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Project Page Link: <https://review.jove.com/account/file-uploader?src=21198768>

Title: Single-Plate Runs of Mito Stress and Glycolysis Stress Tests Using Adherent or Non-Adherent Cells

Authors and Affiliations:

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Author Questionnaire

1. **Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

NOTE: Videographer filmed screen shots

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes, the same lab, different rooms**
4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 21

Number of Shots: 52

Interviews

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

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

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

- 1.1. **Alla Ivanova:** We study how stress, sex, inflammation, and mitochondrial impairment during aging alter cell metabolism, inflammatory responses, and stress resilience.

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

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

- 1.2. **Alla Ivanova:** Existing methods poorly capture dynamic metabolic changes; this protocol simultaneously measures mitochondrial and glycolytic activity in real time.

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

CONCLUSION:

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

- 1.3. **Alla Ivanova:** An important consideration is prior optimization of cell density to ensure accurate, reproducible, and biologically meaningful data.

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

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

- 1.4. **Alla Ivanova:** This protocol simultaneously evaluates the effects of compounds, drugs, and experimental conditions on the dynamics of mitochondrial and glycolytic parameters.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Alla Ivanova:** Based on the results, future studies can develop strategies to restore energy metabolism and improve overall cellular health and resilience.

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

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

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Pre-Analysis Preparation for Mito Stress and Glycolysis Stress Assays

Demonstrator: Jane Tonello

- 2.1. To begin, remove the XFe96/XF Pro (X-F-E-ninety-six or X-F-Pro) Cell Culture Microplate containing the cells of interest, seeded at optimal density from the carbon dioxide incubator [1]. Use a multi-pipettor to remove 160 to 180 microliters of growth medium from each well, working one row or column at a time to prevent the cells from drying [2]. Avoid touching the bottom of the wells during media removal [3].
 - 2.1.1. WIDE: Talent removing the XFe96/XF Pro Cell Culture Microplate from the incubator. ***Videographer:** Please obtain CU shot as well for this shot*
 - 2.1.2. Talent using a multi-pipettor to aspirate media from a microplate row by row.
 - 2.1.3. Close-up of media removal from sides of the wells without touching the well-bottom. **Videographer's NOTE: 2-1-3 Take 2 is tighter**
- 2.2. Use a single tip pipetman to remove the remaining 20 to 30 microliters of media from each well without touching the cell layer [1]. To change the media, gently dispense 180 microliters of Glycolysis or Mito Stress assay media along the side walls of each well, including the cell-free corner wells used as blank controls [2].
 - 2.2.1. CU: Show careful removal of residual media with a single tip pipetman without disturbing the cell layer.
 - 2.2.2. Talent adding assay media into wells along the side walls of the well.
- 2.3. Place the microplate into a 37 degrees Celsius carbon dioxide-less incubator for 40 to 45 minutes prior to starting the assay [1]. Begin preparing the drugs during this time [2].
 - 2.3.1. Talent placing the microplate into an incubator. ***Videographer:** Please capture the digital display on the incubator.*
 - 2.3.2. Talent arranging materials for drug preparation on the bench.
- 2.4. For drug preparation and cartridge loading, use the pre-warmed Mito Stress and Glycolysis assay media [1] to dilute the compounds to the required concentrations for each cell type [2]. Prepare each compound in a separate 15 milliliter tube [3].
 - 2.4.1. Talent pipetting assay media into tubes containing compounds.
 - 2.4.2. TEXT ON A PLAIN BACKGROUND
Compound concentrations for Mito Stress Assay

- Oligomycin 1 μ M
- FCCP 1 μ M
- Rotenone/Antimycin 0.5 μ M

Compound concentrations for Glycolysis Assay

- Glucose 10 mM
- Oligomycin 1 μ M
- 2-DG 50 mM

2.4.3. Shot of multiple labeled 15 milliliter tubes prepared with different compounds.

2.5. Remove the sensor cartridge and utility plate from the incubator [1]. After removing the lid, align the provided XF loading guide over the cartridge to ensure accurate drug injection [2].

2.5.1. Talent removing a cartridge from the incubator and opening it.

2.5.2. Talent positioning the loading guide onto the cartridge.

2.6. Use a multichannel pipettor and reagent reservoir to slowly add the required volume of each drug into the corresponding port while holding the pipettor upright [1].

