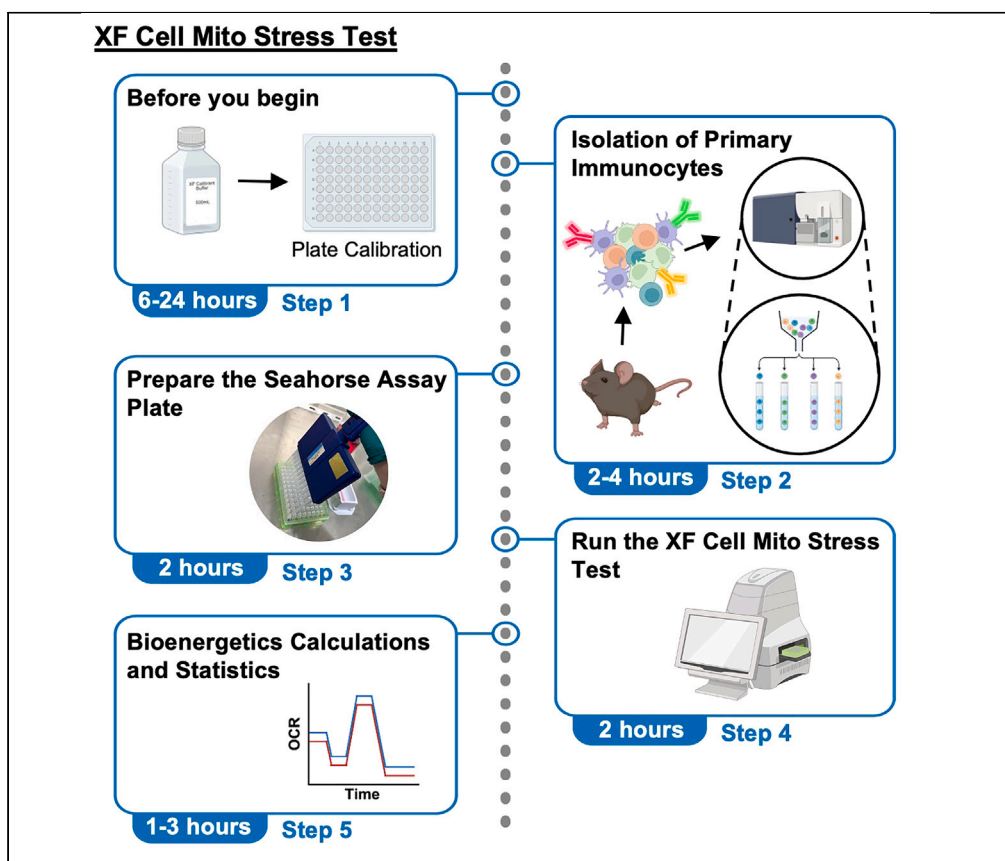# STAR Protocols

Protocol

Protocol to assess bioenergetics and mitochondrial fuel usage in murine autoreactive immunocytes using the Seahorse Extracellular Flux Analyzer

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Highlights

Instructions for isolation of primary immunocytes for bioenergetics analysis

Step-by-step guidance on preparation of the Seahorse assay

Measurement of mitochondrial fuel use using the Cell Mito Stress Test

Guidance on calculations for bioenergetics and statistics

Efficient metabolism, or the means by which cells produce energy resources, is critical for proper effector function. Here, we present a protocol for examining the bioenergetics and mitochondrial fuel utilization of primary murine autoreactive immunocytes using cellular metabolism-modulating drugs. We describe steps for plate calibration, isolation of primary immunocytes, and Seahorse assay plate preparation. We then detail procedures for performing the XF Cell Mito Stress Test followed by bioenergetics calculations and statistics.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Protocol to assess bioenergetics and mitochondrial fuel usage in murine autoreactive immunocytes using the Seahorse Extracellular Flux Analyzer

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SUMMARY

Efficient metabolism, or the means by which cells produce energy resources, is critical for proper effector function. Here, we present a protocol for examining the bioenergetics and mitochondrial fuel utilization of primary murine autoreactive immunocytes using cellular metabolism-modulating drugs. We describe steps for plate calibration, isolation of primary immunocytes, and Seahorse assay plate preparation. We then detail procedures for performing the XF Cell Mito Stress Test followed by bioenergetics calculations and statistics.

For complete details on the use and execution of this protocol, please refer to Wilson et al.[1]

BEFORE YOU BEGIN

Cells maintain homeostasis and functionality via metabolic regulation, which is modified in response to external cues.[2,3] In this process, external resources, such as glucose, other carbohydrates, fats, and proteins, are broken down into products that fuel energy demand, including ATP. Two major pathways that fuel cells are glycolysis, occurring in the cytosol of cells, and oxidative phosphorylation (OXPHOS), in the mitochondria. Typically, in oxygen-replete conditions, pyruvate, a product of glycolysis, is shunted into mitochondria for use in the tricarboxylic acid (TCA) cycle, while in oxygen-deprived, or hypoxic, conditions, pyruvate is largely converted to lactate. Glycolysis also generates intermediates that can serve as fuel substrates for metabolic pathways, including pyruvate in the TCA cycle, and electrons to the electron transport chain (ETC), to generate ATP in OXPHOS. Alternative fuels can be incorporated into the TCA cycle, including the generation of acetyl-CoA in fatty acid oxidation (FAO), and certain amino acids, such as glutamine, which can drive the ETC and promote OXPHOS. Overall, metabolism is comprised of contributions of multiple pathways, and can significantly contribute to many pathologies[1,4,5]; thus, a detailed understanding of metabolism may contribute to improved therapies.

The Seahorse XF Cell Mito Stress Test (Acute Injection) by Agilent, is an assay that measures cellular bioenergetics by assessing components of mitochondria function, including basal respiration, ATP-linked respiration, proton leak, and maximal respiration (Figure 1). An Agilent Seahorse XF Analyzer tracks subtle changes in oxygen levels (oxygen consumption rate; OCR) and the release of free protons, represented by extracellular acidification (extracellular acidification rate; ECAR), in the XF assay medium over time to determine rates of mitochondrial OXPHOS and glycolysis, respectively. Fuel inhibitors and metabolic modulators can be added into the XF assay medium to induce
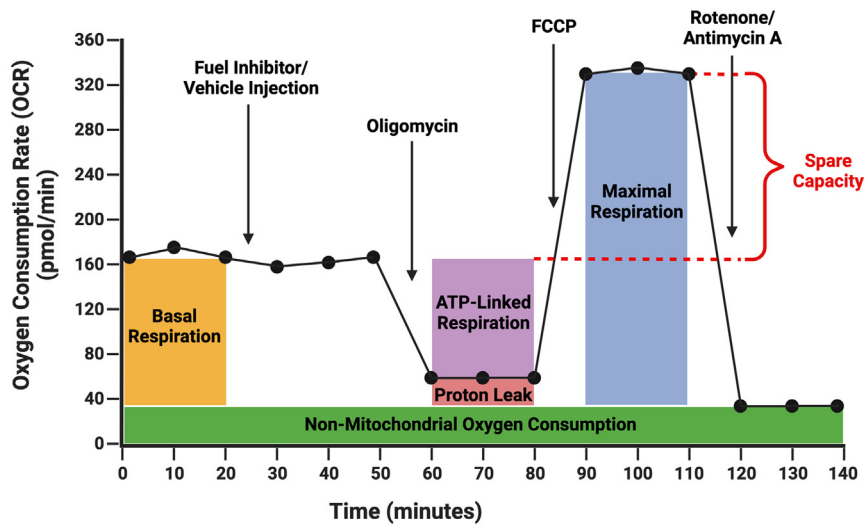
Figure 1. Key components of mitochondrial respiration in the Seahorse XF Cell Mito Stress Test (acute injection)
The Seahorse XF Cell Mito Stress Test measures the reliance of mitochondrial respiration, related to oxidative phosphorylation (OXPHOS), in cellular bioenergetics. The profile of mitochondrial respiration in the test includes basal respiration, ATP-linked respiration, proton leak, maximum respiration (also known as maximal respiratory capacity), and spare capacity. Each of these contributors to mitochondrial respiration are determined following injection of a fuel inhibitor, oligomycin, FCCP, and a combinatory injection of rotenone and antimycin A. Oligomycin reduces the oxygen consumption ration (OCR) through inhibition of ATP synthase, a critical facilitator of oxidation phosphorylation of ADP into ATP. FCCP potently collapses the proton gradient at the inner mitochondrial membrane, simulating a maximal energy demand in the cells. Rotenone and antimycin A are complex I and complex III inhibitors of the electron transport chain (ETC), respectively. The dual injection of these two inhibitors prevents the flow of electrons in the ETC, abolishing mitochondrial respiration for the determination of the degree of non-mitochondrial oxygen consumption. Image adapted from Agilent and created with BioRender.com.

alterations in cellular metabolism. Subsequent OCR measurements after the introduction of FCCP, a proton gradient uncoupler, allow for the determination of the maximal respiratory capacity (MRC) of the cells to elucidate their specific requirements for various fuel substrates for OXPHOS.

