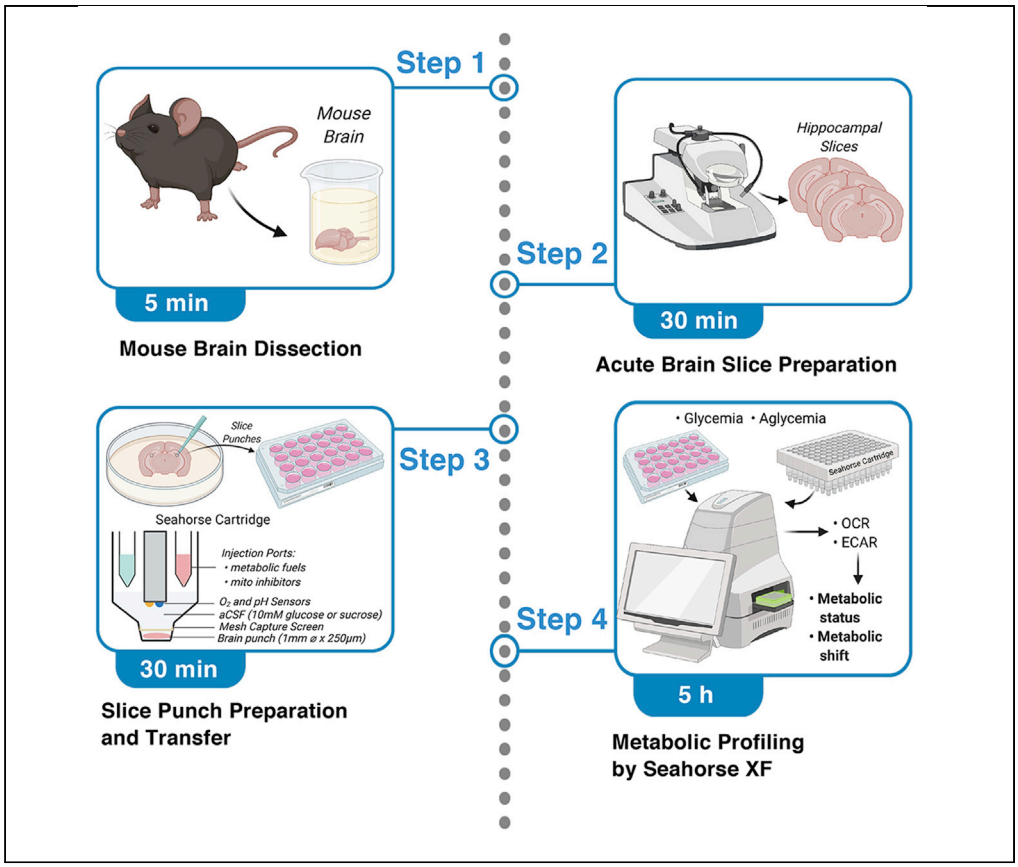
STAR Protocols

CellPress
OPEN ACCESS

Protocol

# Characterizing brain metabolic function ex vivo with acute mouse slice punches




Mitochondrial dysfunction and metabolic reprogramming are implicated in a variety of neurological disorders. Here, we present a protocol that enables complex profiling of brain metabolic function using acute mouse brain slices *ex vivo*. Utilizing differential metabolic conditions, substrates, and inhibitors, this protocol can be broadly applied to determine metabolic shift or reprogramming upon genetic manipulations, pathological insults, or therapeutic interventions and could thus further the understanding of the dynamic role of energy metabolism in brain physiological function and diseases.

Guoyuan Qi, Yashi Mi, Fei Yin

feiyin@arizona.edu

## Highlights

Detailed protocol to determine complex brain metabolic function in mouse

Streamlined method to determine brain metabolic shift or reprogramming

Validated procedure to quantify brain aerobic glycolytic rate *ex vivo*

Qi et al., STAR Protocols *2*, 100559
June 18, 2021 
https://doi.org/10.1016/j.xpro.2021.100559

STAR Protocols

CellPress
OPEN ACCESS

Protocol

# Characterizing brain metabolic function ex vivo with acute mouse slice punches

Guoyuan Qi,[1,4,5] Yashi Mi,[1,4] and Fei Yin[1,2,3,6,*]

[1]Center for Innovation in Brain Science, University of Arizona Health Sciences, University of Arizona, 1230 N Cherry Ave, Tucson, AZ 85724, USA

[2]Department of Pharmacology, College of Medicine Tucson, University of Arizona, Tucson, AZ 85724, USA

[3]Graduate Interdisciplinary Program in Neuroscience, University of Arizona, Tucson, AZ 85721, USA

[4]These authors contributed equally

[5]Technical contact

[6]Lead contact

*Correspondence: feiyin@arizona.edu
https://doi.org/10.1016/j.xpro.2021.100559

## SUMMARY

**Mitochondrial dysfunction and metabolic reprogramming are implicated in a variety of neurological disorders. Here, we present a protocol that enables complex profiling of brain metabolic function using acute mouse brain slices *ex vivo*. Utilizing differential metabolic conditions, substrates, and inhibitors, this protocol can be broadly applied to determine metabolic shift or reprogramming upon genetic manipulations, pathological insults, or therapeutic interventions and could thus further the understanding of the dynamic role of energy metabolism in brain physiological function and diseases.**

