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Cover Letter

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Editors of *JoVE*,

We thank the editor and the reviewers for their careful consideration of our article. We have addressed each editorial and reviewer comment point-by-point in our response. We believe that the process has resulted in a manuscript that is clearer and more useful to the reader and more even in tone, and we thank each reviewer for their contribution. My co-author and I are pleased to submit our revised manuscript, now entitled "Enabling Real-Time Compensation in Fast Photochemical Oxidations of Proteins for the Determination of Protein Topography Changes."

Thank you for your consideration, and we look forward to your response.

Sincerely,

Joshua S. Sharp, Ph.D.

Associate Professor of Pharmacology
School of Pharmacy
Associate Professor of Chemistry and Biochemistry
The University of Mississippi

TITLE:

Enabling Real-Time Compensation in Fast Photochemical Oxidations of Proteins for the Determination of Protein Topography Changes

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KEYWORDS:

Fast photochemical oxidation of proteins (FPOP), protein footprinting, biosimilar, compensation, liquid chromatography-mass spectrometry (LC-MS).

SUMMARY:

Fast photochemical oxidation of proteins is an emerging technique for the structural characterization of proteins. Different solvent additives and ligands have varied hydroxyl radical scavenging properties. To compare the protein structure in different conditions, real-time compensation of hydroxyl radicals generated in the reaction is required to normalize reaction conditions.

ABSTRACT:

Fast photochemical oxidation of proteins (FPOP) is a mass spectrometry-based structural biology technique that probes the solvent-accessible surface area of proteins. This technique relies on the reaction of amino acid side chains with hydroxyl radicals freely diffusing in solution. FPOP generates these radicals in situ by laser photolysis of hydrogen peroxide, creating a burst of hydroxyl radicals that is depleted on the order of a microsecond. When these hydroxyl radicals react with a solvent-accessible amino acid side chain, the reaction products exhibit a mass shift that can be measured and quantified by mass spectrometry. Since the rate of reaction of an amino acid depends in part on the average solvent accessible surface of that amino acid, measured changes in the amount of oxidation of a given region of a protein can be directly correlated to changes in the solvent accessibility of that region between different conformations (e.g., ligand-bound versus ligand-free, monomer vs. aggregate, etc.) FPOP has been applied in a number of problems in biology, including protein-protein interactions, protein conformational changes, and protein-ligand binding. As the available concentration of hydroxyl radicals varies based on many experimental conditions in the FPOP experiment, it is important to monitor the effective radical dose to which the protein analyte is

exposed. This monitoring is efficiently achieved by incorporating an inline dosimeter to measure the signal from the FPOP reaction, with laser fluence adjusted in real-time to achieve the desired amount of oxidation. With this compensation, changes in protein topography reflecting conformational changes, ligand-binding surfaces, and/or protein-protein interaction interfaces can be determined in heterogeneous samples using relatively low sample amounts.

INTRODUCTION

Fast photochemical oxidation of proteins (FPOP) is an emerging technique for the determination of protein topographical changes by ultra-fast covalent modification of the solvent-exposed surface area of proteins followed by detection by LC-MS[1]. FPOP generates a high concentration of hydroxyl radicals in situ by UV laser flash photolysis of hydrogen peroxide. These hydroxyl radicals are very reactive and short lived, consumed on roughly a microsecond timescale under FPOP conditions[2]. These hydroxyl radicals diffuse through water and oxidize various organic components in solution at kinetic rates generally ranging from fast (~10^6 M^{-1} s^{-1}) to diffusion-controlled[3]. When the hydroxyl radical encounters a protein surface, the radical will oxidize the amino acid side chains on the protein surface, resulting in a mass shift of that amino acid (most commonly the net addition of one oxygen atom)[4]. The rate of the oxidation reaction at any amino acid depends on two factors: the inherent reactivity of that amino acid (which depends on the side chain and the sequence context)[4,5] and the accessibility of that side chain to the diffusing hydroxyl radical, which closely correlates to the average solvent accessible surface area[6,7]. All of the standard amino acids except glycine have been observed as labeled by these highly reactive hydroxyl radicals in FPOP experiments, albeit at widely differing yields; in practice, Ser, Thr, Asn, and Ala are rarely seen as oxidized in most samples except under high radical doses and identified by careful and sensitive targeted ETD fragmentation[8,9]. After oxidation, samples are quenched to remove hydrogen peroxide and secondary oxidants (superoxide, singlet oxygen, peptidyl hydroperoxides, etc.) The quenched samples are then proteolytically digested to generate mixtures of oxidized peptides, where the structural information is frozen as a chemical “snapshot” in the patterns of oxidation products of the various peptides (**Figure 1**). Liquid chromatography coupled to mass spectrometry (LC-MS) is used to measure the amount of oxidation of amino acids in a given proteolytic peptide based on the relative intensities of the oxidized and unoxidized versions of that peptide. By comparing this oxidative footprint of the same protein obtained under different conformational conditions (e.g., ligand-bound versus ligand-free), differences in the amount of oxidation of a given region of the protein can be directly correlated with differences in the solvent-accessible surface area of that region[6,7]. The ability to provide protein topographical information makes FPOP an attractive technology for the higher-order structure determination of proteins, including in protein therapeutic discovery and development[10,11].

[Figure 1 here]

Different constituents present in the FPOP solution (e.g., ligands, excipients, buffers) have different scavenging activity towards the hydroxyl radicals generated upon the laser photolysis of hydrogen peroxide[3]. Similarly, a small change in peroxide concentration, laser fluence, and buffer composition may change the effective radical dose, making the reproduction of FPOP

data challenging across the samples and between different labs. Therefore, it is important to be able to compare the hydroxyl radical dose available to react with protein in each sample using one of several available hydroxyl radical dosimeters[12-16]. Hydroxyl radical dosimeters act by competing with the analyte (and with all scavengers in solution) for the pool of hydroxyl radicals; the effective dose of hydroxyl radicals is measured by measuring the amount of oxidation of the dosimeter. Note that "effective hydroxyl radical dose" is a function of both the initial concentration of hydroxyl radical generated and the half-life of the radical. These two parameters are partially dependent on one another, making the theoretical kinetic modeling somewhat complex (**Figure 2**). Two samples could have wildly different initial radical half-lives while still maintaining the same effective radical dose by changing the initial concentration of hydroxyl radical formed; they will still generate identical footprints[17]. Adenine[13] and Tris[12] are convenient hydroxyl radical dosimeters because their level of oxidation can be measured by UV spectroscopy in real-time, allowing for researchers to quickly identify when there is a problem with effective hydroxyl radical dose and to troubleshoot their problem. To solve this issue, an inline dosimeter located in the flow system directly after the site of irradiation that can monitor the signal from adenine absorbance changes in real-time is important. This helps in carrying out FPOP experiments in buffers or any other excipient with widely differing levels of hydroxyl radical scavenging capacity[17]. This radical dosage compensation can be performed in real-time, yielding statistically indistinguishable results for the same conformer by adjusting the effective radical dose.

In this protocol, we have detailed procedures for performing a typical FPOP experiment with radical dosage compensation using adenine as an internal optical radical dosimeter. This method allows investigators to compare footprints across FPOP conditions that have different scavenging capacity by performing compensation in real-time.

PROTOCOL

1. Prepare the Optical Bench and the Capillary for FPOP

CAUTION: KrF excimer lasers are extreme eye hazards, and direct or reflected light can cause permanent eye damage. Always wear appropriate eye protection, avoid the presence of any reflective objects near the beam path when possible, and use engineering controls to prevent unauthorized access to an active laser and to restrain any stray reflections.

1.1. Prepare the FPOP optical bench.

1.1.1. Turn on the laser to warm up. Set the laser to External Trigger, Constant Energy, No Gas Replacement. Set the laser energy per pulse (typically between 80-120 mJ/pulse).

1.1.2. Set up the optical bench with the plano-convex lens (30 mm Dia. x 120 mm FL uncoated) directly in the path of the laser beam and a non-reflective backstop to absorb the light as shown in **Figure 3A**.

[Place **Figure 3** here]

1.2. Cut an appropriate length of the fused silica capillary with (360 µm outer diameter and 100 µm inner diameter) and using a sleeve, connect to the gas-tight syringe using a low dead volume connector.

1.3. Gently burn the polyimide coating of the capillary with a butane torch at the place where the inline dosimeter reads the absorbance signal at 265 nm after laser exposure of the samples. Wipe the debris on the capillary gently using methanol on a lint-free wipe. The polyimide coating at the site of laser incidence can either be similarly burned off with the butane torch or burned off with the excimer laser firing at low power.

NOTE: Wait for the capillary to cool as it is a fire hazard to use the methanol on the hot capillary.

1.4. Place this capillary through the beam path of the laser and into the inline dosimeter.

1.4.1. Press the lever on the top of the inline dosimeter to open the hinge. Remove the magnetic holders. Place the capillary in the machined groove of the inline dosimeter, using the magnetic holders to keep the capillary in place. Close the dosimeter hinge over the capillary, pressing it until the level locks in place.

1.5. Using the dosimetry software, click on the **Start Flash** button to begin firing the excimer laser. Set the preset laser power between 50-100 mJ/pulse on the laser control software itself, and set the preset repetition rate between 10-20 Hz in the Settings tab of the dosimetry software.

1.5.1. Focus the laser beam using a concave lens mounted on a linear motorized stage. Measure the width and the height of the laser spot at the position of the capillary on a sticky note precisely using a caliper to calculate incident fluence (mJ/mm^2) as shown in **Figure 3B**.

1.6. Place an opaque aperture near the capillary to ensure consistent illuminated width of the capillary regardless of changes in the beam size due to movement of the lens or changing the energy per pulse of the laser[18].

1.6.1. With the laser firing, move the motorized stage through its range of motion. Ensure that the beam stays centered on the aperture and the silhouette of the capillary can be observed throughout. The diameter of the aperture must be smaller than the width of the impinging focused beam at every point in the range of the motorized stage.

1.7. Run water through the capillary at 20 µL/min for at least one minute to wash the capillary.

1.7.1. Click the **Start Data + AutoZero** button on the dosimeter software to zero the dosimeter to water and begin data collection.

NOTE: If the buffer system for FPOP has significant UV absorbance at 265 nm, the FPOP system should be zeroed on the buffer, not water.

1.8. Set the calculated flow rate on the syringe pump.

1.8.1. The flow rate of the protein sample depends on the irradiated volume per shot (V_{Irr}), the number of laser shots per second (R), and the desired unirradiated exclusion volume fraction (F_{Ex}) to correct for laminar flow effects and sample diffusion (0.15-0.30 recommended)[2,19,20]. Calculate the V_{Irr} (in µL) based on the inner diameter of the capillary in mm (d) and the width of the laser spot impinging upon the capillary (i.e., the width of the aperture) in mm (w) using the following equation:

$$V_{Irr} = \pi(d/2)^2 w$$

1.8.2. Calculate the desired flow rate (in µL/min) based on the following equation:

$$Flow = 60R[V_{Irr}(1 + F_{Ex})]$$

2. Preparation of the protein solution for FPOP

2.1. Prepare the protein in the two or more different conditions to be compared (e.g., ligand-bound and ligand-free; aggregate and monomer; alone and with a protein-protein binding partner; etc.) for detecting the conformation changes.

2.2. Set the total volume used for FPOP to fit the needs of the experiment. The minimum limit usually depends on the volume of the irradiation capillary and the material required for robust detection and relative quantification, and will vary depending largely on the LC-MS/MS system used and the post-labeling sample processing method. The total volume for FPOP solutions commonly used group is 20 µL after the addition of hydrogen peroxide. The final concentration of the protein is commonly 1-10 µM, with 17 mM glutamine (to limit the lifetime of the hydroxyl radical), 1 mM adenine (to act as a radical dosimeter)[13,17] and 10 mM phosphate buffer (a buffer that is a poor scavenger of hydroxyl radicals). Samples are generally prepared with multiple replicates to allow for statistical modeling of results.

2.2.1. For most general purposes, prepare samples in triplicate in both states, plus at least one sample to use as a no-laser control to measure background oxidation. Prepare 18 µL of this FPOP solution mix.

NOTE: Many buffers and additives commonly used in biochemistry are hydroxyl radical scavengers. These additives and buffers can be used; however, reductions in oxidation due to hydroxyl radical scavenging of the buffer can occur. In general, keep all additives to the minimum required by the biological system to maximize protein oxidation yield. Dimethyl sulfoxide should be avoided due to the propensity to generate secondary radicals;

dimethylformamide has been a useful alternative in our hands. When using buffers that are strong hydroxyl radical scavengers, glutamine can often be excluded from the FPOP solution mix.

