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Title: Investigating Glycolysis in Primary Microglia Using Extracellular Flux Assay

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Author Questionnaire

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Current Protocol Length

Number of Steps: 13

Number of Shots: 40 (12 SC)

Introduction

NOTE to VO: Please record the introductory and conclusion statements

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. This research focuses on investigating glycolysis in primary microglia during inflammation using extracellular flux assays with lab-prepared metabolic inhibitors.

1.1.1. [4.3.2](#)

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. Existing methods utilize expensive kits and lack sufficient detail; this protocol instead offers comprehensive, step-by-step guidance for affordable, reproducible results.

1.2.1. [4.4.2](#)

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. This protocol measures extracellular acidification rate to quantify basal glycolysis, glycolytic capacity, and real time metabolic changes in cultured microglia.

1.3.1. [Figure 4C](#)

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. Precise pH control, optimal cell density, gentle handling, and data normalization are critical to avoid variability and inaccurate ECAR measurements.

1.4.1. [4.1.3](#)

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. This protocol focuses on glycolysis measurement and additionally allows protein content or cell density analysis for normalization after the assay.

1.5.1. [4.2.4](#)

Ethics Title Card

This research has been approved by the Animal Care Committee at Memorial University of Newfoundland.

Protocol

2. Preparation and Treatment of Primary Microglia for Extracellular Flux Analysis

Demonstrator: Aysika Das

2.1. To begin, obtain 10-day-old mixed glial cultures [1-TXT]. Shake the flask at 250 revolutions per minute for 2 hours at 37 degrees Celsius [2]. Collect the entire media containing the floating cells into a 15-milliliter conical tube [3]. Centrifuge at 300 g for 10 minutes [4] and resuspend the pellet in microglia media [5].

2.1.1. WIDE: Show the experimental setup, with the researcher holding the culture flask.

TXT: Source: P0–P2 C57BL/6C mouse cortices

2.1.2. Talent placing the flask on the shaker for incubation at 250 revolutions per min for 2 hrs at 37 °C.

2.1.3. Talent collecting the entire media containing the floating cells into a 15 mL conical tube.

2.1.4. Talent placing the 15 mL conical tube into the centrifuge.

2.1.5. Talent resuspending the pellet in microglia media.

2.2. In the cell culture microplate of the extracellular flux analyzer, mark four wells, with one well in each row, as background controls [1]. Add assay medium to the background control wells [2]. Plate around 250,000 cells per well in the remaining wells [3]. Incubate the microplate at 5 percent carbon dioxide for 30 to 45 minutes [4]. Replace the media with fresh microglia media [5].

2.2.1. Talent identifying 4 background control wells on the microplate, with 1 well in each row. *Video editor: Please show Figure 3C as an inset.*

2.2.2. Talent adding assay media to the background control wells.

2.2.3. Talent plating the required cell suspension into the cell culture microplate wells.

2.2.4. Talent placing the microplate into the incubator at 5% CO₂ for 30 to 45 min.

2.2.5. Talent changing the media in the microplate wells.

2.3. To treat the cells on day 11, first assign the control and lipopolysaccharide or LPS (L-P-S) wells randomly, to rule out positional effects [1]. Then, treat the cells with 100 nanogram per milliliters LPS for 24 hours [2].

2.3.1. Talent assigning control and lipopolysaccharide-treated wells across the plate. *Video editor: Please show Figure 3C as an inset.*

2.3.2. Talent adding lipopolysaccharide to the designated wells.

3. ~~Pre-assay Preparation for Extracellular Flux Assay (Day 11)~~ Extracellular Flux Assay (Day 12)

NOTE: Merge section 3 and 4

- 3.1. One day before the assay, add 1 milliliter calibrant buffer to all wells of the 24-well calibration plate [1]. Keep the plate overnight at 37 degrees Celsius without carbon dioxide [2].
 - 3.1.1. Talent adding 1 mL calibrant buffer to all wells of the 24-well calibration plate.
 - 3.1.2. Talent placing the calibration plate into the incubator overnight at 37 °C without carbon dioxide.
- 3.2. Prepare the stock solutions [1] and store both solutions at 4 degrees Celsius overnight [2]. On the next day, aliquot 30 to 40 milliliters of assay media in a 50-milliliter conical tube for the assays [3].
 - 3.2.1. TEXT ON A PLAIN BACKGROUND:
Glucose stock solution:
90 mg glucose in 1.9 mL extracellular flux assay media
2-deoxy-D-glucose (2-DG) stock:
328 mg 2-DG in 2 mL extracellular flux assay media
 - 3.2.2. Talent placing the stock solutions at 4 °C.
 - 3.2.3. Talent aliquoting 30 to 40 mL of assay media into a 50 mL conical tube.