**For complete details on the use and execution of this protocol, please refer to Qi et al. (2021).**

## BEFORE YOU BEGIN

This protocol below describes the specific steps to characterize brain metabolic function ex vivo with acute mouse hippocampal slice punches. Depending on the research focus, this protocol can also be applied to other brain regions such as cortex and white matter tracts, or other rodent species (processing of rat brains was described previously (Fried et al., 2014; Underwood et al., 2020)).

### Day prior to assay

1. Hydrate Agilent Seahorse XFe24 Sensor Cartridge with water

**Timing: 10 min**

a. Prepare a 24-well Seahorse XFe24 sensor cartridge with utility plate and hydro booster from the Seahorse XFe24 Islet Capture FluxPak.

b. Add 1 mL sterile water to each well of the utility plate, then put the hydro booster on top of the utility plate and the sensor cartridge through the openings on the hydro booster. Make sure the sensors are submerged in water.

c. Place the assembled sensor cartridge and utility plate in a non-$CO_2$ 37°C incubator 12–16 h.

d. Separately, add 40 mL of Seahorse XF Calibrant (included in the FluxPak) into a 50 mL conical tube and place it a non-$CO_2$ 37°C incubator 12–16 h.

**Table 1. Stock solutions of mitochondrial inhibitors**

| Reagent | Stock concentration |
|---|---|
| Oligomycin A | 20 mg/mL in 0.2 mL DMSO |
| FCCP | 10 mM in 0.2 mL DMSO |
| Antimycin A | 10 mM in 0.2 mL DMSO |

*Note:* To prevent evaporation of the water, use a properly humidified incubator or place the assembled sensor cartridge and plate back to its packaging to reduce evaporation.

2. Fasting mice 14–16 h (optional)

**Timing: 5 min**

Transfer the mice to be assessed to new cages with free access to water but not food ~14–16 h before euthanasia.

*Note:* This is an optional step aiming to minimize the impact of variable blood glucose and fatty acid levels across individual mice on brain metabolism of exogenous fuels, especially under aglycemic conditions. If glucose availability to brain is not a concern, unfasted mice can also be used. Make sure an approval is obtained from the Institutional Animal Care and Use Committee prior to the fasting of animals.

3. Reagent and buffer preparation

**Timing: 1 h**

a. Prepare stock solutions of mitochondrial inhibitors listed in Table 1.

*Note:* Store at −20°C for up to 6 months and avoid repeated freeze and thaw cycles.

b. Prepare artificial cerebrospinal fluid (aCSF) without glucose or sucrose (Table 2).

*Note:* Store at 4°C for up to 4 weeks.

c. Agarose block preparation

Mix 3 g agarose powder with 100 mL aCSF in a conical flask, then microwave the mixture until agarose is completely dissolved. Pour the agarose solution into a 100 mm petri dish at 20°C–25°C for 0.5 h for solidification. Then cut out an agarose cube with the side length of ~1 cm (Figure 1). Seal the petri dish with parafilm and store it at 4°C. Prepared agarose gel should be used within 2 months.

**Table 2. Artificial cerebrospinal fluid (aCSF)**

| Reagent | Stock concentration | Final concentration | Amount |
|---|---|---|---|
| NaCl | n/a | 120 mM | 7.00 g |
| KCl | n/a | 3.5 mM | 0.26 g |
| $CaCl_2 \cdot 2H_2O$ | n/a | 1.3 mM | 0.19 g |
| $MgCl_2$ | 1 M | 1 mM | 1 mL |
| $KH_2PO_4$ | n/a | 0.4 mM | 0.054 g |
| HEPES | 1 M | 5 mM | 5 mL |
| $ddH_2O$ | n/a | n/a | ~994 mL |
| Total | n/a | n/a | 1 L |