2.3. Prepare 1 M hydrogen peroxide immediately before the FPOP experiment.

NOTE: 30% hydrogen peroxide as commonly sold by vendors includes a stabilizer, which increases the shelf life. Once diluted, hydrogen peroxide should be used quickly, definitely within the same day. Hydrogen peroxide should also be regularly tested for decomposition by FPOP using a hydroxyl radical dosimeter.

2.4. Prepare microcentrifuge tubes containing 25 µL of quench solution of 0.5 µg/µL of methionine amide and 0.5 µg/µL catalase. If a sample volume greater than 20 µL is used for FPOP, increase the quench solution volume proportionally.

3. Perform the FPOP experiment

3.1. Add 2 µL of hydrogen peroxide in the 18 µL of the FPOP solution mix. Mix the contents gently with a pipette and quickly spin down the solution to the bottom of the microcentrifuge tubes. Immediately collect with a gastight syringe and load into the syringe pump.

3.2. Start the flow on the syringe pump with the flow rate as determined in step 1.8.1 (typically between 8-16 µL/min) by clicking the **Start Pump** button on the dosimeter software.

3.3. Monitor the real-time adenine reading using the inline dosimeter (see **Table of Materials**) and collect the sample in waste. Wait for the Abs_{265} signal to stabilize.

3.4. Click on the **Start Flash** button in the dosimeter software to start firing the laser at the preset repetition rate and energy.

3.5. Monitor the real-time adenine reading using an inline dosimeter (see **Table of Materials**); the difference in Abs_{265} with the laser off and the laser on is the ΔAbs_{265} reading.

NOTE: The appearance of highly unstable Abs_{265} readings upon firing the laser in the presence of hydrogen peroxide is due to the generation of bubbles in solution. Reduce the fluence of the laser and/or the concentration of hydrogen peroxide to eliminate the bubbles.

4. Perform Compensation

NOTE: Different ligands, buffers, etc. may have different scavenging capacity towards hydroxyl radicals. It is important to ensure that comparable hydroxyl radicals across are available to react with protein across different samples. This is accomplished by ensuring equal hydroxyl radical dosimeter response between samples. Using adenine dosimetry, the change in UV

absorbance at 265 nm (ΔAbs_{265}) reflects the effective hydroxyl radical dose; the larger the ΔAbs_{265}, the higher the effective hydroxyl radical dose.

4.1. Compare the ΔAbs_{265} reading obtained with the inline dosimeter with the desired ΔAbs_{265} reading obtained by prior experiments or controls. A ΔAbs_{265} reading lower than the desired reading indicates an insufficient effective dose of hydroxyl radicals; a ΔAbs_{265} reading indicates an effective radical dose that is too high. If the ΔAbs_{265} reading is at the desired level, collect the sample immediately after laser irradiation in the quench buffer[17].

4.2. Compensate the effective radical dose to equalize the ΔAbs_{265}. This compensation can be performed in three ways: change hydrogen peroxide concentration, increase laser fluence by changing the laser energy per pulse, or increase laser fluence by changing the focal plane of the focusing lens.

4.2.1. To make a large change (>10 mAU) in ΔAbs_{265} reading, remake the sample with more or less hydrogen peroxide and rerun the sample as per Section 3.

4.2.2. To make a small change in ΔAbs_{265} reading in real-time, adjust the focal plane of the incident beam by adjusting the position of the focusing lens using the 50 mm Motorized Stage. Bringing the focal plane closer to the position of the capillary will increase the ΔAbs_{265} reading; bringing the focal plane farther from the position of the capillary will decrease the ΔAbs_{265} reading.

4.3. Monitor the adenine ΔAbs_{265} to measure the effective amount of hydroxyl radical present in the sample after laser irradiation[13]. Real-time monitoring with an inline UV capillary detector allows for real-time compensation as described in 4.2.2; adjust the lens position using the motorized stage until the ΔAbs_{265} reading is equal to the desired reading. Post-experimental absorbance measurements with a UV spectrophotometer are also accurate, but require new samples to be used for each effective radical dose.

5. Digest the protein samples

NOTE: Trypsin is most commonly used to digest protein samples for FPOP, and is the protease used in this protocol. It is a reliable protease that generates peptides with basic sites both at the N- and C-terminus, promoting multiply charged peptide ions in MS. Moreover, it cleaves after lysine and arginine, two amino acids that are only moderately reactive to hydroxyl radicals; therefore, changes in the digestion pattern due to analyte oxidation is rare. Other proteases have been successfully used with FPOP[21], but care should be taken to ensure digestion patterns are comparable between unoxidized and oxidized samples.

5.1. Measure the final volume of quenched FPOP sample. Add 500 mM Tris, pH 8.0 with 10 mM $CaCl_2$ containing 50 mM dithiothreitol (DTT) to the protein solution after quenching to a final concentration of 50 mM Tris, 1 mM $CaCl_2$ and 5 mM DTT.

5.2. Heat the protein sample at 95 °C for 15 minutes.

5.3. Immediately cool the sample on ice for 2 min.

5.4. Add the 1:20 trypsin/protein weight ratio to the samples.

5.5. Digest the protein overnight at 37 °C with mixing.

5.6. Stop the digestion reaction by the addition of 0.1% formic acid and/or heating the sample to 95 °C for 10 min.

5.7. Add 2 mM DTT to the samples and heat at 60 ^{0}C for 15 min immediately before LC-MS/MS.

NOTE: While other groups have reported alkylation of thiols in FPOP experiments, in our hands we have noted side products upon alkylation of oxidized proteins (possibly due to reaction with nucleophilic carbonyls formed as a minor oxidation product). Therefore, we choose to avoid alkylation of thiols when possible.

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

6.1. Prepare the mobile phase A consisting of water containing 0.1% formic acid and mobile phase B consisting of acetonitrile with 0.1% formic acid.

6.2. Load the sample first onto a C18 trap column (300 µm I.D. x 5 mm 100 Å pore size, 5 µm particle size) trapping cartridge and wash with 2% solvent B for 3 minutes at a flow rate of 5.0 µL/min to remove salts and hydrophilic small molecules.

6.3. Then separate the peptides on C18 nanocolumn (0.75 mm x 150 mm, 2 µm particle size, 100 Å pore size) at a flow rate of 300 nL/min. The gradient consists of a linear increase from 2 to 35% solvent B over 22 min, ramped to 95% solvent B over 5 min and held for 3 min to wash the column, and then returned to 2% B over 3 min and held for 9 min to re-equilibrate the column.

NOTE: This gradient is sufficient for LC-MS/MS of most one- and two-protein FPOP mixtures seeking to do peptide-level quantification. The percent of solvent B may need to be altered to increase peptide resolution in rare cases where peptides interfere with one another due to similar retention times and m/z values. Proteome-scale FPOP[22] or experimental designs seeking to separate peptide oxidation product isomers[1,23-25] may require longer LC gradients and are beyond the scope of this report.

6.4. Elute the peptides directly into the nanospray source of a high-resolution mass spectrometer using a conductive nanospray emitter.

6.5. Acquire the data in positive ion mode. Set the spray voltage to 2400 V, and the temperature of the ion transfer tube to 300 °C.

6.6. Acquire the full MS scans from m/z 250 to 2000 at a nominal resolution at *m/z* 200 of 60,000 followed by eight subsequent data-dependent linear ion trap MS/MS scans on the top eight most abundant peptide ions using collision-induced dissociation at 35% normalized energy to identify the peptides. Fragment the peptides up to five times within 30 s and then transfer to an exclusion list for 60 s.

7. Data processing and calculation of average oxidation of peptides

7.1. Determine the sequence coverage of the protein, *m/z* values, and retention times of unoxidized peptides using the MS/MS proteomics search engine.

7.2. Set the precursor mass tolerance to 10 ppm and allow up to two missed cleavage sites for the trypsin digested samples, using standard trypsin cleavage specificity.

7.3. Set the peptide mass fragment mass tolerance to 0.4 Daltons.

7.4. Based on the *m/z* ratio of the unmodified peptides detected and the known mass shifts of the major oxidation products, calculate the *m/z* of the various theoretical oxidation products of each peptides[4,26-29].

7.5. Identify the extracted ion chromatogram of these *m/z* values using software to view the mass spectrometric run (**Figure 4**). Identify the peptide oxidation products based on their *m/z*, their charge state, and the similarity in elution time to the unmodified peptide. In our hands, peptide oxidation products elute between 240 seconds before to 180 seconds after the unmodified peptide using the LC gradient above. As oxidation will often result in multiple isomeric oxidation products, it is common to observe multiple partially resolved peaks in the extracted ion chromatograms of peptide oxidation products, as shown in **Figure 4.** Peptide oxidation products are quantified based on the area of the peak(s) in the extracted ion chromatograms.

[Place **Figure 4** here**]**.

7.6. Calculate the average oxidation of the peptides using the following equation.

$$P = [I(singly\ oxidized)\ X\ 1 + I\ (doubly\ oxidized)\ X\ 2 + I(triply\ oxidized)\ X\ 3 + \ldots / [Iunoxidized + I(singly\ oxidized) + I(doubly\ oxidized) + I(triply\ oxidized) \ldots]$$

where *P* denotes the average number of oxidation events per peptide molecule, and *I* represents the peak area of of the unoxidized peptide (*Iunoxidized*) and the peptide with *n* oxidation events. Note that *I(singly oxidized)* would include not only additions of a single oxygen atom but also other less-common single oxidation events that the investigator may choose to measure (e.g., oxidative decarboxylation, carbonyl formation, etc.)[4,26-29].

REPRESENTATIVE RESULTS

Comparison of the heavy chain peptide footprint of the adalimumab biosimilar in phosphate buffer and when heated at 55 °C for 1 h show interesting results. Student's t-test is used for the identification of peptides that are significantly changed in these two conditions ($p \leq 0.05$). The peptides 20-38, 99-125, 215-222, 223-252, 260-278, 376-413, and 414-420 show significant protection from solvent when the protein is heated to form aggregates (**Figure 5**)[30]. This experiment identified the peptide regions that experience topographical changes upon heating and aggregation.

[Place **Figure 5** here]

FPOP experiment of myoglobin was performed in the presence of 10 mM phosphate and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer. MES buffer acts as a good scavenger of the hydroxyl radicals generated upon the photolysis of the hydrogen peroxide after the sample is exposed to the laser irradiation. The difference in the absorbance of the adenine is monitored using an inline dosimeter in real-time. The laser fluence is adjusted in a way to have a comparable change in the adenine absorbance level in MES buffer as compared to the phosphate buffer (**Figure 6**)[17]. The average oxidation of peptides was lower in the presence of MES buffer as compared to the phosphate buffer. However, as the laser fluence was increased to have an equal adenine dosimetry response, the average peptide oxidation values were almost the same after FPOP in MES buffer and phosphate buffer (**Figure 7**)[17]. This experiment shows the importance of compensation of the signal to be able to compare the footprint with two FPOP conditions that have different scavenging capacity. Similar experiments have successfully used adenine-based compensation to probe structural changes of common excipients in adalimumab preparations[30].

Figure 1: **Overview of FPOP**. The surface of the protein is covalently modified by highly reactive hydroxyl radicals. The hydroxyl radicals will react with amino acid side chains of the protein at a rate that is strongly influenced by the solvent accessibility of the side chain. Topographical changes (for example, due to the binding of a ligand as shown above) will protect amino acids in the region of interaction from reacting with hydroxyl radicals, resulting in a decrease in the intensity of modified peptide in the LC-MS signal.

Figure 2: Kinetic simulation of dosimetry-based compensation. 1 mM adenine dosimeter response is measured in 5 µM lysozyme analyte with a 1 mM initial hydroxyl radical concentration (▪OH $t_{1/2}$=53 ns), and set as a target dosimeter response (black). Upon the addition of 1 mM of the scavenger excipient histidine, the dosimeter response (blue) decreases along with the amount of protein oxidation in a proportional manner (cyan). The half-life of the hydroxyl radical also decreases (▪OH $t_{1/2}$=39 ns). When the amount of hydroxyl radical generated is increased to give an equivalent yield of oxidized dosimeter in the sample with 1 mM histidine scavenger as achieved with 1 mM hydroxyl radical in the absence of scavenger (red), the amount of protein oxidation that occurs similarly becomes identical (magenta), while

the hydroxyl radical half-life decreases even further (▪OH $t_{1/2}$=29 ns). Adapted with permission from Sharp J.S., *Am Pharmaceut Rev* **22,** 50-55, 2019.

