

Complementary Protein and Peptide OFFGEL Fractionation for High-Throughput Proteomic Analysis

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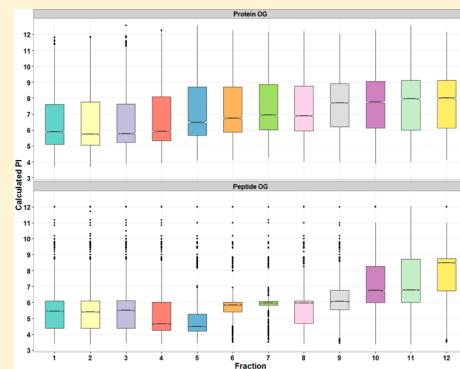
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Supporting Information

ABSTRACT: OFFGEL fractionation of mouse kidney protein lysate and its tryptic peptide digest has been examined in this study for better understanding the differences between protein and peptide fractionation methods and attaining maximum recruitment of this modern methodology for in-depth proteomic analysis. With the same initial protein/peptide load for both fractionation methods, protein OFFGEL fractionation showed a preponderance in terms of protein identification, fractionation efficiency, and focusing resolution, while peptide OFFGEL was better in recovery, number of peptide matches, and protein coverage. This result suggests that the protein fractionation method is more suitable for shotgun analysis while peptide fractionation suits well quantitative peptide analysis [isobaric tags for relative and absolute quantitation (iTRAQ) or tandem mass tags (TMT)]. Taken together, utilization of the advantages of both fractionation approaches could be attained by coupling both methods to be applied on complex biological tissue. A typical result is shown in this article by identification of 8262 confident proteins of whole mouse kidney under stringent condition. We therefore consider OFFGEL fractionation as an effective and efficient addition to both label-free and quantitative label proteomics workflow.



The complexity of proteome analysis in terms of its size and wide dynamic range requires multiple orthogonal fractionation approaches.¹ This fractionation enables identification of more peptides per protein (particularly low-abundant molecules), which ultimately provides reliable results on both qualitative and quantitative levels.² OFFGEL fractionation (OG) technology enables efficient and reproducible separation based on immobilized pH gradient (IPG) strips.³ Unlike the traditional workflow of two-dimensional gel electrophoresis (2DE), amphoteric macromolecules (protein/peptide) present in the liquid phase are forced to migrate through the gel from one compartment to another until reaching their isoelectric point (pI).³ These macromolecules are then retained in the solution because their net charge equals zero (reached isoelectric stability point) and therefore could be recovered from the liquid phase.^{4–6} In fact, the recovery of proteins/peptides in a liquid phase not only facilitated subsequent separation platforms but also opened versatile applications on both label-free quantification and isobaric tags quantitative proteomics as well.⁶ Since introduced by Ros et al.,³ OFFGEL fractionation became more popular because of its applicability on both proteins^{1,4,7–10} and peptides.^{5,11–13} However, choosing between protein or peptide OG fractiona-

tion remains ambiguous. This begs a fundamental question for several researchers of whether there are any differences between both fractionation methods in terms of identification efficiency, protein scoring, fractionation and repartition profiling, and redundancy (under the same condition), a question that has not been answered so far. The initial impetus of this work is to disclose the differences between both fractionation methods to attain maximum recruitment of this modern methodology for in-depth proteomic analysis.

MATERIAL AND METHODS

Animals. Male 8 week old C57BL/6J mice ($n = 3$) were used in this study. Animal handling was in accordance to the ethics approved by the animal committee at Niigata University School of Medicine.

Preparation of Kidney Protein Lysate. The renal capsule was removed, and the right kidney was quickly rinsed with cold PBS supplied with protease inhibitor cocktail (complete ultratablets, mini, Roche, Mannheim) to remove blood and

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inhibit protease activity. Protein extracts were obtained by placing dissected kidney tissues in protein OFFGEL fractionation (OG) buffer supplied by the manufacturer [containing urea, thiourea, DTT, glycerol, and buffer with ampholytes pH (3–10)]. Complete ultra proteases (Roche, Mannheim) was added to the buffer. A Precellys 24 tissue homogenizer was used for protein extraction by adding 2.8 mm ceramic beads (zirconium oxide) to tubes and homogenizing at 6500 rpm for 20 s three times at 4 °C (Precellys, Bertin technologies). Protein extract was precipitated with acetone and assayed using the bicinchoninic acid (BCA) method (Pierce, Rockford IL) at 562 nm. The experimental setup employed in this analysis is shown schematically in Figure 1.

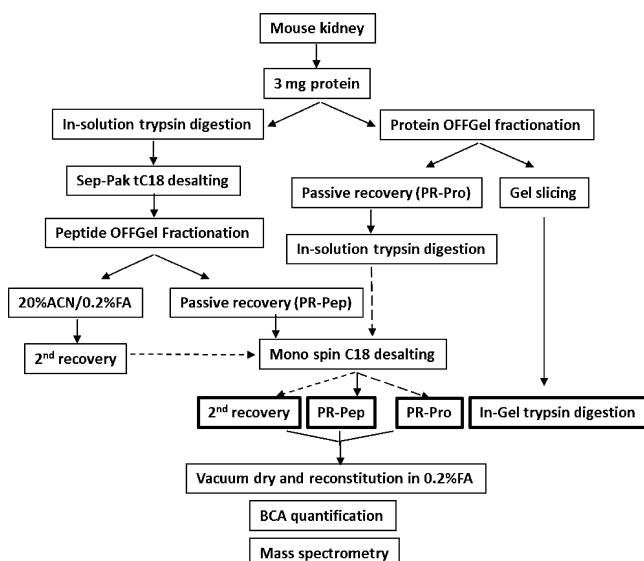


Figure 1. Schematic overview of experimental design. Equal amount of mouse protein kidney lysate was processed for protein OFFGEL fractionation or subjected to in-solution trypsin digestion and desalting followed by peptide OFFGEL fractionation. Additionally, second recovery buffer was used to recover possible peptides retained in the IPG strip or on the compartments wall. For protein OFFGEL fractionation, in-gel trypsin digestion was applied to the IPG strip. Samples were then subjected to C18 cleanup, quantification, and analysis by mass spectrometry.

