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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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Author Questionnaire

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

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

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

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

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

Current Protocol Length

Number of Steps: 13
Number of Shots: 29

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Dante Johnson:** This method provides solutions for today's demanding biological questions. From an in-cell FPOP experiment, we can study protein-ligand interactions, protein-protein interaction sites, and regions of conformational change.

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

1.2. **Dante Johnson:** The advantage of this technology is the use of an automated six-well plate-based IC-FPOP platform that makes it possible to grow cells and perform in cell FPOP studies right at the laser.

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

OPTIONAL:

1.3. **Dante Johnson:** This method can be used in pharmaceutical and biotechnology industries as well as major academic and clinical research institutes, especially by biological investigators in universities studying protein dynamics.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Setting up the platform incubator for IC-FPOP

2.1. To begin, unscrew the platform incubator from the positioning stage **[1]** and disconnect the temperature, gas, and humidifier lines **[2]**.

2.1.1. Talent unscrewing the platform incubator.

2.1.2. Talen disconnecting the lines of temperature, gas and humidifier.

2.2. Spray the incubator with 70% ethanol **[1]** and place it in the cell culture hood **[2]**.

2.2.1. Talent spraying the incubator with ethanol.

2.2.2. Talent placing the incubator in the cell culture hood.

2.3. Remove the six-well plate from the platform incubator **[1]**, secure the plate's original lid **[2]**, and confirm cell confluency using a microscope **[3]**. Then, place the six-well plate with confluent cells back in the platform incubator inside the cell culture hood **[4]**.

2.3.1. Talent removing the six-well plate from the platform incubator.

2.3.2. Talent securing the plate's lid.

2.3.3. Talent at the microscope.

2.3.4. Talent placing the plate in the cell culture hood.

2.4. Insert three precut tygon tubes in each well via the embedded ports by flushing the tubes to the well walls and secure them with custom 3D printed rings **[1-TXT]**. *Videographer: This step is important!*

2.4.1. Talent inserting precut tygon tubes in each well held by 3D printing rings.
TEXT: 15 cm, 1.59 ID tygon tubes

2.5. Prepare an integration software script for pump withdrawal **[1]**. Use one peristaltic pump to completely remove cell media from all six wells **[2]**.

2.5.1. Talent preparing integration software script for pump withdrawal.

2.5.2. Talent removing cell media using peristaltic pump.

2.6. Prime solvents in each channel of the other three peristaltic pumps **[1]**, then infuse hydrogen peroxide and quench buffer in their respective alternating tubes until the reagents reach the incubator ports **[2]**.

2.6.1. Talent priming solvents in each channel of three peristaltic pumps.

2.6.2. Talent infusing hydrogen peroxide and buffer in tubes.

2.7. Confirm that the laser beam has been angled correctly and reaches the incubator uninhibited. Check laser energy using an external sensor **[1-TXT]**. *Videographer: This step is difficult and important!*

2.7.1. Talent checking the laser beam angle. **TEXT: Single laser pulse at 160 mJ at 50 Hz and 27 kV**

~~2.7.2. Talent checking the laser energy~~. ~~**TEXT: single laser pulse of 160 mJ at 50 hz and 27 kV**~~

3. IC-FPOP in the platform incubator

3.1. Prepare the integration software script for pump infusion after confirming beam alignment **[1]**. Infuse 200 millimolar hydrogen peroxide at 35 milliliters per minute into the first well **[2]**. *Videographer: This step is important!*

3.1.1. SCREEN: 62153_screenshot_1. 0:00 – 0:15.

3.1.2. Talent infusing hydrogen peroxide into first well.

3.2. 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 **[1]**. Infuse 125 millimolar quench solution at 35 milliliters per minute into the first well immediately after the laser pulse **[2]**.

3.2.1. SCREEN: 62153_screenshot_2. 0:00 – 0:13.

3.2.2. Talent infusing the quench solution into the first well.

3.3. Manually move the positioning stage to align the next well with the laser beam **[1]** and repeat the process for all the wells **[2]**.

