Journal of Visualized Experiments

In vivo hydroxyl radical protein footprinting for the study of protein interactions in Caenorhabditis elegans

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60910R1
Full Title:	In vivo hydroxyl radical protein footprinting for the study of protein interactions in Caenorhabditis elegans
Section/Category:	JoVE Biochemistry
Keywords:	Caenorhabditis elegans, hydroxyl radical protein footprinting, FPOP, mass spectrometry, in vivo, proteomics
Corresponding Author:	Lisa Jones UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	ljones@rx.umaryland.edu
Order of Authors:	Lisa M. Jones
	Jessica A. Espino
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Baltimore, Maryland, United States

Cover Letter

UNIVERSITY of MARYLAND
SCHOOL OF PHARMACY
MASS SPECTROMETRY CENTER

Lisa M. Jones
Associate Professor
Department of Pharmaceutical Sciences
20 North Pine St N725
Baltimore, MD 21201
410 706 3380

ljones@rx.umaryland.edu

October 15, 2019

Benjamin Werth, Senior Science Editor
Journal of Visualized Experiments

Dear Benjamin Werth,

Please find enclosed the manuscript entitled "*In vivo* hydroxyl radical protein footprinting for the study of protein interactions in Caenorhabditis elegans" authored by Jessica A. Espino and Lisa M. Jones which we are submitting to the *Journal of Visualized Experiments*. This manuscript highlights the use of the hydroxyl radical protein footprinting method *in vivo* fast photochemical oxidation of proteins (IV-FPOP) for studying protein structure in *C. elegans*, an animal model for human disease. FPOP utilizes an excimer laser to photolyze hydrogen peroxide to generate hydroxyl radicals for protein labeling. Mass Spectrometry is used to detect and quantitate protein labeling. In this manuscript, we describe the procedure for carrying out this new method. This includes a description for assembling a custom flow system to flow the worms in a single file. We feel the production of a video with the flow system construction and IV-FPOP method would greatly benefit the readers of JOVE who want to use the method.

Sincerely,

Lisa M. Jones
Associate Professor
Department of Pharmaceutical Sciences
School of Pharmacy
University of Maryland

TITLE:

In Vivo Hydroxyl Radical Protein Footprinting for the Study of Protein Interactions in *Caenorhabditis elegans*

AUTHORS AND AFFILIATIONS:

Jessica A. Espino[1], Lisa M. Jones[1]

[1]Department of Pharmaceutical Sciences, University of Maryland, Baltimore, Maryland 21201, United States

Email address of co-authors:

Jessica A. Espino (jespino@umaryland.edu)

Corresponding author:

Lisa M. Jones (ljones@rx.umaryland.edu)

KEYWORDS:

Caenorhabditis elegans, hydroxyl radical protein footprinting, FPOP, mass spectrometry, in vivo, proteomics

SUMMARY:

In vivo fast photochemical oxidation of proteins (IV-FPOP) is a hydroxyl radical protein footprinting technique that allows for mapping of protein structure in their native environment. This protocol describes the assembly and set-up of the IV-FPOP microfluidic flow system.

ABSTRACT:

Fast oxidation of proteins (FPOP) is a hydroxyl radical protein footprinting (HRPF) method used to study protein structure, protein-ligand interactions, and protein-protein interactions. FPOP utilizes a KrF excimer laser at 248 nm for photolysis of hydrogen peroxide to generate hydroxyl radicals which in turn oxidatively modify solvent-accessible amino acid side chains. Recently, we expanded the use of FPOP of in vivo oxidative labeling in *Caenorhabditis elegans* (*C. elegans*), entitled IV-FPOP. The transparent nematodes have been used as model systems for many human diseases. Structural studies in *C. elegans* by IV-FPOP is feasible because of the animal's ability to uptake hydrogen peroxide, their transparency to laser irradiation at 248 nm, and the irreversible nature of the modification. The assembly of a microfluidic flow system for IV-FPOP labeling, IV-FPOP parameters, protein extraction, and LC-MS/MS optimized parameters are described herein.

INTRODUCTION:

Protein footprinting coupled to mass spectrometry (MS) has been used in recent years to study protein interactions and conformational changes. Hydroxyl radical protein footprinting (HRPF) methods probe protein solvent accessibility by modifying protein amino acid side chains. The HRPF method, fast photochemical oxidation of proteins (FPOP)[1], has been used to probe protein structure in vitro[2], in-cell (IC-FPOP)[3], and most recently in vivo (IV-FPOP)[4]. FPOP utilizes a 248 nm wavelength excimer laser in order to rapidly generate hydroxyl radicals by photolysis of hydrogen

peroxide to form hydroxyl radicals[1]. In turn, these radicals can label 19 out of 20 amino acids on a microsecond time scale, faster than proteins can unfold. Although, the reactivity of each amino acid with hydroxyl radicals extends 1000-fold, it is possible to normalize side chain oxidation by calculating a protection factor (PF)[5].

Since FPOP can oxidatively modify proteins regardless of their size or primary sequence, it proves to be advantageous for in-cell and in vivo protein studies. IV-FPOP probes protein structure in *C. elegans* similarly to in vitro and in-cell studies[4]. *C. elegans* are part of the nematode family and are widely used as a model to study human diseases. The ability of the worm to uptake hydrogen peroxide by both passive and active diffusion allows for the study of protein structure in different body systems. In addition, *C. elegans* are suited for IV-FPOP due to their transparency at the 248 nm laser wavelength needed for FPOP[6]. Coupling of this method to mass spectrometry allows for the identification of multiple modified proteins using traditional bottom-up proteomics approaches.

In this protocol, we describe how to perform IV-FPOP for the analysis of protein structure in *C. elegans*. The experimental protocol requires the assembly and set-up of microfluidic flow system for IV-FPOP adapted from Konermann et al[7]. After IV-FPOP, samples are homogenized for protein extraction. Protein samples are proteolyzed and peptides are analyzed by liquid chromatograph (LC) tandem MS, followed by quantification.

PROTOCOL:

1. *C. elegans* maintenance and culture

1.1. Grow and synchronize worms colonies to their fourth larvae (L4) stage following standard laboratory procedures[8].

1.2. The day of IV-FPOP experiment, wash L4 worms from bacterial lawns (OP50 *E. coli*) with M9 buffer (0.02 M KH_2PO_4, 0.08 M Na_2HPO_4, 0.08 M NaCl, 1 mM $MgSO_4$).

1.3. Obtain 500 µL aliquots of ~10,000 worms per IV-FPOP sample.

2. Microfluidic flow system assembly

2.1. Start the flow system assembly by cutting a 2 cm piece of fluorinated ethylene propylene (FEP) tubing (1/16 in. outer diameter (o.d.) x 0.020 in. inner diameter (i.d.)).

2.2. Using a clean dissecting needle, widen the i.d. of the FEP tubing in order to make a small crater at one end only, ~50 mm in length. The crater should be big enough to fit two 360 µm o.d. pieces of fused silica.

NOTE: Do not widen the opposite end as this end will only be used to fit the outlet capillary.

2.3. With a ceramic cutter, cut two 15 cm pieces of 250 µm i.d. fused silica. These two pieces will become the infusing lines of the flow system.

2.4. Using self-adhesive tape, tape the two 250 µm i.d. capillaries together ensuring they are parallel to each other and their ends are 100% flushed.

NOTE: To ensure the fused silica ends are not crushed after cutting, and are straight and 100% flushed against each other, it is recommended to examine them using a magnifying glass or under a stereo microscope.

2.5. Insert the two taped capillaries into the handmade crater of the FEP tubing. Push the capillaries up to the very edge of the handmade crater.

CAUTION: To maintain the efficacy of the flow system, do not push past the handmade crater (**Figure 1a**).

2.6. Place a small dot of epoxy resin on a clean surface and mix with a dissecting needle.

2.7. Quickly, using the same needle, place a small drop of resin at the end of the infusing capillaries where they connect with the FEP tubing and allow the resin to dry, hanging outlet-side up, for a few minutes (**Figure 1a**).

2.8. While the resin dries, binding the FEP tubing and two capillaries together, cut a new 250 µm i.d. capillary. The new capillary will become the outlet capillary of the flow system. The desired length of the capillary can be calculated using the equation below:

$$\ell = \frac{f \times t}{\pi \left(\frac{i.d.}{2}\right)^2}$$

Where ℓ is length of the capillary to be cut in centimeters, t is the desired reaction time in minutes, f is the flow rate in mL/min, and i.d. is the inner diameter of the capillary in centimeters.

NOTE: After cutting the outlet capillary, examine the ends ensuring they are straight and not crushed.

2.9. Once the resin has dried, insert the new capillary through the FEP tubing outlet end. The inside ends of the outlet capillary and the two infusing capillaries should be flush against each other inside the FEP tubing, this creates the mixing-T (**Figure 1b**).

