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Simple In-Cell Processing Enables Deep Proteome Analysis of Low-Input *Caenorhabditis elegans*

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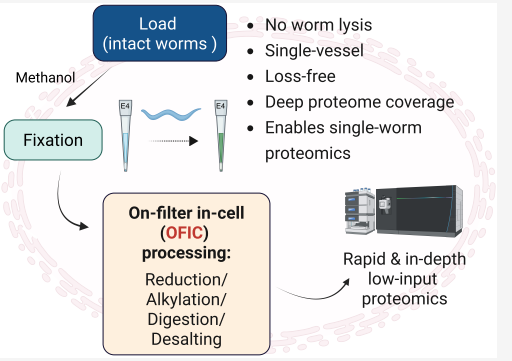
ABSTRACT: *Caenorhabditis elegans* is a widely used genetic model organism; however, the worm cuticle complicates extraction of intracellular proteins, a prerequisite for typical bottom-up proteomics. Conventional physical disruption procedures are not only time-consuming but can also cause significant sample loss, making it difficult to perform proteomics with low-input samples. Here, for the first time, we present an on-filter in-cell (OFIC) processing approach that can digest *C. elegans* proteins directly in the cells of the organism after methanol fixation. With OFIC processing and single-shot LC-MS analysis, we identified over 9400 proteins from a sample of only 200 worms, the largest *C. elegans* proteome reported to date that did not require fractionation or enrichment. We systematically evaluated the performance of the OFIC approach by comparing it to conventional lysis-based methods. Our data suggest superior performance of OFIC processing for *C. elegans* proteome identification and quantitation. We further evaluated the OFIC approach with even lower-input samples, including single worms. Then, we used this method to determine how the proteome is impacted by loss of superoxide dismutase *sod-1*, the ortholog of human *SOD1*, a gene associated with amyotrophic lateral sclerosis. Analysis of 8800 proteins from only 50 worms as the initial input showed that loss of *sod-1* affects the abundance of proteins required for stress response, ribosome biogenesis, and metabolism. In conclusion, our streamlined OFIC approach, which can be broadly applied to other systems, minimizes sample loss while offering the simplest workflow reported to date for *C. elegans* proteomics.



INTRODUCTION

The nematode *Caenorhabditis elegans* is one of the most widely used organisms to study fundamental biological processes and model human diseases due to its ease of culture and genetic manipulation, short lifespan, and high reproductive rate.[1] Direct analysis of *C. elegans* proteins in genetic mutants or strains grown under different conditions can lead to a better understanding of complex cellular processes and conserved pathways. Technologies in mass spectrometry (MS)-based proteomics have advanced tremendously in the past two decades, enabling simultaneous identification and quantification of thousands of proteins in complex proteomes.[2,3] To examine the *C. elegans* proteome, worms are typically lysed to extract proteins and then subjected to proteolytic digestion followed by liquid chromatography (LC) and tandem mass spectrometry (MS/MS) analysis.[4−7] Since worms have an exoskeleton, a tough cuticle largely comprised of cross-linked collagens,[8] the animals are usually flash-frozen in harsh lysis buffer followed by grinding or sonication to obtain sufficient protein yield.[9,10] However, such physical disruption causes significant loss of material,[6] which increases the number of worms required and introduces technical variation in sample preparation that can impact downstream data interpretation.

To combat such challenges, we recently developed an efficient, effective, and economical (E3) technology for global proteome analysis using on-filter in-cell (OFIC) processing.[11] Compared with other existing methods, it bypasses the need for cell lysis by directly digesting proteins in methanol-fixed cells. This significantly reduces the number of steps and amount of time required for sample preparation and streamlines all proteomic processing in a single device.[11] Cell fixation by methanol is a common practice in histochemical and cytochemical studies[12,13] and is the best preservation method for scRNA-seq analyses of neural cells.[14] Methanol arrests metabolism, denatures proteins, solubilizes lipids on the cell membrane, and removes free water,[15] allowing cells after fixation to become porous and permeable to proteolytic enzymes. Among the bottom-up proteomic sample preparation methods, in-cell digestion is quite new. Kelly and co-workers showed the first experimental evidence where cells were fixed by formaldehyde followed by methanol permeabilization and in-cell digestion.[16] Hatano et al. avoided unwanted protein

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cross-linking by formaldehyde by digesting cells treated with methanol alone and obtained equivalent proteomic performance compared to SDS-based lysis.[17] We further advanced this method by performing all steps on chromatographic filter devices instead of in microtubes. As the reagents after each reaction on the filters can simply be depleted via centrifugation, this prevents chemical carryover that could affect subsequent analysis. The functional resins in the filter also enable immediate peptide cleanup and desalting after digestion, which reduces sample loss associated with conventional "drying−resuspension" procedures.[11]

In our original study, we tested OFIC processing using yeast and mammalian cells and demonstrated proof-of-concept evidence for low-cell number proteome analysis.[11] However, the similarity between proteomes derived from OFIC digestion compared with lysate-based digestion methods was not evaluated. Further, whether OFIC digestion could be used to process proteins in cells of an intact multicellular organism was not assessed. Here, we utilized this advanced sample preparation technology for proteomic analysis of *C. elegans*, which is notoriously difficult to process, we compared OFIC processing side-by-side with other common lysis-based methods and showed the efficiency and effectiveness of the OFIC approach for in-depth proteomics of *C. elegans*. We further tested the sensitivity of our method with an even lower input, down to the single worm, using a tip-based, enhanced E3 device, the E4tip. We then utilized E4tips to compare the proteomes of wild-type and mutant *C. elegans*, focusing on how the proteome is impacted by loss of evolutionarily conserved Cu/Zn superoxide dismutase 1 (SOD1), which plays a critical role in cellular defense via catalysis of superoxide radicals into less harmful oxygen and hydrogen peroxide.[18,19] Gain-of-function mutations in *SOD1* cause familial amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease,[20−23] and children homozygous for loss-of-function *SOD1* mutations have motor system deficits.[24] Using OFIC processing, we identified previously unknown changes in the proteome resulting from loss of *sod-1*, which could potentially shed light on mechanisms underlying these debilitating phenotypes.