Preparation of Kidney Peptide Digests. Large-scale protein in-solution digestion was applied as a substrate for the peptide OFFGEL fractionation (peptide OG). In brief, 3 mg of protein lysate was denatured in 8 M urea, 500 mM Tris-HCl pH 8.5 with protease inhibitor cocktail (complete ultratablets, mini, Roche, Mannheim) followed by reduction of disulfide bonds with 5 mM tris(2-carboxyethyl)phosphine (TCEP) for 30 min. Thiol groups were blocked by alkylation with 10 mM iodoacetamide for 20 min in the dark. Samples were diluted to final concentration 2 M urea with 100 mM Tris-HCl, pH 8.5 prior to digestion with trypsin. For endopeptidase digestion, modified trypsin (Promega, Madison, WI) was added at 50:1 (protein/protease mass ratio) along with 1 mM CaCl₂, and the mixture was incubated overnight in a thermoshaker at 600 rpm at 37 °C.⁷ Proteolytically digested peptide sample was acidified using 90% formic acid (FA) to a final pH of 3 and desalting using Sep-Pak tC18 plus short cartridge (cat no. WAT036810, Waters). Efficiency of peptide digestion was confirmed (Supporting Information 1).

Protein and Peptide OFFGEL Fractionation. Isoelectric point-based OFFGEL fractionations for protein extracts and peptide digest were carried out as specified by manufacturer. Briefly, 3 mg of both preparations were used for OFFGEL fractionation using a 3100 OFFGEL fractionator (Agilent Technologies, Japan).⁷ Macromolecules (protein or peptides) were separated under fully denaturing condition as previously described and according to the manufacturer's protocol using a 12-multiwells format device encompassing IPG strips (pH 3–10, 13 cm, Agilent Technologies, Japan), focused for 20 kV·h with a maximum current of 50 μA and maximum voltage set to 4500 V. Samples were then shifted to a hold step at a voltage (500 V) and current of 20 μA. During the whole fractionation period that lasts for 68 h, oil was added to the electrodes to prevent evaporation and electrode pads were replaced daily. After successful fractionation, 12 liquid fractions [named passive recovery, protein (PR-Pro), peptide (PR-Pep)] were collected.

Protein and Peptide Quantification and Cleanup.

Precise measurements of protein/peptide recovery in retrieved fractions were done using the BCA method (Pierce, Rockford IL).¹⁴ Basically, the yield of recovery was determined by measuring the volumes and protein concentrations in different fractions.¹⁵ In addition, a densitometric method was also verified (see Supporting Information 1). To achieve accurate quantification, detergents and other interfering materials were removed from the PR-Pro fractions using Vivacon 500 spin ultrafiltration units of nominal molecular weight cutoff 10.000 (cat no. VN01H01, Sartorius Stedim) followed by BCA assay and in-solution digestion similar as shown earlier (Preparation of Kidney Peptide Digests section).¹⁶ In a next step, digests from PR-Pro, PR-Pep, and second recovery fractions were acidified and enriched using MonoSpin C18 columns (GL Sciences, Japan). Eluted fractions were vacuum-dried and reconstituted in 0.2% formic acid prior to mass analysis.

IPG Strip Peptide Extraction (Second Recovery).

OFFGEL fractionation separates macromolecules in a two phases; liquid upper phase (passive recovery) and IPG strip trapped phase. To extract peptides retained in the IPG strips, 150 μL of 20% acetonitrile (ACN)/0.2% formic acid was added in each compartment and shaken for 30 min at 600 rpm.⁵ The solution (second recovery) was retrieved and enriched using MonoSpin C18 columns (GL Sciences, Japan), then vacuum-dried and reconstituted again in 0.2% formic acid prior to mass analysis.

In-Gel Trypsin Digestion. Gel bound to IPG strip (protein OG) was sliced carefully into 12 pieces, and proteins retained in the IPG strips were processed by in-gel digestion protocol as shown previously.^{17–20}

Nano-Reversed-Phase Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) Analysis. LC–MS/MS analysis was performed on TripleTOF 5600 (AB Sciex, Japan) interfaced at the front end with chip-based platform (cHiPLC-Nanoflex) combined with a nanoLC 400 (Eksigent, Japan). Peptides were separated using a nano-cHiPLC column (75 μm × 15 cm ChromXP C18-CL 5 μm 300 Å) on direct mode at flow rate of 300 nL/min. MS and MS/MS ranges were 400–1250 *m/z* and 100–1600 *m/z*, respectively. The peptides were separated with a linear gradient of 5–60% solution B (95% ACN, 0.1% formic acid). The 10 most intense ions were sequentially selected under data-dependent acquisition (DDA) mode with a charge state of 2–5. For each cycle, survey full scan MS and MS/MS spectra were acquired at resolution of

