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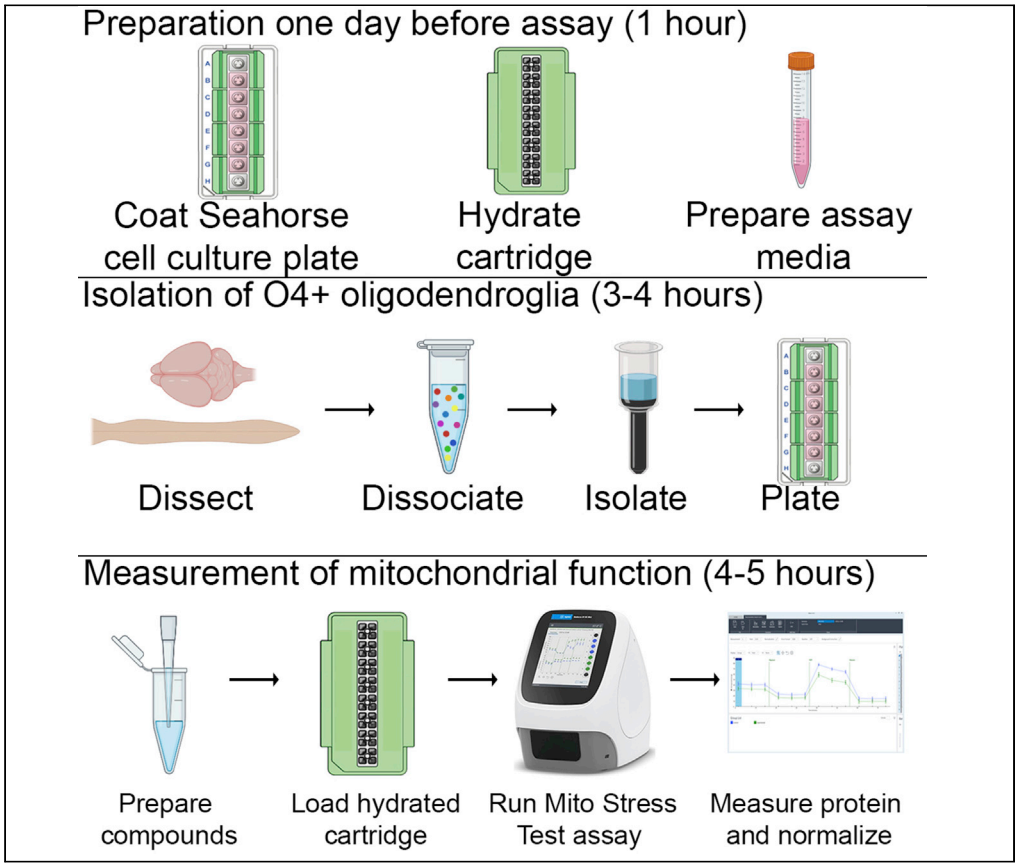
Protocol

Live-cell metabolic analysis of oligodendroglia isolated from postnatal mouse brain and spinal cord



This protocol describes isolation and live-cell metabolic analysis of O4+ oligodendroglia from brain and spinal cord of postnatal mice. We have optimized existing protocols for O4+ isolation from neonatal brain and expanded the protocol to include isolation of highly viable oligodendroglia from spinal cords of postnatal mice up to 18 days of age. Isolated oligodendroglia can be used in multiple downstream analyses, and here we describe an optimized real-time metabolic assay using Agilent Seahorse Analyzer to measure mitochondrial respiration.

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

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Highlights

Isolation of highly viable oligodendroglia from postnatal mouse spinal cord and brain

High-efficiency isolation of O4+ oligodendroglia from mice of up to 18 days of age

Measurement of real-time cellular bioenergetics of isolated mouse oligodendroglia

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Live-cell metabolic analysis of oligodendroglia isolated from postnatal mouse brain and spinal cord

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SUMMARY

This protocol describes isolation and live-cell metabolic analysis of O4+ oligodendroglia from brain and spinal cord of postnatal mice. We have optimized existing protocols for O4+ isolation from neonatal brain and expanded the protocol to include isolation of highly viable oligodendroglia from spinal cords of postnatal mice up to 18 days of age. Isolated oligodendroglia can be used in multiple downstream analyses, and here we describe an optimized real-time metabolic assay using Agilent Seahorse Analyzer to measure mitochondrial respiration.

For complete details on the use and execution of this protocol, please refer to Khandker et al. (2022).

BEFORE YOU BEGIN

Isolation of mouse oligodendroglia allows us to study cellular functions through downstream *in vitro* and molecular assays; using genetically modified mice allows for investigation of the genes regulating myelin development and function. In this protocol we isolate oligodendrolia during the dynamic process of developmental myelination, and we do so from both the brain and spinal cord. Downstream metabolic analyses are particularly important since myelination is an energetically demanding process (Harris and Attwell, 2012; Rinholm et al., 2011). Agilent Seahorse analyzers measure cellular bioenergetics in real-time and protocols are established for measuring mitochondrial respiration and glycolysis in other cell types (Gotoh et al., 2021; Gu et al., 2021; Qing et al., 2021). We have established a protocol to measure metabolic function in primary mouse oligodendroglia using Seahorse Analyzers.

> ***Note:*** The protocol below describes the specific steps for isolation of O4+ oligodendroglia from mice at Postnatal day 10 (P10). We have also used this protocol at P6, P10, P14, and P18. Here we describe analyzing isolated cells by Agilent Seahorse Cell Mito Stress Test. We have also used the cells for other downstream analyses including single-cell RNA sequencing, protein extraction and western blotting, RNA extraction and RT-PCR, and cholesterol measurement (Khandker et al., 2022).

Institutional permissions

All animal protocols described here were conducted in accordance with the guidelines set forth by Rutgers University and are in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines and the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Before using this protocol, ensure that all animal studies are approved by IACUC or relevant local institution.

Prepare for O4+ cell isolation and Agilent Seahorse Cell Mito Stress Test

Timing: 1 h

The steps below are performed on the day before the experiment is performed.

