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Metabolic Analysis of Lymphatic Endothelial Cells

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Abstract

Metabolism is pivotal for formation of the lymphatic vasculature. Understanding metabolism in lymphatic endothelial cells (LECs) requires quantitative characterization of specific metabolic pathways. Here we describe methods for using radioactive tracers to assess flux rates of glycolysis, fatty acid β -oxidation, glucose oxidation, and glutamine oxidation. We also provide a detailed method for utilizing mass spectrometry (MS) to measure glycolytic intermediates and ATP.

Keywords

Lymphatic endothelial cells; Cellular metabolism; Glycolysis; Fatty acid β -oxidation; Glucose oxidation; Glutamine oxidation; Mass spectrometry

1 Introduction

Cellular metabolic pathways including glycolysis and fatty acid β -oxidation play critical roles in lymphatic vascular development [1]. Glycolysis refers to a metabolic process involving glucose conversion to pyruvate and it serves as a major source of ATP in LECs [2]. Disruption of glycolysis in LECs by ablation of hexokinase 2 (HK2), the first rate-limiting glycolytic enzyme, reduces ATP generation and suppresses LEC proliferation, migration, and sprouting, thereby causing impaired lymphangiogenesis [2, 3]. HK2 expression is regulated, in part, by continuous fibroblast growth factor receptor signaling via transcription factor MYC [2]. Fatty acid β -oxidation is another metabolic process critical for lymphangiogenesis [4]. In LECs, acetyl-CoA, generated in the course of fatty acid β -oxidation, supports de novo nucleotide synthesis, and promotes PROX1-dependent transcription regulation of vascular endothelial growth factor receptor 3 (*VEGFR3*) through an epigenetic mechanism [4, 5]. Other functionally significant, but less explored, LEC metabolic processes are glucose oxidation and glutamine oxidation [2]. Their functional significance in lymphatic vessel development, however, remains to be defined.

In this chapter, we describe methods for measuring glycolysis, fatty acid β -oxidation, glucose oxidation, and glutamine oxidation in LECs. Tritium labeling of glucose and palmitic acid at specific positions is used to measure rates of glycolysis and fatty acid β -oxidation respectively, by means of assessment of $^3\text{H}_2\text{O}$ [6, 7] in both of these reactions. We also take advantage of ^{14}C -labeled glucose and glutamine to assess flux rates of glucose and glutamine oxidation, during which $^{14}\text{CO}_2$ is generated [8, 9]. By capturing and measuring $^3\text{H}_2\text{O}$ or $^{14}\text{CO}_2$ produced by LECs, we are able to evaluate relative flux rates of glycolysis, fatty acid β -oxidation, glucose oxidation, and glutamine oxidation.

In addition to radioactive tracer-based approaches, we have also developed a method for using MS to measure the concentrations of glycolytic intermediates [10]. MS is an excellent technique to measure metabolite intermediates due to its high sensitivity. This technique relies on the ionization, fragmentation, and detection of each metabolite based on their mass-to-charge ratio (MS/MS). For this reason, MS is often coupled to a liquid chromatography step that separates metabolites prior ionization in the mass spectrometer (LC–MS/MS). Because the glycolytic intermediates are very similar in mass and very unstable in solution (due to the high-energy phosphate bond), the following methodology focuses on metabolite stability, separation, and detection using an LC–MS/MS system.

2 Materials

2.1 Radiochemicals

1. D-[5-³H(N)]-Glucose.
2. [9,10-³H(N)]-Palmitic acid.
3. D-[6-¹⁴C]-Glucose.
4. L-[¹⁴C(U)]-Glutamine.

2.2 Chemical Reagents and Solutions

1. RIPA lysis and extraction buffer: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail.
2. Scintillation liquid.
3. Carnitine stock solution: dissolve L-Carnitine inner salt in water to make 50 mM stock solution.
4. Bovine serum albumin (BSA) stock solution: dissolve fatty acid-free BSA in water to make 5 mM stock solution.
5. Sodium palmitate stock solution: dissolve sodium palmitate in ethanol to make 10 mM stock solution.
6. Perchloric acid: 70%; ACS reagent.
7. Hyamine hydroxide: 10× solution.