Detailed here is the protocol by which our laboratory measures the bioenergetics of primary murine lymphocytes, beginning with:

1. Calibrate the Seahorse probe plate (also known as the sensor cartridge), by adding 200 μL of XF Calibrant buffer to each well of the plate after the removal of the probe insert from the wells. For troubleshooting, see problem 2.

   Note: Avoid contact to the sensitive probe tips of the sensor cartridge during this process as it can cause damage and compromise their accuracy and functionality. To safeguard the integrity of the probes during the addition of the calibrant buffer, it is recommended that the probe is inverted so that it rests on the lid with the probe tips sticking up.

   Note: Ensure that the Seahorse probe plate is not expired. It is always recommended to use Seahorse plates prior to the expiration date listed for optimal results.

2. Replace the probe plate on top of the lower plate so that each probe is submerged in the XF Calibrant buffer in each well.
3. Incubate the Seahorse probe plate in a 37°C incubator, without CO_2, for a minimum of 6 h.

   ⏸ Pause point: If necessary, this step can be performed the previous day, though the duration of the calibration should not exceed 24 h.

Institutional permission

This protocol involves the utilization of mice. Users who intend to follow this protocol must obtain the necessary permissions from their respective institutions and conduct experiments in strict compliance with relevant institutional and national guidelines and regulations. It is important to note that all animal procedures outlined in this protocol have received approval from The Jackson Laboratory Institutional Animal Care and Use Committee and were carried out in full accordance with the Animal Care and Use Guidelines established by the U.S. National Institutes of Health.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45R/B220 BV711 (dilution 1:400)	BioLegend	RRID:AB_2563491
CD4 BV650 (dilution 1:400)	BioLegend	RRID:AB_2562098
CD95/FAS PE-Cy7 (dilution 1:300)	BD Biosciences	RRID:AB_396768
Anti-MU/HU GL7 AF647 (dilution 1:400)	BioLegend	RRID:AB_2562185
CXCR5 BV421 (dilution 1:300)	BioLegend	RRID:AB_2562128
PD-1 PE (dilution 1:400)	BioLegend	RRID:AB_1877231
Chemicals, peptides, and recombinant proteins		
2-Deoxy-D-glucose	Thermo Fisher Scientific	Cat#111980250
UK5099	GlpBio	Cat#GC11865
BPTES	Cayman Chemical	Cat#19284
Thioridazine	Cayman Chemical	Cat#14400
FCCP	Sigma	Cat#370-86-5
Rotenone	Sigma	Cat#557368
Antimycin A	Sigma	Cat#A8674
Oligomycin	Sigma	Cat#1404-19-9
Etomoxir	Cayman Chemical	Cat#11969
Fetal bovine serum	Gibco	Cat#SH3091003
RPMI medium 1640	Life Technologies	Cat#11875-093
Ammonium chloride	Sigma	Cat#A9434
Potassium bicarbonate	Sigma	Cat#237205
EDTA tetra sodium salt	Sigma	Cat#15708-41-5
Powered non-buffered RPMI 1640 without glucose	Thermo Fisher Scientific	Cat#31800105
L-glutamine (200 mM)	Thermo Fisher Scientific	Cat#25030081
D-(+)-glucose	Sigma	Cat#G7021
Seahorse XF Calibrant buffer	Agilent Technologies	Cat#100840-000
MP Biomedicals Poly-D-lysine hydrobromide attachment factors	Fisher Scientific	Cat#ICN15017510
Trypan blue solution, 0.4%	Thermo Fisher Scientific	Cat#15250061
Sodium pyruvate solution	Sigma	Cat#11360070
Critical commercial assays		
Seahorse XFe96/XF Pro FluxPak	Agilent Technologies	Cat#103792-100
Experimental models: Organisms/strains		
C57BL/6J (male homozygous mice, 11 weeks of age)	The Jackson Laboratory	Cat#000664
BXSB.Cg-$Cd8at^{tm1Mak}$ $Il15t^{tm1lmx}$/Dcr Yaa (male homozygous mice, 11 weeks of age)	In-house breeding	N/A
Software and algorithms		
Prism	GraphPad	https://graphpad.com/features
Seahorse Wave	Agilent Technologies	https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
Other		
Seahorse Analyzer XFe96	Agilent Technologies	N/A
FACSAria II	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Seahorse XF Analyzer

This assay is optimized for utilization of the Agilent Seahorse XFe96, requiring 96-well Seahorse assay plates. Basic user information for the Seahorse XFe96 can be found from the manufacturer: https://www.agilent.com/en/product/cell-analysis/how-to-run-an-assay?sh_0009=.

Alternatives: Seahorse XF (8 well, 24 well, or 96 well systems) or Seahorse XF HS Mini (8 well) Analyzers can be alternatively used.

Cell sorting

Isolation of primary murine cells, for the example provided, was performed using a FACSAria II; alternative cell sorters capable of multi-color detection are also suitable.

Additional required materials:

$37°C$ incubator (without CO_2 supplementation).

Cell counter/Hemacytometer.

Preparation of metabolic modulators and other reagents

Reconstitute all metabolic modulators listed below in DMSO, unless otherwise indicated, following the specified concentrations. Once dissolved, store all chemical stocks, in single use aliquots, at $-20°C$; they remain stable for a minimum of 6 months, with exceptions noted below. Mechanisms of action of fuel inhibitors are depicted in Figure 2. For troubleshooting, see problem 4.

Metabolic modulators

- 1 mM oligomycin

  ⚠ CRITICAL: Oligomycin carries health hazards for toxicity and the potential for organ damage if swallowed. It is recommended to utilize appropriate PPE when handling this compound, including the use of protective gloves, protective eyewear, protective clothing, and appropriate engineering controls including a certified fume hood.

- 1.5 mM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP)

  Note: While we have not encountered any issue with solubility at this concentration, the solution can be warmed in a $37°C$ bead bath, if needed, to promote a homogenous mixture.