2.6.1. Talent loading drugs into cartridge ports with a multichannel pipettor.

2.7. Change or reuse the loading guide after rinsing and drying for each port, as needed, and discard pipette tips after each load [1].

2.7.1. Talent rinsing and drying the loading guide and discarding used tips.

Videographer's NOTE: 2-7-1 XTRA is tight on the washing

2.8. After loading all compounds into their respective ports, remove the guide and visually confirm that all ports are filled [1]. Secure the cartridge with its lid, ensuring drugs correspond to the assay layout [2].

2.8.1. Talent inspecting filled ports on the cartridge.

2.8.2. Talent placing the lid back onto the cartridge.

3. Set up of Seahorse XFe96 Analyzer

3.1. Turn on the analyzer [1] and open the **WAVE** software [2]. Select **XF Mito Stress Test** in the software [3].

3.1.1. Talent turning on the Seahorse XFe96 Analyzer.

3.1.2. SCREEN: Display the analyzer startup and opening of WAVE software.

3.1.3. SCREEN: Show selection of **XF Mito Stress Test** option.

Videographer's NOTE: 3-1-2 to 3-3-2 & 3-4-2 to 3-4-3

Researchers could not do screen recordings, so we shot the screen with the camera. This is in 4K. Researcher narrates as she works through the steps.

- 3.2. Select the number of 'Groups' based on the plate setup and label each group [1]. Proceed to the next tab to assign wells for the plate map to perform both assays in the same plate [2]. Highlight wells for each group based on the plate loading [3].
 - 3.2.1. SCREEN: Show group selection and labeling interface.
 - 3.2.2. SCREEN: Assign wells on the plate map.
 - 3.2.3. SCREEN: Highlight selected wells corresponding to groups.
- 3.3. Adjust the program settings according to the cell type and experimental purpose [1]. Select **Run Assay** and enter the required information to save the data file [2].
 - 3.3.1. SCREEN: Modify assay program parameters.
 - 3.3.2. SCREEN: Show clicking **Run Assay** and entering file details.
- 3.4. After removing the lid, load the pre-hydrated cartridge containing the drugs into the machine when prompted for initialization [1]. Confirm proper hydration and functionality of sensors during initialization [2]. Start the assay, and load and unload plates as prompted during the program [3].
 - 3.4.1. Talent placing the cartridge into the analyzer after removing the lid.
Videographer's NOTE: 3-4-1 ALT is wider and static
 - 3.4.2. SCREEN: Display initialization status indicating proper sensor hydration and readiness.
 - 3.4.3. SCREEN: Show assay start and prompt-guided plate handling steps.

4. Cellular Normalization Using Sulforhodamine B Assay

- 4.1. After real-time metabolic analysis, remove the plate from the analyzer [1]. Add one quarter of the media volume of fixation solution to each well as per kit instructions [2] and incubate overnight at 4 degrees Celsius. [3].
 - 4.1.1. Talent removing the plate from the analyser.
 - 4.1.2. Talent adding fixation solution into each well.
 - 4.1.3. Talent placing the plate at 4 °C.
- 4.2. Remove the fixative solution [1] and wash the wells three times with 200 microliters of RNase (*R-N-ace*) and DNase (*D-N-ace*)-free water without disturbing the cell layer [2]. After removing the wash solution, allow the plate to air-dry completely at room temperature [3]. Add 25 microliters of sulforhodamine B, or SRB (*S-R-B*) solution to each well and stain for 15 minutes at room temperature in the dark [4].

- 4.2.1. Talent aspirating the fixing solution.
- 4.2.2. Talent adding water for washing the wells.
- 4.2.3. Shot of plate left uncovered for drying.
- 4.2.4. Talent adding staining solution to wells and covering plate.
- 4.3. Remove the staining solution [1] and wash each well two to three times with 100 microliters of 1X washing solution [2]. Use vacuum suction to remove as much wash solution as possible to prevent bleaching [3].
 - 4.3.1. Talent aspirating the staining solution.
 - 4.3.2. Talent adding wash solution to the wells.
 - 4.3.3. Talent using vacuum suction to rapidly remove the wash solution.
- 4.4. Add 100 microliters of 1X solubilization solution to each well [1] and place the plate on a shaker for 10 minutes at room temperature [2].
 - 4.4.1. Talent adding 1X solubilization solution to each well.
 - 4.4.2. Talent placing the plate on shaker.
- 4.5. Use a microplate reader to measure the optical density at 565 nanometers to determine well density based on color saturation [1]. Export the data as an Excel file for processing in WAVE software [2].
 - 4.5.1. Talent placing plate into the microplate reader. Videographer's NOTE: 4-5-1 XTRA Is tighter
 - 4.5.2. SCREEN: Show OD measurement at 565 nm and exporting the data to Excel. Videographer's NOTE: 4-5- XTRA Is tighter

