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Water Droplet-in-Oil Digestion Method for Single-Cell Proteomics

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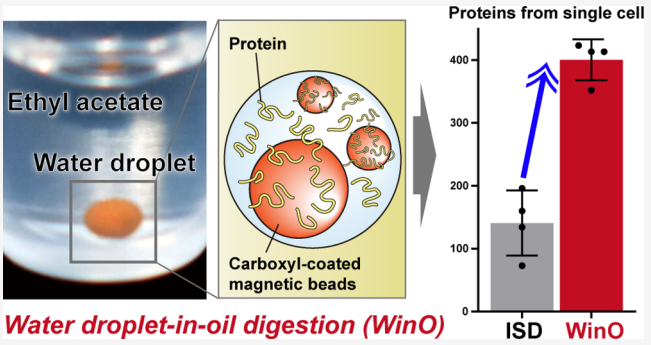
ABSTRACT: Recent advances in single-cell proteomics highlight the promise of sensitive analyses in limited cell populations. However, technical challenges remain for sample recovery, throughput, and versatility. Here, we first report a water droplet-in-oil digestion (WinO) method based on carboxyl-coated beads and phase transfer surfactants for proteomic analysis using limited sample amounts. This method was developed to minimize the contact area between the sample solution and the container to reduce the loss of proteins and peptides by adsorption. This method increased protein and peptide recovery 10-fold. The proteome profiles obtained from 100 cells using the WinO method highly correlated with those from 10,000 cells using the in-solution digestion method. We successfully applied the WinO method to single-cell proteomics and quantified 462 proteins. Using the WinO method, samples can be easily prepared in a multi-well plate, making it a widely applicable and suitable method for single-cell proteomics.



1. INTRODUCTION

In recent decades, single-cell omics has become an important analytical technique in several research fields that has brought new perspectives to cancer genomics,[1,2] tissue development,[3] and cellular differentiation.[4,5] The genome and transcriptome are currently the main targets of single-cell omics studies. Quantitative amplification and next-generation sequencing enable high-throughput single-cell epigenetic and transcriptional analyses. Proteins are important biomolecules playing a major role in biological phenomena. Furthermore, because protein expression levels are reportedly difficult to predict based solely on mRNA expression levels,[6,7] there remains a need to measure protein expression directly with proteomics.

For single-cell proteomics, high recovery of proteins and peptides, as well as high throughput, is required. To quantify proteins by proteomics, extracted proteins are digested into peptides by enzymes and then analyzed by nano-liquid chromatography−tandem mass spectrometry (nanoLC-MS/MS). Additionally, no current method can amplify proteins. Hence, it is critical to reduce adsorption losses during sample preparation and to enhance protein extraction and digestion in single-cell proteomics. Several sample preparation methods, such as single-cell proteomics by mass spectrometry (SCoPE-MS),[8−10] nanodroplet processing in one-pot for trace samples (nanoPOTS),[11,12] and automated processing in one pot for trace samples (autoPOTS),[13] can dramatically improve the sample recovery rate and sensitivity of MS for single-cell proteomics. Using these approaches combined with state-of-the-art LC-MS systems, the number of proteins identified from a single cell was dramatically increased. SCoPE-MS is based on multiplexing with a tandem mass tag (TMT) reagent where small amounts of samples are mixed with a carrier containing large amounts of peptides, thus reducing sample loss during LC injection. In addition, the greater signal intensity of peptides from the carrier proteome can increase to the number of MS/MS triggers. SCoPE-MS has recently been upgraded to SCoPE2;[14] this upgrade has improved peptide recovery and throughput owing to reduced sample volume in the pretreatment step[9] or by pretreatment in a 300 pL solution formed on a slide.[10] The nanoPOTS method uses a specially fabricated nano-well chip and a liquid handling system for digestion. These devices were designed to process the sample in a small volume to reduce protein and peptide adsorption loss.[11,12,15] The performance of NanoPOTS has improved over time in terms of versatility, sensitivity, and reproducibility by combining a nested nano-well chip with a cellenONE commercial liquid dispensing instrument.[15] The cellenONE system has also been used in proteoCHIP-based single-cell

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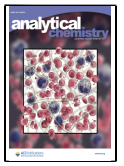

