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Corresponding Author:	Scot Weinberger Agilent Technologies Inc Montara, California UNITED STATES
Corresponding Author's Institution:	Agilent Technologies Inc
Corresponding Author E-Mail:	sweinberger@gxntech.com
Order of Authors:	Scot Weinberger Emily E Chea Joshua S Sharp Sandeep K Misra
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TITLE:

Laser-free Hydroxyl Radical Protein Footprinting to Perform Higher Order Structural Analysis of Proteins

AUTHORS AND AFFILIATIONS:

Scot R Weinberger¹
Emily E Chea¹
Joshua S Sharp^{1,2,3}
Sandeep K Misra²

¹GenNext Technologies Inc., Half Moon Bay, CA

²Department of Biomolecular Sciences, School of Pharmacy, University of Mississippi, Oxford, MS

³Department of Chemistry and Biochemistry, University of Mississippi, Oxford, Mississippi

Email addresses of co-authors:

Emily E Chea (echea@gnxtech.com)
Joshua S Sharp (jsharp@gnxtech.com)
Sandeep K Misra (skmisra@olemiss.edu)

Corresponding author:

Scot R Weinberger (sweinberger@gnxtech.com)

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SUMMARY:

This protocol presents a method to use inline radical dosimetry and a plasma light source to perform flash oxidation protein footprinting. This method replaces the hazardous UV laser to simplify and improve the reproducibility of fast photochemical oxidation of protein studies.

ABSTRACT:

Hydroxyl Radical Protein Foot-printing (HRPF) is an emerging and promising higher order structural analysis technique that provides information on changes in protein structure, protein-protein interactions, or protein-ligand interactions. HRPF utilizes hydroxyl radicals ([•]OH) to irreversibly label a protein's solvent accessible surface. The inherent complexity, cost, and hazardous nature of performing HRPF have substantially limited broad-based adoption in biopharma. These factors include: 1) the use of complicated, dangerous, and expensive lasers that demand substantial safety precautions; and 2) the irreproducibility of HRPF caused by background scavenging of [•]OH that limit comparative studies. This publication provides a protocol to perform a laser-free HRPF system. This laser-free HRPF system utilizes a high energy, high-pressure plasma light source flash oxidation technology with in-line radical dosimetry. The plasma light source is safer, easier to use, and more efficient in generating hydroxyl radicals, and

the in-line radical dosimeter increases the reproducibility of studies. Combined, the laser-free HRPF system addresses and surmounts the mentioned shortcomings and limitations of laser-based techniques.

INTRODUCTION:

Protein conformation and associated higher order structure (HOS) are the principal determinants of proper biological function and aberrant behavior¹. The same applies to biopharmaceuticals, whose structure and functional activity is dependent on various aspects of their production and environment. Biopharmaceutical change in HOS have been linked to adverse drug reactions (ADR) attributed to undesirable pharmacology and patient immunological response^{2,3}. The appearance of ADRs has alerted the biopharmaceutical industry to the critical role that protein HOS plays in the safety and efficacy of biotherapeutics, and they have established the need for new and improved HOS analytics⁴.

Hydroxyl Radical Protein Foot-printing (HRPF) is a promising technique to track the change in protein HOS. HRPF involves the irreversible labeling of a protein's exterior with $\cdot\text{OH}$ followed with mass spectrometry (MS) analysis to identify the solvent accessible surface of the protein⁵⁻⁷. HRPF has successfully been used to detect defects in protein HOS and its function^{8,9}, characterize the HOS of monoclonal antibodies (mAb)¹⁰⁻¹³, determine the binding K_d of a ligand¹⁴, and much more¹⁵⁻¹⁹. A common method to generate the $\cdot\text{OH}$ for HRPF is Fast Photochemical Oxidation of Proteins (FPOP), which employs high-energy, fast UV lasers to produce $\cdot\text{OH}$ from photolysis of H_2O_2 . For the most part, FPOP uses expensive excimer lasers employing hazardous gas (KrF) that demands substantial safeguards to avoid respiratory and eye injury²⁰. To avoid inhalation hazards, others have used frequency quadrupled neodymium yttrium aluminum garnet (Nd:YAG) lasers²¹, which eliminates the use of toxic gas but are still costly, require significant operational expertise, and demand extensive stray light controls to protect users from eye injury.

Although ample information can be obtained using HRPF, broad adoption in biopharma has not been met. Two barriers for the limited HRPF adoption include: 1) the use of dangerous and expensive lasers that demand substantial safety precautions²⁰; and 2) the irreproducibility of HRPF caused by background scavenging of $\cdot\text{OH}$ that limit comparative studies²². To supplant laser use, a high-speed, high energy plasma flash photolysis unit was developed to safely perform FPOP in a facile manner. To improve on the irreproducibility of HRPF experiments, real-time radical dosimetry is implemented.

The practice of HRPF has been limited by irreproducibility attributed to background scavenging of $\cdot\text{OH}$ ²². While $\cdot\text{OH}$ are excellent probes of protein topography, they also react with many constituents found in preparations, making it necessary to measure the effective concentration of radical available to oxidize a target protein. Variations in buffer preparation, hydrogen peroxide concentration, ligand properties, or photolysis can result in oxidation differences between control and experimental groups that create ambiguity in HOS differential studies. The addition of real-time radical dosimetry enables the adjustment of the effect $\cdot\text{OH}$ load and therefore increases the confidence and reproducibility during an HRPF experiment. The use of radical dosimetry in FPOP has been described elsewhere²³⁻²⁵, and is further discussed in detail in

a recent publication²⁶. Here, we describe the use of a novel flash photolysis system and real-time dosimetry to label equine apo-myoglobin (aMb), comparing levels of peptide oxidation in an FPOP experiment to that of obtained when using an excimer laser.

PROTOCOL:

1. Installing the capillary tube

1.1. Using a silica cleaving stone, cleave 250 µm inner diameter (ID) silica capillary to 27 inches. Check the capillary ends for a clean, straight cut.

1.2. Create two windows roughly 15 mm in length by burning away the polyimide coating. Starting from the “lower end” make the first photolysis window 90 mm away from the “lower end” and the second dosimeter window 225 mm away from the “lower end”.

NOTE: Once the coating is burned away the capillary is very fragile.

1.3. Unscrew the nut and ferrule at port 5 and insert the “lower end” of the capillary just beyond the conical end of the ferrule (**Figure 1A**).

1.4. On the photolysis module, remove the photolysis cell cap by pulling it straight out and then remove the magnetically mounted metal mask which will hold the capillary in place.

CAUTION: Inside the photolysis cell cap is a curved mirror, do not allow anything to touch the mirror.

1.5. On the dosimeter module, open the dosimeter cell by pushing on the tab on the left and swing the dosimeter cell open to the right. Remove the magnetically mounted clips which will hold the capillary in place.

1.6. Place the capillary into the grooved channel in the base of the photolysis cell. Center the capillary window with the photolysis cell window. While holding the capillary in position with one hand, add the magnetic mask to hold the capillary in place. Place the photolysis cap back in position.

1.7. Place the capillary into the grooved channel in the base of the dosimeter cell. Center the second capillary window on the small aperture at the center of the dosimeter cell base. While holding the capillary in position with one hand, place the two magnetic clips in position to hold the capillary in place. Close the dosimeter cell until it clicks closed.

1.8. Insert the capillary through the knurled nut atop the capillary lift of the product collector (**Figure 1B**). Extend the capillary to just above the bottom of the vial.

NOTE: It is important for the capillary to reach the bottom of the vial to ensure that the capillary

is submerged in the quench solution while running an experiment.

2. Installing an injection loop

2.1. Use Teflon tubing with an outer diameter 1/16" and an inner diameter of 0.015" (381 μm). Follow equation 1 to calculate the length of tubing needed for the desired volume using equation 1.

$$L = \frac{V}{\pi \left(\frac{d}{2}\right)^2}$$

Where L is the length of the tubing in millimeters, V is the desired volume in microliters and d is the tube inner diameter in millimeters. For an injection volume of 25 μL and an inner diameter of 381 μm, the tubing length needs to be roughly 219.3 mm.