EXPERIMENTAL SECTION

***C. elegans* Maintenance.** All *C. elegans* strains were maintained on nematode growth medium (NGM) plates with an OP50 *E. coli* food source at 20 °C. The wild-type worm used in evaluation experiments was Bristol N2; the *sod-1(hen25)* null allele is a 1496-bp deletion with a 3-bp insertion that removes all *sod-1* coding sequences (flanking sequences are ttctatagaaacgattctcc and taaactatcaacaagttctg). Details for *C. elegans* synchronization and microscopy experiments can be found in the Supplementary Methods.

OFIC Processing and Protein Digestion of *C. elegans* with E3filters and E4tips. Live, staged *C. elegans* were rinsed three times with M9 buffer and then once with water. Then, the worms were either processed with OFIC digestion or first lysed with SDS buffer and TFA before on-filter digestion. For OFIC processing, a previously described protocol was followed[11] with minor modifications as described in the Supplementary Methods.

LC-MS/MS Analysis. LC-MS/MS analysis was performed on an Ultimate 3000 RSLCnano system coupled to an Orbitrap Eclipse mass spectrometer and FAIMS Pro Interface (Thermo Scientific). Data-independent acquisition (DIA) mass spec data were processed using Spectronaut software (version 19.1)[25] and a library-free DIA analysis workflow with directDIA+ and the *C. elegans* protein database (UniProt 2024 release; 27,448 sequences). Bioinformatics analyses were performed using Perseus software (version 1.6.2.3), GraphPad Prism (version 10), and InstantClue (version 0.12.2). MS raw files associated with this study have been deposited to the MassIVE server (https://massive.ucsd.edu/) with the dataset identifier MSV000096763. Detailed experimental procedures are in the Supplementary Methods.

RESULTS AND DISCUSSION

Evaluation of OFIC Processing for *C. elegans* Proteome Analysis. Since physical disruption methods are usually employed to break *C. elegans* tissues and extract proteins for digestion and proteomics analysis, we first sought to assess the feasibility of OFIC processing for *C. elegans* proteome analysis. Because the OFIC processing method is naturally loss-free, we used only 200 worms in our initial evaluation experiment. The methanol-treated *C. elegans* remained intact, although many organs were disrupted (Figure S1). For comparative purposes, we processed worms in parallel using two conventional methods (Figure 1); lysis with SDS buffer followed by vortex and water-bath sonication, and lysis with pure TFA. E3filters were used for protein digestions in these experiments to minimize technical variation.[11] To increase detection sensitivity in downstream LC-MS analysis, we employed high-field asymmetric-waveform ion mobility spectrometry (FAIMS), a gas-phase fractionation technique that reduces chemical background and enhances the identification rate for low-input samples.[26] DIA has recently evolved as a powerful alternative to the conventional data-

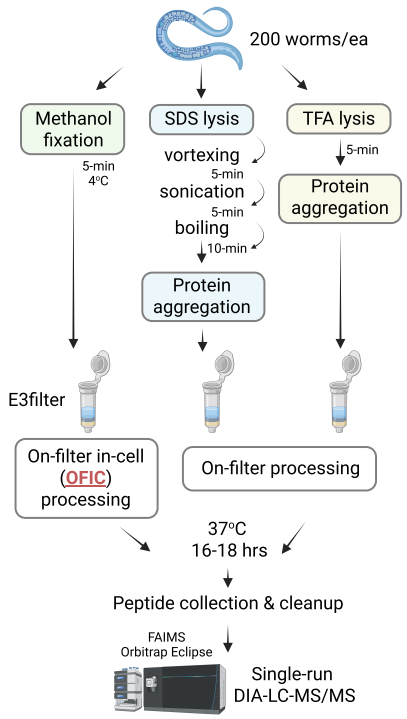

Figure 1. Illustrative flowchart for rapid and in-depth proteome profiling of *C. elegans*. For the OFIC processing method, worms were fixed with methanol followed by protein digestion using E3filters. For comparison, SDS- and TFA-lysed worms were processed on E3filters in parallel.

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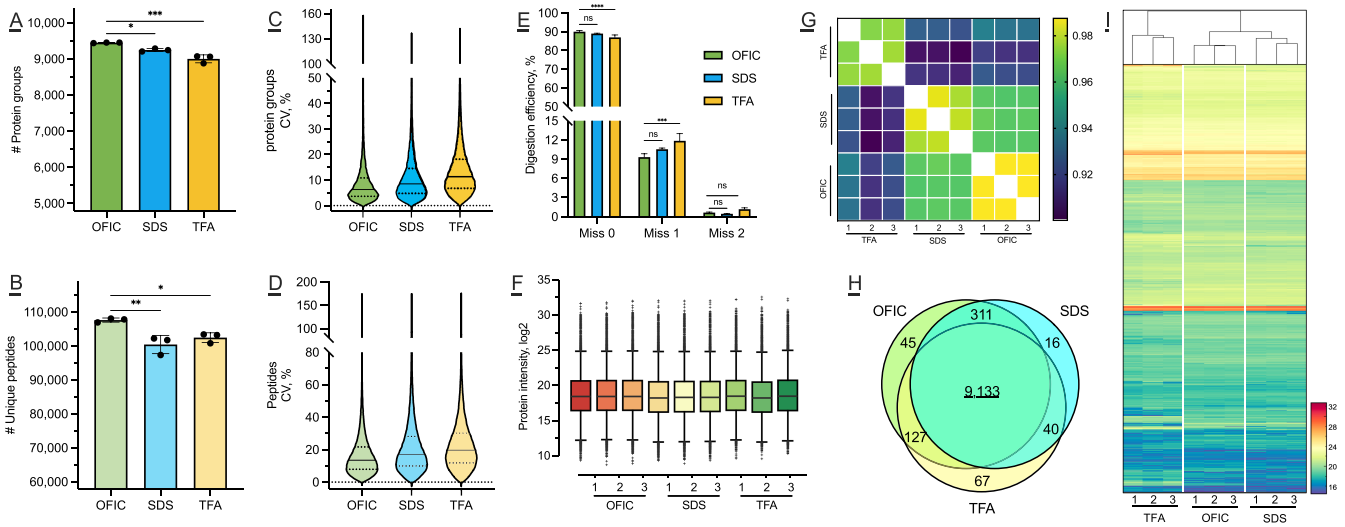