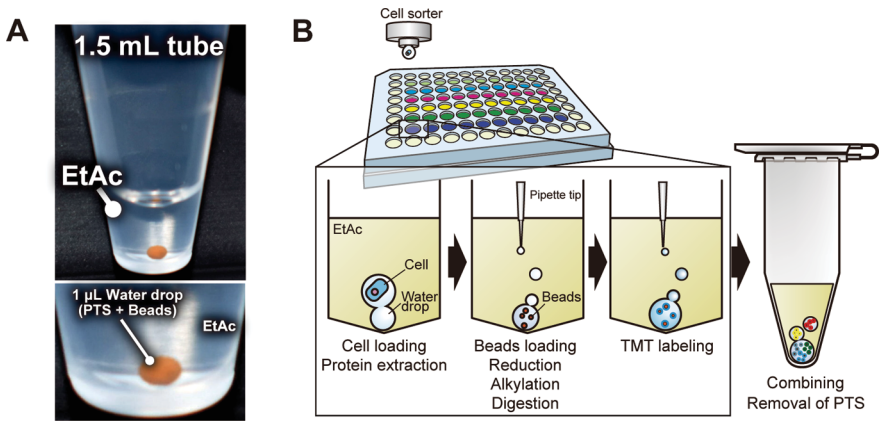

Figure 1. Water droplet-in-oil digestion (WinO) method. One microliter of water droplet containing 0.125 units of benzonase, 3.3 μg of magnetic beads, phase transfer surfactants (PTS, 12 mM sodium deoxycholate and 12 mM sodium lauroyl sarcosinate), and 100 mM TEAB (pH 8.5) formed in ethyl acetate (EtAc) (A). The workflow of the WinO method is presented in panel (B); the cells are loaded into water droplets in ethyl acetate using a cell sorter. The solutions for reduction, alkylation, and digestion are added to the ethyl acetate. The peptides are labeled with TMT reagents and then combined. The peptides are purified using the StageTip and injected into the nanoLC-MS/MS.

proteomics.[16] In this method, the sample solution—in the nanoliter range—is covered with hexadecane to prevent evaporation. The autoPOTS methods rely on commercial devices and equipment to eliminate all sample transfer steps.[13] Sample preparation is performed in the presence of an MS-compatible surfactant,[17] *n*-dodecyl-β-D-maltoside (DDM), to reduce sample loss via adsorption by blocking the adsorption of proteins on plastic surfaces.[18] DDM has also been used in other approaches.[11,16,18,19] The nanoPOTS and autoPOTS methods eliminate all sample transfer steps, thereby reducing sample loss through adsorption. While these advances enable single-cell proteomics, technical challenges remain with respect to increased recovery rate, versatile application, and increased throughput.

Herein, we report a simple and highly efficient sample preparation method for single-cell proteomics that prepares samples in a water droplet, termed water droplet-in-oil digestion (WinO). This new method reduces sample loss during single-cell protein preparations and increases the number of identified proteins compared with the in-solution digestion (ISD) method. The WinO method improves current single-cell proteomic methods and can enhance the throughput and protein identification from single-cell sampling.

2. METHODS

2.1. Sample Preparation Using the ISD Method. First, 1 μL of extraction buffer (50 mM triethylammonium bicarbonate (TEAB), 12 mM SDC, 12 mM SLS, and 0.125 units benzonase) was added into low protein-binding 96-well plates (Sumitomo Bakelite, Tokyo, Japan) or into low protein-binding 1.5 mL tubes (Watson, Tokyo, Japan). Cells were sorted into each well and centrifuged at 300*g* for 1 min at 25 °C to mix the extraction buffer and the cells. The reduction and alkylation were performed by adding 1 μL of 100 mM dithiothreitol (DTT) and 550 mM iodoacetamide (IAA) solution (each in 50 mM TEAB), respectively. Proteins were digested with Lys-C for 3 h at 37 °C, followed by trypsin incubation for 16 h at 37 °C. Enzymes were prepared with 50 mM TEAB; 50 ng of the enzyme was used to digest 100 cells, and 0.5 ng was used for a single cell (1 μL of solution used). The plates were sealed with an adhesive plate seal for every incubation. After digestion, 4 μL (40 μg) of the TMT reagent in 0.5% acetic acid and 50% acetonitrile was added, and samples were incubated for 60 min at 25 °C. TMT10-plex or TMT11-plex was used. The experimental design for TMT labeling is shown in Table S1. The pH of the sample solutions during TMT labeling was approximately pH 8. To quench the TMT reaction, 1 μL of 30% hydroxylamine was added to each well, and samples were incubated for 15 min at 25 °C. Subsequently, the surfactants were removed using the phase transfer method.[20,21] Briefly, sample solutions, including ethyl acetate, were combined and acidified with trifluoroacetic acid (TFA) to give a final concentration of 0.5%. The combined samples were mixed by vortexing and then centrifuged at 15,600*g* for 2 min. Ethyl acetate, including surfactants, was discarded. The peptides were purified using an SDB-XC StageTip.[22,23] The peptide solution eluted from the StageTip was dried and redissolved in 10 μL of 0.1% TFA, and 5 μL was used for the measurement. For peptide fractionation, the sample solution was dried and then dissolved in 300 μL of 0.1% TFA. Fractionation was performed using a High pH Reversed-Phase Peptide Fractionation Kit in accordance with the instructions for TMT-labeled peptides provided along with the kit. The peptides on the column were sequentially eluted into nine fractions, including a wash fraction. All fractions were dried, and the peptides were dissolved in 0.1% TFA. The peptides in the wash fraction were purified using an SDB-XC StageTip.[22,23]

