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Video Article

A Method for Measuring Metabolism in Sorted Subpopulations of Complex Cell Communities Using Stable Isotope Tracing

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Abstract

Mammalian cell types exhibit specialized metabolism, and there is ample evidence that various co-existing cell types engage in metabolic cooperation. Moreover, even cultures of a single cell type may contain cells in distinct metabolic states, such as resting or cycling cells. Methods for measuring metabolic activities of such subpopulations are valuable tools for understanding cellular metabolism. Complex cell populations are most commonly separated using a cell sorter, and subpopulations isolated by this method can be analyzed by metabolomics methods. However, a problem with this approach is that the cell sorting procedure subjects cells to stresses that may distort their metabolism.

To overcome these issues, we reasoned that the mass isotopomer distributions (MIDs) of metabolites from cells cultured with stable isotope-labeled nutrients are likely to be more stable than absolute metabolite concentrations, because MIDs are formed over longer time scales and should be less affected by short-term exposure to cell sorting conditions. Here, we describe a method based on this principle, combining cell sorting with liquid chromatography-high resolution mass spectrometry (LC-HRMS). The procedure involves analyzing three types of samples: (1) metabolite extracts obtained directly from the complex population; (2) extracts of "mock sorted" cells passed through the cell sorter instrument without gating any specific population; and (3) extracts of the actual sorted populations. The mock sorted cells are compared against direct extraction to verify that MIDs are indeed not altered by the cell sorting procedure itself, prior to analyzing the actual sorted populations. We show example results from HeLa cells sorted according to cell cycle phase, revealing changes in nucleotide metabolism.

Video Link

The video component of this article can be found at https://www.jove.com/video/55011/

Introduction

Higher organisms contain complex communities of distinct cell types that collaborate to bring about more complex functions. For example, tumors contain not only cancerous cells, but also fibroblasts, cells that constitute blood vessels, and often immune cell infiltrates[1]; blood contains a complex mixture of dozens of immune cell subtypes[2]; and even cultured cell lines may consist of multiple subpopulations, such as the luminal and basal subtypes of breast cancer cells[3]. Moreover, distinct cell types that coexist can exhibit metabolic "collaboration". For example, in the brain, astrocytes are thought to convert glucose to lactate, which is then "fed" to neurons that oxidize this substrate[4]; T lymphocytes are in some contexts dependent on adjacent dendritic cells as a source of cysteine[5]; and cancer cells may collaborate with associated fibroblasts in tumors[6]. To understand the metabolic behavior of such systems, it is essential to separate and measure the metabolic activities of the various cell types present.

By far the most widely used method for separating cell types is fluorescence-activated cell sorting. This method is broadly applicable, provided that the cell type or state of interest can be "labeled" using fluorescent antibodies, expression of engineered fluorescent proteins, or other dyes. One option is to initially separate cells types through a cell sorter, re-culture the individual cell types obtained, and then perform metabolism studies of these cultures[7]. However, this is only feasible if the cell type or phenotype is stable in culture conditions, and cannot capture transient behavior such as cell cycle states, nor the metabolic cooperation in co-cultures. For such cases, metabolism must be measured directly on sorted cells. This is challenging since the cell sorting procedure subjects cells to stresses that may distort their metabolism[8], and we are aware of only a few studies taking this approach[9,10]. In particular, we have found that major metabolites such as amino acids may leak from cells kept in cell sorting buffer, so that measurements of absolute metabolite abundance are no longer reliable[11] (although relative comparison between sorted fractions may still be valuable).

To circumvent these issues, we label cells with stable isotopes prior to sorting, and focus on the MIDs in cellular metabolites, rather than metabolite abundances. Since MIDs are formed over longer time scales, they should be less affected by short-term exposure to sorting conditions. We quantify MIDs using full-scan high-resolution mass spectrometry, which is sensitive enough to provide data on hundreds of

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metabolites starting from around 500,000 sorted cells, requiring about 30-60 min of cell sorting time. A comparison between a "mock sorted" control (cells passed through the cell sorter instrument without gating any specific population) and metabolite extraction directly from the culture dish is made to ensure that the observed MIDs are representative of those in the original culture. Depending on the choice of stable isotope tracers, various metabolic pathways can be studied with this method.