2.10. Bind the outlet capillary and FEP tubing with fresh epoxy resin as described in steps 2.6 and 2.7.

2.11. Allow the resin to dry overnight binding the flow system together. Set up the microfluidic

flow system the next day (**Figure 1c**).

3. Microfluidic flow system for in vivo FPOP

3.1. Insert four magnetic stirrers inside one 5 mL syringe. This syringe becomes the sample syringe. The magnetic stirrers prevent the worms from settling inside the syringe during IV-FPOP (**Figure 2A**).

3.2. Fill the two 5 mL syringes with M9. Make sure there are no air bubbles inside each syringe as they can affect the flow rate and mixing efficiency.

3.3. Connect a Luer adapter to each 5 mL syringe ensuring they are finger tight and secured in place.

3.4. Attach each syringe to a single 3-2 valve. The syringe should be attached to the middle port of the valve (**Figure 2B**).

3.5. Secure each 5 mL syringe to the dual syringe pump and adjust the mechanical stop collar to prevent over pressure to the syringe from the pusher block. Keep in mind the addition of the magnetic stirrers when setting the stop collar.

3.6. Attach each infusing capillary end of the homemade microfluidic flow system to the top port of each 3-2 valve using a super flangeless nut, FEP sleeve, and super flangeless ferrule (**Figure 2B**).

3.7. To the remaining opened port of the 3-2 valve (bottom port), attach a 10 cm 450 µm i.d. capillary using a super flangeless nut, FEP sleeve, and super flangeless ferrule (**Figure 2B**). These capillaries become the withdrawing sample capillaries.

3.8. Start the pump flow and check all connections of the microfluidic flow system for visual leaks. Flow at least three syringe volumes using the experimental flow rate. The final flow rate is dependent on the laser irradiation window, laser frequency, and a zero-exclusion fraction volume.

NOTE: For this protocol, a final flow rate of 375.52 µL/min was calculated from a laser irradiation window of 2.55 mm, 250 µm i.d. fused silica, zero exclusion fraction, and 50 Hz frequency.

3.8.1. The flow path is marked by the arrows on the 3-2 valve handle. Each syringe can be refilled manually by moving the valve handle from the expelling position to the withdrawing position.

NOTE: Leaks at the 3-2 valve can be fixed by re-screwing the super flangeless nut or replacing any of the valve connections (super flangeless nut, FEP sleeve, or super flangeless ferrule). Leaks at the mixing-T require a reassembly of the microfluidic flow system (steps 2.1 to 2.11).

3.9. Move the microfluidic flow system to the experimental bench and secure the outlet capillary to the radiating stage using a 360 µm o.d. stainless steel union (**Figure 2C,D**).

3.10. Using a long-reach lighter, burn the coating of the fused silica at the laser irradiation window. Clean the burned coating using lint-free tissue and methanol.

NOTE: Alternate between burning cycles and methanol cleaning cycles to avoid over-heating the capillary as excess heat can melt and/or break the capillary.

3.11. Position the magnetic stirrer block above the 5 mL syringe containing the magnetic stirrers. Adjust the speed and position of the magnetic stirrer block so that the magnetic stirrers inside the 5 mL syringe are rotating slowly and constantly.

4. In vivo FPOP

4.1. Turn on the KrF excimer laser and allow the thyratron to warm-up.

CAUTION: The laser emits visible and invisible radiation at 248 nm wavelength that can damage eyes. Proper eye protection should be worn before turning on the laser and opening the beam exit window.

4.2. Measure the laser energy at a frequency of 50 Hz for at least 100 pulses by placing the optical sensor at the beam exit window.

NOTE: For this protocol, a 2.55 mm irradiation window was used with a laser energy of 150 ± 2.32 mJ.

4.3. Obtain approximatively 10,000 worms (500 µL) and manually withdraw the sample into the sample syringe.

4.4. Fill the sample syringe with 2.5 mL of M9 buffer for a final sample volume of 3 mL. Make sure that no air bubbles are introduced into the sample syringe.

4.5. Fill the second syringe with 3 mL of 200 mM H_2O_2, again ensuring no air bubbles are introduced into the syringe.

4.6. At the end of the outlet capillary, place a 15 mL conical tube wrapped in aluminum foil containing 6 mL of 40 mM N'-Dimethylthiourea (DMTU), 40 mM N-tert-Butyl-α-phenylnitrone (PBN), and 1% dimethyl sulfoxide to quench excess H_2O_2, hydroxyl radicals, and inhibit methionine sulfoxide reductase activity, respectively.

4.7. Start the excimer laser from the software window, wait for the first pulse, and start the sample flow from the dual syringe.

NOTE: Samples should be run in biological duplicates in technical triplicates under 3 samples conditions: laser irradiation with H_2O_2 (sample), no laser irradiation with H_2O_2 *and* no laser irradiation *no* H_2O_2 (controls), totaling to 9 samples per biological set.

4.8. Collect the entire sample in the 15 mL tube, while actively monitoring the sample flow for any visual leaks as some back pressure from the sample syringe can sometimes build-up.

4.9. Following IV-FPOP, pellet worms by centrifugation at 805 x *g* for 2 min. Remove quench solution, add 250 µL of lysis buffer (8 M urea, 0.5% SDS, 50 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Transfer the sample to a clean microcentrifuge tube, flash freeze, and store at -80 °C until sample digestion.

5. Protein extraction, purification, and proteolysis

5.1. Thaw frozen samples on ice and homogenize by sonication for 10 s, followed by a 60 s ice incubation. Multiple rounds of sonication may be required, homogenate can be observed under a stereomicroscope by placing a 2 µL sample aliquot on a microscope slide. Sample homogenization is complete when small to no pieces of worms are seen.

5.2. Centrifuge homogenized worms at 400 x *g* for 5 min at 4 °C and collect the supernatant into a clean microcentrifuge tube.

5.3. Determine sample protein concentrations using a bicinchoninic acid assay (BCA assay) using the manufacturer's instructions.

NOTE: Sample concentration can be determined using any biochemical protein concentration assay of choice. Keep in mind reagent compatibility with lysis buffer (8 M urea, 0.5% SDS, 50 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM PMSF). Additionally, a buffer dilution may be necessary.

5.4. Obtain 100 µg of protein from each sample and place in clean microcentrifuge tubes.

5.5. Add dithiothreitol (DTT) to a 10 mM final concentration to reduce disulfide bonds in all samples. Vortex, spin down, and incubate samples at 50 °C for 45 min.

5.6. Cool samples to room temperature for 10 min.

5.7. Add iodoacetamide (IAA) to a 50 mM final concentration to alkylate reduced residues. Vortex, spin down, and incubate samples for 20 min at room temperature protected from light.

5.8. Immediately after alkylation, precipitate the protein sample by adding 4 volumes of 100% pre-chilled acetone and incubate at -20 °C overnight.

5.9. The next day, pellet protein precipitate by centrifugation at 16,000 x *g* for 10 min. Wash sample pellet with 90% acetone, remove supernatant, and allow pellet to dry for 2–3 min.

5.10. Re-suspend the protein pellet in 25 mM Tris-HCl (1 µg/µL). Add trypsin at a final protease to protein ratio of 1:50 (w/w) and digest samples at 37 °C overnight.

5.11. The next day quench the trypsin digestion reaction by adding 5% formic acid.

NOTE: A minimum of 0.5 µg of peptides per sample is needed for LC-MS/MS analysis (steps 6.1-6.5). Determine sample peptide concentrations using a quantitative colorimetric peptide assay (see the **Table of Materials**) as described by the manufacturer's protocol.

6. High performance liquid chromatography-tandem mass spectrometry (LC-MS/MS)

6.1. Use the following LC mobile phases: water in 0.1% FA (A) and ACN in 0.1% FA (B).

6.2. Load 0.5 µg of peptides onto a trap column (180 µm × 20 mm) containing C18 (5 µm, 100 Å) and wash sample with 99% solvent A and 1% B for 15 min at 15 µL/min flow rate.

6.3. Separate peptides using an in-house packed column (0.075 mm i.d. × 20 mm) with C18 reverse phase material (5 µm, 125 Å).

6.4. Use the following analytical separation method: the gradient was pumped at 300 nL/min for 120 min: 0−1 min, 3% B; 2−90 min, 10−45% B; 100−105 min, 100% B; 106−120 min, 3% B.

6.5. Perform data acquisition of peptides in positive ion mode nano-electrospray ionization (nESI) using a high-resolution mass spectrometer.

NOTE: For this protocol, an ultra-performance LC (UPLC) instrument couple to a high-resolution mass spectrometer was utilized. Data dependent acquisition was utilized. The m/z scan range for MS1 was 3750–1500 at 60,000 resolution and 60 s dynamic exclusion. Ions with charge states of +1 and >6 were excluded. An automatic gain control (AGC) target of 5.5e5 was used with a maximum injection time of 50 ms and an intensity threshold of 5.5e4. Ions selected for MS2 were subjected to higher-energy collisional dissociation (HCD) fragmentation using 32% normalized collision energy. Fragment ions were detected in the spectrometer with 15,000 resolution and a 5.0e4 AGC target.