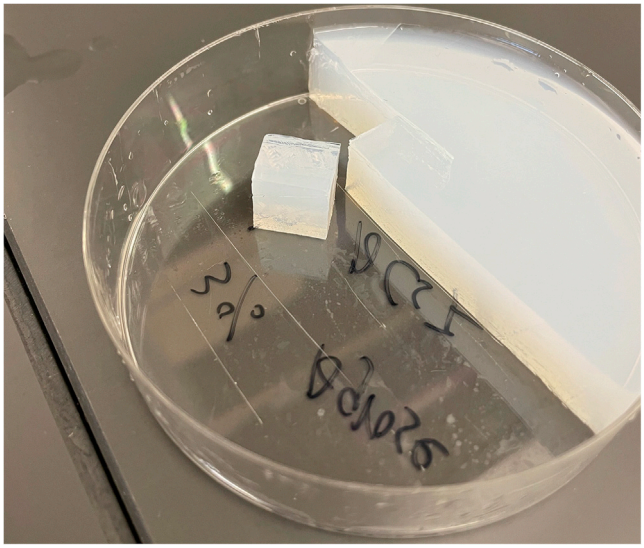


**Figure 1. Agarose block preparation**
Prepare an agarose cube with side length of ~1 cm.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| Antimycin A | Sigma-Aldrich | Cat#A8674-50MG |
| Oligomycin A | Sigma-Aldrich | Cat#75351-5MG |
| FCCP | Sigma-Aldrich | Cat#C2920-10MG |
| Fatty acid-free BSA | Fisher Bioreagents | Cat#BP9704-100 |
| NaCl | Sigma-Aldrich | Cat#S7653 |
| KCl | Sigma-Aldrich | Cat#793590 |
| $CaCl \cdot 2H_2O$ | Sigma-Aldrich | Cat#7902 |
| $MgCl_2$ | Sigma-Aldrich | Cat#M1028 |
| $KH_2PO_4$ | Sigma-Aldrich | Cat#P9791 |
| HEPES | Sigma-Aldrich | Cat#H0887 |
| Sucrose | Sigma-Aldrich | Cat#S9378 |
| PBS (phosphate-buffered saline) | Gibco | Cat#10010023 |
| BSA-Oleate | Cayman Chemical | Cat#29557 |
| Glucose | Sigma-Aldrich | Cat#G7021 |
| Agarose | VWR | Cat#N605-500 g |
| Critical commercial assays | | |
| Seahorse XFe24 Islet Capture FluxPak | Agilent Technologies | Cat#103518-100 |
| Software and algorithms | | |
| Seahorse Wave | Agilent Technologies | N/A |
| Other | | |
| Biopsy Punches with Plunger System | Integra LifeSciences | Cat#33-31AA-P/25 |
| Seahorse XFe24 Analyzer | Agilent Technologies | XFe24 Analyzer |
| Dissecting forceps | Stoelting | Cat#5210206 |
| Vibratome | Leica | VT1200S |
| Super Glue | Krazy | Cat#KG517 |
| 100 mm Petri dish | Thermo Fisher Scientific | Cat#172931 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Experimental models: Organisms/strains | | |
| 3-Month male hApoE3 knockin mouse | The Jackson Laboratory | Stock No: 029018 |
| 3-Month male hApoE4 knockin mouse | The Jackson Laboratory | Stock No: 027894 |

*Note:* Seahorse XFe24 Analyzer is absolutely required and the XFe24 Islet Capture FluxPak is a critical assay item.

## STEP-BY-STEP METHOD DETAILS

### Hydrate Agilent Seahorse XFe24 sensor cartridge with XF calibrant

Timing: 10 min

1. Discard the water from the utility plate prepared previously and fill each well with 1 mL pre-warmed XF calibrant prepared the day before.
2. Place the sensor cartridge utility plate in a non-$CO_2$ 37°C incubator for 45–60 min.

### Preparation of aCSF with different substrates

Timing: 30 min

3. Freshly add fatty acid-free BSA to previously made aCSF (Table 2) to a final concentration of 4 g/L. Then prepare 200 mL aCSF-glucose and 500 mL aCSF-sucrose by dissolving glucose or sucrose in aCSF (with BSA) to a final concentration of 10 mM glucose or 10 mM sucrose, respectively. Adjust pH to 7.4 using 1 M NaOH.

⚠ CRITICAL: When assessing fatty acid-induced oxygen consumption rate (OCR) in brain slices, aCSF-sucrose (aglycemic condition) has to be used to minimize the interference of glucose in the medium. Similarly, for glycolytic activity assessment, Extracellular acidification rate (ECAR) values at aglycemic condition (aCSF-sucrose) will be used as the baseline representing glucose-independent pH changes. aCSF-glucose (glycemic condition) will be used to determine glucose-induced OCR and ECAR. Substrate-free aCSF (no glucose or sucrose) can be prepared and stored at 4°C for up to 4 weeks (Table 2). BSA, glucose or sucrose must be added freshly on the day of experiment.

### Vibratome setup

Timing: 5 min

4. Place a new razor blade in the clamp and fill the vibratome tray with pH 7.4 aCSF-sucrose and set the temperature to 4°C. Adjust the sectioning parameters as follows (Leica VT 1200S): section thickness: 250 μm; cutting speed: 0.20 mm/s; cutting amplitude: 1.50 mm (Figure 2A).