  ⚠ CRITICAL: FCCP carries health hazards for toxicity and the potential for skin-associated allergic reactions. It is recommended to utilize appropriate PPE when handling this compound, including the use of protective gloves, protective eyewear, protective clothing, and appropriate engineering controls including a certified fume hood.
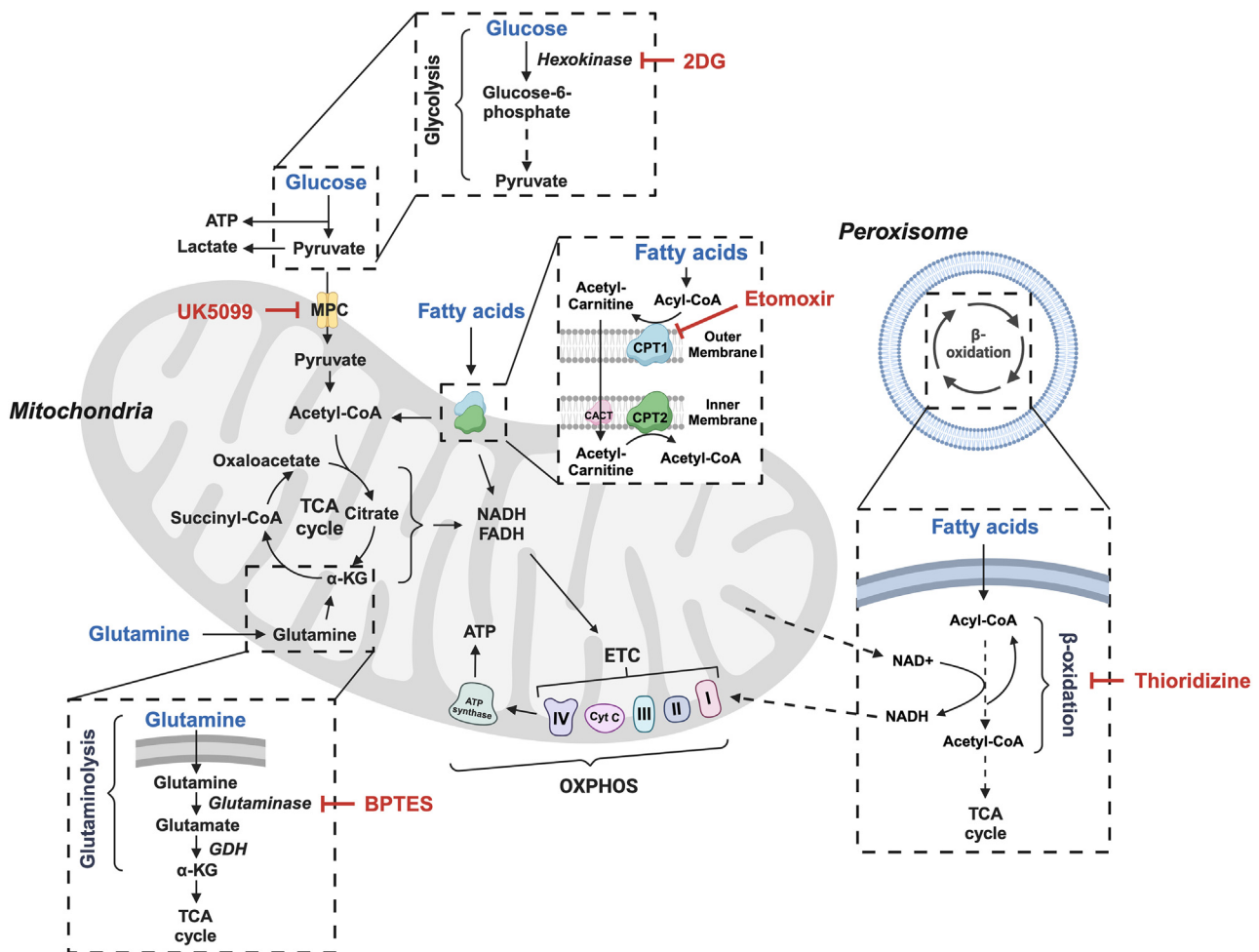
- 100 mM rotenone

  ⚠ CRITICAL: Rotenone carries health hazards for toxicity, skin, eye, and respiratory irritation. It is recommended to utilize appropriate PPE when handling this compound, including the use of protective gloves, protective eyewear, protective clothing, and appropriate engineering controls including a certified fume hood.

Figure 2. Metabolic pathways influenced by fuel inhibitors utilized in this protocol

Metabolism incorporates the products from several intracellular pathways for the production of cellular fuel. These networks, including glycolysis (beginning with glucose), fatty acid oxidation (beginning with long-chain fatty acid), and glutaminolysis (beginning with glutamine) terminate in the production of metabolites that can feed directly into the tricarboxylic acid (TCA) cycle. Selective inhibition of these individual pathways can provide crucial insight into the metabolic reliance and flexibility of differing cell types. 2DG is a glucose mimic and selective inhibitor of hexokinase activity, playing a key role in regulating glucose oxidation. Hexokinase catalyzes the phosphorylation of glucose into glucose-6-phosphate, thus ultimately preventing the conversion of glucose into pyruvate. UK5099 is a selective inhibitor of pyruvate import into the mitochondria for utilization in the TCA cycle. BPTES is a specific inhibitor of glutaminase activity, inhibiting the breakdown of glutamine into α-ketoglutarate (α-KG). Thioridazine inhibits peroxisomal β-oxidation, impeding peroxisomal fatty acid oxidation (FAO) and halting the breakdown of long-chain fatty acids into acetyl-CoA for the TCA cycle. Etomoxir, as a mitochondrial FAO inhibitor, specifically disrupts the action of CPT1, preventing the conversion of acyl coenzyme A (CoA) to acetyl-carnitine, thereby inhibiting the mitochondrial-derived utilization of long-chain fatty acids to fuel the TCA cycle. Image created with BioRender.com.

- 1 mM antimycin

   ⚠ CRITICAL: Antimycin carries health hazards for toxicity, and can be fatal if swallowed, It is recommended to utilize appropriate PPE when handling this compound, including the use of protective gloves, protective eyewear, protective clothing, and appropriate engineering controls including a certified fume hood.

- 1 M 2-deoxyglucose (dissolve in PBS and store at 4°C, stable for 1 week)
- 20 mM UK5099
- 10 mM BPTES

⚠ CRITICAL: BPTES carries health hazards for skin, eye, and respiratory irritation. It is recommended to utilize appropriate PPE when handling this compound, including the use of protective gloves, protective eyewear, protective clothing, and appropriate engineering controls including a certified fume hood.

- 40 mM etomoxir

⚠ CRITICAL: Etomoxir carries health hazards for toxicity, and is toxic if swallowed. It is recommended to utilize appropriate PPE when handling this compound, including the use of protective gloves, protective eyewear, protective clothing, and appropriate engineering controls including a certified fume hood.

- 100 mM thioridazine

⚠ CRITICAL: Thioridazine carries health hazards for toxicity, skin, eye, and respiratory irritation. It is recommended to utilize appropriate PPE when handling this compound, including the use of protective gloves, protective clothing, and appropriate engineering controls including a certified fume hood.

R1 medium (Store at 4°C (stable for 2–4 weeks))

Reagent	Final concentration	Amount
Fetal Bovine Serum	1%	5 mL
RPMI Medium	N/A	495 mL
Total	N/A	500 mL

Red blood cell lysis buffer (autoclave, and store at 4°C (stable to up to 6 months))

Reagent	Final concentration	Amount
Ammonium chloride	155 mM	4.15 g
Potassium bicarbonate	10 mM	0.5 g
EDTA tetra sodium salt	0.35 mM	0.067 g
ddH$_2$0	N/A	500 mL
Total	N/A	500 mL

Seahorse XF Assay medium (pH to 7.4, sterilize through a 0.22 μM filter and store at 4°C (stable for up to 1 month))

Reagent	Final concentration	Amount
Powered non-buffered RPMI 1640 without glucose	N/A	4.15 g
L-Glutamine (200 mM)	2 mM	5 mL
D-Glucose	25 mM	2.25 g
Sodium Pyruvate Solution (100 mM)	1 mM	5 mL
ddH$_2$O	N/A	490 mL
Total	N/A	500 mL

STEP-BY-STEP METHOD DETAILS

The protocol detailed below provides instruction for bioenergetic analysis of primary autoreactive germinal center B (GCB) and T follicular helper (Tfh) cells. This protocol has been optimized for metabolic analyses of *ex vivo* mouse immunocytes, though may be adapted to other relevant primary cell types.

Isolation of primary murine immunocytes

⏱ Timing: approximately 2–4 h

To perform the Seahorse Assay on primary murine cell types, the cells first need to be harvested from the mouse. Then, the target cell populations of interest must be identified and purified. In this step, isolation of primary murine splenocytes is described, as well as sort purification of GCB and Tfh cells from the bulk splenocyte population.

1. Isolate GCB and Tfh cells from lupus-prone mice by:
   a. Euthanize BXSB.Cg-Cd8a[tm1Mak] Il15[tm1Imx]/Dcr *Yaa* (*Yaa* DKO) male mice with active lupus-like disease (11-week-old) via CO_2 asphyxiation on the day of the Seahorse Assay.

   Note: For the analysis of the *ex vivo* immunocytes, the immune cells must be harvested on the same day that the Seahorse Assay is to be completed to promote cell viability and accuracy of the measurements within the Seahorse Assay. Overnight culture of the primary immunocytes is not recommended.

   b. Harvest the spleen and inguinal, axillary, and cervical lymph nodes.

   ⚠ CRITICAL: With regard to overall human health and the success of this assay, it is recommended to wear PPE, including protective clothing, eye wear, and gloves when handling mice and tissues or fluids from mice. It is essential that all techniques involving the use of laboratory animals has received the necessary approval from Institutional Animal Care and Use Committees and are carried out in full accordance with the Animal Care and Use Guidelines established by the U.S. National Institutes of Health.

   c. Mechanically disassociate spleens and lymph nodes in approximately 2 mL of R1 medium until only fibrous tissue remained.
   d. Pass the disassociated cells in R1 medium through a 70 μm cell strainer into a 15 mL conical tube.

   Note: A piece of 70 μm nytex can also be used in place of the 70 μm cell strainer.

   Note: To maximize the yield of isolated cells, 1 mL of R1 medium can be used to wash the dish and pass through the filter after the disassociated cells.

   e. Add 5 mL of red blood cell lysis buffer to the cell suspension. Gently invert the tube to mix and incubate at 20°C–22°C for 5 min.
   f. After the 5-min lysis, fill the conical tube to the top with R1 medium and then centrifuge the tube at 4°C for 5 min at 400 × *g*.
   g. Resuspend the cell pellet in an appropriate amount of R1 medium and determine the total live cell number via hemacytometer using trypan blue or another viability dye.

   ⚠ CRITICAL: To maintain the highest level of cell viability, it is recommended to maintain the cells that are to be sort purified on ice.

2. Purify the target cell populations by:
   a. Prepare the cell stain antibody cocktail comprising the combination of antibodies for identification of immune cell sub-populations.

   Note: In the example data shown, identification of GCB and Tfh cells was necessary.

Table 1. Example antibody staining used for identification of GCB and Tfh cells

Target cell population	Antibody	Fluorophore	Source	Dilution factor
GCB cells	B220	BV711	BioLegend	1:400
	CD4 (negative)	BV650	BioLegend	1:400
	CD95	PE-Cy7	BioLegend	1:300
	GL7	AF647	BioLegend	1:400
Tfh cells	B220 (negative)	BV711	BioLegend	1:400
	CD4	BV650	BioLegend	1:400
	CXCR5	BV421	BioLegend	1:300
	PD1	PE	BioLegend	1:400

Note: Example fluorophores used in this assay for isolation of the GCB and Tfh cells are described ([Table 1](#)). The recommended dilution factors have been verified by our laboratory for isolation of the indicated immune cell populations from lupus-prone mice.

Note: The optimal number of cells per hemocytometer square is between 30 and 300 to yield an accurate count. If cells are not within this range, adjust the volume of R1 medium in which the cells are resuspended.

Note: 500 mL of antibody stain cocktail is recommended for every 100 million cells.

b. Sort the target cell populations using a 70 μm nozzle.

Note: Due to the stress of sorting on the cells, it is important to either sort into ice cold 100% FBS or RPMI medium containing 50% FBS to ensure maximum cell viability.

c. Centrifuge the tubes to pellet the cells, aspirate the supernatant, and resuspend in Seahorse XF Assay medium. Cells should be resuspended at a concentration of ~250,000 cells per 100 μL of Seahorse XF Assay medium.
d. Calculate the total number of live cells with a hemocytometer using trypan blue or alternative viability dye.

⚠ CRITICAL: An accurate measurement of the total cell number is essential for the overall success of this assay and the accuracy of the resultant data collected. Counting the cells several times independently is recommended, followed by the averaging of the individual count replicates for the overall determination of cell number.

e. Dilute the target cells to the ideal concentration of cells for plating.

Note: While the recommended range for cell density in a standard Seahorse assay is between 50,000–200,000 cells for each individual well, we have determined that 100,000 *ex vivo* GCB and Tfh cells from Lupus-prone mice per well results in the ideal basal respiratory rates for analysis in this assay. Because of this, for the example described in this protocol, the cells were diluted to 100,000 cells per 40 μL of Seahorse XF Assay medium.

Preparation of Seahorse Assay

⏱ Timing: approximately 2 h

One of the most critical components of this Seahorse Assay is setting up the Seahorse Assay cell and injection plates. In this step of the protocol, we describe how to optimally seed the sort purified primary murine autoreactive immunocytes into the Seahorse cell culture plate. Here we also delineate

the protocol for preparing and pipetting the injection solutions into the Seahorse XF Cell Mito Stress Test injection plate.

3. Prepare the Seahorse Assay cell culture plate. For troubleshooting, see problems 1, 3, 6.
   a. Coat the Seahorse cell culture plate with poly-D-lysine (20 µL/well of a 50 µg/mL stock solution). Incubate the culture plate at 20°C–22°C for 1–2 h.
   b. Aspirate the poly-D-lysine coating and allow the plate to dry, uncovered, in a sterile tissue culture hood, 20°C–22°C, for approximately 45 min–1 h or until completely dry.

⚠ CRITICAL: The primary *ex vivo* immune cells from Lupus-prone mice are non-adherent. It is essential that the plate is coated with the poly-D-lysine to promote the adherence of the cells at the bottom of the well for the most accurate measurements. This is a critical component of our adaptation of this protocol as the Seahorse XF Cell Mito Stress Test (Acute Injection) protocol from the manufacturer is designed for adherent cell lines.

   c. After determining the total number of cells in each sample followed by dilution of the cells to the appropriate density in Seahorse XF Assay medium, seed the cells of interest.

Note: For the example shown here, GCB and Tfh cells were seeded into the Seahorse Assay cell culture plate.

Note: While the recommended range for cell density in a Seahorse assay is between 50,000–200,000 cells for each individual well, we have determined that 100,000 *ex vivo* GCB and Tfh cells from Lupus-prone mice per well results in the ideal basal respiratory rates for analysis in this assay. As such, here the cells were diluted to 100,000 cells per 40 µL of Seahorse XF Assay medium, and thus, 40 µL of cells were plated per well.

Note: The cell density per well may vary depending on the cell type, size, and baseline levels of metabolism. Typically, the ideal cell density is between 50,000–200,000 cells per well.

Note: The total number of wells recommended for reliable determination of cellular bioenergetics is a minimum of 5–8 per target cell sample, per treatment. The final determination for the number of wells per cell sample is dependent on both how many wells are available for use, as well as the total number of cells that were acquired in sorting. For the example shown here, a minimum of 6 wells were used for each replicate of GCB or Tfh cells.

Note: Increasing the number of wells allows for more accurate data analysis because of potential well-to-well variability, or failure of some wells.

⚠ CRITICAL: The background wells should only contain Seahorse XF Assay medium; no cells should be added to these wells.

⚠ CRITICAL: When only a partial plate will be used, the remaining unused wells must contain Seahorse XF Assay medium. These wells (without cells) should be included as additional background wells.

   d. Centrifuge the cell plate for 5 min at 400 × *g* to promote cell adhesion to the poly-D-lysine coating at the bottom of the plate.
   e. Top off each well to the maximum volume of 180 µL with Seahorse XF Assay medium.

Note: In the example shown, 140 µL of Seahorse XF Assay medium was added to each well (equates to 180 µL of total volume when added to the 40 µL of medium containing cells already in the plate).

Table 2. Concentrations of metabolic modulators and reagents utilized in this assay

Injection Port	Drug	Stock [x]	Final [x] after injection	Volume of drug diluted	Volume of seahorse XF assay medium
A	Vehicle (no drug)	N/A	N/A	0 μL	2.8 mL
	2DG	1 M	25 mM	700 μL	2.1 mL
	UK5099	20 mM	20 μM	28 μL	2.8 mL
	BPTES	10 mM	10 μM	28 μL	2.8 mL
	Etomoxir	40 mM	40 μM	28 μL	2.8 mL
	Thioridizine	100 μM	100 nM	28 μL	2.8 mL
B	Oligomycin	1 mM	1 μM	28 μL	2.8 mL
C	FCCP	1.5 mM	1.5 μM	28 μL	2.8 mL
D	Rotenone	100 μM	100 nM	28 μL	2.8 mL
	Antimycin A	1 mM	1 μM	28 μL	

Note: It is important that the pelleted cells are not disturbed. Slowly pipette the Seahorse XF Assay medium down the side of each well using a multichannel pipette (to minimize pipetting error). Following the supplemental Seahorse XF Assay medium addition if there is concern of cell lifting or disturbance due to pipetting, the cell plate can be centrifuged again, for 5 min at 4°C, 400 × g.

f. Place the cell plate in a 37°C incubator, without CO_2, for a minimum of 45 min.

⚠ CRITICAL: This incubation is essential for de-gassing the cells, Seahorse XF Assay medium, and plate. The presence of CO_2 will result in inaccurate ECAR measurements, as CO_2 can increase acidification of the Seahorse XF Assay medium.

4. Prepare the Seahorse XF Cell Mito Stress Test injection plate. For troubleshooting, see problem 4.
   a. Prepare the injection solutions for the Seahorse XF Cell Mito Stress Test injection plate. The intended port for each of the drugs/inhibitors, the final concentrations, as well as recommended dilutions for each of the drugs/inhibitors are described (Table 2).

Note: Volumes were calculated based on utilization of the full Seahorse plate. Alterations can be made dependent on the number of wells utilized in the plate. i.e., divide the volumes of each component in half when only half of the Seahorse plate will be used.

⚠ CRITICAL: While we have recommended concentrations that have been validated by our laboratory for *ex vivo* GCB and Tfh cells derived from lupus-prone mice, for a given cell type under investigation, titration of the drugs used is crucial to ensure optimal experimental conditions.

   b. After the 6-h incubation of the Seahorse probe plate in the XF Calibrant Buffer has elapsed, pipette the drugs into the injection plate. The volumes of each diluted drug (volumes for dilutions delineated in Table 2) to pipette into each port is as follows:
      Port A (Vehicle, 2DG or other fuel inhibitor as in Table 2): 18 μL per injection.
      Port B (Oligomycin): 20 μL per injection.
      Port C (FCCP): 22 μL per injection.
      Port D (Rotenone/Antimycin A): 24 μL per injection.

⚠ CRITICAL: For the Acute Injection strategy in the Seahorse XF Cell Mito Stress Test, fuel inhibitors of metabolic pathways (Figure 2) are <u>individually</u> included in Port A of the injection plate, followed by Oligomycin, FCCP, and then Rotenone + Antimycin A in Ports B, C,

and D, respectively. The fuel inhibitor included in Port A is determined by the metabolic pathway of interest (Figure 2), the assay can be completed either multiple times or with additional wells devoted to test each individual fuel inhibitor for each cell population of interest compared to vehicle-treated wells. The stock and final concentration for each drug or inhibitor following injection have been optimized by our laboratory for this protocol for investigation of *ex vivo* isolated primary immune cells (Table 2). The volume of each drug utilized and the volume of Seahorse XF Assay medium for dilution are based on both the initial and final intended concentration of each reagent, as well the use of a full 96-well plate assay. Pipette the metabolic modulators into the injection plate in the following order: vehicle, or 2DG (or alternative fuel inhibitor as described in Table 2), Oligomycin, FCCP, and then Rotenone/Antimycin A.

Note: The volumes for each injection are optimized to account for the increasing volume in each well after each injection. It is important to account for this volume change to avoid altering the final drug concentrations following injection.

⚠ CRITICAL: When only a partial plate will be used the remaining injection ports must contain unaltered Seahorse XF Assay medium (without cells).

⚠ CRITICAL: Control wells should have unaltered Seahorse XF Assay medium included in Port A for injection in place of the inhibitor or drug used for the experimental wells.

Run the Seahorse Assay

⊙ Timing: approximately 2 h

After preparing both the cell plate as well as the injection plate, performing the Seahorse Assay requires the use of the Seahorse XF Analyzer and associated Wave application software. Here, we describe how to run the Seahorse Assay.

5. Adjust settings in the XF Analyzer.
   a. Open the Wave application software.
   b. Select XF 'Cell Mito Stress Test (Acute Injection)'.
   c. Select 'Add Groups' to define experimental test groups (Figure 3).

Note: In the example data shown, groups for each of the target cell populations (GCB and Tfh cells) were created. Two groups each were generated (experimental or control) for each of the tested metabolic modulators as described in Table 2 (Port A).

   d. Use the 'Plate Map' feature to identify location of wells pertaining to each experimental group defined (Figure 4).

Note: It is important that a minimum of 4 background wells are designated. These wells should contain no cells, but the injection components should still contain inhibitors/drugs.

Note: For confidence in the results, at least 5–8 wells, per target cell group, per metabolic modulator or control group tested, should be included.

   e. Select 'Protocol' to modify the injection strategy or measurement frequency (measurements are defined in Table 3; Figure 5). The assay steps include (in order of treatment/measurement) the baseline measurements, the measurements for control or experimental wells, Oligomycin, FCCP, and lastly Rotenone/Antimycin A measurements.
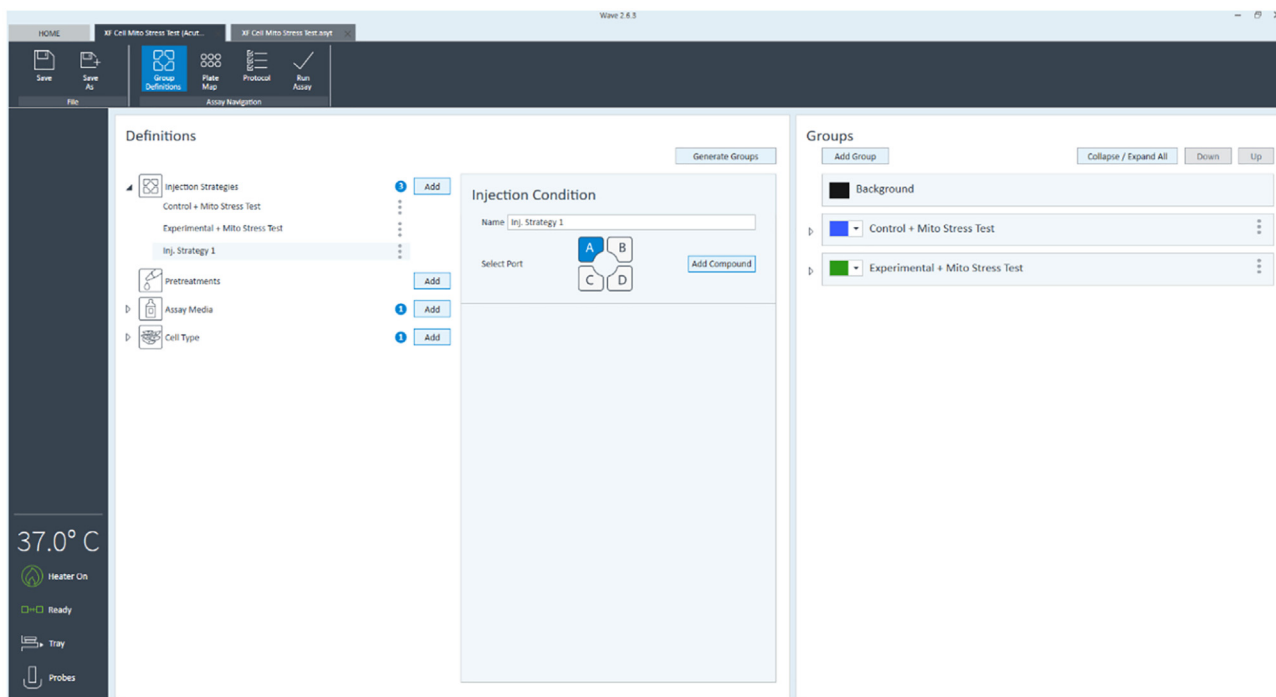
Figure 3. Group definitions in Seahorse wave software
The 'Group Definitions' section of the Seahorse XF Cell Mito Stress Test allows for the designation of experimental groups or cell types in the assay. Modifications to the injection compounds can also be included in this section.

> *Note:* The standard protocol includes 3 measurements for each assay step, each including a 3-min mix, a 0-min wait, followed by a 3-min measurement. The only recommended alteration performed in the protocol described here for *ex vivo* primary immunocytes from lupus-prone mice includes an additional measurement cycle for the control/experimental assay step. The remaining assay step measurement cycles are unaltered in this protocol. Without modifications to the measurement frequency or duration, approximate run time is 1 h and 40 min. For the example data shown here, the approximate run time is 1 h and 50 min.

> *Note:* The number of measurement cycles for the Control or Experimental Assay Step was increased from 3 cycles (according to the Agilent Seahorse recommendation) to 4 cycles to allow for maximal effect and results following treatment of the cells with 2DG or other fuel modulator.

f. Select "Run Assay".

Bioenergetics calculations and statistics

© Timing: approximately 1–3 h

After acquiring the raw data using the Seahorse XF Analyzer and Wave application software, the raw data is then subjected to calculations to determine the maximal respiratory capacity.