7. Data analysis

7.1. Search tandem MS files with a bottom-up proteomics analysis software against the *C. elegans* database.

7.2. Set the protein analysis search parameters as follow: one trypsin missed cleavage, 375–1500 m/z peptide mass range, fragment mass tolerance of 0.02, and precursor mass tolerance of 10 ppm. Carbamidomethylation is set as a static modification and all know hydroxyl radical side-chain modifications[9,10] as dynamic. Peptide identification is established at 95% confidence

(medium) and residue at 99% confidence (high). The false discovery rate (FDR) is set a 1%.

7.3. Export data to an electronic database and summarize the extent of oxidation per peptide or residue using the equation below[11]:

$$\frac{\sum \text{EIC area modified}}{\sum \text{EIC area}}$$

NOTE: Where EIC area modified is the extracted ion chromatographic area (EIC) of the peptide or residue with an oxidative modification, and EIC area is the total area of the same peptide or residue with and without the oxidative modification.

REPRESENTATIVE RESULTS:

In the microfluidic flow system used for IV-FPOP, H_2O_2 and the worms are kept separated until just prior to laser irradiation. This separation eliminates breakdown of H_2O_2 by endogenous catalase and other cellular mechanisms[12]. The use of a 250 µm i.d. capillary shows a total sample recovery between 63–89% across two biological replicates, while the 150 µm i.d. capillary only shows 21–31% recovery (**Figure 3A**). The use of a larger i.d. capillary (250 µm) leads to better worm flow during IV-FPOP and single worm flow (**Figure 3B**) when compared to a smaller i.d. capillary (150 µm) (**Figure 3C**). The 150 µm i.d. capillary does not allow for single worm flow (**Figure 3C**) and multiple worms are seen flowing together at the laser irradiating window which decreases the amount of laser exposure per single worm.

IV-FPOP is a covalent labeling technique that probes solvent accessibility in *C. elegans*. **Figure 4A** shows a representative extracted ion chromatograms (EIC) of a FPOP modified and unmodified peptide. The hydroxyl radical label changes the chemistry of oxidatively modified peptides, thus making FPOP modified peptides more polar. In reverse phase chromatography, IV-FPOP modified peptides have earlier retention times than unmodified peptides. MS/MS fragmentation of isolated peptides allows for the identification of oxidatively modified residues (**Figure 4B**).

IV-FPOP has shown to oxidatively modified a total of 545 proteins across two biological replicates within *C. elegans* (**Figure 5A,B**). An advantage of IV-FPOP as a protein footprinting method relies on the technique's ability to modify proteins in a variety of body systems within the worms (**Figure 5C**). This method would allow to probe protein structure and protein interactions regardless of body tissue or organ within the worm. Further, tandem MS analysis confirms IV-FPOP probes solvent accessibility in vivo. The oxidation pattern of the heat shock protein 90 (Hsp90) in complex with the myosin chaperon protein UNC-45 was analyzed (**Figure 6**). MS/MS analysis for Hsp90 shows four oxidatively modified residues (**Figure 6C,D**), the normalized extent of FPOP modification $(\ln(PF))^5$ indicates Hsp90's residue M698 to be less solvent accessible than residues R697, E699, and E700 when bound to UNC-45 (**Figure 6C**). These differences in oxidation are validated by literature solvent accessible surface area (SASA) calculations (PDB 4I2Z[13]). Residue M698 has a SASA value of 0.03 which is consider to be a buried residue when compared to residues R697, E699, and E700 with higher SASA values (**Figure 6C**).[14]

FIGURE LEGENDS:

Figure 1. In vivo FPOP microfluidic flow system schematic. (**A**) The two infusing lines (orange) of the IV-FPOP flow system are shown inside the FEP tubing (yellow), the correct binding position of the epoxy resin is represented by the light blue circle. (**B**) Complete assembled mixing-T formed by the three 250 µm i.d. capillaries. The correct resin binding position of the outlet capillary to the FEP tubing is represented by the light blue circle. (**C**) The complete assembled flow system for in vivo covalent labeling of *C. elegans*. Prior to FPOP, worms are kept separated from H_2O_2 until just prior to labeling; the laser irradiation window is shown in light blue and the laser beam is represented by the purple lightning bolt. Figures are not to scale. This figure has been modified from Espino et al.[4].

Figure 2. Microfluidic system during IV-FPOP. (**A**) Representative picture of *C. elegans* inside the 5 mL syringe. Without stirring, the worms settle at the bottom of the syringe (left). The magnetic stirrers and stirrer block keep the worms in suspension during the IV-FPOP experiments (right). (**B**) Representative picture of a 5 mL syringe, infusing capillary, and withdrawing capillary connected to the 3-2 valve. The 3-2 valve handle is shown in the withdrawing position. (**C**) Microfluidic flow system during IV-FPOP, the magnetic stirrer block is position above the worms' 5 mL syringe. (**D**) Outlet capillary secured to the radiating stage.

Figure 3. Comparison of *C. elegans* flow and recovery using two i.d. capillaries. (**A**) Percent recovery of worms after IV-FPOP for two biological replicates (BR) with 250 (gray) and 150 (black) µm i.d. capillaries. Error bars are calculated from the standard deviation across technical triplicates. *C. elgans* flowing through the laser irradiating window through a 250 µm (**B**) and 150 µm (**C**) i.d. capillaries. The worms are more tightly compacted in the smaller capillary. The 150 µm i.d. capillary shows clumping of worms. This figure has been modified from Espino et al.[4].

Figure 4. Representative LC-MS/MS results following IV-FPOP. (**A**) EIC of a FPOP modified peptide (red) and unmodified (blue). The selected peptide belongs to the actin-1 protein. (**B**) MS/MS spectrum of doubly charged unmodified actin-1 peptide 317-327. (**C**) MS/MS spectrum of doubly charged FPOP modified actin-1 peptide 317-327, in this example P323 was oxidatively modified (y_5^+ ion, red).

Figure 5. IV-FPOP oxidatively modifies proteins within *C. elegans.* (**A**) Venn diagram of oxidatively modified proteins in the presence of 200 mM hydrogen peroxide at 50 Hz across two biological replicates (BR), BR1 is in blue and BR2 is in yellow. (**B**) Venn diagram of oxidatively modified proteins identified in irradiated samples, hydrogen peroxide control, and worm-only control in BR2 across technical triplicates. (**C**) Pie chart of oxidatively modified proteins within different *C. elegans* body systems. This figure has been modified from Espino et al.[4].

Figure 6. Correlating IV-FPOP modifications to solvent accessibility. (**A**) Myosin chaperon protein UNC-45 (gray) (PDB ID 4I2Z[13]) highlighting two modified peptides identified by LC/MS/MS analysis, 669−680 and 698−706 (green, left inset). UNC-45 is bound to the Hsp90 peptide fragment (blue). Oxidatively modified residues within this fragment are shown in sticks (red), and

UNC-45 is rendered as a surface (right inset). (**B**) Tandem MS spectra of UNC-45 peptide 669−680 (top) and 698−706 (bottom) showing b- and y-ions for the loss of CO_2, an FPOP modification. (**C**) The calculated ln(PF) for the Hsp90 oxidatively modified residues, R697, M698, E699, and E700. Calculated SASA values for Hsp90 are denoted above each residue. (**D**) Tandem MS spectra for R697, M698, E699, and E700 showing a +16 FPOP modification. This figure has been modified from Espino et al.[4].

DISCUSSION:

The current benchmark for the study of in vivo protein-protein interactions (PPI) is fluorescence resonance energy transfer (FRET). In its most simple form, this technique studies PPI by energy transfer between two molecules when they are in close proximity to one another[15]. Unlike MS techniques, FRET does not have the resolution to characterize conformational changes and interaction sites at the amino-acid level. MS based techniques have been increasingly utilized for the study of PPI[16]. IV-FPOP is a HRPF method that allows for the in vivo protein structural analysis in *C. elegans.* In order to successfully label *C. elegans* by IV-FPOP, it is important to properly assemble the microfluidic flow system to reduce sample loss. The 250 µm i.d. capillary has shown to maximize sample recovery when compared to smaller i.d. capillaries[4]. Larger i.d. capillaries have not been tested, however the microfluidic flow system is designed using a capillary with the same i.d. as a commercially available flow cytometry system for the sorting of *C. elegans*.[17] The worm sample size is also important, a sample size of less than ~10,000 per sample prior to FPOP does not yield protein concentrations high enough for LC-MS/MS analysis. Higher samples sizes (>10,000 worms) can also be used by adjusting the initial starting volume (step 4.5).