NOTE: For volumes less than 20 μL, use PTFE tubing with an inner diameter of 0.01".

2.2. Cut the Teflon tubing to the necessary length using a tube cutter. Check the ends of the tube for a clean, straight cut.

2.3. Insert one end of the new injection loop through one of the nuts and place a new ferrule onto the end of the tube. Hold the ferrule and nut in place and insert the tube into port 3 of the injection valve until it bottoms out in the valve. Hold the tube firmly and tighten the nut finger tight. Using a wrench, tighten the nut ¼ turn further. Remove and inspect the assembly.

NOTE: If the ferrule is not fixed in position, reinstall and tighten 1/8 turn further. Repeat with the other end of the loop.

2.4. Once both ends have a nut and fixed ferrule, loosely screw one end to port 3 and the other end to port 6 (**Figure 1C**). Once in position tighten both sides to finger tight then ¼ turn past finger tight with a wrench.

3. Initialize the photolysis system

3.1. Turn on the photolysis modules in the following order: (1) Fluidics Module (2) Photolysis Module (3) Dosimetry Module (4) Product Collector, and lastly (5) the system computer and launch the control software

NOTE: Allow the Dosimeter Module at least a half-hour to warm up from a cold start.

3.2. Fully submerge tubing from the "Valve" position on the syringe pump (**Figure 1D**) into 10 mL of buffer. Place the tubing from the "Waste" position (**Figure 1D**) and tubing from port 2 on the syringe port (**Figure 1C**) to an empty container to collect waste.

NOTE: Use the buffer your protein will be suspended in. A recommended buffer is 10 mM NaPO₄.

3.3. On the Product Collector carousel, place 1.5 mL microcentrifuge tubes at the position marked H and 6.

3.4. If using 250 µm ID capillary, set the load flow rate to 500 µL/min, processing flow rate to 7.5 µL/min, and waste flow rate to 500 µL/min.

3.5. Calculate the flash delay, product time, and waste time to use the Semi-Automatic Mode using equation 2.

$$t = s \frac{\pi \left(\frac{d}{2}\right)^2}{f}$$

Where t is the time in minutes, s is the capillary distance in millimeters, d is the inner diameter of the capillary in millimeters, and f is the flow rate in µL/min.

3.5.1. For the flash delay, the distance is from the “lower end” of the capillary to the first window, which should be roughly 90 mm. Using 250 µm ID capillary and a processing flow rate of 7.5 µL/min the sample will arrive to the photolysis window in about 35 seconds. Allow two flashes at 1 Hz to occur before the samples arrive, so set the flash delay to 33 seconds.

3.5.2. For the product time, enter the amount of time injected solution will take to flow from the injection valve to the end of the capillary. For a 27” long 250 µm ID capillary, set the product time to 4.5 minutes.

3.5.3. For the waste time, enter the amount of time the total volume of injected solution will take to be collected. At this time, the product collector will move from the product position to the waste vial. For an injection volume of 25 µL and a flow rate of 7.5 µL/min, set the waste time to 7.8 minutes.

3.6. Rinse out the injection loop five times by injecting 25 µL of HPLC-grade water into the injecting port with the injection valve set to the load position.

3.7. Manually turn the injection valve up to ‘inject position’ to flush the rest of the system. Select **Process (Out)** on the control software to begin flowing water. While flowing, raise the product collector capillary lift by selecting **Up** under the product collector so that you can see the end of the capillary. Flow water through the capillary until a droplet forms.

NOTE: If a droplet does not form, check the injector valve for leaks. If there is a leak, loosen the nut, reseal the capillary, and retighten.

218 4. **Determine actual $\cdot\text{OH}$ yield to test radical scavenging effects from the buffer.**

219
220 4.1. In the Control Software, start the flow by selecting **Process (Out)**. In the settings tab, set
221 the flash voltage to 0 V. Under the **Manual Control** tab in the dosimeter data section, select
222 **Start Data + AutoZero**.

223
224 4.2. Select the position for the product vial (H) and waste vial (6).

225
226 4.3. Select **Ready**, manually turn the injection valve down to the load position, and inject 25
227 μL of 1 mM Adenine with 100 mM H_2O_2 into the injection port. Once injected, manually turn
228 the injection valve up to the inject position.

229
230 NOTE: This automatically triggers the system to begin the flow, turn on the plasma source, and
231 acquire the dosimetry data.

232
233 4.4. Ramp the voltage up by navigating to the settings tab, and changing the flash voltage.
234 Repeat steps 4.2 and 4.3 using 500 V, 750 V, 1000 V, and 1250 V. Perform the reading of
235 adenine absorbance at each voltage in triplicates.

236
237 4.5. Select calculations tab to determine the average absorbance of each sample. First, click
238 **Select**, then manually select the beginning and end of the peak absorbance. In the available
239 space, write in a description of the sample. Repeat for all acquired data.

240
241 NOTE: Bubbles may form causing a spike in dosimeter reading. When selecting data to determine
242 average absorbance, omit areas with bubbles.

243
244 4.6. Copy and paste data in Excel to calculate the average change in adenine absorbance for
245 each voltage, thus determining the effective $\cdot\text{OH}$ yield.

246
247 4.7. Repeat steps 4.1-4.6 if multiple sample conditions (different buffer/additives) are being
248 used to normalize the effective $\cdot\text{OH}$ yield for each condition.

249
250 **5. Modification of protein to detect changes in higher order structure.**

251
252 5.1. Mix 4 mM adenine with 20 μM of protein in a one-to-one ratio to make a solution
253 containing 2 mM adenine and 10 μM protein.

254
255 5.2. Make the quench solution using 0.3 mg/mL catalase to break down excess hydrogen
256 peroxide and 35 mM methionine amide to quench any remaining radicals. Aliquot 25 μL of
257 quench solution into a 200 μL microtube so that an equal volume of quench and the labeled
258 product is mixed.

259
260 5.3. Dilute H_2O_2 to 200 mM and keep on ice.

261

262 5.4. On the Control Software in the settings tab, start the flash voltage at 0 V to determine
263 any background oxidation.

265 5.5. In the manual control tab, select **Start Data + AutoZero**, followed by **Process (Out)**, then
266 **Ready**, and finally turn the injection valve down to the load position.

268 5.6. Place a quench solution in position 1 on the product collect carousel. On the System
269 Control Software change the product vial to 1.

271 5.7. Immediately before injection, mix 12.5 μL of the adenine and protein mixture with 12.5
272 μL of H_2O_2 to make a final concentration of 1 mM Adenine, 5 μM protein, and 100 mM H_2O_2 .
273 Gently pipette up and down to mix, quickly spin down, and inject 25 μL using the injection port
274 within 10 seconds of mixing.

276 5.8. Switch the injection valve to the inject position and wait while sample is being
277 processed.

279 5.9. Repeat acquisition with 500 V, 750 V, 1000 V, and 1250 V. Perform each voltage
280 measurement in triplicate.

282 5.10. Calculate the average absorbance as described in step 4.5. Copy and paste all data to
283 Excel.

285 6. Shut the system down

287 6.1. After all samples have been collected, flush out the syringe port and sample loop by
288 setting the injection valve down to the load position and inject 25 μL of HPLC water five times.

290 6.2. Turn the injection valve up to the inject position to flush the rest of the system with
291 HPLC water.

293 6.3. Stop the flow, close the system control software, and turn off the modules starting with
294 the product collector, dosimeter module, photolysis module, then finally the fluidics module.