Figure 2. Evaluation of OFIC processing for *C. elegans* proteome analysis. (A, B) Comparison of the number of proteins and peptides derived from the three processing methods. Three replicate experiments were performed for each method. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (C, D) CV analysis for protein and peptide identifications. (E) Digestion efficiency calculated based on peptide intensity. (F) Box plot of protein intensity. (G) Pearson correlation of replicate experiments between all three methods. (H) Venn diagram of protein identifications. (I) Heatmap of all the proteins quantified by the three methods.

dependent acquisition (DDA)-based shotgun approach for highly reproducible proteome profiling.[27] However, to the best of our knowledge, single-shot LC-DIA MS/MS in combination with FAIMS has not been tested for *C. elegans* proteomics.

Using the OFIC processing approach with a single-shot LC-FAIMS-MS/MS 2.5 h acquisition run, we identified over 9400 nonredundant protein groups and 110,000 unique peptides from the 200-worm samples (Table S1). Only 154 of these were *E. coli* proteins, accounting for on average only 0.2% of total protein intensity, showing minimal contamination from the *E. coli* food source (Figure S2A,B, Table S1). All of the following analyses were focused solely on *C. elegans* proteins (Figure 2). The identification rate was very consistent among triplicate experiments, demonstrating the feasibility of digesting proteins directly in methanol-fixed *C. elegans*. When compared with the two lysis-based methods, OFIC processing provided better performance in the number of protein and peptide identifications (Figure 2A,B). Among all the protein and peptide hits identified by OFIC, approximately 96% and 85% of them were also identified by both lysis-based processing methods, respectively (Figure 2H, Figure S2C). For OFIC-derived proteins, the median coefficient of variation (CV) was 6.4%, better than those for the SDS (8.6%) and TFA (10.3%) methods. On the peptide level, OFIC also showed the least variation, 13.5% versus 17.1% for SDS and 19.6% for TFA (Figure 2C,D). We attribute these improvements to the single-device nature of the OFIC method, which not only reduces sample loss but also minimizes variations during sample handling.

We next assessed the digestion efficiency of the OFIC method. By peptide counts, around 23% and 3% of the peptides derived from OFIC digestion carried one and two missed cleavage sites, respectively (Figure S2D). This distribution is not significantly different from the SDS- and TFA-lysed samples and similar to recent tip-based[28] and filter-based digestion of cell lines and muscle tissue samples.[29,30] By peptide intensities, these miscleaved peptides contributed to less than 10% of the overall intensity of the OFIC-derived peptides (Figure 2E), suggesting a high quantitative yield of fully cleaved peptides by in-cell digestion. Quantitatively, the intensity distributions of the proteomes derived from the three processing methods were largely similar (Figure 2F). Unsupervised clustering analysis showed some distinctness of the TFA-based processing, while clustering of OFIC and SDS methods was tighter (Figure 2I). Overall, the proteome-level reproducibility of OFIC digestion (Pearson >0.98) outperformed the other two lysis-based methods (Figure 2G).

Methionine oxidation, which can occur during sample preparation for bottom-up proteomic experiments,[31] can hinder protein digestion and electrospray ionization, complicating investigation of this biologically important modification that occurs *in vivo*.[32] Consistent with previous on-filter digestion experiments, we found that about 10% of methionine-containing peptides were oxidized in the OFIC samples.[33] The OFIC processing produced the least summed intensity of oxidized peptides (Figure 3A), accounting for less than 0.7% of the overall precursor intensity, compared with the two lysis-based methods, which exhibited significantly more methionine oxidation. We then examined whether methanol induced artificial methylation and found only a limited number of identified peptides (760 out of over 157,000) with methylation modification, with intensities accounting for only ~0.6% of the total peptide intensity (Figure 3B). Thus, compared with lysate-based sample preparation methods, methanol fixation followed by OFIC digestion did not introduce excess artificial methylation modification, which agrees with previous findings.[17] Lastly, we did not observe significant differences in the size of the proteins identified between the three methods, although OFIC detected slightly more proteins smaller than 20 kDa (Figure S3).