Protocol

1. Metabolite Extraction

1. **Extraction from dish**
    1. Culture cells in a 6-well plate in triplicates in stable isotope labeled culture media + dialyzed supplements (serum or other growth supplements) until cells become 75% confluent.
    NOTE: Here culture HeLa cells for 48 h in RPMI containing 40% U-^{13}C-Glucose and 70% U-^{13}C, ^{15}N2-Glutamine and 5% dialyzed FBS (Fetal Bovine Serum). Dialyzed FBS is used to get rid of the small molecular weight metabolites which might contaminate the labeled media. Culturing cells in dialyzed supplement prior to the real experiment is recommended to ensure cells are growing normally in the medium. Supplements are dialyzed in 0.15 M NaCl solution overnight using snake skin dialysis tubing.
    2. At the day of extraction discard culture media, rinse wells twice with 500 µL cold HBSS and then discard it.
    NOTE: Here use HBSS containing 40% U-^{13}C-Glucose since it is also in the culture media.
    3. Add 600 µL 100% methanol pre-cooled on dry ice.
    4. Transfer the dish to dry ice and remove cell material with a cell scraper.
    5. Carefully pipette the cell extracts to a microcentrifuge tube and store at -80 °C until mass spectrometry analysis.

2. **Extraction of mock sorted cells**
    1. Culture cells in a 100 mm dish in triplicates in stable isotope labeled culture media + dialyzed supplements.
    NOTE: Here culture HeLa cells for 48 h in 100 mm dish because a high number of cells (~4 x 10^6) are needed to obtain 500,000 sorted cells. This number of HeLa cells extract was required to obtain good measurement of metabolites. The culture media used was RPMI containing 40% U-^{13}C-Glucose and 70% U-^{13}C, ^{15}N2-Glutamine and 5% dialyzed FBS.
    2. At the day of extraction, discard culture media, rinse wells with 1.5 mL warm HBSS and then discard it.
    3. Detach cells by adding 1.5 mL trypsin/EDTA for 4 min at 37 °C. Perform the following steps in 4 °C or in ice.
    4. Deactivate trypsin by adding 3 mL ice cold HBSS (Hank's Balanced Salt Solution) + dialyzed supplement.
    5. Collect cells in a 15 mL tube and centrifuge at 750 x g for 3 min.
    6. Resuspend the pellet in HBSS + dialyzed supplement + 1 mM EDTA at a concentration 1-2 x 10^6 cells/mL, pass through 40 µm cell strainers to obtain single cells, and transfer to a 5 mL tube.
    7. Sort cells through cell sorter gating only for singlets, and centrifuge sorted cells at 750 x g for 3 min at 4 °C.
    NOTE: Here sort HeLa cells at a rate 1,000 events/s, with instrument pressure 27 psi, and using a 100 µm nozzle. HeLa cells are big so they should be sorted at a slow event rate and large nozzle to get as intact pellet as possible. Keep the cells in cold blocks throughout sorting to decrease the metabolism. A thorough description of sorting procedure is described previously[12].
    8. Discard the supernatant and resuspend the pellet in 50 µL ice cold dH_2O to obtain a homogenous pellet before adding methanol. Addition of cold methanol directly to pellet forms a solid pellet which is hard to resuspend.
    9. Extract metabolites by adding 540 µL methanol kept in dry ice and keep extracts at -80 °C until liquid chromatography – high resolution mass spectrometry (LC-HRMS) analysis.

