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TITLE:

A comprehensive high-efficiency protocol for isolation, culture, polarization, and glycolytic characterization of bone marrow-derived macrophages

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Real-time glycolysis, macrophage polarization, M1-like, and M2-like Macrophages, Extracellular flux analysis, immunometabolism, Oxidative phosphorylation (OXPHOS), Bone Marrow-Derived Macrophages (BMDM)

SUMMARY:

This protocol provides detailed and comprehensive methods for the isolation, culture, polarization, and measurement of the glycolytic metabolic state of live bone marrow-derived macrophages (BMDMs). This paper provides step-by-step instructions with realistic visual illustrations for workflow and glycolytic assessment of BMDMs in real-time.

ABSTRACT:

Macrophages are among the most important antigen-presenting cells. Many subsets of macrophages have been identified with unique metabolic signatures. Macrophages are commonly classified as M1-like (inflammatory) and M2-like (anti-inflammatory) subtypes. M1-like macrophages are pro-inflammatory macrophages that get activated by LPS and/or pro-inflammatory cytokines such as INF-γ, IL-12 & IL-2. M1-like polarized macrophages are involved in various diseases by mediating the host's defense to a variety of bacteria and viruses. That is very important to study LPS induced M1-like macrophages and their metabolic states in inflammatory diseases. M2-like macrophages are considered anti-inflammatory macrophages, activated by anti-inflammatory cytokines and stimulators. Under the pro-inflammatory state, macrophages show increased glycolysis in glycolytic function. The glycolytic function has been actively investigated in the context of glycolysis, glycolytic capacity, glycolytic reserve, compensatory glycolysis, or non-glycolytic acidification using extracellular flux (xf) analyzers.

This paper demonstrates how to assess the glycolytic states in real-time with easy-to-follow steps when the bone marrow-derived macrophages (BMDMs) are respiring, consuming, and producing energy. Using specific inhibitors and activators of glycolysis in this protocol, we show how to obtain a systemic and complete view of glycolytic metabolic processes in the cells and provide more accurate and realistic results. To be able to measure multiple glycolytic phenotypes, we provide an easy, sensitive, DNA-based normalization method for polarization assessment of BMDMs. Culturing, activation/polarization and identification of the phenotype and metabolic state of the BMDMs are crucial techniques that can help to investigate many different types of diseases.

In this paper, we polarized the naïve M0 macrophages to M1-like and M2-like macrophages with LPS and IL4, respectively, and measured a comprehensive set of glycolytic parameters in BMDMs in real-time and longitudinally over time, using extracellular flux analysis and glycolytic activators and inhibitors.

INTRODUCTION:

Macrophages are one of the most critical cells of the innate immune system M1-like. They are involved in clearing infectious diseases, phagocytosis, antigen presentation, and inflammation regulation[2]. Furthermore, macrophages are required to regulate other immune cells via various cytokines they release[3]. There is a big spectrum in macrophage phenotypes[4]. Depending on the signals that macrophages are exposed to, they polarize toward different inflammatory and metabolic states[5]. Macrophages manifest metabolic alterations in various diseases, depending on what tissue the macrophages reside[6]. Polarized macrophages have the capability to reprogram or switch their glycolytic metabolism, lipid metabolism, amino acid metabolism, and mitochondrial oxidative phosphorylation (OXPHOS)[7,8]. Classically activated M1-like macrophages and alternatively activated M2-like macrophages are the two most studied phenotypes of macrophages[3]. Non-activated quiescent macrophages are referred to as M0 macrophages. Polarization of M0 macrophages towards an M1-like phenotype can be induced by stimulation of naive BMDMs with bacterial lipopolysaccharide (LPS)[9]. The PI3K-AKT-mTOR-HIF1a signaling pathway can be activated in macrophages in the presence of inflammatory cytokines, interferon-gamma (IFN γ,) or tumor necrosis factor (TNF)[10]. M1-like macrophages have increased levels of glycolysis metabolism, decreased levels of oxidative phosphorylation (OXPHOS), producing inflammatory cytokines involved in infectious and inflammatory diseases[8]. On the other hand, polarization towards the M2-like phenotype can be induced by Interleukin (IL)-4, via the JAK-STAT, PPAR, and AMPK pathways, or by (IL)-13 and TGFβ pathays[11,12].

In contrast to M1-like macrophages, M2-like macrophages have decreased glycolysis and increased OXPHOS and are involved in anti-parasitic and tissue repair activities[8,13]. BMDMs are a widely used system for the study of macrophages that are derived from bone marrow stem cells. Glycolysis and OXPHOS are the two leading energy production pathways in the cells[14]. Based on their microenvironment, BMDMs can choose to use either of these pathways; in some cases, switch from one to another, or use both pathways[14]. In this study, we focused on glycolysis metabolism in activated pro-inflammatory macrophages. When the glucose in the cytoplasm is

converted to pyruvate and then lactate, the cells produce protons in the medium that cause an elevation in the acidification rate in the surrounded medium of M1-like cells[5]. An extracellular flux analyzer was used to measure the acidification rate of the cell media. Results are reported as Extracellular Acidification Rate (ECAR) or as Proton Efflux Rate.

An optimized quick and easy method to access glycolysis levels in polarized macrophages is essential to determine the glycolytic phenotype, metabolite changes, and the effects of inhibitors/activators and drugs on the polarized macrophages. The method described in this manuscript has been optimized to give information about specific glycolysis factors (Glycolysis, Glycolytic capacity, Glycolytic reserve, and Non-glycolytic acidification), as well as the metabolic reprogramming of glycolytic metabolism. The inhibitor (2DG) that has been used in this study explicitly targets the glycolysis pathway.

This optimized protocol has been modified and improved based on the combination of a published protocol[16], extracellular flux analysis of glycolytic assays of manufacturer's user guides, and direct communication with manufacturer's R&D scientists.

PROTOCOL:

Mice were humanely sacrificed according to Assessment and Accreditation of Laboratory Animal Care (AAALAC) and American Association for Laboratory Animal Science (AALAS) guidelines and using protocols approved by the Texas A&M University institutional animal care and use committee (IACUC).

1. Mice bone marrow harvest and culture of BMDMs

1.1. Sacrifice mouse (6-10 weeks of age C57Bl/6 mice were in this protocol) and lay it on its ventral side, cut the skin and peritoneal layer and gently peel off the legs.

NOTE: Use CO_2 gas exposure to euthanize the mouse.

1.2. Separate both hind legs from the hip down, being careful not to cut the bone.

1.3. Place the whole leg in an empty 50 mL conical tube (with feet facing up to have an easy grip to pull out later) on ice and proceed with harvesting both legs from the mouse.

2. Femur exposure

NOTE: Perform the following steps in a biosafety cabinet.

2.1. Harvest the femur by cutting off the tibia from each leg and remove as much tissue surrounding the femur as possible with scissors and laboratory paper.

2.2. Place harvested, “cleaned” femurs in a 10 cm plate containing a piece of laboratory paper saturated with tissue culture (TC) medium or PBS. Place them on ice.

2.3. Continue harvesting femurs and removing tissue from all femurs before proceeding to the flushing stage (**Figure 1A**).

3. Marrow flush

3.1. To flush marrow from femurs, use a 3 mL syringe filled with TC medium or PBS with a 23G needle. Fill the syringe before exposing the marrow.

3.2. Use scissors to expose the marrow by cutting the very end of the femur at both epiphyses.

3.3. Insert needle tip into the femur and gently flush marrow out into a 10 cm dish.

3.4. Run the needle through the entire length of the femur, and flush until the bone color turns white. Usually, most marrow can be flushed out with 2-3 mL of media.

3.5. Flush all femurs and pool bone marrow in the dish. Use a needle to break up any visible clumps. Strain marrow into a 50 mL conical tube (**Figure 1A**).

4. RBC lysis

4.1. Spin marrow at 190 x g for 10 min. Aspirate the supernatant.

4.2. Resuspend the pellet in 4 mL of ACK lysis buffer with a pipette. Allow RBC lysis buffer to work for 5 min at room temperature.

4.3. Add 4 mL of TC medium RPMI-C 10% (RPMI 1640 –GlutaMAX) supplemented with 2-mercaptoethanol, gentamicin, streptomycin, and 10% FCS to the marrow suspension and spin at 1300 x g for 10 min.

4.4. Strain again to remove RBC debris and re-suspend in a small volume of RPMI-C 10% for counting.

4.5. Count cells with a cell counter (**Figure 1B**). A Vi-Cell Counter was used to determine the count and viability of cells in the suspension.