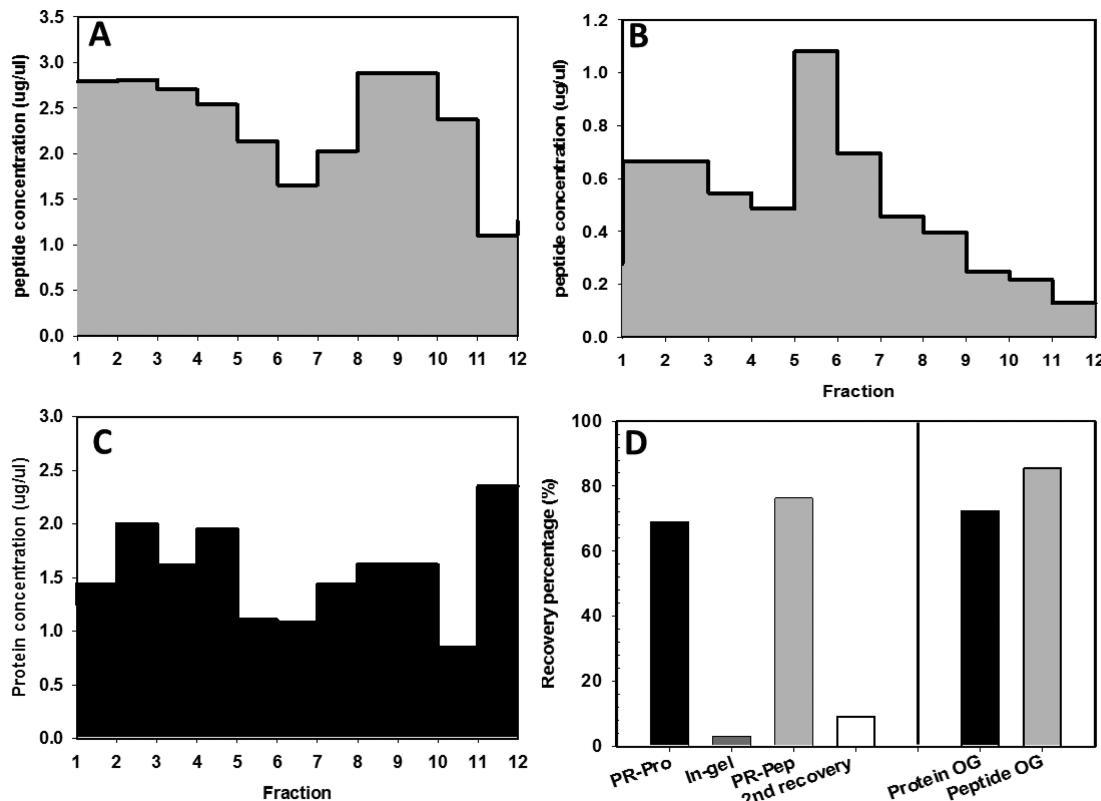


Figure 2. BCA-based protein and peptide quantification. (A) Peptide concentration ($\mu\text{g}/\mu\text{L}$) of passive recovery from peptide OG fractionation. (B) Peptide concentration of second recovery from peptide OG fractionation. (C) Protein concentration ($\mu\text{g}/\mu\text{L}$) retrieved from passive fraction of protein OG fractionation. (D) Overall recovery percentage for each recovery method (left) or overall protein/peptide recovery of each fractionation method (right).

35.000 and 15.000, respectively. To ensure accuracy, external calibration was scheduled and run during sample batches to correct possible time-of-flight (TOF) deviation.

Data Analysis. Mascot generic format (mgf) data files were generated from raw files using script supplied by AB Sciex. MS/MS spectra were searched using Mascot Daemon 2.4 (Matrix Science, Japan) against *Mus musculus* UniProtKB/TrEMBL database (101.614 entries; released in February, 2015). The search space included all fully and semitryptic peptide candidates (up to two missed cleavages with at least seven amino acids). Precursor mass and fragment mass were identified with an initial mass tolerance of 10 ppm and 0.2 Da, respectively. Carbamidomethylation of cysteine (+57.02146 amu) was considered as a static modification and oxidation at methionine (+15.995) as a variable modification. To ensure high-quality result, peptide candidates were selected for protein assembly if the expectation value (*E*-value) was less than 0.01 with rank 1. The false discovery rate (FDR) was kept at 1% at the protein level. For data integration, protein OG fractions were searched independently then merged with score correction. On the other hand, peptide OG fractions were searched with merging using Mascot daemon 2.4 (Matrix Science, Japan). Final assembly of sample replicates was applied to generate the final outputs of fractionation methods.

Bioinformatics and Biostatistical Analysis. Unlike most web-based calculators, we developed in-house Perl scripts for unrestricted mass processing to predict isoelectric point (pI), molecular weight, number of cleavage sites, grand average of hydrophobicity (GRAVY), aliphatic index, protein length, amino acid composition, percentage of basic and acidic

amino acids for protein and peptide sequences identified confidently in both fractionation methods (see script details and usage in [Supporting Information 2](#)). In addition, the scripts count protein redundancy (based on accession number) or peptide redundancy (based on peptide sequence) within fractions and replicates. We adopted two further parameters in the scripts for evaluating the macromolecule shifting from its correct place; one is the Δ fraction, which represents the difference between the experimental fraction (fraction where the protein/peptide is found) and calculated fraction (based on the sequence), and Δ pI, which represents difference between the experimental pI (average pI of the fraction where the protein/peptide was found based on the IPG strip supplier data and the fractions size) and calculated pI (based on the sequence). Statistical analysis of this study was performed using Mann–Whitney's U test. $P < 0.05$ was considered a statistically significant threshold. Both statistics and graphics were done using with R (v 3.1.2).

