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TITLE:

Platform Incubator with Movable XY stage: A New Platform for Implementing In-Cell Fast Photochemical Oxidation of Proteins

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KEYWORDS:

Fast Photochemical Oxidation of Proteins (FPOP), protein footprinting, hydroxyl radicals, proteome wide structural biology, proteomics, mass spectrometry.

SUMMARY:

A new static platform is used to characterize protein structure and interaction sites in the native cell environment utilizing a protein footprinting technique called in-cell fast photochemical oxidation of proteins (IC-FPOP).

ABSTRACT:

Fast Photochemical Oxidation of proteins (FPOP) coupled with mass spectrometry (MS) has become an invaluable tool in structural proteomics to interrogate protein interactions, structure, and protein conformational dynamics as a function of solvent accessibility. In recent years, the scope of FPOP, a hydroxyl radical protein foot printing (HRPF) technique, has been expanded to protein labeling in live cell cultures, providing the means to study protein interactions in the convoluted cellular environment. In-cell protein modifications can provide insight into ligand induced structural changes or conformational changes accompanying protein complex formation, all within the cellular context. Protein footprinting has been accomplished employing a customary flow-based system and a 248 nm KrF excimer laser to yield hydroxyl radicals via photolysis of hydrogen peroxide, requiring 20 minutes of analysis for one cell sample. To facilitate time-resolved FPOP experiments, the use of a new 6-well plate-based IC-FPOP platform was

pioneered. In the current system, a single laser pulse irradiates one entire well, which truncates the FPOP experimental time frame resulting in 20 seconds of analysis time, a 60-fold decrease. This greatly reduced analysis time making it possible to research cellular mechanisms such as biochemical signaling cascades, protein folding, and differential experiments (i.e., drug-free vs. drug bound) in a time-dependent manner. This new instrumentation, entitled Platform Incubator with Movable XY Stage (PIXY), allows the user to perform cell culture and IC-FPOP directly on the optical bench using a platform incubator with temperature, CO_2 and humidity control. The platform also includes a positioning stage, peristaltic pumps, and mirror optics for laser beam guidance. IC-FPOP conditions such as optics configuration, flow rates, transient transfections, and H_2O_2 concentration in PIXY have been optimized and peer-reviewed. Automation of all components of the system will reduce human manipulation and increase throughput.

INTRODUCTION:

Protein footprinting techniques can reveal profound information on the organization of proteins. These essential structural biology MS-based techniques are a component of the mass spectrometry toolbox. These methods probe protein higher order structure (HOS) and synergy via covalent labeling[1-4]. Fast photochemical oxidation of proteins (FPOP) employs hydroxyl radicals to oxidatively modify solvent accessible side chains of amino acids[5,6] (**Table 1**). The method utilizes an excimer laser at 248 nm for photolysis of hydrogen peroxide (H_2O_2) to generate hydroxyl radicals. Theoretically, 19 of the 20 amino acids, can be oxidatively modified with Gly being the lone exception. However, owing to the variant reactivity rates of amino acids with hydroxyl radicals, modification of only a subset of these has been observed experimentally. Still, the method does have the potential for analysis over the length of a protein sequence[5]. FPOP modifies proteins on the microsecond timescale, making it useful in studying weak interactions with fast off rates. Solvent accessibility changes upon ligand-binding or a change in protein conformation, thus, the power of the method lies in the comparison of the labeling pattern of a protein in multiple states (i.e., ligand-free compared to ligand-bound). As a result, FPOP has been successful in identifying protein-protein and protein-ligand interaction sites and regions of conformational change[7-10]. The FPOP method has been extended from the study of purified protein systems to in-cell analysis. In-cell FPOP (IC-FPOP) can oxidatively modify over a thousand proteins in cells to provide structural information across the proteome[11,12]. The conventional IC-FPOP platform utilizes a flow system to flow cells single file past the laser beam. The development of this system allowed individual cells to have equal exposure to laser irradiation. This led to 13-fold rise in the number of oxidatively labeled proteins[12]. However, a limitation of the flow system is the length of a single sample experiment consisting of a 10-minute irradiation interval during which modification takes place and an additional 10-minute wash cycle. The time constraints of IC-FPOP makes it unsuitable for studying short lived protein folding intermediates or changes that exist among interaction networks in biochemical signaling cascades. This temporal limitation inspired the design of a new IC-FPOP platform equipped with higher throughput.

To accurately measure protein higher order structure in the native cell environment, the new design allows cell culture to be accomplished directly at the laser platform, which enables IC-FPOP to be high throughput. This setup also allows minimized perturbations to the cellular

environment, in contrast to IC-FPOP using flow where adherent cells must be removed from the substrate. The new platform permits IC-FPOP to occur in a sterile incubation system using a CO_2 and temperature-controlled stage top chamber while utilizing configured mirror optics for laser beam guidance, a positioning system for XY motion, and peristaltic pumps for chemical exchange. The new platform for conducting IC-FPOP is entitled Platform Incubator with Movable XY Stage (PIXY) (**Figure 1**). In PIXY, IC-FPOP is carried out on human cells grown in six-well plates within the platform incubator chamber. For this configuration, the laser beam is reflected downward onto the plate using beam compatible mirrors as a positioning stage that holds the incubator is moved, in the XY-plane, so the laser beam is strategically aligned to only irradiate one well at a time. Validation studies show that IC-FPOP can be performed faster in PIXY than in the flow system and leads to increased amino acid modifications per protein. The development of this new IC-FPOP platform will expound upon the knowledge that can be gained from cellular experiments[13].

PROTOCOL:

1. Assembly of Platform Incubator with Movable XY stage

NOTE: The new platform includes the incubation system, the positioning stage and controllers, the peristaltic pumps, the 248 nm KrF excimer laser, and the optical mirrors assembled on an Imperial optical breadboard.

1.1. Assemble the incubation system: the temperature unit, carbon dioxide unit, humidifier, air pump, and touch monitoring system (**Figure 2A-E**).

NOTE: Detailed assembly instructions provided by the manufacturer. The incubation system must stabilize to 5% CO_2, 37 °C, and 85% humidity before growing cells within the incubator. These parameters may depend on the cell line.

1.2. Assemble the custom six-well plate incubator, nanopositioning drive stage, and XY drive stage. The former screws into the latter, respectively.

NOTE: Detailed assembly instructions provided by the manufacturer.

1.3. Connect the four peristaltic pumps in a “daisy chain” sequence via RS-232 cables, with an RS-232 to USB cable connected to the controlling computer. Connect 3.18 mm ID polymer tubing (e.g., Tygon) to every channel on each pump channel roller.

NOTE: Each pump has four rollers. Direction and flow rate of rollers are manually manipulated.

1.4. Insert 1/16" x 1/8” connectors at the end of each 3.18 mm ID polymer tube. Insert the 1/16’’ end of the connector into 1.59 ID polymer tubing, then insert the polymer tubing into the incubator via custom ports. Place the other end of the tube in the solution that will be infused during IC-FPOP experimentation.

NOTE: For the perfusion lines, the incubator has 36 custom ports for tubing lines all around the periphery to accommodate all the reagents used.

1.5. Screw one 50 mm, 248 nm, 45° excimer laser line mirror within a kinematic mirror mount for Ø2" optics into the breadboard 10-11 grid points from the 248 nm laser aperture. Place the second mirror at a 90° angle to the first on the other side of the incubator. Angle the second mirror downward at approximately 45° for laser beam guidance to the incubator (**Figure 2F-J**).

2. Synchronization and initial automation of system via integration software

2.1. Install the latest version of the integration software needed to control the drivers and pumps.

2.2. Referring to the pump manual, rename the pumps starting with '5' and increasing in value. The commands can be sent to the pump system using the **Manual Control** sub-program in the integration software.

NOTE: Setting a pump to Channel Mode automatically changes the pump naming convention into the set, 1, 2, 3 and 4, corresponding to the four pump channels.

2.3. Open the integration software program for the automation of the platform incubator.

2.4. Choose the appropriate USB comport device name (e.g., COM4) corresponding to the pump system USB cable from the connection dropdown menu labelled **Comport for pumps**.

2.5. Click the **Script Builder** button to edit/create a script for the automated platform incubator platform. Click **Save Script** to save the sequence as a text file (**Figure 3A**).

NOTE: The script builder has a user interface for defining the script with definitions of pump number, direction, rate, volume, tubing diameter, channel mode, and timed delay.

2.6. If channel mode is desired in the making of the script, a step in the script must be dedicated to changing the pump in and out of channel mode, and the following steps corresponding to the pump channels must be labelled as pumps 1-4.

NOTE: This ensures that no two pumps can simultaneously be in channel mode during a platform incubator script and that each pump is switched back to legacy mode after the commands have been sent to the needed channels.

2.7. Click the **Read Script** button and choose the appropriate script file desired for the platform incubator operation.

2.8. Switch the **Run Sequence** button to the ON position (green) to run the script and then click the **START** button (**Figure 3B**)**.**

3. Grow cells in the platform incubator

NOTE: Cells must be placed in the platform incubator under sterile conditions in a cell culture hood the day before experimentation.

3.1. Unscrew the platform incubator from the nanopositioning stage and disconnect the temperature, gas, and humidifier lines. After spraying the incubator with 70% ethanol, place it in a cell culture hood.

3.2. Grow cells in a T-175 to about 80-90% confluency prior.

NOTE: The term confluency is used as a measure of the number/coverage of the cells in a cell culture dish or a flask.

3.3. Remove media and rinse with buffer.

3.4. Detach cells using trypsin-EDTA using manufacturer's protocol.

3.5. Resuspend in 8 mL of media and count the cells.

3.6. Seed fibronectin/collagen[14] coated six-well plates with approximately 90-95 μL of resuspended cells in each well. This volume will be appropriate to achieve 80-90% confluency (~1.2 million cells) in each well on the next day.

NOTE: Six-well plates must be coated with collagen prior to seeding. The reagents used during IC-FPOP are infused with fast flow rates. Coated plates ensure cells are not prematurely detached due to infusion of reagents.

3.7. Place the seeded plate into the platform incubator and cover with the quartz lid.

NOTE: During design and development, a glass incubator lid was changed to quartz, so it is compatible with the ultraviolet laser light.

3.8. Replace and secure the platform incubator back on the nanopositioning drive stage. Reconnect the temperature, gas, and humidifier lines.

3.9. Let cells grow to confluency overnight.

NOTE: The following cell culture steps are optional and are intended for experiments in which human cells are transiently transfected. These steps assess transfection efficiency under cell culture conditions.

3.10. Transfect the plasmid containing the gene for the chimeric protein GCaMP2 into HEK 293 cells using a commercial cationic-lipid transfection kit.

3.11. Perform transient transfections of GCaMP2 in HEK293T cells in two six-well plates.

3.12. Incubate one six-well plate in the platform incubator, and the second six-well plate in a standard CO_2 incubator.

3.13. Compare transfection efficiency within each plate by fluorescence imaging using a fluorescence microscope.

4. Make quench buffer and H_2O_2

4.1. Make 100 mL of quench buffer containing 125 mM N-tert-Butyl-α-phenylnitrone (PBN) and 125 mM N, N'-Dimethylthiourea (DMTU).