2.3 Consumables

1. Serum vials: borosilicate glass; 10 mL capacity; clear color.
2. Rubber stoppers with fold over skirts: bottom diameter 15.9 mm.
3. Center wells for incubation flasks: polypropylene; length 70 mm; diameter 10 mm.
4. Filter paper: grade 3 MM chr cellulose chromatography papers.
5. Disposable scintillation vials: 8 mL and 10 mL.

2.4 Cell Culture

1. Human dermal lymphatic endothelial cells (HDLECs).
2. Cell culture plates.
3. Endothelial cell growth medium that contains 5.55 mM glucose and 10 mM L-glutamine.
4. Penicillin–streptomycin solution.
5. Dulbecco's phosphate-buffered saline (DPBS).

2.5 LC-MS/MS Analysis

Prepare all solutions using ultrapure water. Solutions used in MS must be prepared using HPLC-grade solvents.

1. Glycolysis quenching solution: 20% methanol, 0.1% formic acid, 1 mM phenylalanine, 3 mM NaF, 100 μ M EDTA, 10 μ M $^2\text{H}_4$ -taurine (CDN Isotopes; as a loading control). Keep solution at 4 °C (*see Note 1*).
2. ATP extraction solution: 50% acetonitrile, 10 mM spermine, 100 μ M EDTA.
3. HPLC aqueous mobile phase: 15 mM ammonium formate.
4. HPLC organic mobile phase: 60% acetonitrile, 35% isopropyl alcohol, 15 mM ammonium formate.
5. Metabolite standard solution: standard mix containing 200 μ M of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6bP), dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3PG), 2-phosphoglycerate (2PG), phosphoenolpyruvate (PEP), lactate, ATP, and taurine. Keep solution at 4 °C.

2.6 Specialized Materials for LC-MS/MS Analysis

1. Freeze Dryer.
2. Falcon™ 96-well V-bottom plate (*see Note 2*).
3. Mass Spectrometer, ABSCIEX 5500 QTRAP® equipped with SelexION® for Differential Mobility Spectrometry (DMS) (*see Note 3*).
4. HPLC (Shimadzu) equipped with autosampler (SIL-20 AC) and a column oven (CTO-20A).
5. Chromatography: C18 column (5 μ m particle size, 4.6 mm $\times$ 25 cm, Vydac, 218TP54).

3 Methods

3.1 Measurement of Glycolytic Flux Using D-[5- ^3H (N)]-Glucose

1. One day before experiments, plate HDLECs (*see Note 4*) into 12-well plates (four wells for each experimental group). Prepare two background control wells,

which are filled with cell culture medium but without any cells. Include the two control wells throughout experiments.

2. On the day of experiments, prepare radioactive medium by adding D-[5-³H(N)]-Glucose into culture medium (0.4 μCi/mL).
3. Incubate HDLECs in the radioactive medium (1 mL/well) for 2 h in a cell culture incubator (37 °C).
4. Assemble rubber stoppers with center wells (Fig. 1a). Fold and place a filter paper (1 cm × 6 cm) soaked with 200 μL of water into each center well (Fig. 1a).
5. At the end of the 2 h incubation, carefully transfer 800 μL of radioactive medium from each well to glass serum vials (*see Note 5*); seal vials tightly with rubber stoppers (Fig. 1b).
6. Remove residual radioactive medium from each well, wash cells with ice-cold DPBS, add 100 μL of ice-cold RIPA buffer per well to lyse HDLECs, and collect lysates into tubes for protein concentration measurement.
7. Incubate glass vials in a cell culture incubator (37 °C) for at least 2 days to allow the evaporated ³H₂O to be captured by filter paper in hanging wells.
8. Carefully remove rubbers stoppers, transfer filter paper into scintillation vials (volume 7 mL) prefilled with 6 mL of scintillation liquid, and measure radioactivity of ³H.
9. Measure protein concentration of lysates from each well.
10. Calculate relative flux rates of glycolysis (*see Note 6*).

