FINAL SCRIPT: APPROVED FOR FILMING

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Title: Automated Sample Preparation for the Multiplexed Analysis of Single-Cell Histone Post-Translational Modifications (sc-hPTM2)

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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. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? Two labs are across the street, 5 min walk.

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length
Number of Steps: 17
Number of Shots: 33

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

NOTE: The authors edited the wording throughout the script

1.1. **Giulia Barotti:** We're advancing single-cell proteomics with an automated, multiplexed method to study global chromatin heterogeneity through histone post-translational modifications.

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

1.2. **Ronald Cutler:** What makes this protocol challenging is the picogram-scale proteomic preparation, multiplexing, as well as resolving complex combinatorial modified histone peptides.

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

CONCLUSION:

1.3. **Giulia Barotti:** This protocol closes the gap in epigenetics research, allowing scientist to study chromatin states at single-cell resolution.

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

1.4. **Ronald Cutler:** This workflow enables high throughput sample preparation, sample multiplexing, and unbiased mass spectrometry to produce both sensitive and quantitative single-cell epigenetic data.

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

1.5. **Giulia Barotti:** Future research will explore how the regulation of chromatin is altered in disease states, such as cancer, metabolic disease, and aging.

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

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Automated Single Cell Workflow for Quantitative Histone Post Translational Modification Analysis Using Nano Liquid Handling

Demonstrators: Giulia Barotti, Ronald Cutler

2.1. To begin, reconstitute HepG2/C3A *(Hep-G-Two-C-3-A)* hepatocellular carcinoma cells **[1]** at a final concentration of 200 to 500 cells per microliter in ice-cold PBS **[2]**. Set each nozzle's pulse width and drive voltage to the values provided by the manufacturer **[3]**.

2.1.1. WIDE: Talent handling cells in hood

2.1.2. Added shot: Talent counting cells on cell counter

2.1.3. SCREEN: 69588_2.2.1-2.2.3.mp4 00:00-00:20.

2.2. For maximal pick-up efficiency, ensure that the z-offset between the two nozzles differs by less than 50 micrometers **[1]**. Identify the optimal contact position for each nozzle and compare the Z-height readouts in the **Nozzle Setup** tab **[2]**.

2.2.1. SCREEN: 69588_2.2.1-2.2.3.mp4 00:50-01:10.

2.2.2. SCREEN: 69588_2.2.1-2.2.3.mp4 01:12-01:25.

2.3. For a two-plex multiplexing scheme, load a single slide onto the slide holder in the hood **[1]** and then transfer it to the instrument **[2]**.

2.3.1. Added shot: Talent loading a slide into block in hood.

2.3.2. Talent loading block into the instrument.

2.4. Using a pipette, aliquot 150 microliters of LC-MS *(L-C-M-S)*-grade DMSO into a new PCR tube **[1].** Place it into position 2 of the wash station with the tube cap facing the instrument door **[2]**. Start the DMSO run, and perform the head camera wizard setup to align the camera **[3]**.

2.4.1. Talent pipetting 150 µL dimethyl sulfoxide into a PCR tube.

2.4.2. Talent placing it in position 2 with the cap facing the instrument door.

2.4.3. SCREEN: 69588_2.4.3.mp4 00:25-00:40

2.5. Dispense DMSO and allow the instrument to automatically flush the nozzles and capture images across all slides for quality control **[1]**. Review these images to verify a uniform DMSO droplet on every slide and to check for missing or off-target droplets **[2]**.

2.5.1. Talent operating the instrument to dispense the DMSO and Shot of instrument performing DMSO dispense. **NOTE**: This was shot in 2 parts 2.5.1A and 2.5.1B

2.5.2. SCREEN: 69588_2.5.3.mp4 01:20-01:40.

2.6. In the **Nozzle Setup** tab, launch the cellenONE module. Perform a background capture and then run the mapping routine to define the ejection zone **[1]**. Run the analysis and then gate the particles to select for cells based on size and elongation **[2]**.