NOTE: DMTU and PBN are free radical scavengers and are cell permeable.

4.2. Dilute H_2O_2 to 200 mM. Each sample requires 6 mL of H_2O_2 to fully immerse the layer of cells at the bottom of each well.

NOTE: The quench buffer is made the day before and stored at 4 °C overnight, protected from light. Dilute the H_2O_2 on the day of the experiment.

5. Set up the platform incubator for IC-FPOP

NOTE: The platform incubator must be assembled under sterile conditions. Assemble the incubator in a sterile cell culture hood.

5.1. Unscrew the platform incubator from the positioning stage and disconnect the temperature, gas, and humidifier lines.

5.2. After spraying the incubator with 70% ethanol, place it in the cell culture hood.

5.3. Remove the six-well plate from the platform incubator, secure the plate's original lid, and confirm cell confluency using a microscope.

5.4. After cell confluency has been confirmed, replace the six- well plate with confluent cells back in the platform incubator inside the cell culture hood.

5.5. Insert three precut (15 cm) 1.59 ID polymer tubes in each well via the embedded ports. Flush the tubes to the well walls and hold with custom 3D printed rings.

NOTE: Six 33 mm PLA filament 3D printed rings were custom designed to secure the tubing to the walls of the plate without disturbing the cells or getting in the way of the laser pulse.

5.6. Cover the platform incubator with its quartz lid. Replace and secure the stage top incubator on the positioning system. Reconnect the temperature, gas, and humidifier lines.

5.7. Connect 1.59 ID polymer tubing to 1/16" end of the 1/16x1/8" connectors.

6. Performing IC-FPOP in the platform incubator

6.1. Prepare an integration software script for pump withdrawal. Use one peristaltic pump (Pump 8) to completely remove cell media from all six wells.

6.2. Prime solvents in each channel of the other three peristaltic pumps (Pumps 5-7). Infuse H_2O_2 and quench buffer in their respective alternating tubes until the reagents reach the incubator ports.

6.3. Confirm that the laser beam has been angled correctly by the mirrors and reaches the incubator uninhibited.

NOTE: Laser safety goggles must be worn whenever the laser is in use. Do not prematurely irradiate the cells during the angling/alignment process. Use cardboard to completely cover the quartz lid when aligning the beam. Also, use a printed outline on white paper of a six-well plate to further confirm the laser beam is hitting the center of each well. Use the **Continuous** pulse setting at the lowest frequency and energy for alignment.

6.4. Check laser energy using an external sensor. Use one single laser pulse of 160 mJ at 50 Hz and 27 kV.

NOTE: A timer is needed for the following steps.

6.5. Prepare integration software script for pump infusion after confirming beam alignment.

6.6. Begin the timer and press the **Start** button on the integration software pump script at the same time.

6.7. Infuse 200 mM H_2O_2 at 35 mL/min into the first well (6-10 second mark on timer).

NOTE: There is a five second delay before a pump begins to infuse. It also takes the laser seven seconds before the pulse is triggered. The laser pulse must come immediately after H_2O_2 infusion.

6.8. Press the **Start** button on the laser software at the 5 second mark to trigger the pulse at the 11 second mark on the timer.

6.9. Infuse 125 mM quench solution at 35 mL/min into the first well immediately after the laser pulse. Manually move the positioning stage to align the next well with the laser beam.

6.10. Repeat the above steps 6.5-6.9 until each well has been processed.

NOTE: IC-POP is performed in technical triplicate of three laser and three non-laser samples. One processed six-well plate serves as one biological replicate.

6.11. In a cell culture hood, use a cell scraper to transfer the cells from each well into individual 15 mL conical tubes. Centrifuge cells at 1,200 x g for 5 minutes.

6.12. Discard the supernatant and resuspend cells in 100 µL of RIPA lysis buffer.

6.13. Transfer cells to individual 1.2 mL polypropylene tubes.

6.14. Flash freeze all samples in liquid nitrogen and place in a -80 °C freezer until use.

NOTE: The protocol can be paused here.

7. Protein extraction, purification, and proteolysis

7.1. Thaw the samples and heat at 95 °C in a heat block for 5 minutes.

7.2. After heating, cool on ice for 5 minutes.

7.3. Add 25 units of nuclease to the cell lysate to degrade single-stranded, double-stranded, linear and circular DNA and RNA and incubate at room temperate for 15 minutes.

7.4. Centrifuge samples at 16,000 x g for 10 minutes at 4 °C.

7.5. Collect the supernatant and transfer to a clean polypropylene tube.

7.6. Check the protein concentration using a colorimetric protein assay.

7.7. Transfer 100 µg of sample to a clean polypropylene tube and bring to 100 µL with cell lysis buffer.

7.8. Reduce samples with 10 mM dithiothreitol (DTT) at 50 °C for 45 minutes.

7.9. Cool samples at room temperature for 10 minutes.

7.10. Alkylate with 50 mM iodoacetamide (IAA) at room temperature for 20 minutes.

NOTE: Protect IAA from light

7.11. Add 460 µL of pre-chilled (-20 °C) acetone. Vortex samples and place at -20 °C overnight.

NOTE: The protocol can be paused here.

7.12. Next day, centrifuge samples at 16,000 x g for 10 minutes at 4 °C.

7.13. Remove acetone without disrupting the protein pellet.

7.14. Add 50 µL of 90% pre-chilled (-20 °C) acetone. Vortex samples to mix and centrifuge at 16,000 x g for an additional 5 minutes at 4 °C.

7.15. Remove acetone and let samples dry for 2-3 minutes.

7.16. Resuspend protein pellet with 10 mM Tris buffer pH 8.

7.17. Resuspend MS grade trypsin (20 µg stock) in 40 µL of 10 mM Tris buffer pH 8 and add 2.5 µg of trypsin to each sample.

7.18. Incubate samples at 37 °C overnight.

NOTE: The protocol can be paused here.

7.19. Assess peptide concentration using a colorimetric peptide assay.

7.20. Quench the samples with 5% formic acid.

7.21. Transfer 10 µg of sample to a clean polypropylene tube.

7.22. Dry the sample using a vacuum centrifuge and resuspend with 20 µL of MS grade 0.1% formic acid (FA) in water.

7.23. Transfer each sample to clean autosampler vials with pre-slit caps.

8. High performance Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

8.1. To localize FPOP modifications, analyze the digested cell lysate using LC-MS/MS analysis.

8.2. Use mobile phases of 0.1% FA in water (A) and 0.1% FA in acetonitrile (ACN) (B).

8.3. Load 0.5 µg of sample onto a 180 µm x 20 mm C18 (5 µm and 100 Å) trapping column and wash the column with 99% (A) and 1% (B) for 15 minutes.

8.4. Using a 75 µm x 30 cm C18 (5 µm and 125 Å) analytical column, elute and separate

digested peptides with a flow rate of 0.300 µL/min for 120 minutes.

8.5. Run LC gradient as follows: 0−1 min, 3% solvent B; 2−100 min, 10−45% B; 100−110 min, 45-100% B; 110−115 min, 100% B.

8.6. Recondition the column at 3% (B) from 115-116 minutes and hold at 3% (B) from 116-120 minutes.

8.7. Analyze eluted peptides in positive ion mode with nano electrospray ionization.

8.8. Acquire MS1 spectra over a m/z scan range of 375-1500 at a resolution of 60,000.

8.9. Set the automatic gain control (AGC) target to $5.0e^5$ with a maximum injection time of 50 ms and $5.0e^4$ intensity threshold.

8.10. Isolate precursor ions with charge states 2-6 via data dependent acquisition (DDA) with an isolation window of 1.2 m/z and a cycle time of 4 seconds.

8.11. Subject MS2 ions to high-energy collisional dissociation (HCD) (32% normalized energy).

8.12. Exclude peptides after 1 MS/MS acquisition for 60 seconds.

8.13. Set MS/MS resolution to 15,000 with an AGC target of $5.0e^4$ and a maximum injection time of 35 ms.

9. Proteome discoverer/data processing

9.1. Search tandem raw data files on available bottom-up proteomics analysis software against a relevant Homo sapiens protein database and digest enzyme.

9.2. Set the protein analysis search parameters.

9.3. Set fragment tolerance to 0.02 Da and parent ion tolerance to 10 ppm.

9.4. Set enzyme specificity to trypsin and allow for one missed cleavage.

9.5. Set mass range to 375-1500 m/z.

9.6. Establish peptide confidence at 95 % (medium) and residue confidence at 99 % (high).

9.7. Accept proteins if at least two distinct peptides are identified with the 5% discovery rate (FDR) filter.

9.8. Set carbamidomethylation as a static modification and all known hydroxyl radical side-

chain modifications[15, 16] as dynamic modifications.

9.9. Once files are finished searching, export sequence, modification locations, protein accession, spectrum file, precursor abundance, and retention time information to an electronic database.

9.10. Calculate the extent of modification per peptide or residue from this equation: $\frac{\Sigma EIC\ area\ modified}{\Sigma EIC\ area}$

NOTE: EIC area modified is the extracted ion chromatographic area (EIC) of the peptide or residue with an oxidative modification, and EIC area is the total area of the same peptide or residue with and without the oxidative modification. Over time, protein in the presence of hydrogen peroxide will oxidize, resulting in background oxidations. To calculate the extent of modification, the area of the modified peptide is divided by the total area. A non-irradiated control sample accounts for background oxidation. The background oxidation from the control sample is subtracted out from the laser treated sample to identify an FPOP modification.

REPRESENTATIVE RESULTS:

To confirm the platform incubator conditions are sufficient for cell culture at the laser platform, GCaMP2 was transiently transfected into HEK293T and transfection efficiency for both plates was assessed via fluorescence imaging (**Figure 4A**). GCaMP2 is a calcium sensing fluorescent protein used as a genetically encoded intracellular calcium indicator. It is a fusion of green fluorescent protein (GFP) and the calcium-binding protein, calmodulin. A luciferase assay was performed using transfected prl-TK in HEK 293 cells to quantitate transfection efficiency (**Figure 4B**). Results support the platform incubator exceeded the performance of the standard incubator, with a 1.13-fold increase in transfection efficiency, providing a quantitative benchmark for optimal cell culture environment.

FPOP modifications in HEK293T cells labeled in the flow system were compared to those labeled in the platform incubator and showed that the platform incubator outperforms the flow system both in the number of proteins modified (**Figure 5**) and the total FPOP coverage in those proteins. The number of FPOP modified proteins acquired in the platform incubator was approximately 1051, 2.2- fold more than those acquired in a typical experiment. Modifications were combined between two biological replicates for each experiment. Furthermore, PIXY provides higher throughput.