5. Plating and culture

5.1. Add 10 mL of RPMI-C 10% + 10 ng/mL M-CSF (Macrophage Colony Stimulating Factor, an essential regulator of monocyte/macrophage proliferation, differentiation, and survival) to as many 10 cm plates as desired.

5.2. Add an appropriate volume of counted cells so that each 10 cm plate contains 1 x 10^6 cells. Put the plates in a 37 °C incubator (defined as day 0).

5.3. On day 3, gently add 5 mL of fresh RPMI-C 10% + 10 ng/mL M-CSF to each plate.

NOTE: On day 7, BMDMs should be ready for testing (**Figure 1C**).

6. Harvest from plates

6.1. Use a light microscope to confirm that most cells have adhered to plates.

6.2. Gently aspirate media. Then add 3 mL of PBS and gently swirl the plate. Aspirate this well to remove any remaining non-adherent cells.

6.3. Add 7-10 mL of cold PBS to the plate, use a P1000 pipette to wash the bottom of plates, and harvest all remaining cells into a collection tube.

NOTE: Keep tubes on ice as macrophages are very tightly adherent and will adhere to the inside of the tube. If the cells kept cold, they would be less tightly adherent.

6.4. Centrifuge, count, and plate cells for experiments (**Figure 1D**). Using Flow cytometry, resulting cells should be >95% positive for CD11b and F4/80. (macrophage polarization was determined by staining with M1-like markers of CD38, TNF-a, and MCP-1 and M2-like marker of CD206.

NOTE: Perform steps 6.1-6.3 in the biosafety cabinet and perform step 6.4 on the benchtop. Maintain aseptic techniques throughout the procedure.

[Place **Figure 1** here].

7. The day before the metabolic flux analyzer assay: seeding and polarization of the cells for the glycolytic test

7.1. Warm up the Metabolic Flux Analyzer to 37 °C by turning on the instrument.

7.2. Hydrate a cartridge by adding 200 µL of a Calibrant Solution and incubate the cartridge in a non-CO_2 incubator overnight (**Figure 2A**). The humidity of non-CO_2 incubator is not important for cartridge hydration.

7.2.1. An hour prior to the experiment, dip the plate a few times up and down, which will help to remove air bubbles.

7.3. Design the plate map on the software in the default glycolysis stress test-acute injection, by following the instruction of the test.

7.3.1. Click on the software icon, and then click on **Glycolysis stress-acute injection test**. On the group definition icon, generate group names.

7.4. There are five measurement cycles with a duration of 18 minutes and four injections. Change the injection of port A to Glucose, port B to Oligomycin, Port C to Rotenone and antimycin A (Rot/AA), and Port D to 2DG.

7.5. Re-suspend the cells in RPMI-C 10% medium and seed 50k cells per well except for the four edges of the plate (A1, A12, H1, and H12; Add media only, no cells) in a Metabolic Flux Analyzer microplate to a final volume of 100 µL. Normally a minimum of 40k cells is required to conduct this assay.

7.6. Allow the cells to sit at room temperature for 45 min to avoid the edge effect of the cells. The edge effect is when the medium from around the perimeter of the plate evaporates partly, which causes volume and concentration changes and reduces cell viability.

7.7. Add 10 ng/mL LPS to polarize the naïve macrophages towards M1-like cells and add 20 ng/ml of IL-4 to polarize them towards M2-like cells. Use at least 3 to 6 wells per condition (**Figure 2B**).

7.8. Check the cells under the microscope and place the plate in an incubator at 37 °C and 5% CO_2 for 24 hours.

[Place **Figure 2** here].

8. Day of the assay: XF Medium and compound preparation

8.1. Complement 100 mL of XF RPMI (pH 7.4) assay medium with 2 mM glutamine.

8.2. Filter-sterilize the media using a 0.2 µm vacuum filter system.

8.3. Place the assay media in a 37 °C water bath for 20 min.

8.4. Remove the plated cells from the 37 °C, 5% CO_2 incubator. Wash the cells with assay media twice and replace the previous media with assay media to the final volume of 180 µL.

8.5. Use a microscope to make sure that all the wells have confluent cells and mark any wells that have any scratches from pipetting. If there are any scratches, remove that plate before analyzing.

8.6. Position the cell-containing plate in a non-CO_2 incubator for 45 min (**Figure 3A**).

8.7. Using the compounds and the assay media to make stock solutions of Glucose (100 mM), Oligomycin (100 µM), Rot/AA (50 µM), and 2DG (500 mM) (**Table 1**).

8.8. Make a 10x injection mixture of each compound using assay media (**Table 2**).

Place **Table 1** here].

Place **Table 2** here].

9. Day of the assay: Running the acute glycolytic test on polarized macrophages

9.1. Open the saved Glycolysis stress assay (Acute injection) template from the software. The default Acute Glyco-Stress Test has 3 minutes of mix and measurement before each injection.

9.2. Check the template and the assay details, and when ready, click on **Run** and follow the instruction of the default assay. However, all parameters can be customized.

9.3. Remove the Sensor Cartridge from the non-CO_2 incubator, remove the lid, and insert in the instrument in a way that the A1 well of the cartridge plate falls into the top left corner of the insertion panel of the machine. Usually, calibration takes between 20 to 45 min.

9.4. After finishing the calibration, the device will eject the plate containing the calibrant solution and will hold the sensor cartridge. Remove the calibrant containing the plate.

9.5. Remove the cell plate from the non-CO_2 incubator, remove the plate lid, and insert it in the machine. Click on Run (**Figure 3B**).

9.6. When the assay is done, the machine will eject the cell plate and the sensor cartridge.

9.7. Remove the media from the plate and freeze it at -20 °C for further normalization.

9.8. Use the commercial cell proliferation assay kit (e.g., CyQUANT) for normalizing the cells.

9.9. Add 1 mL of Compound B or lysis buffer to 19 mL of nuclease-free distilled water.

9.10. Add 100 µL of Compound A or GR working solution to the abovementioned solution.

9.11. Make sure the cells in the plate are thawed and then add 200 µL of the solution to each well.

9.12. Incubate for 5 min at room temperature (RT).

9.13. Measure the fluorescence in 480 nm excitation and 520 nm emission wavelengths using a plate reader.

9.14. Normalize the cells on the normalization panel of the software.

9.15. Normalize cells based on naïve macrophages cell count (**Figure 3C**). Consider the average of the naïve macrophages as 1 (by dividing the cell number of each well by the average cell number of naïve macrophages) and apply them to all macrophages.

[Place **Figure 3** here].

REPRESENTATIVE RESULTS:

Glycolysis and mitochondrial oxidative phosphorylation are the two major ATP production pathways in the cells (**Figure 4A**). Some cells have the capability to switch between these two pathways to meet their energy demands. The conversion of glucose to pyruvate in the cytoplasm is called glycolysis. Pyruvate has two fates; it will either get converted to lactate or further metabolized through the TCA cycle and eventually through the electron transport chain (ETC) to produce more ATP. In order to obtain the best understanding of the glycolytic parameters of cells, we minimize the OXPHOS pathways by using oligomycin, which is an inhibitor for mitochondrial ATP synthase. We also inject Rot/AA to completely shut down the ETC to assess the maximal glycolytic capacity and the compensatory glycolysis in the cell (**Figure 4A**). Glucose is the primary fuel of the glycolysis. Since the XF assay medium does not have any glucose or glutamine, the first three measurements in the assay will be an indicator of non-glycolytic acidification rate (**Figure 4B,C**), which is indicative of acidification but not related to the conversion of glucose to lactate. After injection of glucose from port A, increased ECAR levels are indicators of glycolysis rates (measurements 4,5 & 6). Next, by injection of oligomycin from port B and injection of Rot/AA from port C, the ETC is inhibited, and increased amounts of ECAR are an indicator of glycolytic capacity and the compensatory glycolysis rates of the cells (**Figure 4B,C**). Compensatory glycolysis rates of BMDMs demonstrates the cellular energy management capability under mitochondrial stress conditions. In other words, this parameter indicates compensation for energy demand when mitochondrial respiration is inhibited. The last injection is 2 deoxyglucose or 2DG from port D which is a competitive inhibitor of the glucose.

In **Figure 4B,C** an alternative calculation, especially if there are big errors between 3 measurements, is that one can measure the last data point before each injection and prevent unnecessary errors and variations in the data.

Place **Figure 4** here].