Since OFIC processing does not require lysis steps before digestion and involves minimal sample transferring, we hypothesized that it would reduce sample loss and enhance proteome identification. To test this, we determined the protein and peptide yields of the *C. elegans* samples mechanically lysed with grinding, bead beating, and sonication

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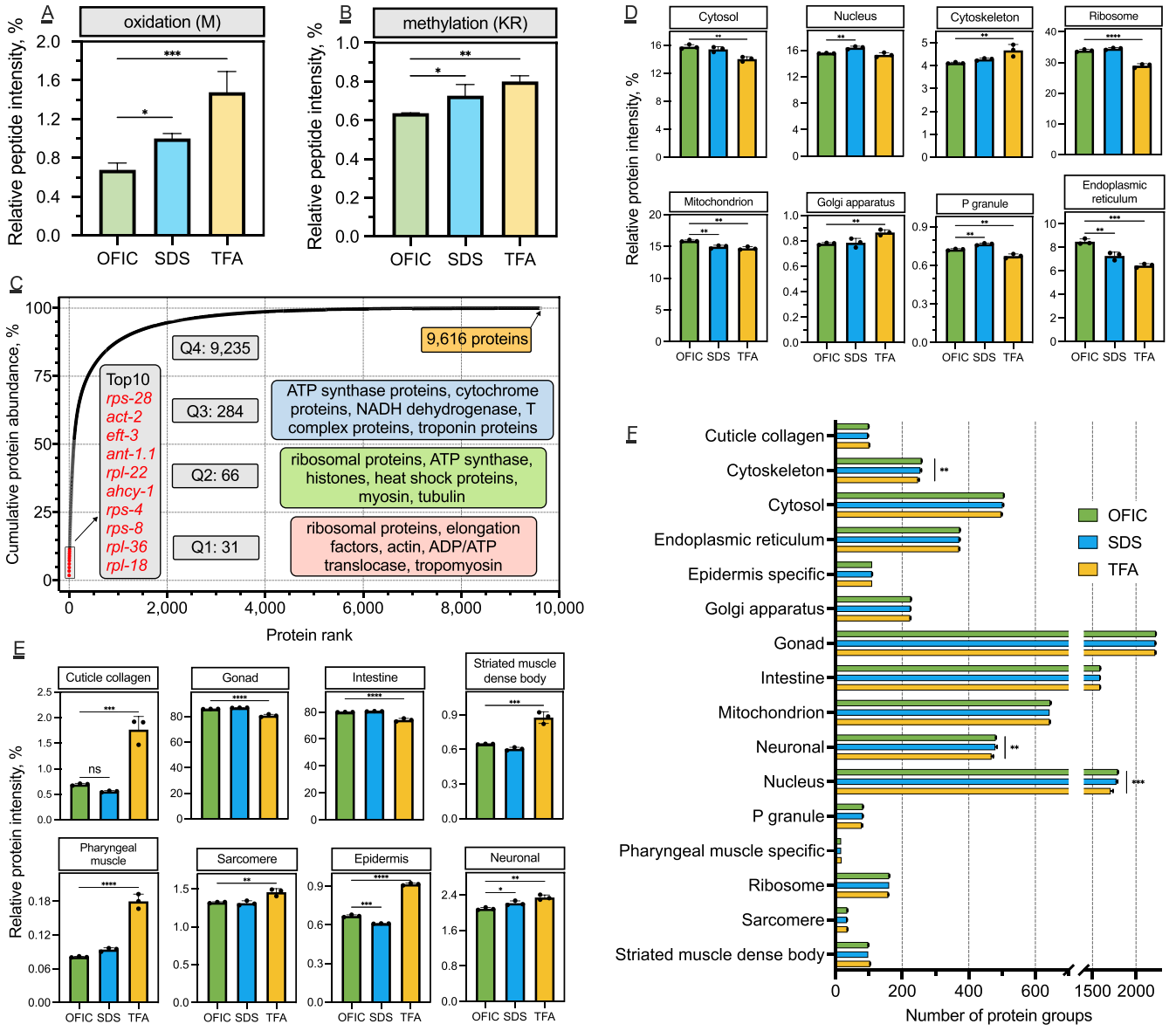

Figure 3. Dissecting the *C. elegans* proteome identified using the OFIC approach. (A) Intensity distribution of methionine oxidation and (B) lysine and arginine methylation among the three processing methods. The sum intensity of modification-containing peptides was divided by the total intensity of precursors for each run. (C) Total quantified *C. elegans* proteins identified by OFIC processing, ranked by abundance (iBAQ intensity). The top 10 most abundant proteins, number of proteins in each quartile, and functional classifications are as indicated. (D) Comparison of the intensity distribution of eight different groups of proteins based on subcellular localization for the three different processing methods. (E) Similar to (D), classes of proteins related to muscle, gonad, and intestine as well as neuronal proteins were compared among the three methods. (F) Number of protein hits classified in each category. Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

methods prior to LC-MS; for OFIC-processed samples, only peptide yield was determined. From an input of 200 worms, the amount of protein varied widely, depending on the lysis condition (Figure S4A). Manual grinding yielded the least amount of protein, likely due to significant sample loss in processing a relatively small number of worms. Determination of peptide output showed that the OFIC digestion gave the highest yield among the five processing methods, provided the largest number of protein and peptide hits after LC-MS, and also showed the least variability (Figure S4, Table S2). These data show that OFIC processing reduces sample loss and enhances the quantitation accuracy.

