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Corresponding Author:	Lisa Jones UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	ljones@rx.umaryland.edu
Order of Authors:	Lisa M Jones
	Emily E. Hart
	Aimee Rinas
	Jessica A. Espino
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TITLE:

Characterizing Cellular Proteins with In-cell Fast Photochemical Oxidation of Proteins

AUTHORS AND AFFILIATIONS:

Emily E. Chea[1], Aimee Rinas[2], Jessica A. Espino[1], Lisa M. Jones[1]

[1]Department of Pharmaceutical Sciences, University of Maryland Baltimore, Baltimore, MD, USA
[2]AIT Bioscience, Indianapolis, IN, USA

Corresponding Author:

Lisa M. Jones, ljones@rx.umaryland.edu

Email Addresses of Co-authors:

Emily E. Chea, emily.hart@umaryland.edu
Aimee Rinas, arinas@umail.iu.edu
Jessica A. Espino, jespino@umaryland.edu

KEYWORDS:

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

SUMMARY:

Here, we characterize protein structure and interaction sites in living cells using a protein footprinting technique termed in-cell fast photochemical oxidation of proteins (IC-FPOP).

ABSTRACT:

Fast photochemical oxidation of proteins (FPOP) is a hydroxyl radical protein footprinting method used to characterize protein structure and interactions. FPOP uses a 248 nm excimer laser to photolyze hydrogen peroxide producing hydroxyl radicals. These radicals oxidatively modify solvent exposed side chains of 19 of the 20 amino acids. Recently, this method has been used in live cells (IC-FPOP) to study protein interactions in their native environment. The study of proteins in cells accounts for intermolecular crowding and various protein interactions that are disrupted for in vitro studies. A custom single cell flow system was designed to reduce cell aggregation and clogging during IC-FPOP. This flow system focuses the cells past the excimer laser individually, thus ensuring consistent irradiation. By comparing the extent of oxidation produced from FPOP to the protein's solvent accessibility calculated from a crystal structure, IC-FPOP can accurately probe the solvent accessible side chains of proteins.

INTRODUCTION:

Hydroxyl radical protein footprinting (HRPF) is a method that probes the solvent accessibility of a protein through covalent modifications produced from hydroxyl radicals. When protein structure or protein interactions change, it will alter the solvent exposure of amino acids, thus altering the extent of modification of residues. With HRPF, protein interactions[1-3] and protein conformational changes[4-6] have successfully been interrogated in vitro. There are several

methods that generate hydroxyl radicals for HRPF experiments, one being fast photochemical oxidation of proteins (FPOP). FPOP was developed by Hambly and Gross in 2005 and utilizes a 248 nm excimer laser to produce hydroxyl radicals through the photolysis of hydrogen peroxide (H_2O_2)[7].

Recently, Espino et al. extended the use of FPOP to probe protein structure in live cells, a method termed in-cell FPOP (IC-FPOP)[8]. In contrast to in vitro studies, studying proteins in cells accounts for molecular crowding along with various protein interactions that could potentially influence structure. Additionally, it presents the advantage of providing a snapshot of the full proteome potentially providing structural information of numerous systems at once to perform proteome wide structural biology. Furthermore, this technique is ideal for proteins that are difficult to study in vitro, like membrane proteins.

Initial studies of IC-FPOP successfully probed 105 proteins ranging in protein abundance and cellular localization. To improve the IC-FPOP method, Rinas et al. developed a microflow system for single cell flow[9]. The enhancement of the original flow system limits cell aggregation and increases the available H_2O_2 available for irradiation. In the initial flow system, cells clumping in the silica tubing resulted in clogs and uneven irradiation. The incorporation of two streams of a sheath buffer hydrodynamically focuses the cells, allowing them to flow individually past the laser. The incorporation of a separate syringe for the H_2O_2 enables more controlled and optimizable exposure time allowing higher H_2O_2 concentrations without adverse effects. Also, limiting the incubation time limits the breakdown of H_2O_2 by endogenous catalase. By incorporating this new flow system, the detected number of proteins with an FPOP modification increased 13-fold, thus expanding the capabilities of this method to probe a multitude of proteins in living cells. In this protocol a general IC-FPOP experiment is described focusing on the assembly of the IC-FPOP flow system.

PROTOCOL:

1. Set up IC-FPOP flow system

1.1 To begin the assembly of the flow system, cut the fused silica using a cleavage stone to size. The IC-FPOP flow system requires four 12 cm and one 17 cm fused silica with an inner diameter (ID) of 450 µm and outer diameter (OD) of 670 µm, two 24 cm and one 40 cm with an ID of 75 µm and an OD of 360 µm, and finally two 57 cm with an ID of 150 µm and an OD of 360 µm.

NOTE: When cutting the silica tubing, gently scrape away the polyimide coating, and bend to get the cleanest cut. Check to make sure it is a straight cut (this is necessary to ensure no blockages or leaks form).

1.2 Set up 15 connections using nano-tight sleeves (0.0155” ID X 1/16” OD for 360 µm OD silica tubing and 0.027” ID X 1/16” OD for the 670 µm OD silica tubing) with super flangeless nut PEEK 1/4-28 flat-bottom for 1/16” OD and super flangeless ferrule w/SST ring, Tefzel (ETFE), 1/4-28 flat-bottom, for 1/16” OD. Construct connections as per the manufacturer’s protocol (**Figure 1**).

1.3 Place 6 small cylindrical magnets in one 500 µL syringe. Fill this syringe along with another 500 µL syringe and two 5 mL syringes with buffer and remove air. Position on syringe pump as shown on **Figure 2A**.

NOTE: The 5 mL syringes are larger than the 500 µL syringes, so a spacer is needed to tighten all the syringes simultaneously in place (**Figure 2B**).

1.4 Tighten syringe pump stopper so that the cell syringe has roughly 50 µL left when the motor stalls. This will leave room for the magnetic stirrers. (**Figure 2C and 2D**).

CAUTION: If the syringe pump puts pressure on the magnets, they will jam the syringe and can cause the syringe to break.

1.5 Using a Luer adapter, connect a manual valve to each syringe. Assemble the silica tubing as shown in **Figure 3**.

NOTE: Thread the line with the cells + H_2O_2 all the way through the cross to the other side. Then insert it into the 450 µm ID silica tubing. The sheath buffers in the 5 mL syringe will be flowing at a faster rate than the cells and H_2O_2. With the sheath buffers on both sides, the cells will be hydrodynamically focused into a single line for irradiation.

1.6 Position flow system next to laser. Using a lighter, burn away the silica coating on the 450 µm ID tubing to make a window for laser irradiation.

1.7 Place a magnetic stirrer above the cell syringe containing the six magnets.

1.8 Set syringe pump to 492.4 µL/min for a final flow rate of 1,083.3 µL/min. Flow buffer through the system three times to flush the system and test for any leaks.

1.9 Focus the excimer laser onto the silica tubing using a convex lens. Once focused, test the irradiation window by placing a small piece of paper behind the silica tubing and turn the laser on. Measure the region burnt from the irradiation. Calculate the needed laser frequency by using the irradiation window and flow rate to obtain an exclusion fraction of zero.

NOTE: The recommended laser energy for an IC-FPOP experiment is ≥120 mJ. To get an exclusion fraction of 0 with an irradiation window of 2.58 mm and a flow rate of 1083.3 µL/min, the frequency needs to be 44. Below are the equations that are needed to calculate the laser frequency if the irradiation window, flow rate, and exclusion fraction is known.

Equation 1: $w = \pi * x * 100{,}000 * \left(y * 5 * 10^{-5}\right)^2$

Equation 2: $v = \frac{w}{((1-b) * 1000)}$

Equation 3: $f = \frac{a}{60*v}$

where w is the volume in nL, x is the laser spot width in mm, y is the silica tubing ID in µm, v is the total volume in µL, b is the exclusion fraction, a is the flow rate in µL/min, and f is the frequency in Hz.

2. Make quench and H_2O_2

2.1 Make quench containing 100 mM N-tert-Butyl-alpha-phenylnitrone (PBN) and 100 mM N,N'-dimethylthiourea (DMTU). Aliquot 11 mL of quench for each sample into a 50 mL conical tube.

2.2 Dilute H_2O_2 to 200 mM. Each sample requires 500 µL of H_2O_2.

NOTE: The quench can be made the day before and stored at 4 °C overnight, protected from light. H_2O_2 should be made fresh the day of experimentation.

3. Collect cells

3.1 Grow cells in a T175 flask to about 70-90% confluency.

3.2 Remove media and rinse with buffer.

NOTE: Typical buffers to use are cell culture grade Dulbecco's phosphate-buffered saline (DPBS) or Hank's balanced salt solution (HBSS).

3.3 Detach cells using either trypsin-EDTA or with a scraper.

3.4 Once detached resuspend in 10 mL of buffer and count the cells.

3.5 Spin down, remove the buffer and trypsin-EDTA, and resuspend to make 2 x 10^6 cells/mL.

3.6 Aliquot 500 µL of the cells per sample.

NOTE: For each condition, make a minimum of 3 laser samples, and 3 no laser controls.

4. Performing IC-FPOP

4.1 Fill the two 5 mL syringes with buffer, the 500 µL syringe containing the magnets with the cells, and the final 500 µL syringe with H_2O_2. Turn the magnetic stirrer on.

4.2 Spike in 220 µL of dimethyl sulfoxide (DMSO) to one aliquot of quench, gently mix, and place behind flow system to collect irradiated samples. The addition of DMSO will inhibit endogenous

methionine sulfoxide reductase.

4.3 Turn on laser, wait 7 s, and then turn on flow system.

4.4 Once the sample finishes flowing, turn the laser off, and gently mix the quench with the collected sample. Place this to the side during steps 4.5 and 4.6.

4.5 Fill all four syringes with the buffer the cells are suspended in and flow it through the flow system.

4.6 After the system finishes flushing, repeat steps 4.1 and 4.2. Start the flow without irradiation. This is the no laser control to account for background oxidation in the cells.

4.7 While the next sample is running, spin down the previous sample at 450-800 x *g* for 5 min, remove the solvent, and resuspend in 100 µL of a cell lysis buffer like radioimmunoprecipitation assay (RIPA) buffer.

4.8 Transfer the sample to a microcentrifuge tube and flash freeze in liquid nitrogen.

4.9 When all samples have finished running, disassemble the flow system for cleaning. Discard the used silica tubing and clean all the other connections by sonicating for 1 h in 50% water: 50% methanol. Clean the syringes as per manufacturer's instructions.

5. Digest

5.1 Digest the whole cell lysate. Begin by thawing the samples and heat at 95 °C for 10 min.

5.2 After heating, cool the lysate on ice for 15 min.

5.3 Add 25 units of nuclease to digest DNA and RNA and incubate at room temperate for 15 min.

5.4 Spin samples using a table-top centrifuge at 16,000 x *g* for 10 min at 4 °C.

5.5 Collect the supernatant and transfer it to a clean microcentrifuge tube.

5.6 Check the protein concentration using a protein assay kit.

5.7 Transfer 20-100 µg of sample to a clean microcentrifuge tube and bring to 100 µL.

5.8 Reduce samples with 20 mM dithiothreiotol (DTT) at 50 °C for 45 min.

5.9 Cool the samples at room temperature for 15 min.

5.10 Alkylate with 20 mM iodoacetamide (IAA) at room temperature for 20 min protected from

light.