Generally, polarized macrophages have more glycolytic activities compared to naïve M0 macrophages. LPS induced M1-like macrophages possess the highest glycolytic activity. Although polarized macrophages have more distinct separations in their OCR spare respiratory capacity (16), which has not been shown here, their glycolytic metabolism is also wholly distinguishable. It is important to note that an increase in ECAR in LPS induced M1-like polarized BMDMs is not a definite characteristic for other types of M1-like polarized macrophages (such as LPS + INF-γ or PAMP induced M1-likes macrophages), and they may not increase or change the ECAR without

glycolytic stress. As expected, in the first three measurements, which is the indicator of the non-glycolytic activity, polarized macrophages do not show a significant difference because the media do not have any sources of glucose or pyruvate (**Figure 5**). After injection of glucose, polarized BMDMs indicate higher levels of glycolysis than naïve BMDMs, and M1-like BMDMs demonstrate the highest levels of glycolysis compared to M0 and M1-like.

Typically, glycolytic metabolism is the preferred ATP production pathway used by M1-like macrophages, and OXPHOS is the main ATP production pathway for M2-like macrophages. After the injection of Oligomycin, the ATP synthase complex in the mitochondrial electron transport chain of BMDMs will shut down; thus, the cells will start to rely on glycolysis to meet their energy demands. Since glucose is present in the media, the glycolytic capacity of the polarized BMDMs will be comparable. Again, LPS-induced M1-like BMDMs will have the highest glycolytic capacity. M2-like and M0 will have lower glycolytic capacities, respectively (**Figure 5**). Injection of Rot/AA will inhibit the Complex I and III of the mitochondrial ETC and completely shut down the OXPHOS, and there will be a slightly higher increase in the ECAR levels, which is an indicator of compensatory glycolysis. Again, M1-like BMDMs will have the highest ECAR levels in this step; finally, 2DG, a competitive inhibitor of glucose and negative control for glycolysis, will completely shut down the Glycolysis pathway.

[Place **Figure 5** here].

FIGURE AND TABLE LEGENDS:

Figure 1: Graphical workflow of mouse bone marrow culture of BM-Derived Macrophages. (A) Leg harvest, Femur exposure, and marrow flush; **(B)** RBC Lysis; **(C)** Plating and culture; **(D)** Cell harvest from the plates.

Figure 2: Graphical demonstration of seeding and polarization of the cells. (A) Extracellular flux analyzer set up and cartridge hydration; **(B)** Polarization of the cells and overnight incubation.

Figure 3: Day of the assay: medium and compound preparation and running the assay. (A) Cells preparation for assay; **(B)** Compounds preparation, calibration, and running the assay; **(C)** Normalization and data analysis.

Figure 4: Energy production in the cell and glycolytic parameters. (A) schematic view of the two most important energy production pathways in the cell; Glycolysis (left) and mitochondrial oxidative phosphorylation (right). Glycolysis is the conversion of glucose to pyruvate. XF analyzer can detect the protons that are produced by conversion of pyruvate to lactate as ECAR (mpH/min) levels. Inhibition of the ATP synthase followed by inhibition of complex I and II the in mitochondrial electron transport chain will eliminate the ATP production and proton efflux through OCR. **(B)** calculation of glycolytic parameters. **(C)** Glycolytic function parameters after each compound injection.

Figure 5: Glycolytic functions of naïve M0 and polarized M1-like (LPS-induced) and M2-like (IL4-induced) BMDMs. Glycolytic parameters of polarized macrophages indicated as ECAR (mpH/min). (A) Non-glycolytic acidification rate, Glycolysis, Glycolytic capacity, and compensatory glycolysis as ECAR (mpH/min) in M0, M1-like, and M2-like BMDMs. Injections of the ports are as follow→ port A: Glucose, Port B: Oligomycin, Port C: Rotenone plus antimycin A, and port D: 2 Deoxy Glucose (B) Bar graphs of each parameter for M0, M1-like, and M2-like BMDMs. Data shown are from 4-6 culture wells per experiment. Measurements are based on means + SEM. Statistical significance between groups are based one-way ANOVA with Tukey's multiple comparison test at "*" $p < 0.05$, significance at "**" $p < 0.01$, significance at "***" $p < 0.001$, significance at "****" $p < 0.0001$. Error bars are derived from standard deviation.

Table 1. Injection stocks

Table 2. Final Injection Concentrations

DISCUSSION:

As mentioned earlier, the extracellular flux analyzer machine can provide real-time information about two major energy-producing pathways of the cells by measuring OCR (oxygen consumption rate), an indicator of mitochondrial OXPHOS activity, and ECAR (extracellular acidification rate) which is an indicator of glycolysis. Macrophages can use both pathways, depending on their microenvironment. They can also switch their energy production pathways[17,18]. Understanding the macrophages' energetic states and their responses to different drugs, cytokines, inhibitors, activators, etc. will provide a better understanding of the metabolic states of these cells. Since glycolysis is one of the most critical pathways that get activated in M1-like types of macrophages, real-time glycolysis related information can help track the changes of M1-like polarized BMDMs in different in vitro conditions[19]. The extracellular flux analysis of the ATP real-time rate assay is highly regarded as a way to assess the ATP production of the polarized BMDMs[20]. The protocol presented herein provides technical details and approaches with a visual demonstration of the workflow to serve as a comprehensive protocol that can be adapted to experimental needs.

This assay provides accurate measurements of glycolytic levels for basal conditions and compensatory glycolysis following mitochondrial inhibition. It is important to note that some of the acidification in extracellular media can have a mitochondrial source[21]. The Krebs cycle or TCA cycle produces CO_2 that can acidify the media through its reaction with water molecules[22]. When the mitochondrial activity is inhibited, the acidification rates are indicators of lactate accumulation in the media. The advantage of the glycolytic stress test is the injection of glucose in a medium that does not have any glucose or pyruvate sources to assess glycolysis levels before and after the treatment of glucose in the media.

On the other hand, the glycolytic rate assay provides specific information about the distinct sources of glycolysis by blocking the mitochondrial activity. In other words, glycolytic proton efflux can be calculated by subtracting OXPHOS proton efflux from total proton efflux. In this protocol, we combined glycolytic assays in one assay and maximized the glycolytic data to obtain glycolysis, glycolytic capacity, glycolytic reserve, compensatory glycolysis, and non-glycolytic

acidification results. These parameters will give a better understanding of the metabolic states and glycolytic phenotype of the cells. With an optimized quick and easy normalization method, it would be possible to get more accurate information about glycolytic metabolism and metabolic reprogramming[21-23].

It is important to note that although the implication of glycolytic reserve does not change in the new combined system, the calculation scheme in the method has been altered slightly. In the combined system (**Figure 4B**), the glycolytic reserve is estimated by Avg. ECAR (10,11,12)-Avg. ECAR (4,5,6). However, in non-combined methods, the glycolytic reserve is calculated by Avg. ECAR (7,8,9)-Avg. ECAR (4,5,6) formula. Both calculations provide very similar results and reflect the glycolytic reserve.

Furthermore, it is worth mentioning that there are different metrics of extracellular acidifications in extracellular flux analyzers. The results of the glycolytic assays in extracellular flux analyzers can be analyzed based on ECAR (mpH/min), PPR (pmol H+/min), and PER (pmol H+/min). There are advantages and disadvantages, but generally, ECAR is the most typical way of displaying extracellular acidification data.

Our lab studies the role of microbiota metabolites on immune cells. Since macrophages are one of the key components of the immune system in chronic inflammatory diseases such as atherosclerosis[23-25], we are interested in studying the role of microbiome metabolites on the polarization of macrophages, especially inflammatory polarized macrophages that have been induced by different proatherogenic signals such as saturated fatty acids, modified LDLs, and harmful gut microbiota-derived or dependent metabolites. We confirm the polarization of the BMDMs by M1-like and M2-like surface and intracellular markers using flow cytometry and qPCR. We consider the extracellular flux assays as functional readouts in the studies. We perform complementary studies by measuring the non-real-time glycolysis factors with a lactate assay.

LPS induced M1-like polarization or LPS + IFNγ induced M1-like polarization are the most common classic M1-like activation way in macrophages. In M1-like polarization, adding IFNγ to LPS or increasing LPS concentration will increase the reduction of spare respiratory capacity in the mitochondrial electron transport chain. IFNγ is known to induce an M1-like phenotype, but, usually, IFNγ by itself is not enough and requires additional TLRs agonists to induce the phenotype. But this is dependent on the diseases and M1-like polarization concerning a specific condition. For example, IFNγ- induced M1-like macrophages cannot produce NO and inflammatory cytokines similar to LPS or LPS/ IFNγ induced macrophages[26].

Drugs that prevent macrophages from inflammatory polarization have the potential to prevent and control atherosclerosis. Understanding the metabolic pathways, energetics, and phenotypic characteristics of the M1 macrophages is essential for studying the role of different endogenous and exogenous drugs. Glycolysis is the dominant energy-producing pathway in M1-like macrophages[24,27].