1. Coat Seahorse Plate.
   a. Remove the foil seal from the Seahorse cell culture plate that you will be using.
   b. Add 50 µL Poly-D-Lysine (PDL)/well (20 mg/L PDL solution made in water).
   c. Add water to moats with multi-channel pipette set at 200 µL.
   d. Incubate at 20°C–22°C for 30 min.
   e. Store in original packaging at 4°C for 12–20 h.
2. Hydrate Seahorse Cartridge.
   a. Remove Sensor Cartridge from foil pack.
   b. Separate Plate and Cartridge and place Cartridge upside down on lab bench.
   c. Fill each well of plate with 200 µL of sterile water using multi-channel pipette.
   d. Fill moats with water at same pipette setting.
   e. Close cartridge over plate. Do this slowly to avoid creating bubbles.
   f. Incubate in non-CO^2 37°C incubator for 3–20 h. Make sure incubator is humidified. If doing same day hydration, use calibrant instead of water.
   g. On the day of the assay switch from water to pre-warmed calibrant 45–60 min before running assay.
3. Prepare Assay Media following recipe below.
4. Prepare BSA buffer following recipe below.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
FcR blocking reagent, mouse	Miltenyi	130-092-575; RRID: AB_2892833
Anti O4 microbeads	Miltenyi	130-094-543; RRID: AB_2847907
RIPA buffer	Thermo Scientific	89900
Halt Protease/Phosphatase Inhibitor Cocktail	Thermo Scientific	78440
L-glutamine	Gibco	25030081
Poly-D-Lysine	Sigma-Aldrich	P1024
Bovine serum albumin	Sigma-Aldrich	A7906
Seahorse base medium	Agilent	103193-100
Sodium pyruvate	Gibco	11360070
Glucose	Agilent	103577-100
EDTA disodium salt	Santa Cruz	sc-29092
PBS, pH 7.4	Thermo Fisher Scientific	10-010-023
Trypan blue solution	Thermo Fisher Scientific	15250061
Critical commercial assays		
Neural Dissociation Kit (P)	Miltenyi	130-092-628
RC DC Protein Assay Kit II	Bio-Rad	5000122
Seahorse XF Cell Mito Stress Test Kit	Agilent	103010-100
Seahorse XF Mini FluxPak	Agilent	103723-100
Experimental models: Organisms/strains		
Mouse: *mTOR$^{fl/fl}$/Cre−/−*, *mTOR$^{fl/fl}$/Cre+/−* (postnatal day 10, males and females)	Wahl et al., 2014	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Seahorse Wave Desktop Software	Agilent	https://www.agilent.com/en/products/cell-analysis/software-download-for-wave-desktop
Other		
Seahorse XF mini analyzer	Agilent	N/A
OctoMACS Starting Kit	Miltenyi	130-042-108
MS Columns	Miltenyi	130-042-201
70 μm filters	Miltenyi	130-095-823
30 μm filters	Miltenyi	130-041-407

MATERIALS AND EQUIPMENT

Equipment alternatives: The protocol here uses a Seahorse XF mini analyzer which can simultaneously analyze up to 8 cell culture wells. The protocol can be scaled up for up to 24 wells in a Seahorse XFe24 analyzer and up to 96 wells in a Seahorse XFe96 analyzer, both of which we have tested.

We have used manual dissociation for tissue dissociation in this protocol. Using a gentleMACS automatic dissociator with heaters is an alternative for brain dissociation. However, we have found that using an automatic dissociator does not yield a high number of viable spinal cord cells.

Assay Media		
Reagent	Final concentration	Amount
Sodium pyruvate (100 mM)	1 mM	100 μL
L-Glutamine (200 mM)	2 mM	100 μL
Glucose	10 mM	100 μL
Seahorse base medium	N/A	9.7 mL
Total	**N/A**	**10 mL**

Adjust pH to 7.4 using 1 N NaOH. Use small volumes (microliters) since very little NaOH is required to change the pH. Store at 4°C until assay is run the next day. Unused Assay Media can be stored for up to 2 weeks, but pH should be checked before reusing.

BSA Buffer		
Reagent	Final concentration	Amount
BSA	0.5%	2.5 g
EDTA (1 M, pH8)	2 mM	1 mL
PBS	N/A	to 500 mL
Total	**N/A**	**500 mL**

Adjust pH to 7.4. Store at 4°C until assay is run the next day. Unused BSA buffer can be stored for up to 2 weeks, but pH should be checked before reusing.

STEP-BY-STEP METHOD DETAILS

Dissociation of brain and spinal cord

Timing: 2–3 h

This section of the protocol describes dissociation of neonatal mouse brain and spinal cord tissues into single cells using Miltenyi's Neural Tissue Dissociation Kit (P). We have modified the manufacturer's protocol https://www.miltenyibiotec.com/upload/assets/IM0001320.PDF in two major ways. First, the original protocol is for brain tissues from mice ≤ 7 days of age, and we have optimized for

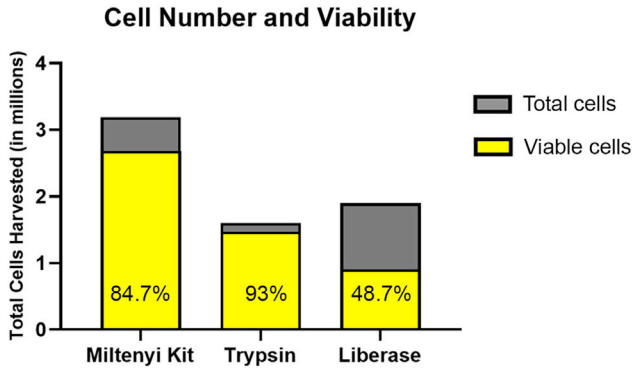


Figure 1. Optimization of enzymatic dissociation of neonatal spinal cords
One P10 spinal cord was dissociated using each condition.

up to 18 days of age. Secondly, we have expanded the protocol to include dissociation of spinal cord. We tested different dissociation protocols and found that the one detailed below gave the best cell yields (Figure 1).