### Brain dissection

Timing: 5 min

5. Euthanize the mouse by cervical dissociation.

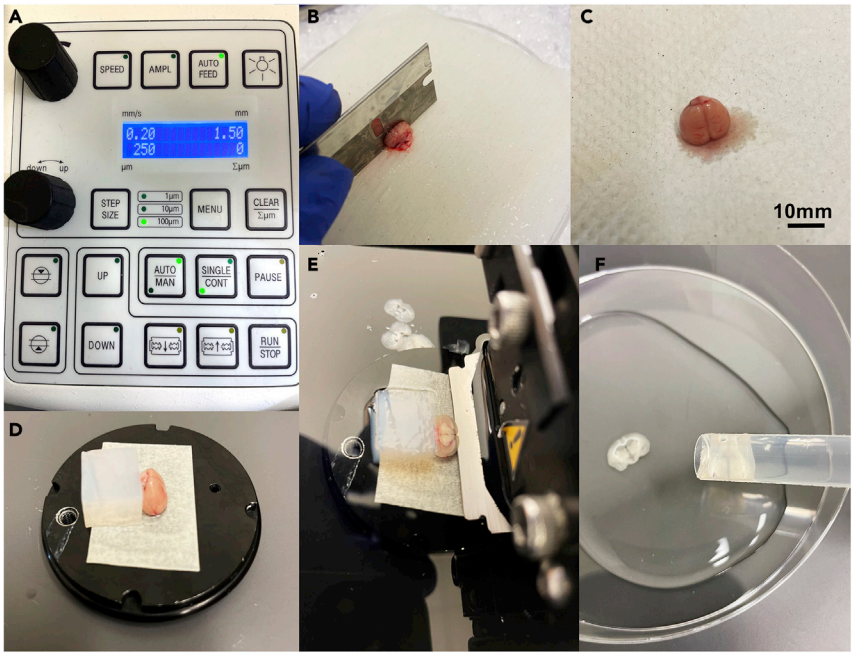



**Figure 2. Brain slices preparation**

(A) Experimental settings of the vibratome for sectioning.

(B) Cerebellum and brain stem are removed and discarded from the brain freshly dissected from the skull.

(C) Dap the bottom surface of the brain gently with paper towel to remove excess liquid before mount the brain onto the specimen plate.

(D) A supporting piece of agarose block is added behind the brain to provide structural support during sectioning.

(E) Sectioning a brain.

(F) Collect brain slice containing the region of interest from the buffer tray of the vibratome to a petri dish prefilled with aCSF-sucrose using a transfer pipet.

*Optional:* Anesthesia of mice by isoflurane in an anesthetizing chamber before dissection is optional and should be used with caution, because anesthesia is known to affect brain metabolism.

⚠ CRITICAL: If anesthesia is used, isoflurane should be handled in a fume hood and the exposure time of mice to isoflurane should be minimized.

6. Decapitate mouse and dissected the brain rapidly from the skull.
7. Transfer the brain onto phosphate buffered saline (PBS)-soaked paper towel placed in a petri dish on ice. Remove the cerebellum and brain stem (Figure 2B), put the remaining brain into a 50 mL beaker containing ice-cold aCSF-sucrose.

⚠ CRITICAL: Brain should be rapidly collected within 30 s–60 s of decapitation.

### Vibratome sectioning

Timing: 30 min (per mouse brain)

8. Tape the center of specimen plate and place a small dot of superglue in the center of the tape.

*Note:* The use of lab tape greatly helps the cleaning of the specimen plate afterwards.

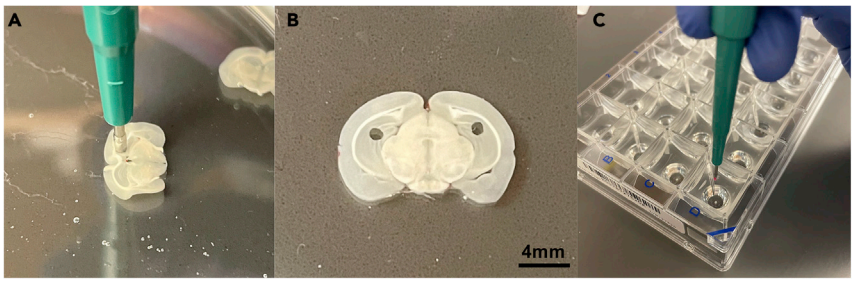



**Figure 3. Slice punch preparation and transfer**

(A) Lower 1 mm stainless steel biopsy puncher into the petri dish and press gently overtop the region of interest of the brain slice.

(B) Brain slice after punch collection of the dentate gyrus of both hemispheres.

(C) Eject the punched piece into a well of XFe Islet Capture Microplate directly and adjust the brain punches to make sure it's located in the center of each well using the biopsy puncher.

9. Take out the brain from aCSF-sucrose from step 7 with forceps and dap the dorsum of the brain gently with paper towel to remove excess buffer (Figure 2C). Carefully place the brain on the superglue (dorsum down), then slide a stabilizing agarose block to the ventral side of the brain (opposite to the blade side) to provide structural support during slicing (Figure 2D).

   *Note:* Remove excess buffer with paper towel will help the brain stick more firmly to the specimen plate.

10. Mount and orient the specimen plate to make sure the dorsal side of the brain faces the blade.
11. Set the cutting window to define the start and stop positions of the slicing. Make sure the blade cut completely through the tissue so slices will come off freely.
12. Adjust the position of the buffer tray until the razor blade is in the cutting place. Start sectioning (Figure 2E).
13. Identify and transfer brain slices that contain the region of interest from the buffer tray to a 100 mm petri dish prefilled with 37°C aCSF-sucrose using a transfer pipet (Figure 2F).