2.2. Sample Preparation Using the WinO Method. For the WinO digestion, 50 μL of ethyl acetate was added into the wells of a 96-well plate before adding 1 μL of extraction buffer (50 mM TEAB, 12 mM sodium deoxycholate (SDC), 12 mM sodium lauroyl sarcosinate (SLS), and 0.125 units of benzonase). Cells were sorted into each well and spun at 300*g* for 1 min at 25 °C to mix the extraction buffer droplets and cell droplets. After 30 min of incubation at RT, 1 μL (3.3 μg) of carboxyl-coated Magnosphere beads equilibrated with 50 mM TEAB was added to each well. From the reduction step with 100 mM DTT, the sample preparation was performed as per the ISD method. The solutions were added into ethyl acetate.

For preparing the carrier sample, 10,000 cells were sorted into a 1.5 mL tube. To extract proteins from sorted cells, equal volume of 2 times concentration of phase transfer surfactant

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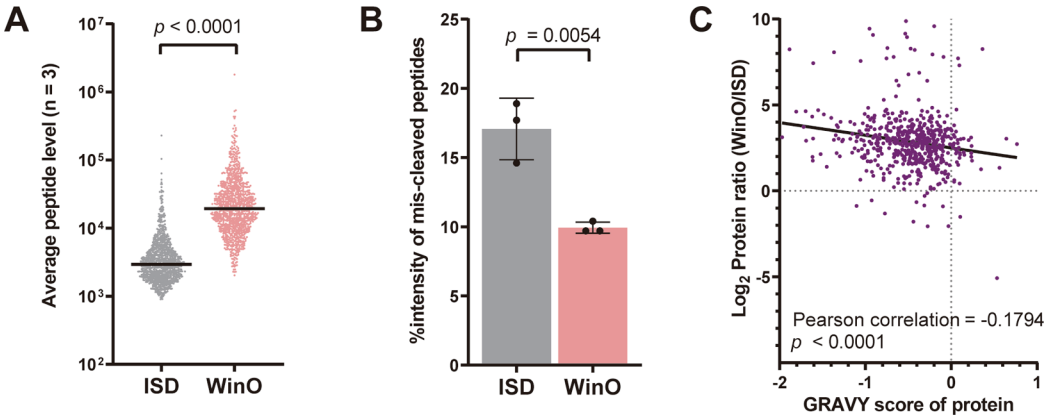

Figure 2. Comparison of the in-solution digestion (ISD) and WinO methods. As the starting material, 100 RPMI8226 cells were sorted and digested in triplicate using ISD and WinO methods. Half of the prepared samples were analyzed by nanoLC-MS/MS. The scatter plot shows the levels of 1015 peptides quantified using these digestion methods (A). Peptide levels are represented as the average of triplicate data. Each bar shows the median. The reporter ion intensity for each TMT channel was used as the peptide level. This peptide level did not include the intensity from the carrier channel. The proportion of mis-cleaved peptides in the ISD and WinO methods is shown in panel (B). These proportions were calculated based on peptide levels and averaged across triplicate samples. Error bars indicate standard deviation. The correlation between the GRAVY protein score and relative protein levels from the WinO to the ISD method is shown in panel (C). In this correlation, 561 proteins commonly quantified by both methods are shown.

(PTS) solution (100 mM TEAB, 24 mM SDC, 24 mM SLS, and 0.25 units of benzonase) was added to cells, and samples were incubated at room temperature for 30 min. The reduction, alkylation, and digestion were performed in accordance with the ISD method. The peptides were labeled with 120 μg of the TMT reagent, and the labeling reaction was quenched by adding 5% hydroxyl amine; this resulted in a final concentration of 0.5% of hydroxyl amine. The carrier sample corresponding to 5000 or 50 cells for 100 or single-cell proteomics, respectively, was mixed with the ISD and WinO samples.

3. RESULTS AND DISCUSSION

3.1. Effect of the WinO Method on the Recovery of Small Samples. In this study, SDC and SLS were used for protein extraction. These surfactants have been reported to enhance protein extraction and digestion efficiencies of Lys-C and trypsin.[20,21] In addition, SDC and SLS are known as PTSs, which can be removed from peptide solutions by a phase transfer method.[20,21] To enhance the protein extraction from cells, heating, ultrasonication, and freezing/thawing are generally used. In the WinO method, these treatments are not possible because ethyl acetate is volatile or because sample solution and ethyl acetate are mixed during these treatments. Hence, we assessed differences in extraction efficiency between ultrasonication and heating in the PTS solution and a solution prepared by mixing cells in the PTS solution (Figure S1A). The protein concentration was quantified using the BCA assay. The peptide and protein analyses were performed on a TripleTOF 5600 using DIA. The protein amount, number, and intensity of quantified proteins and peptides were comparable between the two methods. Therefore, ultrasonication and heat treatment were not used for the protein extraction process in this study. Importantly, the efficiency of protein extraction by the sonication and heating free extraction was decreased in fixed cells (Figure S1B). Magnosphere beads, DTT, IAA, Lys-C, and trypsin solutions prepared in 50 mM TEAB were added to ethyl acetate (Figure 1B). These solutions formed water droplets in ethyl acetate, which merged with the sample droplet.