Evaluation of the *C. elegans* Proteome Derived from OFIC Digestion. Next, we took a closer look at the overall *C. elegans* proteome obtained by OFIC processing, totaling 9616 proteins after taking the mean quantity of the three biological replicates (Table S1). Notably, an in-depth study that used thousands of worms in combination with extensive peptide-level fractionation and a total of 320 h of LC-MS acquisition time identified only about 9400 proteins,[4] suggesting that our single-shot LC-MS analysis of OFIC-processed samples, even without any prefractionation, is capable of profiling the *C. elegans* proteome with similar depth. The OFIC-derived *C. elegans* proteome spanned over 7 orders of magnitude (Figure S5) and was dominated by a small proportion of proteins. The 31 most abundant proteins comprised 25% of the total proteome mass, while the most abundant 97 proteins comprised half of the total protein mass (Figure 3C). The

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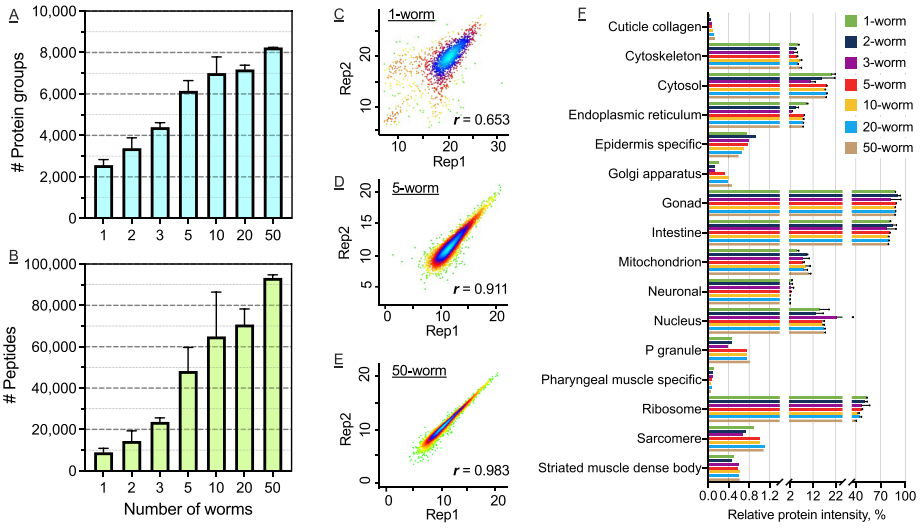

Figure 4. Using OFIC digestion to study the *C. elegans* proteome with ultralow input samples. (A) Protein and (B) peptide identifications summarized for different inputs of worms. (C–E) Representative Pearson correlation analysis for 1-, 5-, and 50-worm samples. (F) Relative intensities of proteins classified in different cellular and tissue types.

most abundant proteins include small and large ribosomal subunit proteins (e.g., RPS-28, RPL-22, RPS-4, and RPS-8), cytoskeleton proteins (e.g., actin ACT-2 and tubulin TBA-2, TBB-2), and nuclear proteins such as histones.

We then sought to determine whether there was a quantitative bias toward any subcellular protein groups or tissues. The overall intensities of the proteins in different classes varied depending on the processing method (Figure 3D–E). For example, the collagen protein intensities from the TFA lysate were significantly higher compared with those of the OFIC-digested samples. However, out of 173 predicted cuticle collagen proteins,[8] 100 were identified in OFIC-processed samples, showing no drastic difference from the other two lysis-based methods (98 from SDS lysate and 102 from TFA lysate). In fact, all three methods identified a very similar number of proteins for nearly all the classified subcellular compartments and tissue types, although the TFA-based method detected a smaller number of cytoskeletal, neuronal, and nuclear proteins (Figure 3F). Neuronal proteins play critical functional roles, yet a majority are present in relatively low abundance (Figure S5). We compared our data with a recently defined neuronal proteome by Aburaya et al.,[34] and found that nearly half of the 1039 reported neuronal proteins were also detected by OFIC, more than in the TFA lysate. We also looked at other tissue types, including the gonad and intestine. Comparing our study with tissue-specific proteomes[6] showed that OFIC processing offered equivalent coverage of these proteins as the SDS method and outperformed the TFA method. Taken together, we conclude that treatment of *C. elegans* with methanol permits enzyme accessibility to intraorganismal regions, enabling direct digestion of proteins in fixed worms.

OFIC Processing of Ultra-Low-Number *C. elegans* Samples. Having established that OFIC processing of 200 worms achieved a proteome depth similar to that of approaches with larger input and extensive fractionation, we set out to investigate if we could further reduce the initial input for in-cell processing. For this, we utilized the pipettetip-based method, E4tip, which integrates sample preparation, protein digestion, and peptide cleanup in a single device[11] to further reduce nonspecific sample loss. With a single LC-MS/MS run, we identified on average 2500 proteins from a single worm, nearly 4400 proteins from three worms, and over 6000 proteins from five worms (Figure 4A,B). As the input increased, we consistently gained more identifications such that from 50 worms, we were able to detect over 8800 proteins and nearly 110,000 peptides (Table S3). Less variability was also seen in the higher-input samples (Figure 4C–E, Figure S6) and the number of proteins from different subcellular compartments and tissues that was identified increased according to the input (Figure S7). However, the contributions of these proteins to the overall proteome by intensities remained largely similar among the various input experiments (Figure 4F). These data show the feasibility of OFIC processing for analysis of the *C. elegans* proteome with a small number of worms and suggest that, even with low input, the data tends to be unbiased with regard to tissue and subcellular localization, which is in line with the findings from recent low-cell and single-cell proteomics studies.[35,36]

OFIC Processing of *C. elegans* Eggs. We next determined the feasibility of using OFIC digestion on *C. elegans* embryos, which are physically protected by an eggshell and contain a large amount of maternal yolk proteins, presenting significant hurdles for low-abundance protein identification.[37,38] Previous studies utilized harsh lysis conditions and fractionation or enrichment strategies to obtain in-depth proteome coverage.[39–41] Here, we fixed eggs with methanol, performed OFIC digestion, and using single-shot LC-MS, identified nearly 7500 nonredundant proteins and over 75,000 unique peptides (Table S4), representing the largest *C. elegans* embryonic proteome reported to date. The protein and peptide identification rates were consistently better compared to those of the TFA-lysed samples (Figure 5A,B), again highlighting the power of the OFIC method. The abundance of the egg-derived proteins spans over 5 orders of magnitude, with some protein classes particularly relevant to embryogenesis showing much higher expression in the embryos compared to adult worms (Figure 5C). For example, six vitellogenin proteins contributed to almost 7% of the total egg protein mass and were nearly five times higher than in