3.2 Measurement of Fatty Acid β-Oxidation Using [9,10-³H(N)]-Palmitic Acid

1. One day before experiments, plate HDLECs (*see Note 4*) into 12-well plates for measurement of fatty acid β-oxidation (Four wells for each experimental group). In addition, plate the same number of HDLECs into separate 12-well plates for protein extraction and concentration measurement (three wells for each experimental group). Moreover, prepare two background control wells, which are filled with cell culture medium but without any cells. Include the two control wells throughout experiments.
2. On the day of experiments, prepare radioactive medium through the following steps: (1) add stock solutions for carnitine (final concentration: 50 μM), BSA (final concentration: 50 μM), and sodium palmitate (final concentration: 100 μM) into endothelial cell growth medium (contain every component except serum); (2) incubate the solution at a 37 °C water bath for 1 h; (3) add [9,10-³H(N)]-Palmitic acid into the solution (2 μCi/mL).
3. Incubate HDLECs in the radioactive medium (1 mL/well) for 6 h in a cell culture incubator (37 °C).

4. Wash cells plated in 12-well plates (for protein extraction) with ice-cold DPBS, add 100 μ L of ice-cold RIPA buffer per well to lyse HDLECs, and collect lysates into tubes for protein concentration measurement.
5. Assemble rubber stoppers with center wells (Fig. 1a). Fold and place a filter paper (1 cm $\times$ 6 cm) soaked with 200 μ L of water into each center well (Fig. 1a).
6. At the end of the 6 h incubation, carefully transfer 800 μ L of radioactive medium from each well to glass serum vials (*see Note 5*); seal vials tightly with rubber stoppers (Fig. 1b).
7. Incubate glass vials in a cell culture incubator (37 $^{\circ}$ C) for at least 2 days to allow evaporated $^3\text{H}_2\text{O}$ to be captured by filter paper in hanging wells.
8. Carefully remove rubbers stoppers, transfer filter paper into scintillation vials (volume 7 mL) prefilled with 6 mL of scintillation liquid, and measure radioactivity of ^3H .
9. Measure protein concentration of lysates from each well.
10. Calculate relative flux rates of fatty acid β -oxidation (*see Note 6*).

3.3 Measurement of Glucose Oxidation Using D-[6- ^{14}C]-Glucose

1. One day before experiments, plate HDLECs (*see Note 4*) into 12-well plates (*see Note 7*) for measurement of glucose oxidation (3–4 wells for each experimental group). In addition, plate the same number of HDLECs into separate 12-well plates for protein extraction and concentration measurement (three wells for each experimental group). Moreover, prepare two background control wells, which are filled with cell culture medium but without any cells. Include the two control wells throughout experiments.
2. On the day of experiments, prepare radioactive medium by adding D-[6- ^{14}C]-Glucose into culture medium (0.5 $\mu\text{Ci/mL}$).
3. Incubate HDLECs in the radioactive medium (1 mL/well) for 6 h in a cell culture incubator (37 $^{\circ}$ C).
4. Wash cells plated in 12-well plates (for protein extraction) with ice-cold DPBS, add 100 μ L of ice-cold RIPA buffer per well to lyse HDLECs, and collect lysates into tubes for protein concentration measurement.
5. Use water to dilute 70% perchloric acid and 10 $\times$ hyamine hydroxide into 12% perchloric acid and 1 $\times$ hyamine hydroxide, respectively.
6. Perform the following steps in a chemical fume hood.
7. Prepare and place filter paper (2.6 cm $\times$ 2.6 cm) on lids of 12-well plates and make sure each well with the radioactive medium can be fully covered by a filter paper. Use 1 $\times$ hyamine to wet filter paper ($\sim$ 300 μ L hyamine/filter paper).

8. Add 250 μ L of 12% perchloric acid into each well with the radioactive medium to promote release of $^{14}\text{CO}_2$ from the medium. Immediately cover wells with 12-well plate lids (with hyamine-saturated filter paper).
9. Gently seal the marginal space between 12-well plates and their lids with plastic paraffin films.
10. Place 12-well plates in a chemical fume hood overnight.
11. Carefully open 12-well plates, transfer filter paper into scintillation vials (volume 20 mL) prefilled with 18 mL of scintillation liquid, and measure radioactivity of ^{14}C .
12. Measure protein concentration of lysates from each well.
13. Calculate relative flux rates of glucose oxidation (*see Note 6*).