Figure 3: **Optical bench for the FPOP experiment.** (**A**) The sample is mixed with H_2O_2, adenine radical dosimeter, and glutamine scavenger and loaded into the syringe. The sample is pushed through the fused silica capillary through the focused beam path of a KrF excimer UV laser. The UV light photolyzes H_2O_2 into hydroxyl radicals, which oxidizes the protein and adenine dosimeter. The syringe flow pushes the illuminated sample out of the path of the laser before the next laser pulse, with an unilluminated exclusion volume between illuminated regions. Immediately after oxidation, the sample is passed through an inline UV spectrophotometer, which measures the UV absorbance of adenine at 265 nm. The sample is then deposited into a quench buffer to eliminate the remaining H_2O_2 and secondary oxidants. (**B**) The spot size is measured after irradiating a colored sticky note affixed behind the capillary with the laser at 248 nm. The width of the spot is used for calculating the sample flow rate, and the silhouette of the capillary in the center of the spot is used to align the optical bench.

Figure 4: **Extracted ion chromatogram of a peptide and its oxidation products after FPOP**. The *m/z* of the peptide oxidation products are calculated based on the *m/z* of the unoxidized peptide and the known oxidation products; and the areas of these peptide products are determined. The area of the peptide products is then used for the calculation of the average oxidation events per peptide.

Figure 5: Peptide level footprint of the heavy chain of adalimumab. The peptide average oxidation of adalimumab at room temperature (blue) and after adalimumab is heated at 55 ^{0}C for 1 hr, then cooled to room temperature. The error bars represent one standard deviation of triplicate measurements. The asterisk represents the peptides that are significantly changed in the two conditions ($p \leq 0.05$).

Figure 6: **Compensation of adenine dosimetry readings**. The adenine reading before and after laser irradiation were recorded for FPOP in phosphate buffer at 265 nm with using inline dosimeter. As MES is a good scavenger of the hydroxyl radicals, the difference in adenine readings was lower. Increased the laser fluence of FPOP solution with MES buffer to "compensate" and overcome the effect of MES buffer to have similar adenine reading as phosphate buffer.

Figure 7: **Real-time compensation of myoglobin oxidation by inline adenine dosimetry**. Myoglobin oxidized in 10 mM phosphate buffer (blue) and 10 mM MES buffer (orange). As noted, the oxidation of the peptides is lower in the MES buffer. As the laser fluence is increased for the samples in the MES buffer to have almost similar adenine dosimetry level as compared to phosphate buffer (grey), the peptide level oxidation is also similar to the oxidation level seen in samples with phosphate buffer. This figure has been adapted with permission from *Analytical Chemistry* 2018, 90, 21, 12625-12630. Copyright 2018 American Chemical Society.

DISCUSSION:

Mass spectrometry-based structural techniques, including hydrogen-deuterium exchange, chemical cross-linking, covalent labeling, and native spray mass spectrometry and ion mobility have been rapidly growing in popularity due to their flexibility, sensitivity, and ability to handle complex mixtures. FPOP boasts several advantages that has boosted its popularity in the area of mass spectrometry-based structural techniques. Like most covalent labeling strategies, it provides a stable chemical snapshot of protein topography that is compatible with most post-labeling processes (e.g., trypsin digestion, deglycosylation, etc.), avoiding issues of back-exchange and scrambling that hinder hydrogen-deuterium exchange. However, unlike traditional covalent labeling technologies that target specific amino acids, FPOP is able to label a broad array of amino acids in a single experiment. Moreover, FPOP is able to complete the primary hydroxyl radical-protein reaction faster than the proteins are able to unfold to freeze a chemical snapshot of the native conformation[14], although some secondary reactions may occur on a slower timescale[20,31,32]. Unlike earlier experiments in hydroxyl radical protein footprinting using X-ray synchrotron beamlines, FPOP allows for this ultra-rapid labeling in a benchtop format[33,34]. The major hurdles in FPOP faced by the typical protein mass spectrometry lab is experience in handling samples for FPOP, the laser-based oxidation, and data analysis. The goal of this report is to help new investigators overcome these hurdles to generate valuable and reproducible results.

Proteins may undergo background oxidation that may incorrectly provide the extent of oxidation on the identified peptides. To better understand this, a control sample is prepared in replicates along with FPOP samples in which all steps are carried out except the laser is not triggered (step 3.4). The level of oxidation in the no-laser control reveals the level of background oxidation. In-source oxidation of peptides may contribute towards the observed peptide oxidation, and can be readily determined by the LC elution profile of the unmodified peptide and the modification product. If the elution profiles overlap identically, the oxidation product is almost certainly due to post-column in-source oxidation. In-source oxidation may commonly be reduced by lowering the ionization voltage and/or increasing the distance between the emitter and the ion transfer tube. Other background oxidation may either be present in the protein prior to FPOP treatment, or it can be induced by exposure to hydrogen peroxide. The latter can be minimized by decreasing the concentration of hydrogen peroxide used and/or decreasing the time the protein is in the hydrogen peroxide prior to quenching. A key problem often encountered by experimenters attempting FPOP for the first time is high background oxidation. This high background oxidation is usually due to the addition of hydrogen peroxide to the sample prior to analysis. While two-electron oxidation by hydrogen peroxide is much slower than one-electron oxidation by hydroxyl radicals, hydrogen peroxide is still easily able to oxidize certain amino acids (most notably methionine and cysteine) on a timescale of minutes. While the other components in the FPOP mixture are much more stable, hydrogen peroxide should be added directly prior to oxidation by FPOP. As a rule of thumb, the time between the addition of hydrogen peroxide and complete deposition of the sample into the quench solution should be kept to under five minutes. It is crucial to always collect a no-laser control (where the protein is handled as normal for FPOP but the laser is not fired) to detect any problems with sample oxidation, either due to prolonged peroxide exposure or due to protein purification and storage. For cases where the protein is particularly sensitive to

hydrogen peroxide, on-line mixing with hydrogen peroxide prior to irradiation with the excimer laser can limit exposure to seconds or less[31,35]. However, for most proteins, online mixing is unnecessary.

The next difficult hurdle for many novice FPOP experimenters is the setup of the optical path. It is important that the laser impinges squarely upon the capillary in order to get good photolysis of the hydrogen peroxide. While the UV laser light is invisible, it will cause many dyes present in colored paper to fluoresce in the visible range. Therefore, using a piece of colored construction paper on the backstop can help in the alignment of the lens and capillary. The paper can be used to ensure that the laser is squarely striking the center of the focusing lens, and then a piece of colored paper on the laser backstop can help tell when the capillary is successfully positioned in the center of the focused beam, as the diffraction of the capillary will cause a silhouette in the beam profile (**Figure 2B**).

It is also often not appreciated by novice investigators that the beam cross-sectional area changes based on the pulse energy. Therefore, if an investigator calculates the syringe pump flow rate based on a laser energy of 100 mJ/pulse, and then increases the laser energy to 120 mJ/pulse, the width of the laser beam will similarly increase causing the calculations to be incorrect. In order to prevent this issue, the use of an opaque aperture is recommended. For commercial excimer lasers we have worked with, when the laser energy per pulse is increased the largest change is in the cross-section of the beam, not the laser fluence. Since the concentration of hydroxyl radicals is based in part on the fluence of incident UV light, merely changing the laser energy per pulse is often inefficient at increasing effective hydroxyl radical dosage.

Reproducibility is another common hurdle for novice investigators to overcome. Most commonly, a lack of reproducibility is caused by a failure to generate equivalent hydroxyl radical doses across different replicates. This can be due to improper optical bench alignment, the unwitting use of different levels of hydroxyl radical scavenging agents, or the use of aged hydrogen peroxide. For all of the cases, the use of an internal dosimeter allows for the rapid identification of problems with effective hydroxyl radical dose. Hydroxyl radical dosimeters act by competing with the analyte (and with all scavengers in solution) for the pool of hydroxyl radicals; the effective dose of hydroxyl radicals is measured by measuring the amount of oxidation of the dosimeter. Note that “effective hydroxyl radical dose” is a function of both the initial concentration of hydroxyl radical generated, and the half-life of the radical. These two parameters are partially dependent on one another, making the theoretical kinetic modeling somewhat complex (**Figure 2**). Two samples could have wildly different initial radical half-lives while still maintaining the same effective radical dose by changing the initial concentration of hydroxyl radical formed; they will still generate identical footprints[17]. Adenine[13] and Tris[12] are convenient hydroxyl radical dosimeters because their level of oxidation can be measured by UV spectroscopy in real-time, allowing for researchers to quickly identify when there is a problem with effective hydroxyl radical dose and to troubleshoot their problem.

Data analysis remains the most time-intensive part of any FPOP experiment. While reports exist using commercial packages to quantify oxidation products, these quantification algorithms have difficulties in properly defining peak areas in the partially resolved, asymmetrical peaks generated from groups of oxidation isomers[21]. In our hands, while current available automated software packages can usually correctly identify changes in oxidation, they often do not correctly quantify the magnitude of those changes (unpublished data), requiring post-analysis auditing and correction. Given the difficulties presented by FPOP data, the current status of available data analysis software able to handle FPOP quantification at all is remarkable; however, continued software development will benefit the field with increases in accuracy and reliability.

The protocol described here generates a peptide-level spatial resolution of hydroxyl radical protein footprints. It is possible to generate spatial resolution up to the amino acid level; however, disagreements in the field remain regarding the absolute accuracy of different methods of generating this high resolution FPOP data. A recent study comparing hydrogen-deuterium exchange and FPOP found that FPOP data can probe solvent accessibility at sub-amino acid level[36]. One method uses HPLC to resolve oxidation isomers as much as possible, and then to quantify each isomer by peak area[1,23-25]. However, when a simple mixture of synthetic peptide oxidation isomers was quantified by this method, errors were found in the absolute quantification and previous reports have indicated that collision-induced dissociation MS/MS can misidentify sites of oxidation[37,38]. Quantification by electron transfer dissociation (ETD) has been shown to be accurate on synthetic standards and proteins, but direct application of this method requires co-elution of all oxidized peptide isomers which cannot be accomplished using reversed phase HPLC and generally requires size exclusion chromatography or HILIC[7,39-41]; otherwise, complicated and time-consuming targeted ETD analyses must be used[7-9]. The current consensus in the field seems to be that LC peak area-based amino acid level quantification seems to at least correctly identify sites of oxidation that change and correctly identify the relative amount of change (i.e., oxidation of amino acid X decreases by Y% in conformation A compared to conformation B), but the accuracy of quantification of the amount of oxidation (i.e., amino acid X is Y% oxidized) remains in dispute.

The strengths of FPOP as a flexible benchtop method for probing protein topography at many sites in a single experiment is driving continued interest in this technology, despite the current hurdles for the novice investigator. Commercial options for performing FPOP are just starting to come on the market; however, it remains quite possible for the interested investigator to develop their own FPOP optical bench and perform experiments using commonly available data analysis software. As the field grows and improvements to the available tools continue, the ease of access to FPOP technology will increase.

DISCLOSURES:

Joshua S. Sharp discloses a significant financial interest in GenNext Technologies, Inc., a small company seeking to commercialize technologies for protein higher order structure analysis including hydroxyl radical protein footprinting.