RESULTS AND DISCUSSION

Protein and Peptide Recovery. In the current study, the same starting amounts of protein lysate or peptide digests were used for OFFGEL fractionation. To obviate the potential poor recovery of OG fractionation, passive recovery for protein and peptide was coupled with a second recovery wash (for peptide OG) or in-gel IPG strip digestion (for protein OG). BCA-based protein/peptide quantification was used to calculate total protein/peptide recovery. [Figure 2](#) shows that macromolecule concentration per fraction was variable as a result of isoelectric focusing. In general, passive recovery in both fractionation

methods (protein OG and peptide OG) yielded 69.2% and 76.3% of the initial protein/peptide load (3 mg), respectively. Second recovery using 20% ACN/0.2% FA succeeded to recover an additional 9.2% of peptides trapped in the IPG strip or known to be adhered to the compartment polyurethane wall.²¹ This finding is coincidental with earlier studies.^{6,15,22} On the other hand, in-gel trypsin digestion of IPG strips from protein OG recovered modest peptide yield (3.1%). Ultimately, protein OG recovery (PR-pro and in-gel digestion) was 72.3% (2.17 mg) while peptide OG recovery (PR-pep and second recovery) was 85.5% (2.56 mg). The modest increase in peptide OG recovery might be attributed to its higher amenable solubility to isoelectric focusing solution than protein molecules. Further investigations are required to disclose this point.¹¹

Protein and Peptide Identification. Nonredundant protein and peptide lists were assembled after removing duplicates between fractions and replicates (*Supporting Information 3*). All raw files (.Wiff) and Mascot generic files (.Mgf) are accessible from the Peptide Atlas repository at URL: <ftp://PASS00674:JT5654jp@ftp.peptideatlas.org/>. Figure 3A shows that protein OG fractionation identified significantly more proteins (7206) than peptide OG (4087). Overlaps between different fractionation methods revealed that protein OG covered 74% of protein identification in peptide OG,

whereas peptide OG covered only 42% of protein OG identifications (Figure 3C1). We further parsed these data after extracting proteins with at least one unique peptide (Figure 3C2) where results showed the same pattern. Surprisingly, peptide identification was not proportional to protein identifications (Figure 3D). Peptide OG clearly identified 2-fold increase of nonredundant peptides (19 133) than in protein OG (9340) with the same frequency of missed cleavage. For the first glance, it highlights the increase in protein coverage. This observation is coincidental with the fact that several peptides with different pI's were fractionated into different compartments which increases the chance of identifying the candidate protein with several peptide matches. To ensure the fidelity of this result, we retrieved protein candidates corresponding to unique peptides in both fractionation methods (see Figure 3, parts B and D). Results deduced that it is almost certain that protein identifications in peptide OG were identified with more than four peptides in average, whereas, it was one to two peptides in protein OG.

Protein and Peptide Score and Counts. To give more credence to our finding, we investigated in details the protein and peptide score between experimental groups. As illustrated in Figure 4, peptide scores were almost similar in both fractionation methods denoting similar spectral quality (Figure 4A). In contrast, a prominent increase in global protein scoring was characteristic in peptide OG rather than protein OG (Figure 4B). The substantial superiority of peptide OG in terms of protein scoring was further confirmed when each protein was plotted with its score retrieved from both fractionation methods as shown in Figure 4C. In addition, the majority of proteins were identified by one peptide in protein OG while number of proteins identified with four peptides or more were higher in peptide OG (Figure 4D), a result that aligned with the earlier finding.

Protein Coverage. As a result of a high number of peptide identifications and matched peptides in the peptide OG method, but not protein identifications, protein coverage was higher in the peptide OG than in the protein OG method (Figure 5 and *Supporting Information 1*). Taking all earlier results together, a distinct identification capability between both fractionations methods could be discerned.

Efficiency of Fractionation (Correct Placement Based on pI). Fractionated protein/peptides were confirmed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as illustrated in Figure 6, parts A and B. In viewing the calculated pI of macromolecules in each compartment for both groups, fractionation preponderance was obvious in protein OG but not in the counter group (Figure 6C). Notwithstanding, for peptide OG, basic molecules were better focused especially toward the cathode. Several deviated pI outlier molecules were detected in peptide OG fractionation (Figure 6C). One probable reason for imperfect peptide fractionation might be the narrow pI range between peptide molecules compared to protein as a result of low peptide net charge based on negatively or positively charged amino acids.

Efficiency of Fractionation (Unique Placement and Redundancy). We further examined whether the macromolecules were uniquely placed in one compartment or redundant within several fractions. As shown in Figure 7, uniquely placed molecules (only in one compartment) were higher in protein OG than in peptide OG. Over 68% of identified proteins were singly located in one compartment compared to peptide OG (46.2%). On the other hand,

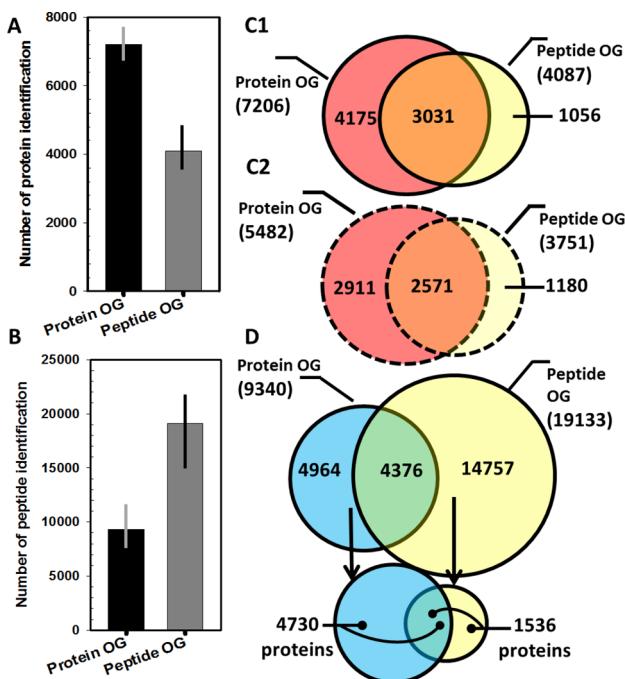


Figure 3. Protein and peptide identification. (A) Total protein identification number (nonredundant) from both fractionation methods with protein FDR (0.01) and stringent corresponding peptide filter (*E*-value < 0.01 and rank 1). (B) Total number of nonredundant peptides identified from both fractionation methods. (C1) Venn diagram showing protein overlapping between both fractionation methods. Panel C2 illustrates overlapping of identified proteins between both groups with at least one unique peptide. (D) Venn diagram showing peptide overlapping between both fractionation methods. Corresponding proteins in panel D demonstrate approximate protein identifications from unique peptides in both groups taking in account possible protein sharing. Error bars represent standard deviation at $p < 0.05$.