1. Tissue harvest.
   a. Rapidly decapitate mouse. For mice P10 and older, administer chemical anesthetics prior to decapitation.
   b. Dissect brain.
      i. Place carcass dorsal side up.
      ii. Separate and cut away skin going from base of neck to nose.
      iii. Cut skull from brainstem to olfactory bulb. Remove skull by pulling away to the sides.
      iv. Scoop out brain and place in petri dish with cold PBS.
   c. Dissect spinal cord.
      i. Cut and peel back skin to access spinal column.
      ii. Dissect out spinal column by cutting through ribs and freeing entire length of the column.
      iii. Starting at the caudal (tail) end of the cord, pinch with blunt ended forceps with wide, serrated tips, and squeeze the cord out by progressively moving the forceps (2 pairs) one over the other until the entire cord comes out of the top of the spinal column.
      iv. Place in petri dish with cold PBS.
   d. When all dissections are complete, transfer tissues to empty petri dishes. Pool and work with 3 brains or 3 spinal cords at a time.
   e. Chop up tissues to a paste-like consistency using razor blade (Figure 2A).

   Note: To avoid small differences in developmental stage, we exclusively used littermates within experiments.

2. Dissociation using Miltenyi's Neural Tissue Dissociation Kit (P).
   a. Add 1,950 µL preheated Enzyme mix 1 (50 µL Enzyme P + 1,900 µL Buffer X) to 3 pooled P10 spinal cords. Double the volume for 3 pooled brain samples. Transfer to 15 mL conical tubes.
   b. Incubate 15 min at 37°C. Mix every 2–3 min by inverting tubes.
   c. Add 30 µL Enzyme mix 2 (20 µL Buffer Y + 10 µL Enzyme A) to spinal cord samples. Double the volume for brain samples.
   d. Mix by inverting tubes.
   e. Pipette up and down 10× with 5 mL pipette.
   f. Incubate 10 min at 37°C. Mix every 2–3 min by inverting tubes.
   g. Pipette up and down 10× with 1 mL tip.

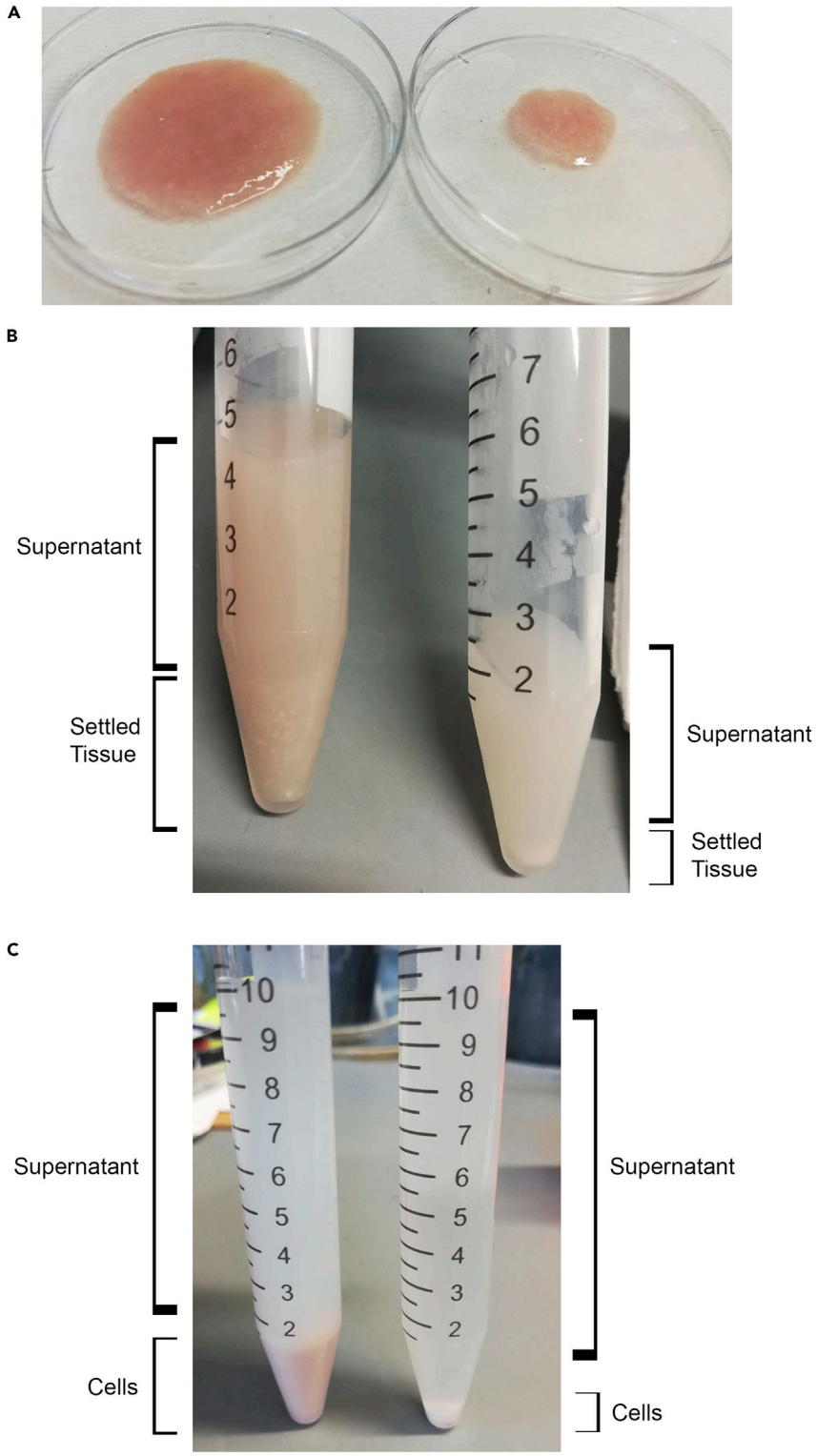


Figure 2. Dissociation of brain and spinal cord

(A) Tissues chopped to a paste-like consistency using razor blade. Left shows brain samples, right shows spinal cord samples.