To examine whether the proteins and peptides were retained in the water droplet in ethyl acetate, 10 μg of HEK293 whole cell lysate or 10 μg of digested peptide solution was added into ethyl acetate and incubated for 24 h. Ethyl acetate and water droplets were collected, and the distribution of each fraction was confirmed by SDS-PAGE and nanoLC-MS/MS for proteins and peptides, respectively. As controls, whole-cell lysates or peptide solutions were also used for SDS-PAGE or nanoLC-MS/MS along with the treated samples. In addition, unloaded samples without proteins or peptides were prepared as negative control samples. To examine protein retention in water droplets suspended in ethyl acetate using SDS-PAGE, smears larger than 100 kDa and smaller than 25 kDa were detected in the ethyl acetate fraction in the CBB-stained gel (Figure S2A). These smears were also detected in the ethyl acetate fraction in the unloaded negative control samples. No smear was detected at a position corresponding to a molecular weight of 25 kDa in the negative staining (Figure S2A). In addition, no protein band was detected at a position corresponding to this smear. No other protein bands were detected in the ethyl acetate fraction of the protein-loaded group. Next, we examined the distribution of the peptides in ethyl acetate and the sample droplet (Figure S2B). No significant difference was observed in the total peak area of the peptides detected in the control and water droplet fractions (p = 0.5734). The percent composition of the peptide peak area quantified in the ethyl acetate fraction was only 0.12%. The total peak area of peptides in this fraction showed no significant difference from that in the ethyl acetate fraction of the negative control (p = 0.0561). These results suggest that proteins and peptides are retained in water droplets for at least 24 h.

The WinO method was performed as described above, but with the addition of beads to evaluate the effect on sample recovery. In the ISD method, surfactant solution was added into the well of a 96-well plate before 100 cells were injected into the solution using a cell sorter. After digestion, TMT-labeled peptides corresponding to 5000 cells as carriers were combined with the 100-cell samples from the ISD and WinO methods. The carrier was used to increase peptide and protein

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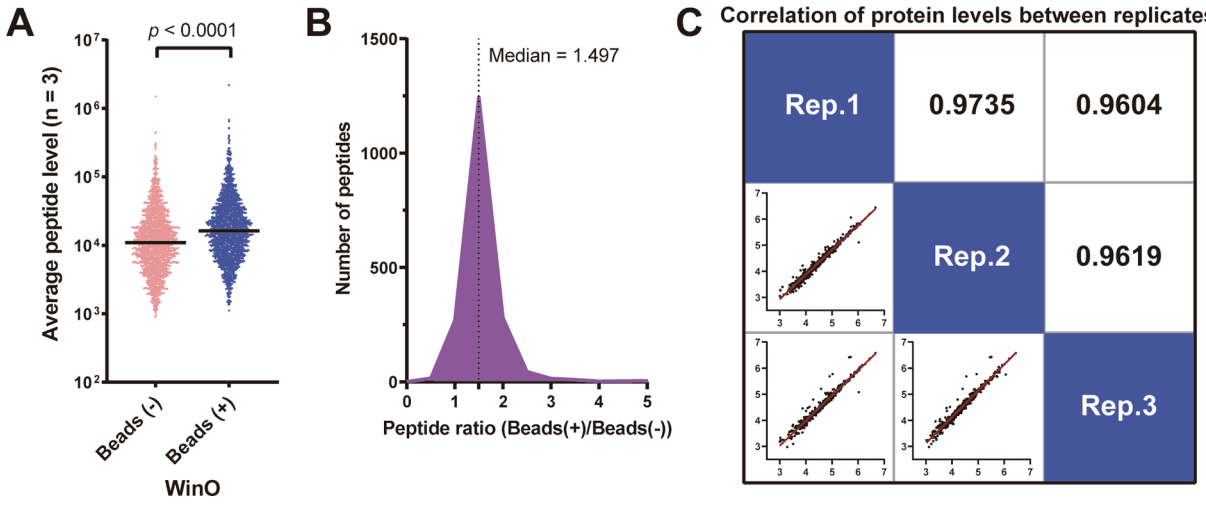

Figure 3. Effect of carboxyl-coated magnetic beads on the WinO method efficiency. One hundred RPMI8226 cells were sorted and digested in triplicate. The WinO method was performed with and without magnetic beads. Half of the prepared samples were analyzed by nanoLC-MS/MS. Peptide levels are presented as the average of triplicate data. The scatter plot shows the levels of 1898 peptides quantified using both methods (A). Each bar shows the median. The reporter ion intensity for each TMT channel was used as the peptide level. This peptide level did not include the intensity from the carrier channel. The distribution of relative peptide levels between the WinO method with and without beads is shown in panel (B). Correlations of protein levels between replicates are shown in panel (C).