   ⚠ CRITICAL: Using this protocol, brain slices can maintain their metabolic stability for about 7–8 h after sectioning. The entire procedure needs to be completed within this time frame.

## Preparation of brain punches and transfer to seahorse plate

Timing: 30 min

14. Place the mesh inserts of the Seahorse XFe24 Islet Capture Microplate in a 100 mm petri dish containing fresh 37°C aCSF-sucrose, make sure the mesh inserts are completely immersed in the liquid without any bubble under the mesh.

   ⚠ CRITICAL: XFe24 Islet Capture Microplate rather than the standard XFe24 cell culture microplate should be used. The XFe Islet Capture Microplate contains depressed chamber and mesh that will help hold the brain punches in place at the center of each well throughout the measurement.

15. Add 700 μL per well 37°C aCSF-sucrose to half of the XFe24 Islet Capture Microplate wells for aglycemic condition and add aCSF-glucose to the other half of wells for glycemic condition.
16. Position 1 mm stainless steel biopsy puncher over the area of interest on the brain section from step 13 and press down (Figures 3A and 3B).

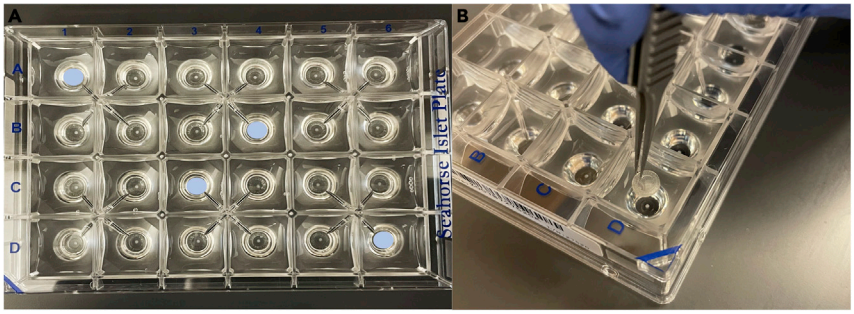



**Figure 4. Layout of XFe24 Islet Capture Microplate and insertion of the mesh insert**

(A) Four background correction wells (A1, B4, C3, and D6) in the XFe24 Islet Capture Microplate should contain aCSF only (no punches).

(B) Gently place the mesh inserts into each well of the XFe24 Islet Capture Microplate with fine-tipped tweezers above the punch to hold the latter in the center of each well.

⚠ CRITICAL: Mechanical damages to the brain tissue during biopsy and transfer can substantially affect tissue respiration. It is recommended to only use brain punches without damage.

17. Expel each punch (1mm diameter) into one well of XFe24 Islet Capture Microplate and adjust the position of brain punch using biopsy puncher to make sure it is located in the center of each well (Figure 3C). Each punch should be submerged in the liquid and centered at the bottom of the wells.

⚠ CRITICAL: Do not place brain punches into the four background correction wells (A1, B4, C3, and D6) in the XFe24 Islet Capture Microplate (Figure 4A).

Place the mesh inserts into each well of the XF Islet Capture Microplate with fine-tipped tweezers which will help keep the brain punches in the center of each well throughout the experiment (Figure 4B).

18. Incubate the XF Islet Capture Microplate with punches in a 37°C incubator without $CO_2$ for 1 h to allow temperature and pH equilibration.

## Metabolic profiling by seahorse XFe24 analyzer

Timing: 5 h

19. Prepare injection solutions of BSA-oleate (1.65 mM), oligomycin A (240 μg/mL), FCCP (130 μM) and antimycin A (280 μM) by diluting their stock solutions (prepared as in Table 1) using either aCSF-sucrose or aCSF-glucose (Table 3), corresponding to aglycemic or glycemic conditions assigned in step 15.
20. Load the hydrated sensory cartridge (from step 2) ports with 70 μL of 1.65 mM BSA-oleate in port A, 70 μL of 240 μg/mL oligomycin A in port B, 70 μL of 130 μM FCCP in port C, and 70 μL of 280 μM Antimycin A in port D using a 200 μL pipette and reagent reservoir (Table 4).

⚠ CRITICAL: aCSF-sucrose should be used to prepare the injection solutions of substrates and inhibitors for aglycemic conditions. In parallel, for glycemic conditions, aCSF-glucose should be used as diluent of injection solutions.

**Table 3. Preparation of injection solutions of BSA-Oleate and mitochondrial inhibitors**

| Reagent | Volume of reagent (μL) | Volume of aCSF (μL) | Injection concentration |
|---|---|---|---|
| BSA-Oleate | 330 | 670 | 1.65 mM |
| Oligomycin A | 12 | 988 | 240 μg/mL |
| FCCP | 13 | 987 | 130 μM |
| Antimycin A | 28 | 972 | 280 μM |

21. Set up the Seahorse XFe24 program as follows: baseline, 4 cycles; inject port A (BSA-oleate), 5 cycles; inject port B (oligomycin), 6 cycles; inject port C (FCCP), 5 cycles; inject port D (Antimycin A), 15 cycles. Each cycle is composed of mix 3 min, wait 3 min, and measure 2 min (Table 5).

