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PERSPECTIVE

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Heejin Lim[1] and DaeWon Moon[1,2,a)]

AFFILIATIONS

[1]Department of New Biology, DGIST, 333, Techno Jungang-daero, Dalsung, Daegu 711-873, South Korea
[2]School of Undergraduate Studies, DGIST, 333, Techno Jungang-daero, Dalsung, Daegu 711-873, South Korea

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[a)]**Electronic mail:** dwmoon@dgist.ac.kr

ABSTRACT

Single protein imaging and understanding their interactions are of paramount importance to understand the life phenomena. Recently reported multiplex protein SIMS imaging methodology using metal-oxide nanoparticle conjugated antibodies can be extended to a single protein imaging methodology using He ion microscopy (HIM). It is proposed here that single protein can be imaged in the microscale and the nanoscale by the complementary use of SIMS and HIM.

I. INTRODUCTION

After the development of cluster ion beam technology, lipid imaging by SIMS using gas cluster ions and the MS/MS capability has been successful.[1,2] The pioneering protein SIMS imaging using an isotope-labeled antibody has been successfully applied for cancer tissues,[3,4] which requires high ion dose oxygen ion beam bombardment that causes extensive ion beam damage. Recently, we reported multiplex protein SIMS imaging using metal-oxide nanoparticle conjugated antibodies that allow multiplex protein imaging on cell membranes at a low ion dose without any ion beam damage problem.[5]

In this report, we propose that protein SIMS imaging using antibody-conjugated nanoparticles can be extended to nanoscopic helium ion microscopy (HIM) imaging for single protein imaging. Complementary microscopic and nanoscopic protein imaging by SIMS and HIM can be a new and powerful bioimaging platform for innovative biological and medical science.

II. EXPERIMENT

A. Cell culture and QD (Quantum Dot) labeling of ErbB2 on A549 lung cancer cells and SKBR3 breast cancer cells

All materials for sample preparations were purchased from Thermo Fisher Scientific (Life Technologies) unless indicated otherwise. A549 human lung carcinoma cells (ATCC, CCL-185) and SKBR3 human breast cancer cells (ATCC, HTB-30) were cultured on a 12 mm diameter cover glass at 37 °C under 5% CO_2 in an incubator and grown overnight in a culture medium [Roswell Park Memorial Institute (RPMI-1640) containing L-glutamine supplemented with 10% fetal bovine serum (FBS), 50 μg/ml penicillin–streptomycin and 100 μg/ml neomycin (Sigma Aldrich) for A549, and ATCC-formulated McCoy's 5a Medium modified (ATCC) containing 10% FBS for SKBR3]. The QD labeling of ErbB2 on cells was performed with reference to the protocols described by Dahmke *et al.*[6] Cells were rinsed once in phosphate buffered saline (PBS) and fixed with 10% formalin (Sigma Aldrich) at RT for 10 min. After being washed twice with PBS, the cells were rinsed once in GS-BSA-GEL-PBS [1% goat serum (GS), 1% bovine serum albumin (BSA) fraction V (Sigma Aldrich), and 0.1% cold water fish skin gelatin (Sigma Aldrich) in PBS (*p*H 7.4)] and then incubated in the same solution for 15 min to block unspecific binding of biotin-conjugated anti-ErbB2-affibodies (Abcam, ab31890, ErbB2-Aff-B). Next, the cells were washed twice with PBS and incubated in 200 nM ErbB2-Aff-B in GS-BSA-GEL-PBS at RT for 30 min followed by three washes with PBS. After being incubated in 10 nM streptavidin-conjugated Qdot 655 (Q10121MP, SV-QD) in 6% BSA-PBS at RT for 1 h and then washed three times in PBS, the cells were imaged by fluorescence microscopy. After that, the cells were washed once with PBS and fixed with 2% glutaraldehyde (Electron Microscopy Sciences) at RT for 10 min. A control sample

was prepared by following the same procedure as above except for the incubation of ErbB2-Aff-B.