5.11 Add pre-chilled acetone at a 1:4 ratio protein: acetone. Mix samples and place in -20 °C overnight.

5.12 The next morning, spin samples at 16,000 x *g* for 10 min at 4 °C.

5.13 Remove the supernatant and add 50 µL of 90% pre-chilled acetone. Mix samples and spin down at 16,000 x *g* for 5 min at 4 °C.

5.14 Remove acetone and let samples dry by leaving the caps of the microcentrifuge open with a lint free wipe covering the top. After the samples have dried, resuspend protein pellet with 10 mM Tris buffer pH 8.

5.15 Resuspend 20 µg of trypsin in 40 µL of 10 mM Tris buffer pH 8. Add 2 µg of trypsin (mass: mass ratio of 1 trypsin: 50 sample). Incubate samples at 37 °Covernight.

5.16 The next morning check the peptide concentration using a peptide assay. After the sample has been removed for the peptide assay, quench the trypsin digestion by adding formic acid to the samples (final concentration is 5% formic acid).

5.17 Once final peptide concentration is determined, transfer 10 µg of each sample to a clean microcentrifuge tube. This ensures the same amount of each sample is analyzed. Dry the sample using a vacuum centrifuge. Once dried resuspend with 20 µL of mass spectrometry grade 0.1% formic acid. Transfer samples to autosampler vials.

6. Liquid Chromatography-Tandem Mass Spectrometry

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

6.2 Use mobile phases of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B).

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

6.4 Using a 75 µm x 30 cm C18 (5 µm and 125 Å) analytical column, run the analytical separation method with a flow rate of 0.300 µL/min starting at 3% (B) for one min then ramp to 10% (B) from 1-2 min. Next ramp to 45% (B) from 2-100 min then 100% (B) from 100-110 min. Clean the column by holding at 100% (B) from 110-115 min. Recondition the column by ramping down to 3% (B) from 115-116 min and hold at 3% (B) from 116-130 min.

6.5 Set the MS acquisition method to have a resolution of 60,000 with an m/z scan range of 375-1500. Set the automatic gain control (AGC) target to 5.0×10^5 with a maximum injection time of 50 ms.

6.6 During the MS acquisition, select the precursor ions with charge states 2-6 for isolation via data dependent acquisition (DDA) with an isolation window of 1.2 m/z and a cycle time of 4 s. Select the peptides with an intensity threshold of 5.0 x 10^4 for HCD activation with a normalized energy set to 32%. Exclude the peptides after 1 MS/MS acquisition for 60 s. Set the MS/MS resolution to 15,000 with an AGC target of 5.0 x 10^4 and a maximum injection time of 35 ms.

7. Data Processing

7.1 Search the RAW files on an available protein analysis software against a relevant protein database and the relevant digest enzyme. Here, use the Swiss-Prot Homo Sapiens database and trypsin.

7.2 Set the precursor mass to search between 350 to 5,000 Da and a mass tolerance of 10 ppm. There can be at most 1 missed cleavage site with a peptide length between 6 to 144 residues. These limitations facilitate database searching.

7.3 Set the fragment ions maximum mass tolerance to 0.02 Da with the carbamidomethyl (+57.021) as a static modification and all FPOP modifications from 17 amino acids as a dynamic modification (**Figure 4** is an example of the protein analysis workflow used to detect FPOP modifications).

NOTE: FPOP modifications on serine and threonine are not included in the search due to their lower reactivity with hydroxyl radicals.

7.4 Search with a decoy database with a false discovery rate of 1% and 5%.

7.5 Once files are finished searching calculate the extent of FPOP modification. Open Proteome Discoverer 2.2, export sequence, modification locations, protein accession, spectrum file, precursor abundance, and retention time information. Calculate the extent of modification from equation 4:

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

The EIC area modified is the chromatographic area of a specific peptide with a hydroxyl radical modification. The EIC area is the total chromatographic area of both modified and unmodified areas of that specific peptide. The extent of oxidation is calculated in the samples with and without irradiation. The sample omitting irradiation (control) accounts for any background oxidation that could have been present in the cells before and after H_2O_2 exposure. The controls are subtracted from the irradiation samples to help isolate FPOP specific modifications.

REPRESENTATIVE RESULTS:

IC-FPOP is a footprinting method to interrogate protein interactions in live cells. In the IC-FPOP flow system, the H_2O_2 exposure time is limited to roughly 3 s, warranting higher H_2O_2

concentrations without detrimental consequences for the cells. The flow system also incorporates two streams of sheath buffer, which hydrodynamically focuses the cells to the center of the tubing producing a single flow of cells to be uniformly irradiated (**Figure 5**)[9]. Fluorescence imaging of orthogonal YZ stacked images (**Figure 5A**) show a clear separation of the sheath buffer (containing a FITC fluorophore) from the cell solution (containing a TMRM fluorophore). To emphasize this separation, **Figure 5B** and **Figure 5C** show three-dimensional average heat maps of either the sheath buffer solution or cell solution, illustrating minimal mixing of the two solutions.

The use of the single cell flow system increases the number of oxidatively modified proteins by 13-fold (**Figure 6A**).[9] In this method, proteins in many of the cellular compartments are labeled with membrane proteins, cytoplasmic proteins, and proteins within the nucleus being the most prevalent.[8,9] To ensure proteins were modified within intact cells, fluorescent images of CellROX treated cells were performed following H_2O_2 treatment and irradiation (**Figure 6B**)[8]. The stability of the cells throughout the labeling process further confirms the efficacy of IC-FPOP to probe proteins in their native cellular environment. By using tandem-mass spectrometry, these modifications can be localized to specific amino acids on a protein. **Figure 7** represents a modification that takes place during IC-FPOP along with its extracted ion chromatogram. The shift observed in the extracted ion chromatogram translates to the change in hydrophobicity caused by the oxidized methionine in the modified peptide.

To test if the FPOP modifications probe solvent accessibility inside the cells, in-cell labeled actin was compared to both an in vitro footprinting study and various crystal structures of actin (**Figure 8**)[8]. The in-cell labeled actin is represented in **Figure 8A** shows comparable extents of oxidation from the in vitro study by Guan et al.[10] (**Figure 8B**), concluding actin has similar solvent accessibility for both in-cell and in vitro studies. To further confirm IC-FPOP was probing the solvent accessibility of actin, the extent of FPOP modifications were compared to the solvent accessibility of the labeled residues calculated from two actin crystal structures (**Figure 8C**). This correlation demonstrates that IC-FPOP probes the solvent accessibility of the monomeric protein well.

FIGURE AND TABLE LEGENDS:

Figure 1: How to properly construct ferules. (**A**) Place ferrules, silica tubing, and sleeve together before tightening. (**B**) Tighten all components together. (**C**) Final product will produce a ferule that has been tightened down on the sleeve.

Figure 2: Setting up IC-FPOP flow system. (**A**) Image of a fully assembled IC-FPOP flow system positioned next to the laser. (**B**) Example of a spacer needed to increase the outer diameter of the 500 µL syringes to successfully tighten all syringes down together. (**C**) Representative pictures showing the space required for the magnetic stirrers. (**D**) Stoppers are necessary to stall the syringe pump without breaking the syringes.

Figure 3: Schematic of the flow system developed for IC-FPOP. Blue lines represent silica tubing

with a 450 µm ID and 670 µm OD, red lines have a 150 µm ID and 360 µm OD, and orange lines have a 75 µm ID and 360 µm OD.

Figure 4: Protein analysis software used to detect FPOP modifications. A typical workflow with the corresponding modifications searched in each node.

Figure 5: The single cell flow system hydrodynamically focuses the cells into a single stream. (**A**) Orthogonal YZ stack illustrating 3D focusing of the cellular analyte (red, TMRM fluorophore) surrounded by the sheath buffer (green, FITC fluorophore). Three-dimensional average intensity heat map of the sheath buffer (**B**) and cellular analyte (**C**). Lower intensities are blue and highest are red (**B-C**). This figure has been modified from Rinas et al.[9]

Figure 6: Utilization of the IC-FPOP Flow System drastically increases FPOP modified proteins in intake cells. (A) Comparison of oxidized proteins identified with and without the flow system. The flow system identified 1391 FPOP modified proteins while only 105 proteins were identified with no flow system with an overlap of 58 modified proteins. This figure has been modified from Rinas et al.[9] (B) Fluorescence imaging of CellROX treated cells after IC-FPOP show the cells are still intact after oxidative labeling. Cells were imaged using an Olympus Fluoview FV1000 MPE multiphoton microscope at 665 nm. Image shown is a single slice. This figure has been modified from Espino et al.[8]

Figure 7: Examples of tandem MS/MS spectra that take place during IC-FPOP. Product-ion (MS/MS) spectra of an (**A**) unmodified peptide, and an (**B**) oxidation detected on residue M8 found on that peptide. A representative EIC of the (**C**) unmodified peptide and (**D**) modified peptide.

Figure 8: IC-FPOP is effective in probing the solvent accessibility of proteins. (**A**) Extent of modification for the 9 oxidatively modified peptides from actin. Values are shown as averages plus and minus standard deviation (n = 3). (**B**) Modification of actin peptides oxidized in vitro by synchrotron radiolysis from Guan et al.[10] (**C**) Correlation of residue FPOP modifications with SASA in the tight (triangles, dashed trend line) and open (circles, solid trend line) states of actin. This figure has been modified from Espino et al.[8]

DISCUSSION:

Several mass spectrometry-based techniques have been developed to study protein structure and protein-ligand complexes in a proteome-wide manner in whole cells or cell lysates. These techniques include but are not limited to stability of proteins from rate of oxidation (SPROX), thermal proteome profiling (TPP), chemical cross-linking (XL-MS), and hydroxyl radical protein footprinting (HRPF). Each technique has unique limitations and advantages compared to one another, which have been extensively reviewed[12]. Each of these methods have been used for proteome wide structural biology to elucidate protein structure and ultimately function within the complex cellular environment. IC-FPOP is a HRPF technique that utilizes hydroxyl radicals to oxidatively modify solvent exposed side chains of amino acids, probing protein structure and protein-ligand interactions within viable cells[13]. IC-FPOP is an improvement to initial HRPF in live

cells that used Fenton chemistry to generate radicals on the minutes timescale[14]. In this study, structural changes in an integral membrane protein in response to lowering the pH or ionic strength of the buffer were successfully characterized with good oxidation coverage across the protein. Compared to Fenton chemistry, IC-FPOP is much faster, modifying proteins on the microsecond timescale, thus enabling the native protein conformation to be studied.