(B) Tubes after tissues have settled on ice for 2 min. Left shows brain samples, right shows spinal cord samples.

(C) Tubes after centrifugation. Left shows brain samples, right shows spinal cord samples.

h. Place tubes on ice and let tissues settle for 2 min (Figure 2B). Transfer supernatant (with cells) to fresh 15 mL conical tube. While transferring, pass through pre-wet 70 μm filter.
i. Break up remaining tissue pellet by pipetting up and down 10× with 200 μL tip. Transfer (through filter) to conical tube with cells from step 2h.
j. Bring up to 10 mL with BSA buffer.
k. Count cells with Trypan blue exclusion. Troubleshooting 1.
l. Spin down cells at 400 × *g* for 5 min and aspirate supernatant to remove enzyme (Figure 2C).
m. Proceed to O4+ cell isolation.

⚠ CRITICAL: Working quickly during dissection and dissociation will yield higher numbers of viable cells.

O4+ cell isolation

Timing: 1 h

This section of the protocol describes isolating cells expressing the O4 antigen. We begin with diverse cell types dissociated from mouse brains and spinal cords and end with an enriched population of oligodendroglia that express the O4 cell surface sulfatide. O4 is expressed on late stage oligodendrocyte precursor cells (OPCs) after the early markers platelet-derived growth factor receptor alpha (PDGFRα) and neural/glial antigen 2 (NG2) are mostly downregulated and continues to be present on pre-myelinating as well as actively myelinating oligodendrocytes (Ornelas et al., 2020; Sommer and Schachner, 1981; Warrington and Pfeiffer, 1992). Isolation efficiency using this protocol is 80%–90% (Khandker et al., 2022). We have modified the manufacturer's protocol https://www.miltenyibiotec.com/upload/assets/IM0002007.PDF for isolation from spinal cords.

3. Add 80 μL of BSA buffer/ 5 × 10^6 cells. If a sample has more than 5 × 10^6 cells, split into multiple 1.5 mL tubes. This prevents clogging of columns.
4. Add 10 μL mouse FcR Blocking Reagent to each sample/tube.
5. Mix well by flicking. Do not vortex. Incubate for 10 min at 4°C.
6. Add 10 μL of Anti-O4 microbeads to each tube.
7. Mix well by flicking. Do not vortex. Incubate for 15 min at 4°C.
8. Add 1 mL of BSA buffer to each tube.
9. Centrifuge at 400 × *g* for 5 min at 4°C. Aspirate supernatant completely.
10. Resuspend cells in 1 mL BSA buffer.
11. During above centrifugation steps, prepare columns.
    a. Separate plungers from MS columns. Put aside plungers for future steps.
    b. Place columns in the magnetic field of OctoMACS Separator. Verify that column is facing the correct way. If incorrect, the column will not be vertically straight.
    c. Prepare columns by rinsing with 500 μL BSA buffer.
    d. Add a pre-wet 30 μm filter to each column.
12. Apply cell suspension onto column filter. Allow entire cell suspension to enter column before proceeding to wash steps. Discard flow-through containing unlabeled cells.
13. Wash column 3× with 500 μL BSA buffer. Allow entire wash volume to enter column before proceeding to next wash. Discard unlabeled cells that pass through. Troubleshooting 2.
14. Remove each column from separator and place on collection tube.
15. Pipette 1 mL BSA buffer into the column.
16. Immediately flush out O4+ enriched cells by firmly pushing plunger into the column. Keep cells onice.
17. Count cells with trypan blue exclusion and proceed to downstream analyses.

Note: If greater than 80%–90% isolation efficiency is desired, steps 4–18 may be repeated. This will achieve higher purity of O4+ cells but will yield lower cell numbers.

Note: Isolation efficiency can be determined by flow cytometry to measure the percentage of O4+ cells (Khandker et al., 2022).

Note: If isolated cells are used for different downstream analysis, verify that presence of BSA and EDTA will not interfere. When using O4+ cells for single-cell RNA sequencing, we centrifuged isolated O4+ cells and resuspended in 0.04% BSA in PBS, with no EDTA. When extracting RNA or protein, we resuspended in appropriate lysis buffer. When performing the Agilent Seahorse Cell Mito Stress Test, we resuspended in Assay buffer as described below.

Agilent Seahorse Cell Mito Stress Test

Timing: 4–5 h

In this step, we will plate the O4+ oligodendroglia isolated above and perform live cell metabolic measurements. The Seahorse Mitochondrial Stress Test evaluates mitochondrial function by measuring oxygen consumption rate (OCR), an indicator of mitochondrial respiration. During the assay, modulators of respiration are added to cells through injection ports, which provides a detailed understanding of cell energetics. Extracellular acidification rate (ECAR), an indicator of glycolysis, is also measured. However, for a complete analysis of energy production through glycolysis, the Seahorse Glycolytic Rate Assay Kit should be used.

We have optimized the manufacturer's protocol https://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Mito_Stress_Test_Kit_User_Guide.pdf specifically for isolated primary oligodendroglia. This included optimization of cell number (Figures 4A and 4B) and FCCP concentration (Figure 4C).

18. Prepare cartridge by removing water from wells and adding pre-warmed calibrant. Do this 45–60 min before running assay.
19. Prepare Seahorse plate.
    a. Remove PDL from plate.
    b. Wash 1× with sterile water.
    c. Remove water completely and allow to dry before plating cells.
20. Plate cells.
    a. Isolate and count O4+ cells as described above.
    b. Centrifuge cells at 400 × *g* for 5 min at 4°C. Aspirate supernatant completely.
    c. Resuspend in 540 μL pre-warmed assay media such that there are 40,000 cells/180 μL.
    d. Add 180 μL of assay medium only (no cells) to wells A and H. These are background correction wells.
    e. Add 180 μL of cell suspension (40,000 cells) to remaining wells B-G.
    f. Centrifuge plate for 1 min at 400 × *g*. This will ensure that cells quickly settle to the bottom of the wells.
    g. Place cell plate in non-CO^2 37°C incubator while completing steps 3–7 below.
21. Prepare stock compounds.
    a. Oligomycin: Add 252 μL assay medium to make 50 μM solution.
    b. FCCP: Add 288 μL assay medium to make 50 μM solution.
    c. Rotenone / Antimycin A: Add 216 μL assay medium to make 25 μM solution.
    d. Pipette each compound up and down 20× to solubilize.
22. Prepare working solutions of compounds for loading in sensor cartridge by following Table 1. For example, add 60 μL of 50 μM oligomycin stock solution to 240 μL of assay media. After addition to cell plate, this will result in a final concentration of 1 μM.