Proper assembly of the microfluidic flow system is important. Leaks in the sample pathway result in an inconsistent flow of the worms or H_2O_2. The ferrules, sleeves, and 3-2 valves can be reused from multiple IV-FPOP experiments if properly cleaned after each experiment. However, we recommend to assemble a new microfluidic flow system for every biological replicate. If the microfluidics is properly assembled, the worms and H_2O_2 will mix at the mixing-T with minimal back pressure. As quality control (QC) of the microfluidic flow system, we recommend testing the mixing efficiency by using colored dyes. It is important to monitor the motion of the magnetic stirrers inside the worm syringe during IV-FPOP, improper sample mixing at the worm syringe or the mixing-T can result in back pressure causing leaks. In addition, poor mixing conditions lead to large sample losses, poor laser exposure of worms at the laser window, and clogging.

C. elegans maintenance is important in order to decrease background oxidation. We recommend growing the worms at low temperatures under low stress conditions as high temperatures can affect total background oxidation. A control sample set of worms only, no H_2O_2 and no laser irradiation, is recommended for all IV-FPOP experiments to account for background oxidation due to laboratory maintenance. One of the current limitations of this technique is total number of oxidatively modified peptides identified and the total number of residues oxidized per peptide in order to gain higher protein structural information. Although not recommended, an increase in oxidative modifications could be achieve by using higher concentrations of hydrogen peroxide. An increase in hydrogen peroxide could significantly alter important biological pathways as well as lead to oxidation-induced unfolding. If the hydrogen peroxide concentration for IV-FPOP is

increased, it is recommended to test worm viability and background oxidation as concentrations higher than 200 mM have not been tested.

The LC-MS/MS protocol described can be optimized and modified to meet the MS QC of other laboratories. The use of 2D-chromatography techniques has been previously shown to increase the identification of oxidatively modified peptides and proteins[18]. Nonetheless, protein enrichment techniques that target a specific protein of interest are not recommend, including but not limited to antibody precipitation or pull-down assays. These techniques can bias towards one protein conformer if the epitope/binding site of the protein has been oxidatively modified by IV-FPOP. New developments in footprinting radical reagents, such as sulfate radical anion[10] or trifluoromethylation[19] could increase the versatility of IV-FPOP. Although the only labeling reagent tested in vivo thus far is hydrogen peroxide. other laser-activated radicals could be optimized. The use of other radicals should prove to be compatible with worm viability, cell permeable, and the 248 nm laser wavelength. Owing to the use of *C. elegans* as a model system for many human diseases, IV-FPOP has the potential to have a strong impact in studying the role of protein structure in disease pathogenesis.

ACKNOWLEDGMENTS:

This work was supported by start-up funds from the University of Maryland, Baltimore and the NIH 1R01 GM 127595 awarded to LMJ. The authors thank Dr. Daniel Deredge for his help in editing the manuscript.

DISCLOSURES:

This publication was written in partial fulfillment of JAE Ph.D. thesis. The authors declare no conflict of interest.

REFERENCES:

1 Hambly, D. M., Gross, M. L. Laser flash photolysis of hydrogen peroxide to oxidize protein solvent-accessible residues on the microsecond timescale. *J Am Soc Mass Spectrom.* **16** (12), 2057-2063, (2005).

2 Jones, L. M., B. Sperry, J., A. Carroll, J., Gross, M. L. Fast Photochemical Oxidation of Proteins for Epitope Mapping. *Analytical Chemistry.* **83** (20), 7657-7661, (2011).

3 Espino, J. A., Mali, V. S., Jones, L. M. In Cell Footprinting Coupled with Mass Spectrometry for the Structural Analysis of Proteins in Live Cells. *Analytical Chemistry.* **87** (15), 7971-7978, (2015).

4 Espino, J. A., Jones, L. M. Illuminating Biological Interactions with in Vivo Protein Footprinting. *Analytical Chemistry.* **91** (10), 6577-6584, (2019).

5 Huang, W., Ravikumar, K. M., Chance, M. R., Yang, S. Quantitative mapping of protein structure by hydroxyl radical footprinting-mediated structural mass spectrometry: a protection factor analysis. *Biophysical Journal.* **108** (1), 107-115, (2015).

6 Keller, C. I., Calkins, J., Hartman, P. S., Rupert, C. S. UV photobiology of the nematode Caenorhabditis elegans: action spectra, absence of photoreactivation and effects of caffeine. *Photochemistry and Photobiology.* **46** (4), 483-488, (1987).

7 Konermann, L., Collings, B. A., Douglas, D. J. Cytochrome c Folding Kinetics Studied by

Time-Resolved Electrospray Ionization Mass Spectrometry. *Biochemistry.* **36** (18), 5554-5559, (1997).

8 Brenner, S. The genetics of Caenorhabditis elegans. *Genetics.* **77** (1), 71-94, (1974).

9 Xu, G., Chance, M. R. Hydroxyl radical-mediated modification of proteins as probes for structural proteomics. *Chemical Reviews.* **107** (8), 3514-3543, (2007).

10 Gau, B. C., Chen, H., Zhang, Y., Gross, M. L. Sulfate radical anion as a new reagent for fast photochemical oxidation of proteins. *Analytical Chemistry.* **82** (18), 7821-7827, (2010).

11 Rinas, A., Espino, J. A., Jones, L. M. An efficient quantitation strategy for hydroxyl radical-mediated protein footprinting using Proteome Discoverer. *Analytical and Bioanalytical Chemistry.* **408** (11), 3021-3031, (2016).

12 Wagner, B. A., Witmer, J. R., van 't Erve, T. J., Buettner, G. R. An Assay for the Rate of Removal of Extracellular Hydrogen Peroxide by Cells. *Redox Biology.* **1** (1), 210-217, (2013).

13 Gazda, L. *et al.* The myosin chaperone UNC-45 is organized in tandem modules to support myofilament formation in C. elegans. *Cell.* **152** (1-2), 183-195, (2013).

14 Willard, L. *et al.* VADAR: a web server for quantitative evaluation of protein structure quality. *Nucleic Acids Research.* **31** (13), 3316-3319, (2003).

15 Broussard, J. A., Green, K. J. Research Techniques Made Simple: Methodology and Applications of Förster Resonance Energy Transfer (FRET) Microscopy. *Journal of Investigative Dermatology.* **137** (11), e185-e191, (2017).

16 Kaur, U. *et al.* Evolution of Structural Biology through the Lens of Mass Spectrometry. *Analytical Chemistry.* **91** (1), 142-155, (2019).

17 Pulak, R. in *C. elegans: Methods and Applications* 10.1385/1-59745-151-7:275 (ed Kevin Strange) 275-286 (Humana Press, 2006).

18 Rinas, A., Jones, L. Fast Photochemical Oxidation of Proteins Coupled to Multidimensional Protein Identification Technology (MudPIT): Expanding Footprinting Strategies to Complex Systems. *Journal of the American Society for Mass Spectrometry.* **26** (4), 540-546, (2015).

19 Cheng, M., Zhang, B., Cui, W., Gross, M. L. Laser-Initiated Radical Trifluoromethylation of Peptides and Proteins: Application to Mass-Spectrometry-Based Protein Footprinting. *Angewandte Chemie International Edition.* **56** (45), 14007-14010, (2017).