A key test for IC-FPOP is to confirm the viability of the cells following exposure to H_2O_2. Using a 40 cm mixing line, the cells are incubated in H_2O_2 for roughly 3 s before irradiation. This time can be adjusted by changing the length of this silica tubing. It is noteworthy that although the use of trypan blue to test cell viability shows the integrity of the cells are sustained following H_2O_2 incubation, the cells could potential be under stress effecting signaling pathways that interact with H_2O_2. Fortunately, the short incubation time is faster than protein synthesis providing confidence the proteins present are not induced by H_2O_2.

The next important step is to confirm proper assembly of the flow system. Once assembled, ensure there are no leaks present after flushing the system with the desired buffer. If leaks are present, make sure that the silica tubing was cut properly and is flush against the ferrule to make a proper seal once tightened down. All parts are hand-tightened, so no tools are necessary. During each IC-FPOP experiment, make sure that the magnetic stirrers in the cell syringe remain in motion. This small agitation limits the number of cells that settle at the bottom of the syringe but is not harsh enough to shear the cells. After one run, there is roughly 50 µL of cells left in the syringe. Always make sure to dilute this out with a rinsing step to limit the number of cells that carry over to the next experiment. It is recommended to use a fresh cell syringe if multiple cell treatments are being compared. It is also important to select an appropriate buffer for the cells being tested. Some buffers quench the hydroxyl radical leading to fewer modifications on proteins. Xu et al. have shown that some commonly used buffers decrease the hydroxyl radical lifetime[11]. DPBS and HBSS are common buffers used for IC-FPOP experiments.

Following IC-FPOP, optimize the digestion protocol based on the parameters needed. Since FPOP produces irreversible covalent modifications, there is ample time available for a thorough digestion and clean-up without losing the labeling coverage. Always test and normalize the protein concentration so uniform peptide concentrations are loaded for tandem mass spectrometry. Finally, be mindful that an immunoprecipitation cannot be performed in conjunction with IC-FPOP. If an FPOP modification targets the region of interaction the affinity of the antibody will be lowered. To help increase the identification of FPOP modifications, 2D-chromatographic separation steps have shown to more than triple the number of oxidized peptides detected.[15]

A challenge of any FPOP experiment is the complicated level of data analysis due to the possible oxidation products that can arise. This is true for both in-cell or in vitro but is drastically increased with the added complexity of analyzing cell lysates. With further optimization of IC-FPOP more proteins with higher modification coverages are arising, thus expeditiously making analysis more arduous. The shear amount of data generated from a single IC-FPOP experiment limits the use of manual validation causing researchers to rely more heavily on software. Due to this, Rinas et al.

developed a quantitation strategy for HRPF using Proteome Discoverer (PD)[16]. This method utilizes a multi-search node workflow on PD combined with a quantitative platform in a spreadsheet. Further improvements on the IC-FPOP platform are underway to increase the number of identified peptides with FPOP modifications along with increased reproducibility and quantitation accuracy.

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DISCLOSURES:

Lisa Jones is an inventor on the flow assembly for cells patent (publication number: 20180079998).

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Figure

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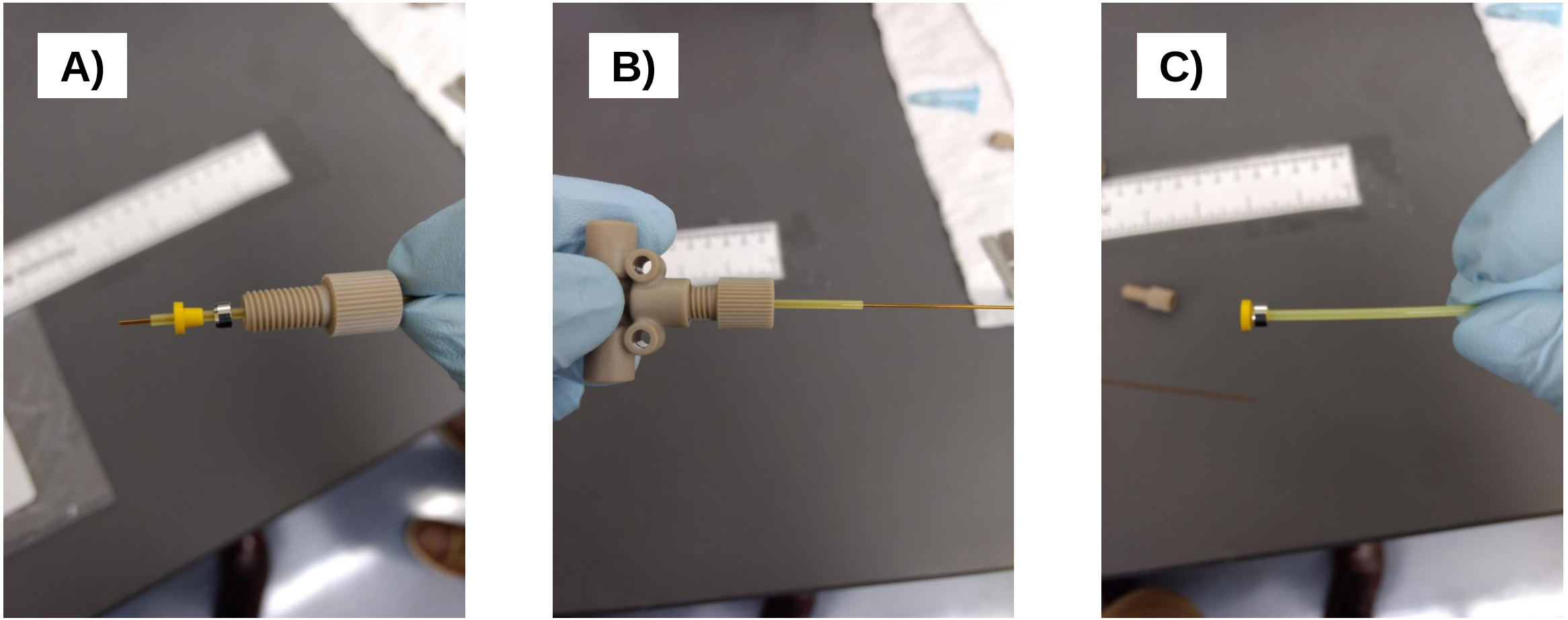

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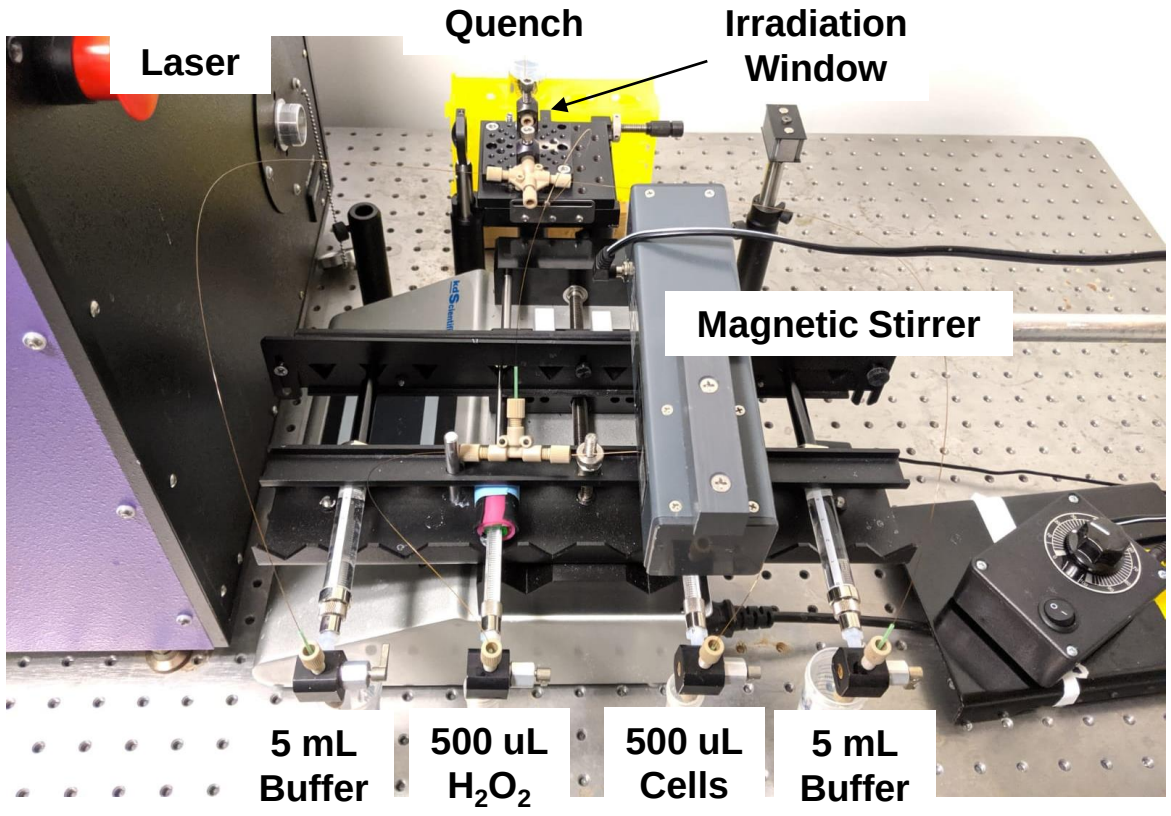