identification and to reduce peptide loss after digestion as in SCoPE-MS.[8] The peptide and protein analyses were performed using an Orbitrap Fusion Tribrid mass spectrometer. The peptide intensity in the WinO method was significantly higher than that in the ISD method ($p < 0.0001$; $n = 3$; Figure 2A). Next, we compared the recovery of the peptides quantified in all data. The intensity of 1018 out of 1177 peptides (86.5%) increased significantly (≥2-fold, $p < 0.05$) in the WinO method, whereas the intensity of none of the peptides increased significantly in the ISD method (Figure S3A). The median relative peptide recovery from WinO was 6.70-fold greater than that from the ISD method. The number of quantified peptides increased only 1.3-fold ($p = 0.0199$) when the WinO method (2071.7 ± 34.4) was used compared to the ISD method (1598.3 ± 215.9). We counted the quantified proteins. The triggering of MS2 was assisted by the carrier, which reduced the difference in the number of detected proteins between the two methods. To examine the reproducibility of the WinO method, we compared the percent coefficient of variations (CVs) of peptide levels in triplicate between the two methods. The %CV distribution pattern using the WinO method was lower than that with the ISD method (Figure S3B), with median values of 39.6 and 14.4% for ISD and WinO methods, respectively.

Next, we evaluated the digestion efficiency of the WinO method by measuring the levels of mis-cleaved peptides. Lys-C and trypsin solutions were delivered to the sample droplets through ethyl acetate in the WinO method. It was previously reported that 8.03 g of ethyl acetate was dissolved in 100 mL of water at room temperature.[24] It was expected that the dissolved ethyl acetate would affect the activity of Lys-C and trypsin. However, mis-cleavage of the total peptides in the WinO method (9.9 ± 0.4%) was significantly lower than that in the ISD method (17.1 ± 2.2%) (Figure 2B). In addition, the improvement in peptide recovery from fully cleaved peptides was significantly higher ($p < 0.0001$) than in the mis-cleaved peptides using the WinO method (Figure S3C). Contrary to expectations, the digestion efficiency of the WinO method was higher than that of the ISD method. The activities of proteolytic enzymes are enhanced in the presence of organic solvents, such as methanol, isopropyl alcohol, and acetonitrile.[25] It is likely that the dissolved ethyl acetate in the sample droplet enhanced Lys-C and trypsin activities in the WinO method. Moreover, the reduction in the adsorption loss of Lys-C and trypsin maintained a high enzyme concentration in the sample droplet. Although the recovery of proteins was overall improved with the WinO method, a significant negative correlation ($r = -0.1794$, $p < 0.0001$) was observed in protein hydrophobicity, as evidenced by the grand average of hydropathy (GRAVY) score and protein recovery (Figure 2C). A greater score indicates a more hydrophobic protein/peptide in the GRAVY score. The results indicated that the WinO method led to a higher recovery of hydrophilic than of hydrophobic proteins.

Based on these results, we speculated that the 100-cell protein and peptide recoveries were enhanced with the WinO method due to the reduced contact surface area between the sample solution and plastic tubes, as well as the improved digestion efficiency of trypsin and Lys-C. However, the improvement in hydrophobic protein recovery was lower than that in hydrophilic proteins, possibly due to the lower retention of these proteins in the ethyl acetate solution than of hydrophilic proteins.

3.2. Effect of Carboxyl-Coated Magnetic Beads on Peptide Recovery Using the WinO Method. To enhance the recovery of hydrophobic proteins and peptides, we tried to retain them in the sample droplet using beads. To select beads with high peptide recovery for the WinO method, we examined six different types of beads: amine-, methyl sulfonate-, sulfopropyl-, and three carboxyl-coated beads. The carboxyl-coated beads tended to show a higher total intensity of proteins than other bead types, and carboxyl-coated Magnosphere beads showed the highest recovery among all beads tested (Figure S4). Single-pot, solid-phase-enhanced sample preparation (SP3)[26] and protein aggregation capture (PAC)[27] have been reported as methods of retaining peptides on beads. In SP3 and PAC, peptides are captured on the beads in an organic solvent based on the hydrophilic interaction. In