5. Data Normalization and Analysis

Videographer's NOTE: 5-1-1 to 5-4-3

Researchers could not do screen recordings, so we shot the screen with the camera. This is in 4K. Researcher narrates as she works through the steps

- 5.1. Open the Excel sheet in WAVE software [1] and click on Normalize [2].
 - 5.1.1. SCREEN: Display results panel showing the OD readings of SRB assay in WAVE software.
 - 5.1.2. SCREEN: Click on Normalize.
- 5.2. In the open window, choose "select all" followed by "paste" to paste the data copied from the Excel spreadsheet [1], then click on Apply to automatically generate the normalized dataset [2].

- 5.2.1. SCREEN: Show pasting data.
- 5.2.2. SCREEN: Show clicking **Apply**.
- 5.3. Next, click on Export tab [1] and from the **Export to** pop-up window, select **XF Cell Mito Stress Test Report Generator** [2] then save the file [3], selecting only the wells assigned to the Mito Stress Test during analysis [4].
 - 5.3.1. SCREEN: Show clicking on the **Export** tab
 - 5.3.2. SCREEN: Show selection of **XF Cell Mito Stress Test Report Generator** from the **Export to** pop-up window
 - 5.3.3. SCREEN: Show saving the file
 - 5.3.4. SCREEN: Show selection of wells assigned for Mito Stress Test during analysis.
- 5.4. To generate a glycolysis report, select **XF Cell Glycolysis Stress Test Report Generator** from the **Export to** pop-up window [1]. Save the file [2], selecting only the wells assigned to the Glycolysis Stress Test during analysis [3].
 - 5.4.1. SCREEN: Show selection of **XF Cell Glycolysis Stress Test Report Generator** from the **Export to** pop-up window.
 - 5.4.2. SCREEN: Show saving the file
 - 5.4.3. SCREEN: Show selection of wells assigned for Glycolysis Stress Test during analysis.

Results

6. Results

- 6.1. The evaluation of metabotropic glutamate receptor 1– T-cell receptor, or mGluR1 (*M-glue-R-one*) – TCR (*T-C-R*) crosstalk using an integrated dual-assay approach revealed that blocking mGluR1 signaling in lipopolysaccharide, or LPS (*L-P-S*)-activated CD8+ (*C-D-eight-positive*) T (*tee*) cells significantly impacts both mitochondrial and non-mitochondrial energy production [1]. This reduced energy production is notable when compared to LPS-activated CD8+ T cells [2], while naive cells without LPS-pretreatment and mGluR1 inhibition remain metabolically unchanged [3].
 - 6.1.1. LAB MEDIA: Figure 6A, B. *Video Editor: Please emphasize the teal line in both figures.*
 - 6.1.2. LAB MEDIA: Figure 6A, B. *Video Editor: Please emphasize the red line in both figures.*
 - 6.1.3. LAB MEDIA: Figure 6A, B. *Video Editor: Please emphasize the orange line in both figures.*
- 6.2. Specifically, Mito Stress tests revealed high basal respiration [1], maximal respiration [2], and reserve capacity in LPS pre-treated activated cells [3].
 - 6.2.1. LAB MEDIA: Figure 6A. *Video Editor: Please emphasize the red line along with the teal bar region.*
 - 6.2.2. LAB MEDIA: Figure 6A. *Video Editor: Please emphasize the red line along with the green bar region.*
 - 6.2.3. LAB MEDIA: Figure 6A. *Video Editor: Please emphasize the red line along with the blue bar region.*
- 6.3. However, the LPS-activated cells treated with the mGluR1 antagonist showed reduced oxygen consumption [1-TXT] and diminished ATP production [2] like naive levels [3].
 - 6.3.1. LAB MEDIA: Figure 6C. *Video Editor: Please emphasize the maroon bar in the X-axis region labelled “CPCCOEt”* **TXT: mGluR1 antagonist CPCCOEt**
 - 6.3.2. LAB MEDIA: Figure 6C. *Video Editor: Please emphasize the purple bar in the X-axis region labelled “CPCCOEt”*
 - 6.3.3. LAB MEDIA: Figure 6C. *Video Editor: Please emphasize the maroon and purple bars in the X-axis region labelled “Naive”*
- 6.4. Furthermore, Glycolysis assays confirmed that only untreated activated cells effectively utilize glycolytic pathways [1], as antagonist treatment reduced glycolytic