To demonstrate the advantage of higher modification coverage across a protein, IC-FPOP modifications were localized on the peptide level and the extent of modification was quantified to distinguish differences in outcomes between the systems for actin, a 375 amino acid protein. In the flow system, two modified peptides were detected, providing limited structural information (**Figure 6A**). However, five modified peptides spanning the actin sequence were detected in the platform incubator. Tandem mass spectra indicate that residue Pro322 was both modified and detected in each experiment (**Figure 6B**). The five peptides modified in the platform

incubator samples contained twelve modified residues, while only four residues were modified with the flow system (**Figure 6C**). The increase in oxidation coverage provides more structural information across the protein.

Espino et al. demonstrated the capacity of FPOP to be performed in vivo (IV-FPOP) within *C. elegans*, a worm model for human disease states[17]. While IV-FPOP is also performed via a flow system, the PIXY system was tested for compatibility with the worms. Approximately 10,000 worms were incubated in each well in the platform incubator at 20 °C. LC–MS/MS analysis revealed that 792 proteins were modified by IV-FPOP in the platform incubator compared to the 545 proteins modified with the flow system (**Figure 7**). These results demonstrate that in addition to 2D cell culture, this new methodology is also compatible with the study of other biological systems such as *C. elegans*.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic of PIXY System. System components: (**A**) stage-top incubator, (**B**) positioning system , (**C**) peristaltic pumps, and (**D**) perfusion lines. Cell culture media is removed from each well via pumps before H_2O_2 and quench solutions are infused at calculated timepoints laser path for irradiation showcased in white. Reprinted with permission from Johnson, D. T., Punshon-Smith, B., Espino, J. A., Gershenson, A., Jones, L. M., Implementing In-Cell Fast Photochemical Oxidation of Proteins in a Platform Incubator with a Movable XY Stage. Analytical Chemistry, 92(2), 1691–1696 2019. Copyright 2020 American Chemical Society.

Figure 2. Fully Assembled PIXY system. (**A**) Touch monitoring system, (**B**) carbon dioxide unit, (**C**) temperature unit, (**D**) air pump, (**E**) humidifier, (**F**) optical mirrors, (**G**) platform incubator, (**H**) positioning stage, (**I**) 248nm KrF excimer laser, and (**J**) peristaltic pumps.

Figure 3. Automation of Peristaltic Pumps. (**A**) Example command script in LABVIEW. Command options include volume, flow rate, pauses, flow direction. Speed, stage distance, and location are currently being automated. (**B**) Script reader in LABVIEW. Here, command scripts are uploaded then Run Sequence and START are pressed to initiate pumps.

Figure 4. HEK cell transfection efficiency. (**A**) Mean fluorescent intensity of GCaMP2 transfection comparison between standard incubator (Control) and stage-top incubator (PIXY). Dots and squares represent each point in a well where a measure was taken. (**B**) Transfection efficiency quantitated and validated with a different vector plasmid, pRL-TK. P-value< 0.005. Reprinted with permission from Johnson, D. T., Punshon-Smith, B., Espino, J. A., Gershenson, A., Jones, L. M., Implementing In-Cell Fast Photochemical Oxidation of Proteins in a Platform Incubator with a Movable XY Stage. Analytical Chemistry, 92(2), 1691–1696 2019. Copyright 2020 American Chemical Society.

Figure 5. Comparison of proteins modified in the single cell flow system and PIXY. Venn diagram of proteins modified using in the flow system (purple) and in PIXY (green). Reprinted with permission from Johnson, D. T., Punshon-Smith, B., Espino, J. A., Gershenson, A., Jones, L. M.,

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Figure 6. Localization of IC-FPOP modifications. Comparison of IC-FPOP modifications between systems. (**A**) Bar graph of oxidatively modified peptides within actin from the flow system (purple) vs platform incubator (green). (**B**) Tandem MS spectra of actin (peptide 316-326) with modified proline in both systems and unmodified actin peptide (C) FPOP modified residues of actin (PDB: 6ZXJ, chain A 11 modified residues in platform incubator (green), 3 modified residues in the flow system (purple), 1 overlapping modified residue (yellow). Reprinted with permission from Johnson, D. T., Punshon-Smith, B., Espino, J. A., Gershenson, A., Jones, L. M., Implementing In-Cell Fast Photochemical Oxidation of Proteins in a Platform Incubator with a Movable XY Stage. Analytical Chemistry, 92(2), 1691–1696 2019. Copyright 2020 American Chemical Society.

Figure 7. Comparison of FPOP modified proteins in *C. elegans* by flow vs PIXY. There is a 1.5-fold increase in oxidatively modified proteins using PIXY when compared to the flow system. Reprinted with permission from Johnson, D. T., Punshon-Smith, B., Espino, J. A., Gershenson, A., Jones, L. M., Implementing In-Cell Fast Photochemical Oxidation of Proteins in a Platform Incubator with a Movable XY Stage. Analytical Chemistry, 92(2), 1691–1696 2019. Copyright 2020 American Chemical Society.

Table 1. Workflow modification distribution and mass shifts (**Da**)**.**

DISCUSSION:

Proteins perform much of the work in living cells. Given this importance, protein function and higher order structure (HOS) in the cellular environment is needed to deepen the understanding of the intricacies in larger complexes and enzymatic reactions in cells as opposed to purified systems. To do this, a hydroxyl radical protein foot printing (HRFP) method was adopted entitled In-Cell Fast Photochemical Oxidation of Proteins (IC-FPOP). Most FPOP studies have been done in vitro in relatively pure protein systems, which markedly contrasts with the crowded molecular environment which affects binding interactions and protein conformational dynamics. As a result, there is a chasm between the findings from in vitro experiments[18] and those that would be obtained in an actual cellular environment. To bridge the gap between the idealized conditions of an in vitro FPOP experiment and the complex nature of the cell, a new automated six-well plate-based in cell-FPOP platform has been developed. This novel FPOP technology is capable of identifying and characterizing these molecular species and tracing their dynamic molecular interactions in both healthy and diseased states. This new platform is called Platform Incubator with Movable XY stage (PIXY).

FPOP has been successfully used to characterize the structural information within the proteome. However, every biological technique has certain limitations that require further improvement. Specific reagents are required during laser photolysis and to efficiently quench unreacted hydroxyl radicals. Separation of digested peptides can require large amounts of time to maximize structural information.This wealth of information can also require extensive quantitation during

post-MS data analysis[1]. The platform incubator, including the peripheral machinery needed for cell culture and IC-FPOP at the laser platform, comes with a large cost that may not be feasible for some labs. As progress continues to be made, robust software and analysis tools should advance the technique further; some of which is showcased in this study. Current studies in this platform incubator have been performed on HEK293T cells and in *C. elegans*. The IC-FPOP method has been shown to be compatible with a wide variety of cell lines including Chinese hamster ovary (CHO), Vero, MCF-7, and MCF10-A cells[19]. Since the general IC-FPOP method is translatable to this static platform, these cell lines should be amenable for study using PIXY as well.

IC-FPOP utilizes H_2O_2 to oxidatively modify solvent accessible side chains of amino acids, to then further discern protein interactions, structure, and metabolic effects within viable cells which is significant in providing biological context. It is essential before an IC-FPOP experiment to confirm that the cells are viable after H_2O_2 addition. Cell viability studies demonstrated that the cells were viable in the presence of H_2O_2 concentrations up to 200 mM [13]. It is also important to make sure H_2O_2 is infused at a final concentration of 200 mM directly on cells after media is removed. Failure to completely remove cell culture media will cause varying concentrations of H_2O_2. Compared to standard conditions, increasing the incubation time to 10 seconds along with increasing the H_2O_2 concentration led to a higher number of proteins modified by IC-FPOP in the platform incubator. It is imperative to prime peristaltic pumps before use to ensure pumps are working properly and liquid is being dispersed. Failure to do so may cause air bubbles in the tubing, insufficient volume of H_2O_2 to immerse cells, and/or insufficient volume of quench hydroxy radicals.

Another issue that may arise is unwanted delays in the system. An example of this is the process of verifying received commands for the pump systems which adds significant delays on the order of 1000 or more milliseconds using the integration software. This problem can be fixed by minimizing the communication with the pumps during the experiment and using pre-set commands ahead of time as much as possible.

In the future, the goal for PIXY is producing a fully automated and integrated system. In addition to the peristaltic pumps, the triggering of the laser pulse will be automated. A new positioning system will also be utilized for the rapid movement of the platform incubator to enhance speed and accuracy. All components of the system will continue to be programmed using the integration software to further increase throughput.

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DISCLOSURES:
The authors declare no competing financial interest.
The research presented herein is associated with the below referenced patent application:
U.S. Non-Provisional Patent Application Number: 17/042,565
Title: “Device and Method for Determining Protein Folding”
UMB Docket Number: LJ-2018-104 UMass Ref: UMA 18-059.

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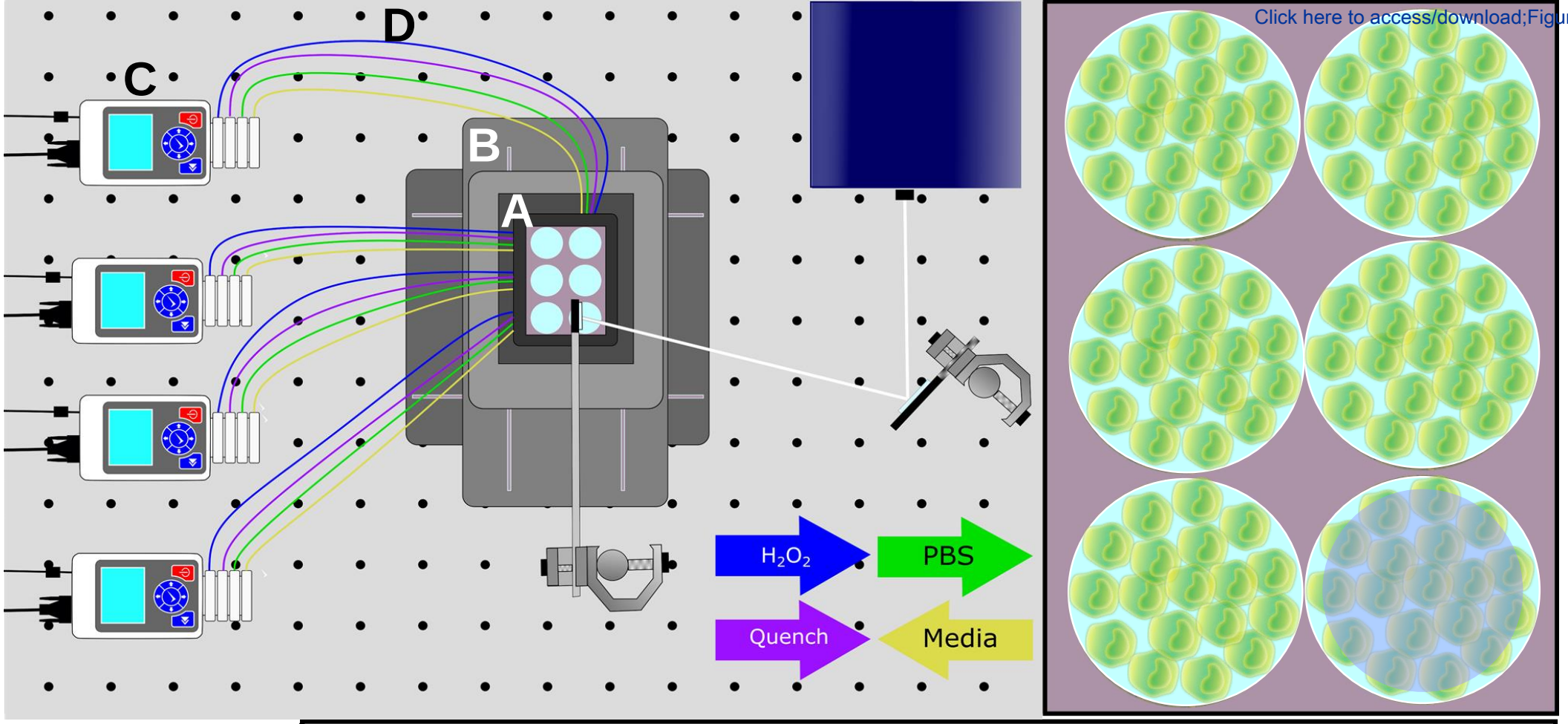
Figure 1