296 7. Sample preparation and liquid chromatography-mass spectrometry

298 7.1. Denature the protein by incubating samples at 80 $^{\circ}\text{C}$ for 20 min in the presence of 50
299 mM Tris and 1 mM CaCl_2 . Cool samples to room temperature and add 1:20 trypsin to protein.
300 Digest the protein overnight at 37 $^{\circ}\text{C}$ with sample mixing. Next morning, terminate trypsin
301 digestion by heating samples to 95 $^{\circ}\text{C}$ for 10 minutes.

303 7.2. Detect peptides using a high-resolution LC-MS/MS system connected with a UPLC
304 system.

7.3. Load the sample first onto trap column (300 µm ID X 5 mm 100 Å pore size, 5 µm particle size) and wash at 5.0 µL/mL for 3 min with water containing 2% solvent B (acetonitrile and 0.1% formic acid).

7.4. Separate the peptides on a C18 nanocolumn (0.75 mm x 150 mm, 2 µm particle size, 100 Å pore size) at a flow rate of 300 nL/min with a gradient between solvent A (water containing 0.1% formic acid) and solvent B. The gradient for peptide elution consists of a linear increase from 2 to 35% B over 22 min, ramped to 95% 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.

7.5. 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.

7.6. Acquire the full MS scans from 250-2000 m/z followed by eight subsequent data-dependent MS/MS scans on the top eight most abundant peptide ions. Use collision-induced dissociation at 35% normalized energy to fragment the peptides.

7.7. Identify all the unmodified peptides detected from MS/MS analysis using an available protein analysis software against the necessary FASTA file containing the protein sequence and the relevant proteolytic enzyme.

7.8. Search and quantify modified peptides using a HRPD Data Processing Software. The extent of oxidation for each identified peptide is calculated by dividing the summed chromatographic peak area of a modified peptide by the total chromatographic peak area of that peptide modified and unmodified using equation 3.

$$P = \frac{I(\text{singly oxidized}) \times 1 + I(\text{doubly oxidized}) \times 2 + I(\text{triply oxidized}) \times 3 + \dots}{I(\text{unoxidized}) + I(\text{singly oxidized}) + I(\text{doubly oxidized}) + I(\text{triply oxidized}) \dots}$$

where P denotes the average number of oxidation events per peptide molecule, and I represents the peak area of the unoxidized peptide ($I_{\text{unoxidized}}$) and the peptide with n oxidation events.

8. For a differential study, repeat steps 5-7 on the second condition.

NOTE: To confirm reproducibility, two biological replicates in addition to technical triplicates for each condition are recommended.

REPRESENTATIVE RESULTS:

The high-pressure plasma source coupled with real-time dosimetry allows better control of $\cdot\text{OH}$ yield to observe changes in higher-order protein structure more accurately. The addition of adenine allows for an effective real-time radical dosimeter. Upon oxidation, adenine loses UV absorbance at 265 nm (**Figure 2A**). The change in adenine absorbance is directly related to the concentration of radicals available for HRPD thus providing a means to effectively monitor

changes in radical concentration in the presence of radical scavengers like buffers, excipients, and ligands (**Figure 2B**).

Apomyoglobin (aMb) was modified in the presence of 100 mM H₂O₂ and 1 mM adenine at four increasing plasma voltages (**Figure 3**). Six peptides were detected with a linear increase in oxidation with the change of adenine UV 265 nm absorbance. Adenine UV absorbance is a surrogate for effective [•]OH concentration. The linear change in oxidation versus the change in radical concentration confirms the absence of artifactual change in the protein's higher-order structure during labeling. Furthermore, the extent of oxidation detected with the high-pressure plasma source was much higher than the laser-based method. This increase arises from the high-pressure plasma source's broad-spectrum UV emission spectrum (**Figure 4**). By producing a broad UV spectrum, the plasma source better overlaps the absorbance domain of H₂O₂ providing more efficient production of [•]OH through the photolysis of H₂O₂ (**Figure 5**). This is in direct comparison to the KrF Excimer laser and Nd:YAG laser, which are common sources that photolyze H₂O₂ in HRP studies^{21,27,28}. These lasers have a very narrow bandwidth at the lower end of H₂O₂'s photometric absorbance range. In comparison to the KrF excimer laser, the high pressure plasma source significantly increases the production of [•]OHs while concomitantly significantly decreasing the demand of required optical fluence (**Figure 6**).

FIGURE LEGENDS:

Figure 1: Components of the flash photolysis platform. (A) Assembled capillary tube, union tube, nut and ferrule. The tip of the "lower end" of the capillary extends barely beyond the union tube. **(B)** Knurled nut on the product collector to insert the end of the capillary. **(C)** Labeled six-port injection valve. **(D)** Syringe pump with the syringe.

Figure 2: Change in adenine absorbance used for dosimetry. (A) Upon oxidation, adenine decreases in UV absorbance at 265 nm. **(B)** Change in adenine absorbance at 265 nm at different flash voltages for two buffers. Buffer 2 contains a radical scavenger which will decrease the change in adenine absorbance compared to buffer 1. To overcome the radical scavenging effects from buffer 2, increased flash voltage is required.

Figure 3: Apomyoglobin dose response curve. (A) Average oxidation of six peptides vs adenine change in absorbance is shown. FPOP oxidation levels are shown in the dashed lines. **(B)** The six oxidized peptides are labeled on a crystal structure of myoglobin (PDB: 3RGK).

Figure 4: Spectral Irradiance of the plasma lamp. The high-pressure Xe gas lamp emits broad spectrum UV radiation from 200-300 nm along with visible light.

Figure 5: H₂O₂ photometric absorbance spectra. The black trace is the UV absorbance spectrum of H₂O₂²⁹. Highlighted in purple is the narrow wavelengths of the KrF excimer laser (248 nm) and Nd:YAG laser (265 nm) produces while the blue arrow represents the broad-spectrum plasma source which extends the range of useful H₂O₂ absorbance.

Figure 6: Efficacy of the plasma source to photolyze H₂O₂. The change in adenine absorbance

over increased plasma fluence demonstrates the concentration of $\cdot\text{OH}$ s generated. Highlighted in purple is a typical max change in absorbance produced from a KrF excimer laser²⁵.

DISCUSSION:

There are several critical steps to ensure proper labeling of proteins during any HRPf experiment. First, an appropriate flow rate and source flash rate are selected to make certain each bolus of the sample is irradiated once. This ensures that the protein is exposed to a single bolus of newly formed $\cdot\text{OH}$. Once a protein is oxidized, the higher order protein structure can be altered. To be confident the native protein structure is probed, each protein molecule must be modified in a single instant. Dosimetry can be used to test if the native protein structure is probed. As the concentration of hydroxyl radical increases the extent of oxidation linearly increases if the native protein structure is probed. However, if a large concentration of hydroxyl radicals causes the protein to partially unfold during labeling, then the extent of oxidation will significantly increase thus providing an effective means to ensure the native protein structure is probed.

It is also critical to ensure the reaction is quenched appropriately. This includes incorporating a proper radical scavenger mixed with the protein during the labeling process. For this method, adenine is not only used for dosimetry but also scavenges $\cdot\text{OH}$ limiting the lifetime of the radical as it labels the protein. By limiting the lifetime of the radical, confidence that the native protein structure is being modified is gained. Following the labeling process, it is important to collect the sample in a quench solution containing catalase and methionine amide which will break down excess H_2O_2 and scavenge $\cdot\text{OH}$. Limiting the protein's exposure to H_2O_2 ensures H_2O_2 induced oxidation is minimized.

It is also critical to appropriately select a protease for peptide digestion. Many enzymes cleave the protein at specific locations. For example, trypsin cleaves on the carboxyl side of arginine and lysine residues. This allows for simple data analysis, but if the protein of interest has a limited number of arginines and lysines, an alternative protease or a mixture of proteases might be required for acceptable peptide coverage during bottom-up proteomics. It is also advised to digest the modified protein before long term storage to avoid loss of oxidized protein due to aggregation and/or solubility issues.

Using HRPf to study changes in protein structure and protein interactions has been very successful^{11,30-33}, but there are added complications while labeling heme-binding proteins or glycoproteins. While both heme binding³⁴ and glycoproteins³⁵ have been successfully probed using HRPf, thorough troubleshooting in the concentration of H_2O_2 , radical scavenger, and the timeframe of H_2O_2 exposure must be balanced. Additionally, some buffers extensively quench $\cdot\text{OH}$ s. Therefore, it is vital to select an appropriate buffer and limit excipients like DTT and DMSO. Typical buffers used during an HRPf experiment include acetate, sodium phosphate, phosphate buffered saline, or low concentrations of Tris buffer. Interestingly, Tris scavenges some $\cdot\text{OH}$, but following oxidation, it increases in photometric absorbance at 265 nm and can be used in place of adenine for dosimetry³⁶. Although some buffers might decrease the effective concentration of $\cdot\text{OH}$, by incorporating an in-line radical dosimeter these challenges can be surmounted.

HRPF utilizes non-specific irreversible labeling by $^{\bullet}\text{OH}$ to probe solvent accessibility. The non-specific nature of the label provides increased overall coverage compared to other more targeted footprinting methods including N-ethylmaleimide³⁷ and glycine ethyl ester³⁸. Since the modification is irreversible, ample time is available during sample handling. This allows for complete protein digestion and a long LC gradient for improved MS/MS analysis. For HRP there are several methods to generate $^{\bullet}\text{OH}$. A popular method utilizes a laser to photolyze H_2O_2 into $^{\bullet}\text{OH}$. Laser based platforms have been very successful in probing native protein structure, but they require extensive safety precautions, rigorous maintenance, and a substantial amount of space.

With the use of a high-pressure plasma source described here, there is no need for harmful gases and no fear of stray UV radiation. The lifetime of each high-pressure plasma source is enough to label between 180-200 samples, and besides monitoring the dosimeter response, no additional maintenance is required. The plasma source is under high-pressure, so while handling the plasma source, wear safety glasses. The plasma source also photolyzes H_2O_2 more efficiently, allowing for increased protein modification. If insufficient labeling is observed, H_2O_2 concentration can be increased along with increasing the lamp energy for additional radical production. Additionally, with the incorporation of real-time dosimetry, one can increase the reproducibility of experiments. Altogether, with the use of a plasma source coupled with real-time dosimetry and software for automated FPOP data calculation provides an advantageous package to perform HRP experiments in a safe, easier to operate, and with improved reproducibility, as compared to standard laser-based methods.

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DISCLOSURES:

E.E.C., J.S.S., and S.R.W. disclose a significant financial interest in GenNext Technologies, Inc., an early stage company seeking to commercialize technologies for higher-order protein structure analysis. The representative data provided has been reviewed by S.K.M., who has no financial conflict of interest.

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549 [properties/default.aspx?pid=65&name=Ultraviolet-Absorption-Spectrum](http://www.h2o2.com/technical-library/physical-chemical-properties/radiation-properties/default.aspx?pid=65&name=Ultraviolet-Absorption-Spectrum)> (2016).

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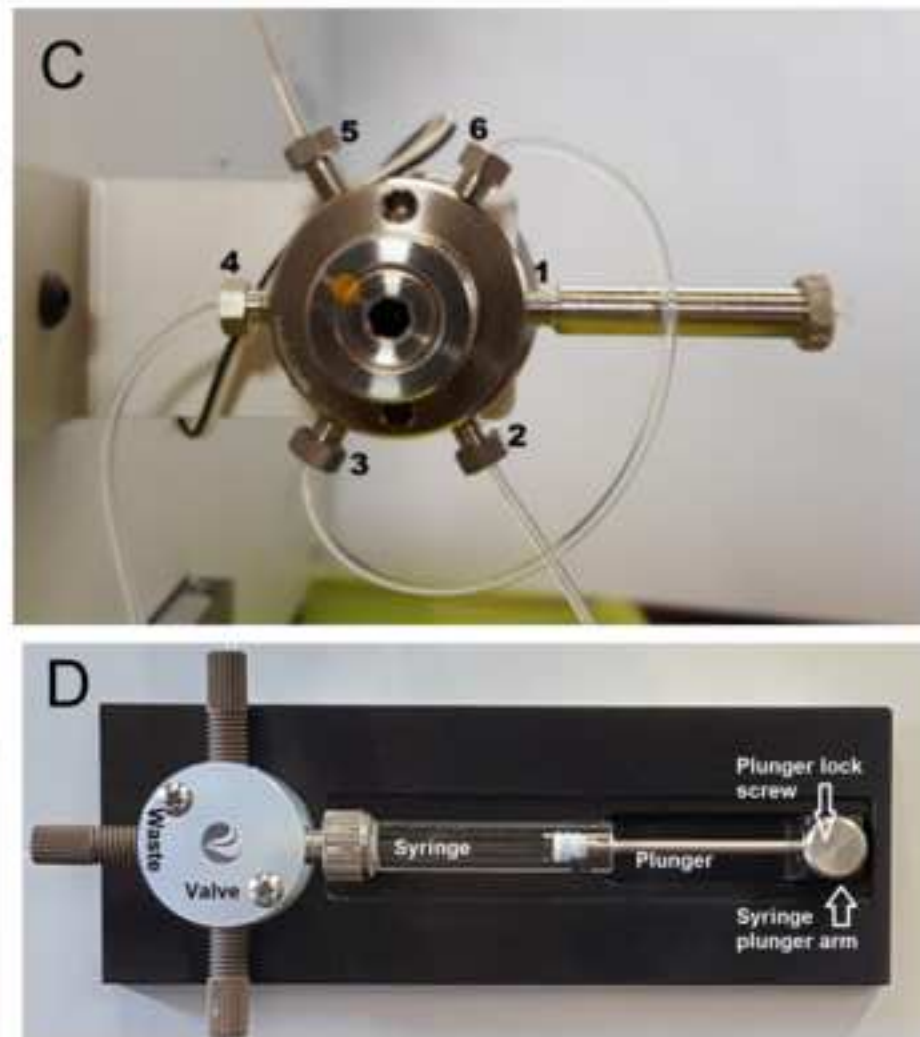
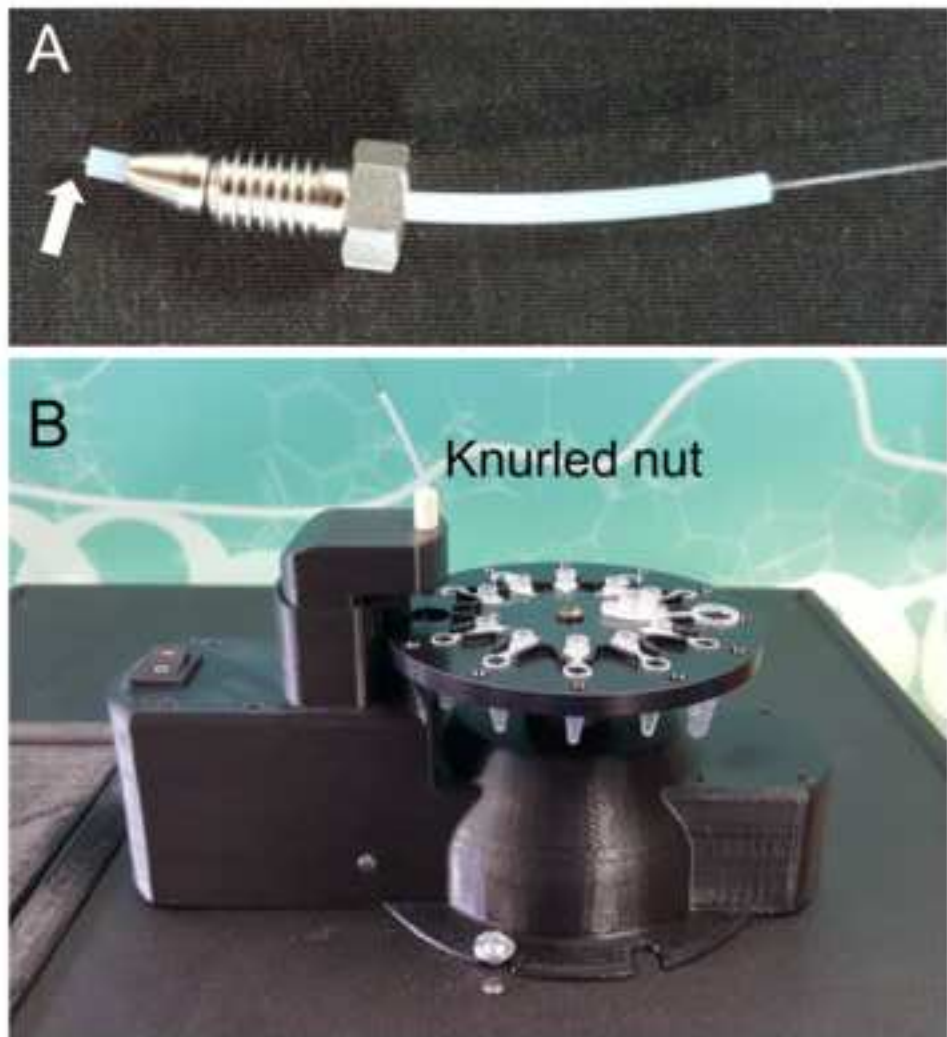
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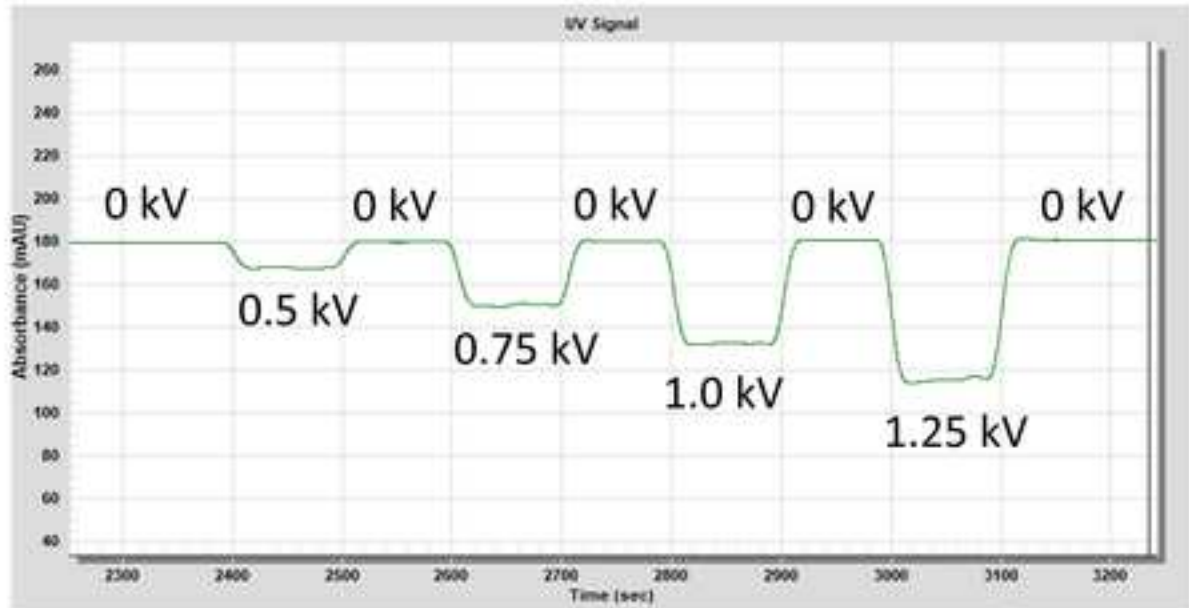
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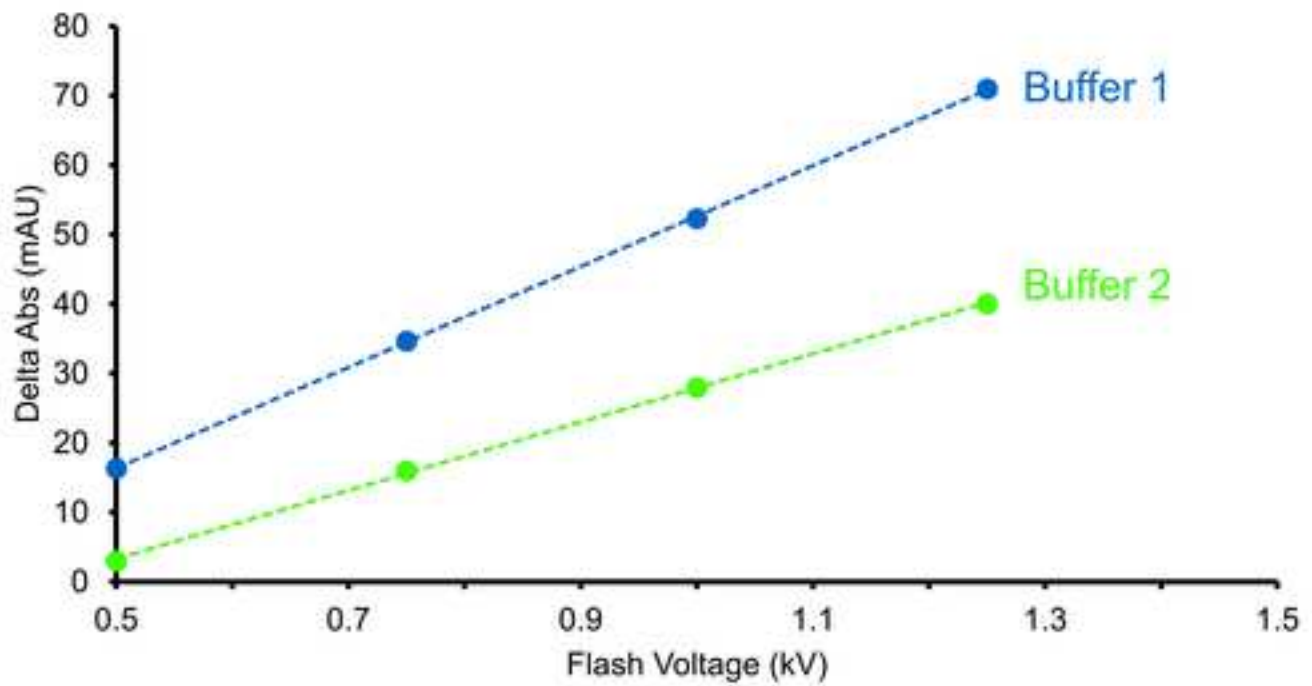
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A



B



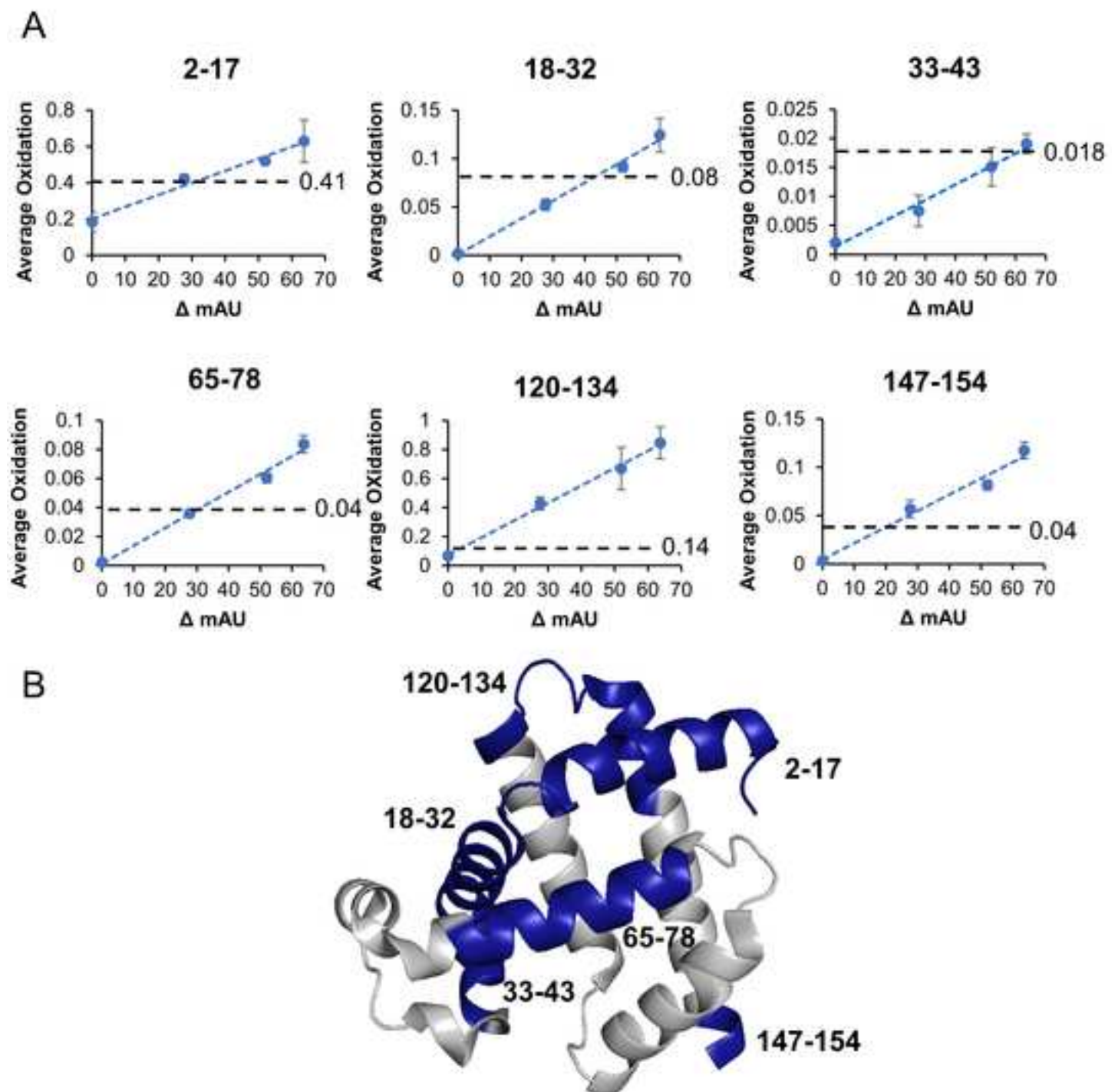


Figure 4

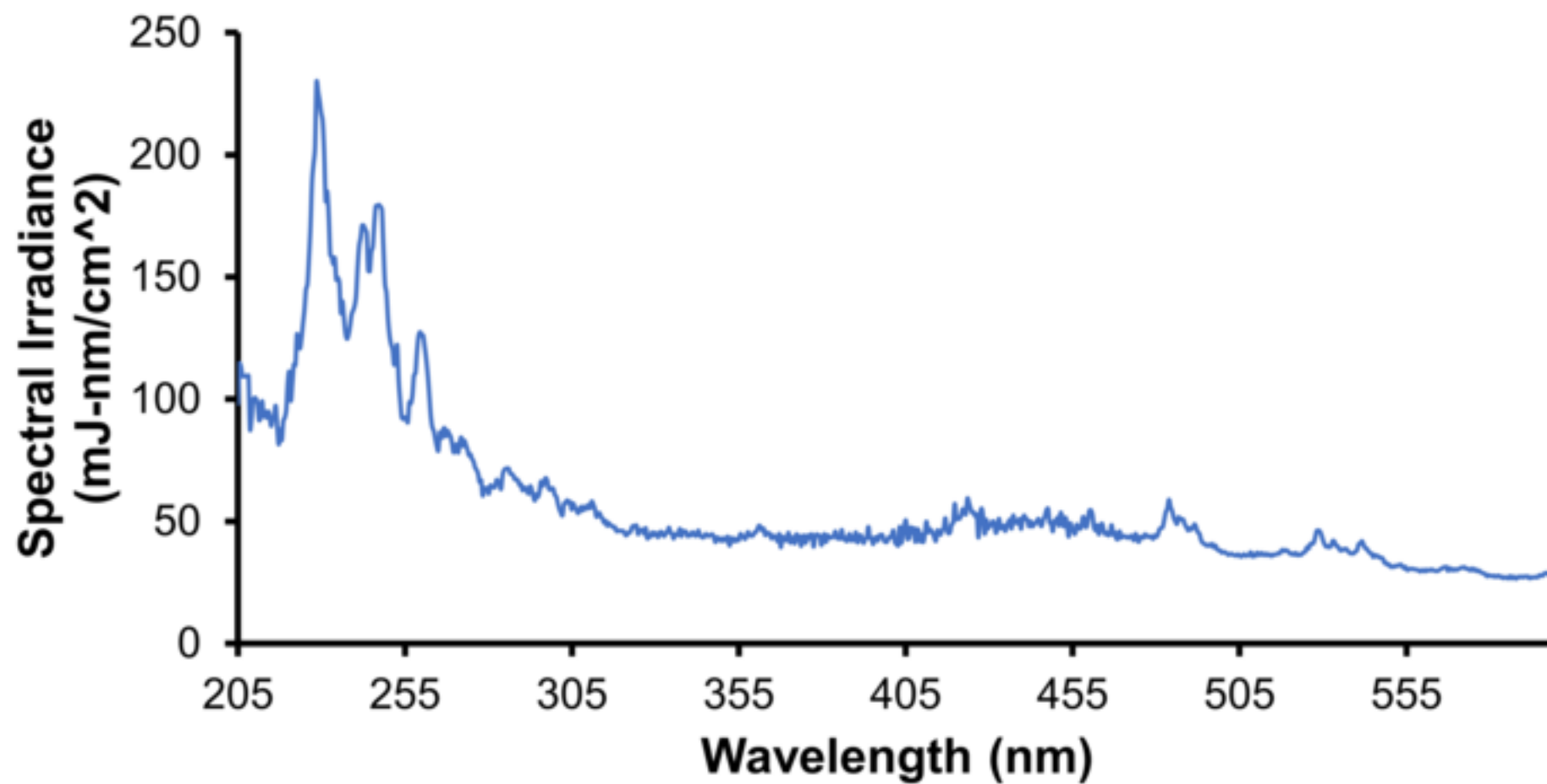
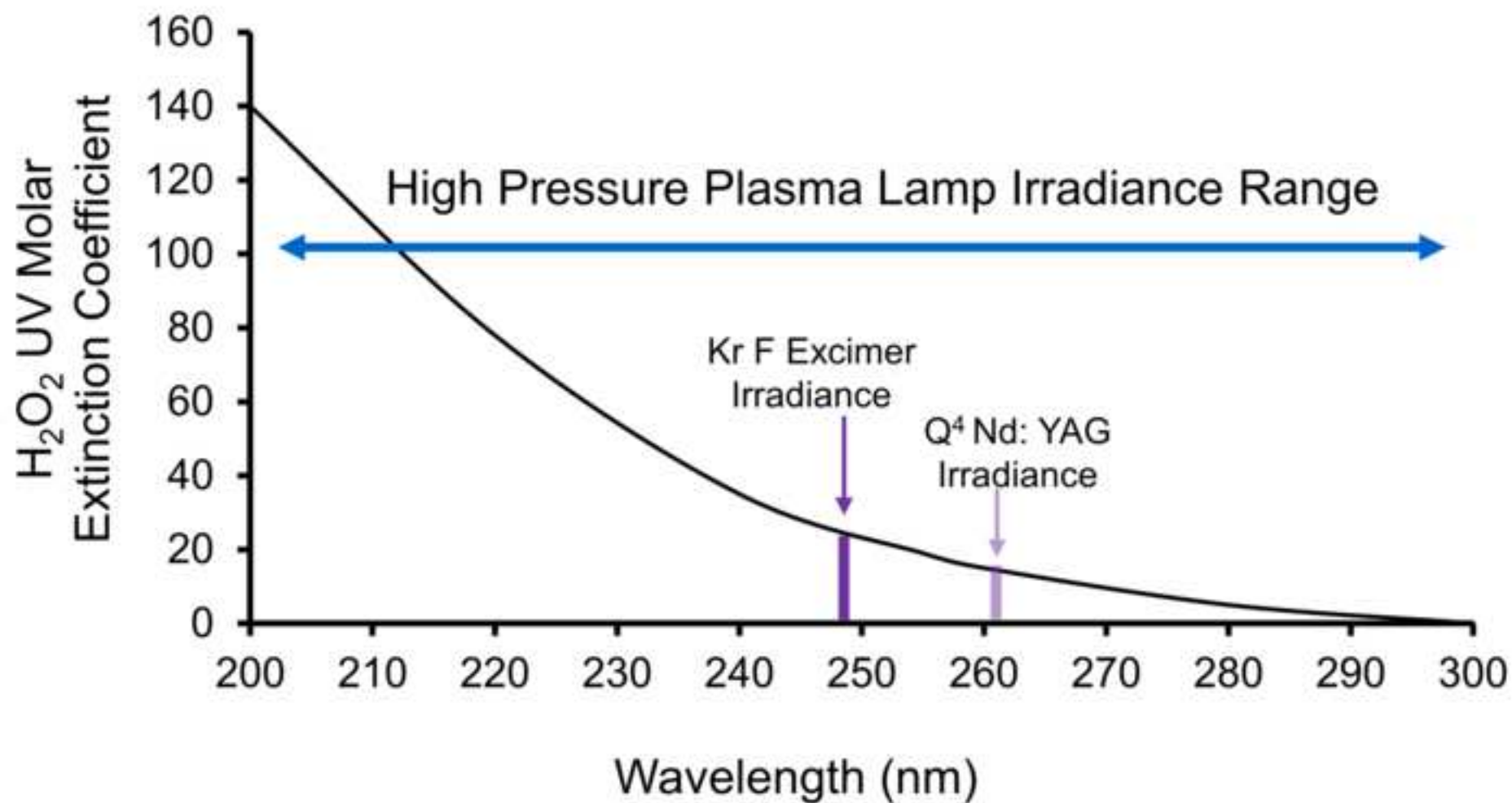
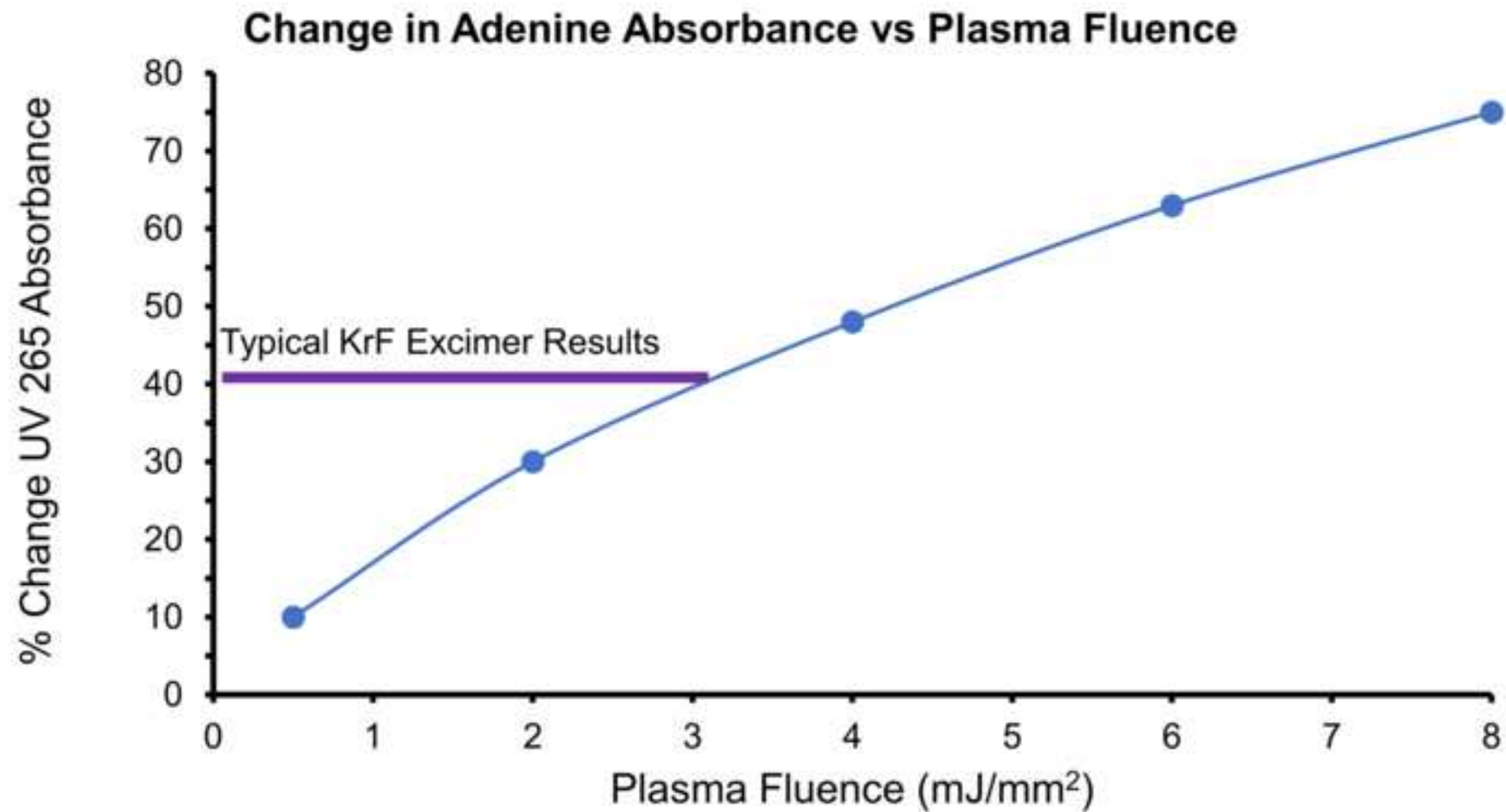


Figure 5





Name of Material/Equipment	Company	Catalog Number	Comments/Description
15 mL Conical Centrifuge Tubes	Fisher Scientific	14-959-53A	any brand is sufficient
50 µL SGE Gastight Syringes	Fisher Scientific	SG-00723	
Acclaim PepMap 100 C18 nanocolumn (0.75 mm X 150 mm, 2 µm)	Thermo Scientific		
Acetonitrile with 0.1% Formic Acid (v/v), LC/MS Grade	Fisher Scientific	LS120-500	
Apomyoglobin	Sigma-Aldrich		
Catalase	Sigma-Aldrich	C9322	
Centrifuge	Eppendorf	022625501	
Delicate Task Wipers	Fisher Scientific	06-666A	

Hydrogen Peroxide	Fisher Scientific	H325-100	any 30% hydrogen peroxide is sufficient
Methionine amide	Chem-Impex	03109	
Microcentrifuge	Thermo Scientific	75002436	
Orbitrap Fusion Lumos Tribrid Mass Spectrometer	Thermo Scientific	Orbitrap Fusion Lumos Tribrid Mass Spectrometer	other high resolution instruments (e.g. Q exactive Orbitrap or Orbitrap Fusion) can be used
Pierce Trypsin Protease, MS Grade	Thermo Scientific	90058	
Polymicro Cleaving Stone, 1" x 1" x 1/32"	Molex	1068680064	any capillary tubing cutter is sufficient
UPLC	Thermo Scientific		
Water with 0.1% Formic Acid (v/v), LC/MS Grade	Fisher Scientific	LS118-500	

Water, LC/MS Grade

Fisher Scientific

W6-4

August 24, 2020

Vineeta Bajaj, M.S., Ph.D., Senior Editor

Journal of Visualized Experiments

Dear Dr. Vineeta Bajaj:

The authors would like to thank you and the editorial team along with the reviewers for their specific and helpful comments regarding our manuscript # JoVE61861, entitled Laser-free Hydroxyl Radical Protein Footprinting to Perform Higher Order Structural Analysis of Proteins.

