for imaging is advantageous compared to fluorescence because SERS generates continuous and strong scattering light without blinking or photobleaching[35,36], which is an inherent bottleneck in fluorescence-based measurement. For multiplex SERS imaging, multiple Raman tags (or reporters) can be conjugated to antibodies on metal NPs, enabling multiplex imaging.

Cho's group developed antibody-conjugated fluorescent SERS (F-SERS) composed of silver NP-embedded silica spheres with fluorescent dye and specific Raman labels[21], which was employed for multiplex targeting and imaging of apoptosis (using BAX and BAD antibodies or Annexin V) in fixed cells and tissues. To effectively detect molecular events in cells, SERS-based multiplex imaging was combined with the tracking ability of fluorescence. This F-SERS method was further investigated to detect multiple targets in bronchioalveolar stem cells (BASCs) in the murine lung, where silver-doped silica NPs encoded with three Raman reporters (mercaptotoluene (MT), benzenethiol (BT), and naphthalenethiol (NT)) and three antibodies against CD34, Sca-1, and SP-C were used to identify BASCs[37]. Because three cellular proteins, are highly expressed in BASCs, F-SERS-based multiplex imaging was used for cellular characterization through quantitative analysis of three biomarkers with high sensitivity, which is not easily assessed using conventional fluorescent probes. Similar work has been reported by Choo's group[38], where dual mode nanoprobes consisting of SERS and fluorescence were fabricated by conjugating CD24 and CD44 antibodies on silica-encapsulated gold nanoparticles (AuNPs) with Raman reporters (MGITC and Rubpy) and fluorescent dyes (FITC and RuITC). With rapid detection by fluorescence, SERS imaging fingerprinted the co-localization of CD24 and CD44 in breast cancer cells (MDA-MB-231), demonstrating that multiplex imaging can be effectively improved by combining the two methods. It was recently reported by Lee and Jeong's groups that the fast imaging capability of fluorescence and multiplex capability of SERS enabled reliable identification of breast cancer cells in a mouse orthotopic model using an optical fiber bundle for intraoperative endoscopic system with F-SERS dots targeting HER2 and EGFR[39].

Independently of fluorescence measurement, the ability of SERS for multiplex imaging was initially validated by Gambhir's group at Stanford University[40]. When 10 different types of Raman active molecules provided by Oxonica Materials were adsorbed onto a 60-nm diameter Au core coated with silica and tested as a function of NP concentration *in vivo*, they obtained 5 different SERS images of natural accumulation in the liver in a living mouse. Notably, there was a linear correlation between the increased Raman signal and increased concentration of SERS nanoparticles in living mice, but antibody-based targeting study was not performed in this work. In addition to the *in vivo* usability of SERS multiplex imaging, Pezacki *et al*. demonstrated duplex SERS imaging of surface proteins at the cellular level[41]. Using 4-(mercaptomethyl) benzonitrile and d7-mercaptomethyl benzene-functionalized silver NPs, they conducted a double surface receptor imaging to detect β_2-adrenergic receptor and caveolin-3 on the surface of rat cardiomyocytes.

Choo's group detected three different types of breast cancer biomarkers (EGFO, ErbR2, and IFG-1) by SERS immuno-NPs[38]. They showed that localization of an electromagnetic field through pinholes in hollow AuNPs increased the SERS imaging properties, leading to improved multiplex imaging with the three types of SERS immuno-NPs, bearing the Raman reporter molecules MGITC, RBITC, and RuITC (Figure 4). When the immuno-NPs were used to treat different breast cancer cell lines (MDA-MB-468, KPL4, and SK-BR-3), SERS mapping imaging allowed for simultaneous rapid identification of cell phenotypes and quantification of marker proteins in the single-cell level simultaneously, which was well-correlated with the western blotting results.

The multiplex capability of SERS was greatly improved by the research groups of Zharov and Bris[42], where circulating tumor cells were successfully detected by a newly designed SERS probe in unprocessed human blood. They developed super-enhanced SERS and photothermal (PT) resonances using silver-gold nanorods (AuNR/Ag) with narrow SERS and high photothermal contrast, which enhanced the SERS signal by more than two orders of magnitude compared to traditional gold nanorods. As shown in Figure 5, immuno-nanorods (NRs) were composed AuNR/Ag, four different unique organic Raman molecules (4-mercaptobenzoic acid [4MBA], *p*-aminothiophenol [PATP],