ACKNOWLEDGMENTS:

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Figure 1

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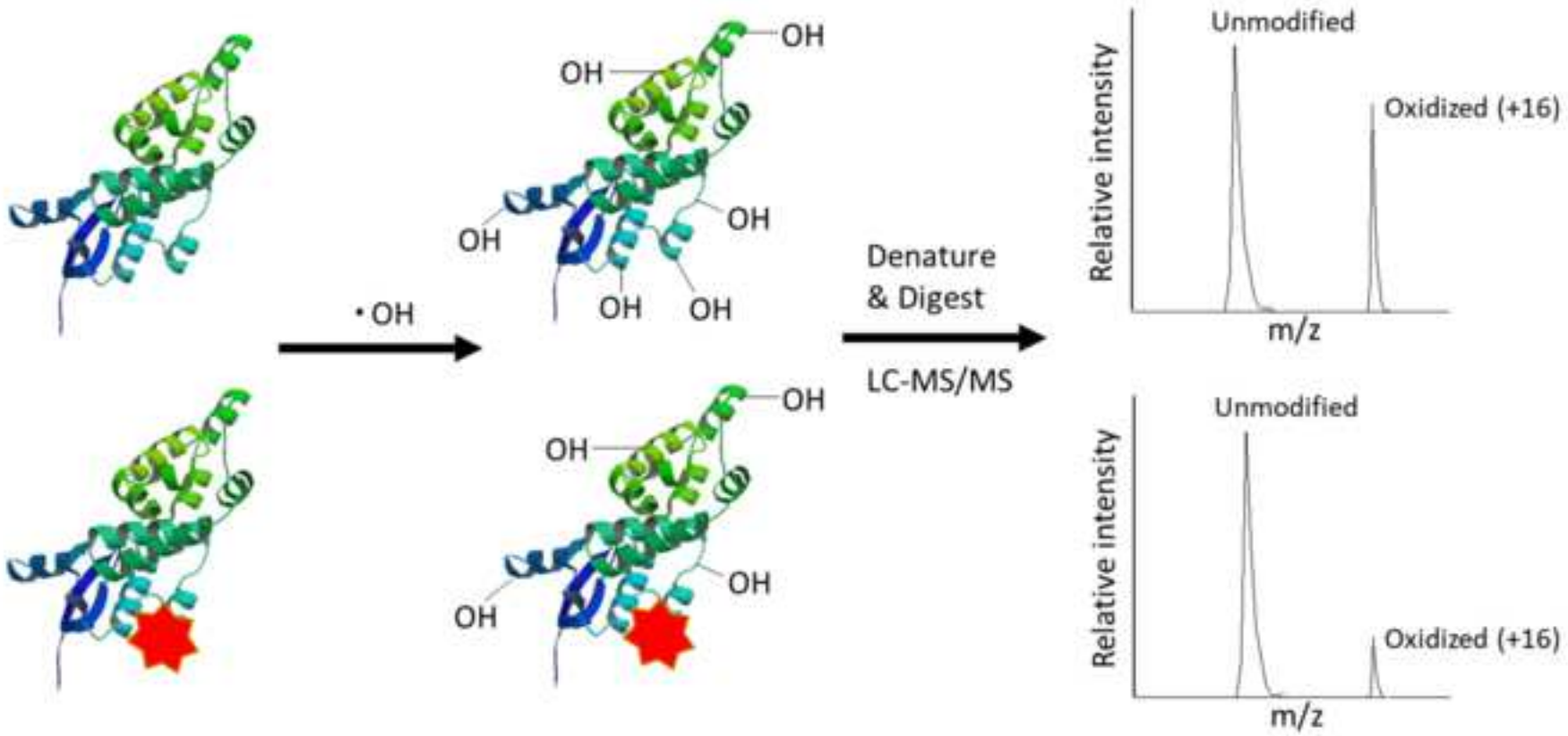