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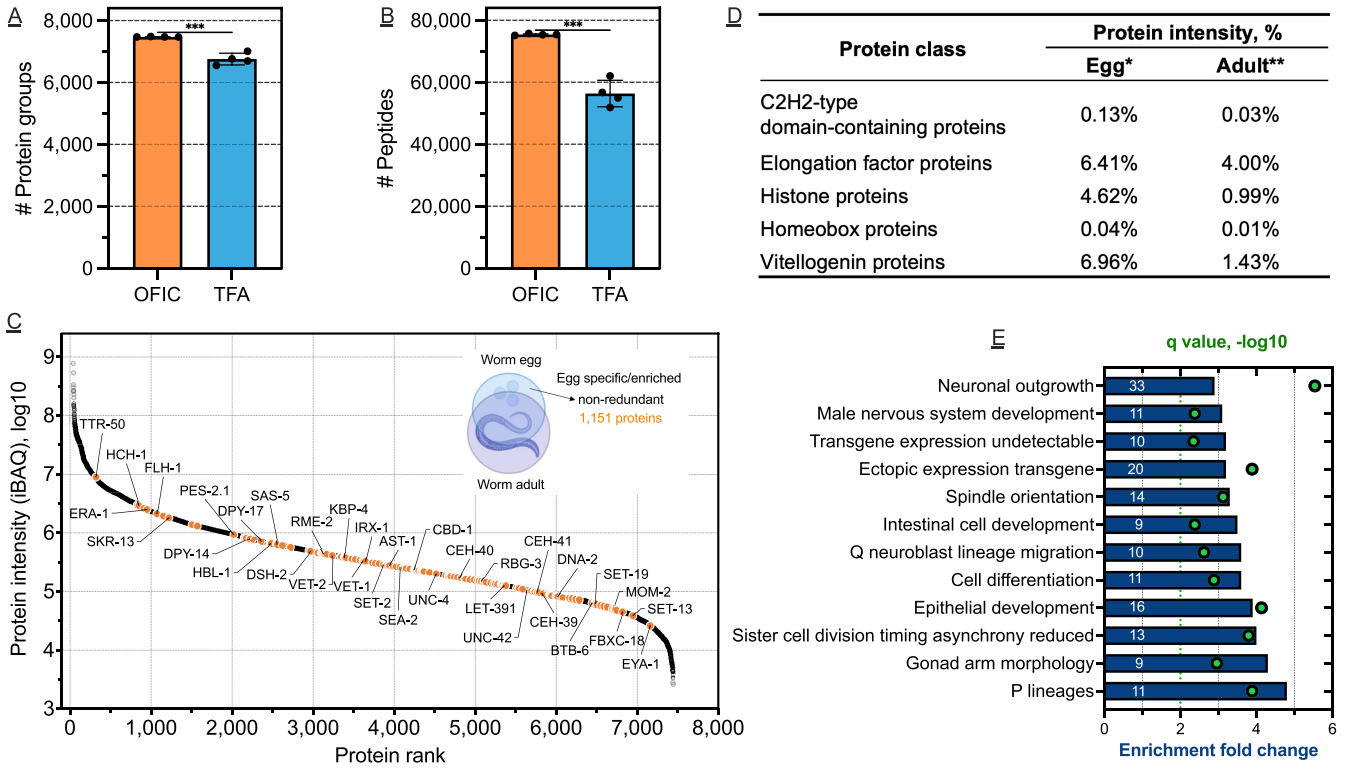

Protein class	Protein intensity, %	
	Egg*	Adult**
C2H2-type domain-containing proteins	0.13%	0.03%
Elongation factor proteins	6.41%	4.00%
Histone proteins	4.62%	0.99%
Homeobox proteins	0.04%	0.01%
Vitellogenin proteins	6.96%	1.43%

Figure 5. Using OFIC digestion to study the *C. elegans* embryo proteome. (A) Protein and (B) peptide identifications obtained with the OFIC digestion and TFA-lysis methods. *** $p < 0.001$. (C) Rank order plot of worm egg proteins. Inset: The Venn diagram shows the overlap between the worm egg proteome and adult worm proteome. Proteins highlighted in orange were exclusively identified in worm embryos. (D) Abundance comparison of representative protein classes. * and ** denote median values of eight and nine biological replicates, respectively. (E) Phenotype enrichment analysis of the egg-specific proteins. Blue bars represent fold enrichment. Green dots represent the *q* value (-log10 transformed). The green line indicates the threshold of the *q* value, i.e., 0.01. The number of proteins in each category is indicated.

adult worms (Figure 5D). We further examined the egg-enriched proteins and found that other functionally important proteins in the *C. elegans* embryo including the yolk receptor protein RME-2[42] and eggshell formation protein CBD-1[43] were identified in the OFIC-digested egg samples with high confidence (Figure 5C, Table S4). Nuclear proteins such as histones, C2H2 zinc finger proteins, and homeobox proteins were also enriched in the embryos compared to the adult worm proteome (Figure 5D). Further, proteins involved in cell division and differentiation were enriched in embryos (Figure 5E). These data show that OFIC digestion can be used for proteomic studies of *C. elegans* eggs and provide the worm embryo proteome, a rich source of data for developmental biology studies (Table S4).