This simplified study focuses on only glycolytic energetic states of the polarized BMDMs. Doses used in this paper are built on the manufacturer's recommendation and make the experiment much easier to follow. Also, most of the compounds used in this study are provided in the standard kit from the manufacturer; this helps to save time and enhance the consistency of the experiments. It is essential to know that slight differences in compound doses, cell numbers, and incubation times can affect the experiment results. Also, each experimenter should run a cell titration, dose-response, and kinetic analysis for their particular cell type and conditions to understand how those conditions perform on the extracellular flux analyzer.

One should note that during the media change, washing, and normalization steps, some cells may come off by pipetting or fluid pressure. The confluency of the cells is always detectable under the microscope. Those wells need to be excluded from the study if they have any signs of scratches or depletion of cells.

We use 96 well microplates with a minimal number of cells per well, allowing positive and negative controls, as well as different conditions to be tested in one plate; thus, this assay is very time-saving and cost-efficient for extracellular flux analysis. This study has been optimized for BMDM, which are different from tissue-resident macrophages, peritoneal macrophages, and macrophage cell lines.

While in this protocol we primarily focused on the application of extracellular flux analysis in the pro-inflammatory state associated glycolysis, extracellular flux analysis can also be used to assess mitochondrial function characteristics such as total respiration, basal mitochondrial respiration, ATP production, proton leak, maximal respiration, and spare respiratory capacity. Mitochondria play an important role in macrophage metabolic reprogramming. Extracellular flux analyzers have been used to assess mitochondrial stress and fatty acid oxidation by measuring the oxygen consumption rate of the cells[16].

In conclusion, here, we have provided a comprehensive protocol for isolation, culture, polarization, and glycolytic functional analysis of BMDMs. Detailed step-by-step procedures and visual demonstrations were provided for all steps. We hope this protocol will help the investigators to streamline their analyses and to assess the glycolytic function of BMDMs with high quality and efficiency.

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Disclosures:
The authors have nothing to disclose.

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Figure 1- Pdf

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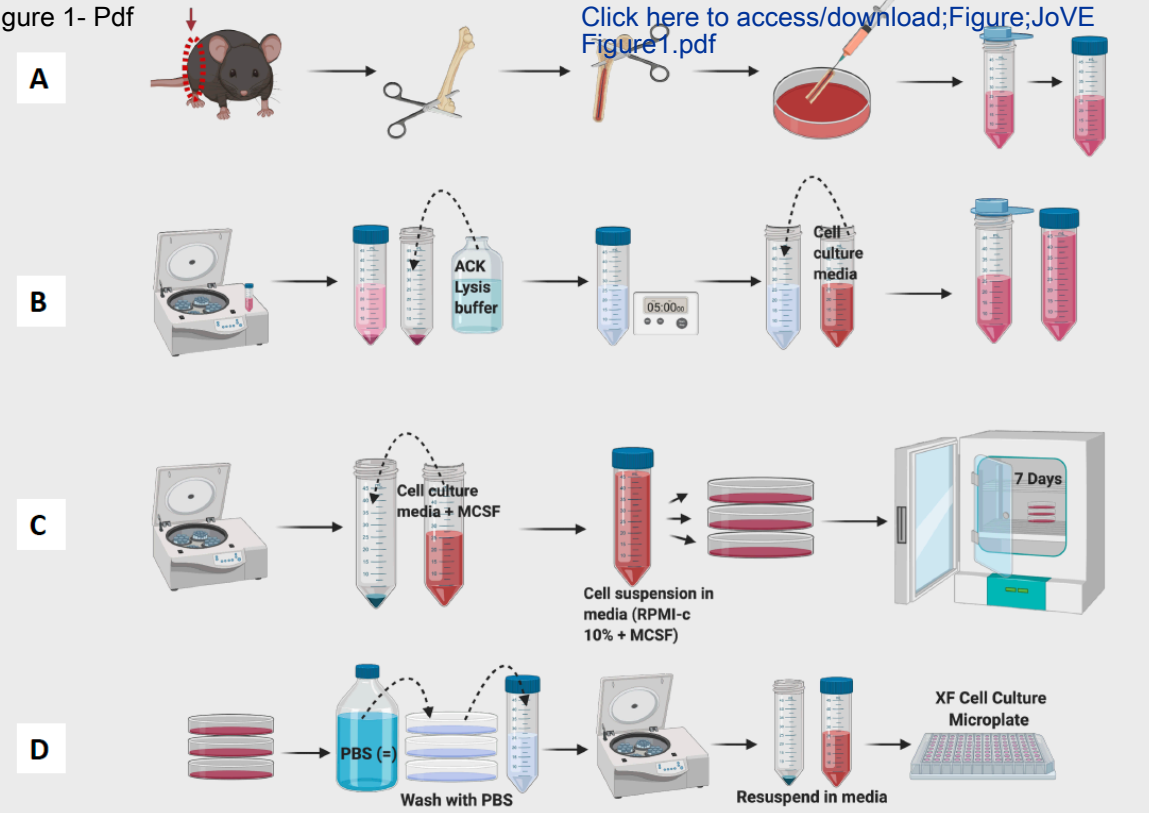

Figure 2- Pdf

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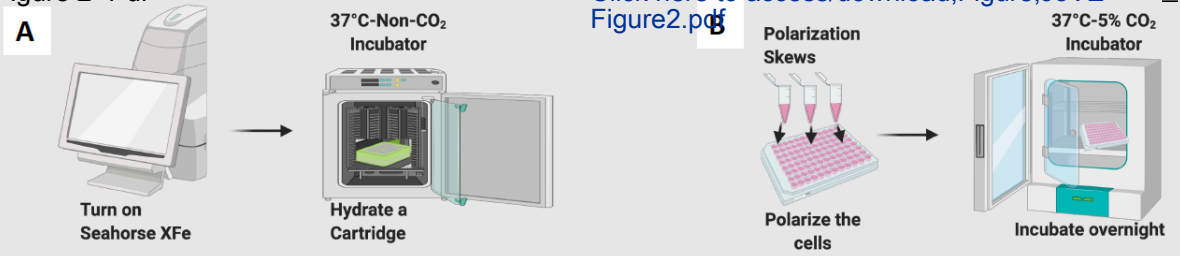

Figure 3- Pdf

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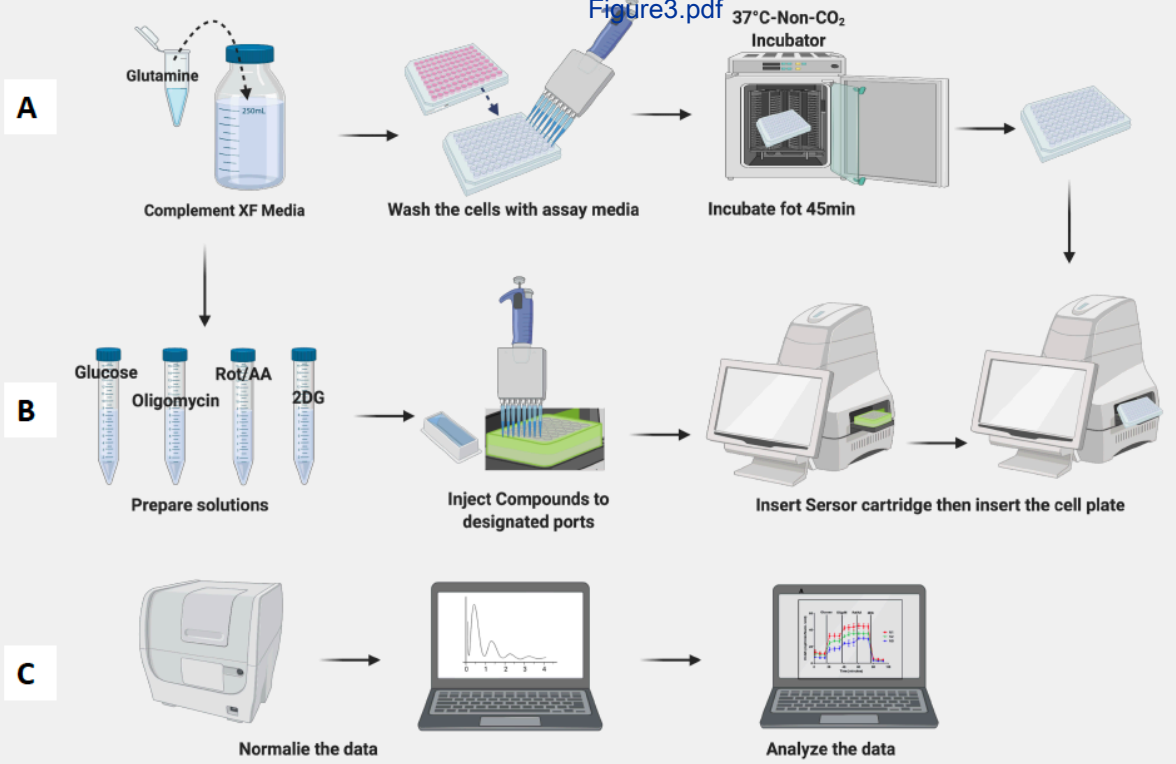
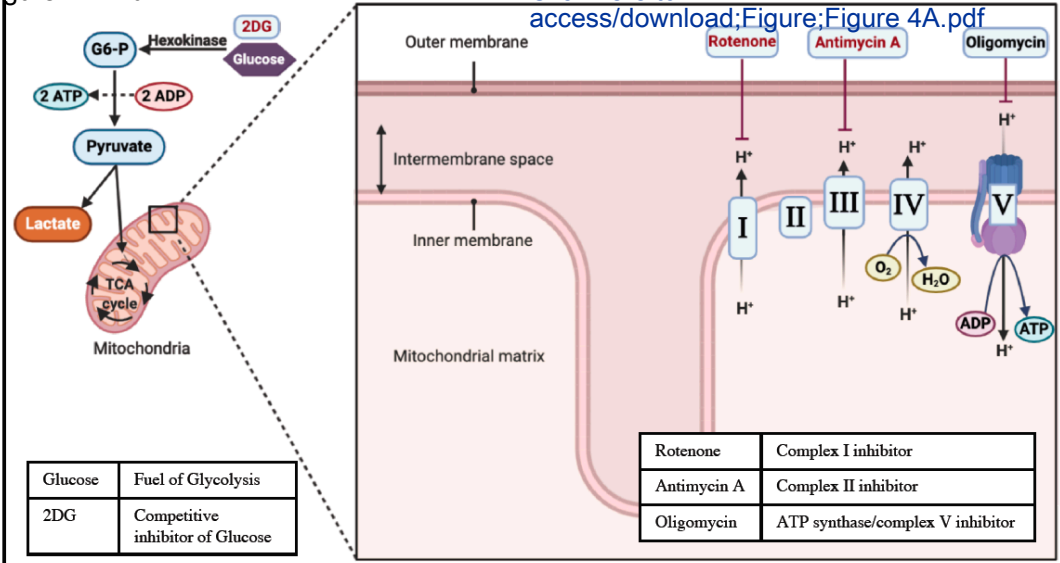