Table 1. Preparation of working stocks of compounds

	Final concentration (μM)	Volume of stock solution	Volume of media	Volume added to port
Oligomycin	1	60 μL	240 μL	20 μL
FCCP	2	120 μL	180 μL	22 μL
Rotenone/Antimycin A	0.5	60 μL	240 μL	25 μL

23. Load Sensor Cartridge with compounds by following Table 1. When dispensing compounds into ports, orient the pipette tips at a very slight angle and dispense towards the side wall or corner of the port. Do not push tips completely into the port holes.
    a. Port A: Oligomycin.
    b. Port B: FCCP.
    c. Port C: Rotenone/antimycin A.
    d. Port D: 20 μL of assay medium or PBS. This port can also be used to test the metabolic effects of an additional drug. If the port is not used for an experimental compound, it cannot be left empty and must be filled with media or buffer in order for all compounds to be dispensed.
24. On the Seahorse XF Mini Analyzer, select XF Cell Mito Stress Test on the Templates window. You can add information about each experimental group on the Plate map tab.
25. For the protocol described here we kept the default parameters for Cell Mito Stress Test (Figure 3). To verify, check that the parameters on the Protocol tab are as follows:
    a. For each stage (Baseline, Oligomycin, FCCP, Rotenone/Antimycin A), there are 3 cycles, 3 mixes, 0 Wait, and 3 Measurements.
26. Start equilibration of Seahorse XF Mini Analyzer by touching "Start Run". Load the cartridge (hydrated and loaded with compounds) onto the tray when prompted. Ensure the cartridge fits properly, the lid is removed from the cartridge, and the direction of the cartridge matches the image on the screen. Analyzer will complete equilibration in 20 min, then pause until cell plate is loaded.

Note: We allow the Seahorse XF Mini Analyzer to always remain powered on. If not, switch on Analyzer before beginning animal dissections. Before beginning assay, ensure that the temperature reading is at 37°C.

27. Run the Seahorse XF Cell Mito Stress Test.
    a. Touch "Continue" when you are ready to load your plate.
    b. The XF Mini Analyzer tray will open and present the utility plate.
    c. Remove the utility plate and load the cell plate. Ensure the lid is removed from the cell plate before loading onto the Analyzer tray.
28. The assay takes about 1.5 h to run. Once the assay is complete, touch Eject to retrieve the sensor cartridge and cell plate. The cell plate can then be used for normalization. This step is critical when working with experimental oligodendroglia which may have different cell viabilities. Troubleshooting 3, 4, 5.
    a. Gently remove media without disturbing cells.
    b. Add 50 μL of RIPA lysis buffer with Halt protease/phosphatase inhibitor cocktail into each well.
    c. Pipette up and down 5× to lyse and mix.
    d. Measure total protein/well using standard protein measurement protocols.
    e. Use total protein measurement to normalize results in Wave as described below.

Pause point: Protein measurement in step 9 can be performed immediately, or the plate can be stored at −80°C after addition of RIPA lysis buffer and analyzed on a later day.