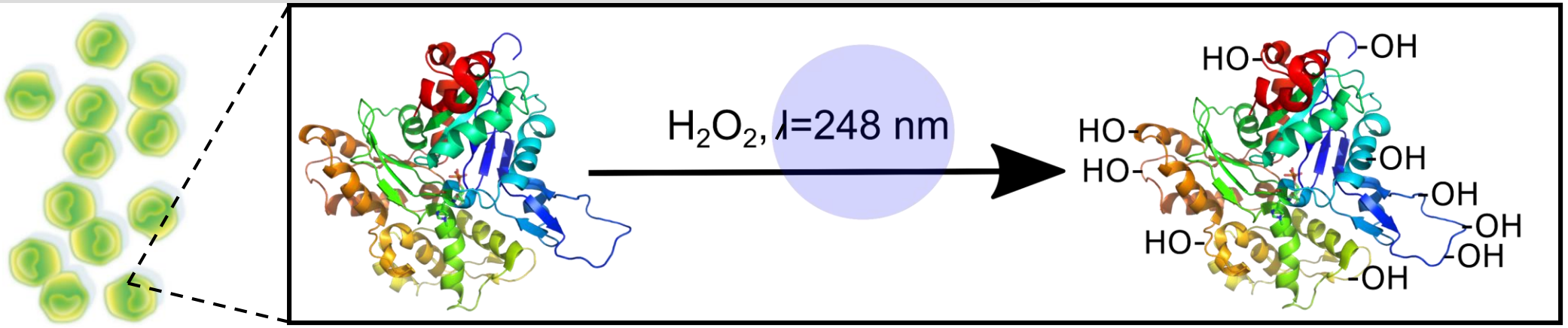


Figure 2

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Figure 3

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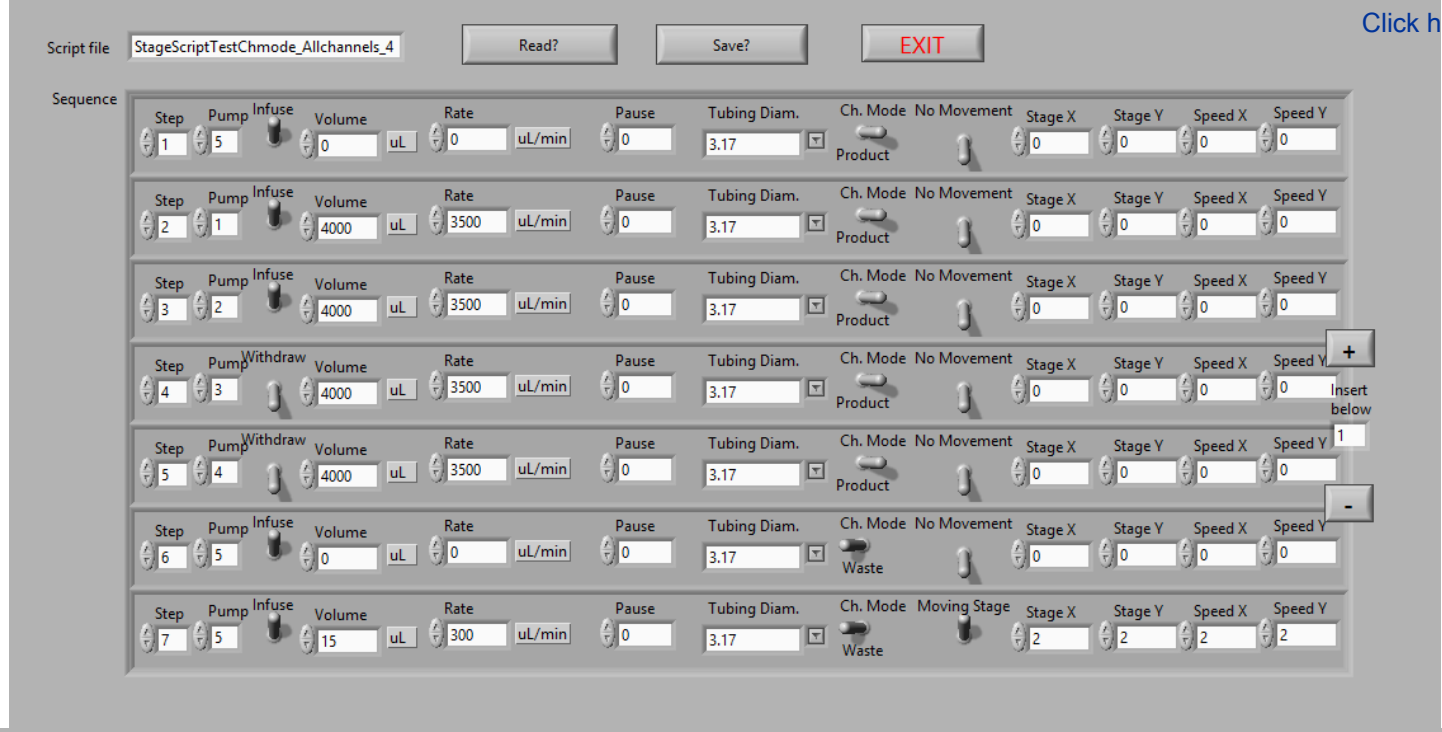
A



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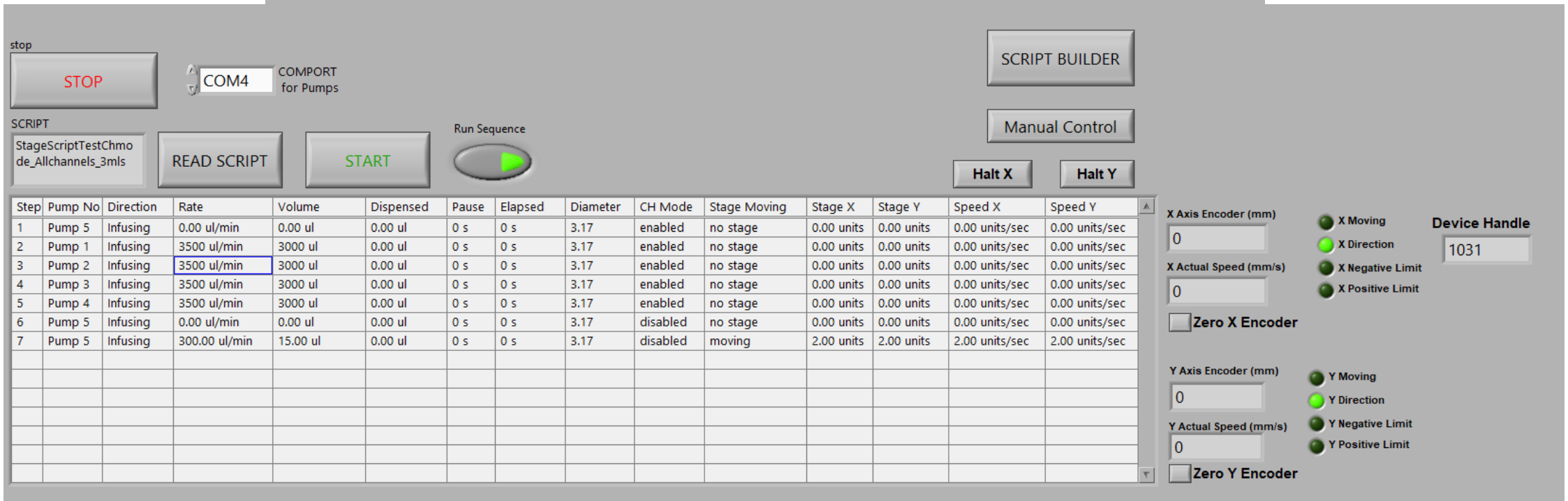