A Figure 4A- Pdf

Click here to access/download;Figure;Figure 4A.pdf



Glucose	Fuel of Glycolysis
2DG	Competitive inhibitor of Glucose

Rotenone	Complex I inhibitor
Antimycin A	Complex II inhibitor
Oligomycin	ATP synthase/complex V inhibitor

B Equations/Formulas:

Figure 4B- Pdf

Click here to access/download;

Non-glycolytic acidification	Avg. ECAR (1,2,3)
Glycolysis	Avg. ECAR (4,5,6) – Avg. ECAR (1,2,3)
Glycolytic capacity	Avg. ECAR (7,8,9)– Avg. ECAR (1,2,3)
Compensatory (COMP) Glycolysis	Avg. ECAR (10,11,12) – Avg. ECAR (1,2,3)
Glycolytic Reserve	Avg. ECAR (11,12,13) – Avg. ECAR (4,5,6)

Figure 4C Pdf

Click here to access/download;

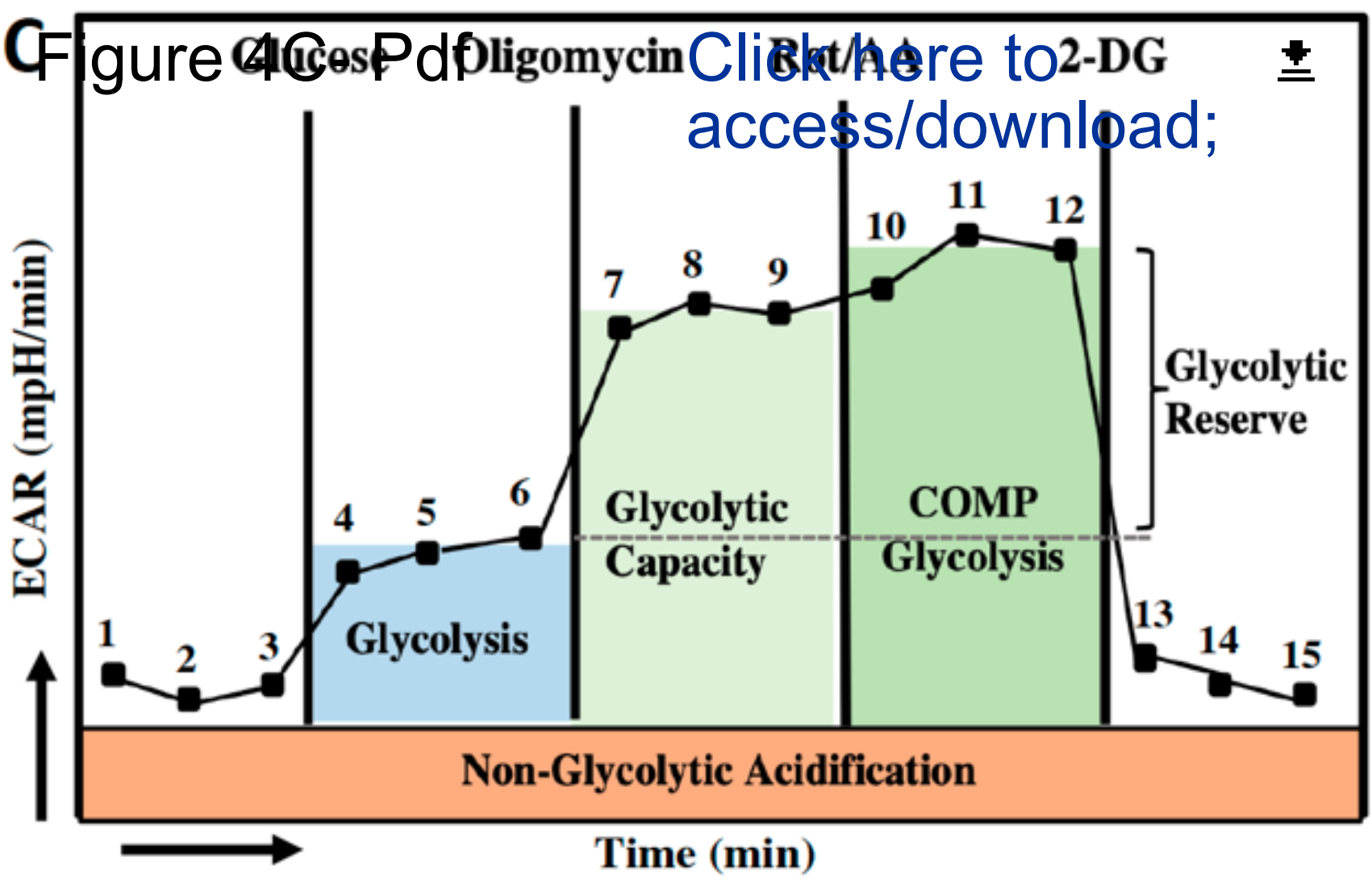
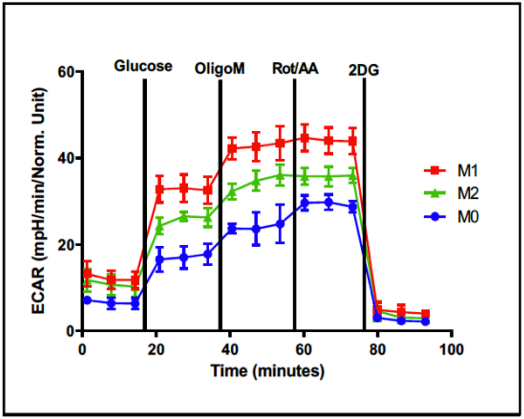