*Note:* Comparing to cellular XF assays, it takes longer time for brain slices to reach stable OCR after oligomycin- and antimycin A injections (Figure 6). Additional cycles are thus needed for measurements after oligomycin (6 cycles) and after antimycin A (15 cycles). However, depending on whether proton leak-related OCR (after oligomycin) and non-mitochondrial OCR (after antimycin A) is of interest, the cycle numbers for these steps can be adjusted.

22. Place the utility plate with the loaded sensor cartridge into the XFe24 instrument tray and start the calibration step, which takes approximately 20 min.
23. After calibration is completed, replace the utility plate with the XFe24 Islet Capture Microplate with brain punches prepared in step 18 and start the XF assay. Assay duration is around 5 h.

## EXPECTED OUTCOMES

Seahorse XFe Analyzer has been widely used in evaluating metabolic function in isolated brain mitochondria and in cultured cells by monitoring OCR and ECAR, indicators of mitochondrial respiration and glycolysis, respectively. As data derived from cultured cells or isolated mitochondria may not recapitulate the complex cellular environment of the brain, and these *in vitro* models cannot reflect the significance of intercellular coordination and metabolic coupling across the heterogenous cell types in the brain (Qi et al., 2019), *ex vivo* methods to measure mitochondrial respiration on glucose-derived substrate have thus been developed with rat brain slices (Fried et al., 2014; Schuh et al., 2011; Underwood et al., 2020). However, over the past decade, the view of brain bioenergetics and its role in diseases has been evolving from the monotonic "respiratory decline" towards a dynamic metabolic reprogramming and adaptation model. Such reprogramming and adaptation involve the differential ability of the mitochondria in utilizing various energy substrates, as well as the allocation of ATP production between glycolysis and OxPhos. To this end, we describe a protocol herein to characterize complex metabolic function in mouse brains beyond glucose-dependent OCR. In our protocol, by incubating punches of acute brain slices under different glucose availability and supplying them with different substrate (*e.g.*, fatty acids), brain metabolic function is quantified and any metabolic shift in the brain's ability to utilize different fuel substrates can be determined. A typical result of OCR curves for brain slice respiration under glycemic or aglycemic conditions is shown in Figure 5. Further, a metabolic shift towards aerobic glycolysis has been well-established in glial cells upon inflammatory activation in aging and pathological conditions (Qi et al., 2019). This protocol also establishes and validates ECAR measurement as an indicator of brain aerobic glycolysis. By including tissues under both glycemic and aglycemic conditions, our protocol further

**Table 4. Port injection volume and final concentrations of BSA-Oleate and inhibitors**

| Reagent (injection concentration) | Final concentration in well | Volume added to port | Port |
|---|---|---|---|
| BSA-Oleate (1.65 mM) | 150 μM | 70 μL | A |
| Oligomycin A (240 μg/mL) | 20 μg/mL | 70 μL | B |
| FCCP (130 μM) | 10 μM | 70 μL | C |
| Antimycin A (280 μM) | 20 μM | 70 μL | D |

**Table 5. XF assay protocol**

| Step | Time | Cycles |
|---|---|---|
| Baseline | | |
| Mix | 3 min | 4 |
| Wait | 3 min | |
| Measure | 2 min | |
| Injection of port A (BSA-Oleate) | | |
| Mix | 3 min | 5 |
| Wait | 3 min | |
| Measure | 2 min | |
| Injection of port B (Oligomycin A) | | |
| Mix | 3 min | 6 |
| Wait | 3 min | |
| Measure | 2 min | |
| Injection of port C (FCCP) | | |
| Mix | 3 min | 5 |
| Wait | 3 min | |
| Measure | 2 min | |
| Injection of port D (Antimycin A) | | |
| Mix | 3 min | 15 |
| Wait | 3 min | |
| Measure | 2 min | |

enables quantitative measurements of ECAR (pH) changes specifically due to glycolytic production of lactic acid.

Using this protocol, we revealed a metabolic shift towards enhanced glucose metabolism and diminished fatty acid metabolism induced by Alzheimer's disease risk gene ApoE4 (Figure 6) (Qi et al., 2021). Hippocampal slices from humanized ApoE4 knockin mice exhibited a higher basal respiration than ApoE3 when glucose, but not FA, was present (Figure 6A). However, upon glucose depletion, ApoE4 slices showed a lower respiration than ApoE3 (Figure 6B). When exogenous FA (oleate-BSA) was supplemented, a lower respiration was seen in ApoE4 slices, but only under aglycemic condition (Figures 6C and 6D). In parallel with increased glucose respiration and decreased FA respiration, ApoE4 brains showed a higher rate of aerobic glycolysis either with or without FA (Figures 6E and 6F).