Figure 4

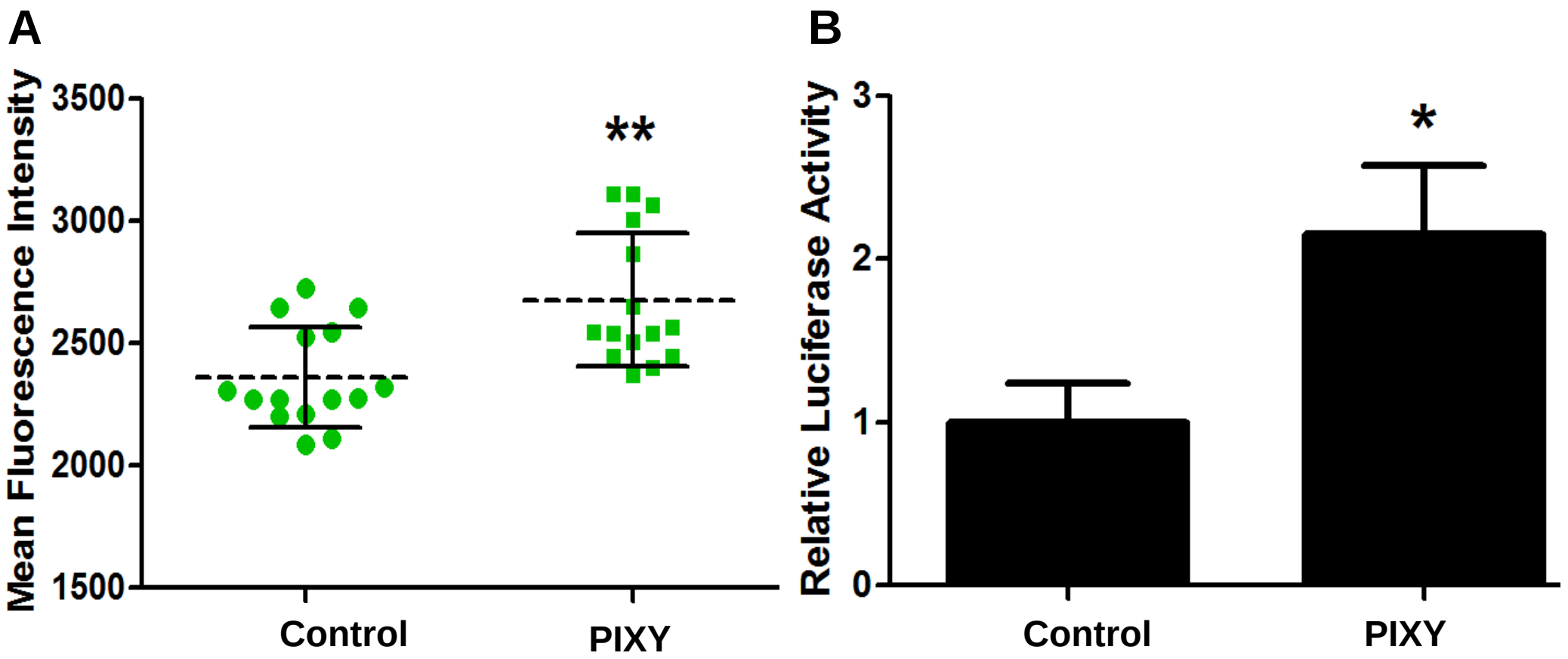

Figure 5

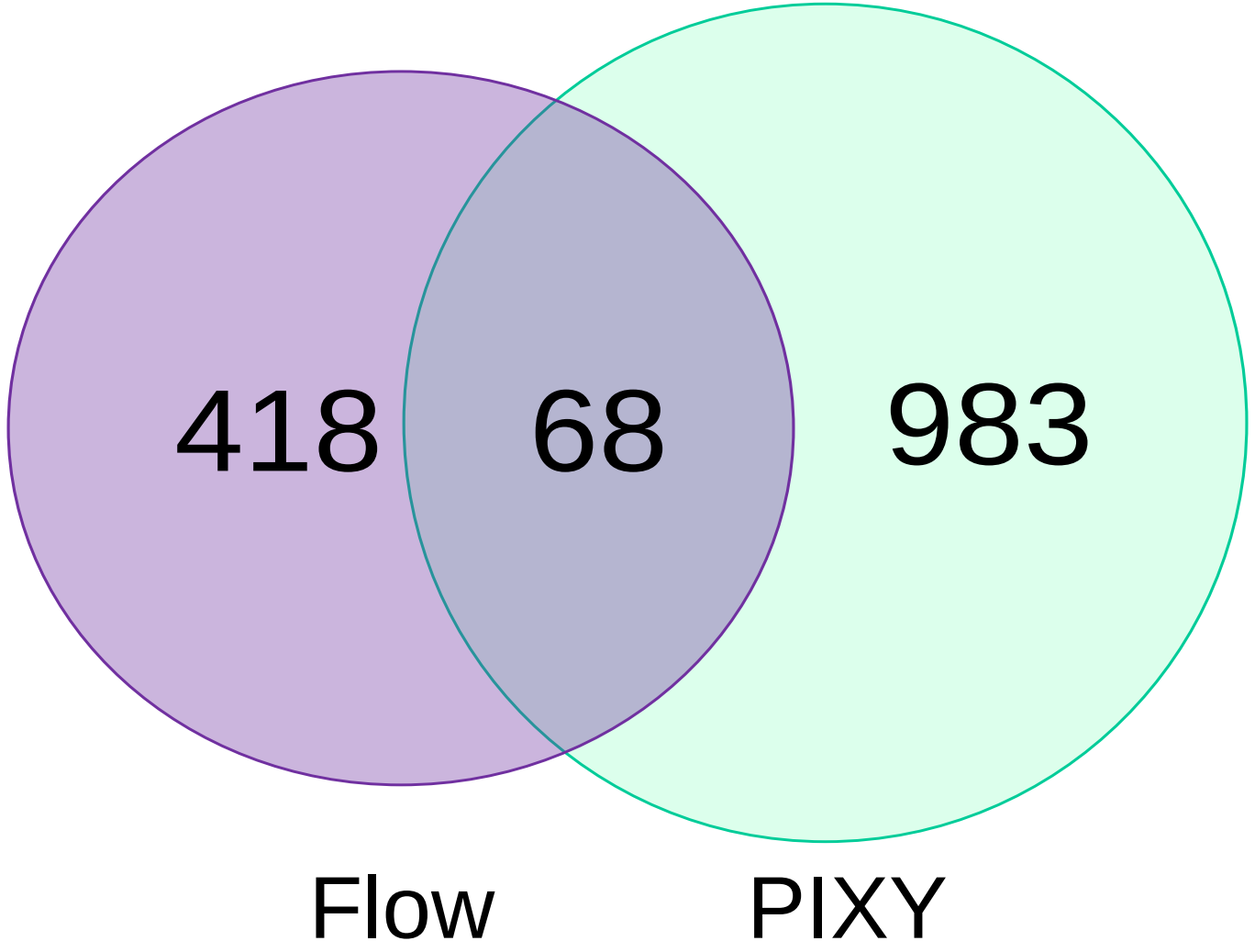

Figure 6

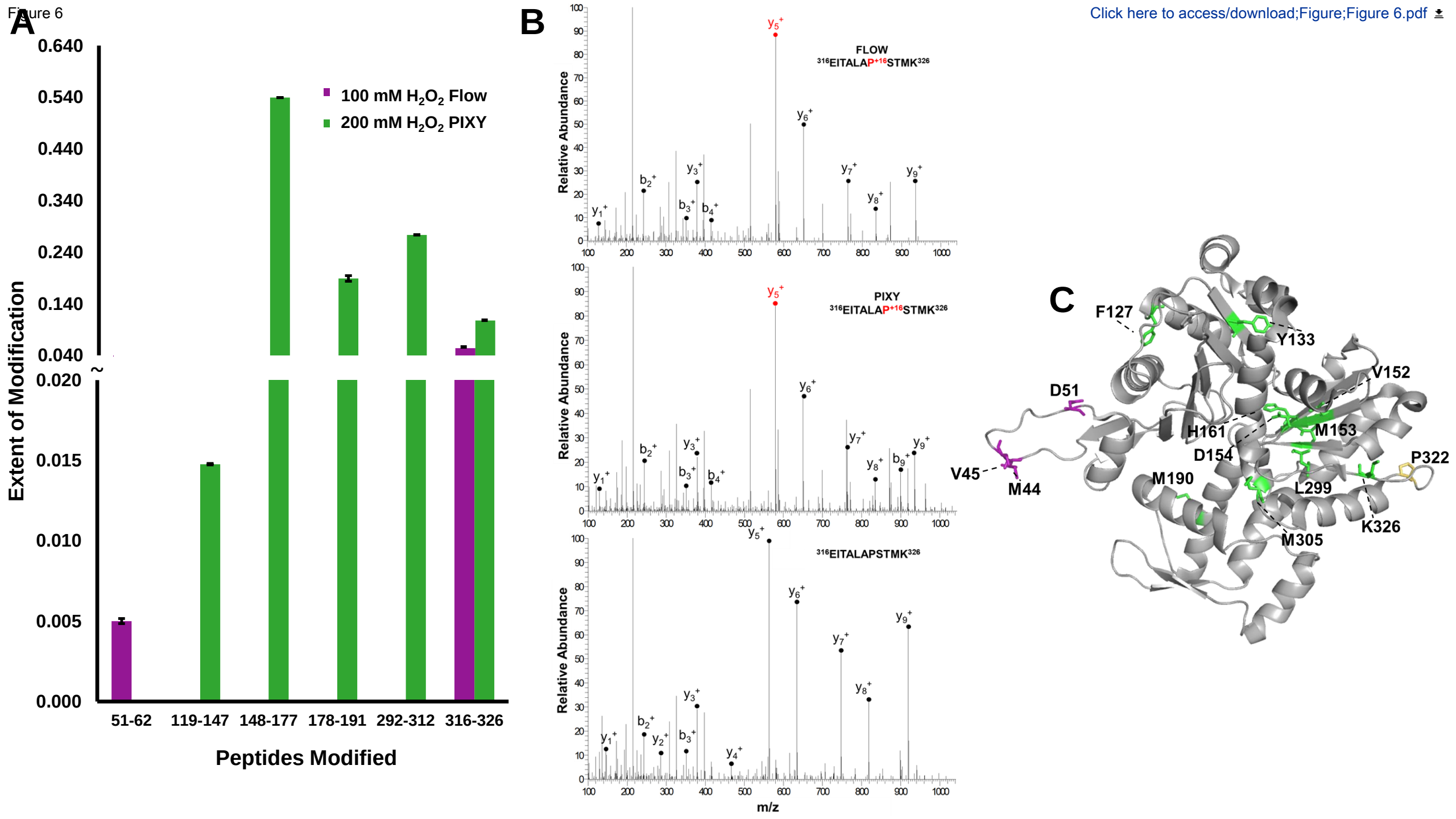

Figure 7

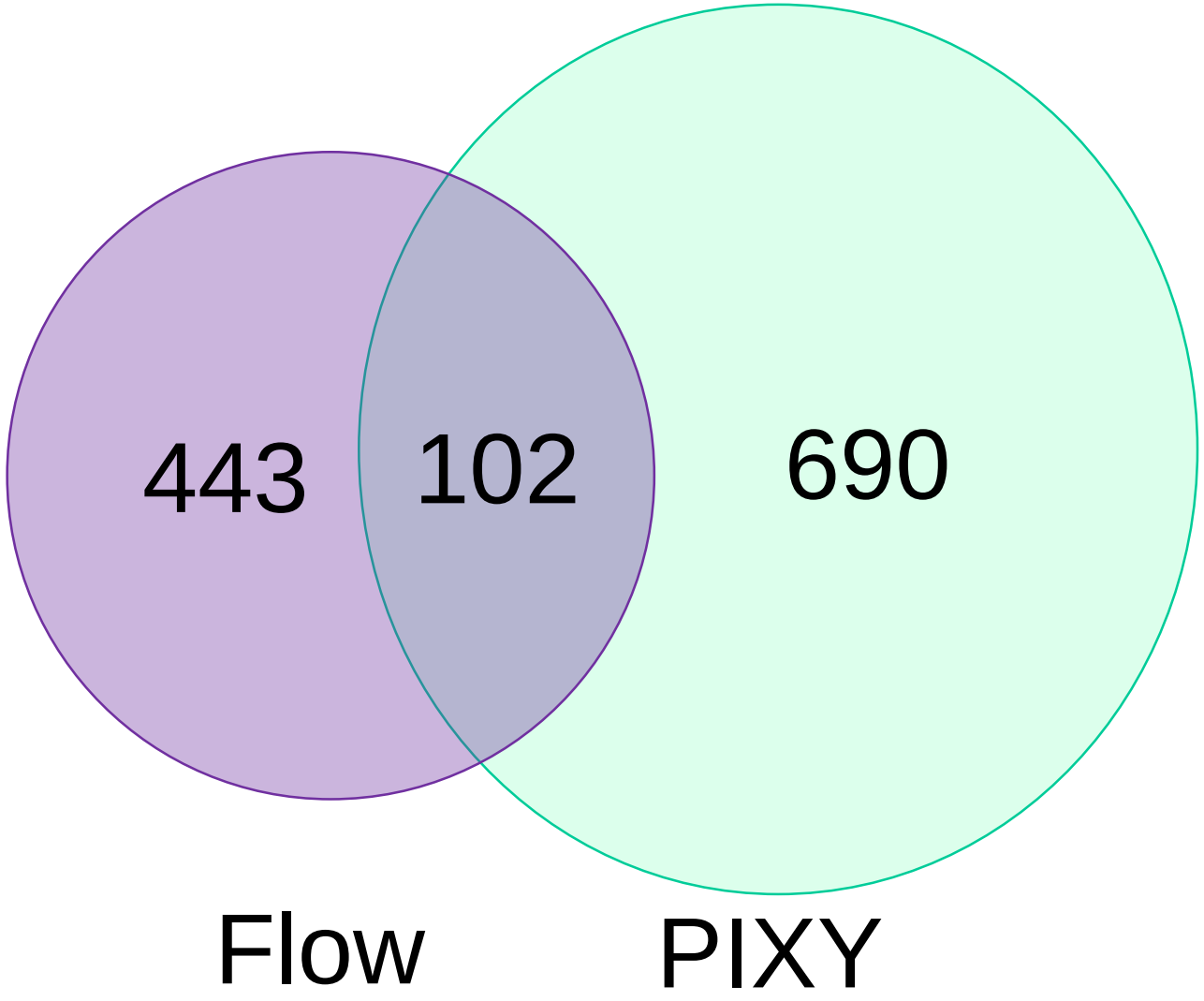

Table 1

Amino Acid	Side chain modification and mass shifts
Cys	sulfonic acid (+48), sulfinic acid (+32+), hydroxyl (-16)
Met	sulfoxide (+16) sulfoxide (+16), sulfone (+32), aldehyde (-32)
Trp	hydroxy- (+16, +32, +48, etc.), pyrrol ring-open (+32, etc.)
Tyr	hydroxy- (+16, +32, etc.)
Phe	hydroxy- (+16, +32, etc.)
His	oxo- (+16), ring-open (-22, -10, +5)
Leu	hydroxy- (+16), carbonyl (+14)
Ile	hydroxy- (+16), carbonyl (+14)
Val	hydroxy- (+16), carbonyl (+14)
Pro	hydroxy- (+16), carbonyl (+14)
Arg	deguanidination (-43), hydroxy- (+16), carbonyl (+14)
Lys	hydroxy- (+16), carbonyl (+14)
Glu	decarboxylation (-30), hydroxy- (+16), carbonyl (+14)
Gln	hydroxy- (+16), carbonyl (+14)
Asp	decarboxylation (-30), hydroxy- (+16)
Asn	hydroxy- (+16)
Ser	hydroxy- (+16), carbonyl (-2- or +16-H2O)
Thr	hydroxy- (+16), carbonyl (-2- or +16-H2O)
Ala	hydroxy- (+16)

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_PIXY.xlsx

Name of Material/Equipment

15 mL Conical Centrifuge Tubes
5 mL Gas Tight Syringe, Removable Luer Lock
50 mL Conical Centrifuge Tubes
500 µL SGE Gastight Syringes: Fixed Luer-Lok Models
Acetone, HPLC Grade
Acetonitrile with 0.1% Formic Acid (v/v), LC/MS Grade
ACQUITY UPLC M-Class Symmetry C18 Trap Column, 100Å, 5 µm, 180 µm x 20 mm, 2G, V/M, 1/pkg
ACQUITY UPLC M-Class System
Aluminum Foil
Aqua 5 µm C18 125 Å packing material
Centrifuge
Delicate Task Wipers
Dithiothreiotol (DTT)
DMSO, Anhydrous
EX350 excimer laser
FEP Tubing 1/16" OD x 0.020" ID
Formic Acid, LC/MS Grade
HV3-2 VALVE
Hydrogen Peroxide
Iodoacetamide (IAA)
Legato 210 syringe pump
Luer Adapter Female Luer to 1/4-28 Male Polypropylene
Methanol, LC/MS Grade
Microcentrifuge
N,N'-Dimethylthiourea (DMTU)
NanoTight Sleeve Green 1/16" ID x .0155" ID x1.6"'
NanoTight Sleeve Yellow 1/16" OD x 0.027" ID x 1.6"
N-tert-Butyl-α-phenylnitrone (PBN)
Orbitrap Fusion Lumos Tribrid Mass Spectrometer
PE50-C pyroelectric energy meter
Pierce Quantitative Colorimetric Peptide Assay
Pierce Rapid Gold BCA Protein Assay Kit

Pierce Trypsin Protease, MS Grade
Pierce Universal Nuclease for Cell Lysis
Polymicro Cleaving Stone, 1" x 1" x 1/32”
Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 150 µm, Outer Diameter 360 µm, TSP150350

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

Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 75 µm, Outer Diameter 360 µm, TSP075375
Potassium Phosphate Monobasic
Proteome Discover (bottom-up proteomics software)
Rotary Magnetic Tumble Stirrer
Rotary Magnetic Tumble Stirrer, accessory kit for use with Syringe Pumps
Super Flangeless Ferrule w/SST Ring, Tefzel™ (ETFE), 1/4-28 Flat-Bottom, for 1/16" OD
Super Flangeless Nut PEEK 1/4-28 Flat-Bottom, for 1/16" & 1/32" OD
Super Tumble Stir Discs, 3.35 mm diameter, 0.61 mm thick
Thermo Scientific Pierce RIPA Buffer
Tris Base
Upchurch Scientific Low-Pressure Crosses: PEEK
Upchurch Scientific Low-Pressure Tees: PEEK
V&P Scientific IncSupplier Diversity Partner TUMBL STIR DISC PARYLENE 1000
VHP MicroTight Union for 360µm OD
Water with 0.1% Formic Acid (v/v), LC/MS Grade