Figure 2

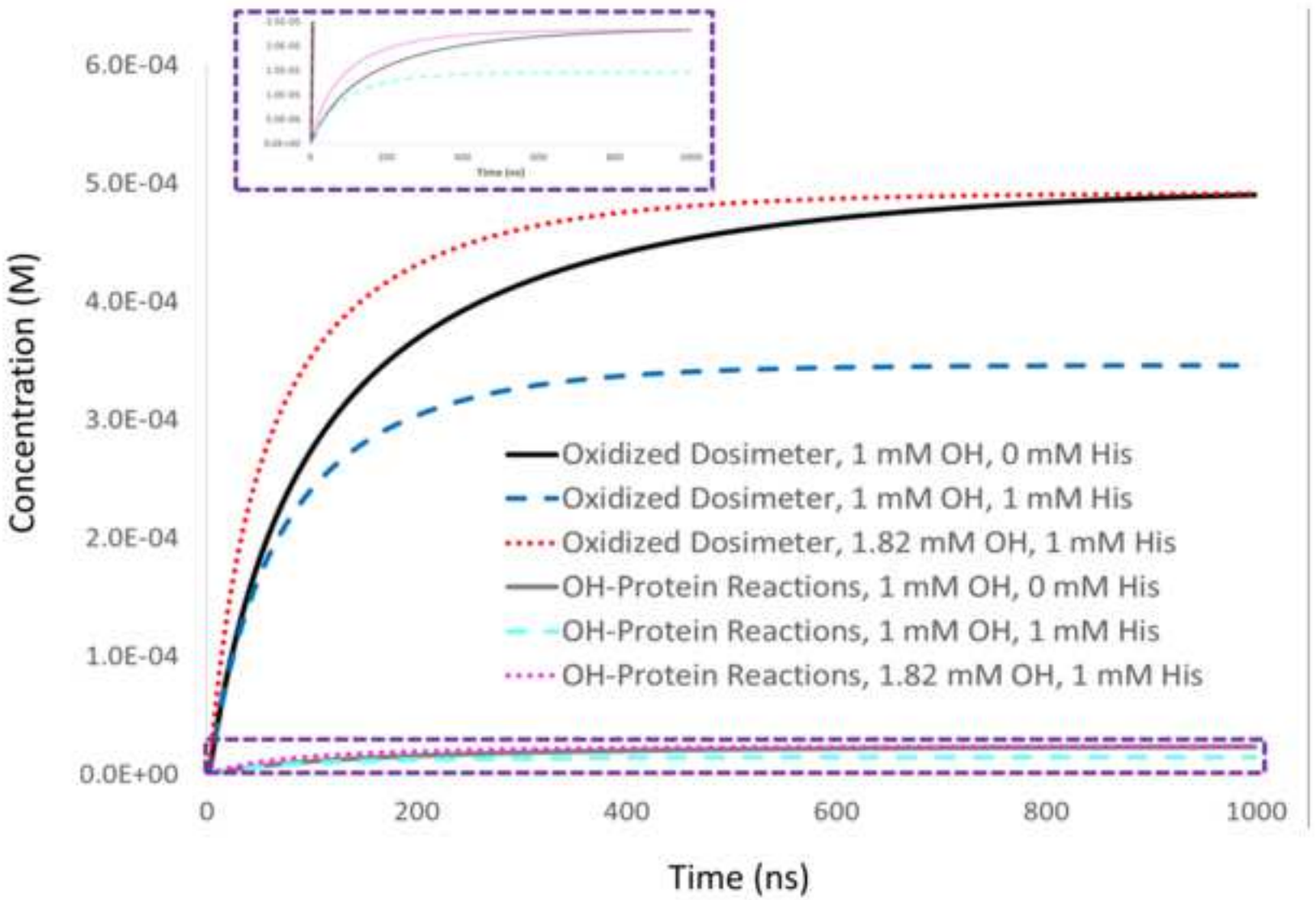

Figure 3

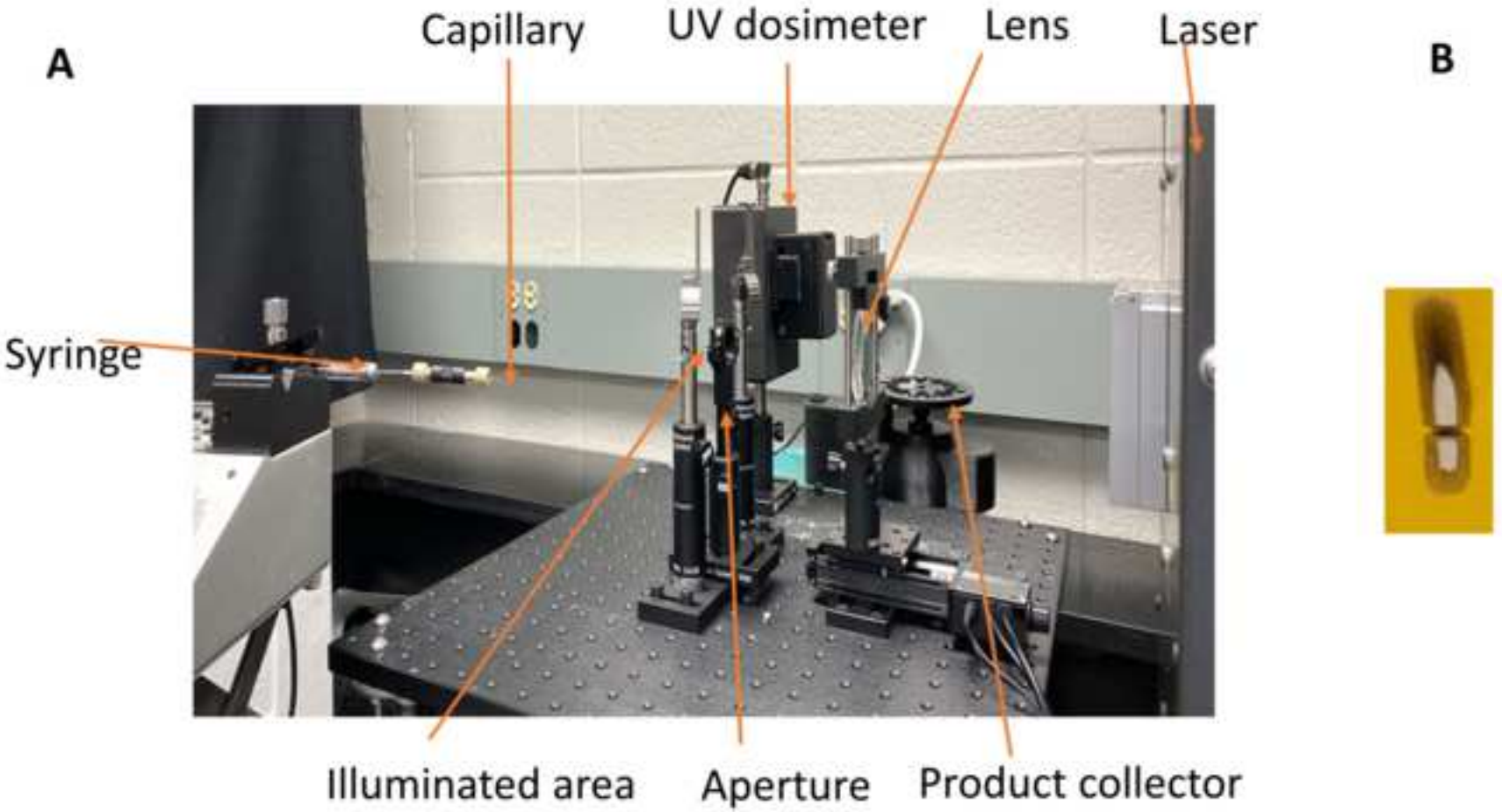

Figure 4

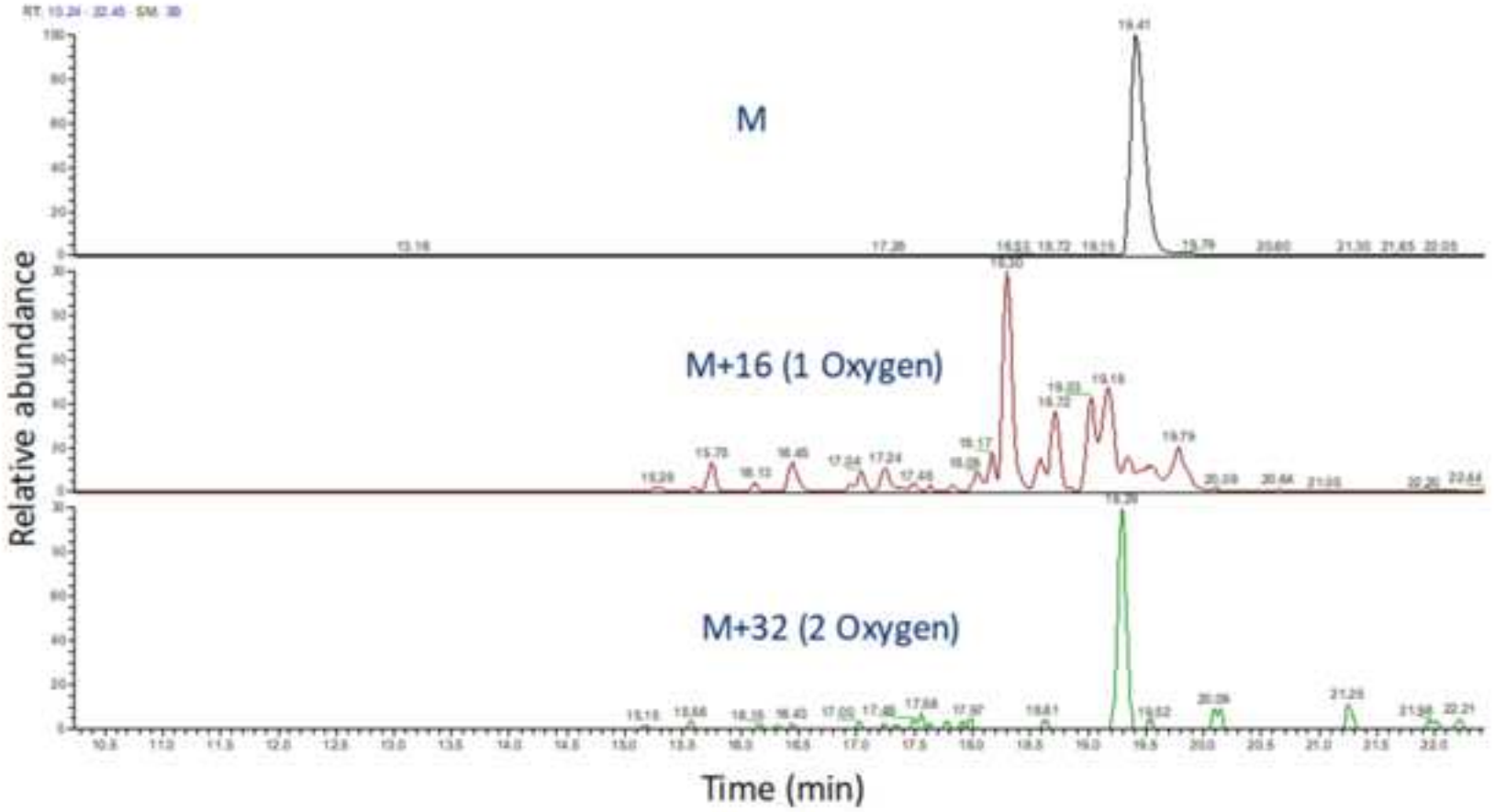

Figure 5

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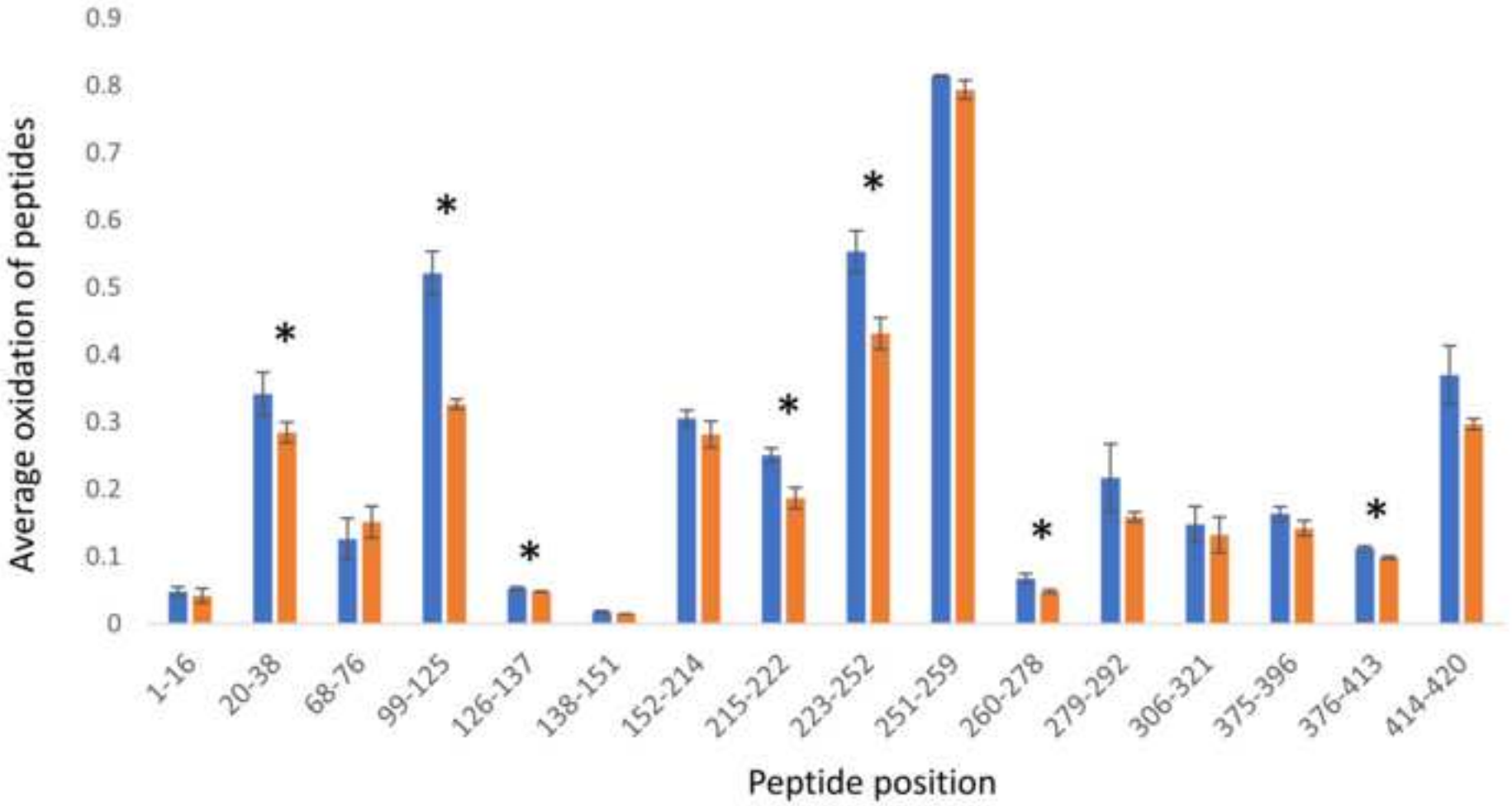

Figure 6

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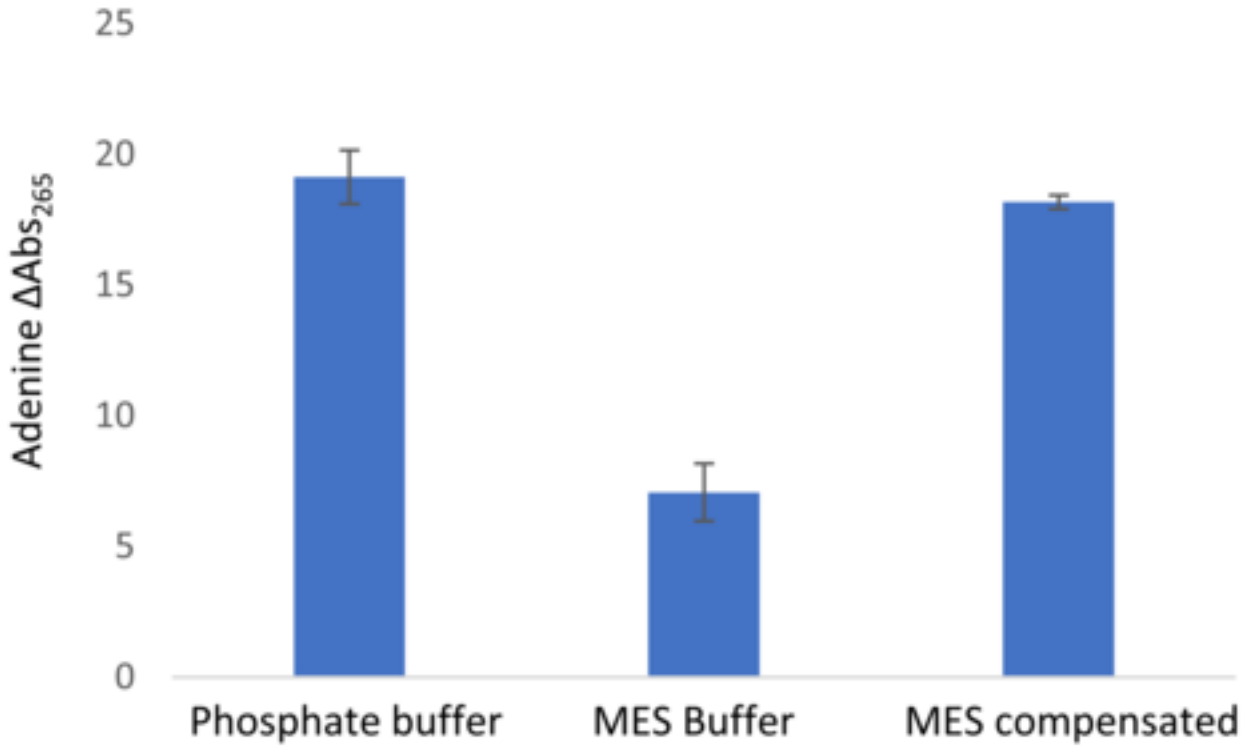

Figure 7

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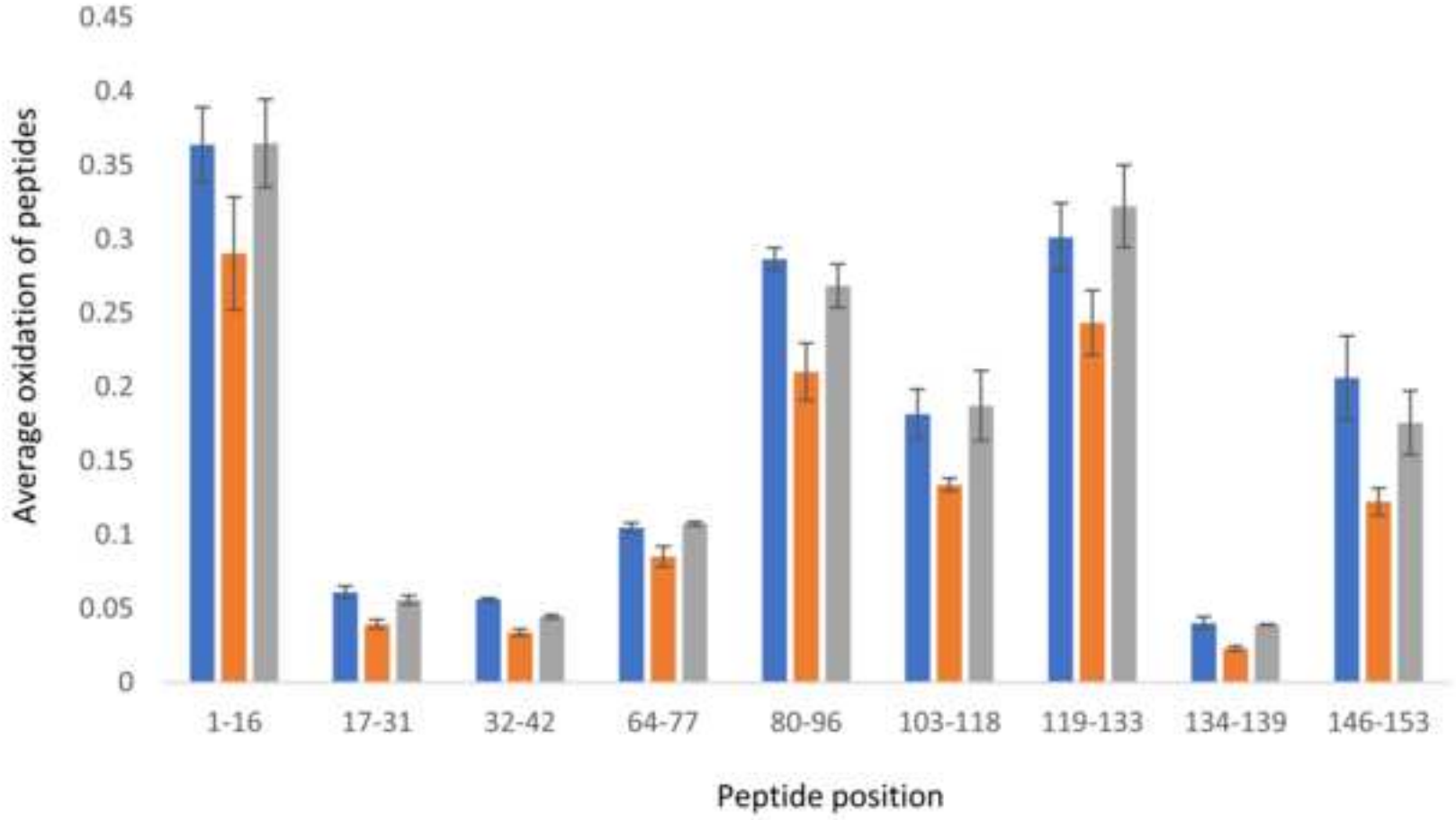

Table of Materials

Click here to access/download;Table of Materials;Misra and Sharp JoVE_Table_of_Materials_revised.xlsx