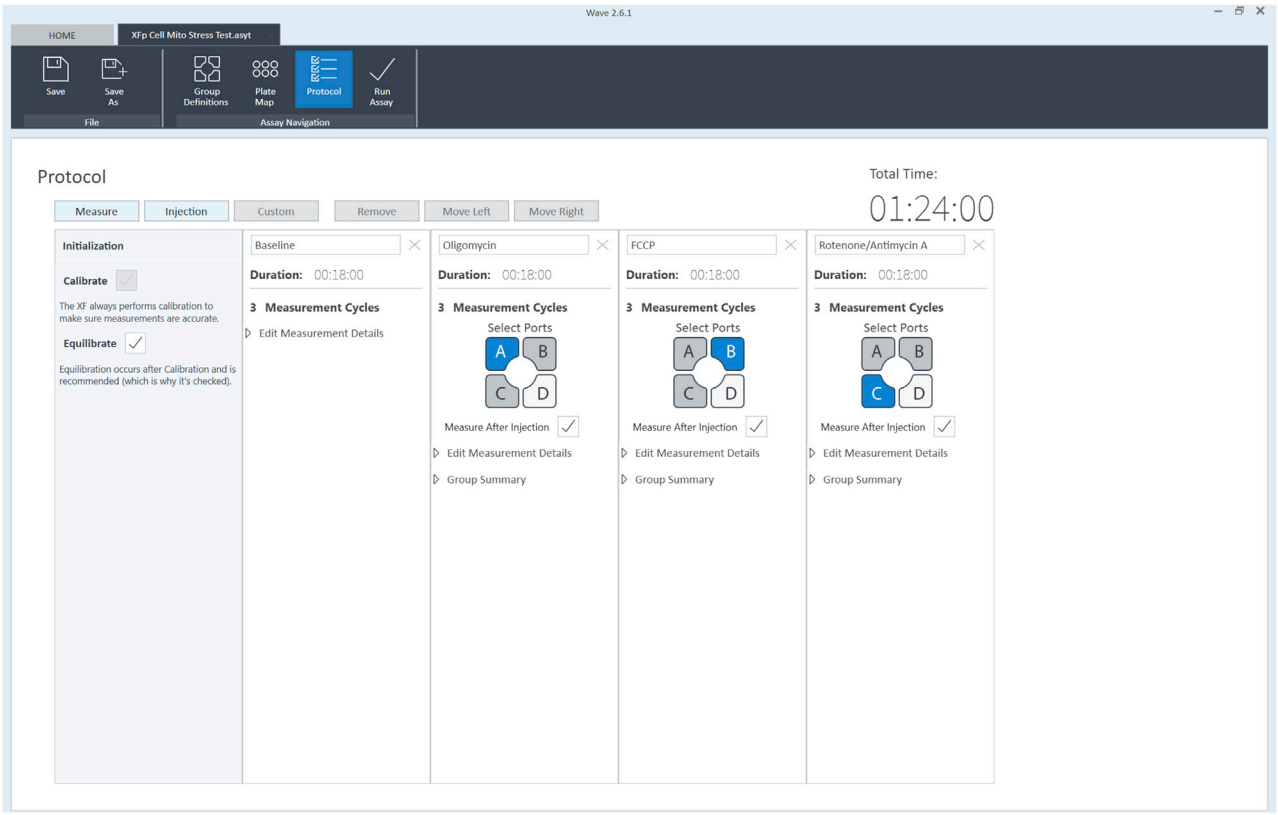


Figure 3. Parameters for Cell Mito Stress Test
For each stage (Baseline, Oligomycin, FCCP, Rotenone/Antimycin A), there are 3 cycles, 3 mixes, 0 Wait, and 3 Measurements.

EXPECTED OUTCOMES

After isolation of O4+ cells from postnatal mouse brain or spinal cord using the protocol described here, samples are expected to be 80%–90% O4+ as determined by flow cytometry (Khandker et al., 2022).

For robust results, raw Basal OCR measurements in the Seahorse Cell Mito Stress Test should fall between 100 and 200 pmol/min. OCR modulation after injection of compounds should be as shown in Figure 4C with a decrease after Oligomycin, increase after FCCP, and decrease after Rotenone/Antimycin A.

QUANTIFICATION AND STATISTICAL ANALYSIS

Perform data analysis using Seahorse Wave desktop software. The software is available for free download from Agilent {https://www.agilent.com/en/products/cell-analysis/software-download-for-wave-desktop}. We have followed the manufacturer's instructions for managing and analyzing data, with the addition of normalization to total protein. https://www.agilent.com/cs/library/usermanuals/public/S7894-10000_Rev_C_Wave_2_6_User_Guide.pdf.

1. Export results from Seahorse XF Mini Analyzer as a Wave file.
2. Copy onto personal computer and save file.
3. Open data file in Wave.
4. Experimental details such as injection strategies, treatments, media, and cell type can be added or modified by clicking "Modify."
5. Normalize results to total protein.