Loss of SOD-1 Impacts the Global *C. elegans* Proteome. Having shown the feasibility and power of the OFIC digestion method for low-input, intact *C. elegans* proteomics sample processing, we sought to use this method to compare the proteomes of wild-type and mutant *C. elegans* (Figure 6A). Autosomal dominant mutations in *SOD1* cause ~1 in 5 cases of familial ALS through a gain-of-function mechanism,[22,23] and a recently approved therapeutic approach aims to reduce *SOD1* expression.[44] However, since complete loss of SOD1 function causes debilitating motor system abnormalities,[24,45,46] it is important to understand the consequences of lowering this gene product. Thus, we sought to use the OFIC digestion approach to determine how loss of *C. elegans sod-1*, which leads to an increase in both cytosolic and mitochondrial superoxide levels,[47] impacts the global proteome. To do so, we utilized E4tips to analyze 50 worms per sample, performing three biological replicates for both WT and *sod-1* mutant strains and quantified 8812 proteins from the six experiments (Table S5). Compared to the wild-type control, the abundance of 508 proteins in the *sod-1* mutant exhibited a ≥1.5-fold change, with significance defined as an adjusted *q* value <0.05 (Figure 6B). We then utilized WormCat, an online tool for analysis of *C. elegans* data sets,[48] to categorize the 241 upregulated and 267 down-regulated proteins (Figure 6C,D, Table S6).

Consistent with redox dysregulation, there was a significant increase in stress response proteins in the *sod-1* mutant, including glutathione S-transferase GST-24, and UDP glycosyltransferases UGT-26, UGT-29, UGT-31, and UGT-41. Notably, other stress response proteins, including heat shock proteins HSP-16.1 and HSP-16.2 and glutathione S-transferases GST-28 and GST-39, were significantly down-regulated, suggesting that loss of *sod-1* causes global changes in stress response (Table S6). Abundance of certain proteins involved in lipid metabolism, including the acyl-CoA synthetases ACS-1 and ACS-3, as well as the stearoyl-CoA desaturase FAT-7, was increased in the *sod-1* mutant. Several proteins involved in fatty acid metabolism are differentially regulated in ALS model mice with a SOD1(G93A) mutation,[49] which causes decreased enzyme activity,[50] suggesting that the impact of SOD-1 on metabolism is conserved. Examination of the proteins with reduced abundance in the *sod-1* mutant showed a highly significant enrichment of ribosomal proteins ($p < 10^{-37}$) including 24 RPL proteins, which are in the large

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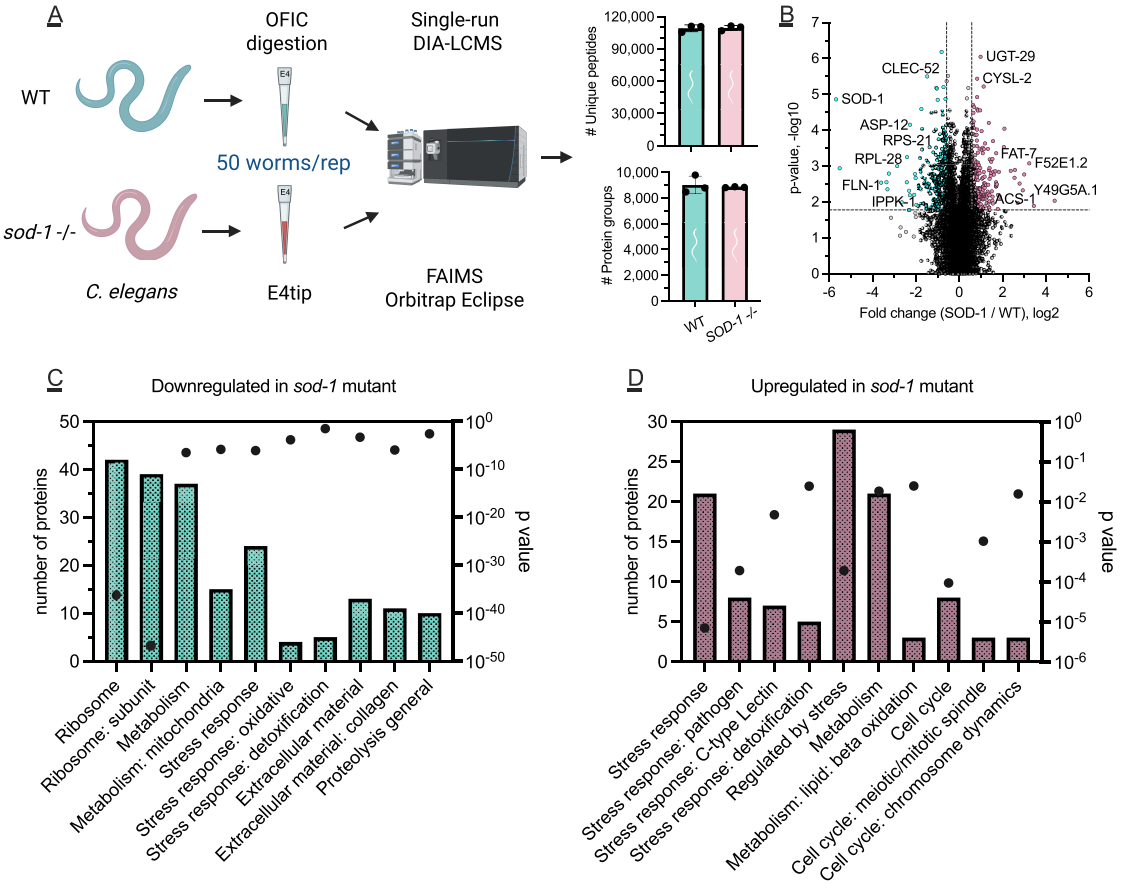

Figure 6. Use of OFIC processing to investigate the impact of SOD-1 on the proteome. (A) Overview diagram of the experimental workflow. (B) Volcano plot shows proteins with decreased (blue) and increased (red) abundance in the *sod-1* mutant; dotted lines show the 1.5-fold change and the adjusted q value of 0.05. (C) Number of upregulated and (D) downregulated proteins in significantly enriched categories ($p < 0.05$; Fisher's exact test) as determined by WormCat analysis. Bars represent the number of proteins in each class; dots indicate the p-value.