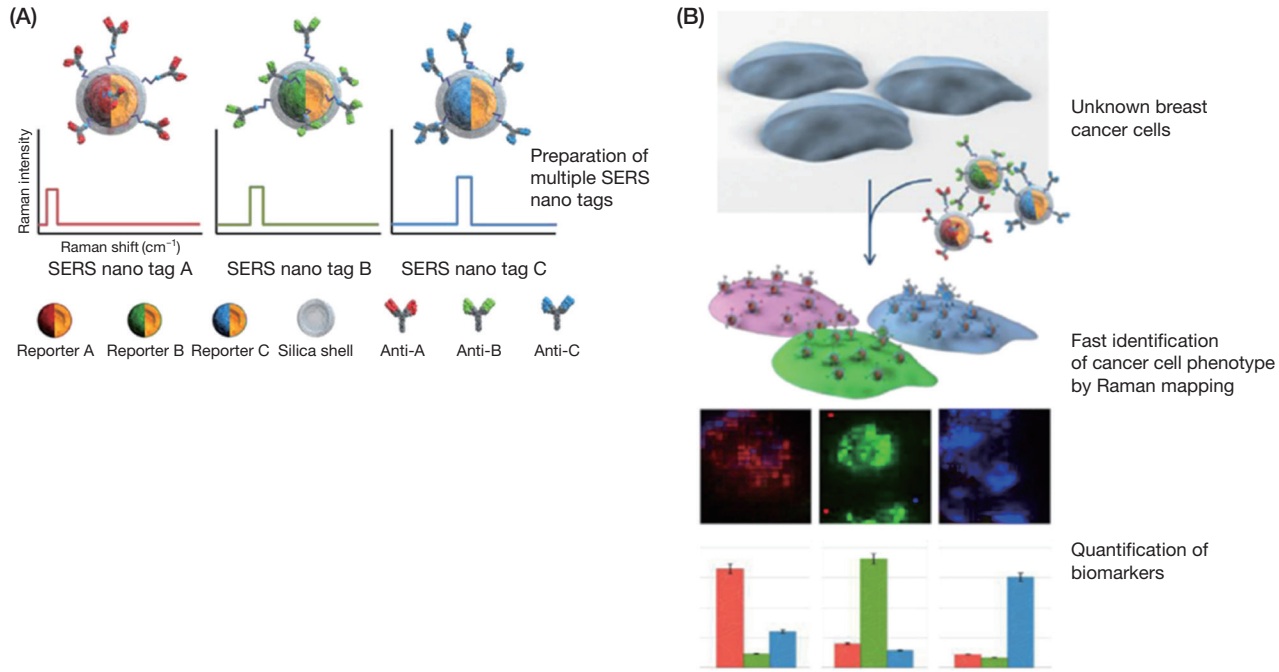

Figure 4. SERS-based multiplex imaging of receptors in breast cancer cells. (A) Preparation of Si-encapsulated SERS nanotags bearing three different Raman reporters and antibodies. (B) Multiplex detection and quantification of protein markers on the cell surface using the SERS imaging technique. Adapted with permission from the reference[38].

p-nitrothiophenol [PNTP], and 4-(methylsulfanyl) thiophenol [4MSTP]) and antibodies of four breast cancer biomarkers (epithelial cells adhesion molecules; EpCAM, CD44, keratin, and insulin-like growth factor antigen (IGF-I receptor β). When immuno-NRs were used to treat MCF-7 cell-containing blood samples, only one MCF-7 cell among 90,000 fibroblast cells was clearly detected without enrichment by simultaneous multicolor SERS multicolor imaging and PT-based super imaging, whereas no signal was observed in blood cells, indicating that this approach is sensitive for detecting circulating tumor cells in the blood with super specificity. Notably, this approach has great potential to distinguish specific and nonspecific binding by enhancing spectral recognition using nonoverlapping, ultra-sharp SERS multispectral 2D mapping and PT plasmonic resonance. However, the relatively long process time and requirement for equipment for multimodal and multiplex imaging must be improved.

In addition to identifying cancer cells, the multiplex SERS technique has been implemented to differentiate mouse embryonic stem cells (mESCs) and detect teratoma, as recently reported by Chang's group[43]. Among 54 different Raman reporters based on cyanine derivatives from CyR library compounds, they selected three reporters (CyRLA-572, Cy7LA, and Cy7.5LA) from strong near-infrared SERS intensity using AuNPs. These Raman reporters were strongly absorbed on the surface of AuNPs by chemisorption, which was followed by conjugating CD34, CD184, and Notch1 antibodies that specifically target their respective receptors on the cell surface of the mesoderm, endoderm and ectoderm. Using multiplex SERS mapping imaging, mESCs as well as teratoma formed in mESCs were simultaneously identified even in a mouse xenograft model. Because this study was focused on the new design of a near-infrared Raman reporter, developed multiplex imaging is expected to be more useful for *in vivo* analysis rather than the intracellular detection of proteins.