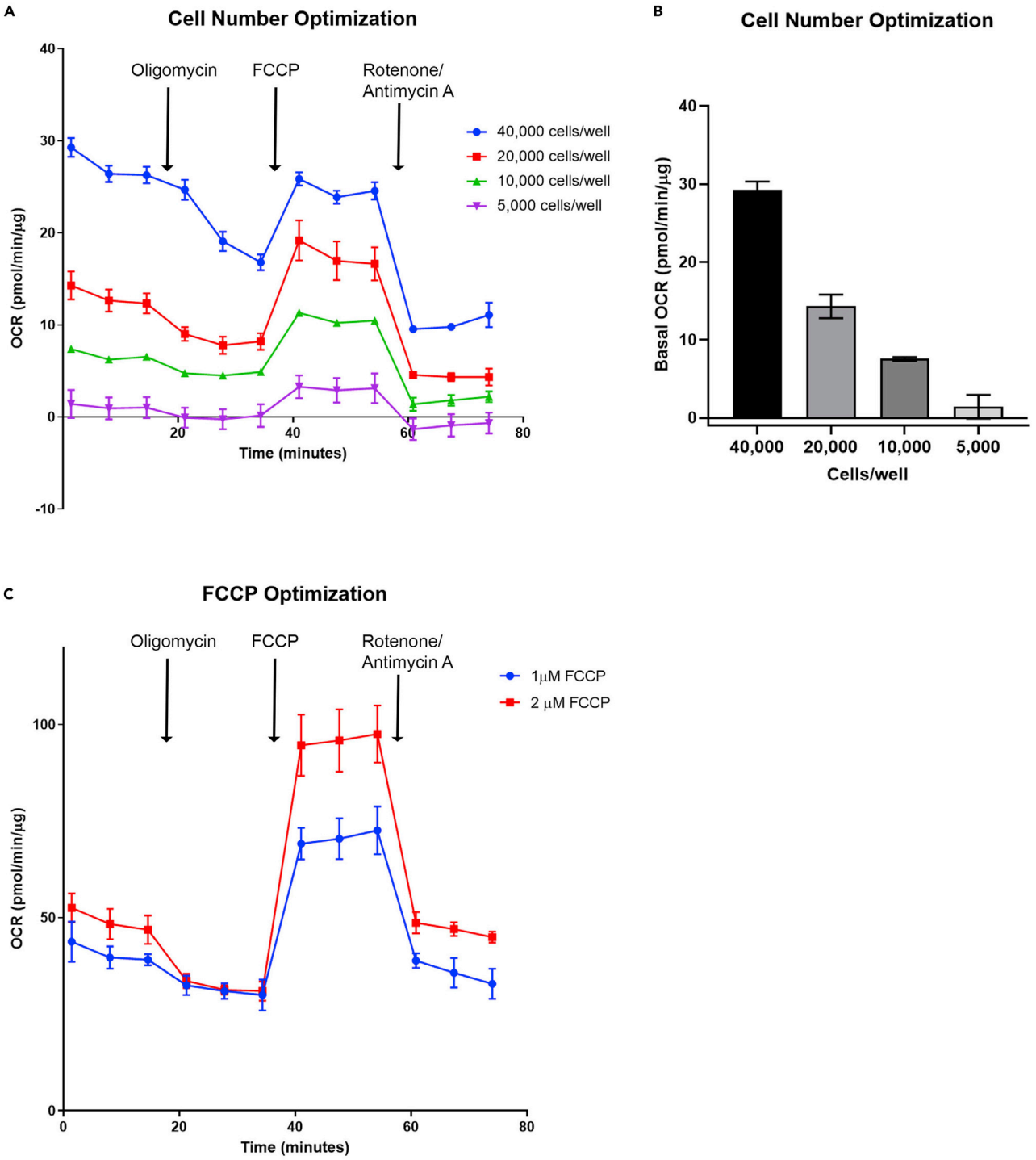


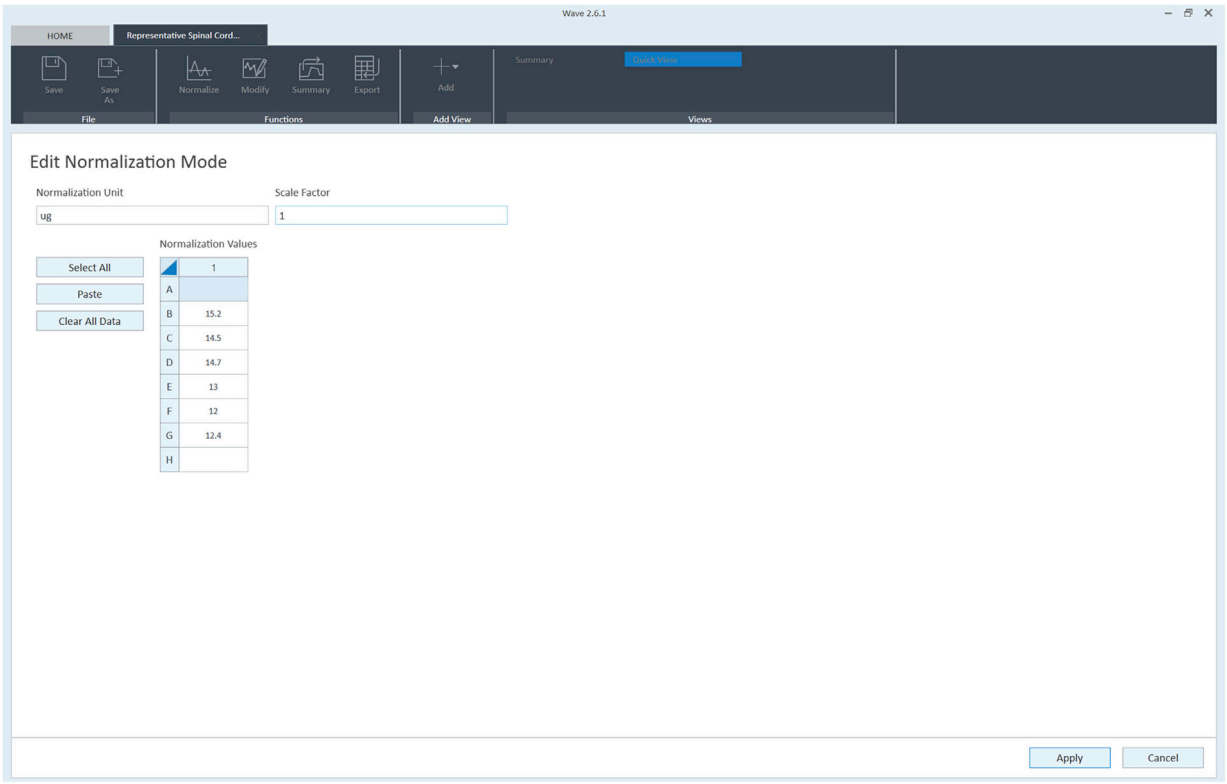
Figure 4. Optimization of O4+ cell number and FCCP concentration for Seahorse Mito Stress Test

(A) Live measurements of Basal OCR followed by OCR measurements after acute injections of Oligomycin, FCCP, and Rotenone/Antimycin A. Cells were plated at 40,000, 20,000, 10,000 and 5,000 cells/well Values expressed as mean ± SEM.

(B) Basal OCR measurements when O4+ cells are plated at different numbers. Values expressed as mean ± SEM.

(C) Optimization of FCCP concentration for maximal respiration. 40,000 cells/well were used. All values were normalized based on total protein content measured after completion. Values expressed as mean ± SEM.

A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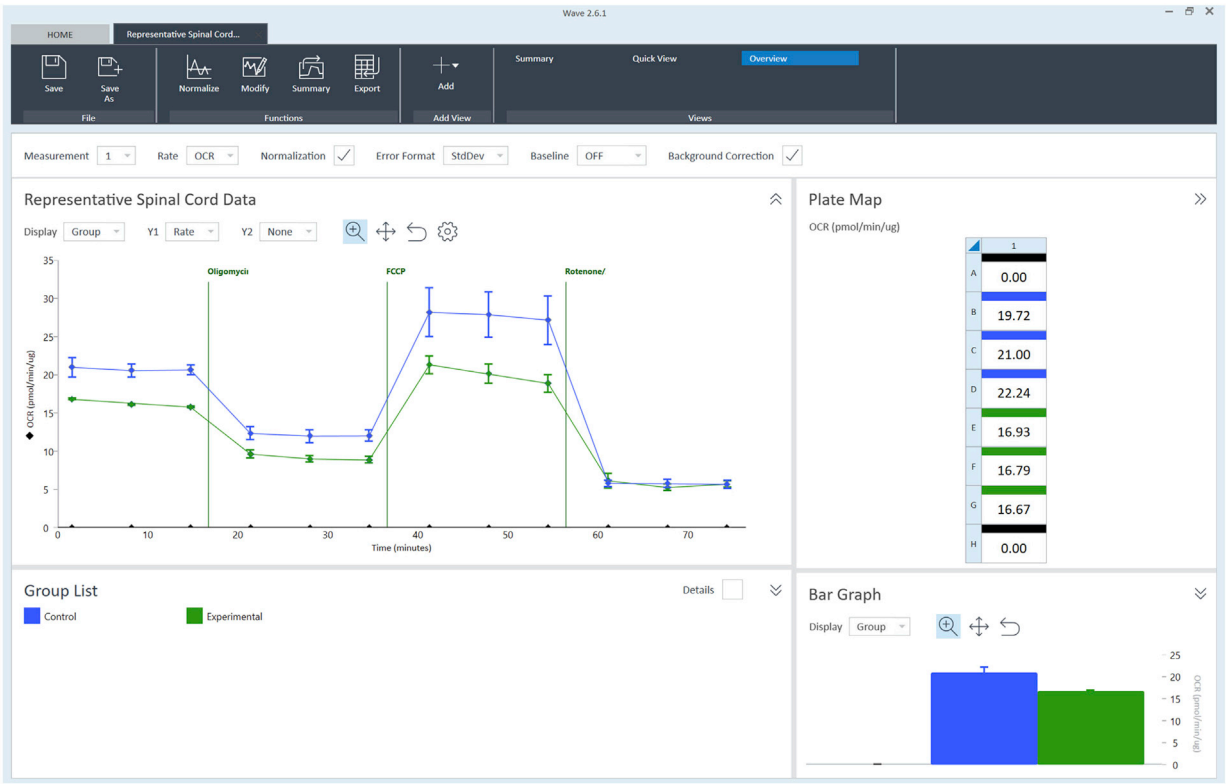
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Add View
Views
Measurement 1
Rate OCR
Normalization
Error Format StdDev
Baseline OFF
Background Correction
Representative Spinal Cord Data
Display Group
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Y2 None
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FCCP
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C 21.00
D 22.24
E 16.93
F 16.79
G 16.67
H 0.00
Group List
Details
Control
Experimental
Bar Graph
Display Group
OCR (pmol/min/ug)