Collectively, this protocol complements the existing toolbox assessing brain metabolism by measuring multiple aspects of bioenergetics, namely glucose-dependent mitochondrial respiration, alternative-fuel-dependent respiration, and aerobic glycolysis in one streamlined procedure. Utilizing bioenergetic fuels such as ketone body, fatty acids, lactate and amino acids, and pharmacological enhancers or inhibitors of substrate transport, glycolysis, ketolysis, β-oxidation and tricarboxylic

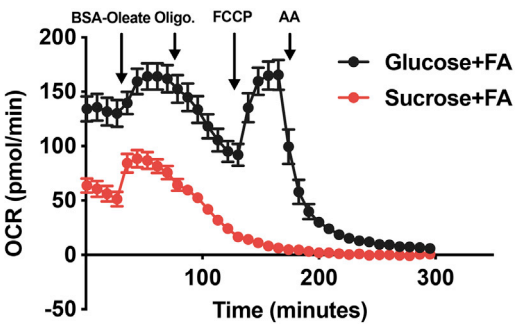



**Figure 5. Representative oxygen consumption rate (OCR) with acute hippocampal slice punches under aglycemic (sucrose) and glycemic (glucose) conditions**

FA, fatty acid; Oligo., oligomycin; AA, antimycin A.

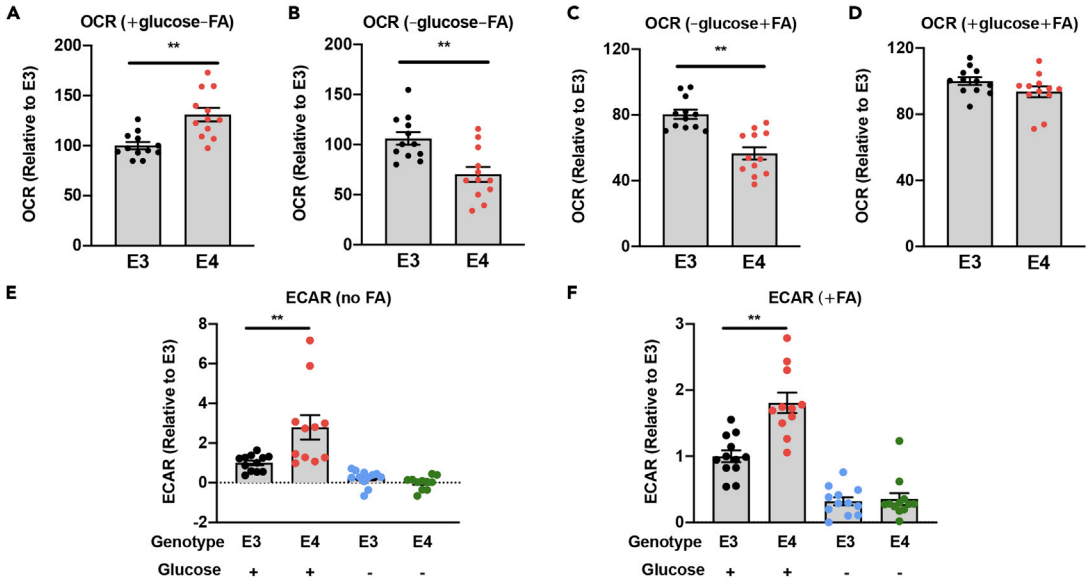



**Figure 6. ApoE4 diminishes FA oxidation and promotes glucose metabolism in mouse hippocampus**

Figure reprinted with permission from (Qi et al., 2021).

(A) Mitochondrial oxygen consumption rate (OCR) of hippocampal slices with 10 mM glucose but no exogenous FA.

(B) Mitochondrial OCR of hippocampal slices without exogenous glucose or FA.

(C) Mitochondrial OCR of hippocampal slices with 150 mM oleate-BSA but no glucose.

(D) Mitochondrial OCR of hippocampal slices with 10 mM glucose plus 150 mM oleate-BSA.

(E) Extracellular acidification rate (ECAR) in hippocampal slices without exogenous FA.

(F) ECAR in hippocampal slices with exogenous FA.

acid (TCA) cycle, this protocol can be versatilely modified to characterize the multifaceted brain bioenergetic system, which is often reprogrammed and thereby implicated in the pathogenesis of a wide variety of neurological diseases.

## QUANTIFICATION AND STATISTICAL ANALYSIS

1. Quantification analysis is performed using Seahorse Wave Desktop. More detailed information on data export with Wave software can be found at Agilent website. https://www.agilent.com/cs/pubimages/misc/User_Guide_Wave_Desktop_2-3.pdf
2. Assay results can be exported to Microsoft Excel or GraphPad Prism for further statistical analyses.