activity [2] to match naive conditions, collectively demonstrating that mGluR1 signaling is vital for CD8+ T cell metabolic reprogramming during activation [3].

6.4.1. LAB MEDIA: Figure 6D. *Video Editor: Please emphasize the blue, maroon and purple bars in the X-axis region labelled "Activated"*

6.4.2. LAB MEDIA: Figure 6D. *Video Editor: Please emphasize all the bars in the X-axis region labelled "CPCCOEt"*

6.4.3. LAB MEDIA: Figure 6D. *Video Editor: Please emphasize all the bars in the X-axis region labelled "Naive"*

1. XFe96
Pronunciation link: No confirmed link found
IPA: /ˌɛksˌɛfˈiːˌnaɪntiˈsɪks/
Phonetic Spelling: eks·ef·ee·nine·tee·siks
2. Glycolysis
Pronunciation link: <https://www.merriam-webster.com/dictionary/glycolysis>
IPA: /ˌɡlaɪˈkɑːləsɪs/
Phonetic Spelling: gly·kaa·luh·suhs
3. Pipettor
Pronunciation link: <https://www.merriam-webster.com/dictionary/pipette>
IPA: /paɪˈpɛtər/
Phonetic Spelling: pie·pet·er
4. Oligomycin
Pronunciation link: <https://www.howtopronounce.com/oligomycin>
IPA: /ˌɑːlɪɡoʊˈmaɪsɪn/
Phonetic Spelling: aa·lih·goh·my·sin
5. FCCP
Pronunciation link: No confirmed link found
IPA: /ˌɛfˌsiːˌsiːˈpiː/
Phonetic Spelling: ef·see·see·pee
6. Rotenone
Pronunciation link: <https://www.merriam-webster.com/dictionary/rotenone>
IPA: /ˈroʊtəˌnoʊn/
Phonetic Spelling: roh·tuh·nohn
7. Antimycin
Pronunciation link: <https://www.howtopronounce.com/antimycin>
IPA: /ˌæntɪˈmaɪsɪn/
Phonetic Spelling: an·tee·my·sin
8. Multichannel
Pronunciation link: <https://www.merriam-webster.com/dictionary/multichannel>

- IPA: /ˌmʌltiˈtʃænəl/
Phonetic Spelling: mul·tee·chan·uhl
9. Reagent
Pronunciation link: <https://www.merriam-webster.com/dictionary/reagent>
IPA: /riˈeɪdʒənt/
Phonetic Spelling: ree·ay·juhnt
10. Seahorse
Pronunciation link: <https://www.merriam-webster.com/dictionary/seahorse>
IPA: /ˈsiːˌhɔːrs/
Phonetic Spelling: see·hors
11. Analyzer
Pronunciation link: <https://www.merriam-webster.com/dictionary/analyzer>
IPA: /ˈænəˌlaɪzər/
Phonetic Spelling: an·uh·ly·zer
12. Pre-hydrated
Pronunciation link: No confirmed link found
IPA: /ˌpriːˈhaɪd্রেɪtɪd/
Phonetic Spelling: pree·hy·dray·tid
13. Metabolic
Pronunciation link: <https://www.merriam-webster.com/dictionary/metabolic>
IPA: /ˌmetəˈbɑːlɪk/
Phonetic Spelling: met·uh·baa·lik
14. Sulforhodamine
Pronunciation link: No confirmed link found
IPA: /ˌsʌlfoʊˈroʊdəmiːn/
Phonetic Spelling: sul·foh·roh·duh·meen
15. RNase
Pronunciation link: <https://www.merriam-webster.com/dictionary/ribonuclease>
IPA: /ˈɑːrˌenˌeɪs/
Phonetic Spelling: ar·en·ace
16. DNase
Pronunciation link: <https://www.merriam-webster.com/dictionary/deoxyribonuclease>
IPA: /ˈdiːˌenˌeɪs/
Phonetic Spelling: dee·en·ace
17. Solubilization
Pronunciation link: No confirmed link found
IPA: /ˌsɑːljəbələˈzeɪʃən/
Phonetic Spelling: saal·yuh·buh·luh·zay·shuhn
18. Nanometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/nanometer>
IPA: /ˈnænəʊˌmiːtərz/
Phonetic Spelling: nan·oh·mee·terz
19. Metabotropic
Pronunciation link: <https://www.howtopronounce.com/metabotropic>