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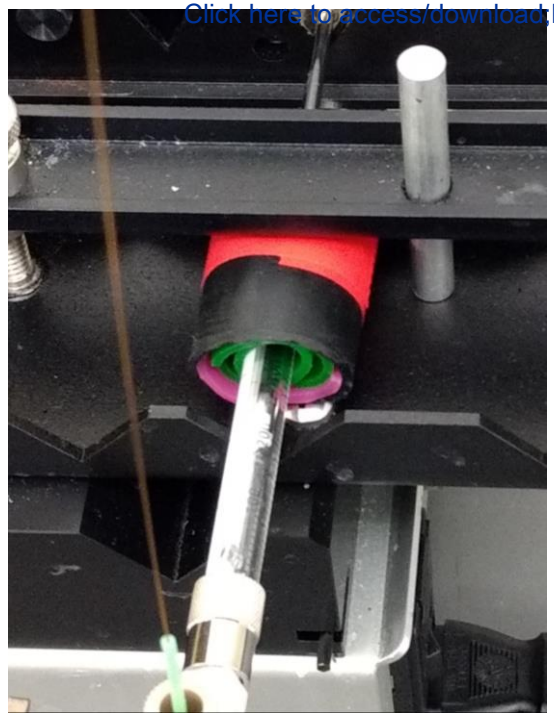
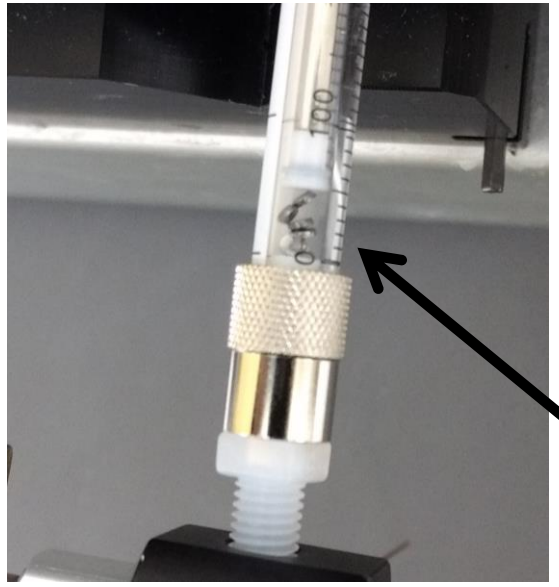
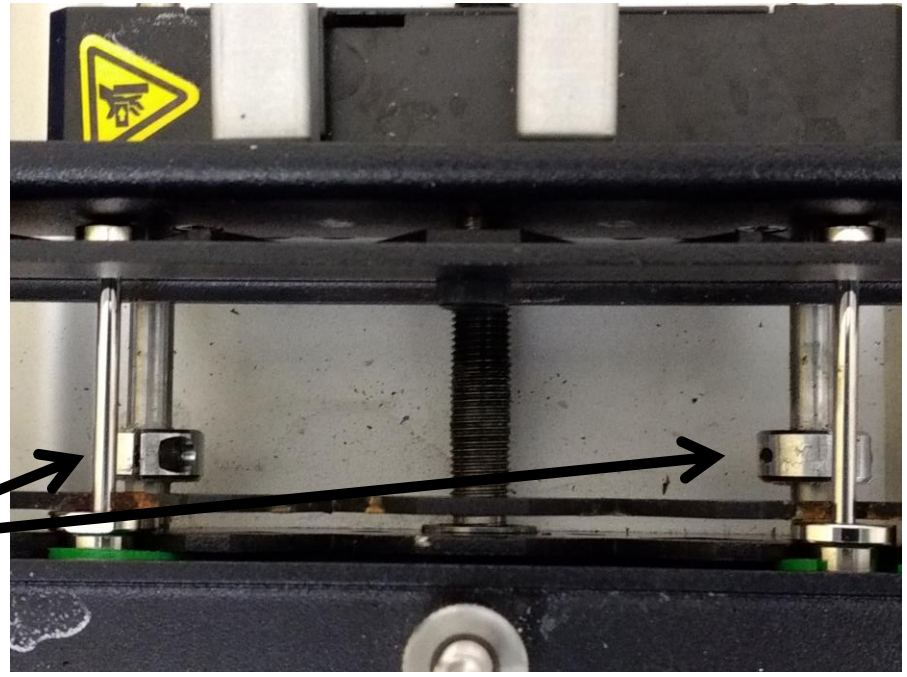


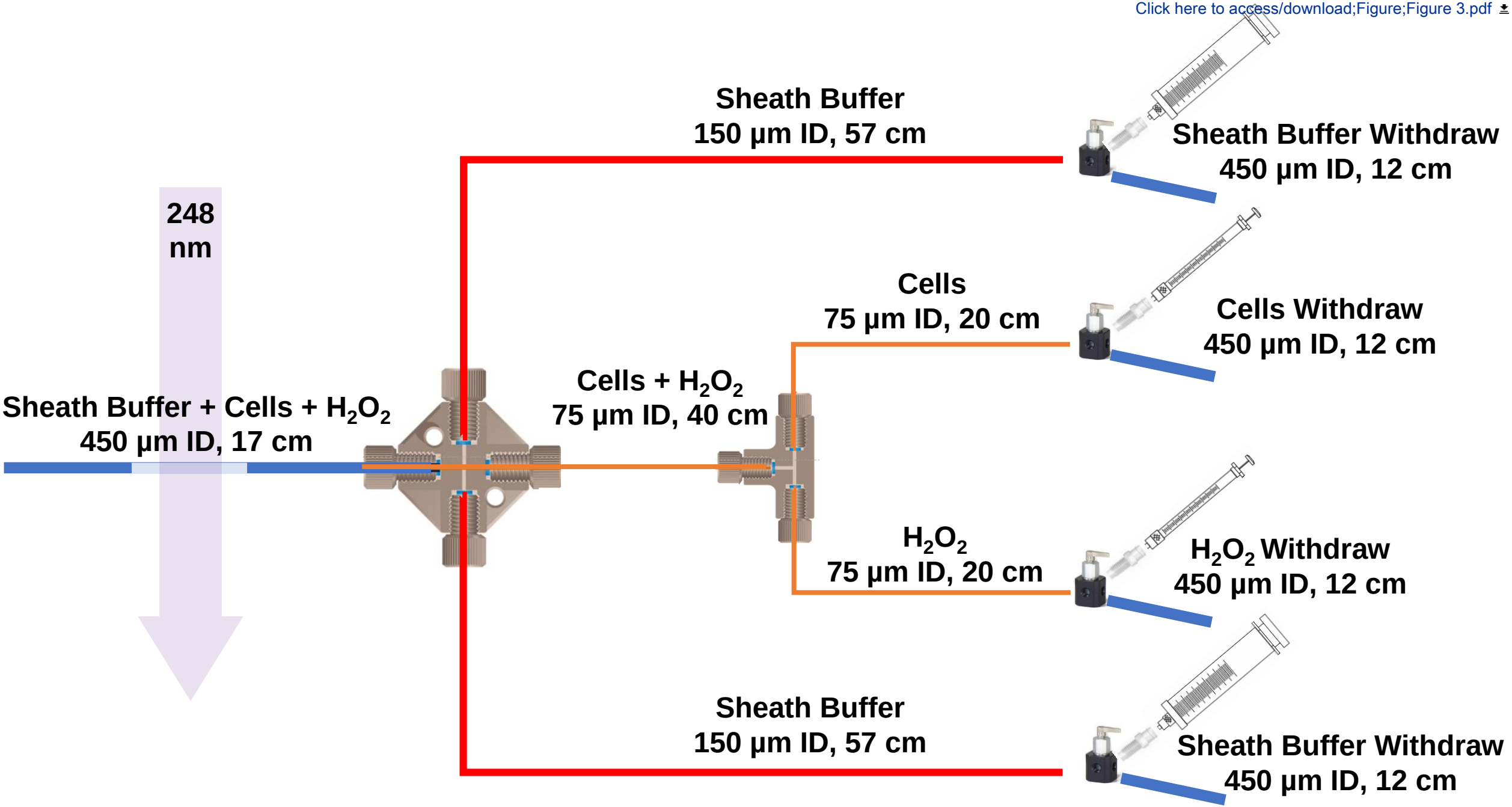
C)

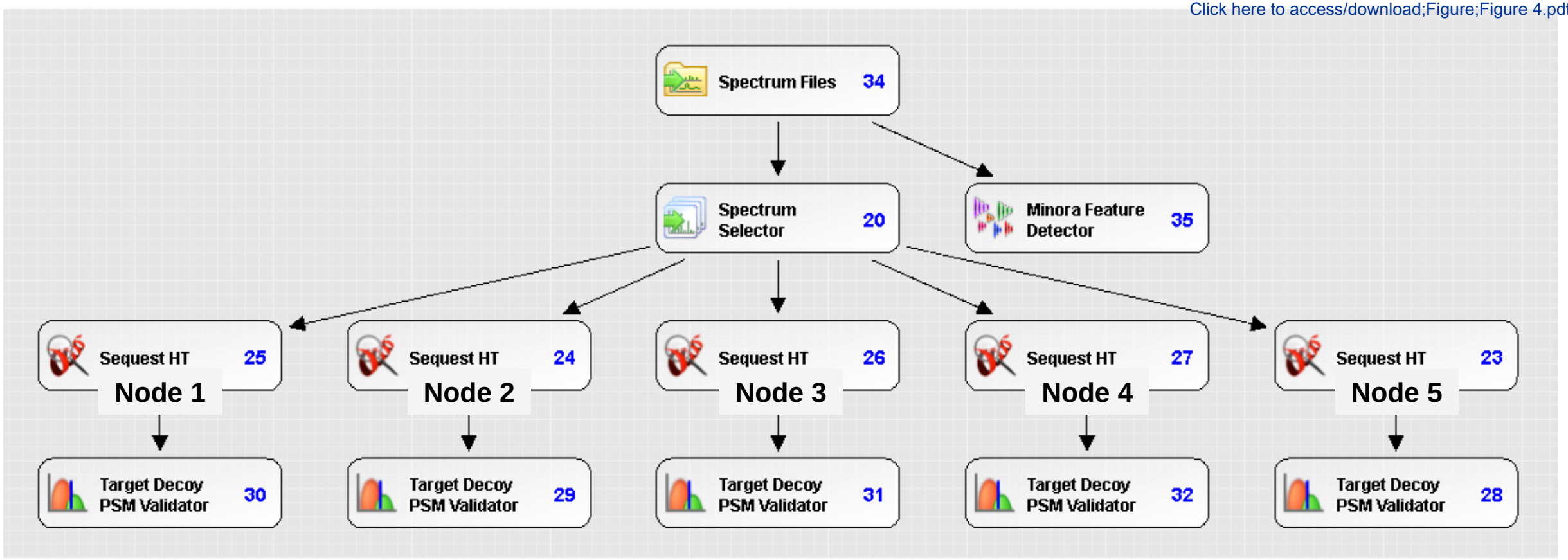


D)



