Submission ID #: 68995
Scriptwriter Name: Poornima G
Project Page Link: https://review.jove.com/account/file-uploader?src=21043523

Title: Sample Preparation for Single Cell Mass Spectrometry Metabolomics Studies: Combined Cell Washing, Quenching, Drying, and Storage

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

   If **Yes**, how far apart are the locations? 3rd floor and 1st floor in the same building (Room number: SLSRC 3550, 1300, 3770)

Current Protocol Length
Number of Steps: 24
Number of Shots: 51 (12 SC, 1 LAB MEDIA)

Introduction

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

1.1. **Deepti Bhusal:** We have developed protocols to preserve the integrity of cellular metabolites for single-cell mass spectrometry studies. We investigated how washing, quenching, and storage conditions affect cell metabolites.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.3*

What are the most recent developments in your field of research?

1.2. **Deepti Bhusal:** New sample preparation methods and mass spectrometry techniques have been recently developed to advance single-cell metabolomics studies to better understand cellular heterogeneity and disease mechanisms.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.2*

What are the current experimental challenges?

1.3. **Deepti Bhusal:** Researchers need to overcome multiple key challenges, including limited sample amount, reduced detection sensitivity due to sample complexity, and altered metabolites during sample preparation and analysis.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.1*

What research gap are you addressing with your protocol?

1.4. **Shakya Wije Munige:** We demonstrate comprehensive sample preparation techniques that can preserve cellular metabolites and cell integrity for single-cell mass spectrometry metabolomics studies.

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

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What advantage does your protocol offer compared to other techniques?

1.5. **Shakya Wije Munige :** Our protocols could offer a simple and effective way for preparation, storage, and transportation of samples for single-cell metabolomics studies using different mass spectrometry techniques.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 6.2.1*

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

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Dan Chen, Graduate Student, University of Oklahoma**: (authors will present their testimonial statements live)

(Publishing with JoVE will enhance the visibility and impact of our work because it provides visualized, clear demonstration of experimental details, allowing readers to reproduce and adapt our protocols to improve studies in single cell mass spectrometry field.)

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

Protocol

2. Culturing and Washing the Cells

Demonstrators: Shakya Wije Munige and Deepti Bhusal

2.1. To begin, incubate the cells at 37 degrees Celsius in a humidified incubator containing 5 percent carbon dioxide **[1]**. Monitor the confluency of the cells daily **[2]** and passage them when cultures reach 80 percent confluency **[3]**.

2.1.1. Talent placing the culture dish inside a humidified incubator.

2.1.2. Talent placing the cells under a microscope.

2.1.3. LAB MEDIA: 68995_2.1.4.jpg

2.2. For passaging, aspirate the medium from the dish **[1]** and rinse once with 5 milliliters of PBS **[2]**. Incubate the cells with 2 milliliters of 0.25 percent trypsin-EDTA at 37 degrees Celsius for 3 minutes to facilitate cell detachment **[3]**.

2.2.1. Talent aspirating the medium from the culture dish.

2.2.2. Talent rinsing the dish with phosphate-buffered saline.

2.2.3. Talent placing the culture dish inside the incubator.

2.3. Neutralize the trypsin by adding 8 milliliters of pre-warmed complete medium **[1]** and gently pipette to disperse the cells **[2]**. Transfer 1 milliliter of the suspension into 9 milliliters of fresh complete medium **[3]** for counting the cells **[4]**.

2.3.1. Talent pipetting complete medium into the dish.

2.3.2. Talent pipetting the cell suspension up and down to disperse the cells.

2.3.3. Talent transferring 1 milliliter of the cell suspension into a new tube with 9 milliliters of fresh complete medium.

2.3.4. Talent placing the sample in a cell counter.

2.4. To seed the cells, dilute them to a final concentration of 1×10^6 cells per milliliter **[1]**. Place individual 18-millimeter glass coverslips into the wells of a 12-well plate **[2]**. Add 2 milliliters of complete medium and 200 microliters of the cell suspension into each well to achieve 2×10^5 cells per well **[3]**. Incubate the cells overnight to allow attachment **[4]**.