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this study, proteins and peptides were retained on the beads in aqueous solution. Hence, we assumed that proteins and peptides were retained on the beads via ionic interactions rather than hydrophilic interactions. In addition, it is likely that the hydrophobic parts of proteins and peptides had a high affinity with the beads because of the hydrophobic material of Magnosphere beads. We performed proteomic analysis of the 100 sorted cells using the WinO method with or without beads in triplicate (Tables S5 and S6 for peptides and proteins, respectively). In the desalting step, the beads were loaded onto the SDB-XC StageTip with sample solution together to purify the peptides. The Orbitrap Fusion Tribrid mass spectrometer was used for peptide and protein analyses. The peptide level significantly increased in WinO samples combined with beads compared to WinO samples without beads ($p < 0.0001$; Figure 3A). Figure 3B shows the distribution of relative peptide levels ($n = 1898$) in the WinO samples processed with beads compared to those without. The median peptide ratio was 1.497. The peptide ratio of 1825 out of 1898 peptides (96.2%) was higher in samples prepared with the beads than in those without beads. Moreover, there was no significant difference in the percentage of cleaved peptides (Figure S5A), suggesting that the addition of beads did not affect Lys-C or trypsin activities. The reproducibility of the WinO method was evaluated by comparing the protein levels from triplicate analyses (Figure 3C). The Pearson correlations for all pairs were higher than 0.96, indicating that the reproducibility of peptide quantification was unaffected by the presence of beads. Thus, we combined the beads with the WinO method in subsequent experiments.

We characterized proteins and peptides that exhibited improved recovery after addition of beads. The hydrophobicity of proteins did not correlate with their recovery (Figure S5B); in other words, the protein recovery rate improved independently of their hydrophobicity. Next, the Spearman correlation coefficient was calculated by comparing the recovery rate of peptides with the frequency of each amino acid and its GRAVY score (Figure 4). A significant positive correlation was observed between the GRAVY score and peptide recovery ($r = 0.0810$, $p = 0.0004$). In addition, the frequency of basic amino acids (H, K, and R) showed the highest coefficient ($r = 0.1067$, $p < 0.0001$; Figure 4 and Figure S5C), whereas the frequency of acidic amino acids (D and E) showed the lowest coefficient ($r = -0.1162$, $p < 0.0001$; Figure 4 and Figure S5C). The recovery of basic and hydrophobic peptides, which had a high affinity for beads under basic conditions, was improved by addition of beads. Although acidic amino acids showed a negative correlation coefficient with peptide recovery, the peptide and protein recoveries improved overall in the WinO samples combined with beads (Figure 3A).

3.3. Comparison of the Proteome Profiles Obtained with the ISD and WinO Methods. We evaluated whether the proteome profiles obtained by the WinO method were comparable to those obtained using the conventional ISD method. ProteoCHIP technology has been reported as a method for single-cell proteomics using oil.[16] In this method, the oil is layered over samples to prevent evaporation, and the pretreatment reagents, such as trypsin, are added through the oil. However, it is still not clear whether protein solubilization and digestion occur in water droplet-in-oil, as in the ISD method. To examine the similarity of the proteome profiles between these two methods, we compared the proteome

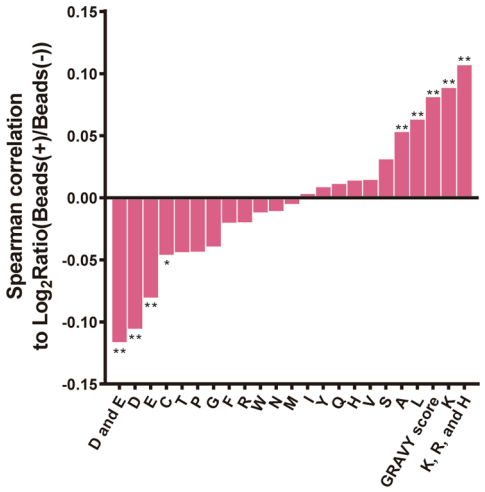

Figure 4. Relationship between peptide sequence and recovery in the WinO method with carboxyl-coated magnetic beads. The Spearman correlation coefficient was calculated by comparing the recovery rate of peptides with the WinO method with or without beads. The frequency of each amino acid and GRAVY score is presented for the corresponding peptides. The one-letter amino acid code is indicated on the *X* axis. ** indicates $p < 0.01$; * indicates $p < 0.05$.

profile of 100 cells processed with the WinO method with that of 10,000 cells processed with the ISD method using 15 multiple myeloma cell lines (Figure S6). As starting materials, 10,000 or 100 cells from 15 multiple myeloma cell lines were sorted and digested in triplicate or quadruplicate. The digested peptides were labeled with TMT reagents and mixed. The peptides and proteins were analyzed using the Orbitrap Fusion Tribrid mass spectrometer. From the 100-cell group, 2183.6 ± 74.5 peptides were quantified on average (Table S7), whereas 29,293.0 ± 561.2 peptides were quantified from the 10,000-cell group (Figure S7A and Table S8). From these peptides, an average of 592.9 ± 13.2 proteins was quantified from the 100-cell group (Table S9), whereas an average of 4651.6 ± 91.6 proteins was quantified from the 10,000-cell group (Figure S7B and Table S10). In total, 798 proteins were quantified from the 15 strains using the WinO method, among which 387 proteins were found in all cell lines. Using the ISD method with 10,000 cells, 5545 proteins were identified, with 3584 proteins found in all cell lines. Next, we compared the expression profiles of the 377 proteins that were quantified in both methods. Normalized expression levels were plotted using the UMAP algorithm (Figure 5). As the preparation methods and cell counts used in the proteomic method were different, it was possible that the ion count detected on MS, the ratio of enzyme to substrate, the contact area with the vessel, and the difference in the vessel material affected the proteome profile. However, the proteome profiles of 100 and 10,000 cells were plotted close to each other and formed populations among the same cell line, suggesting that the proteome data obtained with the WinO method were comparable to those obtained using the ISD method.