3. **Extraction of cell cycle sorted cells**
    1. Culture cells in a 100 mm dish in triplicates in culture media + dialyzed supplements.
    NOTE: Here culture HeLa cells containing Geminin Fucci Green (mAG1-hGem) probe[13] which allows for sorting of G1 (negative) and SG2M (positive) cells. Cells can be cultured in stable isotope tracing media for as long as required. Here we have cultured them for 46 h in unlabeled media, then we have switched to RPMI containing 40% U-^{13}C-Glucose and 70% U-^{13}C, ^{15}N2-Glutamine 2 h before starting sorting. This was done in order to be able to study the cell cycle phases which requires a short pulse labeling.
    2. At the day of extraction, discard culture media, rinse wells with 1.5 mL warm HBSS and then discard it.
    3. Detach cells by adding 1.5 mL trypsin/EDTA for 4 min at 37 °C. Perform the following steps in 4 °C or in ice.
    4. Deactivate trypsin by adding 3 mL ice cold HBSS + dialyzed supplement.
    5. Collect cells in a 15 mL tube and centrifuge at 750 x g for 3 min.
    6. Resuspend the pellet in HBSS + dialyzed supplement + 1 mM EDTA at a concentration 1-2 x 10^6 cells/mL, pass through 40 µm cell strainers to obtain single cells, and transfer to a 5 mL tube.
    7. Sort cells through the cell sorter gating out debris and doublets, then gating for the cell marker of interest, and centrifuge sorted cells at 750 x g for 3 min at 4 °C.
    NOTE: Here sort HeLa cells at a rate 1,000 events/s, with instrument pressure 27 psi, and using a 100 µm nozzle. Keep the cells in cold blocks throughout sorting to decrease the metabolism rate.
    8. Discard the supernatant and resuspend the pellet in 50 µL ice cold dH_2O to obtain a homogenous pellet before adding methanol. Addition of cold methanol directly to pellet forms a solid pellet which is hard to resuspend.
    9. Extract the metabolites by adding 540 µL methanol kept in dry ice and keep extracts at -80 °C until LC-HRMS analysis.

2. Mass Spectrometry Analysis

Note: Here we describe the protocol for analyzing cell extracts on a LC-HRMS system. Any metabolomics methods for analysis of cell extracts can be used. Full scan analysis might be useful for detecting a wide range of metabolites.

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1. Calibrate the instrument using a mass spectrometry reference calibration mix.
2. Thaw cell extracts on ice for 30 min and vortex for 15 s.
3. Transfer 100 µL of the cell extract to a spin filter and centrifuge for 10 min at 13,000 x g at 4 °C.
4. Inject 12.5 µL of the filtrate onto the LC-HRMS system.
5. Separate metabolites using a Zwitterionic Hydrophilic Interaction Liquid Chromatography (ZIC-HILIC) column (150 mm x 4.6 mm, 5 µm particle size) fitted with a ZIC-HILIC guard column (20 mm × 2.1 mm) using a gradient elution of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). Start the gradient elution at 20% of solvent A and increase up to 80% in 17 min. Maintain this percentage during 4 min with a flow of 400 µL min^{-1} and column temperature and sample tray at 23 °C and 4 °C, respectively.
6. Use an instrument coupled to the chromatographic separation for metabolites detection, a heated electrospray (H-ESI II) in both positive and negative modes as ionization source, and a full scan acquisition mode at a mass resolving power of 70,000 Full Width Half Maximum (FWHM) (m/z 200).
7. Use nitrogen (purity >99.995%) for the sheath gas and auxiliary gas at a flow rate of 45 and 10 a.u. (arbitrary units) and set vaporizer temperature at 350 °C and the electrospray voltage at 4 kV in positive mode and -3.5 kV in negative mode.

3. Data Analysis

1. Select a number of metabolites for which standards are available and which show good quality peaks in the samples. Good quality peaks have high signal to noise ratio. It is important to verify peak quality and make sure not to include false isotopes. Isotopic peaks which differ in shape and/or retention time are likely false.
2. For isotope-labeled samples calculate mass isotopomer (MI) fractions by dividing the peak area of each MI with total peak areas of all MIs.
3. Calculate the labeled carbon/nitrogen fractions, enrichment of ^{13}C and ^{15}N, respectively, as

$$Enrichment = \frac{\sum_{x=0}^{n} x * MIx}{n}$$

where *n* is the total number of carbons (or nitrogens, respectively) in the metabolite, and MIx is the MI fraction of x.
NOTE: All calculations can be performed using programming languages.

Representative Results

As an example, here we describe an experiment investigating the metabolism of HeLa cells sorted according to cell cycle phase. To label a wide range of central metabolites on both carbons and nitrogens, we cultured cells for 48 h using U-^{13}C-glucose and U-^{13}C, ^{15}N-glutamine as tracers. To obtain rich MIDs for the validation experiment, we here chose a mixture of 40% U-^{13}C-Glucose and 70% U-^{13}C, $^{15}N2$-Glutamine, as intermediate levels of isotopes tend to generate more varied MI patterns[14].