Water, LC/MS Grade

Company	Catalog Number	Comments/Description
Fisher Scientific	14-959-53A	any brand is sufficient
SGE Analytical Science	008760	
Fisher Scientific	14-432-22	any brand is sufficient
Fisher Scientific	SG-00723	
Fisher Scientific	A929-4	4 L quantity is not necessary
Fisher Scientific	LS120-500	
Waters	186007496	
Waters		
Fisher Scientific	01-213-100	any brand is sufficient
Phenomenex		
Eppendorf	022625501	
Fisher Scientific	06-666A	
AmericanBio	AB00490-00005	
Invitrogen	D12345	
GAM Laser		
IDEX Health & Sciene	1548L	
Fisher Scientific	A117-50	
Hamilton	86728	
Fisher Scientific	H325-100	any 30% hydrogen peroxide is sufficient
ACROS Organics	122270050	
KD Scientific	788212	
IDEX Health & Sciene	P-618L	
Fisher Scientific	A454SK-4	4 L quantity is not necessary
Thermo Scientific	75002436	
ACROS Organics	116891000	
IDEX Health & Sciene	F-242X	
IDEX Health & Sciene	F-246	
ACROS Organics	177350250	
Thermo Scientific		other high resolution instruments (e.g. Q exactive Orbitrap or Orbitrap Fusion) ca
Ophir Optronics	7Z02936	
Thermo Scientific	23275	
Thermo Scientific	A53225	

Thermo Scientific	90058	
Fisher Scientific	88702	
Molex	1068680064	any capillary tubing cutter is sufficient
Polymicro Technologies	1068150024	
Polymicro Technologies	1068150025	
Polymicro Technologies	1068150019	
Fisher Scientific	P382-500	
Thermo Scientific	OPTON-30799	
V&P Scientific, Inc.	VP 710D3	
V&P Scientific, Inc.	VP 710D3-4	
IDEX Health & Sciene	P-259X	
IDEX Health & Sciene	P-255X	
V&P Scientific, Inc.	VP 722F	
Fisher Scientific	PI89900	
Fisher Scientific	BP152-500	
Fisher Scientific	05-700-182	
Fisher Scientific	05-700-178	
V&P Scientific, Inc.	VP724F	
IDEX Health & Sciene	UH-436	
Fisher Scientific	LS118-500	
Fisher Scientific	W6-4	

n be used

Name of Material/Equipment

Ismatec Reglo ICC Peristaltic Pumps
Kinematic Mirror Mount for Ø2" Optics
10X trypsin−EDTA
50.0mm 248nm 45°, Excimer Laser Line Mirror
Acetone, HPLC Grade
Acetonitrile with 0.1% Formic Acid (v/v), LC/MS Grade
ACQUITY UPLC M-Class Symmetry C18 Trap Column, 100Å, 5 µm, 180 µm x 20 mm, 2G, V/M, 1/pkg
ACQUITY UPLC M-Class System

Afinia H480 3D Printer
air pump
Aluminum Foil
Aqua 5 µm C18 125 Å packing material
carbon dioxide unit
Centrifuge
connectors (Y PP 1/16” and 1/16x1/8”)
Delicate Task Wipers
Dithiothreiotol (DTT)
DMSO, Anhydrous
Dulbecco’s phosphate-buffered saline (DPBS)
EX350 excimer laser
Fetal bovine serum (FBS)
Formic Acid, LC/MS Grade
HEK293T cells
humidifier
HV3-2 VALVE
Hydrogen Peroxide
Iodoacetamide (IAA)
LABVIEW Professional 2018
MadMotor Positioning system and controllers
Methanol, LC/MS Grade
Microcentrifuge
N,N’-Dimethylthiourea (DMTU)
Nanopositioinging stage
N-tert-Butyl-α-phenylnitrone (PBN)
OKO Touch Monitoring System
Orbitrap Fusion Lumos Tribrid Mass Spectrometer
PE50-C pyroelectric energy meter
Penicillin-streptomycin
Pierce Quantitative Colorimetric Peptide Assay
Pierce Rapid Gold BCA Protein Assay Kit
Pierce Trypsin Protease, MS Grade
Pierce Universal Nuclease for Cell Lysis

pressure gauge
PTFE filter

Six 33 mm PLA filament rings
sterile incubator
temperature unit
Thermo Scientific Pierce RIPA Buffer

TiNKERcad
Tris Base
tubing (Tygon 3.18 and 1.59 ID)
Water with 0.1% Formic Acid (v/v), LC/MS Grade
Water, LC/MS Grade