Gene ontology (GO) analysis of 798 proteins identified ribosomal proteins, proteasome-related proteins, and enzymes of the central carbon metabolism system (Figure S8). In addition to these abundant proteins, transmembrane proteins (TMPs) and cell adhesion-related proteins were identified. To examine the effect of the WinO method on the recovery of TMPs, we compared the distribution of the number of

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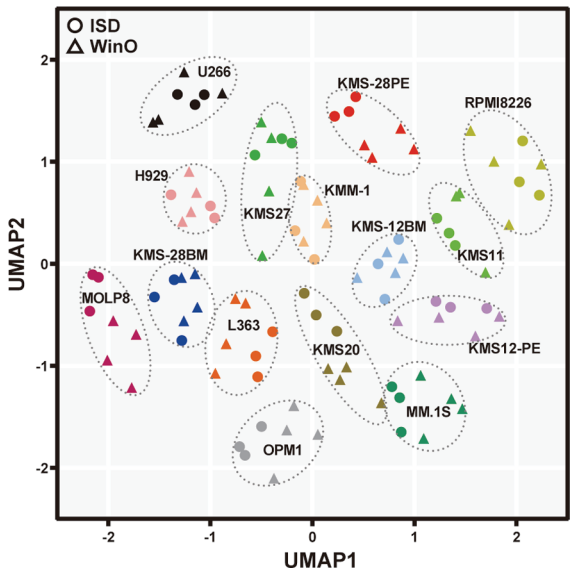

Figure 5. Comparison of the proteome profiles obtained with the ISD and WinO methods. The proteome data obtained with the ISD and WinO methods were plotted using UMAP. UMAP was performed using the umap package in R. Circles and triangles show the 10,000-cell and 100-cell proteomics data, respectively.

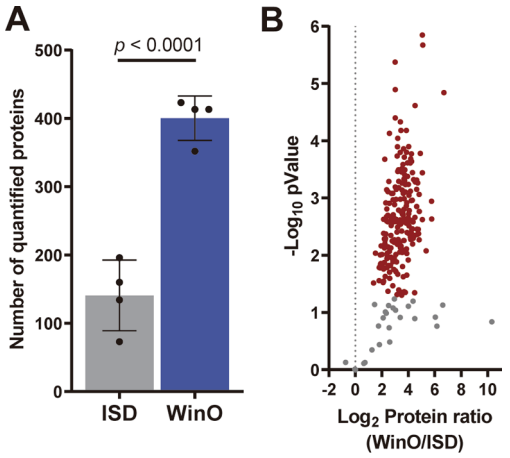

Figure 6. Application of the WinO method to single-cell proteomics. Number of proteins quantified using the ISD or WinO method (A). The graphs plot the average number of quantified proteins and the standard deviation of quadruplicate data. Protein levels detected from the WinO and ISD samples are compared in a volcano plot (B). Red dots indicate proteins with a significant ($p < 0.05$) change of 2-fold or more.

transmembrane domains (TMDs) between the 832 TMPs identified with the ISD method using 10,000 cells and the 70 TMPs identified by the WinO method using 100 cells (Figure S9). TMPs are some of the most difficult proteins to identify using proteomics, and proteins with more TMDs are generally more difficult to extract and identify.[28,29] The WinO method uses the PTS as protein extraction developed for membrane proteomics.[20,21] WinO identified not only proteins with a single TMD but also those with more than 10 TMDs. These results indicated that the WinO method using PTS can identify both soluble and membrane proteins.