2.6.1. SCREEN: 69588_2.6.1-2.6.2.mp4. 00:00-00:20

2.6.2. SCREEN: 69588_2.6.1-2.6.2.mp4. 00:35-00:45

2.7. Now, prepare a fresh digestion master mix in LC-MS-grade water **[1-TXT]**. Degas the solution with a vacuum pump for 10 minutes on ice **[2]**.

2.7.1. Talent preparing reagents and combining them to make the digestion master mix. **TXT: Digestion mix: ArgC Ultra 60 ng/µL, HEPES 6 mM, DDM 0.03%, DTT 10 mM**

2.7.2. Talent degassing the prepared mix with a vacuum pump on ice for 10 minutes.

2.8. After dispensing the digestion mix onto the slides, let the instrument automatically flush the nozzles and photograph every slide. Review the images to verify that each droplet received the digestion mix uniformly **[1]**.

2.8.1. ~~Talent operating the instrument to dispense the digestion mix~~.

2.8.2. SCREEN: 69588_2.8.1.mp4. 00:00-00:30

2.9. Incubate the slides at room temperature overnight to perform digestion **[1]**.

2.9.1. SCREEN: 69588_2.9.1.mp4 00:00-00:25 *Video editor: Please speed up*

2.10. Inspect the images after overnight digestion **[1]**. If the droplets are sufficiently dry, stop the run **[2]**. If not, click **Continue** and dry for another 5 minutes **[3]**.

2.10.1. SCREEN: 69588_2.10.1.mp4.

2.10.2. SCREEN: 69588_2.10.2.mp4.. 00:35-00:40

2.10.3. SCREEN: 69588_2.10.3.mp4.. 00:00-00:25

2.11. For labelling, first prepare stock solutions for multiplexed histone derivatization **[1]**.

2.11.1. Talent preparing the two labeling stock solutions in separate vials.
AND
TEXT ON PLAIN BACKGROUND:
Stock 1: 25% (v/v) propionic anhydride in DMSO

Stock 2: 25% (v/v) propionic anhydride-d_{10} in DMSO
Video Editor: Please play both shots side by side in a split screen

2.12. After dispensing, allow the instrument to automatically flush the nozzles and photograph every slide. Review the images to confirm even distribution and correct

droplet placement across all slides. After incubation is complete, dispense hydroxylamine solution for quenching **[1]**.

2.12.1. SCREEN: 69588_2.12.1.mp4..

2.12.2. ~~SCREEN: Talent verifying correct droplet placement of HA solution on the instrument monitor~~.

2.13. To pick up the sample, select the run in the **Main** tab **[1]** and then start the run in the **Run** tab **[2].** After the pickup is finished, review the images to check for any errors **[3]**.

2.13.1. SCREEN: 69588_2.13.1.mp4. 00:00-00:10

2.13.2. Shot of robot doing pickup. **NOTE**: This is called 2.13.3A.

2.13.3. SCREEN: 69588_2.13.3.mp4. 00:00-00:25

2.14. Once done with pickup, remove the cover from the pickup plate **[1].** Then dry the samples at low heat in a vacuum concentrator **[2]**.

2.14.1. Talent removing foil seals.

2.14.2. Talent placing slides in a vacuum concentrator for drying.

2.15. For data acquisition, program the high-performance liquid chromatography method. Set the MS2 experiment to contain isolation windows of 50 mass over charge, higher-energy collisional dissociation energy of 27 percent, and automatic gain control target of 1000 percent **[1]**.

2.15.1. SCREEN: 69588_2.15.1.mp4. 00:35-01:00.

2.16. Cover the plate with a rubber seal mat and place it inside the autosampler **[1]**.

2.16.1. Talent sealing the plate with a rubber mat and loading it into the autosampler.

2.17. After obtaining raw files, briefly check the chromatogram and then run the raw data in EpiProfile version 2.1 *(Epi-Profile-Version-Two-Point-one)* **[1].** Review the output table of normalized histone PTM abundances and use it for downstream analyses **[2]**.