Figure 5- Pdf

A



B

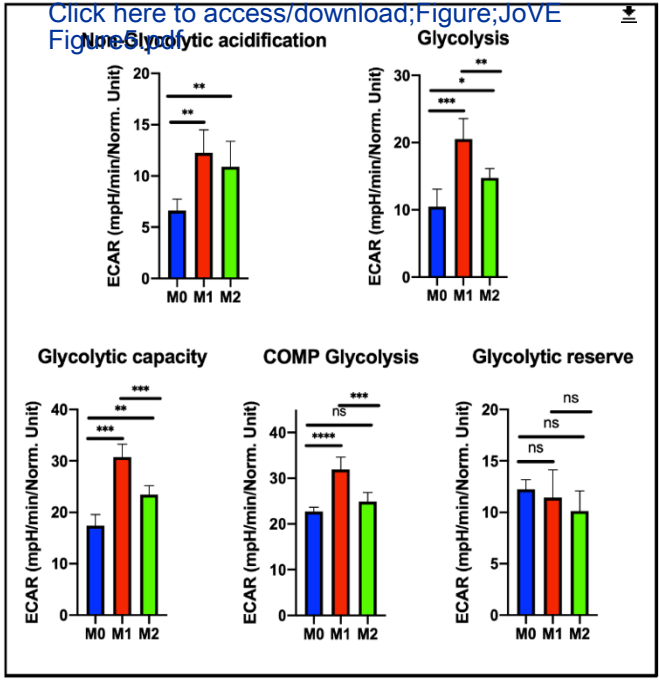


Table 1

Injection Stocks (Provided in the kits)	Add Complete assay media (mL)	Final Stock concentration (µM)
Glucose	3	100K
Oligomycin	0.72	100
2-DG	3	100k

Table 2

Ports on the Cartridge	Stock solutions	Add stock volume	Add assay media
A	Glucose (100 mM)	3000 µL + 0 µL	
B	Oligomycin (100 µM)	300 µL + 2700 µL	
C	Rotenone/ Antimycin A (50 µM)	300 µL + 2700 µL	
D	2-DG (500 mM)	300 µL + 0 µL	

Final concentration of injections (10x)	Add this volume to designated port (µL)	Final concentration after injection in each well
100 mM	20	10 mM
10 µM	22	1.0 µM
5 µM	25	0.5 µM
500 mM	28	50 mM

Name of Material/ Equipment	Company	Catalog Number
23G needles	VWR	BD305145
2-mercaptoethanol	Life Technologies	21985023
50ml Conical Tube	VWR	21008-951
ACK lysis buffer	Thermo Fisher Scientific	A1049201
Agilent Seahorse XF glycolysis stress test kit	Agilent Technologies	103020-100
Agilent Seahorse XF Glycolysis Stress Test Kit User Guide	Agilent Technologies	103020-400
Agilent Seahorse XF Glycolytic Rate Assay Kit	Agilent Technologies	103344-100
Agilent Seahorse XF Glycolytic Rate Assay Kit User Guide	Agilent Technologies	103344-100
Alexa Fluor 488 anti-mouse CD206 (MMR) Antibody	BioLegend	141710
anti-mouse CD11b eFluor450 100ug	eBioscience	48-0112-82
BD 3ML - SYRINGE	VWR	BD309657
Cell counter-Vi-CELL- XR Complete System	BECKMAN COULTER Life Sciences	731050
Cell Strainer-70µm	VWR	10199-656
CyQUANT Cell Proliferation Assay Kit	Thermo Fisher Scientific	C7026
F4/80 monoclonal antibody (BM8) pe-Cyanine7	eBioscience	25-4801-82
Fetal Bovine Serum	Life Technologies	16000-044
Flow cytometer: BD LSFRFortessa X-20	BD	656385
Kim Wipes	VWR	82003-822
LPS-SM ultrapure (tlrl-smpls) 5 mg	Invivogen	tlrl-smlps
MCSF	Peprotech	315-02
Murine IL-4	Peprotech	214-14
PE Rat Anti-Mouse CD38	BD Biosciences	553764
Penicillin-Streptomycin (10,000 U/mL)	Life Technologies	15140122
Petri Dish 100mm x 15 mm	Fisher Scientific	F80875712
RPMI, Glutamax, HEPES	Invitrogen	72400-120
Seahorse Calibrant Solution	Agilent Technologies	103059-000
Seahorse XF 200mM Glutamine Solution	Agilent Technologies	103579-100
Seahorse XF Glycolytic Rate Assay Kit	Agilent Technologies	103344-100
Seahorse XFe96 FluxPaks	Agilent Technologies	102416-100
XF Glycolysis Stress Test Kit	Agilent Technologies	103020-100
XF RPMI Medium, pH 7.4 without phenol Red	Agilent Technologies	103336-100

Comments/Description
It can be lab-made
Other syringes are acceptable too Cells can be manually counted too

Dear JoVE editor & reviewers,

We greatly appreciate your insightful critiques, thank you for the opportunity to revise. We have carefully revised our manuscript; below please see our point-by-point responses to the reviewers' comments (in blue text). To note, some of the procedure numbers may be shifted due to the revision.

Thank you so much!

Changes recommended by the JoVE Scientific Review Editor:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Addressed. All abbreviations are defined at first use.

2. Please make the title concise: Isolation, Culture, Polarization, and Real-Time Glycolytic Functional Analysis of Bone Marrow-Derived Macrophages

Addressed. The title word number is not exceeding the JoVE standard word number.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Agilent Seahorse XF glycolysis stress test kit (cat #103020-100), Glycolytic rate assay (cat # 103344-100), Agilent Seahorse XF Glycolytic Rate Assay Kit User Guide (#103344-100), Agilent Seahorse XF Stress Test Kit User Guide (#103020-400), Ack lysis buffer (Thermo Fisher Scientific), Vi-Cell Counter (Beckman-Counter), Seahorse Metabolic Flux Analyzer, Seahorse XFe, Seahorse Calibrant Solution (Cat # 103059-000), Wave software, CyQUANT cell proliferation assay kit, etc

Addressed. All commercial products are referenced in the Table of Materials and Reagents.

4. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of/remove "Seahorse" and “Agilent” within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

Addressed. Seahorse and Agilent words are removed from the text.

5. Please move the ethics statement (lines 112-115) to the beginning of the protocol, before you start the numbered steps.

Addressed. The ethics statement moved to the beginning of the protocol.

6. Please specify the method of euthanasia, without highlighting it.

Addressed. Euthanasia method specified.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Addressed. Details added. Also, some other details are included in the figure's illustrations as well.

With regards to numerical values for settings, microscopic magnification is not mentioned because as long as people can see the consistency of the cells under microscope the magnification does not matter. Numerical values are mentioned in the figure 5 A&B.

8. 2.2: Please define the abbreviation TC (tissue culture) medium at its first use and specify its composition at its first use.
Addressed.

9. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
Addressed; "rpm" speed converted to "x g".

10. 9.8: How do you use a cell proliferation assay to normalize cells? To what are they being normalized?
Addressed in the text. The concern is addressed and updated test is added in section 9.16. Cells are normalized based on naïve macrophages cell count. The average number of the naïve macrophages is considered as 1 (by dividing the cell number of each well by the average cell number of naïve macrophages) and applying them to all macrophages.

11. 9.15: How do you determine the naïve macrophage cell count and how do you normalize cells based on this count?
It is explained in section 9.16 - we determine the naïve macrophage cell count by Adding 1ml of Compound B or lysis buffer to 19ml of nuclease-free distilled water. (This is a DNA based normalization method). We add 100µl of Compound A or GR working solution to the abovementioned solution, then by adding 200µl of the solution to each well (cell containing 96 well microplate without media on top) to measure the fluorescence in 480nm Excitation and 520nm Emission wavelengths (We used Cytation1 plate reader).

12. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
Around 2.75 pages are highlighted.

13. Line 296: Do you mean M1 BMDMs demonstrate the highest levels of glycolysis compared to MO and M2 (not M1)?
M1 BMDMs demonstrate the highest levels of glycolysis compared to M0 BMDMs and M2 BMDMs. M0, M1 and M2 macrophages in this study are all BMDMs.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol→ Explained in first (Line 360 to 373) and 10th (430 to 434) paragraph of discussion.
b) Any modifications and troubleshooting of the technique→ Explained in the 4th (Line 391-395),7th paragraph(Line 391 to 395) of discussion,
c) Any limitations of the technique→ Explained in the 2nd line (374 to 381) and 9th (paragraph (Line 426 to 429) of discussion.
d) The significance with respect to existing methods→Explained in the 3rd (Line 382 to 390) and 8th (Line 418 to 425) paragraph of the discussion.
e) Any future applications of the technique→ Explained in the 5th (Line 396 to 404) &7th (Line 413 to 417) paragraph of the discussion.

15) Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Reference is performed using endnote after following JoVE journal format.

16. Please indicate whether error bars are derived from standard deviation or standard error of the mean.
The error bars are derived from the standard deviation that has been noted.

17. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).
Will be upload accordingly.

18. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
Will be upload accordingly.

19. Please add a table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.
Will be upload accordingly.

Reviewer #1

The manuscript entitled "A comprehensive high efficiency protocol for isolation, culture, polarization and real-time glycolytic functional analysis of bone marrow-derived macrophages" by Eshghjoo et al brings forward an interesting concept of measuring the metabolic status of cell in real-time of their polarization process. Authors have given a comprehensive protocol and will be useful for people in multidisciplinary research area studying metabolic profile of other cell types as well.