For the validation experiment we performed a targeted analysis of 85 metabolites. After quality controls steps to remove poor quality LC-MS peaks, we were able to detect 69 peaks in the direct cell extracts, of which 66 were present in the mock sorted cells (96%). 60 of these appeared to be labeled (isotope enrichment above natural abundance), and in most cases their MIDs were similar between dish extracts and mock sorted cells (**Figure 2A**). For example, the MID of glutamate (which includes both ^{13}C and ^{15}N MIs) is similar between dish and mock sorted extracts (**Figure 2B**), indicating that the glutamate MID is reliable also in sorted cell populations. However, some metabolites are clearly affected by the sorting procedure: for example, lactate had less $^{13}C_3$ in mock sorted cells, for unknown reasons. Such metabolites should be viewed with caution when analyzing data from actual sorted fractions. In mock sorted cells, 91% of the measured MI fraction had a standard deviation less than 1% across biological triplicates, indicating that MIDs were highly reproducible in sorted cells.

We next analyzed HeLa sorted into either G0/G1 or S/G2/M cell cycle stages using the FUCCI Geminin probe[12]. Because these cell cycle stages last only ~10 h, we here "pulse" labeled cultures for 2 h to achieve different isotope labeling between stages. For example, we noted that cytidine is labeled in both populations, but to a higher enrichment in the S/G2/M stage, consistent with increased *de novo* synthesis of nucleotides during the S phase (**Figure 3**). In this case, the MID shows the same pattern in both fractions, suggesting that same synthesis pathway is used, but is more active in the S/G2/M stage. These data show that metabolic differences are readily detectable by this method even between closely related subpopulations.

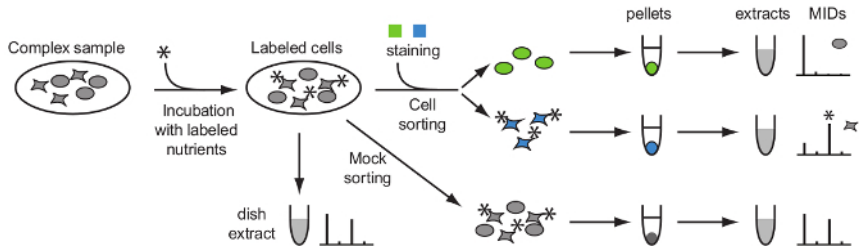

Figure 1: Experimental design. Workflow for preparation of dish, mock sorted and sorted subpopulation extracts. Dish and mock sorted extracts are used in the validation experiment MIDs of dish and mock sorted extracts are compared to test for possible changes due to sorting. Complex population of cells is sorted based on marker of the cell type of interest and then extracted. Please click here to view a larger version of this figure.

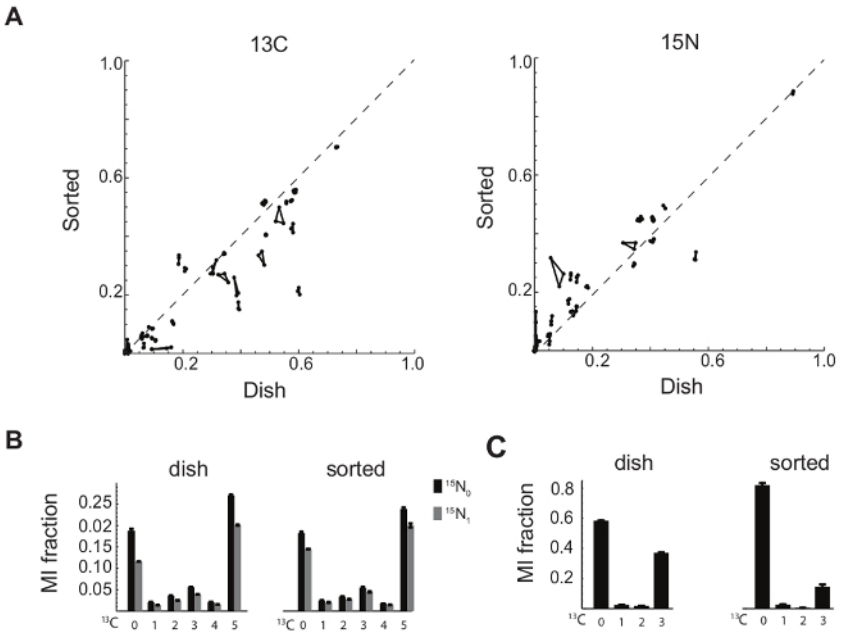

Figure 2: Validation of MIDs against direct extractions. Scatter plots of (**A**) ^{13}C and ^{15}N enrichment in dish and mock sorted samples. Each dot represents a replicate. Triplicates are joined with lines. (**B**) ^{13}C-^{15}N MIDs of glutamate. (**C**) ^{13}C MIDs of lactate. The error bars are standard deviations of triplicate measurements. Please click here to view a larger version of this figure.