Figure1

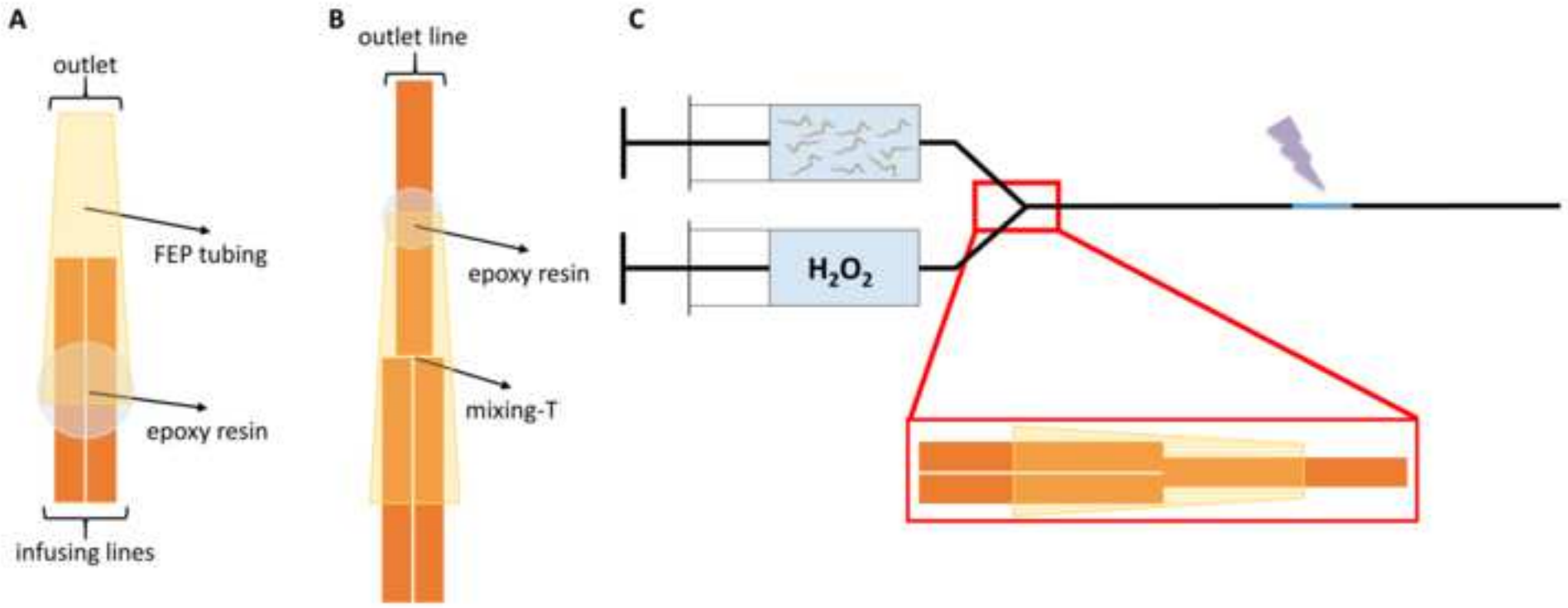


Figure2

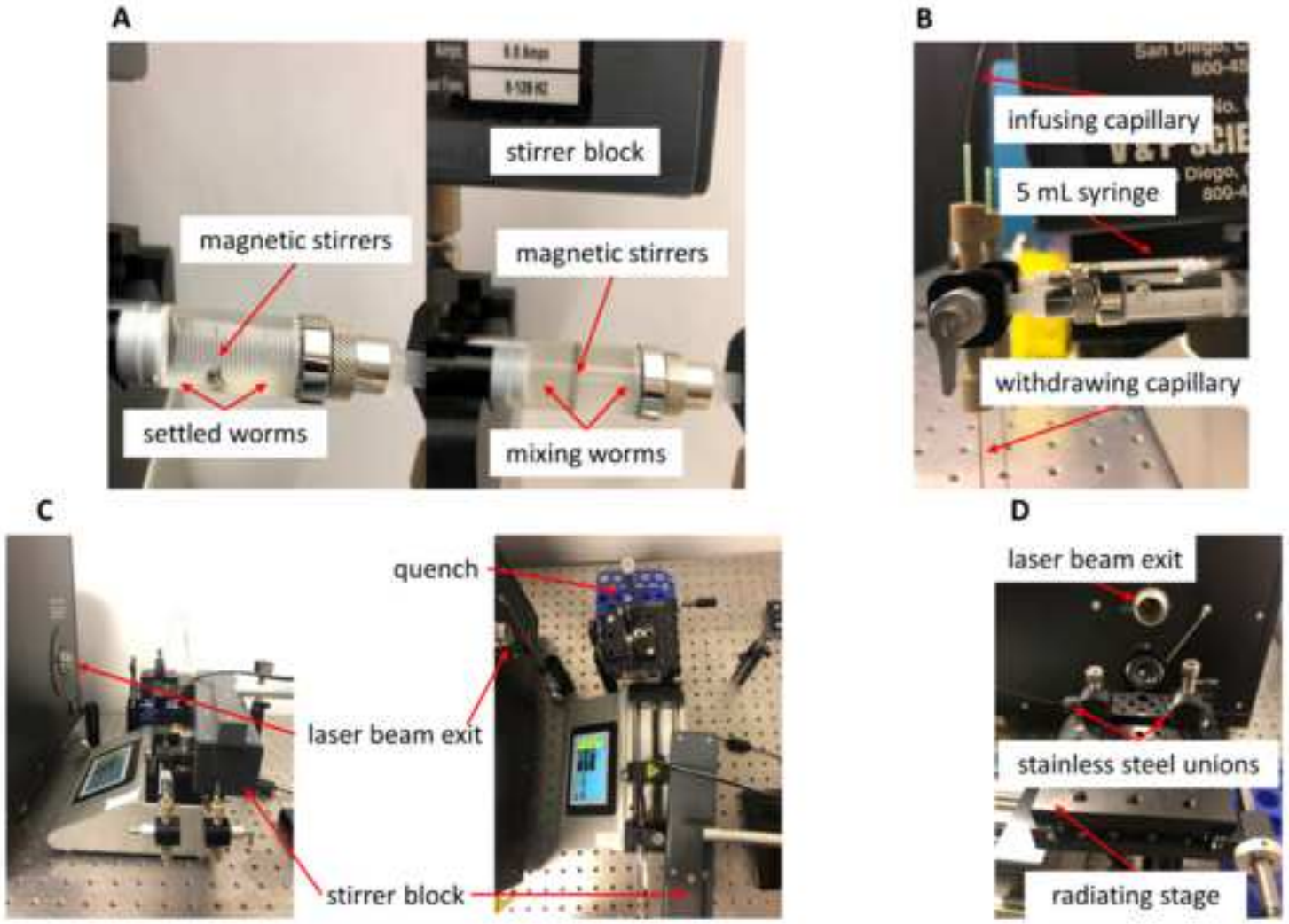


Figure3

Click here to access/download;Figure;Figure3.TIF

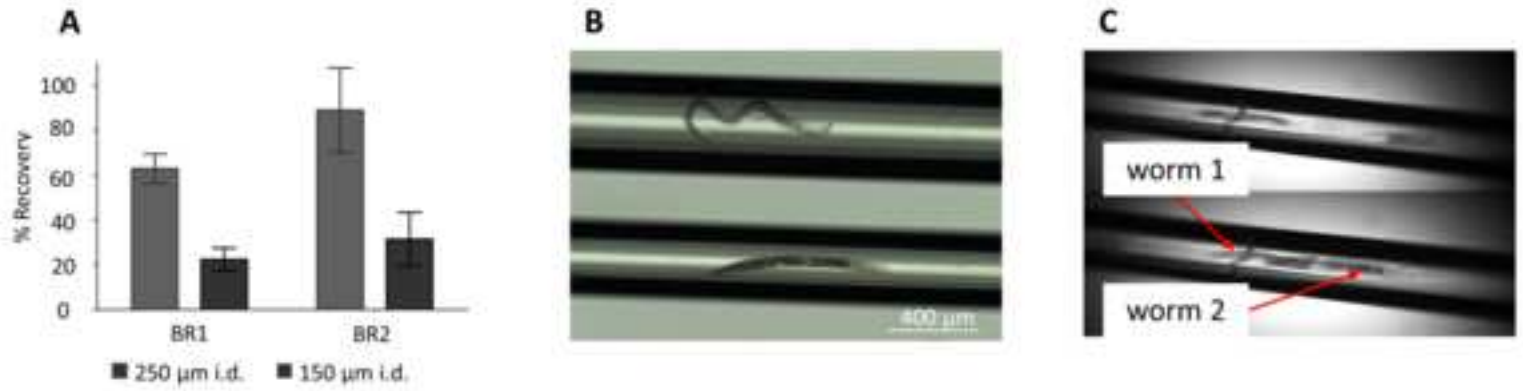