Minor Concerns:

1. Line 183: What does locate the cartridge means ? Do you mean incubate/position the cartridge ?
Appreciate the reviewer point it the confusion, we changed the word “locate” to “incubate”.

2. Line 215: Locate the cell-containing plate ? Is it possible to replace the word locate with any other synonym. This term is a bit confusing while reading the sentence.
We changed the word “locate” to “position”.

3. What is the total no. of cells seeded in plate? What is the minimum number of cells required to conduct this assay (sensitivity) ?
Resolved. Normally a minimum of 40K cells is required to conduct this assay.

4. There is no detailed materials list provided in the manuscript.
The detailed material list was uploaded in JoVE.

Reviewer #2:

The authors claim to provide real-time glycolytic functional analysis of bone marrow-derived macrophages.

Major Concerns:

"Although there are many methods to test the Glycolytic state of a cell, most published protocols measure glycolysis as a snapshotusing fixed cells." This statement in the introduction is not correct and misleading since it suggests this paper will provide an alternative method to excisting protocols. This is not the case an the authors just replicate the supplier's protocol. This would not be a problem is this was not done earlier and more extensively (including OCR etc) by others in JoVE and other journals. As such, this paper would not be helpful for the field as it would rather bring confusion.

We appreciate the reviewer's insightful comment; the inaccurate statement has been deleted in the revision.

While there are other protocols on Seahorse, our paper specifically focus on glycolysis, and discuss ECAR in a broader context, while previously publication mostly focus on OCR. Our protocol is complementary to the previously published paper, yet more inclusive and comprehensive. Also, the methods are more current and updated, because our protocol is developed based on the most current literature and by working in close communication with the R&D scientists of the manufacturer of extracellular flux analyzer.

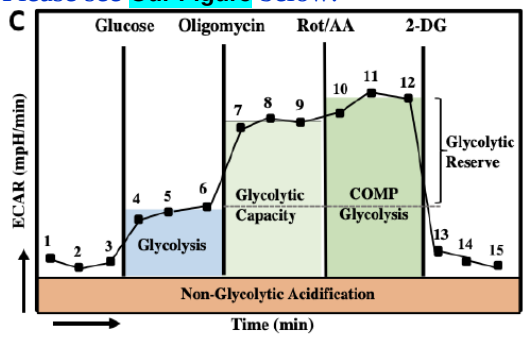
The supplier's protocol does not combine the assay and does not provide the protocol for BMDM culture and their polarization. We have optimized the paper based on the supplier's protocols to make the paper simple and easy to follow and added in the application aspect of BMDM.

In recent high-profile publications nowadays, most investigators use live cells in Seahorse. Seahorse technology is in great demand, but many have trouble to execute it well, we feel our protocol provides unique insight and detailed instructions that would be beneficial to many investigators.

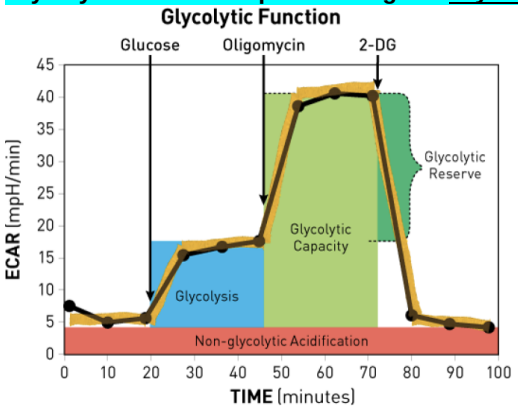
Difference between our paper and previously published JoVE papers on a similar topic:
1- The earlier (JoVE) paper published in 2015 (Reference number16) is more general and relatively incomplete in explaining glycolysis parameters. They did not use 2DG as a negative control for their assay. And because they combined the mito-stress test and glycolysis stress test, the FCCP and Rotenone and antimycin injection do not produce any ECAR related data.

Minor Concerns:
-The reason for injecting 2DG in the last step is not obvious since measurements 14, 15, 16 are not used in the calculations of the different glycolysis parameters.
2DG is a negative control. It is not used in the calculations. It helps us to understand if the assay worked or not. 2DG specifically inhibits glycolysis.
-In the calculations, the authors use the average of 3 measurements while according to the latest Agilent recommendations, once should use one particular data point (e.g. last before OM, highest after injection etc. Averaging can introduce unnecessary errors and variation in the data.
The alternative calculations are now mentioned in lines 289-291.
The bone marrow-derived macrophages normally show a strong response to each injection.
-CD38 is a poor marker to demonstrate "M1" polarization.
In addition to CD38, we also used other markers such as MCP-1 and TNF to define M1-like macrophages.
-M1/M2 nomenclature should not be used and using the guidelines proposed by Murray et al would be more appropriate
We agree M1/M2 nomenclature is not concise. We replaced M1and M2 with M1-like (Inflammatory) or M2-like (anti-inflammatory) just for the convenience of the discussion.
-After hydration, the sensor cartridges should not be directly placed in the Seahorse as written in the protocol. Dipping the plate a few times up and down is crucial to remove air bubbles to obtain useful data. This is a key step that was introduced when moving to distinct plates around 2016.
The details are added in line 188.
-The glycolytic capacity was always interpreted as the compensatory glycolysis in response to mitochondrial inhibition with OM. Now the authors introduce another "compensatory glycolysis" in response to Rot/AA injection. What is the biological/metabolic relevance of this "new" parameter? For example, how should scientist explain a difference in "compensatory glycolysis" in cases where glycoltyic capacity is not altered?

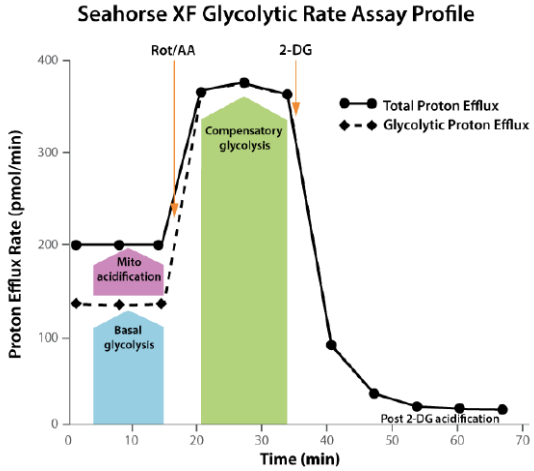
Glycolysis capacity is defined after inhibition by oligomycin, and compensatory glycolysis is defined after inhibition by Rot/AA. These are not "new" parameters, apologize for not make it clearer. Please see **Our Figure** below:



Glycolysis stress test protocol Figure: Glycolysis capacity is defined after injection of Oligomycin



Glycolytic Rate assay protocol Figure: Compensatory Glycolysis is defined after injection of Rot/AA



Reviewer #3:

This manuscript developed protocols for the bioenergetic analysis of bone marrow derived macrophages.

Major Concerns:

The bioenergetics of macrophages are fairly well established in many fields; this study potentially, represents a small incremental advance.

We agree with the reviewer that the bioenergetic of macrophages have been extensively studied. We would like to emphasize our paper is not a research paper to add new knowledge about macrophages, but a comprehensive protocol paper to improve the method and practical application. While some techniques are reported in various publications, any researchers are struggling to find an easy to follow work flow and optimized condition for the assay. Also, in this protocol, we have brought all aspects together covering isolation, culture, polarization and functional glycolytic analysis of live BMDMs in real time, which provide researchers a comprehensive and completes guide for BMDB glycolysis analysis.

I have 2 major concerns. Firstly, the authors use ECAR and not the GRA as indicator of glycolysis. In my opinion, this is (unfortunately) flawed and represents a concept that emerged during the early development of the Seahorse. Several papers thoroughly addressed this issue; using ECAR as indicator of of glycolysis should be avoided - numerous papers conclusively demonstrated that carbonic acid production via the TCA cycle contributes to acidification. Hence, the authors addition of Rot/AA to the ECAR assay is of little use as these compounds inhibit OXPHOS, but not the TCA cycle, which generates carbon dioxide.

We agree with the viewer that ECAR may include acidification from TCA cycle as well. However, since many scientists are still using ECAR as an indicator of glycolysis, in order to perform parallel studies to compare to previous reports, we included this assay, we agree the role of TCA acidification needs to be considered.

In the discussion part of this paper, we now have added a statement that carbonic acid production via the TCA cycle contributes to acidification. In this assay we have used 2DG which is an inhibitor of glycolysis and not TCA. While this negative control may help us to estimate the reduction of protons after glycolysis, it will not (directly) shut down TCA, which is a caveat of the assay.