Figure 5. Representative results analyzed in Wave

(A) Normalization screen with total protein/well added to normalize OCR data.

(B) Overview screen with sample results. In this experiment, we had three wells with Control spinal cord O4+ cells and three wells with Experimental spinal cord O4+ cells. The Experimental animals with the *Mtor* gene deleted specifically in oligodendrocytes (Wahl et al., 2014) had lower mitochondrial respiration (Khandker et al., 2022). The top right panel shows OCR measurement 1 from each well. The bottom right panel shows a bar graph of OCR measurement 1. The Measurement dropdown menu at the top left can be changed to other measurements, and the right-side panels will update accordingly.

a. Click "Normalize" button and add normalization values as shown in Figure 5A.
b. The values should be total protein (μg) in each well as measured after the assay is complete.
c. Enter "μg" for Normalization Unit.
d. Enter "1" for Scale factor.
e. Click Apply button.
f. Click "Overview" button and verify that the Normalization box is checked. Sample results shown in Figure 5B.

LIMITATIONS

The tissue dissociation method described here successfully yields high numbers of viable oligodendroglia from mouse brain and spinal cord up to 18 days of age. We have isolated O4+ cells from these dissociated samples with high isolation efficiency and in sufficient numbers for many different downstream analyses. However, in our experience, it is difficult to isolate other OPC stages such as PDGFRα+ or NG2+ cells when the mice are older than 10 days. It is possible that these antigens are not well-preserved using this enzymatic dissociation method. There are also fewer early stage OPCs later in development.

The Agilent Seahorse Mitochondrial Stress Test protocol described here evaluates metabolic function in O4+ oligodendroglia by measuring oxygen consumption rate (OCR), an indicator of mitochondrial respiration. When normalized to protein as described above, the results are robust with low variability. However, relatively high cell numbers are required to achieve this robustness. While harvesting and isolating sufficient O4+ cells from brain tissue is not difficult, it is challenging to get enough cells from single spinal cords, especially from mice as young as 6 days of age. To overcome this challenge, we have pooled tissues and cells from multiple animals within a litter. This means that we required larger numbers of littermates to begin the experiment. This can be a limiting factor when different genotypes are necessary within a litter.

TROUBLESHOOTING

Problem 1

Low cell number and/or viability after dissociation (step 2k).

Potential solution

We have harvested a variable number of cells from mouse spinal cords at postnatal day 10. When pooling 3 spinal cords, we harvested anywhere between 20×10^6 and 65×10^6 cells, with viability between 80% and 95%. If results are lower, complete the dissection and dissociation steps as quickly as possible. It may help to have two people working side by side to achieve this.

Problem 2

MS columns becoming clogged during O4+ cell isolation (step 13). The washes should take a few seconds each. If it takes longer than a minute, the column is clogged.

Potential solution

The solution for clogged columns is to have fewer cells/column. If the samples are very precious and you want to retrieve cells that are already in the column, place your thumb on top of the column to make a seal. Push down to force fluid through the column. Skip further washes and proceed to elution. This will result in low isolation efficiency but will allow retrieval of the cells in the column.

Problem 3
No OCR signal detected in Seahorse Mitochondrial Stress Test (step 28).

Potential solution
The cartridge is probably not hydrated. Repeat with a hydrated cartridge.

Problem 4
Basal OCR measurements are not within or close to the range of 100–200 pmol/min (step 28).

Potential solution
Adjust cell number plated. If OCR is below the range, increase cell number. If OCR is above the range, decrease cell number.

Problem 5
After injection of one of the compounds, one measurement is very different from the others. For example, Measurement 4 (after injection of Oligomycin) measures two wells with a decrease in OCR, but one that stays at basal levels. This can occur if one of the wells did not get compound added. After assay is complete, look at the cartridge. Ports will be empty if all compounds were added successfully. If one was missed, you can visualize compound remaining in the port (step 28).

Potential solution
If you can confirm that the cause of the variability is compound not being added, you can remove the well from your analysis in Wave by clicking on the well in the Plate Map.

RESOURCE AVAILABILITY

Lead contact
Requests for further information should be directed to and will be fulfilled by the lead contact, Teresa L. Wood (terri.wood@rutgers.edu).

Materials availability
This study did not generate new unique reagents.

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

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AUTHOR CONTRIBUTIONS
L.K. optimized the protocols, performed the experiments, and wrote the manuscript. T.L.W. reviewed and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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