## LIMITATIONS

Theoretically, the present method measures the overall metabolic function of the mixed population of cells including neurons, glia, and other cell types present in the tissue punches. Therefore, it cannot stratify the contribution from each cell type to the observed metabolic phenotype. Also, the *ex vivo* setting of this protocol, while advantageous to cell cultures, may not fully recapitulate brain metabolic function *in vivo*. A combination of this protocol with *in vitro* studies of different cell types, and other *in vivo* assessment of brain metabolism, such as metabolomics, should be used to address these limitations.

Technically, the entire procedure, including brain dissection, slicing, punch preparation, and eventually the Seahorse assay, takes approximately 7 h. Helpful practices were discussed previously to keep brain punches metabolically active and obtain reliable and reproducible results

(Underwood et al., 2020). Lastly, the availability of the Seahorse XFe24 Analyzer may limit the usage of this protocol.

## TROUBLESHOOTING

### Problem 1

High variance in results between punch replicates.

### Potential solution

High variance between experimental replicates could arise from the quality control of brain punches. Each biopsy puncher should be used for no more than 4 punches. Brain punches of poor quality or irregular shape should not be used (step 16).

Another step that could cause damage to slice punches is step 17. Make sure the mesh insert is gently placed into each well of the microplate with fine-tipped tweezers and the mesh should only lightly touch the punches. Punches could be damaged due to excessive pressure on the mesh insert.

### Problem 2

Lack of response in OCR to substrate or inhibitor injections.

### Potential solution

If punches in some wells show normal baseline readouts but fail to respond to the injections of inhibitors or substrates, this may suggest incomplete injections of inhibitors into those wells. It is thus important to always check all injection ports upon completion of the XFe24 assay to make sure all inhibitors or substrates have been injected (steps 20 and 23).

Moreover, the responsiveness of punches to substrates or inhibitors may vary by brain region, as well as the sex, age, and/or genotype of the animal. If a lack of response is found in most wells across multiple experimental groups, a titration should be conducted for the substrate or inhibitor in question to determine the optimal dose (step 19).

### Problem 3

Poor metabolic activity due to extended punch preparation

### Potential solution

Poor metabolic activity (OCR and ECAR) may be observed if the procedure takes more than 7 h due to the extended time to process additional brains for punches (steps 8–18). In this case, brain sections can be placed in oxygenated aCSF prior to slice punching and Seahorse assays to help preserve their metabolic activity (step 13). In addition to antimycin A, complex I inhibitor rotenone (20 μM final concentration in well) can be included in port D injection (steps 19 and 20) which may help to reduce the time reaching complete electron transport chain (ETC) inhibition and thus reduce the measurement cycles.

### Problem 4

High OCR and ECAR values under aglycemic condition

### Potential solution

OCR values under aglycemic condition should be lower than glycemic groups (Figure 5) and the ECAR values should be minimal (Figure 6). If higher than expected values are observed for aglycemic wells, there might be interference from endogenous glucose remaining in the brain tissue. In this case, a 14–16 h starvation to mice can help control the endogenous glucose available to brain tissues and reduce variance across animals (Day prior to assay step 2 in before you begin). Also make sure

aCSF-sucrose is used for punches assigned for aglycemic condition throughout the entire protocol including the fatty acid and inhibitor solutions for these wells (steps 3, 4, 7, 13, 14–18, and 19).

### Problem 5

Difficulty in obtaining good-quality brain slices

### Potential solution

It is important to ensure the integrity and quality of each brain slice. If it is difficult to obtain brain slice with good quality using the method described (step 9), an option is to solidify the brain with 4% low-melting-point agarose before sectioning as previously described (Fried et al., 2014).

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fei Yin (feiyin@arizona.edu)

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate or analyze any datasets or code.

## ACKNOWLEDGMENTS

This work was supported by the University of Arizona Center for Innovation in Brain Science startup fund to F.Y., NIH/NIA P01AG026572 to Roberta Diaz Brinton (Analytic Core to F.Y.), and Arizona Alzheimer's Consortium Pilot Project to F.Y.

## AUTHOR CONTRIBUTIONS

Conceptualization, F.Y.; methodology, G.Q., Y.M., and F.Y.; investigation, G.Q. and Y.M.; writing, G.Q., Y.M., and F.Y.; funding acquisition, F.Y.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

Fried, N.T., Moffat, C., Seifert, E.L., and Oshinsky, M.L. (2014). Functional mitochondrial analysis in acute brain sections from adult rats reveals mitochondrial dysfunction in a rat model of migraine. Am. J. Physiol. Cell Physiol. *307*, C1017–1030.

Qi, G., Mi, Y., Shi, X., Gu, H., Brinton, R.D., and Yin, F. (2021). ApoE4 impairs neuron-astrocyte coupling of fatty acid metabolism. Cell Rep. *34*, 108572.

Qi, G., Mi, Y., and Yin, F. (2019). Cellular specificity and inter-cellular coordination in the brain bioenergetic system: implications for aging and neurodegeneration. Front. Physiol. *10*, 1531.

Schuh, R.A., Clerc, P., Hwang, H., Mehrabian, Z., Bittman, K., