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

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as possible.

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 25
Number of Shots: 53

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Emily Chea:** Laser free hydroxyl radical protein footprinting makes identifying protein interaction sites and regions of conformational change much easier, accelerating research on protein aggregation, structure, and stability studies **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 7.2.*

1.2. **Emily Chea:** With in-line radical dosimetry, users can adjust the effective hydroxyl radical load in real time. Real time radical dosimetry saves experimental time and precious sample while improving labeling reproducibility **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.5.1.*

Protocol

2. Installing the Capillary Tube

2.1. To begin, cleave a 250-micrometer inner diameter silica capillary to 27 inches with a silica cleaving stone, checking the ends of the capillary for a clean and straight cut **[1]**. *Videographer: This step is important!*

2.1.1. Talent cleaving a silica capillary with cleaving stone.

2.2. Create two windows of roughly 15-millimeter length. From the lower end of the capillary burn away the polyimide coating 90 millimeters up to create the photolysis window **[1]** and 225 millimeters for the dosimeter window **[2]**. *Videographer: This step is difficult!*

2.2.1. Talent creating photolysis window at 90 mm.

2.2.2. Talent creating dosimeter window at 225 mm.

2.3. Insert the lower end of the capillary just beyond the conical end of a nut and ferrule **[1]**. Add the capillary to port 5, tightening just beyond finger tight with a wrench **[2]**.

2.3.1. Talent inserting the capillary to the union tube.

2.3.2. Talent screwing the nut and ferrule at port 5.

2.4. Remove the photolysis cell cap of the photolysis module by pulling it straight out **[1]**, then remove the magnetically mounted metal mask which will hold the capillary in place **[2]**.

2.4.1. Talent removing the photolysis cell cap.

2.4.2. Talent removing the metal mask for holding the capillary.

2.5. Open the dosimeter cell by pushing on the tab on the left and swing the dosimeter cell open to the right **[1]**. Remove the magnetically mounted clips that will hold the capillary in place **[2]**.

2.5.1. Talent opening the dosimeter cell.

2.5.2. Talent removing the mounted clips for holding the capillary.

2.6. Then, place the capillary into the grooved channel at the base of the photolysis cell **[1]** and center the capillary window with the photolysis cell window **[2]**. *Videographer: This step is important!*

2.6.1. Talent placing the capillary in the photolysis cell.

2.6.2. Talent centering the capillary window with the photolysis window.

2.7. Hold the capillary in position **[1]** and add the magnetic mask **[2]**. Place the photolysis cap back in position **[3]**.

2.7.1. Talent holding the capillary tube.

2.7.2. Talent adding the magnetic mask.

2.7.3. Talent placing the photolysis cap.

2.8. Place the capillary into the grooved channel at the base of the dosimeter cell **[1]** and center the second capillary window on the small aperture at the center of the dosimeter cell **[2]**. *Videographer: This step is important!*

2.8.1. Talent placing the capillary into the dosimeter cell.

2.8.2. Talent centering the second capillary window with dosimeter window.

2.9. While holding the capillary in position with one hand, place the two magnetic clips in position to hold the capillary in place **[1]**. Close the dosimeter cell until it clicks closed **[2]**. Finally, insert the capillary through the knurled nut atop the capillary lift of the product collector, extending the capillary to just above the bottom of the vial **[3]**. *Videographer: This step is important!*

2.9.1. Talent placing the magnetic clips.

2.9.2. Talent closing the dosimeter cell.

2.9.3. Talent inserting the capillary into the product collector.

3. Installing an Injection Loop

3.1. Cut the desired length of Teflon tubing using a cutter **[1]** and check the ends for a clean, straight cut **[2]**. Insert one end of the new injection loop through one of the nuts **[3]** and place a new ferrule onto the end of the tube **[4]**.

3.1.1. Talent cutting the Teflon tube.

3.1.2. Talent checking the ends of the cut tube.

3.1.3. Talent inserting the injection loop through one of the nuts.

3.1.4. Talent placing the ferrule onto the end of the tube.

3.2. Hold the nut and ferrule in place while inserting the tube in port 6 of the injection valve until it bottoms out **[1]**. Tighten the nut with a wrench **[2]**, then confirm that the nut and ferrule are securely in place **[3]**.

3.2.1. Talent inserting the tube into the port 6 of injection valve.

3.2.2. Talent tightening the nut using wrench.

3.2.3. Talent checks the proper position of nut and ferrule.

3.3. Once both ends have a nut and fixed ferrule, loosely screw one end to port 3 and the other end to port 6 **[1]**. Once in position, tighten both sides to finger tight, then a quarter turn past finger tight with a wrench **[2]**.

3.3.1. Talent screwing the ends of the injection loop.

3.3.2. Talent tightening one side with a wrench.

4. Initializing the Laser Free HRPF System

4.1. Turn on the laser free HRPF system by starting the **Fluidics module,** followed by the **Photolysis module, Dosimeter module, Product Collector,** and the **System Computer [1-TXT]**. Launch the system software **[2]**. *Videographer: Please film the computer screen for shots labeled SCREEN as backup.*

4.1.1. Talent turning each module. **TEXT: HRPF- Hydroxyl Radical Protein Footprinting.**

4.1.2. LAB MEDIA: 4.1.2.Screen.mkv. 00:04-00:18.

4.2. Fully submerge the tubing into 10 milliliters of buffer from the valve position on the syringe pump **[1]**. Direct the tubing from the waste position and the tubing from port 2 on the syringe port to an empty container to collect waste **[2]**.

4.2.1. Talent submerging the tubing.

4.2.2. Placing the tubes from waste and port 2 into the empty container.

4.3. Place 1.5-milliliter microcentrifuge tubes at the position marked H and 6 on the product collector Carousel **[1]**. Rinse out the injection loop five times by injecting 25 microliters of HPLC-grade water with the valve set to the load position **[2]**.

4.3.1. Talent placing the microcentrifuge tubes to the product collector carousel.

4.3.2. Talent rinsing the injection loop with water.

4.4. Manually turn the injection valve to flush the rest of the system **[1]**. Select **Process - Out** on the control software to begin flowing water **[2]** until a droplet forms **[3]**.

4.4.1. Talent turning the injection valve.

4.4.2. LAB MEDIA: 4.4.2.Screen.mkv. 00:02-00:09.

4.4.3. Talent flowing water through the capillary.

5. Modification of Protein to Detect Changes in Higher Order Structure

5.1. Make a solution containing 2 millimolar adenine and 10 micromolar protein **[1]**, then make a quench solution using 0.3 milligrams per milliliter catalase and 35 millimolar methionine amide **[2]**.

5.1.1. Talent making adenine and protein solution.

5.1.2. Talent making quench solution.

5.2. Aliquot 25 microliters of quench solution into a 200-microliter microtube **[1]**. Dilute hydrogen peroxide to 200 millimolar, keeping it on ice **[2]**.

5.2.1. Talent aliquoting quench solution in microtube.

5.2.2. Talent diluting hydrogen peroxide.

5.3. Start the flash voltage at 0 volts on the control software. This zero-volt control will determine any background oxidation on the protein of interest. Select **Start Data + AutoZero** followed by **Process-Out**, then **Ready [1]**. Finally turn the injection valve down to the load position **[2]**.

5.3.1. LAB MEDIA: 5.3.1.Screen.mkv. 00:02-00:13.

5.3.2. Talent turning down the injection valve.

5.4. Place the quench solution in position 1 on the product collector carousel **[1]**, then change the product vial to 1 on the system control software **[2]**.

5.4.1. Talent placing the quench solution in position 1.

5.4.2. LAB MEDIA: 5.4.2.Screen.mkv. 00:03-00:12.

5.5. Immediately before injection, mix 12.5 microliters of the adenine and protein mixture with 12.5 microliters of hydrogen peroxide **[1-TXT]**. Pipette the mixture up and down to mix **[2]**, then quickly spin it down **[3]**. Inject 25 microliters of this solution using the injection port within 10 seconds of mixing **[4]**.

5.5.1. Talent mixing the solutions. **TEXT: Final Concentrations: 1 mM Adenine, 5 µM protein, and 100 mM H_2O_2**

5.5.2. Talent pipetting to mix the solution.

5.5.3. Talent spinning the solution.

5.5.4. Talent injecting the solution using injection port.

5.6. Switch the injection valve to the inject position and wait while the sample is being processed **[1]**.

5.6.1. Talent switching the injection valve to inject position.

5.7. Turn up the flash voltage to 750 volts on the control software and repeat the labeling steps, then record the absorbance of each sample. First, click **Select**, then manually select the beginning and end of the peak absorbance. In the available space, write in a description of the sample. Repeat this for all acquired data **[1]**.

5.7.1. LAB MEDIA: 5.7.1.Screen.mkv. 00:02-00:54.

5.8. Copy and paste the data into a spreadsheet to calculate the average change in adenine absorbance for each voltage **[1]**.

5.8.1. LAB MEDIA: 5.8.1.Screen.mkv. 00:02-00:20.

6. Shutting Down the Laser Free HRPF System

6.1. After all samples have been collected, flush out the syringe port and sample loop by setting the injection valve down to the load position **[1]** and inject 25 microliters of HPLC water five times **[2]**.

6.1.1. Talent flushing out the syringe port and sample loop.

6.1.2. Talent injecting HPLC water.

6.2. Turn the injection valve up to the inject position to flush the rest of the system with HPLC water **[1]**. Once flushed, stop the flow **[2]**, exit the system control software and turn off the modules, starting with the product collector, dosimeter module, photolysis module, and finally the fluidics module **[3]**.

6.2.1. Talent flushing the system with HPLC water.

6.2.2. LAB MEDIA: 6.2.2.Screen.mkv. 00:02-00:21.

6.2.3. Talent turning off the modules.

Results

7. High Order Structural Analysis of Proteins Using Laser-free Hydroxyl Radical Protein Footprinting

7.1. Upon oxidation, adenine used for dosimetry decreases in UV absorbance at 265 nanometers **[1]**. Buffer 2 contains a radical scavenger, which decreases the change in adenine absorbance compared to buffer 1 **[2]**.

7.1.1. LAB MEDIA: Figure 2A.

7.1.2. LAB MEDIA: Figure 2B.

7.2. Apomyoglobin was modified in the presence of hydrogen peroxide and adenine at 4 increasing plasma lamp voltages **[1]**. Six peptides were detected with a linear increase in oxidation with respect to the change of adenine absorbance at 265 nanometers **[2]**. These six oxidized peptides are labeled on a crystal structure of myoglobin **[3]**.

7.2.1. LAB MEDIA: Figure 3A. *Video Editor: Emphasize the 4 data points in each of the 6 graphs.*

7.2.2. LAB MEDIA: Figure 3A.

7.2.3. LAB MEDIA: Figure 3B.

7.3. The extent of oxidation detected from the high-pressure plasma lamp is much higher than the laser-based method. This increase arises from the high-pressure plasma lamp's broad-spectrum UV emission spectrum **[1]**.

7.3.1. LAB MEDIA: Figure 4.

7.4. By producing a broad UV spectrum, the high-pressure plasma lamp better overlaps the absorbance domain of hydrogen peroxide, resulting in a more efficient production of hydroxyl radicals through the photolysis of hydrogen peroxide **[1]**, as compared to the KrF *(spell out)* Excimer laser and Nd:YAG *(spell out)* laser **[2]**.

7.4.1. LAB MEDIA: Figure 5.

7.4.2. LAB MEDIA: Figure 5. *Video editor emphasize the purple and blue arrow.*

7.5. The high-pressure plasma lamp significantly increases the production of hydroxyl radicals beyond what is typically observed using a KrF excimer laser **[1]**.

7.5.1. LAB MEDIA: Figure 6. *Video editor emphasize on point coinciding the purple line.*

Conclusion

8. Conclusion Interview Statements

8.1. **Emily Chea:** Using 100 mM peroxide can cause high levels of background oxidation. If the protein system is susceptible to peroxide induced oxidation, decrease the concentration to as low as 10 mM **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.2.*

8.2. **Emily Chea:** HRPF's irreversible label allows for downstream sample handling, including long protease digestion, adding tandem mass tags for multiplexing, and performing 2D chromatography, thus improving peptide detection and concordant structural information **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 7.2.2.*