3.3.1. Talent positioning the stage to align well to the laser beam.

3.3.2. Talent repeating the infusion in another well.

3.4. In a cell culture hood, use a cell scraper to transfer the cells from each well into individual 15-milliliter conical tubes **[1]**. Centrifuge cells at 1200 times *g* for 5 minutes **[2]**. Discard the supernatant **[3]** and resuspend the cells in 100 microliters of RIPA lysis buffer **[4-TXT]**.

3.4.1. Talent scrapping and transferring cells from wells to conical tubes.

3.4.2. Talent centrifuging the cells.

3.4.3. Talent discarding the supernatant.

3.4.4. Talent adding RIPA lysis buffer to the cells. **TEXT: RIPA: Radio immunoprecipitation assay**

3.5. Transfer cells to individual 1.2-milliliter polypropylene tubes **[1]**. Flash freeze all samples in liquid nitrogen and place them in a minus 80-degree freezer until use **[2]**.

3.5.1. Talent transferring cells into tubes.

3.5.2. Talent flash freezing samples in liquid nitrogen.

3.6. To localize FPOP modifications, analyze the digested cell lysate using liquid chromatography-tandem mass spectrometry analysis **[1-TXT]**. Then, calculate the extent of modification **[2]**.

3.6.1. Talent performing LC-MS/MS. **TEXT: FPOP-Fast photochemical oxidation of proteins**

3.6.2. Talent at the computer calculating the extent of modification.

Results

4. Analysis of Cell Transfection Efficiency, Proteins Modified in Single Cell flow-system and PIXY, and Localization of IC-FPOP Modifications

4.1. To confirm that the platform incubator conditions are sufficient for cell culture at the laser platform, GCaMP2 *(pronounce 'G-camp-2')* was transiently transfected into HEK293T *(H-E-K-2-9-3-T)* and transfection efficiency for both plates was assessed via fluorescence imaging **[1-TXT]**.

4.1.1. LAB MEDIA: Figure 4A.**TEXT: HEK-Human embryonic kidney** *Video editor focus on the green circular points in the control and then gradually focus on the increase shown by green square points in the PIXY.*

4.2. A luciferase assay was performed to quantitate transfection efficiency **[1]**.

4.2.1. LAB MEDIA: Figure 4B. *Video editor focus on the control graph and gradual increase in the PIXY graph.*

4.3. FPOP modifications in HEK293T cells labeled in the flow system were compared to those labeled in the platform incubator. The platform incubator outperformed the flow system both in the number of proteins modified and the total FPOP coverage in those proteins **[1]**.

4.3.1. LAB MEDIA: Figure 5.

4.4. In the flow system, two modified peptides were detected, providing limited structural information **[1]**. However, five modified peptides spanning the actin sequence were detected in the platform incubator **[2]**.

4.4.1. LAB MEDIA: Figure 6A. *Video editor focus on the purple graph.*

4.4.2. LAB MEDIA: Figure 6A. *Video editor focus on the green graph.*

4.5. Tandem MS spectra of actin with modified proline in both systems and unmodified actin peptide are shown here **[1]**.

4.5.1. LAB MEDIA: Figure 6B.

4.6. The FPOP modified residues in the platform incubator samples contained twelve modified residues **[1]**, 3 modified residues in the flow system **[2]**, and 1 overlapping modified residue **[3]**.

4.6.1. LAB MEDIA: Figure 6C. *Video editor focus on the green labels.*

4.6.2. LAB MEDIA: Figure 6C. *Video editor focus on the purple labels.*

4.6.3. LAB MEDIA: Figure 6C. *Video editor focus on the yellow labels.*

4.7. LC−MS-MS analysis revealed that 792 proteins were modified by in vivo-FPOP in the platform incubator compared to the 545 proteins modified with the flow system **[1]**.

4.7.1. Figure 7.

Conclusion

5. Conclusion Interview Statements

5.1. **Dante Johnson:** It is imperative to keep cells under sterile conditions. Always bring the platform incubator into the sterile cell culture hood to insert all necessary six-well plates, tubing, and rings. This ensures the integrity of the experiment.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.2.*