2.17.1. SCREEN: 69588_2.17.1.mp4. 00:10-00:30

2.17.2. SCREEN: 69588_2.17.2.mp4.

Results

3. Results

3.1. The first 20 histone H3 *(H-Three)* peptidoforms were quantified, displaying their relative abundances in control and sodium butyrate–treated conditions **[1]**. Both H3K9ac *(H-Three-K-Nine-A-C)* and H3K14ac *(H-Three-K-Fourteen-A-C)* displayed higher relative abundance upon sodium butyrate treatment **[2]**, while H3K9acK14ac *(H-Three-K-Nine-A-C-K-Fourteen-A-C)* appeared even more enriched in treated cells **[3]**.

3.1.1. LAB MEDIA: Figure 7A.

3.1.2. LAB MEDIA: Figure 7A. *Video editor: Highlight the column showing H3K9ac and H3K14ac in the pink column.*

3.1.3. LAB MEDIA: Figure 7A. *Video editor: Highlight the entry for H3K9acK14ac in the pink column.*

3.2. Total acetylation levels increased following sodium butyrate treatment **[1]**, with treated cells showing a broader error distribution compared to controls reflecting the higher heterogeneity captured at the single-cell level **[2]**. The quantification of single and co-occurring histone modifications demonstrated that combinatorial acetylation states were most strongly affected by sodium butyrate **[3]**.

3.2.1. LAB MEDIA: Figure 7B.

3.2.2. LAB MEDIA: Figure 7B. *Video editor: Highlight the pink bar labeled "NaBut 5 mM,"*

3.2.3. LAB MEDIA: Figure 7C. *Video editor: Highlight the pink bar corresponding to K9acK14ac in the sodium butyrate–treated condition.*

3.3. The wider dispersion of sodium butyrate–treated cells indicated increased biological heterogeneity among individual cells within the spheroids **[1]**.

3.3.1. LAB MEDIA: Figure 7D. *Video editor: Please highlight the pink dots*

- **Hepatocellular carcinoma**

Pronunciation link: https://www.howtopronounce.com/hepatocellular-carcinoma How To Pronounce+2Forvo.com+2
IPA (American): /ˌhɛp.ə.toʊˈsɛl.jə.lə˞ kɑrˌsɪ.noʊ.mə/
Phonetic spelling (US-style): hep-uh-TOH-sel-yuh-LER kar-SIN-noh-muh

- **Dimethyl sulfoxide**

Pronunciation link: https://www.howtopronounce.com/dimethyl-sulfoxide How To Pronounce+1
IPA (American): /daɪˈmaɪ.θəl ˌsʌlˈfɑːk.saɪd/
Phonetic spelling: dye-MY-thuhl sul-FOK-sahyd

- **HepG2** (as in the cell line name)
  - "Hep" → like "hep" in "hepatocyte"
  - "G" → letter "G" (jee)
  - "2" → "two"
    So: **hep-G-two**

- **C3A** (in cell line name "HepG2/C3A")
  - "C" → letter "C" (cee)
  - "3" → "three"
  - "A" → letter "A" (eh)
    So: **cee-three-eh**

- **LC-MS** (as in "LC–MS-grade DMSO")
  - "L" → letter "L" (el)
  - "C" → letter "C" (see)
  - "MS" → letters "M" "S" (em-ess)
    So: **el-see em-ess**

- **DMSO** (dimethyl sulfoxide abbreviation)
  - "D" → "dee"
  - "M" → "em"
  - "S" → "ess"
  - "O" → "oh"
    So: **dee-em-ess-oh**

- **HEPES** (common buffer, appears in your digestion mix)

Pronunciation link: No confirmed link found (could not locate reliable audio in the checked dictionaries)
Suggested phonetic spelling: **HEE-peez** — based on common usage in lab contexts

- **DTT** (in context of "DTT 10 mM")
  - "D" → "dee"
  - "T" → "tee"
  - "T" → "tee"
    So: **dee-tee-tee**

- **Propionic anhydride**

Pronunciation link: No confirmed link found for full phrase in checked sources.
Suggested phonetic spelling: **proh-PEYE-on-ik an-HY-dride**