3.4 Measurement of Glutamine Oxidation Using L-[$^{14}\text{C}(\text{U})$]-Glutamine

1. One day before experiments, plate HDLECs (*see Note 4*) into six-well plates (*see Note 7*) for measurement of glutamine oxidation (3–4 wells for each experimental group). In addition, plate the same number of HDLECs into separate six-well plates for protein extraction and concentration measurement (three wells for each experimental group). Moreover, prepare three background control wells, which are filled with cell culture medium but without any cells. Include the three control wells throughout experiments.
2. On the day of experiments, prepare radioactive medium by adding L-[$^{14}\text{C}(\text{U})$]-Glutamine into culture medium (0.5 $\mu\text{Ci/mL}$).
3. Incubate HDLECs in the radioactive medium (1 mL/well) for 6 h in a cell culture incubator (37 $^{\circ}\text{C}$).
4. Wash cells plated in six-well plates (for protein extraction) with ice-cold DPBS, add 150 μ L of ice-cold RIPA buffer per well to lyse HDLECs, and collect lysates into tubes for protein concentration measurement.
5. Use water to dilute 70% perchloric acid and 10 $\times$ hyamine hydroxide into 12% perchloric acid and 1 $\times$ hyamine hydroxide, respectively.
6. Perform the following steps in a chemical fume hood.
7. Prepare and place filter paper (3.9 cm $\times$ 3.9 cm) on lids of six-well plates and make sure each well with the radioactive medium can be fully covered by a filter paper. Use 1 $\times$ hyamine to wet filter paper ($\sim$ 680 μ L of hyamine/filter paper).
8. Add 500 μ L of 12% perchloric acid into each well with the radioactive medium to promote release of $^{14}\text{CO}_2$ from the medium. Immediately cover wells with six-well plate lids (with hyamine-saturated filter paper).
9. Gently seal the marginal space between six-well plates and their lids with plastic paraffin films.
10. Place six-well plates in a chemical fume hood overnight.

11. Carefully open six-well plates, transfer filter paper into scintillation vials (volume 20 mL) prefilled with 18 mL of scintillation liquid, and measure radioactivity of ^{14}C .
12. Measure protein concentration of lysates from each well.
13. Calculate relative flux rates of glutamine oxidation (*see Note 6*).

3.5 Measurement of Glycolytic Intermediates Using LC-MS/MS

3.5.1 Glycolysis Quenching

1. Remove plates from a cell culture incubator (37 °C) and place them on ice (*see Note 8*).
2. Quickly aspirate cell culture medium and add 2 mL of ice-cold PBS.
3. Aspirate PBS and add 150 μL of ice-cold quenching solution.
4. Quickly scrape cells in the quenching solution.
5. Tilt the plate and carefully collect the solution containing the cell extract in the bottom of each well.
6. Transfer the content of each well onto a 96-well plate previously placed on dry ice.
7. Freeze immediately to prevent metabolite degradation.
8. Freeze dry samples overnight and resuspend them in 50 μL of ultrapure water (*see Note 9*).
9. Prepare a concentration curve by serial diluting the metabolite standard solution.
10. Place the samples in the temperature controlled HPLC autos-amplifier previously set at 4 °C.

3.5.2 ATP Extraction

1. Remove plates from a cell culture incubator and place them on ice.
2. Quickly aspirate cell culture medium and add 2 mL of ice-cold PBS.
3. Aspirate PBS and add 150 μL of ice-cold ATP extraction solution (*see Note 10*).
4. Quickly scrape cells in the quenching solution.
5. Tilt the plate and carefully collect the solution containing the cell extract in the bottom of each well.
6. Transfer the content of each well onto a 96-well plate.
7. DO NOT freeze and store plate to be analyzed at a later date. DO keep plate refrigerated and run samples as soon as possible to avoid ATP degradation.

3.5.3 Sample Analysis with MS

1. Inject samples onto a C18 column at a flow rate of 1 mL/min.

2. Elute metabolites isocratically with an 85% aqueous/15% organic solvent mixture.
3. Detect metabolites using multiple reaction monitoring (MRM) in negative mode. Use the following source parameters: CUR = 30, CAD = high, IS = -1500, TEM = 625, GS1 = 50, and GS2 = 55.
4. DMS is an effective separation of metabolites with similar mass. The separation of glucose-6-phosphate and fructose-6-phosphate is best achieved with no modifier. Use isopropyl alcohol as modifier for the DMS-based separation of the remaining metabolites. The parameters to be used with DMS are: DT = low, MDC = low, DMO = 3, and DR = off. The pair of separation voltage (SV) and compensation voltage (CoV) must be optimized for each metabolite before each experiment.
5. Use the following MRM transition pairs (Q_1/Q_3) for metabolite detection: 259/97 for G6P, 259/97 for F6P, 339/97 for F1,6bP, 169/97 for DHAP, 185/79 for 3PG and 2PG, 89/89 for lactate, 506/159 for ATP, and 124/80 for endogenous taurine.
6. Integrate peaks using MultiQuant®.
7. Calculate metabolite concentration by interpolating the area of each metabolite against its standard curve.
8. Use endogenous taurine as internal control for cell density as previously described [11].