Secondly, the use of macrophages is probably not the best choice as several studies have shown that they function at, or very close to their maximal respiration.

This paper has focused on glycolysis rather than respiration (OCR). M2-like macrophages have been shown to have higher levels of maximal respiration.

Dear JoVE editor & reviewers,

We greatly appreciate your insightful critiques, thank you for the opportunity to revise. We have carefully revised our manuscript 2nd time; below please see our point-by-point responses to the reviewers' comments (in blue text). To note, some of the procedure numbers may be shifted due to the revision.

Thank you so much!

Changes recommended by the JoVE Scientific Review Editor:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript entitled "A comprehensive protocol for isolation, culture, polarization and real-time glycolytic functional analysis of bone marrow-derived macrophages" by Eshghjoo et al provides detailed protocol for a visual demonstration of the workflow to assess the glycolysis in bone marrow derived macrophages, however a brief account on measurement of mitochondrial function characteristics (total respiration, basal mitochondrial respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity) will add to this comprehensive protocol.

We appreciate the reviewer's insightful comment. Mitochondria function is indeed a very relevant aspect of macrophages. However, our main purpose of this protocol is to assess the pro-inflammatory state of macrophages, so we only discussed glycolysis aspect in detail.

Per the reviewer's suggestion, we now have added following paragraph in the discussion lines 442- 448:

Major Concerns:

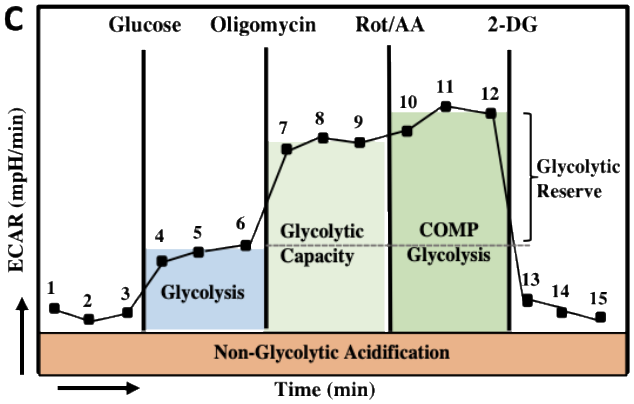
- How would you differentiate if the proton leakage in analysis is due to error in an assay or a real phenomenon?

This is a great point. In figure 4C, the orange box indicates non-glycolytic acidification, we interpret the basal acidification detected by the Extracellular flux analyzer as non-glycolysis related acidification which is due to Proton leak, TCA cycle, or phenomenon other than glycolysis. Since the basal media does not have any glucose, by adding glucose to the media during the first injection we rule out the basal acidification (which reflects proton leakage, assay error, or other phenomena) from total acidification (as mentioned in glycolysis equation formula in figure 4B). The comparison of these data would allow one to determine the source of the proton leakage.

Figure 4 B&C.

B **Equations Formulas:**

Non-glycolytic acidification	Avg. ECAR (1,2,3)
Glycolysis	Avg. ECAR (4,5,6) – Avg. ECAR (1,2,3)
Glycolytic capacity	Avg. ECAR (7,8,9)– Avg. ECAR (1,2,3)
Compensatory (COMP) Glycolysis	Avg. ECAR (10,11,12) – Avg. ECAR (1,2,3)
Glycolytic Reserve	Avg. ECAR (10,11,12) – Avg. ECAR (4,5,6)



- What is the sensitivity of the assay in terms of number of cells?

Addressed in line 196-199. Normally a minimum of 40K cells is required to conduct this assay. The highest number that has been used for BMDMs extracellular flux assays is 80K cells per well. Normally the increased number of cells requires increased doses of the injections. Our protocol has been optimized for 50K cells. We have tested 40K as well in the same experiment, the results with 50K were more consistent.

-How do you cell number normalization?

Addressed in the text in lines 245 to 256 Section.9.8 to 9.15). Cells are normalized based on naïve macrophages cell count. Using a DNA based CyQuant proliferation kit, we lysed the cells and measured the fluorescence 480nm Excitation and 520nm Emission wavelengths using a plate reader. We then divided the cell number of each well of the polarized cells by the average cell number of naïve M0 BMDMs. We will make sure to show the normalization panel of the wave software in the video.

Minor Concerns:
- Is this protocol applicable to suspension cells also, like T-cells and B-cells?
Yes, but since T cells and B cells are not as adhesive as macrophages to the extracellular flux microplates, the microplates need to be pre-treated or precoated, so T or B cells can attach to the bottom of the plates.

- Author's contribution need to be added.

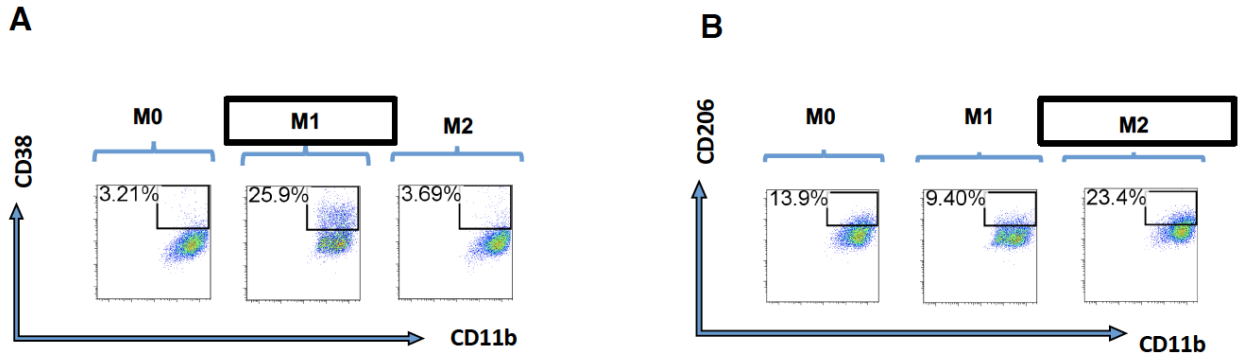
Revised, the authors contribution added to lines 456 to 460.

Reviewer #4:
Manuscript Summary:
This manuscript provides a simple approach to assess a set of glycolytic parameters in the polarized bone marrow derived macrophages (BMDMs). There are two separate kits provided by Agilent Technology: Agilent Seahorse XF glycolysis stress test kit and Glycolysis rate assay kit, which uses ECAR and PER, respectively as metrics of extracellular acidifications in Seahorse XF analyzers. Although PER additionally accounts for the buffer factor in its calculation, the implication of the compensatory glycolysis is not altered using ECAR instead of PER. The authors arranged the sequence of the inhibitors appropriately so that glycolytic capacity and compensatory glycolysis could be measured at once. The treatment of oligomycin prior to Rot/AA does not interfere with the implications of each parameter. Overall, their findings are significant in simplifying the method that has originally been measured separately. Following minor points are recommended to be corrected before considering the manuscript for publication:

Minor Concerns:
1. Since this manuscript mainly elucidates the glycolytic states of the polarized BMDMs, the comparison of the BMDM polarization status with the glycolytic parameters is critical. Although the authors mentioned the confirmation in the manuscript, a figure demonstrating BMDM polarization status by flow cytometry may be required.

While the principle is the same, BMDM derived from different animals may show a different profile. Thus, we feel including data figures from our experiments in this method paper may be misleading.

However, here we share two figures of polarized BMDMs of our unpublished data with the reviewer; CD38 was used as an M1 marker, and CD206 was used as an M2 Marker.



BMDM Polarization demonstration by flow cytometry.

Day 7 BMDMs were stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) M2 (20ng/ml IL-4). Flow cytometric analysis of BMDMs from WT mice, (2x 10^5) cells stained extracellularly for CD11b and F4/80 as macrophage markers and CD38 as M1 marker (A) and CD206 as M2 marker (B) using FACS. The percentage of CD38-positive and CD206-positive cells are indicated on plots.

2. The interpretation of most glycolytic parameters does not interfere with the suggested protocol. However, although the implication of glycolytic reserve does not change in the new combined system, the calculation scheme is altered slightly. In the Agilent Seahorse XF Glycolysis Stress Test, Glycolytic reserve is calculated by Avg.ECAR(7,8,9)-Avg.ECAR(4,5,6), while it is estimated by Avg.ECAR(10,11,12)-Avg.ECAR(4,5,6) in the new combined system(Figure 4B). It is recommended to discuss on the altered calculation scheme in order for readers not to confuse the definition of the glycolytic reserve.

Thank you for the suggestion. The clarification is addressed in the discussion line (386 to 391).