- IPA: /məˌtæbəˈtrɑːpɪk/
Phonetic Spelling: muh·tab·uh·traa·pik
20. Glutamate
Pronunciation link: <https://www.merriam-webster.com/dictionary/glutamate>
IPA: /ˈɡluːtəˌmeɪt/
Phonetic Spelling: gloo·tuh·mayt
21. Receptor
Pronunciation link: <https://www.merriam-webster.com/dictionary/receptor>
IPA: /rɪˈseptər/
Phonetic Spelling: rih·sep·ter
22. mGluR1
Pronunciation link: No confirmed link found
IPA: /ˌɛmˌɡluːˌɑːrˈwʌn/
Phonetic Spelling: em·gloo·ar·wun
23. T-cell
Pronunciation link: <https://www.merriam-webster.com/dictionary/T-cell>
IPA: /ˈtiːˌsel/
Phonetic Spelling: tee·sel
24. Crosstalk
Pronunciation link: <https://www.merriam-webster.com/dictionary/crosstalk>
IPA: /ˈkrɒsˌtɔːk/
Phonetic Spelling: kraws·taawk
25. Lipopolysaccharide
Pronunciation link: <https://www.howtopronounce.com/lipopolysaccharide>
IPA: /ˌlɪpooˌpɑːliˈsækəˌraɪd/, /ˌlaɪpooˌpɑːliˈsækəˌraɪd/
Phonetic Spelling: lip·oh·pol·ee·sak·uh·ride / lie·poh·pol·ee·sak·uh·ride
26. CD8+
Pronunciation link: No confirmed link found
IPA: /ˌsiːˌdiːˈetˈpɑːzətɪv/
Phonetic Spelling: see·dee·eight·poz·uh·tiv
27. Mitochondrial
Pronunciation link: <https://www.merriam-webster.com/dictionary/mitochondrial>
IPA: /ˌmaɪtəˈkɑːndriəl/
Phonetic Spelling: my·tuh·kaan·dree·uhl
28. Naive
Pronunciation link: <https://www.merriam-webster.com/dictionary/naive>
IPA: /naɪˈiːv/
Phonetic Spelling: ny·eev
29. Pretreatment
Pronunciation link: <https://www.merriam-webster.com/dictionary/pretreatment>
IPA: /ˌpriːˈtriːtmənt/
Phonetic Spelling: pree·treet·muhnt
30. Metabolically
Pronunciation link: <https://www.merriam-webster.com/dictionary/metabolically>

IPA: /ˌmɛtəˈbɑːlɪkli/

Phonetic Spelling: met·uh·baa·lik·lee

31. Basal

Pronunciation link: <https://www.merriam-webster.com/dictionary/basal>

IPA: /ˈbeɪsəl/

Phonetic Spelling: bay·suhl

32. Respiration

Pronunciation link: <https://www.merriam-webster.com/dictionary/respiration>

IPA: /ˌrɛspəˈreɪʃən/

Phonetic Spelling: res·puh·ray·shuhn

33. Antagonist

Pronunciation link: <https://www.merriam-webster.com/dictionary/antagonist>

IPA: /ænˈtæɡənɪst/

Phonetic Spelling: an·tag·uh·nist

34. ATP

Pronunciation link: No confirmed link found

IPA: /ˌeɪˈtiːˈpiː/

Phonetic Spelling: ay·tee·pee

35. Glycolytic

Pronunciation link: <https://www.merriam-webster.com/dictionary/glycolytic>

IPA: /ˌɡlaɪkəˈlɪtɪk/

Phonetic Spelling: gly·kuh·lit·ik

36. Reprogramming

Pronunciation link: <https://www.merriam-webster.com/dictionary/reprogram>

IPA: /ˌriːˈproʊɡræmɪŋ/

Phonetic Spelling: ree·proh·gram·ing