4 Notes

1. This solution is designed to burst cells and stop metabolism quickly. Methanol and formic acid ensure the rupture of the cell membranes and precipitation of enzymes. Phenylalanine is an inhibitor of pyruvate kinase, NaF is an inhibitor of enolase, and EDTA inhibits any enzymes that need divalent ions as cofactors. The use of an ice-cold quenching solution prevents the (non)enzymatic degradation of metabolites.
2. These plates are resistance to organic solvents and prevent the contamination of the samples with small plastic by-products that could interfere with the HPLC performance. Additionally, the V-bottom allows the collection of the samples in the bottom of each well, which is ideal for smaller volume samples.
3. The accurate detection of metabolites using LC-MS/MS is dependent on the chromatographic separation provided by the HPLC columns and the detection of unique fragments (using the MRM methodology). The use of DMS adds an additional layer of separation to sample detection through the physical interaction between the metabolite and a volatile solvent (i.e., the modifier). This method of separation is highly efficient and reduces the need for long and complex chromatographic separations.

4. Adjust the number of cells plated to ensure cell confluency of ~80% on the day of experiments.
5. To prevent bacterial contamination, autoclave glass serum vials.
6. To calculate the relative metabolic flux rates, first subtract the average readings of background control wells from the readings of experimental wells. Then divide the adjusted reading of each experimental well by its corresponding protein concentration, and normalize it to a group of data of interest (data average of this group is defined as 1).
7. Plate cells into wells which are not adjacent to each other to avoid interference of neighboring wells.
8. The main preoccupation during the quenching step is to stop metabolism as quickly as possible. This will ensure that the concentrations of each metabolite remain similar to what they were during the incubation. Removing the plates from the incubator will affect the temperature and the oxygen tension experienced by cells, which can in turn affect metabolism. By placing the plates on ice, using ice-cold solutions and quickly executing the procedure one ensures minimal impact of these changes on metabolism.
9. Freeze-drying the samples achieves two main goals: the removal of organic solvent that could affect the chromatographic separation and the creation of a more concentrated solution. The concentration of glycolytic intermediates is typically very low and resuspending the cell extract in a smaller volume ensures a greater signal-to-noise ratio and therefore a more accurate detection of these metabolites.
10. ATP is a highly charged molecule, which promotes unspecific binding to the metal components of the LC-MS/MS system. The resulting poor elution impacts the quality of the chromatograms and the accuracy of the quantification. The polycationic character of spermine helps stabilize ATP preventing its degradation and nonspecific binding. As a result, the chromatographic quality and quantitation are dramatically improved.

References

1. Rohlenova K, Veys K, Miranda-Santos I, De Bock K, Carmeliet P (2018) Endothelial cell metabolism in health and disease. *Trends Cell Biol* 28(3):224–236. 10.1016/j.tcb.2017.10.010 [PubMed: 29153487]
2. Yu P, Wilhelm K, Dubrac A, Tung JK, Alves TC, Fang JS, Xie Y, Zhu J, Chen Z, De Smet F, Zhang J, Jin SW, Sun L, Sun H, Kibbey RG, Hirschi KK, Hay N, Carmeliet P, Chittenden TW, Eichmann A, Potente M, Simons M (2017) FGF-dependent metabolic control of vascular development. *Nature* 545(7653):224–228. 10.1038/nature22322 [PubMed: 28467822]
3. Yu P, Tung JK, Simons M (2014) Lymphatic fate specification: an ERK-controlled transcriptional program. *Microvasc Res* 96:10–15. 10.1016/j.mvr.2014.07.016 [PubMed: 25132472]
4. Wong BW, Wang X, Zecchin A, Thienpont B, Cornelissen I, Kalucka J, Garcia-Caballero M, Missiaen R, Huang H, Bruning U, Blacher S, Vinckier S, Goveia J, Knobloch M, Zhao H, Dierkes C, Shi C, Hagerling R, Moral-Darde V, Wyns S, Lippens M, Jessberger S, Fendt SM, Luttun A, Noel A, Kiefer F, Ghesquiere B, Moons L, Schoonjans L, Dewerchin M, Eelen G, Lambrechts D,