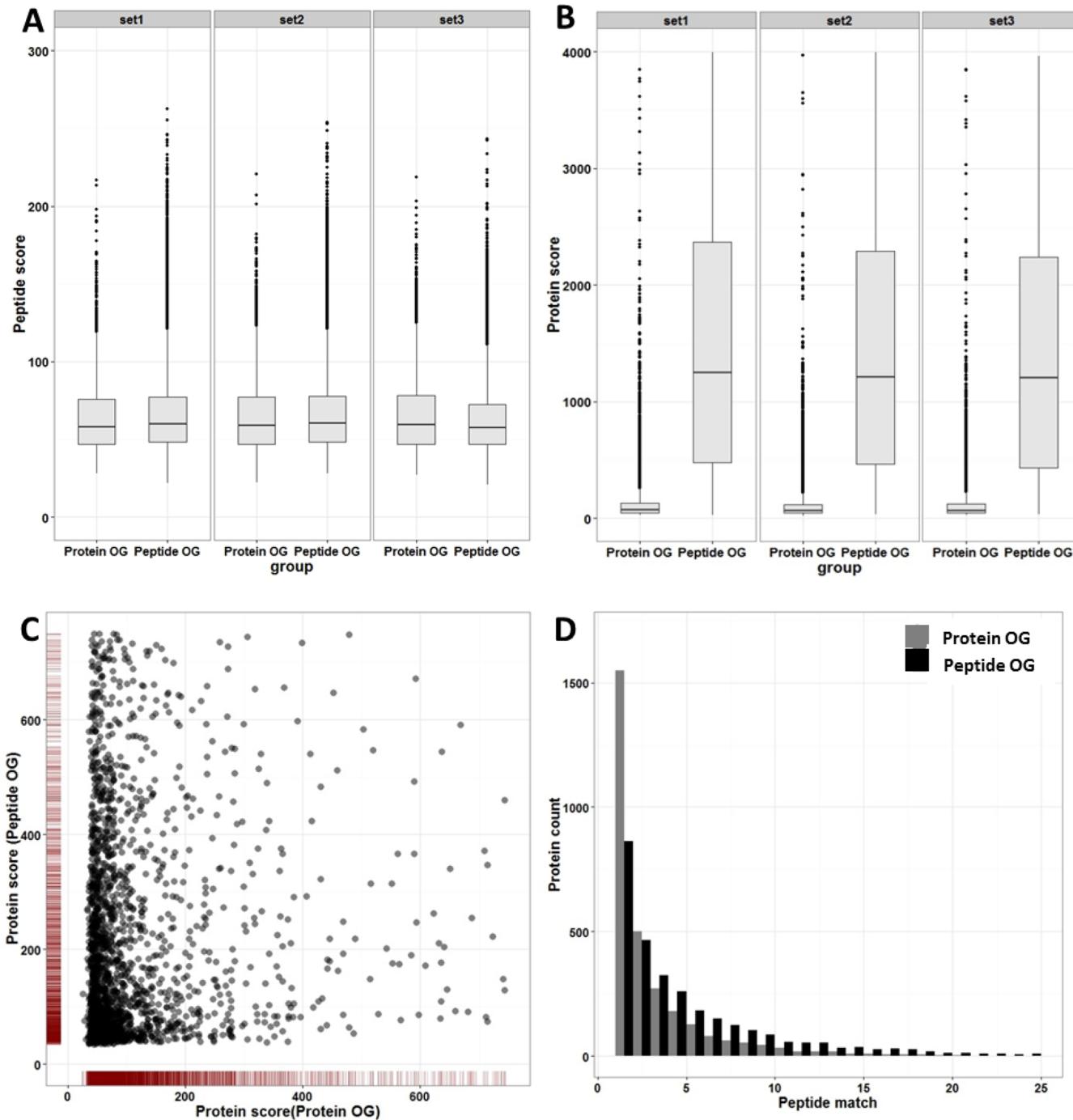


Figure 4. Whiskers and box plot of macromolecules scores and counts: panel A represents peptide scoring between both fractionation methods and replicates; panel B shows statistically significant differences ($p < 0.001$) of protein scoring between both fractionation methods in each replicate; panel C shows the protein score scatter plot of the same shared protein hits between both fractionation methods; panel D illustrates a histogram of peptide match counts for identified proteins in both fractionation methods.

redundancy in peptide OG was cumulatively higher compared to protein OG. We also took in consideration the possible high redundancy in OG fractionation due to methionine oxidation as a common post-translational modification (PTM). However, this was not a case as shown earlier in N-linked glycosylated proteins.²³ We therefore assume that imperfect peptide focusing might be due to higher trafficking occurring in the IPG strip than in protein OG.