Future Perspective

In addition to multiplex imaging using immuno-QDs and SERS immuno-NPs, cyclic immunofluorescence methods[44] and multiplex epitope-based imaging techniques[45] have been developed as powerful methods for improving multiplex analysis in cells and tissues. Despite the rapid advances in multiplex protein imaging techniques, a fundamental limitation is that these methods can be applied only to fixed cells rather than to living cells. The methods described in this review can be combined effectively with well-defined fixed samples to improve the sensitivity and multiplex capability

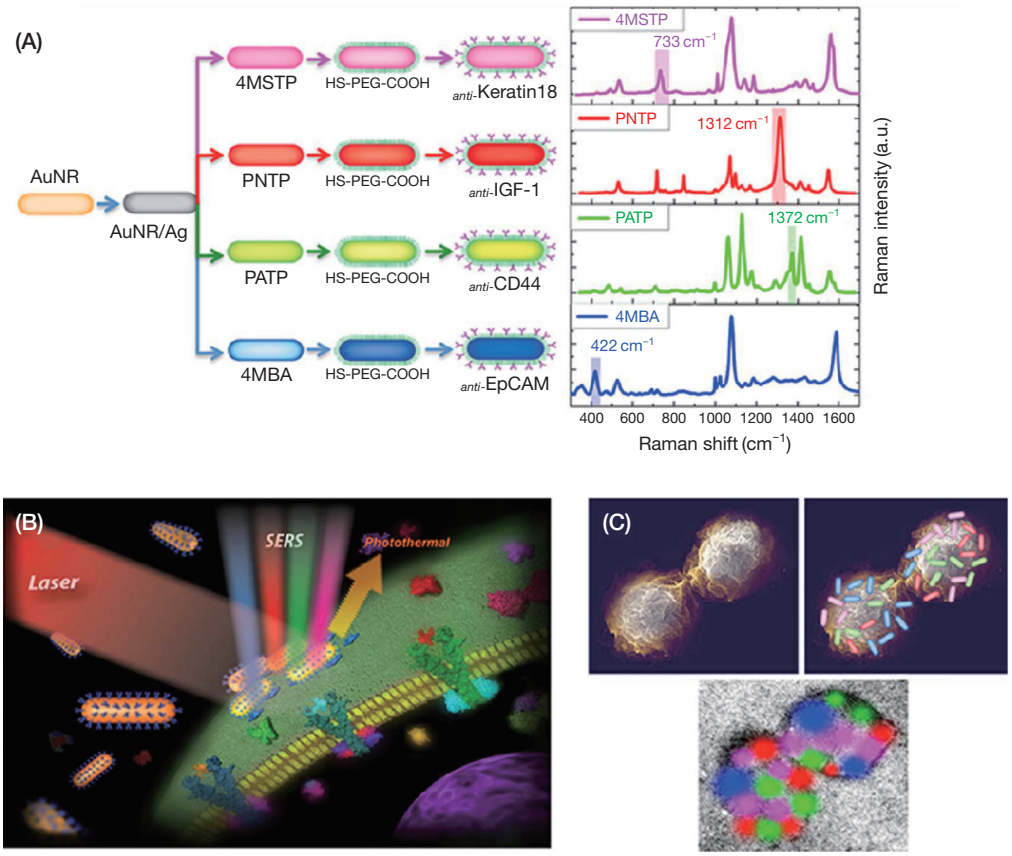

Figure 5. (A) Schematics of preparation steps and Raman spectra for four SERS immuno-NRs integrating AuNR/Ag/Raman tags with antibodies against 4 different biomarkers: AuNR/Ag/4MBA/anti-EpCAM (blue), AuNR/Ag/PNTP/anti-IGF-1 receptor β (red), AuNR/Ag/PATP/anti-CD44 (green), and AuNR/Ag/4MSTP/anti-Keratin18 (magenta). (B) Schematics of multiplex SERS/PT imaging to target breast cancer cells. (C) Schematics of 2D multi-color SERS data by the distribution of immuno-NRs on the cell surface. Adapted with permission from the reference[42].

compared to classical IHC or western blotting; nonetheless, multiplex imaging in living cells is required for understanding the diversity and dynamics of many proteins during time-resolved processes. Along with improved microscopic techniques at the single-cell level as well as advanced nanotechnology, antibody engineering[46], development of aptamers against cell receptors[47,48], and site-specific labeling methods[49,50], this issue is expected to be resolved in further studies. For example, several strategies for addressing this issue have been developed including optomechanical sensing techniques[51,52] and imaging process techniques[53,54], which will have great potential in living cells and *in vivo* imaging. In addition to proteins or their modifications, recent reports of transcriptome imaging by multiplex RNA profiling are notable achievements in the multiplex imaging of single cells[55,56], which will have a great effect on understanding mRNA or non-coding RNA profiling in response to environmental cues[57]. Through current and further studies, we anticipate that multiplex imaging techniques will facilitate fundamental analysis of molecular dynamics as well as clinical applications for disease diagnosis.

Conclusion

In this review, we described immuno-NPs with unprecedented optical properties, which have led to remarkable improvements in multiplex protein imaging in cells and tissues. Although their multiplicity must be evaluated in living cells, QD-based fluorescence and SERS imaging has enabled simultaneous detection of multiple proteins with high sensitivity and selectivity even in a small number of cells, which is not generally achieved by using classical IHC and western blotting. We anticipate that multiplex imaging techniques based on immuno-NPs will provide new opportunities for studying the dynamics and clinical implications of biomolecules in cells and tissues.

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