Company	Catalog Number
Cole-Palmer	122270050
Thor Labs	
Corning	AB00490-00005
Edmond Optics	
Fisher Scientific	
Fisher Scientific	
Waters	23275
Waters	A53225
(Innovation Space University of Maryland Baltimore Campus Library)	
OKOlabs	D12345
Fisher Scientific	Stock #63-120
Phenomenex	
OKOlabs	MadMotor®-UHV
Eppendorf	
Cole-Palmer	116891000
Fisher Scientific	022625501
AmericanBio	
Invitrogen	LS118-500
Corning	SK-78001-82
GAM Laser	PI89900
Corning	SK-12023-78
Fisher Scientific	A929-4
Paul Shapiro Lab (University of Maryland Baltimore)	
OKOlabs	Nano-LPMW stage
Hamilton	BP152-500
Fisher Scientific	LS120-500
ACROS Organics	
National Instruments	86728
Mad City Labs	W6-4
Fisher Scientific	01-213-100
Thermo Scientific	H301-T Unit-BL-PLUS
ACROS Organics	
Mad City Labs	
ACROS Organics	
OKOlabs	
Thermo Scientific	7Z02936
Ophir Optronics	
Corning	
Thermo Scientific	
Thermo Scientific	
Thermo Scientific	75002436
Fisher Scientific	06-666A

OKOlabs	OKO-AIR-PUMP-BL
OKOlabs	CO2-UNIT-BL
(Innovation Space University of Maryland Baltimore Campus Library)	
OKOlabs	
OKOlabs	OKO-TOUCH
Fisher Scientific	A117-50
(Innovation Space University of Maryland Baltimore Campus Library)	
Fisher Scientific	H325-100
Cole-Palmer	177350250
Fisher Scientific	A454SK-4
Fisher Scientific	88702
	90058
	KM200
	186007496

Comments/Description

other high resolution instruments (e.g. Q exactive Orbitrap or Orbitrap Fusion) can be used

4 L quantity is not necessary

any brand is sufficient

any 30% hydrogen peroxide is sufficient

4 L quantity is not necessary

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TITLE:

Platform Incubator with Movable XY stage: A New Platform for Implementing In-Cell Fast Photochemical Oxidation of Proteins

AUTHORS AND AFFILIATIONS:

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The authors thank the reviewers for their comments. We detail our response to these comments below.

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R: The manuscript has been proofread and all abbreviations have been defined

2. Please revise the following lines to avoid overlap with previously published work: 80 (cell culture…)-83 (…CO2), 85 (for…)-88 (bench), 93 (each…)-96, 307-321, 331-338, 345-348, 356-371, 383-387, 395-401, 438 (incubator…)-441, Figure 1 legend (445-449 irradiation), Figure 6 legend (471-477)

R: All above lines have been revised.

3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

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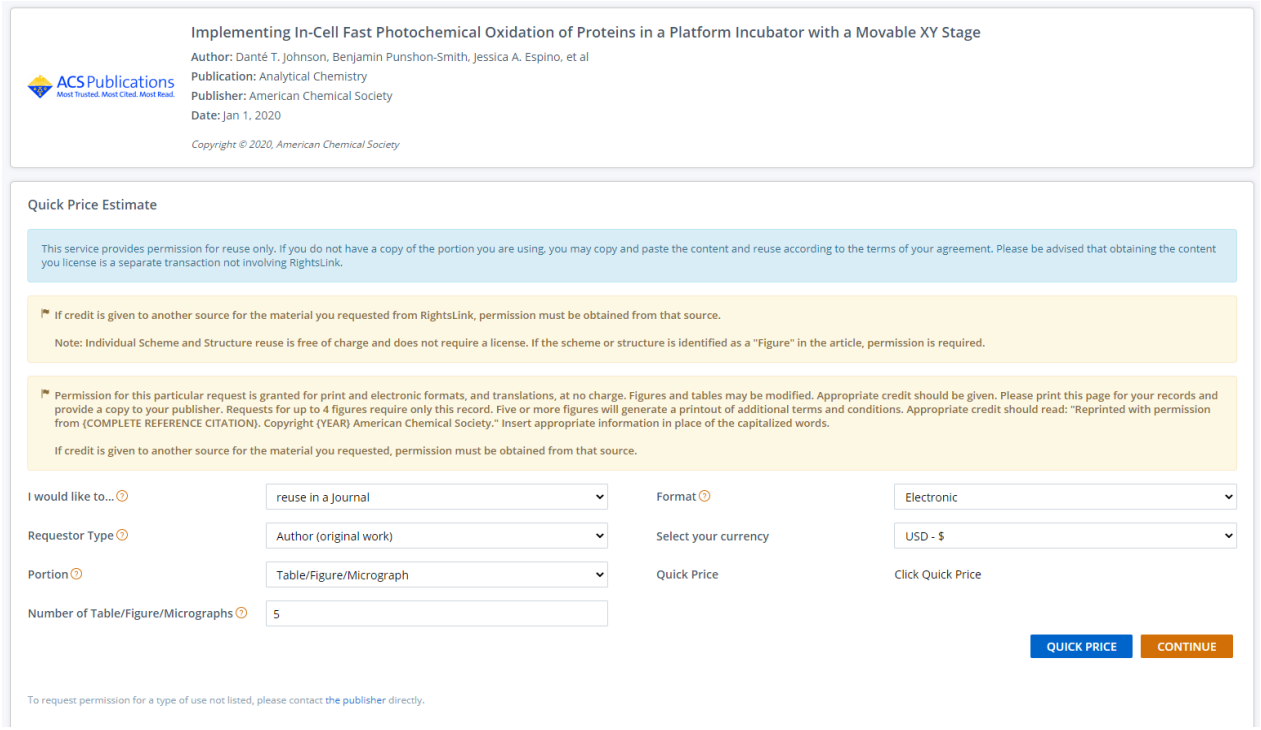

Implementing In-Cell Fast Photochemical Oxidation of Proteins in a Platform Incubator with a Movable XY Stage

Author: Danté T. Johnson, Benjamin Punshon-Smith, Jessica A. Espino, et al
Publication: Analytical Chemistry
Publisher: American Chemical Society
Date: Jan 1, 2020

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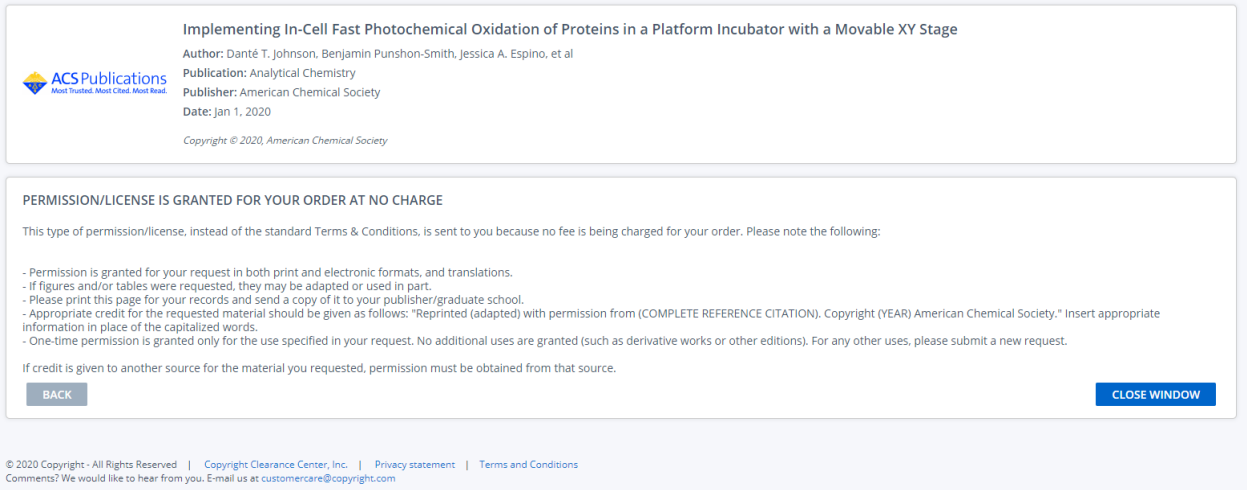

Implementing In-Cell Fast Photochemical Oxidation of Proteins in a Platform Incubator with a Movable XY Stage

Author: Danté T. Johnson, Benjamin Punshon-Smith, Jessica A. Espino, et al
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BACK CLOSE WINDOW

12. As we are a methods journal, please add to the Discussion with citations:
a) Any limitations of the technique
b) The significance with respect to existing methods

R: The above aspects have been included in the discussion section.

13. Please ensure that the references appear as the following: [Last name, F.I., Last Name, F.I., Last Name, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.

R: All references have been revised accordingly as above.

<u>Reviewer #1:</u>

The authors are laboratory is well known for performing cutting-edge FPOP experiments that report on protein structure and dynamics, for proteins in solution and also within living cells. The PIXY system described in this manuscript appears to be a useful addition to this overall toolbox, and it will be of interest to the JOVE readership. I only have a few comments, all of which will be easy to address:

p. 3: Introduction, first sentence. In addition to reference 1 add cast a wider net and include studies from other labs (e.g. Michael Gross' recent review in Chem. Rev.)

R: The following citations were added.

Liu, X. R.; Zhang, M. M.; Gross, M. L., Mass Spectrometry-Based Protein Footprinting for Higher-Order Structure Analysis: Fundamentals and Applications. *Chemical Reviews.*, *120* (10), 4355-4454, 2019.

Li, J.; Chen, G., The use of fast photochemical oxidation of proteins coupled with mass spectrometry in protein therapeutics discovery and development. *Drug Discovery Today.*, *24* (3), 829-883, 2019.

Zhao, B.; Zhuang, J.; Xu, M.; Liu, T.; Limpikirati, P.; Thayumanavan, S.; Vachet, R. W., Covalent Labeling with an α,β-Unsaturated Carbonyl Scaffold for Studying Protein

Structure and Interactions by Mass Spectrometry. ***Analytical Chemistry. 92(9), 6637–6644,*** **2020.**

p. 3: "of 19 of the 20" which one is missing? Gly? Realistically, this statement is too optimistic, because typically the modifications are preferentially seen for the most reactive residues.

R: The following worded was added to the manuscript at lines 66-69.
Theoretically, 19 of the 20 amino acids, can be oxidatively modified with Gly being the lone exception. However, owing to the variant reactivity rates of amino acids with hydroxyl radicals, modification of only a subset of these has been observed experimentally. Still, the method does have the potential for analysis over the length of a protein sequence[5].

Perhaps I missed it, but the authors should discuss what types of cells are amenable to this approach (any cells grown in culture?) How about intact organisms such as C. elegans?

R: Validation studies have only been published in HEK293T cells in PIXY, however, all adherent cells are amenable to the approach. C. elegans are amenable to this approach as well. Evidence is represented in Figure 7 and well as in the text in the representative results section. The revisions are restated below for quick reference.

Espino et al. demonstrated the capacity of FPOP to be performed in vivo (IV-FPOP) within C. elegans, a worm model for human disease states. While IV-FPOP is also performed via a flow system, the PIXY system was tested for compatibility with the worms. Approximately 10,000 worms were incubated in each well in the platform incubator at 20°C. LC–MS/MS analysis revealed 792 proteins were modified by IV-FPOP in the platform incubator compared to the 545 proteins modified with the flow system (Figure 7). These results demonstrate that in addition to 2D cell culture, this new methodology is also compatible with the study of other biological systems such as C. elegans.