Name of Material/Equipment	Company	Catalog Number
Adenine	Acros Organics	147440250
Aperture	Edmund Optics	39-905
Aperture holder	Edmund Optics	53-287
Catalse	Sigma Aldrich	C-40
COMPex Pro laser	Coherent	1113836
Dithiotheitol (DTT)	Promega	V3151
Fraction collector	GenNext Technologies, Inc.	N/A
Fused silica capillay	Molex	1068150023
Glutamine	Acros Organics	119951000
Holder for lens	Edmund Optics	03-668
Hydrogen peroxide	Fisher Scientific	H325-100
LC-MS/MS system	Thermo Scientific	IQLAAEGAAPFADBMBCX
Mas spec grade Acetonitrile	Fisher Scientific	A955-1
Mass spec grade formic acid	Fisher Scientific	A117-50
Mass spec grade water	Fisher Scientific	W6-4
MES buffer	Sigma Aldrich	M0164
Methionine amide	Bachem	4000594.001
Micro V clamp	Thor Labs	VK250
Motorized stage	Edmund Optics	68-638
Nano C18 colum	Thermo Scientific	164534
Optical bench	Edmund Optics	56-935
Pioneer FPOP Module System	GenNext Technologies, Inc.	N/A
Post holder	Edmund Optics	58-979
Sodium phosphate dibasic	Fisher Scientific	BP331-500
Sodium phosphate monobasic	Fisher Scientific	BP330-500
Syringe	Hamilton	81065
Syringe pump	KD Scientific	788101
Trap C18 column	Thermo Scientific	160454
Tris	Sigma Aldrich	252859
Trypsin	Promega	V5111
UV plano convex lens	Edmund Optics	84-285

Comments/Description

Soluble in water upto 3.5 mM
1000 µm Aperture Diameter, Gold-Plated Copper Aperture
25.8mm Outer Diameter, Precision Pinhole Mount
Catalase from bovine liver, lyophilized powder, ≥10,000 units/mg protein
COMPexPRO 102, F-Vversion, KrF laser, No XeCl
DTT, Molecular Grade (DL-Dithiothreitol)
Automated fraction collector
Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 100 µm, Outer Diameter 375 µm, TSP100375
L(+)-Glutamine, 99%
53 mm Outer Diameter, Three-Screw Adjustable Ring Mount
Hydrogen Peroxide, 30% (Certified ACS), Fisher Chemical
Dionex Ultimate 3000 coupled to Orbitap Fusion Tribrid mass spectrometer
Acetonitrile, Optima LC/MS Grade, Fisher Chemical
Formic Acid, 99.0+%, Optima™ LC/MS Grade, Fisher Chemical
Water, Optima LC/MS Grade, Fisher Chemical
MES hemisodium salt
H-met-NH_2.HCl
Micro V-clamp with stainless steel blades
50mm Travel Motorized Stage System with Manual Control
Acclaim PepMap 100 C18 HPLC Columns
18" x 18" breadboard
Inline FPOP Radical Dosimetry System
3" Length, ¼-20 Thread, Post Holder
Sodium Phosphate Dibasic Heptahydrate (Colorless-to-White Crystals), Fisher BioReagents
Sodium Phosphate Monobasic Monohydrate (Colorless-to-white Crystals), Fisher BioReagents
100 µL, Model 1710 RN SYR, Small Removable NDL, 22s ga, 2 in, point style 3
Legato 101 syringe pump
Thermo Scientific Acclaim PepMap 100 C18 HPLC Columns
Tris(hydroxymethyl)aminomethane
Sequencing Grade Modified Trypsin
30 mm Dia. x 120 mm FL Uncoated, UV Plano-Convex Lens

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Abstracts:** Add a 50 word summary.

Response: We have added a summary to the manuscript.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Entire section 1 and most of the other sections need re-writing.

Response: We have made the suggested changes to the wording.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have attempted to include an appropriate level of detail, in line with published JoVE articles on similar topics.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

Response: We have made the requested formatting changes.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tel;l the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces

when calculating the final highlighted length.
5) Notes cannot be filmed and should be excluded from highlighting.

Response: We have highlighted the parts that should be used for making the video.

• **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.

Response: We have limited Discussion to the areas requested, and moved description of the technique to other sections where appropriate.

• **Figures:** Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

Response: Figures are in separate files now and figure legends are still in the manuscript file.

• **References:**Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

Response: References are formatted as required.

• **Commercial Language:** Delete commercial words. Examples of commercial sounding language in your manuscript are Orbitrap, Thermo Scientific, Byonic, etc. 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.

Response: We have attempted to remove all commercial language while keeping the protocol easy to follow.

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]."

Response: Figures 1 and 3-6 are original figures and are not published elsewhere.

For figures 2 and 7, the permission to reproduce the figure is attached as supplemental file.

Comments from Peer-Reviewers:

Reviewer #1:

Minor Concerns:
In section 1 step 6 and section 3 step 2, authors should provide a typical range used for laser energy, flow rate, and repetition.

Response: After some changes, the old section 1 step 6 is now step 5 and the typical range for laser power and repetition rate has been included. Section 3, step 2 contains the typical flow rate.

There are several grammatical errors, some chemical formulas do not have the appropriate super scripting in them, and the degree symbol is missing in Section 5 step 7. These should be addressed.

Response: We have attempted to address all grammatical errors, and will cooperate with the editorial process if others are found.

Reviewer #2:

Major Concerns:
There are sections of the article where unnecessarily negative views are aimed at other work in the field. This may not be the authors' intentions, but they should be aware of the effect. Examples are pointed out in this review.

Response: We have certainly not intended to be unnecessarily negative towards others' work in the field. However, we also do not want to ignore areas in which established researchers have differences of opinion. We have tried to rewrite the manuscript to summarize the conflict while trying to avoid reaching definitive conclusions about the resolution. We will highlight these edits where examples are pointed out.

1. Title: I suggest that topology is not the correct word here. Topology is defined as MATHEMATICS the study of geometric properties and spatial relations unaffected by the continuous change of shape or size of figures. Or the way in which constituent parts are interrelated or arranged as in "the topology of a computer network." I would vote for high order changes in protein structure. Topology is just one aspect of this.

Response: The word "topology" is not used in the title. A search of the document did not find the word "topology" used anywhere in the text. The word used is "topography." The use of the word "topography" in the context of protein footprinting to describe changes to the protein surface

was to my knowledge coined by Prof. H.A. Scheraga (DOI: 10.1021/bi00622a009) in 1977. It has been used by multiple groups to describe the protein quality probed by hydroxyl radical protein footprinting, including my group (first in DOI: 10.1038/s41598-017-04689-3) and Mark Chance's group (first in DOI: 10.1074/mcp.O114.044362). We believe that "higher order structure" which includes numerous factors including secondary structure, domain folding, interdomain contacts, etc. is not the best descriptor of what HRPF is measuring: namely, the surface of the protein exposed to hydroxyl radicals diffusing through the solvent. We respectfully decline to make the recommended change.

2. In general, the article has an undue emphasis on the dosimetry. Indeed, that is important, but the treatment is not commensurate with the title and theme of the article. Given the principal author's commercial interests, this emphasis should be reduced.

Response: We regret that our title was not sufficiently descriptive of the intended focus of the manuscript. While a general overview of FPOP is included, the focus that was pitched to the editor was a detailed demonstration of the use of real-time dosimetry to enable real-time compensation. This is the portion of the protocol that will be focused on for the filmed portion of the submission. We have edited the title to make this focus clearer: "Enabling Real-Time Compensation in Fast Photochemical Oxidations of Proteins for the Determination of Protein Topography Changes."

3. In the abstract, authors mentioned that FPOP is a technique that can probe "solvent-exposed surface area of proteins". Practically, FPOP cannot afford surface area directly. A better way of presenting this is FPOP can probe "solvent accessible surfaces of proteins".

Response: We disagree with the reviewer that FPOP does not measure solvent exposed surface area of proteins. While such measurements are complex and beyond the scope of this review, our publication in 2017 showed the ability to generate absolute solvent accessible surface areas of amino acids for one protein using an empirical relationship equation derived using an unrelated protein (DOI: 10.1038/s41598-017-04689-3). While not using FPOP, Mark Chance's group made a similar claim for hydroxyl radical protein footprinting at an earlier date (DOI: 10.1074/mcp.O114.044362). We contend as we have previously in the published literature that the rate of hydroxyl radical reaction can be used to determine solvent accessible surface area with a valuable level of precision, once the inherent chemical reactivity has been properly controlled for and the amount of oxidation at the amino acid level have been measured using an accurate and precise method. Moreover, this contention is not unique to our group. We respectfully decline to make the recommended change.

4. At the beginning of the introduction, authors mentioned that "The rate of the oxidation reaction at any amino acid depends on two factors: the inherent reactivity of that amino acid (which depends on the side chain and the sequence context) and the accessibility of that side chain to the diffusing hydroxyl radical, which closely correlates to the average solvent accessible surface area. All of the standard amino acids except glycine have been observed as labeled by

these highly reactive hydroxyl radicals in FPOP experiments, albeit at widely differing yields.". The rate of oxidation reaction depends not only on the inherent reactivity of the that amino acid and its solvent accessibility, but also on the local environment of that specific amino acid (Chem. Phys. Lett. 2014, 613, 5−9. DOI: 10.1016/j.cplett.2014.08.041; Anal. Chem. 2019, 91, 14, 9238-9245. DOI: 10.1021/acs.analchem.9b02134; Anal. Chem. 2019, 91, 15163-15170. DOI: 10.1021/acs.analchem.9b03958). The fluctuation of local H2O2 concentration prior to laser irradiation is part of the reason where the oxidation is only observed in selected residues.

Response: While we admire the experimental work of Gross et al as presented in DOI: 10.1021/acs.analchem.9b02134, we dispute their conclusion that the differences in 18O labeling is due to local hydrogen peroxide concentrations. As cited in this review by Reviewer #2 (DOI: 10.1016/j.cplett.2014.08.041), the amino acid calculated to have the highest binding affinity for hydrogen peroxide is aspartic acid, followed by glutamine in aqueous solution. However, Gross et al found that aspartic acid and glutamine took oxygen exclusively from O_2, along with many other polar amino acids. We have numerous other disputes with the mechanisms presented by Gross and coworkers in this work in light of the time-resolved spectroscopy data that have been presented in the field in radiation biochemistry (e.g. we believe that Gross and coworkers misinterpreted their tyrosine data; spectroscopic data suggest that radical addition to the ring is much more preferred than hydrogen abstraction from the OH group, and the lack of hydroxylation observed by Nukuna, Goshe and Anderson is due to the resolution chemistry of hydroxylated tyrosine radicals in evacuated solutions, see DOI: 10.1021/j150654a030). Additionally, we believe that our data from DOI: 10.1038/s41598-017-04689-3 clearly show a close and predictable relationship between solvent accessible surface area and apparent rate of reaction with hydroxyl radicals in FPOP, without the need to include effects of local binding of H_2O_2 in solution. The work cited by Reviewer #2 from the Ashcroft group does not add any light to this question; the Ashcroft group merely invoke the Gross group's work as a possible alternative explanation for FPOP results. As such, we do not believe the effects of local hydrogen peroxide concentration are sufficiently established to be presented in this review, and we respectfully decline to make the recommended change.

This leads to the concern of number of residues that can be labeled by the FPOP. A common consensus in the field is that 14 out of 20 amino acid can be labeled in a real FPOP experiment (Chem. Rev. 2007, 107, 3514-3543. DOI: 10.1021/cr0682047; Chem. Rev. 2020, ASAP. DOI: 10.1021/acs.chemrev.9b00815). A claim of 19 out of 20 amino acids can be labeled by FPOP is overexaggerating.

Response: The DOI: 10.1021/cr0682047 reference cited by Reviewer #2 is not in respect to FPOP; rather, it is referring to X-ray synchrotron radiolysis-based hydroxyl radical protein footprinting. Unlike FPOP, X-ray HRPF is limited in the amount of hydroxyl radical that the protein can be exposed to without inducing artifactual labeling; under such limited labeling regimes, Chance is correct that the less reactive amino acids are unlikely to be labeled. Part of the genius of Gross' FPOP invention is to free HRPF from these shackles of limited labeling. Regardless, we do not see in the Chance reference cited here by Reviewer #2 any claim that only 14 of 20 amino acids can be oxidized. While the Gross group has claimed that only 14 out of 20 amino acids are amenable to labeling by FPOP (excluding Gly, Ala, Asp, Asn, Ser and Thr), we cite Xie et al (DOI: 10.1038/s41598-017-04689-3) where not only do we identify oxidation of

amino acids not in the Gross list, but we demonstrate the dose-response of their oxidation to increasing amounts of hydroxyl radical (e.g. Asn37 and Asn39 of lysozyme shown in Figure 1 of that reference). We similarly report in that paper oxidation of Ala107, Ala110, Ala122, Thr 47, Thr51, Ser50, and Asp52 of lysozyme, along with Thr66, Thr68, T70, Asn12, Asp126 and Ala125 of myoglobin, along with dose response data for each of these amino acids. These amino acids were successfully used to calculate solvent accessible surface area once they were properly normalized for inherent reactivity using a denatured protein, and these values were used to select the correct structure from a group of computational models. We have similarly found these amino acids that Gross has listed as inactive labeled in other studies (e.g. DOI 10.1074/jbc.M115.648410; DOI 10.1021/acs.biochem.6b00888). Indeed, we note that Gross and coworkers report on the oxidation of Asp, an amino acid they had called inactive, in their recent paper on OH radical reaction pathways (DOI 10.1021/acs.analchem.9b02134) cited by Reviewer #2 above.