3.4. Single-Cell Proteomic Analysis Using the WinO Method. Finally, we examined the applicability of the WinO method for single-cell proteomics. Single RPMI8226 cells were directly sorted into a 96-well plate, and proteins were digested using the ISD or WinO method in quadruplicate. Peptides were labeled with TMT reagents and combined with TMT-labeled peptides corresponding to 50 cells. The combined samples were then analyzed by nanoLC-MS/MS using an Orbitrap Eclipse, identifying 845 proteins and 2493 peptides. Of these identified proteins and peptides, 462 (Table S11) and 1506 (Table S12) were quantified, respectively. The average numbers of quantified peptides were 227.0 ± 114.5 and 1177.8 ± 131.6 for the ISD and WinO methods, respectively (Figure S10A). The average numbers of quantified proteins were 140.8 ± 51.8 and 400.3 ± 32.5 for the ISD and WinO methods, respectively (Figure 6A). The numbers of these peptides and proteins were significantly higher in the WinO method at 5.2-fold ($p < 0.0001$) and 2.8-fold ($p < 0.0001$), respectively, than those quantified for ISD. The fact that only about half of the identified peptides were quantified could be attributed to a decrease in the average S/N of the reporter ions after mixing the ISD and WinO samples. To obtain reliable quantitative data, a minimum average S/N value of the reporter ion in Proteome Discoverer was set at 10. We first compared the S/N value of carrier channel reporter ions between the accepted and rejected spectra for quantification (Figure S11A). Among the rejected spectra, 99.94% of the carrier samples had an S/N lower than 100. Because TMT 10-plex was used in this study, the spectra of carrier samples with S/N values greater than 100 correspond to an average S/N value greater than 10. Next, we examined the protein level obtained by the ISD or WinO method, which was calculated as the total S/N of corresponding peptides. In the ISD method data, 92.8% of protein levels were less than 10, whereas 27.3% of proteins were less than 10 in the WinO method (Figure S11B). These data suggest that filling the TMT channel with only WinO samples would yield higher numbers of quantified proteins. A negative control was not used in this study. Although the WinO data using one cell may include peptides not derived from the sample, the above results suggest that the recovery rate of the WinO method in single-cell samples was higher than that of the ISD method.

Next, to examine the effect of the WinO method on protein recovery, we compared 247 commonly quantified proteins in both methods. Figure 6B indicates that the levels of 221 out of 247 proteins significantly ($p < 0.05$) increased 2-fold or more with the WinO method. There were no proteins that significantly decreased in the WinO method. The median relative recovery of proteins was 10.21-fold greater with the WinO method than the ISD method. The levels of proteins commonly quantified using both methods were significantly higher ($p < 0.0001$) than those uniquely identified using the WinO method (Figure S10B). These results suggested that the number of quantified proteins and peptides increased by increasing their recovery by the WinO method using single cells. In addition, 33 TMPs, including one cluster of differentiation (CD) protein, CD71, were quantified in this study; among them, 24 were uniquely quantified using the WinO method. RapiGest[12] and *n*-dodecyl-*β*-D-maltoside[18] have been used for protein extraction in single-cell proteomics. It has been reported that these additives yield comparable or higher solubility of membrane proteins than SDC,[21] which was used in the WinO method. However, the Lys-C and trypsin activities were higher in the presence of SDC than these additives, resulting in a higher number of hydrophobic proteins

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and peptides identified.[21] In this study, we used a mixture of SDC and SLS, which is known to considerably increase the solubility and the number of membranes as well as soluble proteins compared with SDC alone.[20] These findings suggest that the extraction efficiency of proteins from a single cell is higher in the WinO method than in other single-cell proteomic techniques. Our WinO method enhanced protein recovery and protein identification not only of soluble proteins but also TMPs from single cells, thereby highlighting its application for the single-cell proteomic analysis.

4. CONCLUSIONS

On the WinO method, cells were directly injected into the sample droplet by a cell sorter. The method does not require any specialized equipment. The recovery of proteins and peptides is dramatically increased compared to the ISD method by reducing the contact area between the sample solution and the plastic container. In addition, the pipette tip does not contact the sample solution when the DTT, IAA, Lys-C, trypsin, and TMT solutions are added; thus, protein loss due to adsorption onto the pipette tip is avoided. Although there are still limitations to this method, such as the possibility of a lower peptide recovery rate once ethyl acetate is removed, the recovery of peptides and proteins increased approximately 10-fold for single-cell proteomics by coupling the use of phase transfer surfactants and carboxyl-coated hydrophobic beads. In this study, large amounts of carrier samples were added to the single-cell samples. The addition of a carrier improves the recovery of peptides after injection into the LC system, whereas quantitation was partially affected.[30] As we added the carrier to a mixture of ISD and WinO samples, its effect on quantitation and sensitivity suppression of the reporter ion was thought to have been the same for the two types of samples. Several methods for single-cell proteomics have been previously reported.[8,18,31] It is hardly possible to directly compare these methods in terms of numbers of quantified proteins due to the differences in analytic systems and equipment. Nevertheless, we conclude that, when compared to the ISD method, our novel strategy further improves the sensitivity of single-cell proteomics. In addition, although the WinO method was successfully performed on 96-well plates, we expect that the method is scalable to 384- and 1536-well plates using liquid handling robots, further enhancing the throughput of single-cell proteomics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c05487.

Supporting figures including comparison of the extraction efficiency with and without sonication and heating, recovery of proteins and peptides in a water droplet in ethyl acetate, comparison of the ISD and WinO methods, etc. (PDF)

Experimental design of TMT labeling (Table S1), placement and size of the variable SWATH window (Table S2), and peptide and protein quantification tables (Tables S3−S12) (XLSX)

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Author Contributions

T.M. conceived the project and designed the study. T.M., I.Y., F.A., Y.M., K.M., C.-H.C., and K.D. performed the experi-

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ments. T.M., S.I., and S.O. wrote the paper. All the authors discussed the results and commented on the paper.

Notes

The authors declare no competing financial interest.

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