Efficiency of Fractionation (Macromolecule Shifting). Using Δ fraction and Δ pI parameters, we investigated how far

approximately the macromolecule shifted from its predicted place in both fractionation methods. Taking in consideration 0.52 resolution of compartment pI margin (IPG strip 3–10), speculation has intimated that fraction shifting and pI shifting were higher in peptide OG than in protein OG (Figure 8). This observation is in concordant with our previous finding that protein OG fractionation is better than peptide OG fractionation at least under these experimental conditions. Unfortunately, other protein/peptide metrics (gravy index, aliphatic index, negatively charged peptides, amino acid

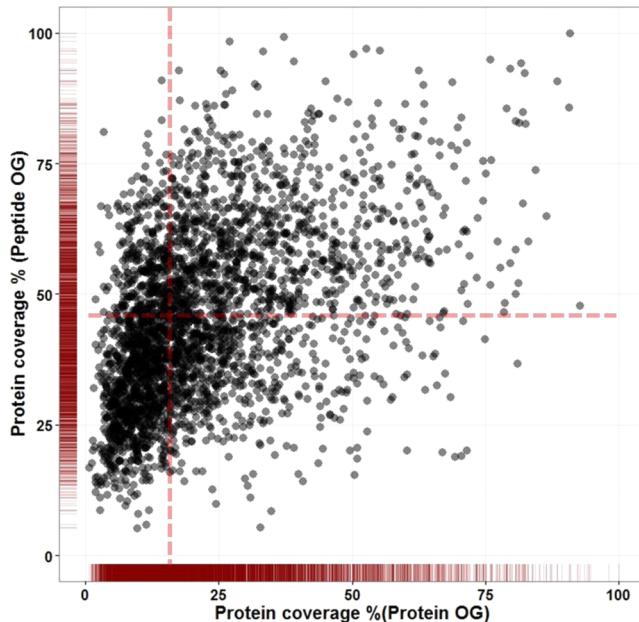


Figure 5. Scatter plot shows protein coverage percentage of the same protein hit between protein OG and peptide OG fractionation. Dashed lines represent average protein coverage for both fractionation methods.

composition, and positively charged peptides) were not significant between both groups (*Supporting Information 1*).

CONCLUSIONS

OFFGEL fractionation is a powerful tool for simplifying complex biological tissues prior to analysis. In the current study, protein OG fractionation, by far, identified more proteins with less matched peptides than in peptide OG methods. In

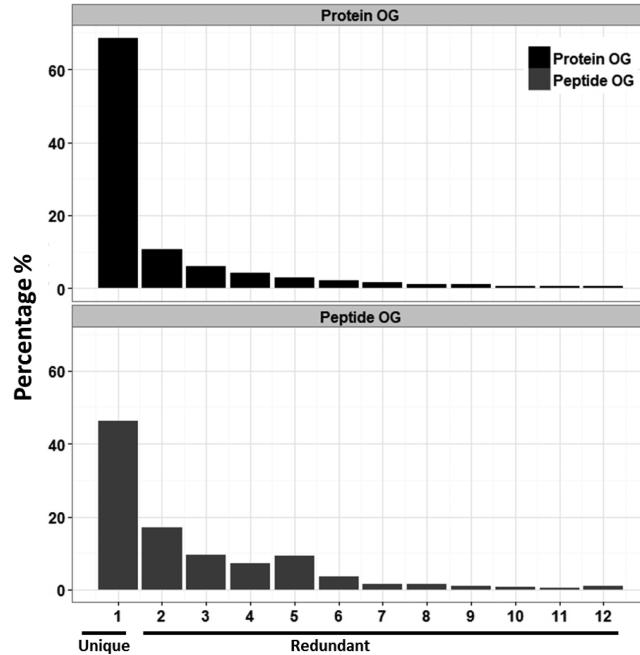


Figure 7. Unique placement and redundancy of protein and peptide OFFGEL fractionation. The X-axis represents protein/peptide found only in one compartment (no. 1), or redundant (nos. 2–12). The Y-axis represents percentage of unique or redundant hits of different OFFGEL fractionation methods.

contrast, peptide OG showed a preponderance of protein coverage due to higher peptide matches on the expense of protein identification. To this end, we conclude that protein OG is appropriate for shotgun analysis while peptide OG is more suitable for quantitative analysis [isobaric tags for relative and absolute quantitation (iTRAQ) or tandem mass tags

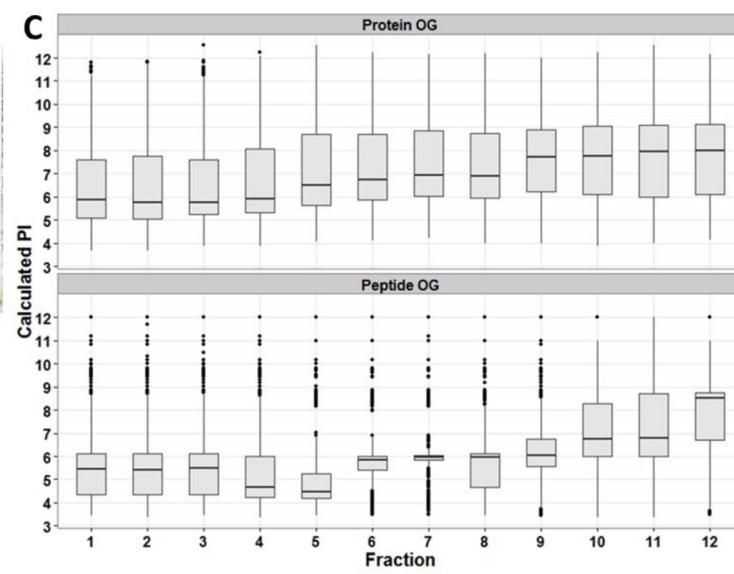
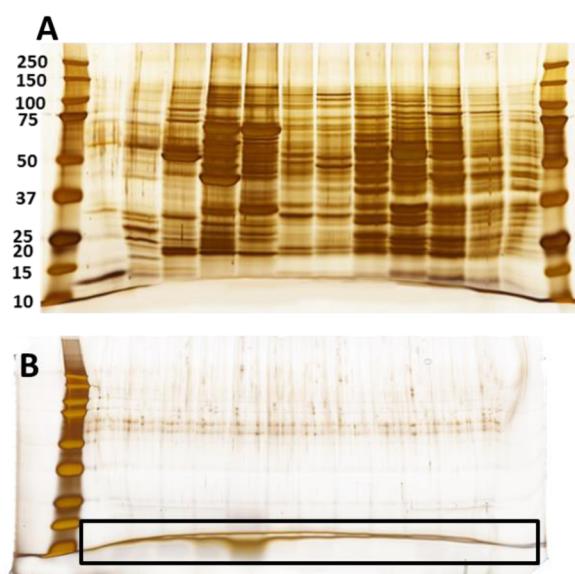