2.4.1. Talent diluting the cells in a tube to the desired concentration by adding media.

2.4.2. Talent placing glass coverslips into the wells of a 12-well plate.

2.4.3. Talent pipetting complete medium and cell suspension into each well.

2.4.4. Talent placing the plate in an incubator for overnight incubation.

2.5. For washing the cells, add 2 milliliters of cold ammonium formate solution to each well of the 12-well plate **[1-TXT]**.

2.5.1. Talent pipetting cold ammonium formate solution into the wells of the plate. **TXT: Ammonium formate: 0.14 M (0.9% w/w)**

2.6. Then, using sterile forceps, gently lift each coverslip containing adherent cells from its well **[1]** and briefly place it for 2 to 3 seconds in a separate well pre-filled with 2 milliliters of cold 0.9 percent ammonium formate solution **[2]**.

2.6.1. Talent carefully lifting a coverslip with sterile forceps.

2.6.2. Talent dipping the coverslip into a well containing ammonium formate solution.

3. Cell Quenching, Freeze-Drying and Storage

3.1. Place each ammonium formate-rinsed coverslip with the cells facing upward into a Petri dish inside an aluminium foil container **[1]**. Slowly pour 10 to 20 milliliters of liquid nitrogen over the coverslips to ensure complete coverage **[2]** and immediately tilt the Petri dish with tweezers to decant any remaining liquid nitrogen **[3]**.

3.1.1. Talent placing rinsed coverslips into a Petri dish lined with aluminum foil.

3.1.2. Talent carefully pouring liquid nitrogen into the Petri dish over the coverslips.

3.1.3. Talent tilting the dish with tweezers to decant excess liquid nitrogen.

3.2. Using cryogenic gloves and insulated tweezers, place the Petri dish containing the liquid nitrogen-chilled coverslips into the vacuum concentrator chamber **[1]**.

3.2.1. Talent wearing cryogenic gloves and using insulated tweezers to carefully place the Petri dish into the chamber.

3.3. Process the samples using the standard vacuum settings for 5 to 7 minutes until the visible liquid nitrogen evaporates and the coverslips appear dry **[1]**. Ensure that the

dried coverslips do not contain any residual liquid nitrogen or ice crystals **[2]**.

3.3.1. Talent adjusting the vacuum concentrator settings.

3.3.2. Close-up of the coverslips inside the chamber, visibly dry with no frost or ice crystals.

3.4. Next, wrap the container with a paper towel **[1]** and transfer it to a minus 80 degree Celsius freezer for 48 hours **[2]**.

3.4.1. Talent wrapping the Petri dish container with a paper towel.

3.4.2. Talent placing the wrapped container into a minus 80 degree Celsius freezer.

3.5. After storage, transfer the coverslips containing the Petri dish into a room-temperature desiccator for 10 minutes **[1]**. Ensure condensed frost or water on the coverslips completely disappears before removing them from the desiccator **[2]**.

3.5.1. Talent placing the Petri dish into a desiccator on a laboratory bench.

3.5.2. Close-up of the coverslips inside the desiccator with frost disappeared.

3.6. Divide the samples into two groups and treat them appropriately **[1-TXT]**.

3.6.1. TEXT ON PLAIN BACKGROUND
Group 1: LN_2 quench → Freeze dry → SCMS analysis (no storage)

Group 2: LN_2 quench → freeze-dry → −80 °C storage 48 h → SCMS analysis

4. Single Cell Mass Spectrometry (SCMS) Setup

4.1. Now, mount the Single-probe onto the XYZ stage **[1]** and affix it to a motorized XYZ manipulator positioned beneath a digital microscope **[2]**.

4.1.1. Talent mounting the Single-probe onto the XYZ stage.

4.1.2. Talent adjusting the manipulator beneath a digital microscope.

4.2. Couple the Single-probe setup to a mass spectrometer for SCMS analysis **[1-TXT]**.

4.2.1. Talent connecting tubing and wiring from the Single-probe to the mass spectrometer. **TXT: SCMS: Single Cell Mass Spectrometry**

4.3. Use acetonitrile containing 0.1 percent formic acid at a purity of at least 99.9 percent as the extraction solvent **[1]** and deliver it at a flow rate of 150 nanoliters per minute using a syringe pump **[2]**.