> *Note:* Drawing from our experience for most studies, it is recommended that experiments should be repeated at least three times to ensure reproducibility. Raw data collected in this assay can be analyzed in either the Seahorse Wave Desktop Software (can be downloaded for free) or in a
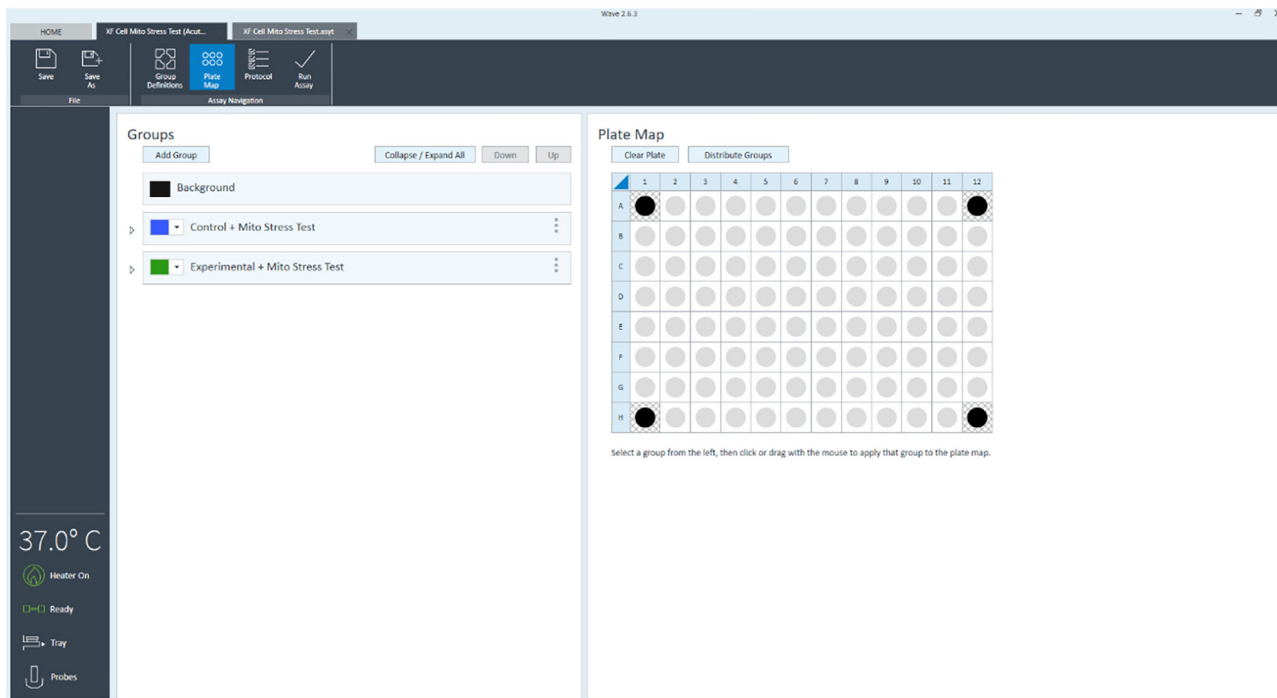
Figure 4. Defining plate layout in Seahorse wave software

After performing the group designations (as described in Figure 2), the 'Plate Map' feature in the Seahorse XF Cell Mito Stress Test protocol is used. This feature maps each experimental group in the plate layout provided. This is critical for the correlation of resulting raw data with the associated specific treatments or cell types used in this assay. Any empty wells remaining in the plate (wells with no plated cells) should still contain Seahorse XF Assay medium and be designated as additional 'Background' wells.

secondary software, including Excel or Prism. For analysis in the Seahorse Wave Desktop Software, export your data using the Seahorse XF Cell Mito Stress Test Report Generator. Details for how to download this report, as well as access to the report generator user guide can be found at: https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-xf-cell-mito-stress-test-report-generators-740899. For analysis in either Excel or Prism, the raw data can be exported in compatible file extensions as well.

6. Determine the maximal respiratory capacity (MRC). The MCR in each sample well is determined using the following calculation:

$$MRC = (maximum\ rate\ measurement\ after\ FCCP\ injection) - (non\text{-}mitochondrial\ respiration)$$

Note: The MRC (also known as maximal respiration) was calculated at the percentage of baseline OCR immediately following injection of FCCP. The injection of FCCP artificially stimulates the respiratory chain in the cells to operate at maximum capacity, facilitating the maximum utilization of available resources for oxidation (i.e., sugars, fatty acids, amino acids). The MRC is calculated following injection of the uncoupler FCCP.

Note: MRC is indicative of bioenergetic potential of target cell populations and, when compared between vehicle-treated wells and those exposed to 2DG or other fuel inhibitors of fuel substrates, can reveal differences in fuel reliance between cell types. Importantly, MRC is influenced by several metabolic networks, including glycolysis, fatty acid oxidation, and glutaminolysis, highlighting the ability of this refined assay to determine the reliance of these contributing networks on mitochondrial oxidation, through the use of metabolic

Table 3. Pre-set settings in the XF Mito stress test (acute injection) in the Seahorse wave software

| Assay step	Number of measurement cycles	Step	Default
Baseline	3	Mix	3 Min
		Wait	0 Min
		Measure	3 Min
Control or Experimental	4	Mix	3 Min
		Wait	0 Min
		Measure	3 Min
Oligomycin	3	Mix	3 Min
		Wait	0 Min
		Measure	3 Min
FCCP	3	Mix	3 Min
		Wait	0 Min
		Measure	3 Min
Rotenone + Antimycin A	3	Mix	3 Min
		Wait	0 Min
		Measure	3 Min

modulators. Percentage of MRC is calculated as the percentage changes in the average MRC between sample wells treated with fuel inhibitors compared to untreated samples. This calculation is used to normalize MRC results from inhibitor-treated samples to vehicle-treated samples and allows for a comparison of fuel reliance between cell types where baseline MRC may differ.

Note: Due to well-to-well variability and the number of replicate wells for each cell type, it is recommended to use a two-tailed Student's *t*-test to determine statistical significance in comparison with the appropriate vehicle controls.

EXPECTED OUTCOMES

This protocol details the steps necessary to measure the bioenergetics of primary immune cells *ex vivo*. Here, we illustrate the XF Cell Mito Stress Test (Acute Injection) assay using *ex vivo*, sort-purified, GCB and Tfh cells derived from lupus-prone mice to better understand the metabolic profiles and fuel utilization within various autoreactive cell populations.[1] To determine the basal metabolic state of lupus-relevant immune cells, the Seahorse XF Cell Mito Stress Test is used and baseline measurements of GCB and Tfh cells from diseased lupus mice are compared with naïve cells isolated from healthy mice. All immune cell populations tested from diseased mice exhibit higher rates of ECAR and OCR in comparison with the naïve cells from non-diseased/healthy lupus-prone mice, indicative of increased metabolic demand (Figure 6A). Upon treatment with 2DG, an inhibitor of hexokinase activity in glycolysis, preventing the production and accumulation of glucose-6-phosphate from glucose (Figure 2),[6] the ECAR of GCB and Tfh cells is significantly reduced (Figure 6B), demonstrating the inhibitory impact of 2DG on glycolysis. Subsequently, the degree of glycolytic reliance in the GCB and Tfh cells for OXPHOS utilization is assessed. For this, utilization of the acute injection strategy in the Seahorse XF Cell Mito Stress Test assay allows for treatment with 2DG prior to injection of Oligomycin, FCCP and R/A for a cell type-specific bioenergetics analysis (Table 3). We demonstrate that MRC is reduced in GCB cells from diseased mice following injection of 2DG in comparison to GCB cells exposed to Seahorse XF Assay medium, following FCCP-mediated mitochondrial decoupling (Figures 6C and 6D). This indicates the necessity of glucose for fueling OXPHOS and glucose oxidation in mitochondria. Also, the reduction in MRC is specific to GCB cells, as Tfh cells are not impacted by 2DG injection (Figures 6C and 6D).