4. ~~Extracellular Flux Assay (Day 12)~~

- 4.1. Warm the stock solutions and the aliquoted assay media at 37 degrees Celsius [1]. Add 1 normal sodium hydroxide in increments and vortex briefly [2] to adjust the pH of each solution to around 7.4 [3].
 - 4.1.1. Talent placing the stock solutions, and the aliquoted assay media at 37 °C.
 - 4.1.2. Talent adding 1 normal sodium hydroxide and vortexing the solution for pH adjustment.
 - 4.1.3. Shot of the solution displaying the final pH close to 7.4 on the pH strip.
- 4.2. Add oligomycin stock solution to 2 milliliters of pH-adjusted assay media [1-TXT]. Gently wash the cells with 1 milliliter of the pH-adjusted assay media to avoid cell detachment [2]. Discard the wash media [3] and add 500 microliters of pH-adjusted assay media [4]. Incubate at 37 degrees Celsius, without carbon dioxide, for 45 minutes to 1 hour [5].
 - 4.2.1. Talent adding 2 µL oligomycin stock solution to 2 mL of pH-adjusted assay media
 - 4.2.2. Talent gently washing the cells with 1 mL of pH-adjusted assay media.
 - 4.2.3. Talent discarding the assay media from the wells.
 - 4.2.4. Talent adding 500 µL of pH-adjusted assay media to the wells.
 - 4.2.5. Talent placing the culture plate into the incubator
- 4.3. Add all the required inhibitors to the extracellular flux analyzer's calibration plate in their respective ports [1]. Load all wells, including background control wells [2].

4.3.1. TEXT IN PLAIN BACKGROUND:

Calibration Plate Inhibitor Loading

Port A (Glucose): Add 55 μ L to top-left wells

Port B (Oligomycin): Add 60 μ L to top-right wells

Port C (2-DG): Add 65 μ L to bottom-left wells

4.3.2. Shot of talent adding inhibitor in the background well.

4.4. Then, remove the top cover from the plate and the pink cover between the plates [1]. Place the calibration plate into the real-time extracellular flux analyzer [2]. Start the run using the Wave desktop platform to calibrate the instrument [3]. Unload the calibration plate and load the culture plate when prompted by the machine [4].

4.4.1. Talent removing the top cover from the plate and the pink cover between the plates.

4.4.2. Talent placing the calibration plate into the real-time extracellular flux analyzer.

4.4.3. SCREEN: SCREEN-4.4.3.MP4: 00:06-00:08, 00:28-00:30, 00:46-00:50

4.4.4. Shot of talent loading the culture plate into the machine.

5. Setting up a Glycolysis Stress Test

5.1. Open the Wave software and select the appropriate assay template from the **Templates** section [1]. Choose **XF Glycolysis Stress Test** on the extracellular flux analyzer [2]. Open the **Group Definitions** tab and create the experimental groups like Background, Control, and LPS [3]. Define the injection strategy by assigning glucose, oligomycin, and 2-DG to the corresponding ports [4].

5.1.1. SCREEN: SCREEN-5.1.1.MP4

5.1.2. SCREEN: SCREEN-5.1.2.MP4

5.1.3. SCREEN: SCREEN-5.1.3.MP4: 00:17-00:39

5.1.4. SCREEN: SCREEN-5.1.4.MP4

5.2. Now, open the **Plate Map** view [1] and assign wells to each group by dragging the group names onto the plate layout [2]. Designate the blank or background wells for accurate normalization [3].

5.2.1. SCREEN: SCREEN-5.2.1.MP4:

5.2.2. SCREEN: SCREEN-5.2.2.MP4: 00:06-00:10, 00:29-00:35

5.2.3. SCREEN: SCREEN-5.2.3.MP4

5.3. Open the Protocol tab and configure the measurement cycles and injections for non-glycolytic acidification, followed by glucose, oligomycin, and 2-DG injections [1]. Define the number of measurement cycles after each injection and adjust the timing or use the software default settings [2]. Review the total assay time before running the assay [3]

- 5.3.1. SCREEN: SCREEN-5.3.1.MP4
- 5.3.2. SCREEN: SCREEN-5.3.2.MP4: 00:00-00:13
- 5.3.3. SCREEN: SCREEN-5.3.3.MP4 *Video editor: Highlight the “total time” displayed on the upper right corner*
- 5.4. Use the built-in normalization option to normalize the data [1].
 - 5.4.1. SCREEN: SCREEN-5.4.1.MP4: 00:03-00:05, 00:27-00:30