Leave room for the magnetic stirrers





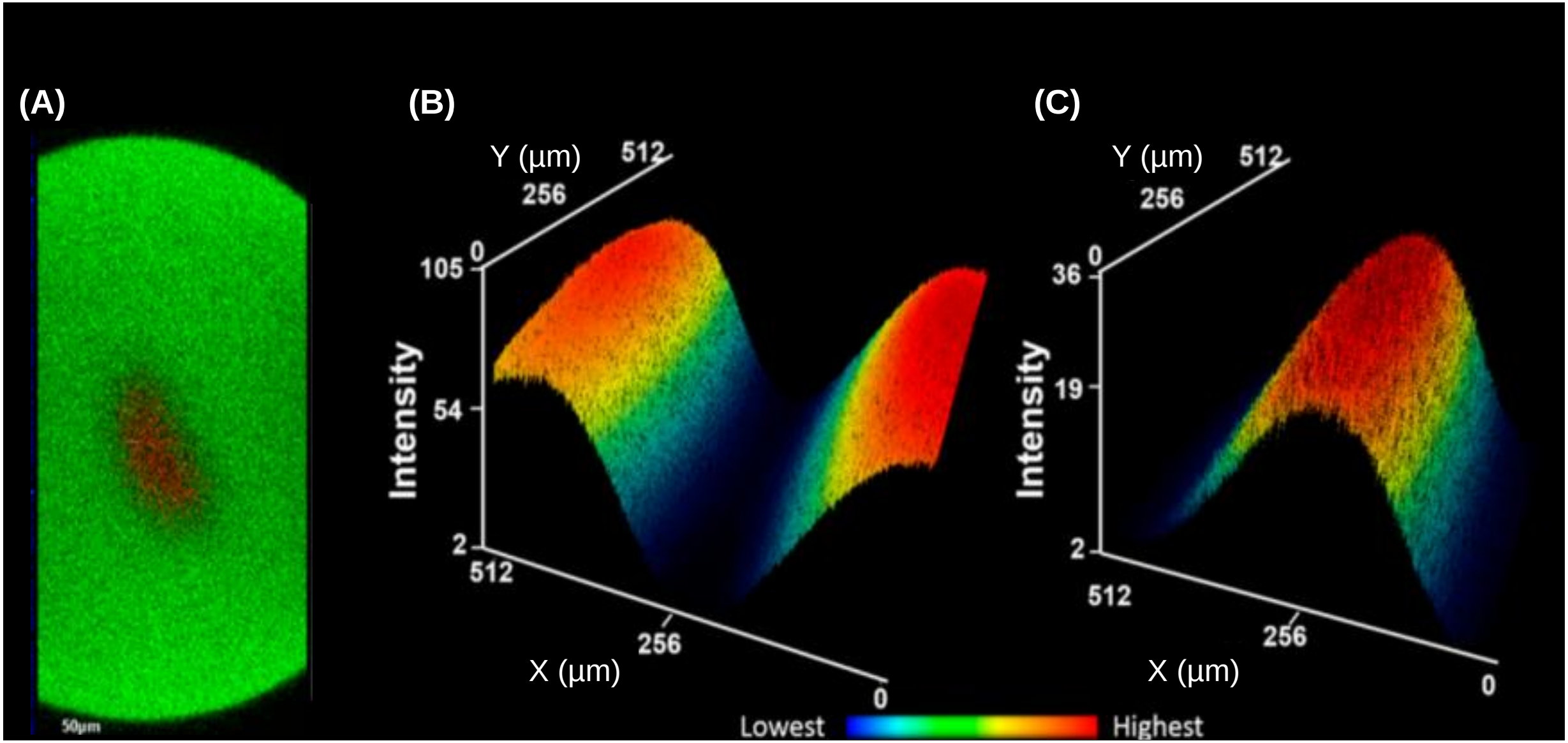
Node 1	
Dynamic Mod	+13.979 Da (E,I,K,L,P,Q,R,V)
Dynamic Mod	+31.990 Da (C,F,M,W,Y)
Dynamic Mod	-10.032 Da (H)

Node 2	
Dynamic Mod	+15.995 Da (F,H,I,L,V,W,Y)
Dynamic Mod	+15.995 Da (A,D,E,K,N,P,Q,R)
Dynamic Mod	+15.995 Da (M)

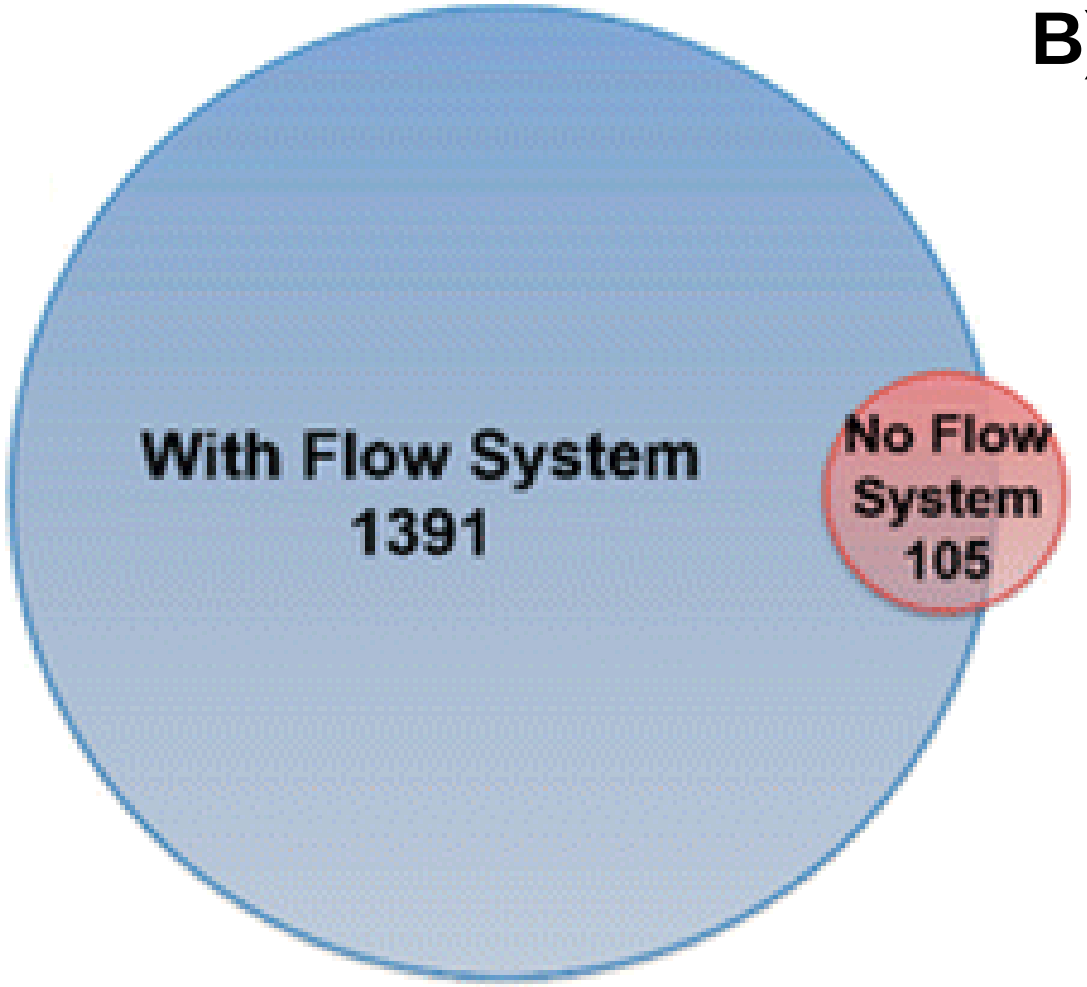
Node 3	
Dynamic Mod	+47.985 Da (C,F,W,Y)
Dynamic Mod	-27.995 Da (D,E)
Dynamic Mod	-30.011 Da (D,E)
Dynamic Mod	+4.979 Da (H)
Dynamic Mod	-43.053 Da (R)

Node 4	
Static Mod	+57.021 Da (C)

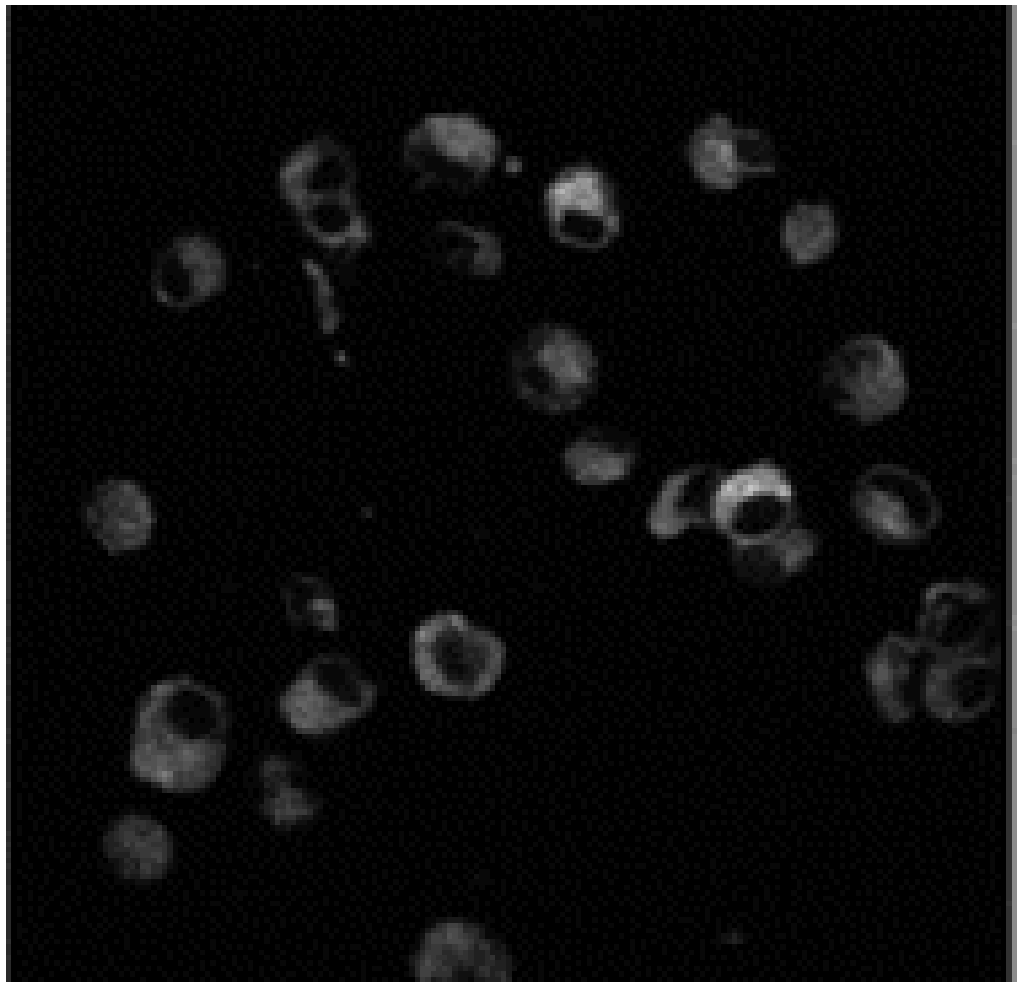
Node 5	
Dynamic Mod	-43.990 Da (D,E)
Dynamic Mod	-22.032 Da (H)
Dynamic Mod	-23.016 Da (H)