Figure4

Click here to access/download;Figure;Figure4.TIF

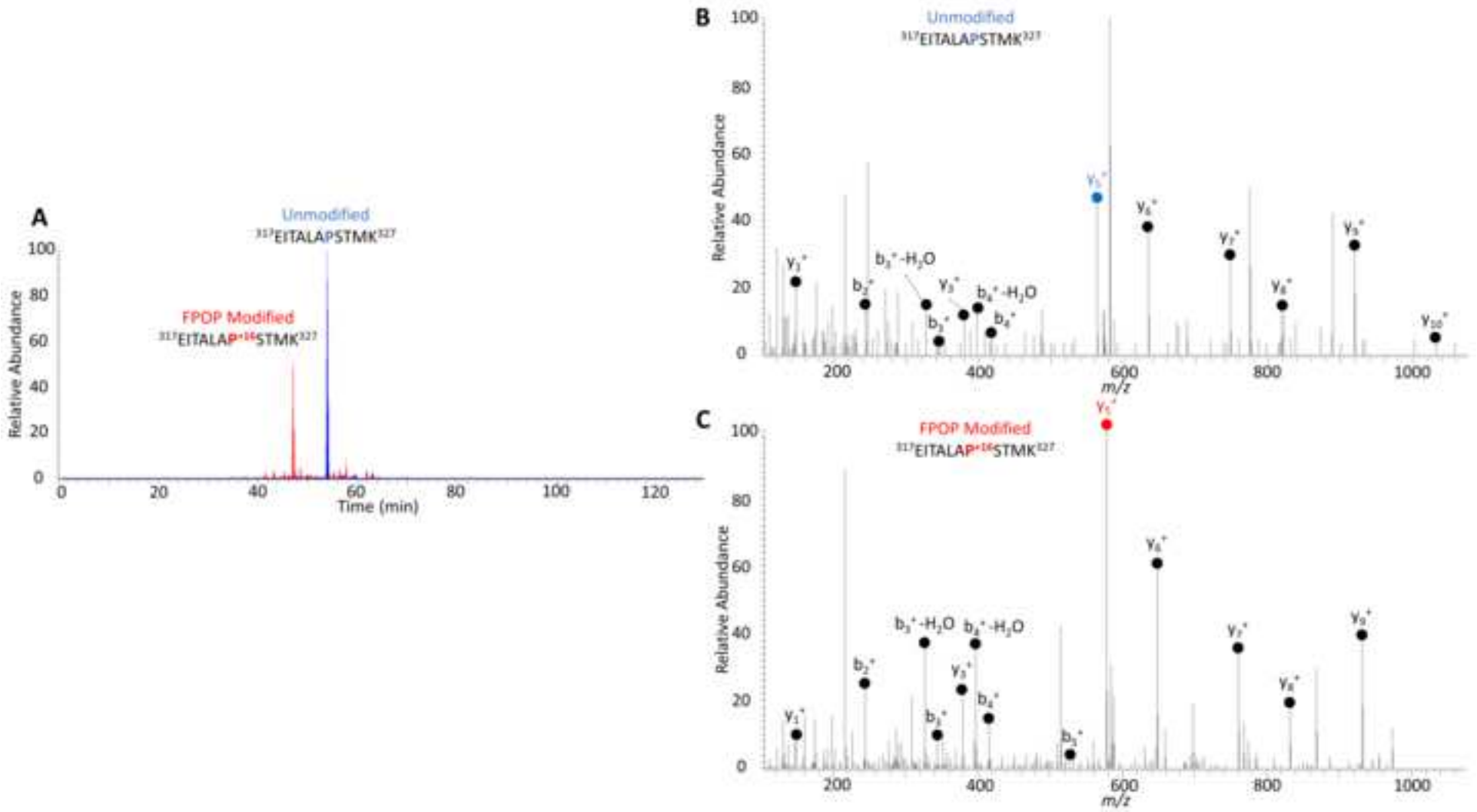


Figure5

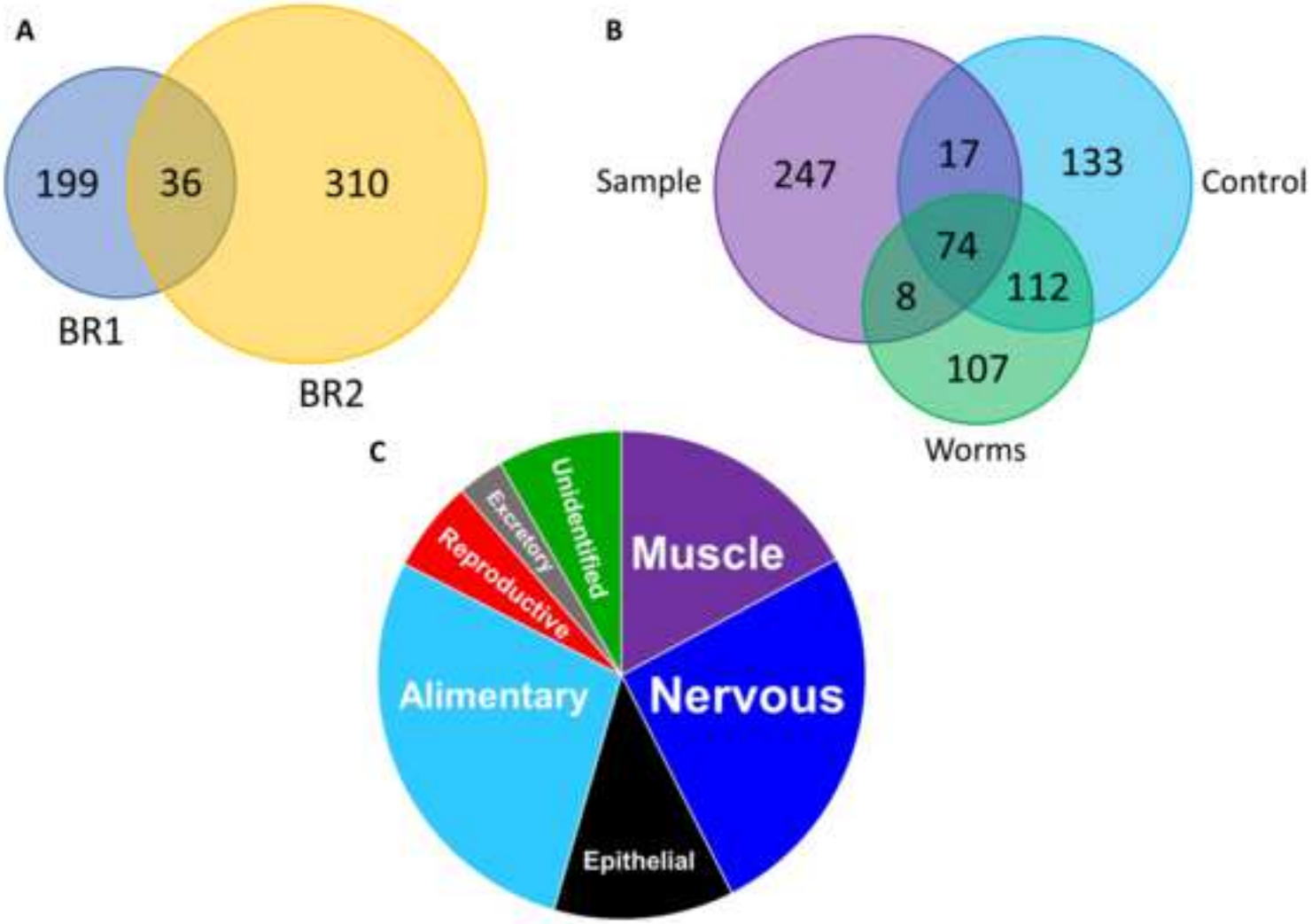
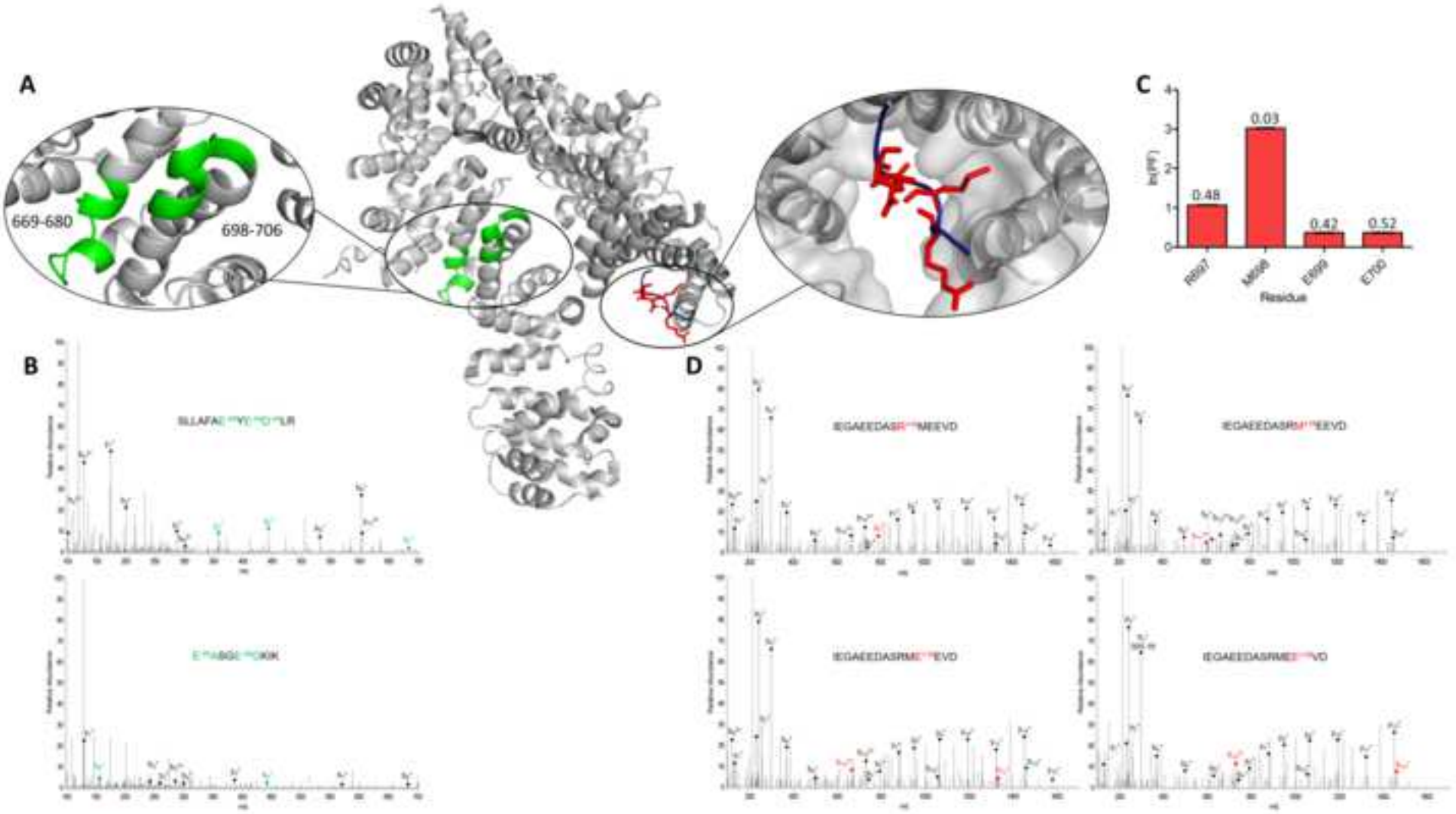