4.3.1. Talent dipping the solvent tubing in the solution.

4.3.2. Talent operating a syringe pump to adjust settings.

4.4. Set the mass spectrometry parameters for positive and negative ion modes **[1]**. **Authors**, we will display this information as text.

4.4.1. TEXT ON PLAIN BACKGROUND:

Positive ion mode:

m/z 200 - 1500

+2.9 kV

Mass resolution 120 k (at m/z 200)

Negative ion mode:

m/z 70 - 900

−2.1 kV

Mass resolution 120 k (at m/z 200)

5. Single-Probe SCMS and Data Analysis

5.1. Now, place the two coverslips from Group 1 and Group 2 together on the motorized XYZ stage of the Single-probe SCMS setup **[1]** and use the microscope to randomly select cells for analysis **[2].**

5.1.1. Talent positioning both coverslips side by side on the XYZ stage under the Single-probe.

5.1.2. Microscopic view appearing on the screen.

5.2. After acquiring mass spectra, analyze approximately 30 cells per group using the same Single-probe, solvent flow rate, and ionization voltage **[1]**.

5.2.1. SCREEN: 68995_5.2.1.mp4 00:00-00:20.

5.3. Preprocess the raw SCMS data using a custom R script and apply background and noise

removal followed by ion intensity normalization to total ion current **[1]**.

5.3.1. SCREEN: 68995_5.3.1.mp4 00:00-0:15 and 00:20-00:25.

5.4. Deisotope the SCMS data using the Python package **ms_deisotope** *(M-S-dee-isotope)* **[1].**

5.4.1. SCREEN: 68995_5.4.1.mp4 01:30-01:41.

5.5. Perform peak alignment using an in-house Python script **[1]**. Apply a bin width of 0.01 mass-to-charge ratio for binning **[2]** and a mass tolerance of 0.01 mass-to-charge ratio or 5 parts per million for peak alignment **[3]**.

5.5.1. SCREEN: 68995_5.5.1.mp4 001:14-01:23.

5.5.2. SCREEN: 68995_5.5.2.mp4 00:00-00:15.

5.5.3. SCREEN: 68995_5.5.3.mp4 00:15-00:25.

5.6. Conduct statistical data analysis using MetaboAnalyst 6.0 *(6-point oh)* **[1]**. Perform a t-test and generate a heat map showing relative metabolite levels **[2]**. Select the option **Use top 100 in T-test/ANOVA** *(T-test or Anova)* to generate the top 100 metabolites ranked by significance **[3]** and plot a heatmap using these top 100 metabolites with significant differences **[4]**.

5.6.1. SCREEN: 68995_5.6.1.mp4.

5.6.2. SCREEN: 68995_5.6.2.mp4.

5.6.3. SCREEN: 68995_5.6.3.mp4.

5.6.4. SCREEN: 68995_5.6.4.mp4.

5.7. Finally, search the accurate mass-to-charge values against the Human Metabolome Database **[1]** using a mass tolerance of 10 parts per million for database searching **[2]**.

5.7.1. SCREEN: 68995_5.7.1.mp4.

5.7.2. SCREEN: 68995_5.7.2.mp4.

Results

6. Results

6.1. Principal Component Analysis in the positive ion mode showed that Group 1 and Group 2 overlapped closely, indicating highly similar metabolite profiles **[1]**.

6.1.1. LAB MEDIA: *Figure 2A. Video editor: Highlight the two overlapping shaded regions from blue for Group 1 and green for Group 2*.

6.2. In the negative ion mode, a comparable pattern was observed, although Group 2 appeared slightly more distinct **[1]**.

6.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the overlapping region between group 1 and 2*.

6.3. Heat map analysis of the top 100 metabolites displayed highly consistent abundance patterns between Group 1 and Group 2 in both ion modes. No major shifts or group-specific clustering are observed, although minor variations in a few metabolite clusters may reflect differences in ionization efficiency or metabolite stability **[1]**.

6.3.1. LAB MEDIA: Figure 3B *Video editor: Sequentially highlight Group 1 and 2 data*

1. Trypsin-EDTA

- **Pronunciation link** (EDTA via Cambridge): Cambridge provides audio for EDTA Cambridge Dictionary
- **IPA**: Trypsin: /ˈtɪɪp.sɪn/ (from Wiktionary) Wiktionary
  EDTA: /i-di-ti-eɪ/ (approx.)
- **Phonetic Spelling**: TRIP-sin ee-dee-TEE-ay

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2. Desiccator

- **Pronunciation link** (HowToPronounce): How To Pronounce
- **IPA**: /dɪˈsɪk.eɪ.tər/ (common American pronunciation)
- **Phonetic Spelling**: dih-SIK-ay-ter

3. Cryogenic

- **Pronunciation link** (Cambridge): Cambridge Dictionary
- **IPA**: /ˌkraɪ.əˈdʒɛn.ɪk/ Collins Dictionary
- **Phonetic Spelling**: kry-oh-JEN-ik

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4. Deisotope

- **Pronunciation link**: No widely available audio found ("No confirmed link found")
- **IPA**: /diːˈaɪ.səˌtoʊp/ (constructed from "de-" + "isotope")
- **Phonetic Spelling**: dee-EYE-suh-tope

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5. Metabolite

- **Pronunciation link** (Cambridge): Cambridge Dictionary
- **IPA**: /məˈtæb.əˌlaɪt/ (American) Cambridge Dictionary
- **Phonetic Spelling**: muh-TAB-uh-lykht

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6. Vacuum

- **Pronunciation link** (Cambridge): Cambridge Dictionary
- **IPA**: /ˈvæk.juːm/
- **Phonetic Spelling**: VAK-yo͞om

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7. Incubator

- **Pronunciation link** (not found) – (No confirmed link found)
- **IPA**: /ˈɪŋ.kjuː.beɪ.tər/
- **Phonetic Spelling**: ING-kyoo-BAY-ter

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8. Confluency

- **Pronunciation link** (not found) – (No confirmed link found)
- **IPA**: /ˈkɒn.flu.ən.si/ (American: /ˈkɑːn.flu.ən.si/)
- **Phonetic Spelling**: KON-floo-en-see

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9. Passage (as a verb)

- **Pronunciation link** (not found) – (No confirmed link found)
- **IPA**: /ˈpæs.ɪdʒ/
- **Phonetic Spelling**: PAS-ij

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10. Trypsin (independently)

- **Pronunciation link** (Merriam-Webster): Merriam-Webster
- **IPA**: /ˈtrɪp.sɪn/ Wiktionary
- **Phonetic Spelling**: TRIP-sin

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11. Phosphate-buffered saline (PBS)

- **Pronunciation link** (not found) – (No confirmed link found)
- **IPA**: /ˈfɒs.feɪt ˈbʌf.ərd ˈseɪ.lɪn/
- **Phonetic Spelling**: FOS-fayt BUF-urd SAY-lin

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12. Trypsinization

- **Pronunciation link** (not found) – (No confirmed link found)
- **IPA**: /ˌtraɪp.sə.nəˈzeɪ.ʃən/
- **Phonetic Spelling**: tryp-suh-nuh-ZAY-shun

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13. Cryogenic Gloves

- **Components already defined; “cryogenic” above
- **Gloves**: /glʌvz/
- **Phonetic**: gluvz

14. Desiccator (plural: *desiccators*)

- Pronunciation is the same as singular
- **Pronunciation link** (HowToPronounce "desiccators"): How To Pronounce
- **IPA**: /dɪˈsɪk.eɪ.tərz/
- **Phonetic Spelling**: dih-SIK-ay-terz

15. Acetonitrile

- **Pronunciation link**: (No confirmed link found)
- **IPA**: /ˌæ.sɪ.toʊˈnaɪ.trəl/
- **Phonetic Spelling**: as-ih-toh-NY-truhl