- Carmeliet P (2017) The role of fatty acid beta-oxidation in lymphangiogenesis. *Nature* 542(7639): 49–54. 10.1038/nature21028 [PubMed: 28024299]
5. Schoors S, Bruning U, Missiaen R, Queiroz KC, Borgers G, Elia I, Zecchin A, Cantelmo AR, Christen S, Goveia J, Heggermont W, Godde L, Vinckier S, Van Veldhoven PP, Eelen G, Schoonjans L, Gerhardt H, Dewerchin M, Baes M, De Bock K, Ghesquiere B, Lunt SY, Fendt SM, Carmeliet P (2015) Fatty acid carbon is essential for dNTP synthesis in endothelial cells. *Nature* 520(7546):192–197. 10.1038/nature14362 [PubMed: 25830893]
6. Dagher Z, Ruderman N, Tornheim K, Ido Y (2001) Acute regulation of fatty acid oxidation and amp-activated protein kinase in human umbilical vein endothelial cells. *Circ Res* 88(12):1276–1282 [PubMed: 11420304]
7. Aragones J, Schneider M, Van Geyte K, Fraisl P, Dresselaers T, Mazzone M, Dirx R, Zacchigna S, Lemieux H, Jeoung NH, Lambrechts D, Bishop T, Lafuste P, Diez-Juan A, Harten SK, Van Noten P, De Bock K, Willam C, Tjwa M, Grosfeld A, Navet R, Moons L, Vandendriessche T, Deroose C, Wijeyekoon B, Nuyts J, Jordan B, Silasi-Mansat R, Lupu F, Dewerchin M, Pugh C, Salmon P, Mortelmans L, Gallez B, Gorus F, Buyse J, Sluse F, Harris RA, Gnaiger E, Hespel P, Van Hecke P, Schuit F, Van Veldhoven P, Ratcliffe P, Baes M, Maxwell P, Carmeliet P (2008) Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nat Genet* 40(2):170–180. 10.1038/ng.2007.62 [PubMed: 18176562]
8. Brand K, Williams JF, Weidemann MJ (1984) Glucose and glutamine metabolism in rat thymocytes. *Biochem J* 221(2):471–475 [PubMed: 6332620]
9. De Bock K, Georgiadou M, Schoors S, Kuchnio A, Wong BW, Cantelmo AR, Quaegebeur A, Ghesquiere B, Cauwenberghs S, Eelen G, Phng LK, Betz I, Tembuyser B, Brepoels K, Welti J, Geudens I, Segura I, Cruys B, Bifari F, Decimo I, Blanco R, Wyns S, Vangindertael J, Rocha S, Collins RT, Munck S, Daelemans D, Imamura H, Devlieger R, Rider M, Van Veldhoven PP, Schuit F, Bartrons R, Hofkens J, Fraisl P, Telang S, Deberardinis RJ, Schoonjans L, Vinckier S, Chesney J, Gerhardt H, Dewerchin M, Carmeliet P (2013) Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell* 154(3):651–663. 10.1016/j.cell.2013.06.037 [PubMed: 23911327]
10. Alves TC, Pongratz RL, Zhao X, Yarborough O, Sereda S, Shirihai O, Cline GW, Mason G, Kibbey RG (2015) Integrated, step-wise, mass-Isotopomeric flux analysis of the TCA cycle. *Cell Metab* 22(5):936–947. 10.1016/j.cmet.2015.08.021 [PubMed: 26411341]
11. Kibbey RG, Pongratz RL, Romanelli AJ, Wollheim CB, Cline GW, Shulman GI (2007) Mitochondrial GTP regulates glucose-stimulated insulin secretion. *Cell Metab* 5(4):253–264. 10.1016/j.cmet.2007.02.008 [PubMed: 17403370]

Fig. 1. Assembly of center wells, rubber stoppers, and serum vials for measurement of glycolytic flux. (a) filter paper (1 cm × 6 cm) saturated with 200 µL of water was placed into a center well, which was assembled with a rubber stopper. (b) A glass serum vial with radioactive medium was tightly sealed with a rubber stopper that was assembled with a center well