Results

6. Results

- 6.1. Slight morphological changes were observed between primary microglia in cultures without LPS after 24 hours [1] and LPS-treated primary microglia after 24 hours [2].
 - 6.1.1. LAB MEDIA: Figure 2D, 2E. *Video editor: Emphasize panel D with the untreated cells after 24 hours.*
 - 6.1.2. LAB MEDIA: Figure 2D, 2E. *Video editor: Emphasize panel E with the lipopolysaccharide-treated cells after 24 hours.*
- 6.2. LPS-treated primary microglia showed increased extracellular acidification rate over time compared to control microglia in the glycolysis stress test profile [1] and in the protein-normalized extracellular acidification rate profile [2].
 - 6.2.1. LAB MEDIA: Figure 4C. *Video editor: Show the line graph labeled "ECAR Data" and emphasize the red line across the time course.*
 - 6.2.2. LAB MEDIA: Figure 4G. *Video editor: Show the line graph labeled "Normalized ECAR Data" and emphasize the red line across the time course.*
- 6.3. LPS-treated primary microglia showed significantly higher glycolysis compared to untreated control microglia in the extracellular acidification rate measurements without normalization [1] and after normalization to protein content [2].
 - 6.3.1. LAB MEDIA: Figure 4D. *Video editor: Highlight the red bar labeled LPS*
 - 6.3.2. LAB MEDIA: Figure 4H. *Video editor: Highlight the red bar labeled LPS*
- 6.4. LPS-treated primary microglia showed significantly higher glycolytic capacity compared to untreated control microglia in extracellular acidification rate measurements without normalization [1] and after normalization to protein content [2].
 - 6.4.1. LAB MEDIA: Figure 4E. *Video editor: Highlight the red bar labeled LPS.*
 - 6.4.2. LAB MEDIA: Figure 4I. *Video editor: Highlight the red bar labeled LPS.*
- 6.5. Glycolytic reserve was not significantly different between control and LPS-treated primary microglia without normalization [1] and after normalization to protein content [2].

- 6.5.1. LAB MEDIA: Figure 4F. *Video editor: Highlight the two bars labeled CONTROL and LPS in the graph titled "Glycolytic reserve," showing similar heights.*
- 6.5.2. LAB MEDIA: Figure 4J. *Video editor: Highlight the two bars labeled CONTROL and LPS in the graph titled "Glycolytic reserve," showing similar heights.*

1. Microglia
Pronunciation link: <https://www.merriam-webster.com/dictionary/microglia>
IPA: /ˌmaɪ.krəˈɡliː.ə/
Phonetic Spelling: my·kruh·glee·uh
2. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsen.trɪˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
3. Conical
Pronunciation link: <https://www.merriam-webster.com/dictionary/conical>
IPA: /ˈkɑː.nɪ.kəl/
Phonetic Spelling: kaa·nuh·kuhl
4. Extracellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/extracellular>
IPA: /ˌɛk.strəˈsɛl.jə.lə/
Phonetic Spelling: ek·struh·sel·yuh·ler
5. Analyzer
Pronunciation link: <https://www.merriam-webster.com/dictionary/analyzer>
IPA: /ˈæn.əl.aɪ.zər/
Phonetic Spelling: a·nuh·ly·zer
6. Lipopolysaccharide
Pronunciation link: <https://www.merriam-webster.com/dictionary/lipopolysaccharide>
IPA: /ˌlaɪ.pouˌpɑː.liˈsæk.əˌraɪd/
Phonetic Spelling: ly·poh·paa·lee·sak·uh·ryde
7. Calibrant
Pronunciation link: <https://www.merriam-webster.com/dictionary/calibrant>
IPA: /ˈkæl.ə.brənt/
Phonetic Spelling: kal·uh·bruhnt
8. Aliquot
Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>

- IPA: /'æɪ.l.kwɑ:t/
Phonetic Spelling: al·ih·kwaat
9. Deoxy
Pronunciation link: <https://www.merriam-webster.com/dictionary/deoxy>
IPA: /di:'ɑ:k.si/
Phonetic Spelling: dee·ok·see
10. Oligomycin
Pronunciation link: <https://www.merriam-webster.com/dictionary/oligomycin>
IPA: /,ɑ:.lɪ.goo'maɪ.sɪn/
Phonetic Spelling: aa·luh·goh·my·sin
11. Sodium hydroxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/sodium%20hydroxide>
IPA: /'soʊ.di.əm haɪ'drɑ:k.sɑɪd/
Phonetic Spelling: soh·dee·uhm hy·draak·syde
12. Vortex
Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>
IPA: /'vɔ:r.teks/
Phonetic Spelling: vor·teks
13. Inhibitor
Pronunciation link: <https://www.merriam-webster.com/dictionary/inhibitor>
IPA: /ɪn'hɪb.ɪ.tə/
Phonetic Spelling: in·hib·ih·ter
14. Glycolysis
Pronunciation link: <https://www.merriam-webster.com/dictionary/glycolysis>
IPA: /glɑɪ'kɑ:.lə.sɪs/
Phonetic Spelling: gly·kaa·luh·sis
15. Normalization
Pronunciation link: <https://www.merriam-webster.com/dictionary/normalization>
IPA: /,nɔ:r.mə.lə'zeɪ.fən/
Phonetic Spelling: nor·muh·luh·zay·shuhn
16. Incubator
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubator>
IPA: /'ɪŋ.kjə.beɪ.tə/
Phonetic Spelling: ing·kyuh·bay·ter