Figure 6. Protein/peptide OFFGEL fractionation. (A) SDS-PAGE image of 3 mg of mouse kidney protein lysate separated by OFFGEL fractionation on a 12 cm pH 3–10 IPG strip. Briefly, 1 μ L from a passive fraction was loaded on 12.5% SDS-PAGE and visualized by silver staining (add marker info). (B) Peptide fractionation could be monitored by variable accumulation at the low molecular weight range (marked bottom). Kaleidoscope prestain marker (BioRad) was loaded. (C) Whiskers and box plot of calculated pI of protein OG (upper panel) and peptide OG (lower panel). The central horizontal line within the box represents the median, whiskers represent highest and lowest observations, and dots represent minimum and maximum observations within fraction.

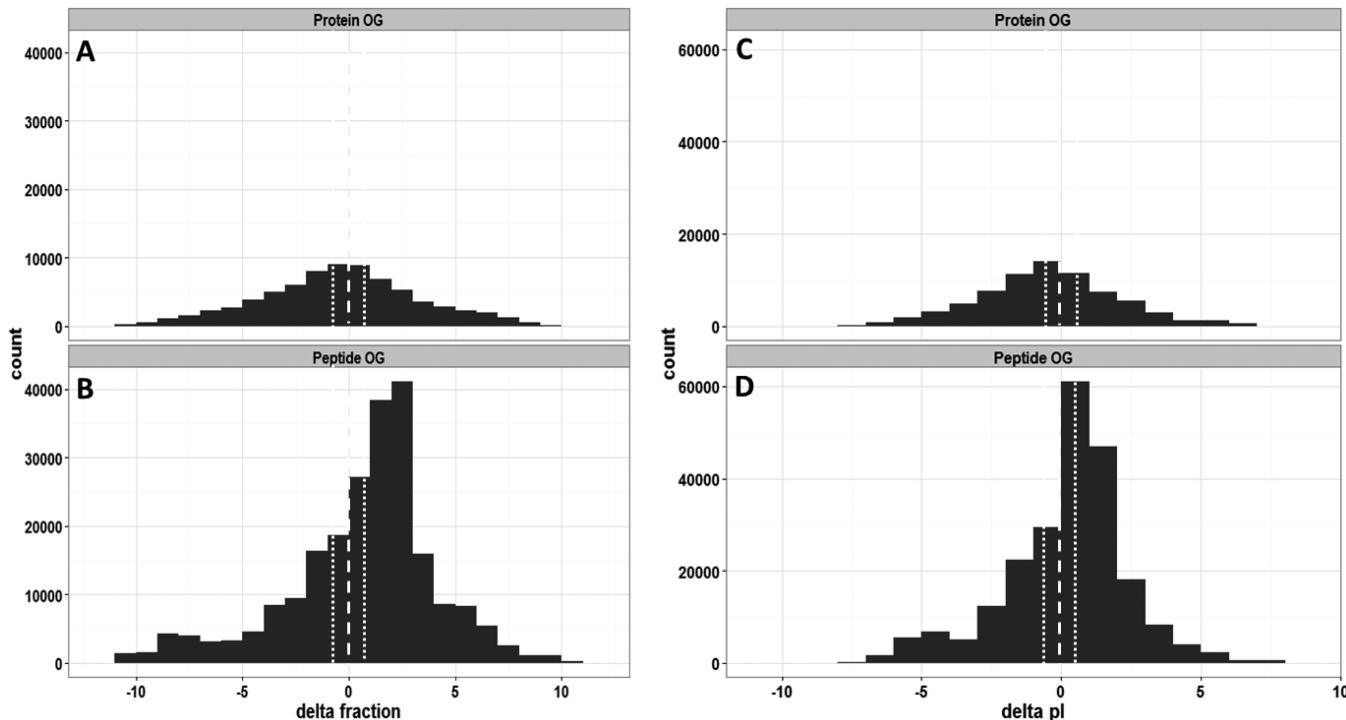


Figure 8. Macromolecule shifting of protein/peptide OFFGEL fractionation. Panels A and B show Δ fraction of protein OFFGEL (A) or peptide OFFGEL fractionation (B), which represents the difference between the experimental fraction (fraction where the protein/peptide is found) and calculated fraction [based on protein ID (protein OG) or peptide sequence (peptide OG)]. Panels C and D represent Δ pI of protein OFFGEL (C) or peptide OFFGEL fractionation (D), which is the difference between the experimental pI (average pI of the fraction where the protein/peptide is found in based on the IPG strip supplier data) and calculated pI [based on protein ID (protein OG) or peptide sequence (peptide OG)]. Thick dashed lines represent center of nonshifted protein/peptide. Thin dashed lines represents ± 0.52 margin of OFFGEL compartment.

Table 1. Summarized Comparison between Protein and Peptide OFFGEL Fractionation

	protein OG	peptide OG
protein identification	more identification	less identification
peptide identification	less identification	more identification
protein scoring	lower	higher
peptide scoring	same	same
protein coverage	less coverage	more coverage
fractionation	more efficient	less efficient
trafficking	less	higher
efficiency of focusing (Δ fraction/ Δ pI)	more efficient	less efficient
redundancy	less redundant	more redundant
acidic/basic residue	same	same
recovery of passive fractions	lower (72.3%)	higher (85.5%)
suitability/applicability	shotgun analysis	quantitative analysis

(TMT)]. Concurrently, a combination of both fractionation methods could be harnessed for simultaneous and maximum utilization of identification and coverage at the same time. As exemplified here, the coupling of both fractionation methods applied to complex biological tissue (mouse kidney) could identify 8262 confidence proteins of mouse kidney under stringent conditions. We also reported that under the same protein/peptide load, the precision of fractionation was better in protein OG over peptide OG. This could be more beneficial for targeted protein identification but not peptide. In addition, although reduction of sample complexity is more important than correct placement based on predicted pI, yet it seems that increased redundancy in peptide OG, due to a shortcoming of focusing or high trafficking, affected final protein identification at least in the current experimental condition. It is therefore

crucial to determine the appropriate OFFGEL fractionation methodology based on the research interest. Table 1 summarizes the major differences between both fractionation methods.