In this protocol, we also provide examples of utilization of alternative metabolic modulators in this assay to better understand the bioenergetics of the cell populations investigated. To mechanistically
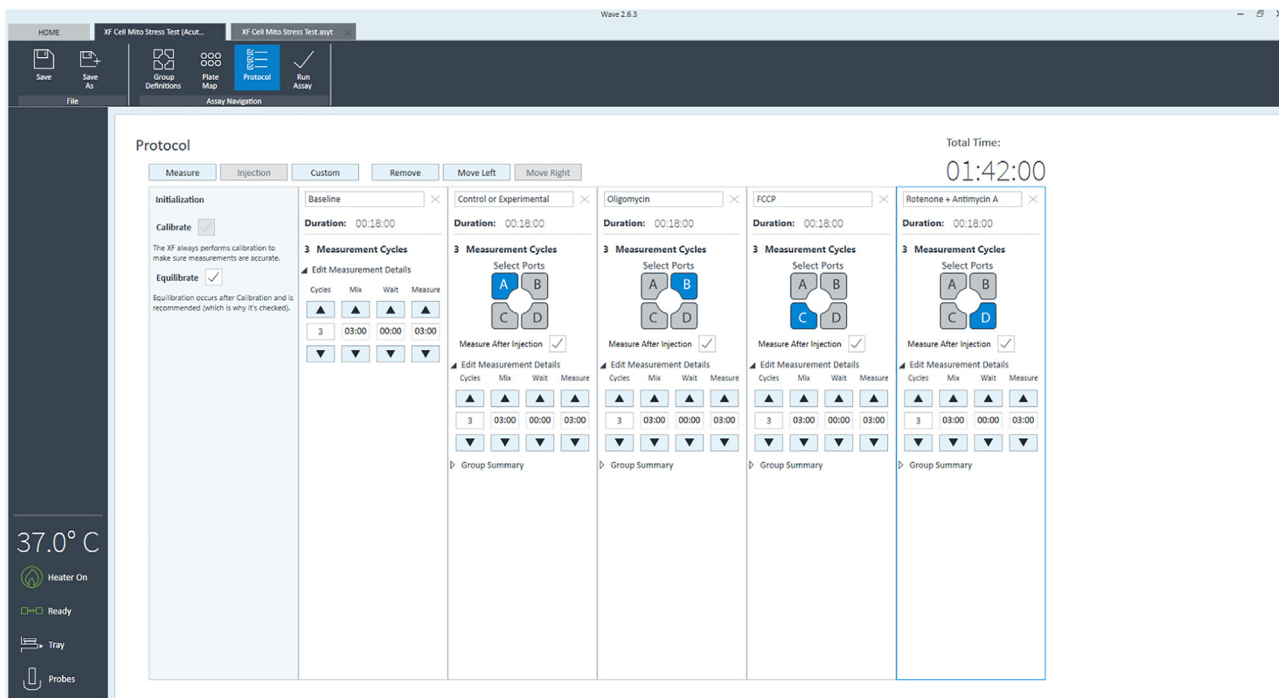
**Figure 5. Settings for measurements and injection strategy in the Seahorse wave software**
Changes to the settings for measurements and the injection strategy can be altered using the 'Protocol' section of the Seahorse Wave software. Here, changes in the duration for the measurement cycles can be altered (See Table 3). Any alterations to the protocol will adjust the approximate total run time calculated for the assay, once initiated (as indicated in the top righthand corner).

understand the importance of glycolysis influence on OXPHOS in GCB bioenergetics in diseased lupus-prone mice, GCB cells are treated with UK5099, via acute injection during the Seahorse assay. UK5099 is an inhibitor of the import of glycolysis-derived pyruvate into the mitochondria, where it can then be oxidized for use in the TCA cycle (Figure 2).[7] The expected outcome is a significant reduction in the MRC of GCB cells following UK5099 treatment, while no observable impact on Tfh cells (Figure 7A). The necessity of alternative mitochondrial fuel sources is also explored through the use of other fuel inhibitors of catabolic pathways in the XF Cell Mito Stress Test. These inhibitors include BPTES, an inhibitor of glutaminase, etomoxir, an inhibitor of mitochondrial fatty acid oxidation (FAO), or thioridazine, a specific inhibitor of peroxisomal FAO (Figure 2).[8–10] Inhibition of glutaminase, via BPTES treatment, results in a moderate, though significant, reduction in MRC in GCB cells while Tfh cells remain unaffected (Figure 7B). Neither etomoxir nor thioridazine treatment impacts the MRCs of either GCB or Tfh cells (Figures 7C and 7D). Together, these data highlight the reliance of autoreactive GCB cells on glycolysis-dependent OXPHOS, while also demonstrating the metabolic flexibility of Tfh cells in diseased lupus-prone *Yaa* DKO mice, information which was gleaned from the protocol described here for the Acute Injection Seahorse XF Cell Mito Stress Test.

LIMITATIONS

The Seahorse XF Cell Mito Stress Test is highly dependent on the ideal plating conditions for your cell type of interest and the appropriate concentrations of the inhibitors or drugs to illicit a cellular response without inducing toxicity. The Seahorse assay indirectly measures cellular bioenergetics through detection of medium acidification (ECAR) and oxygen consumption (OCR) in each well, which correspond to the rate of glycolysis and mitochondrial respiration, respectively. The acidification of the medium correlates with the cellular rates of glycolysis, which typically occurs under aerobic conditions. However, it is important to note that there are several cell types, including
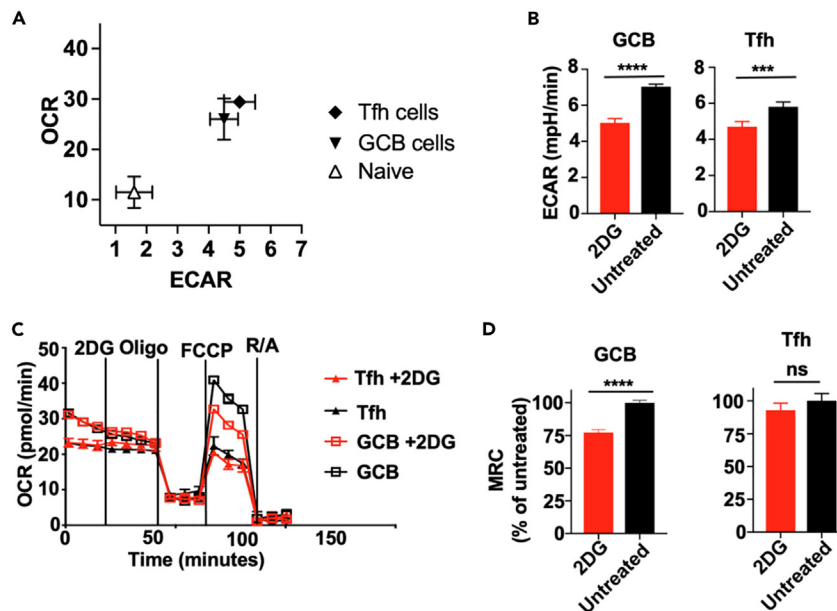
Figure 6. Lupus GCB cells exhibit increased glycolytic reliance compared to Tfh cells in lupus-prone mice

Metabolic analysis of splenocytes from symptomatic *Yaa* DKO mice

(A) Oxygen consumption rate (OCR) versus extracellular acidification rate (ECAR) of GCB and Tfh cells from *Yaa* DKO lupus-prone mice and naïve cells from C57BL/6 mice.

(B) Effects of 2DG on ECAR of GCB and Tfh cells.

(C) Representative graph of OCR in 2DG treated and untreated GCB and Tfh cells.

(D) Maximal respiratory capacity (MRC) of GCB and Tfh cells, with and without 2DG exposure. The effect of 2DG on MRC was normalized to untreated cells after FCCP exposure. Results are representative of at least 3 independent samples or experiments. Error bars represent mean ± SEM; ns, not significant, ***, $p < 0.001$, ****, $p < 0.0001$ by unpaired two-tailed Student's *t*-test. Figure reprinted, with permission, from Wilson et al. (2023).[1]

numerous tumor cells, that can produce lactic acid, a by-product of glycolysis, under hypoxic conditions, also known as 'the Warburg Effect', and should be considered.[11,12] Moreover, alterations in ECAR and OCR are determined in response to the injection of various metabolic modulators. It is important to recognize that while a critical strength of this assay is the ability to determine the bioenergetics of disease-relevant B/T cell types, these are still population-based measurements as a minimum of 50,000 cells is needed per well. Thus, although the assay described here provides critical information for specific cell types, it does not inform on cell-to-cell variability within the same cell type. Because of this, it is extremely important that the strictest conditions are set to limit the amount of variability in this assay, through stringent cell sorting and through rigorously ensuring that the same number of cells are included well-to-well in this assay. Furthermore, given the minimum recommended cell number needed per well, Seahorse assays require that the target cell population of interest for bioenergetics assessment is of high enough frequency to obtain sufficient cell numbers from sorting. Importantly, we have optimized and adapted this protocol from the Seahorse XF Cell Mito Stress Test (Acute Injection) by Agilent to account for the low frequency of several primary immune populations in lupus-prone mice. Of note, while the protocol detailed here is demonstrated as a means of measuring the bioenergetics of select subsets of B/T cells from lupus-prone mice, this protocol can be easily, and with minimal modification, be tailored to almost any primary cell type.