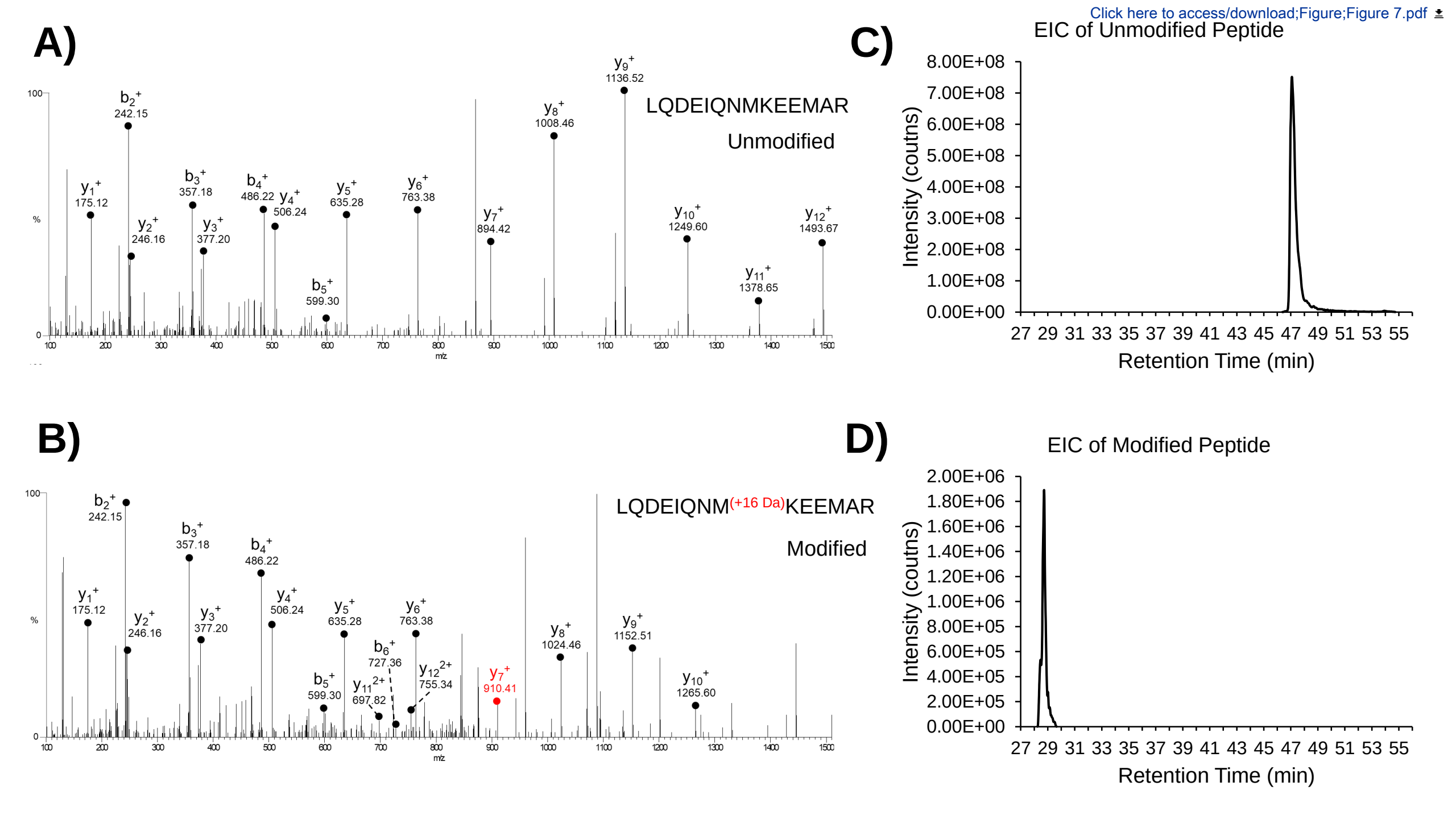


A)



B)





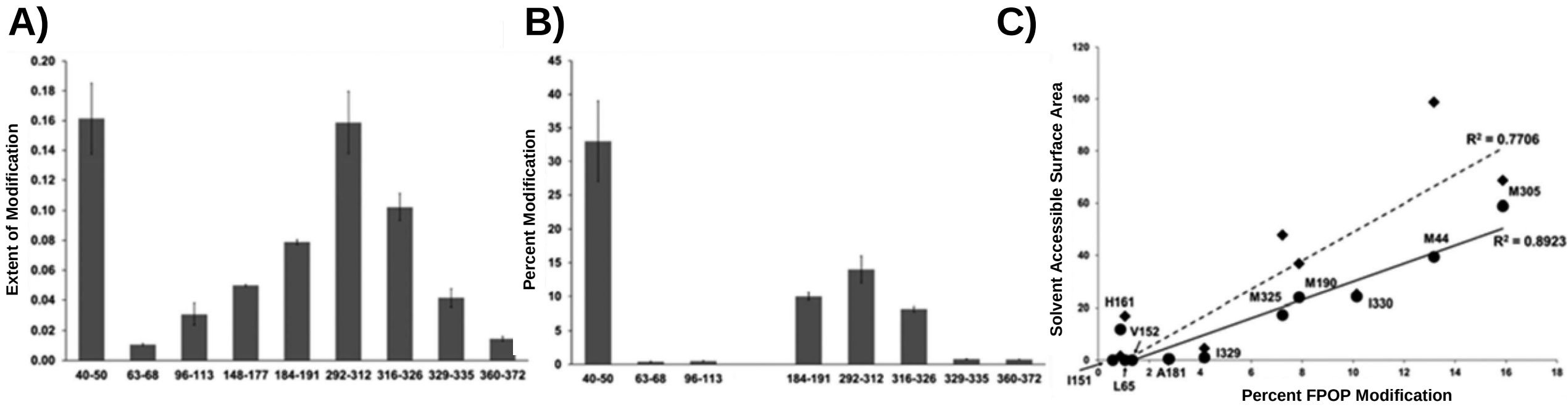


Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
15 mL Conical Centrifuge Tubes	Fisher Scientific	14-959-53A	any brand is sufficient
5 mL Gas Tight Syringe, Removable Luer Lock	SGE Analytical Science	008760	
50 mL Conical Centrifuge Tubes	Fisher Scientific	14-432-22	any brand is sufficient
500 µL SGE Gastight Syringes: Fixed Luer-Lok Models	Fisher Scientific	SG-00723	
Acetone, HPLC Grade	Fisher Scientific	A929-4	4 L quantity is not necessary
Acetonitrile with 0.1% Formic Acid (v/v), LC/MS Grade	Fisher Scientific	LS120-500	
ACQUITY UPLC M-Class Symmetry C18 Trap Column, 100Å, 5 µm, 180 µm x 20 mm, 2G, V/M, 1/pkg	Waters	186007496	
ACQUITY UPLC M-Class System	Waters	ACQUITY UPLC M-Class System	

Aluminum Foil	Fisher Scientific	01-213-100	any brand is sufficient
Aqua 5 µm C18 125 Å packing material	Phenomenex	04A-4299	
Centrifuge	Eppendorf	022625501	
Delicate Task Wipers	Fisher Scientific	06-666A	
Dithiothreiotol (DTT)	AmericanBio	AB00490-00005	
DMSO, Anhydrous	Invitrogen	D12345	
EX350 excimer laser	GAM Laser	EX350 excimer laser	
FEP Tubing 1/16" OD x 0.020" ID	IDEX Health & Sciene	1548L	
Formic Acid, LC/MS Grade	Fisher Scientific	A117-50	

HV3-2 VALVE	Hamilton	86728	
Hydrogen Peroxide	Fisher Scientific	H325-100	any 30% hydrogen peroxide is sufficient
Iodoacetamide (IAA)	ACROS Organics	122270050	
Legato 210 syringe pump	KD Scientific	788212	Any syringe pump that can hold 4, 5 mL syringes, withdraw and expel liquid, and have a way to stale the motor should work
Luer Adapter Female Luer to 1/4-28 Male Polypropylene	IDEX Health & Sciene	P-618L	
Methanol, LC/MS Grade	Fisher Scientific	A454SK-4	4 L quantity is not necessary
Microcentrifuge	Thermo Scientific	75002436	
N,N’-Dimethylthiourea (DMTU)	ACROS Organics	116891000	
NanoTight Sleeve Green 1/16" ID x .0155" ID x1.6"'	IDEX Health & Sciene	F-242X	

NanoTight Sleeve Yellow 1/16" OD x 0.027" ID x 1.6"	IDEX Health & Sciene	F-246	
N-tert-Butyl-α-phenylnitrone (PBN)	ACROS Organics	177350250	
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
PE50-C pyroelectric energy meter	Ophir Optronics	7Z02936	
Pierce Quantitative Colorimetric Peptide Assay	Thermo Scientific	23275	
Pierce Rapid Gold BCA Protein Assay Kit	Thermo Scientific	A53225	
Pierce Trypsin Protease, MS Grade	Thermo Scientific	90058	
Pierce Universal Nuclease for Cell Lysis	Fisher Scientific	88702	
Polymicro Cleaving Stone, 1" x 1" x 1/32”	Molex	1068680064	any capillary tubing cutter is sufficient

Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 150 µm, Outer Diameter 360 µm, TSP150350	Polymicro Technologies	1068150024
Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 450 µm, Outer Diameter 670 µm, TSP450670	Polymicro Technologies	1068150025
Polymicro Flexible Fused Silica Capillary Tubing, Inner Diameter 75 µm, Outer Diameter 360 µm, TSP075375	Polymicro Technologies	1068150019
Potassium Phosphate Monobasic	Fisher Scientific	P382-500
Proteome Discover 2.2 (bottom-up proteomics software)	Thermo Scientific	OPTON-30799
Rotary Magnetic Tumble Stirrer	V&P Scientific, Inc.	VP 710D3
Rotary Magnetic Tumble Stirrer, accessory kit for use with Syringe Pumps	V&P Scientific, Inc.	VP 710D3-4
Super Flangeless Ferrule w/SST Ring, Tefzel™ (ETFE), 1/4-28 Flat-Bottom, for 1/16" OD	IDEX Health & Sciene	P-259X
Super Flangeless Nut PEEK 1/4-28 Flat-Bottom, for 1/16" & 1/32" OD	IDEX Health & Sciene	P-255X

Super Tumble Stir Discs, 3.35 mm diameter, 0.61 mm thick	V&P Scientific, Inc.	VP 722F
Thermo Scientific Pierce RIPA Buffer	Fisher Scientific	PI89900
Tris Base	Fisher Scientific	BP152-500
Upchurch Scientific Low-Pressure Crosses: PEEK	Fisher Scientific	05-700-182
Upchurch Scientific Low-Pressure Tees: PEEK	Fisher Scientific	05-700-178
UV Fused Silica Plano-Convex Spherical Lenses	THORLABS	LA4052
V&P Scientific IncSupplier Diversity Partner TUMBL STIR DISC PARYLENE 1000	V&P Scientific, Inc.	VP724F
VHP MicroTight Union for 360µm OD	IDEX Health & Sciene	UH-436
Water with 0.1% Formic Acid (v/v), LC/MS Grade	Fisher Scientific	LS118-500

Water, LC/MS Grade	Fisher Scientific	W6-4

Editorial comments:
The manuscript has been modified and the updated manuscript, **60911_R0.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- Manuscript has been proofread for spelling and grammar issues.

2. 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].”

- All copyright permissions have been obtained.

3. Please highlight complete sentences (not parts of sentences) for filming.

- The highlighted sections are all complete sentences.

4. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next.

- The highlighted steps form a cohesive narrative with a logical flow.

5. Please use greek characters for SI unit prefixed, e.g. use ‘µL’ instead of ‘uL’.

- All necessary SI units have been replaced with Greek characters.

6. Please use h, min, s for time units.

- All time units have been replaced using h, min, or s.

7. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. 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.

- All commercial language has been removed and replaced with generic terms.

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

- All personal pronouns have been removed.

9. Step 6.5: Please write this step in the imperative tense.

- Step 6.5 has been rewritten in imperative tense.

10. Step 6.6: Please write this step in the imperative tense.

- Step 6.6 has been rewritten in imperative tense.

11. Step 7.2: Please write this step in the imperative tense.

- Step 7.2 has been rewritten in imperative tense.