ASSOCIATED CONTENT

S Supporting Information

Supporting Information 1, additional information about efficiency of peptide digestion, densitometric quantification of protein OG, protein coverage, and physicochemical properties; Supporting Information 2, guide on how to use Perl scripts for analysis of protein/peptide OFFGEL fractionation output data; Supporting Information 3, comprehensive mouse kidney proteome database from complementary protein and peptide OFFGEL fractionation. The Supporting Information is

available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01911.

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Magdeldin, S.; Moresco, J. J.; Yamamoto, T.; Yates, J. R., 3rd *J. Proteome Res.* **2014**, *13*, 3826–3836.
- (2) Zhang, Y.; Li, Y.; Qiu, F.; Qiu, Z. *Electrophoresis* **2010**, *31*, 3797–3807.
- (3) Ros, A.; Faupel, M.; Mees, H.; Oostrum, J.; Ferrigno, R.; Reymond, F.; Michel, P.; Rossier, J. S.; Girault, H. H. *Proteomics* **2002**, *2*, 151–156.
- (4) Tobolkina, E.; Cortes-Salazar, F.; Momotenko, D.; Maillard, J.; Girault, H. H. *Electrophoresis* **2012**, *33*, 3331–3338.
- (5) Franchin, C.; Pivato, M.; Rattazzi, M.; Arrigoni, G.; Millioni, R. *J. Chromatogr. A* **2014**, *1355*, 278–283.
- (6) Moreda-Pineiro, A.; Garcia-Otero, N.; Bermejo-Barrera, P. *Anal. Chim. Acta* **2014**, *836*, 1–17.
- (7) Magdeldin, S.; Yamamoto, K.; Yoshida, Y.; Xu, B.; Zhang, Y.; Fujinaka, H.; Yaoita, E.; Yates, J. R., 3rd; Yamamoto, T. *J. Proteome Res.* **2014**, *13*, 1636–1646.
- (8) O'Qualain, R. D. M.; Hyde, J. E.; Sims, P. F. *Malar. J.* **2010**, *9*, 286.
- (9) Mena, M. L.; Moreno-Gordaliza, E.; Moraleja, I.; Canas, B.; Gomez-Gomez, M. M. *J. Chromatogr. A* **2011**, *1218*, 1281–1290.
- (10) Horth, P.; Miller, C. A.; Preckel, T.; Wenz, C. *Mol. Cell. Proteomics* **2006**, *5*, 1968–1974.
- (11) Abdallah, C.; Sergeant, K.; Guillier, C.; Dumas-Gaudot, E.; Leclercq, C. C.; Renaut, J. *Proteome Sci.* **2012**, *10*, 37.
- (12) Warren, C. M.; Geenen, D. L.; Helseth, D. L., Jr.; Xu, H.; Solaro, R. J. *J. Proteomics* **2010**, *73*, 1551–1561.
- (13) Sudhir, P. R.; Kumari, M. P.; Hsu, W. T.; Massiot, J.; Chen, C. H.; Kuo, H. C.; Chen, C. H. *J. Proteome Res.* **2013**, *12*, 5878–5890.
- (14) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. *Anal. Biochem.* **1985**, *150*, 76–85.
- (15) Heller, M.; Michel, P. E.; Morier, P.; Crettaz, D.; Wenz, C.; Tissot, J. D.; Reymond, F.; Rossier, J. S. *Electrophoresis* **2005**, *26*, 1174–1188.
- (16) Magdeldin, S.; Yamamoto, T.; Tooyama, I.; Abdelalim, E. M. *Stem Cell Rev.* **2014**, *10*, 561–572.
- (17) Enany, S.; Yoshida, Y.; Magdeldin, S.; Zhang, Y.; Bo, X.; Yamamoto, T. *Peptides* **2012**, *37*, 128–137.
- (18) Enany, S.; Yoshida, Y.; Magdeldin, S.; Bo, X.; Zhang, Y.; Enany, M.; Yamamoto, T. *Microbiol. Res.* **2013**, *168*, 504–511.
- (19) Magdeldin, S.; Li, H.; Yoshida, Y.; Enany, S.; Zhang, Y.; Xu, B.; Fujinaka, H.; Yaoita, E.; Yamamoto, T. *J. Proteomics* **2010**, *73*, 2031–2040.
- (20) Magdeldin, S. *Gel Electrophoresis—Principles and Basics*; InTech: Rijeka, Croatia, 2012.
- (21) Keidel, E. M.; Dosch, D.; Brunner, A.; Kellermann, J.; Lottspeich, F. *Electrophoresis* **2011**, *32*, 1659–1666.
- (22) Krishnan, S.; Gaspari, M.; Della Corte, A.; Bianchi, P.; Crescente, M.; Cerletti, C.; Torella, D.; Indolfi, C.; de Gaetano, G.; Donati, M. B.; Rotilio, D.; Cuda, G. *Electrophoresis* **2011**, *32*, 686–695.
- (23) Mulvenna, J.; Hamilton, B.; Nagaraj, S. H.; Smyth, D.; Loukas, A.; Gorman, J. *J. Mol. Cell. Proteomics* **2009**, *8*, 109–121.