TROUBLESHOOTING

Problem 1

There is a wide margin of error between biological replicates or between sample types in baseline measurements, thus hindering final conclusions obtained from data analysis (Step 3).
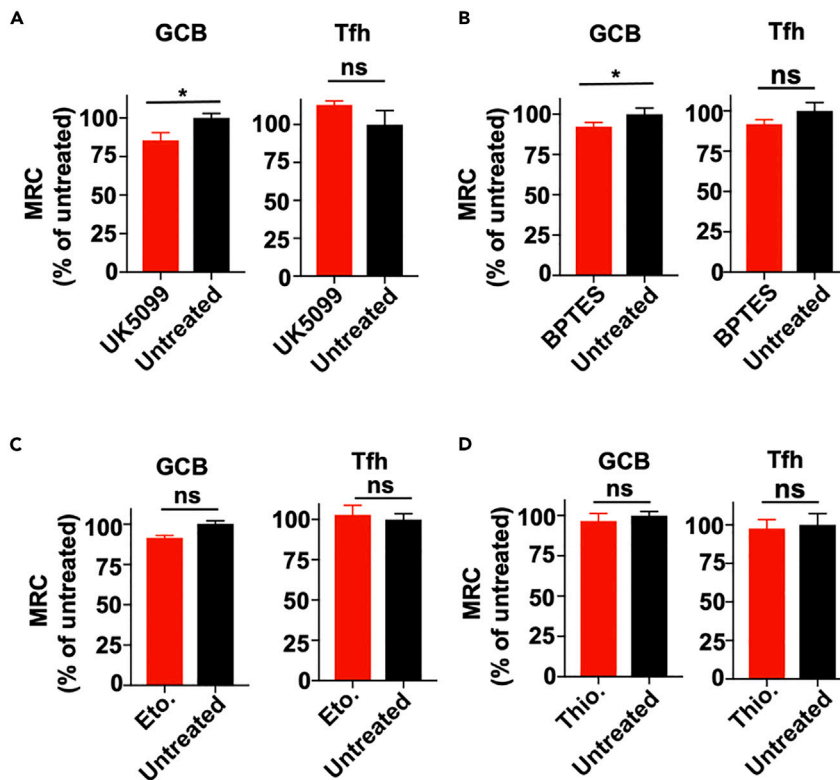
Figure 7. Lupus GCB cells are dependent on glycolysis and glutaminolysis for mitochondrial fueling, whereas lupus Tfh cells are metabolically flexible

Metabolic analysis of splenocytes from symptomatic *Yaa* DKO mice (A–D). Effects of catabolic pathway inhibitors (A) UK5099, (B) BPTES, (C) etomoxir (Eto.), and (D) thioridazine (Thio.), on the maximal respiratory capacity (MRC) (Percentage (%) of untreated) of GCB and Tfh cells. Results are representative of at least 3 independent experiments. Error bars represent mean $\pm$ SEM; ns, not significant, *, $p < 0.05$, by unpaired two-tailed Student's *t*-test. Figure reprinted, with permission, from Wilson et al. (2023).[1]

Potential solution

- There could be inconsistencies between the number of cells plated per well within each biological replicate. It is vital to use a hemacytometer or other cell counter device to accurately count and ensure equal numbers of cells per well. The number of cells should be determined through 2–3 replicate counts and averaged together to garner an accurate representation of the total cell number for each sample. Each count should exhibit a deviation of no more than 10% from the others.
- The absence of a thorough resuspension of the cells could also be a factor here; ensure that the cell pellet is thoroughly resuspended prior to quantification of the number of cells and prior to the loading of the cells into the Seahorse plate.
- The results could be normalized to either total protein or DNA content to account for well-to-well variation in cell number. If one well significantly deviates from the mean of the other wells, as determined by an outlier calculation, then this well can be excluded from analysis as a technical failure.

Problem 2

Wells of the Seahorse plate fail the pH or O_2 calibration prior to running the assay (Step 1 in "before you begin").

Potential solution

- It is important to ensure that the probe plate has been thoroughly hydrated in the XF Calibration Buffer for sufficient time prior to running the assay.
- The expiration date on the Seahorse assay plate should also be checked as plate expiration can influence the function of the probes. It is not recommended to run this Seahorse assay in an expired plate as that can negatively impact the quality of the resulting data collected.
- We recommend that any individual wells that fail calibration should be excluded from final calculations.

Problem 3

The baseline OCR and/or ECAR values in some technical replicate wells exhibit substantial deviation from the group mean (Step 3 in "before you begin", Step 3).

Potential solution

- One possibility is that these readings could be influenced by CO_2 in the plate. For the Seahorse XF Cell Mito Stress Test to run properly, both the cell plate and the probe injection plate must be incubated in the absence of CO_2 supplementation. The presence of CO_2 can contribute additional acidification in the cell medium, ultimately resulting in the failure of the pH test in the quality screen prior to running the assay. If individual wells fail the calibration, it is important that any data collected from those wells are omitted from final calculations.
- Deviations in the data within biological replicates could also be attributed to the number of cells plated in the assay. Check the number of cells added as well as the calculations used to determine the number of cells for potential errors. If too few cells are plated, the pH and O_2 concentrations in the cells may be below the detection limit, preventing accurate or sufficient baseline quantitation. Similarly, if the baseline OCR or ECAR levels are higher than expected, this could be attributed to the seeding of too many cells in the plate. The recommended range for total number of B/T cells per well is 50,000–200,000 per well. Dependent on the cell type and the bioenergetics of the cells in question, the number of cells seeded per well may need to be altered to ensure that the baseline OCR and ECAR values are within the appropriate range.
- Aberrant readings in single wells could also be the result of cells lifting from the bottom of the plate.

Problem 4

Failed, insufficient, or inconsistent response to injection of drugs/inhibitors as anticipated in assay steps (Steps 3, 4, "preparation of metabolic modulators and other reagents").

Potential solution

- If certain wells do not respond, check the drug injection plate to ensure full injection of each drug, ensure that guides were used for drug loading are placed in the right order, and that the corresponding cell plate was loaded into the Seahorse Analyzer in the proper orientation. Prior to running the assay, it is recommended to confirm in the injection plate that every drug was properly loaded. Post-run, you should ensure that each drug was injected into the plate of cells. Any volume of an injection component remaining in the injection plate is indicative of an improper injection for that well; the wells flagged for improper injection should be excluded from final calculations.
- This could be attributed to problems associated with the number of cells in the plate; if too many cells are loaded per well, the responsiveness to the metabolic modulators used in this assay may be insufficient.
- Dependent on the cell type in question, a higher final concentration of the inhibitor or drug may be necessary to achieve the desired response to injection.

- The expiration date for the inhibitors or drugs in questions should be checked to determine if they are within the recommended time of usage as well as if they have been consistently stored at the appropriate temperature. Of important note, multiple freeze thaw cycles are not recommended for inhibitors or drugs used in this protocol.

Problem 5
Error message due to an expired Seahorse plate or cartridge (Step 1 "before you begin").

Potential solution

- Every Seahorse plate ordered arrives labeled with an expiration date. If expired plates are used, the results may be negatively affected, ultimately impacting the final data. It is always recommended to use Seahorse plates prior to the expiration date listed for optimal results.

Problem 6
Inconsistent measurements over time during the assay (Step 3).

Potential solution

- If inconsistent readings are determined following the assay, it is important to check to see if the cells plated are localized to the bottom of the cell culture plate. If cells have lifted or are not properly seated in the cell culture plate this can significantly alter the accuracy of the measurements during the Seahorse assay. In this instance, the incubation time of the poly-D-lysine coating prior to addition of the cells to the plate could be increased to allow for sufficient cell adhesion to the plate.
- It is possible that some of the cells did not adhere to the cell culture plate properly and lifted during the addition of the Seahorse XF Assay medium. To remedy this, the cell culture plate could be additionally centrifuged following the supplementation of the Seahorse XF Assay medium prior to the final incubation of the cell culture plate in the non-CO_2 incubator.

RESOURCE AVAILABILITY

Lead contact
Additional information, requests for resources, or reagents, should be directed to, and will be fulfilled, by the lead contact, Chih-Hao Chang (lucas.chang@jax.org).

Technical contact
Colleen L. Mayberry (colleen.mayberry@jax.org), or John J. Wilson (john.wilson@jax.org).

Materials availability
This study described here did not generate unique plasmids, reagents, or cell lines.

Data and code availability
This study did not generate new datasets or code.

AUTHOR CONTRIBUTIONS

Methodology and investigation, C.L.M., J.J.W., and C.-H.C.; writing – original draft, C.L.M. and B.S.; writing – review and editing, C.L.M., J.J.W., B.S., and C.-H.C.; funding acquisition, C.L.M. and C.-H.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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