12. Step 7.3: Please write this step in the imperative tense.

- Step 7.3 has been rewritten in imperative tense.

13. Step 7.4: Please write this step in the imperative tense.

- Step 7.4 has been rewritten in imperative tense.

14. Please revise the Discussion to 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 discussion has been revised to go into more detail covering the above.

15. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

- All tables have been converted to a .xls file.

16. Figure 2A: Please use µL instead of uL.

- Figure 2A now includes µL instead of uL.

17. Figure 5B: Please add a scale bar.

- Unfortunately, this image was modified from Espino et al., so no scale bar is available.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:

Jones et al. outline the procedure for performing IC-FPOP using a sophisticated instrumentation and experimental process. Overall the procedure is well written, but not without its difficulty to understand. However, the complementary video will likely lessen the confusion.

Major Concerns:
This reviewer has no major concerns with the protocol description, however this reviewer has the following comments.

-How are the authors ensuring there is similar levels of oxidation for each of their irradiated samples being compared? The variability for any giving peptide on a single protein under well controlled in vitro FPOP experiments can be challenging as it is. There is no discussion of the use of a dosimeter for this work, which appears to be gaining traction as a common practice for FPOP analysis.

- This is an excellent point and in sharing the reviewer's concern, this is a challenge we are still working on. But right now, this is beyond the scope of this work.

-In this reviewer's experience, vacuum drying peptides to complete dryness leads to variability in recovery and potentially effecting the % modification calculation, which is not addressed in the current workflow. How are the authors ensuring reproducible recovery of peptides?

- We thank the reviewer for their comment. Analyzing the whole cell lysate using LC-MS/MS there is always some extent of variability between peptides and proteins detected. This variation between two experiments is not well-understood but may be attributed to the cell-to-cell variability in protein expression. Therefore, all experiments run triplicates of each condition along with a biological replicate of the experiment. The effect of vacuum drying the peptides could be adding to the variability, but tests in simpler systems, like purified proteins, the peptide coverage between the triplicate runs are often very similar and reproducible after vacuum drying. Further tests in the effect of vacuum drying needs to be done using the whole cell lysate.

-Proteomic analysis based on peptide identification has high false positive rates. How do the authors ensure accurate interpretation of the search engine data?

- Thank you for your concern, it is very important to ensure we remove all false positives in our search. To remove any peptides with high false positive rates we search all our peptides and FPOP modifications against a decoy database. If the false discovery rate was less than 1% we classify it as a high confidence peptide and if the false discovery rate was less than 5% we classify it as a medium confidence peptide. Step 7.4 in the protocol tells to search against a decoy

database.

This reviewer suggests that the authors address these potential areas of assay variability and data interpretation, or specifically indicate these have been addressed in previous work.

Minor Concerns:
-The authors indicate in the abstract that 19 of 20 AA can be modified but only how 17 in their table (which is not numbered, labeled or refer to in the text)

- We appreciate the reviewer's comment. Although 19 out of the 20 amino acids can react with hydroxyl radicals, serine and threonine have lower reactivity compared to the other 17 amino acids, so we do not search for these modifications. I added this information to the data processing section (7.3 note).

-It is unclear to this reviewer how the author are representing their data in Fig 7 based on their description of the equation on line 265. Graphing "Extent of Modification" does not appear to fit this equation. Some numbers are less than 1 others look like percentages. Please address.

- We appreciate the reviewer's observation. Figure 7A follows the equation on line 265 providing the "extent of modification" while Figure 7B comes from a modified figure from Guan et al. which is the percent extent of modification which multiples equation on line 265 by 100. The figure legend as well as the figure have been corrected to state percent modification.

-It is unclear how the capillary is cut and the window is burned. The use of MicroSolve Window Maker (Cat# 07200-S) and Cleavage Tiles (Cat# 10101-T) are effective for ensuring consistent capillary preparations. If the authors are using other techniques, please explain and consider referring to these items as alternatives.

- Thank you for your suggestion to clarify the protocol. I added that we use a cleavage stone to cut the silica tubing (1.1 NOTE) and as well as using a lighter to burn the window (1.6).

-It would also be helpful if the " Name of the Material/Equipment" section was combined in a single table with the "company, catalog #, comments/description" section so that the audience does not have to match these themselves.

- Thank you for this suggestion. The PDF formatting has been corrected so all sections line up.

Reviewer #2:

Manuscript Summary:

The manuscript by Jones et al. describes in great detail the setup for fast photochemical oxidation of proteins in cells. The work certainly deserves a broad audience and will be of interest to many scientists. After revision, it should be published in JOVE.

Major Concerns:

- p.8/Fig.4: How was the imaging of sheath buffer vs cell suspension performed, i.e. which dyes were used and was the viscosity of the fluids unaltered compared to regular buffer and cell suspension?

    - We appreciate the reviewer's comment. Fluorophores were added to each solution. The sheath buffer was PBS with FITC while the cell and hydrogen peroxide mixture contained TMRM. The viscosity effect of the fluorophores is not significant. The fluorophore information was added to the representative results for clarification.

- p.8: is it possible to recultivate cells after the shorter illumination but higher H2O2 concentration? Please briefly state for the non-expert reader why CELLROX imaging is indicative of non- detrimental consequences. How is this condition defined?

    - We appreciate the reviewer's comment. We have never attempted to recultivate the cells following IC-FPOP. Although the cells are viable before irradiation, I assume the cells lifetime is very limited following irradiation due to the extent of oxidation that not only modifies the protein but also lipids and DNA. The CellRox following IC-FPOP does show the cell remains intact so we can confidently say we modify the proteins in their native cellular environment. The representative results were edited to better convey the significance of the CellROX images.

- p.8, l.284 ff: do you also observe labelled proteins from closed compartments, such as mitochondria, ER/Golgi/lysosomes? In other words, what are the limits in terms of H2O2 diffusion into cellular compartments? It is nevertheless exciting that nuclear proteins can be observed.

    - We thank the reviewer for their question. We do observe proteins from several cellular compartments including closed compartments. The mentioned compartments (cytoplasm, membrane, and nucleus) show the highest number of proteins detected. Slight re-wording in the representative results has been made to state many of the cellular compartments show FPOP modifications.

- References to previous work presented are mostly provided in figure legends, from the text this remains unclear. Please reference correctly.

    - We appreciate the reviewer's observation. The previous work is now properly referenced in the representative results.

Minor Concerns:
- abstract, l.34: FOR IC_FPOP - for not in capitals?

- We thank the reviewer for their observation. The capitalization has been corrected.

- p.4, l.93/94 - the reference to the 5 ml syringe is unclear. The authors only mention 500 µl syringes before.

- We appreciate the reviewer's comment. A reference to the 5 mL syringe has been added earlier in the text (1.3).

- p.4, l.123 - introduce abbreviations of chemicals upon first use.

- We thank the reviewer for their observation. All chemicals mentioned have been written out upon first use.

- p.5, l.171-172: Vol% for water methanol mix?

- We appreciate the reviewer's comment. On page 4, the mixture was corrected to 50 % water: 50 % methanol to indicate the mixture used.

- p.7, l.228 - 0.1% formic acid in acetonitrile (B)?

- We thank the reviewer for their observation. On page 6, the sentence was corrected to 0.1% formic acid in acetonitrile.

- p.10, l.357/358: should read: "...extent of oxidation detected in MS of the intact protein." or sth alike.

- We thank the reviewer for their observation. The discussion section has been edited to remove this specific sentence.

- units of y and x axes missing in Fig.4

- We thank the reviewer for their observation. The X and Y units on Figure 4B and 4C are now included.

- materials list: sometimes catalogue number is missing, e.g. for LC and MS equipment from Waters or Thermo Scientific. If catalogue numbers are not available, please provide the product name, just a brand is insufficient.

- We thank the reviewer for their observation. All materials with a catalogue number are now included. The materials without a catalogue number now states the product name in this section.

Reviewer #3:

Manuscript Summary:
In this manuscript Chea et al., describe the experimental setup for performing in cell fast photochemical oxidation of proteins. The workflow presented here has been pioneered by the Jones lab and as a result they are best placed to write such a detailed protocol. I have no major concerns with the manuscript, and it should be published after minor revisions.

Major Concerns:
None

Minor Concerns:
*What factors determine the suitability of the Excimer laser for FPOP (Laser power etc)? How is the laser set up and is the beam focused? What is the size of the laser irradiation window in the authors' setup?

- Excimer laser can generate a fast pulse at 248 nm can photolyze hydrogen peroxide, but protein and water absorption are limited. The reference to the original FPOP paper is added to the introduction to provide further information on the suitability of the excimer laser for FPOP. We added the information of focusing the laser with the concave lens. The lens we use has been added to the materials list. We also have included the minimal laser energy needed for IC-FPOP. Also, the laser irradiation window in our setup is added in the note following protocol 1.9.

*Line 117. How is the exclusion fraction calculated?

- We thank the reviewer for their question. The calculation to determine the exclusion fraction or frequency have been added as a note below step 1.9.

*Line 152. Why is DMSO added to the quench?

- We appreciate the reviewer's question. The addition of DMSO will inhibit endogenous methionine sulfoxide reductase. This information was added into the protocol.

*Line 155, 139 and 149. Which buffer do the authors recommend to flush the system with, and for resuspending cells?

- We appreciate the reviewer's comment. A note was added stating, "Typical buffers to use are cell culture grade DPBS or HBSS."

*Line 208 - How are the samples dried?

- We appreciate the reviewer's question. The following was added to describe how the samples dried, "by leaving the caps of the microcentrifuge open with a lint free wipe covering the top."

*It may be useful for the reader for the authors to show extracted ion chromatograms in a figure.

- We thank the reviewer for the suggestion and have added extracted ion chromatograms to figure 4.

*In the discussion the authors state it is important to ensure cells are viable after peroxide incubation/laser irradiation. Do the stains used by the authors detect cell viability or cell integrity? I imagine the treated cells are not healthy. Does this matter?

- We appreciate the reviewer's comment. To test various cells integrity following hydrogen peroxide incubation, we have used trypan blue staining which is a very common method for measuring cell viability. Once cells have been compromised the dye can penetrate the membrane. So, with this assay we can be confident the integrity of the cell is sustained, but we do not know how stressed the cell is. With such a quick incubation time we hypothesize there isn't major effects on the proteins present in the cell, but there could be protein interaction changes taking place within signaling cascade of hydrogen peroxide. This could be a potential limitation with this method. The following was added to the discussion to cover this topic: "It is noteworthy that although the use of trypan blue to test cell viability shows the integrity of the cells are sustained following hydrogen peroxide incubation, the cells could potential be under stress effecting signaling pathways that interact with hydrogen peroxide. Fortunately, the short incubation time is faster than protein synthesis providing confidence the proteins present are not induced by hydrogen peroxide."

*Line 356. Is the effect of buffer-induced quenching substantially more than quenching by cellular factors?