Figure6

Click here to access/download;Figure;Figure6.TIF



Name of Material/Equipment

15mL Conical Centrifuge Tubes
5 mL Gas Tight Syringe, Removable Luer Lock
60 Sonic Dismembrator
Acetone, HPLC Grade
Acetonitrile with 0.1% Formic Acid (v/v), LC/MS Grade
ACQUITY UPLC M-Class Symmetry C18 Trap Column, 100Å, 5 µm, 180 µm x 20 mm, 2G, V/M, 1/pkg
ACQUITY UPLC M-Class System
Aluminum Foil
Aqua 5 µm C18 125 Å packing material
Centrifuge
Delicate Task Wipers
Dissecting Needle
Dithiothreiotol (DTT)
DMSO, Anhydrous
Epoxy instant mix 5 minute
Ethylenediaminetetraacetic acid (EDTA)
EX350 excimer laser (248 nm wavelength)
FEP Tubing 1/16" OD x 0.020" ID
Formic Acid, LC/MS Grade
HEPES
HV3-2 VALVE
Hydrochloric Acid
Hydrogen Peroxide
Iodoacetamide (IAA)
Legato 101 syringe pump
Luer Adapter Female Luer to 1/4-28 Male Polypropylene
Magnesium Sulfate
Methanol, LC/MS Grade
Microcentrifuge
N,N’-Dimethylthiourea (DMTU)
NanoTight Sleeve Green 1/16" ID x .0155" ID x1.6"'
NanoTight Sleeve Yellow 1/16" OD x 0.027" ID x 1.6"

N-tert-Butyl-α-phenylnitrone (PBN)
OmniPur Phenylmethyl Sulfonyl Fluoride (PMSF)
Orbitrap Fusion Lumos Tribrid Mass Spectrometer
PE50-C pyroelectric energy meter
Pierce Quantitative Colorimetric Peptide Assay
Pierce Rapid Gold BCA Protein Assay Kit
Pierce Trypsin Protease, MS Grade
Polymicro Cleaving Stone, 1" x 1" x 1/32”
Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 250µm, Outer Diameter 350µm, TSP250350

Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 450µm, Outer Diameter 670µm, TSP450670

Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 75µm, Outer Diameter 375µm, TSP075375
Potassium Phosphate Monobasic
Proteome Discover (bottom-up proteomics software)
Rotary Magnetic Tumble Stirrer
Rotary Magnetic Tumble Stirrer, accessory kit for use with Syringe Pumps
Scissors
Self-Adhesive Label Tape
Snap-Cap Microcentrifuge Flex-Tube Tubes
Sodium Chloride
Sodium Dodecyl Sulfate (SDS)
Sodium Phosphate Dibasic Heptahydrate
Stereo Zoom Microscope
Super Flangeless Ferrule w/SST Ring, Tefzel (ETFE), 1/4-28 Flat-Bottom, for 1/16" OD
Super Flangeless Nut PEEK 1/4-28 Flat-Bottom, for 1/16" & 1/32" OD
Super Tumble Stir Discs, 3.35 mm diameter, 0.61 mm thick
Tris Base
Universal Base Plate, 2.5" x 2.5" x 3/8"
Urea
VHP MicroTight Union for 360µm OD
Water with 0.1% Formic Acid (v/v), LC/MS Grade
Water, LC/MS Grade

Company	Catalog Number	Comments/Description
Fisher Scientific	14-959-53A	any brand is sufficient
SGE Analytical Science	008760	2 minimum
Fisher Scientific	FM3279	This item is no longer available. Any low-volume sonicator will be sufficient
Fisher Scientific	A929-4	4 L quantity is not necessary
Fisher Scientific	LS120-500	
Waters	186007496	
Waters		
Fisher Scientific	01-213-100	any brand is sufficient
Phenomenex		
Eppendorf	022625501	
Fisher Scientific	06-666A	
Fisher Scientific	50-822-525	only a couple are needed
AmericanBio	AB00490-00005	
Invitrogen	D12345	
Loctite	1365868	
Fisher Scientific	S311-100	
GAM Laser		
IDEX Health & Sciene	1548L	
Fisher Scientific	A117-50	
Fisher Scientific	BP310-500	
Hamilton	86728	2 minimum
Fisher Scientific	A144S-500	
Fisher Scientific	H325-100	any 30% hydrogen peroxide is sufficient
ACROS Organics	122270050	
KD Scientific	788101	
IDEX Health & Sciene	P-618L	2 minimum
Fisher Scientific	M65-500	
Fisher Scientific	A454SK-4	4 L quantity is not necessary
Thermo Scientific	75002436	
ACROS Organics	116891000	
IDEX Health & Sciene	F-242X	
IDEX Health & Sciene	F-246	

ACROS Organics	177350250	
Sigma-Aldrich	7110-OP	any protease inhibitor is sufficient
Thermo Scientific		other high resolution instruments (e.g. Q exactive Orbitrap or Orbitrap Fusion) ca
Ophir Optronics	7Z02936	
Thermo Scientific	23275	
Thermo Scientific	A53225	
Thermo Scientific	90058	
Molex	1068680064	any capillary tubing cutter is sufficient
Polymicro Technologies	1068150026	
Polymicro Technologies	1068150625	
Polymicro Technologies	1068150019	
Fisher Scientific	P382-500	
Thermo Scientific	OPTON-30799	
V&P Scientific, Inc.	VP 710D3	
V&P Scientific, Inc.	VP 710D3-4	
Fisher Scientific	50-111-1315	any scissors are sufficient
Fisher Scientific	15937	one roll is sufficient
Fisher Scientific	05-402	any brand is sufficient
Fisher Scientific	S271-500	
Fisher Scientific	15-525-017	
Fisher Scientific	S373-500	
Fisher Scientific	03-000-014	a magnifying glass is sufficient
IDEX Health & Sciene	P-259X	
IDEX Health & Sciene	P-255X	
V&P Scientific, Inc.	VP 722F	
Fisher Scientific	BP152-500	
Thorlabs Inc.	UBP2	
Fisher Scientific	U5378	
IDEX Health & Sciene	UH-436	2 minimum
Fisher Scientific	LS118-500	
Fisher Scientific	W6-4	

n be used

JoVE60910. *In vivo* hydroxyl radical protein footprinting for the study of protein interactions in *Caenorhabditis elegans.*

Editorial comments

• Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write lines 27-33, 289-294, 316-320 to avoid this overlap.

- Text has been edited.

• Protocol Detail:

1) 2.2: mention needle gauge

- We are unavailable to provide a needle gauge as it is not provided by the manufacturer. However, we list the company and part number of the needle use in the table of materials.

• Protocol Numbering: All steps should be lined up at the left margin with no indentations.

- We deleted indentations.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

- The discussion has been modified to include the editor's comment.

• Figures:

1) Fig 3: Define error bars.

- Error bars have been defined (Lines 373-374) (Lines 412-413 with track changes on).

• Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are GAM Laser Inc, Waters Acquity, Thermo Scienctific Orbitrap, Lumos Fusion MS, Proteome Discover (Software Version 2.2), Thermo Scientific, Excel

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

- We have removed the commercial language mentioned above.

JoVE60910. *In vivo* hydroxyl radical protein footprinting for the study of protein interactions in *Caenorhabditis elegans.*

• Please define all abbreviations at first use.

- All abbreviations have been defined.
  - Lines 48, 64, 80-81, 290, 293, and 295. (Lined 60, 78-79, 95-96, 310, 314, 316 with track changes on).

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

- Re-print permission has been uploaded with resubmission. In addition, we have edited all figure captions as described by JoVE and the previous publisher.

JoVE60910. *In vivo* hydroxyl radical protein footprinting for the study of protein interactions in *Caenorhabditis elegans.*

Comments from Peer-Reviewers

Reviewer #1

Manuscript Summary:

Overall, this is an outstanding description of the protocol for performing IV-FPOP of C. elegans. The step-by-step instructions are largely clear and unambiguous. The figures are well-designed and add significantly to the clarity of the manuscript. Safety hazards are noted. Overall, I recommend this manuscript with minor revision to clarify particular areas.