While we strongly dispute the notion that only 14 of 20 amino acids are "active" in FPOP, and stand by our assertion that 19 of 20 can be labeled by the experiment depending on protein structure and oxidation conditions based on published data in the literature, we do not want to leave the reader with the impression that all 19 amino acids are ***commonly*** *labeled. We have added text that indicates that Ser, Thr, Asn and Ala are also rarely seen except in special circumstances. We hope that, given both our multiple reports of oxidized Asp and the reports of the Gross group regarding oxidation of Asp, Reviewer #2 will agree with its inclusion in the list of somewhat commonly oxidized amino acids.*

5. P 4: To solve this issue, an inline dosimeter that can monitor the signal from adenine absorbance changes in real time immediately after the laser irradiation is important. Better to say "located in the flow system after the site of irradiation…"

Response: We have made the recommended changes.

6. P 4, bottom: Not clear what is meant by "engineering controls"

Response: "Engineering controls" is a term common to safety standards, and a key part of OSHA's pyramid of workplace exposure control (https://www.osha.gov/SLTC/hazardoustoxicsubstances/control.html). It refers to physical changes to the workplace to limit exposure. We do not name specific engineering controls here, as they will vary widely from space to space (e.g. laser safety curtains, laser use warning lights, safety interlocks on the access door, blocking all windows, etc.)

7. P 5, top: Place space between units and numerals

Response: We have made the recommended change.

8. In page 4, authors stated "VIrr = π(1/2d)2w" and it can be confusing. It is better to replace it with "VIrr = π(1/2·d)2w".

Response: We have instead simplified the expression in question to (d/2). If the editor would prefer some other numerically equivalent expression to meet the journal style, we are happy to do so.

9. At the beginning of page 5, section 2, step 1 of protocol section, authors mentioned "(e.g. ligand-bound and ligand-free, aggregate and monomer, alone and with a protein binding partner, etc.)". The "alone and with a binding partner" is the same with "ligand-bound and ligand-free".

Response: The term "ligand" in protein biochemistry generally refers to a small molecule; by "binding partner" we meant a protein-protein interaction. We have clarified our intention in the text.

10. P. 5: Clarify: "The diameter of the aperture must be smaller than the smallest impinging focused beam size."

Response: We have clarified the text as follows: "The diameter of the aperture must be smaller than the width of the impinging focused beam at every point in the range of the motorized stage."

11. P 5, bottom: These ideas of flow, exclusion volume, and the governing math were part of the original idea and design. Citation is needed.

Response: We regret the oversight, and have added the citation to Hambly and Gross (2005).

12. P 6, top: "the sensitivity of your MS workup" should be rephrased to clarify.

Response: We have replaced the text in question with "the material required for robust detection and relative quantification, and will vary depending largely on the LC-MS/MS system used and the post-labeling sample processing method." Unfortunately, the range of potential systems and post-labeling sample processing steps are too broad to give a universal answer in the protocol.

13. "Hydrogen peroxide should also be regularly tested for integrity on p 6, bottom." Use a more precise term for integrity

Response: We have replaced "integrity" with "decomposition."

14. At the beginning of page 7, section 5, step 1 of protocol section, please specify the volume of the mixture. Also, give some typical values for item 2.

Response: We have made the addition of digestion buffer more detailed. We are unsure what the reviewer is referring to as "item 2," and so are unable to respond to this comment.

15. Under compensation on p8, point out that the compensation is not in real time. This compensation is a good idea, and it would be useful to know if real-time compensation is possible.

Response: Unfortunately, we must dispute with the reviewer; the compensation is in real time. We first reported real time compensation in 2018 (DOI: 10.1021/acs.analchem.8b02787). In that paper, we compensated in real time by adjusting the laser energy per pulse with an aperture to maintain a constant V_{Irr}. This works for relatively minor differences in effective radical dose, as much of the increased energy goes into increase beam cross-section. In this review, we teach a new method which will be clear in the video. We have added text at the editor's request that should also make this method more explicit in Step 4.3. Namely, we adjust the position of the plano-convex focusing lens using the motorized stage, changing the relative position of the focal plane of the lens and the FPOP capillary to increase or decrease laser fluence at the plane of irradiation of the capillary. We continue adjusting the laser fluence until we reach the desired ΔAbs_{265} reading. The video will make the process quite clear, with shots of the stage movement and the dosimeter response.

16. P 8: "…with a UV spectrophotometer is…" Should be "are"

Response: We have made the recommended change.

17. At the beginning of page 8, section 6, step 3 of protocol section, authors described a 42 min-long LC gradient, which seems to be short. Is this sufficient for sufficient peptide separation? Authors should comment on how to optimize the LC gradient for different samples.

Resposne: This is more than sufficient for simple one- or two-protein samples in experimental designs seeking to do peptide-level quantification, which is what we are teaching here. For groups trying to do the LC-based sub-peptide quantification as performed by Gross, Chance, and other groups, longer LC gradients may be necessary to achieve desired resolution of peptide oxidation isomers but this is beyond the intended scope of this article. We have added a note to section 6.3 giving examples of when altering the LC gradient might be warranted.

18. P 8: What is the purpose of CaCl2?

$CaCl_2$ has been noted to increase the efficiency of trypsin digestion. Reports indicate that this increase is due to stabilization of the trypsin structure (DOI: 10.1002/0470028637.met059). It is

recommended for tryptic solution digests in Tris buffer in the protocols of both Pierce and Promega.

19. P 8: Here and throughout: keep items in a list of parallel construction. For example, each item could be "Heat…", "Digest…" "Quench…". Later, add space between numerals and units.

Response: In response to an editorial comment, we have subdivided the process into individual steps numbered in accordance with the journal's style.

20. P 8, item 5: explain protocol and reason for "rotation"

Response: We have replaced "rotation" with "mixing." In our group, we incubate tryptic digests in a hybridization oven, and mix the reaction by placing the tube in a foam insert attached to the hybridization oven's rotating arm and rotating it. However, other forms of low volume mixing can work as well to increase efficiency of digestion and help prevent undigested protein from precipitating during the incubation.

21. Later this page, step 6, authors mentioned "The peptides were fragmented five times within 30 sec and then it was transferred to an exclusion list for 60 sec.". How was this achieved? Please specify the instrument parameters that was used.

Response: Journal policy as indicated by the editor states that we must avoid commercial terms in our manuscript whenever possible. We are not certain how to more clearly state our method without using Thermo-specific language. We have clarified that the experimenter should fragment peptides ***up to*** *five times before moving them to an exclusion list; we would welcome editorial guidance if more explicit instructions involving Thermo Orbitrap-specific language is required.*

22. P 9, middle: specify the m/z for the resolution (better term is resolving power). Why is it nominal?

Response: In the Thermo Orbitrap instrument used, transient time is selected not by time, but rather by "resolution"; as this is not true resolving power, the resolution is nominal, not actual. If we stated to use a transient time of 128 ms, many readers would be unsure how we performed the experiment. Using the setting term "resolution" allows readers to reproduce our protocol in the settings, while allowing non-Orbitrap users to understand the approximate level of resolution required. The Thermo nominal resolution is calculated for m/z 200; we have added that to the text.

23. P 9: "…trypsin digested samples using standard trypsin…" small point, but the samples are not using anything

Response: We have added a comma to separate the participle clause from the main clause.

24. In page 9, 7th line from the end, authors mentioned "these are not exposed the laser irradiation", and it should be "these are not exposed to the laser irradiation".

Response: Editorial requests have resulted in this section's rewriting. The error is no longer present.

25. P 10, top. "The extracted ion chromatogram of these m/z values.." More accurate to state the chromatograms are of peptides represented by mol ions, not values…

Response: The extracted ion chromatogram (EIC) does not distinguish peptides from non-peptides, or even analytes from non-analytes; it merely shows the intensity of signal at a chosen m/z value. We make note of this fact by recommending readers use the EIC mass as only one factor of three in verifying a given EIC peak's identity as a peptide oxidation product. Defining the EIC as a chromatogram of the protonated molecule of these peptides would be inconsistent with our recommendations for verifying identity. Our usage of EIC is consistent with the definition in the 2013 IUPAC Recommendations (DOI: 10.1351/PAC-REC-06-04-06). We respectfully decline to make the recommended changes.

26. In page 10, beginning of discussion section, authors mentioned the disadvantages and advantages of FPOP. That should be more comprehensive. For example, FPOP platform allows the discovery and application of other radicals (e.g., CO3-●, I●, CF3) in protein footprinting. A more thorough summary is reported in a recent review article (Chem. Rev. 2020, ASAP. DOI: 10.1021/acs.chemrev.9b00815).

Response: While we have great interest in and respect for the work being performed in other labeling technologies, such technologies are beyond the intended scope of this article.

27. P 10: "Using the LC gradient above,…" is a dangling participle.

Response: We have corrected the sentence.

28. P 10: middle: "control samples are prepared…"

Response: We have rewritten the section.

29. P 10: Pls improve the sentence: "After processing these samples in mass spectrometry along with other FPOP samples gives a good idea of the extent of background oxidation."

Response: This section has been entirely rewritten to address editorial comments; the poorly worded sentence is no longer present.

30. In the middle of page 11, authors state "For cases where the protein is particularly sensitive to hydrogen peroxide, on-line mixing with hydrogen peroxide prior to irradiation with the

excimer laser can limit exposure to seconds or less. However, for most proteins, online mixing is unnecessary.". There is another report that describes the online mixing specifically for FPOP to introduce hydrogen peroxide right before laser irradiation (J. Am. Soc. Mass Spectrom. 2015, 26, 526-529. DOI: 10.1021/jasms.8b04986).

Response: We have added the requested reference for completeness.

31. P 11, top: "major hurdles in FPOP for the typical protein mass spectrometry lab is experience in handling". Rephrase this something like "the major hurdles to be faced by a typical mass spectrometry lab…"

Response: We have made the requested change.

32. In the first paragraph of page 12, authors mentioned "Therefore, in order to increase the concentration of hydroxyl radical created, it is often necessary to change the focal plane of the lens either alone or in addition to changing the laser energy per pulse." Is the lens movable? Please specify.

Response: The lens is movable. Please see question 15.

33. Immediately after the previous statement (Point 11), authors state "The use of an aperture allows one to change the size of the incident beam cross-section without changing the width of the illuminated region, allowing the investigator to use a constant flow rate.". The statement is not clear, as changing the aperture opening will change the overall size of the laser spot, and the width of the illuminated region should change as well. Please be more specific/explanatory.

Response: The aperture opening is fixed. The focal plane of the lens is moving, changing the incident beam cross-section in the plane of the capillary without changing the region of the capillary illuminated due to the silhouetting by the aperture.

34. In page 13, the authors state "The current consensus in the field seems to be that peak area-based amino acid level quantification seems to correctly identify sites of oxidation that change, but may not accurately quantify the degree of change.". This is not accurate and too negative. There are many demonstrations that utilizes the peak-area-based quantification to solve biological questions. Some examples are protection factors (Biophys. J. 2015, 108, 107-115. DOI: 10.1016/j.bpj.2014.11.013) and its application in computer-based protein structural prediction (Anal. Chem. 2018, 90, 7721- 7729, DOI: 10.1021/acs.analchem.8b01624), analyzing amyloid beta aggregation (J. Am. Chem. Soc. 2016, 138, 12090-12098. DOI: 10.1021/jacs.6b07543), and FPOP-based titration for protein-ligand binding affinity measurement (Anal. Chem. 2019, 91, 12560-12567. DOI: 10.1021/acs.analchem.9b03491). Authors should tone down their assertion. It's not productive to imply that most of the data in the literature are not to be trusted. One can say that there is room for improvement…and take a positive view.