- We thank the reviewer for their insightful comment. There are many endogenous species within the cell which will quench the hydroxyl radical, posing a good question, how much more would the buffer further quench the radical? To this point, we have attempted at increasing the number of FPOP modifications on proteins by increasing the radicals present, so we have always avoided radical quenching buffers. So, this question is beyond the scope of this paper but how big of an effect radical quenching buffers have on IC-FPOP could be tested.

*It may be useful for the authors to add a discussion of how their IC-FPOP approach is complementary to other in-cell footprinting methods. This will add additional context to their work.

- We appreciate the reviewer's comment. There are very limited studies to date that utilize footprinting methods in live cells, but multiple mass spec-based

techniques are being developed to study protein structure and protein-ligand interactions. A reference to a review has been added along with another reviewer about using HRPF to probe macromolecular interactions in cells.

The manuscript should be thoroughly proofread prior to publication. I found numerous typographical errors when reading. Below is a list of the errors I found in the manuscript, but there may be others.

- We appreciate the reviewer's corrections. All errors mentioned below have been corrected in the manuscript.

*Line 34, FOR should not be capitalised
*Line 34, change passed to past
*Line 43, change alter to altering
*Line 44, change carried out to interrogated
*Line 50, change for to "an approach called" or something similar
*Line 58 change compartments to localisation
*Line 137, change scrapper to scraper
*Line 171, change 50 water:50 methanol to 50 % water: 50 % methanol
*Line 177, Full stop after Thermo Fisher Scientific.
*Line 184, Change 16 XG to 16,000 xg
*Line 203 and 214, Change "Next morning" to "The next morning"
*Line 208, Change After dried… to "After the samples have dried resuspend the protein pellet"
*Line 211, 1trypsin:50 sample. What do the authors mean? Is this a mass:mass ratio?
*Line 228, change can to ACN.
*Line 236, change ramp to ramping
*Line 239, Add "The" to the start of the sentence.
*Line 251, change "and the digest enzyme" to "and the relevant digest enzyme"
*Line 280, change shows to show
*Line 287, remove "to be"
*Line 297, remove adapted

Reviewer #4:
In general I find this to be a nicely detailed and clear protocol that describes how the structures of proteins in cells can be studied by a powerful new tool. Below I have several suggested changes to improve the clarity of the manuscript and to provide better rationale for how and why certain steps are conducted.

1. Is FOR fully capitalized in the abstract for some reason? Is this an acronym for something?

- We thank the reviewer for their observation. The capitalization has been corrected.

2. On page 2, the authors indicate in their NOTE that the 'silica' coating needs to be scraped away. To be clear, are they referring to the polyimide coating on the fused silica that needs to be scraped away? Please clarify.

- We thank the reviewer for their comment. The note is corrected to state the polyimide coating is what needs to be scraped away.

3. Does the type of syringe pump matter for the experiments or will any syringe pump work as long as it has room for 4 syringes?

- We appreciate the reviewer's question. Any syringe pump that can hold at least 4, 5 mL syringes, withdraw and expel liquid, and have a way to stale the motor will work. This clarification is included in the material sheet.

4. For 1.9, it would be useful to show an example calculation.

- We thank the reviewer for their comment. The necessary equations have been added as a note under step 1.9.

5. Please define PBN and DMTU in 2.1.

- We thank the reviewer for the comment. Both PBN and DMTU have been defined when first mentioned.

6. For 3.3, if the cells are detached with trypsin-EDTA, does anything special beyond step 3.5 need to be done to remove trypsin or EDTA before MS analysis? If not, then the authors should add a note that step 3.5 helps remove trypsin.

- We thank for reviewer for their comment. Spin the cells down and removing the solvent also removed the trypsin-EDTA. This sentence now reads, "Spin down, remove the buffer and trypsin-EDTA, and resuspend to make 2e6 cells/mL."

7. In 4.7, define RIPA.

- We thank the reviewer for their comment. RIPA is now defined in the text.

8. In 5.16, what is the purpose of quantifying the peptide concentration? Please explain.

- We thank the reviewer for the question. The peptide assay is a quality control step to ensure all samples for MS are at the same concentration. This is clarified in step 5.17.

9. In 7.2, the authors mention that only 1 missed cleavage site can be considered. What is the rationale for this? Is it to facilitate the database searching, or is it for other reasons?

- We appreciate the reviewer's question. Having the mentioned limitations, including limiting to only 1 missed cleavage, is to facilitate the database searching.

10. At the beginning of the representative results section, the authors mention that H2O2 exposure time is limited to 3 seconds to minimize detrimental consequences for the cells. The second paragraph of this section describes how the authors check the 'stability' of the cells using imaging. By 'stability' I assume that they mean that the cells have not changed shaped or have not burst, but do they also check cell 'viability' to ensure that this H2O2 exposure time is

not damaging/killing the cells? They mention that this is important in the Discussion section, but they do not describe how cell viability is checked beyond observing if cell shapes have changed. Please comment further on how viability is assessed. There are standard approaches for cell viability test, such as resazurin, crystal violet, or other assays.

- We appreciate the reviewer's comment. To test various cells integrity following hydrogen peroxide incubation, we have used trypan blue staining which is a very common method for measuring cell viability. Once cells have been compromised the dye can penetrate the membrane. So, with this assay we can be confident the integrity of the cell is sustained, but we do not know how stressed the cell is. With such a quick incubation time we hypothesize there isn't major effects on the proteins present in the cell, but there could be protein interaction changes taking place within signaling cascade of hydrogen peroxide. This could be a potential limitation with this method. The following was added to the discussion to cover this topic: "It is noteworthy that although the use of trypan blue to test cell viability shows the integrity of the cells are sustained following hydrogen peroxide incubation, the cells could potential be under stress effecting signaling pathways that interact with hydrogen peroxide. Fortunately, the short incubation time is faster than protein synthesis providing confidence the proteins present are not induced by hydrogen peroxide."

11. In the description of Figure 7, the authors use actin as an example. While not stated, I think the authors assume that actin in cells and actin in vitro are similarly solvent exposed. If this is their assumption, then it should be stated more explicitly.

- We appreciate the reviewer's comment. We do believe actin will have similar solvent accessibility for both in-cell and in vitro studies. This is stated within the representative result section.

Reviewer #5:

Manuscript Summary:

The manuscript by Lisa Jones and her coworkers describes the technique of using a microflow system to perform in-cell fast photochemical oxidation of proteins (IC-FPOP), FPOP has been widely adopted in the field of protein footprinting as a useful tool to study protein conformation and interactions, and professor Jones' group pioneered effort that expands the FPOP application to living cells. The improved IC-FPOP technique with sheath buffer flowing alongside cells was communicated in 2016 in an publication on Analytical Chemistry, and this JoVE publication explained in detail about how the in-house built fluidics constructed, how the IC-FPOP experiment is performed, how the sample is prepared for LC-MS, how data analysis is done and how the results demonstrates the viability of the method.

Major Concerns:

From the 2016 Analytical Chemistry paper, the reviewer understands that the flow dynamics had an focusing feature, utilizing a tapered inner capillary and outer capillary, as well as higher flowrate of the sheath flow. Figure 4 seems to be identical to what was in the 2016 publication but it is not clear to the reviewer, in the text, if this feature is still implemented in this JoVE publication, if not, the reviewer would like to know the reason, if yes, please provide more information on the fluidics setup.

- We appreciate the reviewers concern. In the 2016 publication Figure 1 does show tapered capillaries to explain and show a typical way to hydrodynamically focus cells. The IC-FPOP experimental parameters as well as the backpressure limitations of the syringe pump and fittings did not allow for the central capillary to be tapered. Figure 4 shows that the cells were hydrodynamically focused by inserting the silica tubing containing the cells into the larger ID for irradiation and incorporating the two sheath buffers on either side. With the sheath buffers in the 5 mL syringe and the cells and hydrogen peroxide in the 500 µL syringe, the sheath buffer is flowing 10X faster than the cells. IC-FPOP Flow system is set up the same as the original 2016 paper. A clarification is added in the text in a note following step 1.5.

The reviewer is also curious about the mixing efficiency of cell and H2O2 in the experiment, it seems that the flow dynamics of cell/H2O2 mixing stream is in the laminar flow space, similar to the center/sheath flow. If that is the case, what was done to ensure mixing is complete within the short time before cell/H2O2 stream reach the laser? Imaging evidence is welcomed as illustrated for the center/sheath flow.

- In sharing the reviewer's concern, multiple tests were performed in the original 2016 publication to ensure the cells and hydrogen peroxide mix sufficiently. By including the mixing tee connecting, we successfully observed the mixture of two dyes representing the cells and hydrogen peroxide. Following this mixture, they both will flow together in the "Cells + H_2O_2" silica tubing. By testing multiple tubing lengths, we observe with longer incubation times more FPOP modifications are detected. Further testing needs to be done to find the optimal incubation time to get the best FPOP coverage.

The reviewer found the data analysis which is a complicated task for this experiment (caused by number of proteins, number of modifications) worth more description and information, it would be helpful to attach the PD workflow with the publication and explain if manual validation is needed or one can solely rely on software.

- We thank the reviewer for their comment and share their concern. Data analysis is very complicated for any FPOP experiment be that in-cell or in vitro. This challenge of data analysis is now mentioned in the discussion section with a reference to the paper describing the quantitation strategy we use in PD. Also, a figure has been added (Figure 4) to show the PD workflow along with the modifications that are included in each search node.

Minor Concerns:
The reviewer is confused about the Wren diagram in Figure 4 A, it is clear that 1391 proteins are found to be oxidized with the flow system whereas 105 are found for no flow system, however, it looks as if there is a population in the proteins detected with oxidation by the no flow system outside of the population of proteins by flow system. If that is the case, could you please provide both overlapping and outside numbers and explain the differentiation, if that is not the case, could you please modify the wren diagram to clear confusion.

- We thank the reviewer for their comment. Out of the 105 proteins modified with no flow, 58 proteins were detected with the flow while 47 proteins were not detected with the flow system. The variation between the proteins modified in the two experiments is not well-understood but may be attributed to the cell-to-cell variability in protein expression. The overlap of the proteins is now included in the figure legend.

It would be helpful for align the mass range for Figure 6 A and B even if no higher mass fragments were observed.

- We thank the reviewer for their observation. The miss aligned spectra have been removed from the figure.

Title:	Development of a Microflow System for In-Cell Footprinting Coupled with Mass Spectrometry
Author:	Aimee Rinas, Vishaal S. Mali, Jessica A. Espino, et al
Publication:	Analytical Chemistry
Publisher:	American Chemical Society
Date:	Oct 1, 2016

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Title: In Cell Footprinting Coupled with Mass Spectrometry for the Structural Analysis of Proteins in Live Cells

Author: Jessica A. Espino, Vishaal S. Mali, Lisa M. Jones

Publication: Analytical Chemistry

Publisher: American Chemical Society

Date: Aug 1, 2015

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tel. 617.945.9051
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Title of Article:	Characterizing Cellular Proteins with In-cell Fast Photochemical Oxidation of Proteins
Author(s):	Emily E. Chea, Aimee Rinas, Vishaal S. Mali, Jessica A. Espino, and Lisa M. Jones

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