We have revised and modified the text according to the editor's and reviewer's suggestions. Please see below:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- The authors appreciate the editor's suggestion and have thoroughly proofread the manuscript.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

- The authors appreciate the editor's suggestion and have formatted the manuscript properly.

3. Please make the title concise to directly reflect the protocol being presented. Please remove colon from the title.

- The authors appreciate the editor's feedback and has changed the title.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This protocol presents..."

- The authors appreciate the editor's feedback and have rephrased the summary.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

- The authors appreciate the editor's feedback and have rephrased the abstract.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Teflon, etc.

- The authors appreciate the editors' feedback and have removed all commercial language.

7. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. Please remain neutral in tone throughout (e.g., please do not use words like "novel, simple, fast, easy")

- The authors appreciate the editor's feedback and will keep a neutral tone throughout the video.

8. Please ensure the Introduction include all of the following with citation:

- The authors appreciate the editor's feedback and have included the following with citations:

a) A clear statement of the overall goal of this method

- Here we describe the development and use of a new means to perform HRPF based upon a combination of high-speed/high-energy flash oxidation technology with integral, real-time radical dosimetry that addresses laser-based and irreproducibility barriers to broad-scale adoption.

b) The rationale behind the development and/or use of this technique

- Although ample information can be obtained using HRPF, broad adoption in biopharma has not been met. Two barriers for the limited HRPF adoption include: 1) the use of dangerous and expensive lasers that demand substantial safety precautions 8; and 2) the irreproducibility of HRPF caused by background scavenging of $\bullet\text{OH}$ that limit comparative studies

c) The advantages over alternative techniques with applicable references to previous studies

- Two barriers for the limited HRPF adoption include: 1) the use of dangerous and expensive lasers that demand substantial safety precautions 8; and 2) the irreproducibility of HRPF caused by background scavenging of $\bullet\text{OH}$ that limit comparative studies 9. To supplant laser use, a high-speed, high energy plasma flash photolysis unit was developed to safely perform FPOP in a facile manner. To improve on the irreproducibility of HRPF experiments, real-time radical dosimetry is implemented.

d) A description of the context of the technique in the wider body of literature

- Added additional references and short description of the context of the technique in a wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

- Added references for the type of information one can gain from this method.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described

in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

- The authors appreciate the editor’s feedback and ensured that all text in the protocol is written in the imperative.

10. Please ensure you answer the “how” question, i.e., how is the step performed? For this please include button clicks in the software, knob turns, command lines, mechanical actions, etc. If using long scripts, please include as supplementary file.

- The authors appreciate the editor’s feedback and ensured that all text in the protocol is complete with each button click ect.

11. 1.3: Where are the windows created?

- The authors appreciate the editor’s comment, the protocol describes the location of the windows as such: make the first photolysis window 90 mm away from the “lower end” and the second dosimeter window 225 mm away from the “lower end”

12. 2.1: How do you check this? What is being injected?

- The authors appreciate the editor’s comment, better description is included in the protocol.

13. 3.2: Please include Buffer volume and composition.

- The authors appreciate the editor’s comment, the buffer the authors are using is mentioned as a note.

14. 3.5.2: What is the sample being injected?

- The authors appreciate the editor’s comment, better description is included in the protocol.

15. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- The authors appreciate the editor’s comment, at most, 3 pages of protocol are highlighted for the video.

16. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted. Calculations cannot be filmed.

- The authors appreciate the editor's comment, all sub-steps are highlighted for the video production.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

- The authors appreciate the editor's comment, all figures are original works and have not been published elsewhere.

18. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

- The authors appreciate the editor's feedback and the discussion covers each section in detail.

19. Please do not abbreviate the journal titles in the reference section.

- The authors appreciate the editor's feedback and the journal titles are not abbreviated.

20. Please sort the materials table in alphabetical order.

- The authors appreciate the editor's feedback and the materials table is in alphabetical order.

Reviewer #1:

Major Concerns:

The example the authors provide with apomyoglobin does not seem to be relevant to the audience (biopharma) the authors are targeting. The authors discuss the importance of HOS for proper biotherapeutic function, but FPOP is not well suited to demonstrate this, and this messaging is misleading to the readership. There are other technologies (such as NMR) that are the preferred technologies for industry (and the health authorities) to ensure proper HOS. FPOP is best suited for elucidating structural changes in HOS, such as upon ligand binding and from protein-protein interactions. In fact, to this reviewer's knowledge, all of the examples of using FPOP published from industry have been on the latter use of this technology. Therefore, this reviewer suggests demonstrating

how to use this technology to elucidate structural changes rather than focusing on ensuring proper HOS of biotherapeutics. There is not enough information to date to indicate that FPOP can be used to demonstrate proper HOS. This messaging is NOT appropriate for the target readership.

- The authors appreciate the reviewers concern and suggestions. We re-worded the introduction as to not mislead the intention of this protocol. We also added several references relevant to biotherapeutics and relevant to the target readership.
- As our intent is to demonstrate the capabilities of laser-free FPOP system and radical dosimetry, we used a well-studied small protein, apo-myoglobin, that has been extensively used during the evolution of FPOP technology. Asking us to revise our manuscript and perform differential HOS studies of a biopharmaceutical goes beyond the intent and scope of our intended manuscript

Minor Concerns:

1) In the first paragraph of the discussion the authors describe the inherent challenge with ensuring the protein analyte is irradiated only once and there is sufficient quenching of the OH radicals. However, the authors do not provide detail on how a scientist can determine if this is happening for a novice user. I suggest the authors provide some context regarding this.

- The authors appreciate the reviews concern and suggestion. We have included additional information in the discussion how a user can test this using dosimetry.

2) It is apparent that the author have tailored this work to interest the biopharma community. However, the authors do not cite any work on FPOP that has been generated by the leaders of this technology from the industry sector. The credibility for the need of this technology in biopharma is therefore limited.

- The authors appreciate the reviewer's concern. We have added several relevant references.

Reviewer #2:

Minor Concerns:

1. Can the authors comment on possible concerns on the plasma light source that users may have, such as lifetime, maintenance, and any safety concerns.

- The authors appreciate the reviewers concern and suggestion to include additional information. In the discussion section, we included information for each concern.

2. This is a more general suggestion for the HRP methods, especially those targeting industrial applications. It is preferable to calculate protein concentration in mg/mL rather than uM, because the same molar amount of a larger protein will require a higher OH radical concentration for efficient labeling. For example, the labeling condition chosen for myoglobin (17k Da) likely will not suffice mapping of antibody (150k Da). The authors should also discuss strategies that could be taken when insufficient labeling is observed on other protein systems (especially larger proteins).

- The authors appreciate the reviewer's suggestions. We have included additional information on how to increase labeling if insufficient labeling occurs.

3. the X-axis of Fig3 is not clear in its meaning, please clarify.

- The authors appreciate the reviewer's suggestion. The x-axis has been changed to help clarify the meaning.

Sincerely,

A handwritten signature in purple ink, appearing to read 'Scot R Weinberger', is positioned above the typed name.

Scot R Weinberger
President, CEO
GenNext Technologies, Inc.