B. Graphene capping and air-plasma treatment

Based on our established process,[7,8] QD-labeled cells were subjected to graphene capping for scanning electron microscopy (SEM) and HIM imaging and then to subsequent air-plasma treatment for time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging. CVD graphene grown on Cu foil was purchased from Graphene Platform, Japan. Low-quality graphene on the backside of the Cu foil was etched away by O_2 plasma reactive ion etching (RIE) followed by etching the Cu foil in 0.1M ammonium persulfate solution (DAEJUNG, South Korea) for 4 h. The graphene was rinsed in distilled water (DW) several times. After being washed four times in DW, the cells were capped with graphene and dried in air. Graphene and organic impurities on the cell-cultured glass were subsequently removed by air-plasma treatment using a plasma chamber (Femto Science Inc., South Korea) at 50 kHz, 100 W, and 70 sccm of air for 1 min.

C. Time-of-flight secondary ion mass spectrometry

ToF-SIMS analysis was conducted on a ToF-SIMS 5-100 instrument (ION-TOF, Münster, Germany) using a pulsed 30 keV Bi_3^+ primary ion beam for positive ion ToF-SIMS images over a $500 \times 500\,\mu m^2$ area with 256×256 pixels. Low-energy electrons were supplied onto the surface of the sample using an electron flood gun for charging compensation during analysis. Internal mass calibration for ToF-SIMS spectra was performed using the peaks of H^+, H_2^+, CH_3^+, and Na^+ for the positive ion mode before further analysis.

D. Fluorescence microscopy, SEM, and HIM

QD-labeled fixed cells in PBS were imaged using a confocal microscope (Olympus, FV1200, Japan) in a fluorescence mode using a 635 nm laser at 20 mW. For graphene-covered cells, SEM images were acquired using a field emission scanning electron microscope (Hitachi, SU8020, Japan) with an accelerating voltage of 3 keV and a beam current of 10 μA. HIM measurements were performed on an Orion NanoFab instrument (Carl Zeiss, USA) using the following settings: beam energy of 30 keV, beam current of 0.5–1.0 pA, a gas field ion source aperture size of 20 μm, a scan dwell time of 5 μs, and scan number averages of 64. An electron flood gun was used for the charge compensation of the samples.

III. RESULTS AND DISCUSSION

For the analysis of membrane proteins across a wide range of aspects using different analytical techniques, epidermal growth factor receptor ErbB2 in the plasma membrane was specifically labeled with an affibody,[9] which was subsequently coupled to the CdSe–ZnS core-shell QD of 15–20 nm in size via the biotin–streptavidin interaction.[6,10] Cellular samples were prepared based on our established method using graphene capping and subsequent air-plasma treatment for improved ToF-SIMS imaging of cellular membranes as described in Sec. II. In ToF-SIMS analysis, we selected Cd^+ to represent QD on account of its relatively high intensity and low background from both cells and the glass substrate. Figure 1 shows ToF-SIMS images of phosphocholine and Cd^+ for A549 cells on a cover glass. QDs were distributed throughout the surface of both cells and the glass substrate when only treated with QDs without ErbB2 affibody labeling [Fig. 1(a)]. In contrast, both phosphocholine and Cd^+ signals mainly came from cells [Fig. 1(b)], which suggest that ErbB2 protein was successfully labeled with QD and that the distribution of lipids and proteins in the cell membrane could be observed simultaneously using the QD labeling of membrane proteins and ToF-SIMS imaging. However, the protocol should be further optimized for practical applications by reducing the background of cover glass due to the nonspecific binding of QDs to improve the contrast of Cd^+ signals.

The spatial resolution of ToF-SIMS at hundreds of nanometers means that it has limitations in the nanoscale imaging of single proteins labeled with QDs. Instead, HIM with surface sensitivity and a subnanometer resolution[11,12] was utilized to image the nanoscale distribution of single proteins on the plasma membrane. Since single proteins over a microscale area of the cell membrane are to be imaged by HIM, a series of processes should be performed from the microscopic imaging of a large area by fluorescence microscopy and SEM to the nanoscopic imaging of a small area by HIM for a QD-labeled SKBR3 cell as shown in Fig. 2. QD-labeled fixed cells were observed by fluorescence microscopy for the rapid assessment of QD labeling of ErbB2 on cells as shown