60S ribosomal subunit (Figure 6C, Table S6). Previously, knockout of *SOD1* was shown to affect the biogenesis of the 60S ribosomal subunit in KRAS mutant lung cancer cells,[51] and our data show that loss of SOD-1 has potential to impact ribosome abundance in noncancerous cells *in vivo*. These results also suggest that the loss of *sod-1* may cause reduced translation in older animals, which could have a substantial impact on cell function.

CONCLUSIONS

Here we have demonstrated both the feasibility and power of in-cell digestion for proteome analysis using low-input, methanol-fixed *C. elegans*. Unlike typical bottom-up proteomics that requires a large amount of starting material and long processing procedures, this innovative single-vessel approach is extremely convenient as it bypasses cell lysis and disruption, requires no further sample transferring after loading, and digests proteins directly in the cells, which substantially reduces sample loss and technical variation. Compared to traditional lysis-based methods, OFIC processing does not impact the number of protein and peptide identifications, reproducibility, digestion efficiency, undesired modification, and identification of proteins localized to subcellular compartments. Since OFIC processing only requires a low sample input, genotypes which previously could not be utilized for proteomics experiments, including males and balanced strains, can now be analyzed. Further, OFIC could easily be scaled up and automated for high-throughput experiments using the recently developed E4filter devices (e.g., E4plate),[11] which would enable use for high-throughput drug screening in *C. elegans*.[52]

Although the E4tip and single-shot LC-MS yield unprecedented proteome depth for low-input worms, one limitation of our study is that it only covered less than half of the predicted *C. elegans* proteome, hindering the identification of some low-abundance but important proteins. In the context of the SOD-1 protein, its abundance is relatively high (e.g., ranking top 500) and is detectable even in a single worm. Therefore, quantitative analysis of SOD-1 in various strains or under different pathological conditions is generally feasible. However, for some fertilization-related proteins that tend to have extremely low abundance, such as SPE-36, alternative strategies that can further improve the overall sensitivity of E4tip-based proteomics analysis are desired. For instance, the latest generation of LC-MS instrumentation with online sample processing, higher ion-transmission optics, and/or more sensitive detectors, such as the Evosep LC and Astral Orbitrap MS, will certainly help.[28] Another strategy is to reduce sample complexity through peptide-level fractionation (e.g., multishot LC-MS). An inherited advantage of E4technology is the ability to combine different chromatographic resins, thus providing orthogonal or complementary separation strategies. We have ongoing efforts toward this direction, and with that, we

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optimistically believe the proteome depth of single worms will be greatly enhanced.

Single vessel methods, pipette tip-based vessels in particular, are of particular interest to the proteomics community due to small reaction volume, reduced nonspecific adsorption to surfaces, and great ease of handling, scaling up, and automation.[28,53] In existing methods, digestion-compatible or LC-MS-friendly reagents are used to lyse cells and perform digestion; however, these chemicals may lead to a biased proteome or affect LC-MS analysis.[54,55] In our study, we have convincingly demonstrated that OFIC treatment can not only substantially simplify proteomic sample processing but also enable unbiased deep proteome profiling of samples with low input. The proteomic comparison of wild-type and *sod-1* mutant worms using OFIC-based E4tips showed the great potential of this simple method for answering biologically significant questions. Moving forward, we anticipate that OFIC methods such as E4tips could possibly be applied directly to a downstream data acquisition system such as Evosep LC, thus bypassing the "drying−resuspension" steps, further reducing sample loss and enhancing sensitivity. We feel confident that the OFIC approach will not only benefit the *C. elegans* proteomics community, as we demonstrated here, but also see its application to other systems as we have several ongoing projects focused on neural science, developmental biology, and plant immunity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c05003.

Supplementary Figures S1−S7. Microscopic evaluation of methanol-treated *C. elegans*. Evaluation of OFIC processing method specificity for *C. elegans*. Analysis of the sizes of proteins identified with three different sample processing methods. Comparison of OFIC with different mechanical lysis-based sample preparation methods. The dynamic range of the *C. elegans* proteome derived from OFIC digestion. Coefficient of variation analysis of protein groups identified in OFIC digestion experiments with different numbers of worms. The number of protein hits identified from OFIC digestion of different inputs of worms (PDF)

Supplementary Table S1. Detailed list of proteins identified from adult worm samples processed with the OFIC, SDS and TFA methods (Excel) (XLSX)

Supplementary Table S2. Detailed list of proteins identified from adult worm samples processed with the grinding, bead beating, sonication and OFIC methods (Excel) (XLSX)

Supplementary Table S3. Detailed list of proteins identified from varied inputs of adult worms processed with the OFIC method (Excel) (XLSX)

Supplementary Table S4. Detailed list of proteins identified from worm embryos processed with the OFIC and TFA methods (Excel) (XLSX)

Supplementary Table S5. Detailed list of proteins associated with *sod-1* mutant and wild-type *C. elegans* strains (Excel) (XLSX)

Supplementary Table S6. Detailed WormCat analysis of proteins with altered abundance in the *sod-1* mutant strain (XLSX)

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Notes

The authors declare the following competing financial interest(s): Y.Y. is a named inventor on a patent application (PCT/US2023/020215) for the technologies developed in this study, which has been licensed exclusively to CDS Analytical LLC through the University of Delaware. Other authors declare no conflict of interests.

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