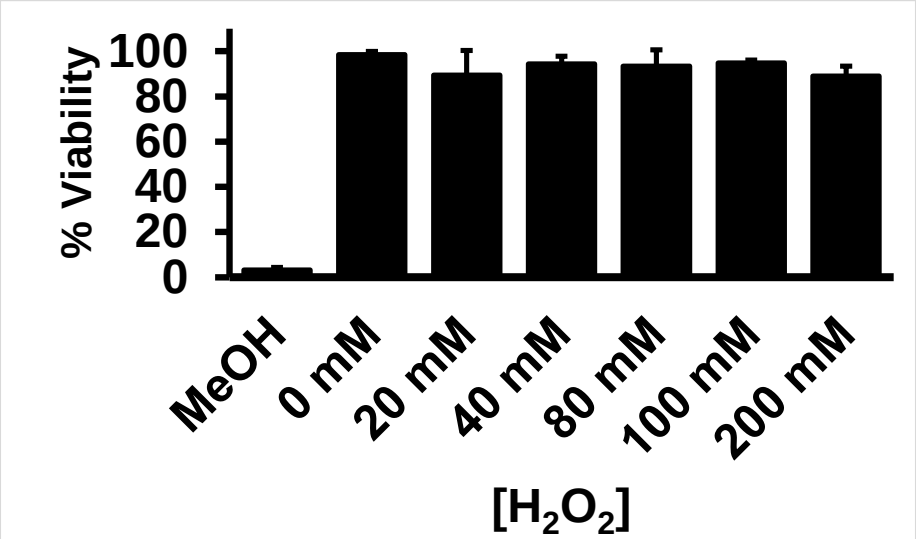
The following wording has also been added to the discussion section (lines 1646-1651) to note the potential of this platform to study other cell lines.

Current studies in this platform incubator have been performed on HEK293T cells and in C. elegans. The IC-FPOP method has been shown to be compatible with a wide variety of cell lines including Chinese hamster ovary (CHO), Vero, MCF-7, and MCF10-A cells. Since the general IC-FPOP method is translatable to this static platform, these cells lines should be amenable for study using PIXY as well.

Are any specific considerations required to allow for H_2O_2 diffusion into the cells? To what extent will the H_2O_2 affect the viability and the physiology of the cells?

R: Hydrogen peroxide readily diffuses through the cell membrane. Generation of hydroxyl radicals by laser induced photolysis of hydrogen peroxide is a

reaction that occurs on a microsecond timescale, theoretically eliminating structural changes induced by labeling. Also, in the PIXY system, cells are only exposed to 200 mM hydrogen peroxide for 10 seconds. Cell viability studies have been done to make sure cells are viable at the concentration as well as time frame. Please see bar graph below including citation.



Johnson, D. T.; Punshon-Smith, B.; Espino, J. A.; Gershenson, A.; Jones, L. M., Implementing In-Cell Fast Photochemical Oxidation of Proteins in a Platform Incubator with a Movable XY Stage. *Analytical Chemistry.* 92(2), 1691–1696 2019.

The following sentence has been added to the discussion section with appropriate reference in lines 2013-2014.

Cell viability studies demonstrated the cell were viable in the presence of H_2O_2 concentrations up to 200 mM.

p. 11: "Results show PIXY significantly outperformed the standard incubator, with a 1.13-fold higher transfection efficiency" To me, this means that PIXY shows about the same performance. "significantly outperformed" sounds like a bold exaggeration? Is this 13% increase even statistically significant?

R: The transfection efficiency difference between PIXY and the standard was statistically significant confirmed by calculating the probability of error (p value) by unpaired t-test. $P<0.05$. The sentence was written in a way not to exaggerate, but to showcase a statistically significant increase of cell culture efficiency. The word significantly was removed from the text at line 1011

Reviewer #2:

Manuscript Summary:

A useful and detailed protocol is presented for the assembly and use of an in-cell photochemical foot printing (oxidative labelling) system.

Major Concerns:

Immobilized cells (adhering to surface coated with specific protein) might behave differently than free-floating cells. This is well studied for bacteria (biofilm vs. floating) but also relevant for other cells - please comment if controls are needed.

R: We have not seen a difference in behavior for cells under constant flow compared under to cells adhered to collagen coated plates. However, cells in suspension may be amenable to the PIXY system and could be studied in the future after specific parameters are sorted.

How quickly are the cells "mixed" with H_2O_2 upon infusion, i.e. what total delay between removal of culture medium and start of the FPOP experiment?

R: The dimension of a single well is 35 mm; the peristaltic pump tubing is 3.14 mm that leads into microtubing tubing of 1.59 mm. However, it is the placement of the tubing along with the fast flowrate that ensures efficient mixing of chemicals. The flow rate used for experimentation is 35 ml/min (35000ul/min) that is being infused over the course of the 20 second experiment. The tubing being flushed to the well wall right above the adhered layer of cells submerged under the 2 ml volume of DPBS under such a fast flowrate helps to ensure proper mixing. The total delay between removal of culture medium and the start of the FPOP experiment is 15 seconds. The infusion of H_2O_2 takes another 10 seconds. This incubation is immediately followed by the laser pulse and 1 second after the pulse, hydroxyl radicals are quenched by an infusion of quench buffer. To support these findings, we performed a simple experiment utilizing red and blue food coloring to watch the dispersion of reagents at their infusion timepoint. A video of this experiment is available and can be sent upon request if necessary. Therefore, we trust that each cell is getting complete exposure to each chemical. We since have moved to using premixed H_2O_2 that we infuse post media removal.

Do the cells die when they are exposed to the peroxide - or are continuous time-resolved experiments feasible?

R: Please see response to Reviewer 1 above

Why are four pumps used, there are six wells?

R: Each pump has four channels. Therefore, 16 tubes can be used to infuse the proper chemicals in each well. In a typical IC-FPOP experiment, each well needs three tubes. One tube for H_2O_2 infusion, one tube for quench infusion, and one tube for cell media withdrawal prior to FPOP. One pump out of the four pumps is solely used for withdrawing media out of the wells so these channels utilize six tubes instead of four using Y-junctions to increase the number of tubes per pump.

Why the specific PBN / DMTU quench used here, briefly explain how it works. Is this the same quench as used in the flow setup?

R: For *in vitro* FPOP, catalase and methionine are added to labeled samples to quench H_2O_2 and hydroxyl radicals, respectively. Since these molecules are not cell permeable, they will not be useful in quenching the reaction inside cells. For IC-FPOP, the cell permeable molecules DMTU and N-tert-butyl-α-phenylnitrone (PBN) were used to quench H_2O_2 and hydroxyl radicals, respectively. These two chemicals are both well studied free radical scavengers only used in our in cell FPOP studies. The cell permeability of these reagents is now noted in the manuscript in Protocol section 4 "Make quench buffer and H_2O_2".

Explain cell confluency.

R: In cell culture biology, confluency is the term commonly used as a measure of the number of the cells in a cell culture dish or a flask and refers to the coverage of the dish or the flask by the cells. In our lab, we follow the Thermo "Useful Numbers for Cell Culture" table to assess cell confluency. We found that experimentation is best done with cells that are 80 % confluent as they are most viable, not undergrown, or over crowed within the flask or well.

A note has been added on line 414 to the cell culture steps to explain confluency.

Can you list all known hydroxyl radical side chain modifications here (with their delta mass)? **R: The following table was added to the document figure file and manuscript introduction as Table 1.**

Amino Acid	Side chain modification and mass shifts
Cys	sulfonic acid (+48), sulfinic acid (+32+), hydroxyl (-16)
Met	sulfoxide (+16) sulfoxide (+16), sulfone (+32), aldehyde (-32)
Trp	hydroxy- (+16, +32, +48, etc.), pyrrol ring-open (+32, etc.)
Tyr	hydroxy- (+16, +32, etc.)
Phe	hydroxy- (+16, +32, etc.)
His	oxo- (+16), ring-open (-22, -10, +5)
Leu	hydroxy- (+16), carbonyl (+14)
Ile	hydroxy- (+16), carbonyl (+14)
Val	hydroxy- (+16), carbonyl (+14)
Pro	hydroxy- (+16), carbonyl (+14)
Arg	deguanidination (-43), hydroxy- (+16), carbonyl (+14)
Lys	hydroxy- (+16), carbonyl (+14)
Glu	decarboxylation (-30), hydroxy- (+16), carbonyl (+14)
Gln	hydroxy- (+16), carbonyl (+14)
Asp	decarboxylation (-30), hydroxy- (+16)
Asn	hydroxy- (+16)
Ser	hydroxy- (+16), carbonyl (-2- or +16-H2O)
Thr	hydroxy- (+16), carbonyl (-2- or +16-H2O)
Ala	hydroxy- (+16)

Please comment in Fig. 6 why some extensive modifications are only seen in PIXY but not in flow, is the side chain reactivity the same? Are background peroxide oxidations (i.e. without laser, as control) causing this?

R: This question is directly addressed in the following publication on which the JOVE submission is based: Johnson, D. T.; Punshon-Smith, B.; Espino, J. A.; Gershenson, A.; Jones, L. M., Implementing In-Cell Fast Photochemical Oxidation of Proteins in a Platform Incubator with a Movable XY Stage. *Analytical Chemistry.* 92(2), 1691–1696 2019.

Please see the excerpt below.

The 5 peptides modified in the PIXY samples contain 12 modified amino acids, while only 4 amino acids were modified with the flow system (Figure 4C). The increase in modification coverage is further demonstrated

with the protein tubulin, where 67 residues were modified with PIXY, while only 15 were modified using the standard ICFPOP conditions with the flow system (Figure S3).
Owing to actin having both a monomeric and polymeric form, we cannot rule out the possibility that the low number of modifications with the flow system are due to the polymerized, more protected, form (F-actin) being the dominant species in these samples. We calculated the solvent accessible surface area (SASA) (Table S2) of the F-actin polymerized structure and found residues M190, P322, M305, and K326 to all be solvent accessible. While all four of these residues are modified with PIXY, only P322 is modified with the flow system.
Interestingly, the highly reactive methionine residues are not modified with the flow system, even though they are solvent accessible. In both cases, we do see residues with low accessibility modified, which could be a result of the difference in solution and static crystal structure conditions. It also could be due to the effects of molecular crowding within the cell that are not considered in the crystal structure.

Minor Concerns:
The term "English optical breadboard" is not wide-spread and might be a bit unclear. Metric instead of English also possible?

R: The term Imperial optical breadboard was confirmed on manufacturer website. This word was changed in the text.

Why is some text marked yellow?

R: Some text was marked in yellow according to the JOVE submission guidelines to highlight experimental steps that would be used for filming.

p26: text fragment
Comments/description: what does this refer to?

R: This error cannot be found in the text. However, all text fragments have been addressed and revised in the text.

Figure 7: this should be a 1.45-fold increase (not 0.45)

R: This error has been addressed and revised in the text.