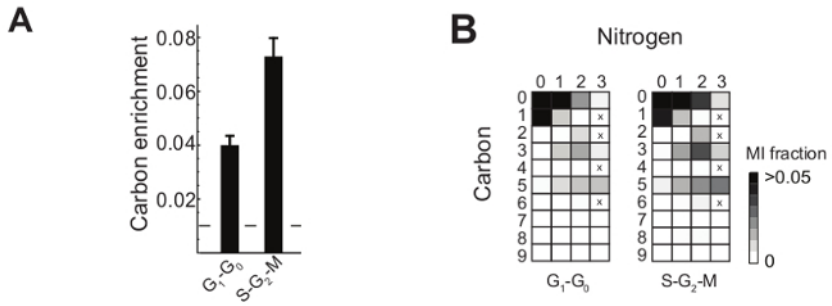

Figure 3: Metabolic differences between cell cycle phases in HeLa cells. Cytidine is labeled differently in G_1-G_0 and S-G_2-M cells. (**A**) Cytidine ^{13}C enrichment in G_1-G_0 and S-G_2-M phases of the cell cycle. Dashed line stands for carbon enrichment from natural mass isotope. (**B**) Cytidine MIDs shown as array plots in G_1-G_0 and S-G_2-M phases. The error bars are standard deviations of triplicate measurements. Please click here to view a larger version of this figure.

Discussion

Our method is based on the principle that MIDs in cellular metabolites reflect the "history" of metabolic activities of a cell. This makes it possible to investigate metabolic activities in subpopulation of cells, as they occurred in the complex community of cells, prior to the cell sorting procedure. In contrast, peak areas of metabolites differ markedly between extracts of sorted cells and direct extraction from the culture dish[11]. In part this is because the different chemical composition alters the signal response in mass spectrometry, a so-called "matrix effect", but we have also shown that amino acids are lost from cells during sorting, while cells are kept in buffer[11]. This may alter the metabolic state and activities of cells during sorting, but this is in itself irrelevant for our method: provided that MIDs are reasonably close to those observed in direct extraction, the data remains a valid measurement of the metabolic state of each subpopulation in the original, complex culture.

We initially tested a method where cells were sorted directly into extraction solution (methanol) to rapidly quench metabolism. Unfortunately, the resulting extracts were difficult to analyze, as they contained high amounts of salts and possibly other contaminants from the cell sorter sheath fluid, resulting in massive ion suppression on our mass spectrometry system. We therefore settled on the protocol described here, where excess fluid deposited by the cell sorter instrument is washed out prior to extraction.

Our protocol entails sorting in HBSS, a physiological salt solution supplemented with glucose to maintain cell viability. In some ways, it may be preferable to sort cells in the actual culture medium to minimize metabolic stress such as amino acid leakage. However, it is difficult to wash the small pellet of sorted cells, and therefore metabolites present in medium would contaminate the sought MIDs of intracellular metabolites. Whenever ^{13}C-labeled glucose is used as a tracer, identical labeled glucose should be used in the HBSS solution as well.

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As seen in **Figure 2**, many, but not all metabolites, maintain their MIDs after the cell sorting procedure. We do not know why certain metabolites (lactate, for example) are specifically altered. This result emphasizes that it is crucial to verify that MIDs of interest are robust towards the cell sorting procedure by mock sorting. Although it is not possible to directly verify the MID of a sorted subpopulation, MIDs that are unaffected by mock sorting (glutamate, for example) should be unaffected by the actual sorting as well, as the procedure is identical except for the cell selection. This validation step should be carried out whenever a new cell type or culture condition is used. It is important to point out that our method is limited to metabolites for which robust MIDs can be verified in this manner.

We anticipate that the method described here will be useful in a number of applications within cell biology and biomedicine. Examples include metabolic phenotypes of co-cultured cells such as stem cell – feeder layer cultures, neuron-astrocyte models[4], subpopulations of blood cells[2], and also complex cell populations isolated from animal models.

Disclosures

The authors have nothing to disclose.

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