Response: The reviewer is correct that the object of the lack of consensus was improperly worded, and we apologize for that; we do not dispute the ability of LC peak area-based

*quantification to accurately determine sites and relative amounts of change, but rather the ability to determine amounts of oxidation at a given amino acid. We believe that it is important to highlight this lack of consensus, especially as groups move into protection factor-style analysis that relies upon accurate determination of the absolute amount of oxidation of a given amino acid. My group has long contended based on results published in 2016, along with cited work from the Vachet group published in 2009, that quantitative results based on LC separation of peptide oxidation isomers and identification by collision-based methods are prone to error in quantification. We demonstrated this using defined mixtures of synthetic oxidation products, which is the typical procedure for validating quantitative analytical methods. We have offered to make these mixtures available to other established researchers in the HRPF field that have published using LC separation-based quantification methods. One such group accepted our offer and received the mixtures; they have not reported results contradicting our 2016 paper. As an established member of the relatively small HRPF field, I think that my concerns reflect a lack of consensus. However, I do not think that my opinion is controlling, and I have tried to make the dispute in the field clear without obviously taking sides: "*The current consensus in the field seems to be that LC peak area-based amino acid level quantification seems to at least correctly identify sites of oxidation that change and correctly identify the relative amount of change (i.e. oxidation of amino acid X decreases by Y% in conformation A compared to conformation B), but the accuracy of quantification of the amount of oxidation (i.e. amino acid X is Y% oxidized) remains in dispute." *Hopefully this corrected wording satisfies the reviewer.*

35. As for item 34, a similar request is made for the comments on software companies. Further, the criticism is a blanket one and may disparage some companies who are working on improvements. Let's not be excessively negative about others to promote one's ideas. Later, the authors say "One method tries to use HPLC…" Again a subtle but "too negative" connotation about other work.

Response: Our critique of current automated software packages are not only indicative of our experiences (where we have manually calculated oxidation of multiple samples and then compared our results with available automated solutions, as well as attempts with defined mixtures of synthetic peptide oxidation products in known quantities), but also anecdotal talks with researchers in leading FPOP groups that ***do*** *commonly use the software; these researchers have repeatedly stated to me that they manually validate and correct all important automated software results because they commonly see misquantification. We do agree with the reviewer's sentiment that the field should be encouraged rather than disparaged; we intentionally left out specific citations to groups using the available software because we do not want to unfairly single out any software solution for criticism or imply that any specific developer is doing a bad job. We have added text to point out how difficult the problem is, and how much laudable progress software solutions have made so far while still pointing out room for improvement. We have also replaced "commercial" with "available", as we have tried non-commercial software made available to us with similar results, and we do not want to single out commercial vendors for criticism.*

We do agree with the reviewer that the phrase "tries to use HPLC to resolve oxidation isomers" is unfair. These groups largely do an excellent job of resolving oxidation isomers. Our data suggest it is not insufficient resolution of these isomers that causes inaccurate absolute

quantification. We have removed the language.

Reviewer #3:

It is quite clear that the lead author wishes to recommend the dosimetry solution which his company also offers commercially. This is fine and the use of dosimetry is justified scientifically, but there are other ways to achieve the same goal, albeit less elegant, by adding an internal molecular dosimeter and using an MS readout at the MS (not MS/MS) level. I would ask the authors to give this option more space in their paper.

Response: We failed to make clear the original intention of the manuscript. The intention was to highlight the use of real-time dosimetry and compensation for FPOP experiments, and this is the part of the protocol that will be highlighted in the video. Currently, UV-responsive dosimetry is the only method that allows for real time dosimetry and compensation in FPOP. We have changed the title of the manuscript to make our intention more clear. We have ensured that references to manuscripts using alternative methods of dosimetry are included in the Introduction; however, we have been editorially directed to limit discussion to the technique illustrated here.

p3 radicals are short-lived at least in part due to the scavenger which limits their lifetime (not inherently so)

Response: We have clarified that the hydroxyl radicals are consumed on a microsecond timescale under FPOP conditions.

p5 using methanol to wipe - small fire hazard when capillary is still hot (I've watched someone make a small harmless fire in this way)

Response: We have added the safety note, and thank the reviewer for suggesting it.

p9 known mass shifts - apart from mentioning +16, would be good to briefly summarize what to look for

Response: While a review of the subject would be beyond the scope of this manuscript, the reviewer is correct that +16 is not the only oxidation product that can be observed. We have added references to both an excellent review of protein-hydroxyl radical chemistry, as well as a later series of papers from Xu and Chance examining MS-observable oxidation products. While we would be willing to add in a table of oxidation products, we worry that it would expand the length of the article beyond acceptable limits; we are happy to follow the editor's discretion on this matter.

p10 is there a reference for the 240/180s window?

Response: This window depends on the LC separation phase and gradient used, and to my knowledge there has been no systematic examination of the topic. We have added language to make it clear that this window is based on observations in our hands only.

p10 background oxidation: important to mention that a significant source for this can be in-source in ESI

Response: We have discussed this phenomenon in the manuscript and included methods for identification and correction of in-source oxidation, as well as other forms of background oxidation.

p11 explain what MES buffer is

Response: The full name of the MES buffer (2-(N-morpholino)ethanesulfonic acid) is now included the first time it appears in the manuscript.

p13 about effective dose, I'm not convinced that high dose for a short period vs. lower dose for longer generate identical footprints. Maybe identical total % oxidation, but the amino acid selectivity might well differ due to the vastly different reactivities, which includes kinetics of the reaction (and possible reversibility!). This is an important point and the statement made not self-evident. If there is evidence however, I would welcome if that was shared here.

Response: The identical amount of oxidation is not only at the protein level, but also at the peptide level (as seen in Figure 7). If compensation resulted in identical total % oxidation but different selectivity, we would observe changes from peptide to peptide as selectivity changed from target residues in one peptide to target residues in another. Every peptide shows identical total oxidation within the entire protein, even though these peptides have vastly different amino acid compositions from one another. We show this in Figure 7 for myoglobin with adenine, but we have also shown it with myoglobin using Tris as a dosimeter (Ref. 12) and with adalimumab using an adenine dosimeter (Ref. 30), showing that the relationship holds regardless of amino acid sequence OR UV-responsive dosimeter used. Additionally, as currently shown in Figure 2, the compensation observed is consistent with what is predicted by kinetic simulations that do not include changes in the rate constants of oxidation of any amino acid; rather, it treats the protein as a non-consumed reactor of hydroxyl radicals with an unchanging second order rate constant of reaction.

Finally, under dissolved atmospheric O2 concentrations, reversibility of the reaction will be negligible. Unstable odd-electron products are stabilized relatively quickly through reaction with O2 and secondary oxygen-based radicals, while even-electron oxidation products are irreversible with the exception of methionine sulfoxide, which generally requires an enzyme catalyst and a reductant to reverse the reaction. Current reference 26 gives a thorough overview of the reaction pathways of amino acids.

p14 spatial resolution: might be appropriate to refer to this paper - https://pubmed.ncbi.nlm.nih.gov/30267362

Response: We have added the recommended reference.

p14 why does ETD require HILIC - please explain

Response: We have added a brief explanation and references in the manuscript.

fig 1 many groups would have a target % of total oxidation, e.g. +16 peak at 50% intensity of unmodified. Also recommendation to run intact, denatured sample to assess this prior to any digest and LC-MS/MS

Response: The level of total oxidation can vary significantly depending on the conformation of the protein; we would argue that while it may be suitable for specific applications, targeting a specific % oxidation intensity is unadvisable as a general practice. Post-oxidation clean-up is required to prevent extensive in-source oxidation due to residual hydrogen peroxide in the electrospray source. Intact protein MS can be challenging depending upon the protein. We have had experiences with some proteins aggregating after oxidation and giving poor to no intact signal of the oxidized protein, leading to underestimation of the amount of oxidation. Based on these experiences and the performance of various dosimetry solutions (including the one described here), we do not recommend tracking oxidation of the intact protein as a general practice.

fig 2 difficult to see detail but video will prob address this

Response: We have taken a new image of the current Figure 3; we hope that this makes the detail a bit more clear. This segment will also be the focus of the video.

fig 5 increasing laser fluence - but with caveats about spot intensity mentioned in the main text...?

Response: We have attempted to make clear the difference between laser <u>fluence</u> and laser <u>energy per pulse</u>. The current Figure 6 was generated by altering the laser fluence, which is the necessary variable in determining peak hydroxyl radical generation. Alteration of laser fluence by altering laser energy per pulse is possible, but care must be taken to account for changes in laser cross-section using the aperture mentioned, along with dosimetry response to report on effective hydroxyl radical dose. The other method of altering laser fluence in real time is altering the focal plane of the lens relative to the capillary. We have added detail about this method to the text, and will demonstrate it in the video.

fig 7 bit unclear what it adds here out of the context of the paper it appeared in - and difficult to understand. "decrease" = "increase"? I recommend leaving this out here

Response: The current Figure 2 is necessary to demonstrate the theoretical underpinnings of both dosimetry and the compensation phenomenon. There is a misconception among some novices that the amount of oxidation of a protein in its buffer is solely a factor of the radical

half-life; this in only true if the peak concentration of hydroxyl radical generated is constant, as shown in Figure 2. While compensation has only been demonstrated using UV-responsive dosimeters, Figure 2 shows that it should work with any accurate dosimeter. We have moved this figure to the Introduction, and have cleaned up the description.

Permission, Figure 2

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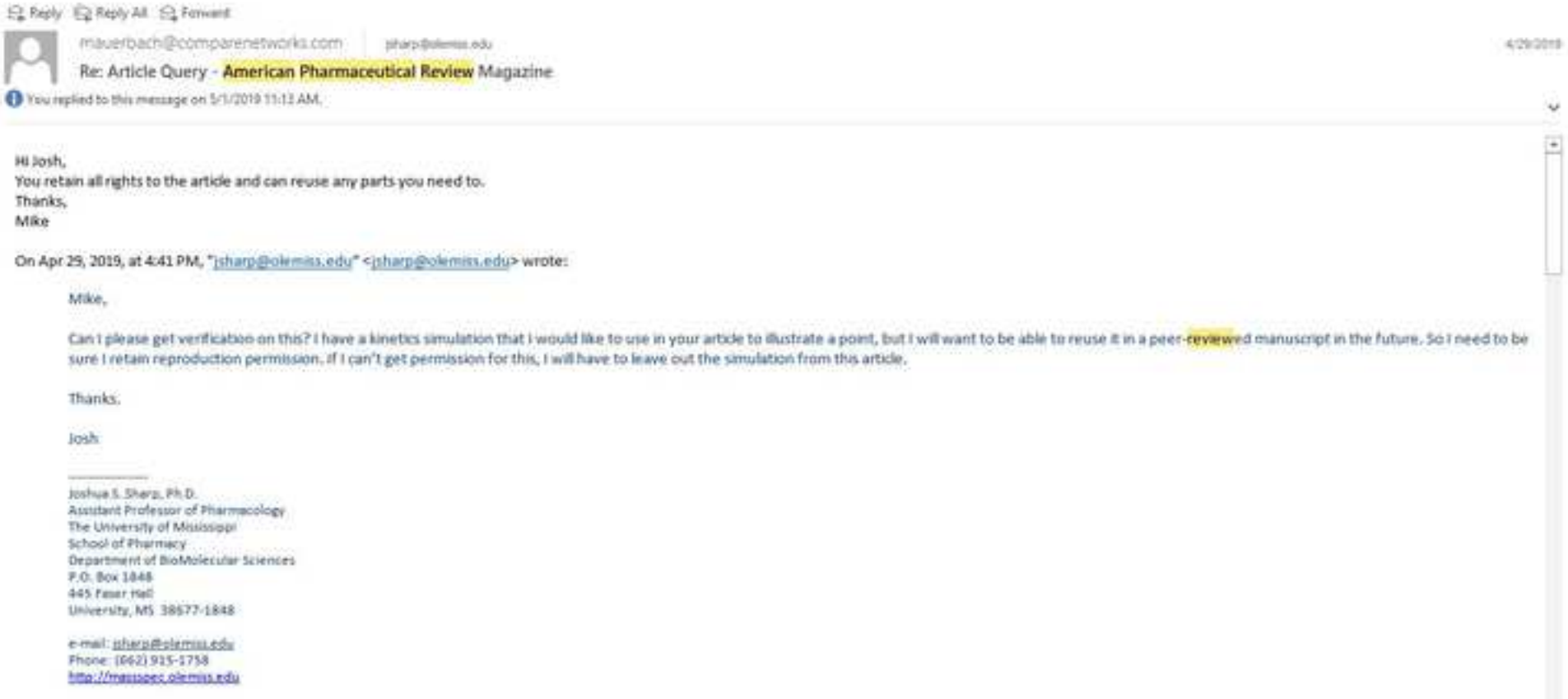

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Thanks.

Josh

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