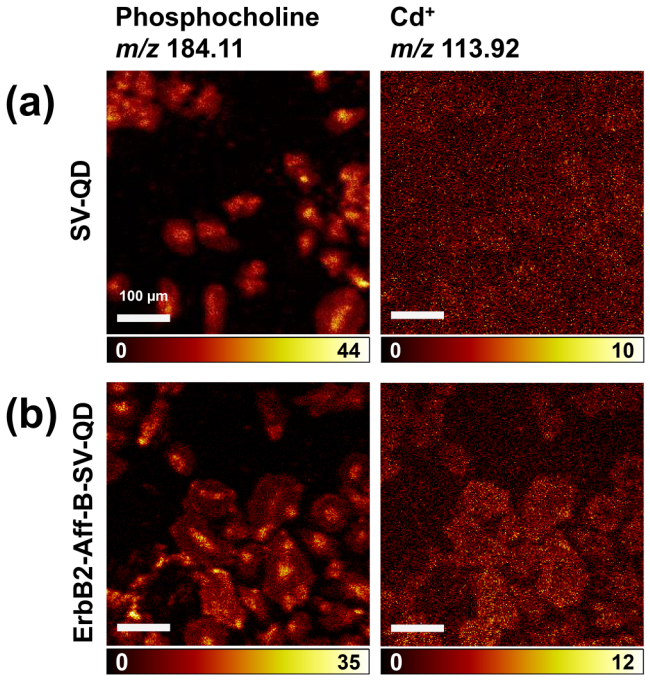

FIG. 1. ToF-SIMS images of phosphocholine at $C_5H_{15}NPO_4$, *m/z* 184.11 (left), and Cd^+ at *m/z* 113.92 (right) for A549 cells only treated with SV-QD as control (a) and for those of which the ErbB2 proteins were labeled by ErbB2-Aff-B-SV-QD (b). Scale bars: 100 μm [(a) and (b)].

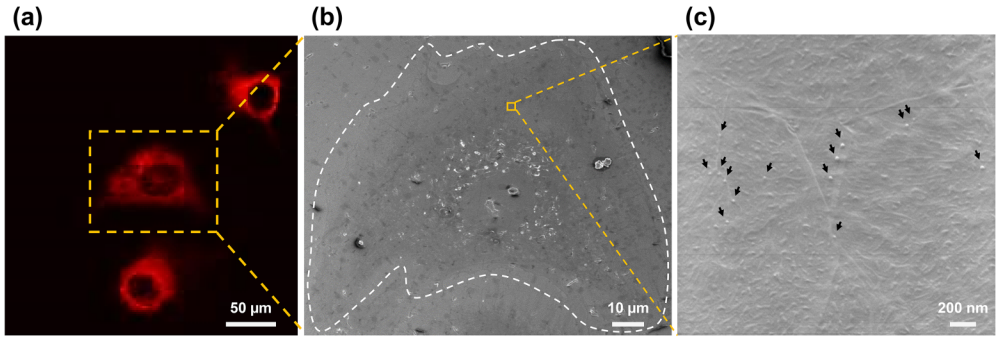

FIG. 2. Correlative imaging of QD-labeled SKBR3 cells using fluorescence microscopy, SEM, and HIM. (a) The fluorescent image shows that QDs were specifically labeled on ErbB2 of the cells. (b) Acquired SEM image for the same cell as indicated with yellow lines in (a) after additional chemical fixation and graphene capping. (c) Magnified HIM image clearly shows that a few 15–20 nm QDs were distributed on the cell membrane indicating a single ErbB2 protein for each QD as indicated with the black arrows. Scale bars: 50 (a), 10 (b), and 200 nm (c).

in Fig. 2(a). After additional chemical fixation and graphene capping, graphene-covered cells were imaged by SEM [Fig. 2(b)] over the same region as in Fig. 2(a), which was zoomed in on by HIM afterward to clearly show both the precise distribution of single QD-labeled ErbB2 proteins with no aggregation of QDs and the detailed surface features of the cell membrane as shown in Fig. 2(c). The observed QD size in the HIM image is consistent with that from the supplier. The implication of the nanoscopic distribution of QDs on the SKBR3 cell membrane is unclear and requires further investigation.

IV. CONCLUSIONS

A QD-labeled affibody was used for microscopic and nanoscopic protein imaging using SIMS and HIM, respectively. A QD-labeled affibody was used for ErbB2 imaging on the SKBR3 cancer cell membrane. HIM images of a QD-labeled SKBR3 cell showed the distribution of single QDs, which shows the position of each ErbB2 protein. Here, we report complementary microscopic protein imaging based on SIMS and confocal fluorescence microscopy and nanoscopic single protein imaging based on HIM for the same cultured cell specimen, which may open a new imaging platform methodology for innovative biomedical science.

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