Major Concerns:

None

Minor Concerns:

Figure 2A: Images of the syringe barrel should be taken against a flat, featureless background to prevent the light diffracting effects that are currently obscuring the contents of the syringe.

- We appreciate all of the reviewer's comments and concerns. We have retaken the picture.

Authors need to define the laser fluence or range of fluences used to generate sufficient FPOP signal. Specifying the laser fluence used to generate results in Figures 4-6 is also desirable.

- We added a note indicating the laser irradiation window and energy used in this protocol (Lines 165-166 and 199-200) (Lines 182-184 and 218-219 with track changes on).

Authors may wish to define the acronym FEP.

- We have defined FEP at first use (Lines 80-81) (Lines 95-96 with track changes on).

JoVE60910. *In vivo* hydroxyl radical protein footprinting for the study of protein interactions in *Caenorhabditis elegans.*

Reviewer #2:

Manuscript Summary:

The manuscript describes the application of in vivo hydroxyl radical protein footprinting using a KrF excimer laser to study the effect oxidatively modified amino acids have on protein protein interaction in C.elegans. The method provides an adaptation of existing technology to the study of whole organism systems. This is useful in view of the fact that C.elegans serves as an excellent model organism for various disease states, in particular neurological disease. The authors describe the assembly of the microfluidic system in great detail, and highlight the pitfalls a user may encounter during installation.

Major Concerns:

Apart from the details provided for the step by step assembly of the microfluidic system, the submitted paper contains the information and a number figures that have already been published in sufficient detail in Anal Chem 2019,91,19, 6577-6584. Although in the current submission the emphasis is on the assembly of the apparatus, provides no information that is not available in the original Anal Chem paper.

- We appreciate all of the reviewer's comments and concerns. We know most of the figures used in this protocol have been published, however our main focus for this manuscript is to provide potential readers with a visualized version of our IV-FPOP method. Since our Anal Chem publication of the IV-FPOP, and also in-cell FPOP, multiple groups have shown interests in both methods and the possibility to do these experiments in their laboratories. Due to this we have focused on the protocol and the major pitfall of the method, in addition to areas of improvement.

JoVE60910. *In vivo* hydroxyl radical protein footprinting for the study of protein interactions in *Caenorhabditis elegans.*

Reviewer #3:

Manuscript Summary:

This manuscript describes the assembly and implementation of IV-FPOP on C. elegans; in particular, a microfluidic set-up with online irradiation that allows the living organism to be footprinted. Representative analyses for selected proteins are shown with tandem MS data interrogating their solvent accessibility.

This work is of great benefit to the wider community and will allow others to set up and develop similar microfluidic systems for IV-FPOP. Such set-ups will hopefully explore different living systems and expand to complementary photochemical probes.

The manuscript is well written and appropriately conveys the described method. I recommend this manuscript for publication in JoVE with the following minor revisions to be addressed, where possible.

Major Concerns:

None

Minor Concerns:

1. In the abstract the authors comment on the study of protein aggregates in neurodegenerative diseases such as Parkinson's and Alzheimer's (Lines 29-32). Given that there is no mention made of these proteins (alpha-synuclein and amyloid beta) in the manuscript among identified peptides, and that these statements are not elaborated in the introduction section -- this is purely speculative and should be removed, as it gives the initial impression that these proteins are discussed in some way. For retention in the manuscript, the authors should make mention of these proteins/diseases in the introduction and also note the published FPOP work on amyloid beta aggregates to support the utility of IV-FPOP for studying these diseases.

- We appreciate all of the reviewer's comments and concerns. Because of their comment and what could lead to misinterpretation of our protocol we have edited the abstract in order to avoid any confusion for the reader, deleting the mention on the study of neurodegenerative diseases such as Parkinson's and Alzheimer's.

2. The authors should clarify their statement regarding hydroxyl radical insertion (Lines 47-48), making mention of the 3-order of magnitude range of reactivity.

- We have added a sentence in order to clarify the difference is amino acid reactivity to hydroxyl radicals (Lines 46-48) (Lines 58-60 with track changes on).

3. (Line 84) The authors should clarify the "~50 mm" number, as currently written this implies that the crater should be that much in diameter rather than length.

JoVE60910. *In vivo* hydroxyl radical protein footprinting for the study of protein interactions in *Caenorhabditis elegans.*

- We have edited the sentence to reflect the 50 mm measurement indicates length rather than diameter (Line 84) (Line 101 with track changes on).

4. (Lines 118 and 161-163) Could the authors give some typical values for flow rates and outlet capillary lengths. This will greatly help readers setting up the instrumentation for the first time.

- We have added a note indicating the typical final flow rate use in IV-FPOP to the protocol (Lines 165-166) (Lines 182-184 with track changes on).

5. (Line 191) Should this say "'Before' turning on the laser..."?

- Sentence has been edited (Line 193) (Line 213 with track changes on).

6. (Line 340 and Figure 5A) Are the authors able to comment on the total number of proteins identified in the Worms-only control for these BRs. If possible, could the authors also show the overlap between both controls and the sample for one of the BRs -- 3-way Venn diagram. This will help put into context the utility of the method for identifying surface-accessibility in vivo.

- We have edited figure 5 to include the reviewer's suggestion in the number of oxidatively modified proteins in all conditions.

7. (Lines 344-345) The authors state that IV-FPOP probes solvent accessibility similar to in vitro studies. Could the authors provide a reference for this statement -- if the authors are referring to their comparison of SASA from PDB 4I2Z and MS/MS data for UNC-45, this should be made clearer. The current form implies that in vitro FPOP data correlate with the IV-FPOP data herein.

- We have removed the statement "similar to *in vitro* FPOP" in the representative results section to avoid any confusion for the reader. Additionally, we added text to indicate the correlation between IV-FPOP experimental oxidation and SASA calculations using the PDB 4I2Z (Line 346-349) (Lines 385-388 with track changes on).

8. (Lines 349 and 396) Are these ln(PF) values calculated from PDB 4I2Z? If so, please state this in the Figure 6 caption. Is it possible to show the ln(PF) for both in crystal structure and IV-FPOP for comparison?

- The ln(PF) is calculated from the extent of oxidation. We can then correlated this to the calculated SASA values if a crystal structure is available. We have added the calculated SASA values of the Hsp90 fragment to **Figure 6C** to show that as ln(PF) increases SASA decreases (Line 346-349) (Lines 385-388 with track changes on).

9. (Figure 6B) If possible, could the corresponding unmodified MS/MS from the controls be shown for either of these peptides. This would show representative changes for multiply oxidized peptides.

- Unfortunately, for these peptides only the oxidized MS/MS was detected. Because this is a methods/protocol manuscript and want to show representative peptide changes of oxidized and unoxidized, we have included **Figure 4A-C.** Changes in peptide chemistry during reverse-phase LC and fragmentation are described.

10. (Line 428) Could the authors clarify that 2D-chromatography increases the 'identification' of oxidatively modified proteins.

- This sentence has been edited to address the reviewer's concern (Line 446) (Line 492 with track changes on).

UNIVERSITY of MARYLAND BALTIMORE

Jessica Espino Perez <jespino@umaryland.edu>

Regarding Incident 3071782 Request to resuse content

1 message

support@services.acs.org <support@services.acs.org> Tue, Oct 15, 2019 at 1:15 AM
To: jespino@umaryland.edu

ACS Chemistry for Life®

Dear Dr. Espino:

Thank you for contacting ACS Publications Support.

Your permission request is granted and there is no fee for this reuse. In your planned reuse, you must cite the ACS article as the source, add this direct link (https://doi.org/10.1021/acs.analchem.9b00244) and include a notice to readers that further permissions related to the material excerpted should be directed to the ACS.

I hope this information helped. Please let me know if I can be of further assistance.

Sincerely,

Ivan Zabala

ACS Customer Services & Information

https://help.acs.org

Incident Information:

Incident #:	3071782
Date Created:	2019-10-15T11:59:52
Priority:	3
Customer:	jespino@umaryland.edu
Title:	Request to resuse content
Description:	Hello ACS,

I would like permission to reuse content of the following article from which I am an author:

Link to the ACS article:

https://pubs.acs.org/doi/10.1021/acs.analchem.9b00244

The portion of content to reuse (e.g., number of figures, entire article for thesis):

-Figure 2

-Figure 4b

-Figure 5

-Supplemental figure S1

-Supplemental figure S4

A description of where the content will be reused (e.g., name of journal, book title, thesis):

-To be resused in the Journal of Visualized Experiments

Thank you,

Jessica Espino

Graduate Research Assistant

University of Maryland, Baltimore

{CMI: MCID787197}

jove VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: In vivo Hydroxyl Radical Protein Footprinting for the Study of Protein Interactions in Caenorhabditis elegans

Author(s): Jessica A. Espino Lisa M. Jones

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.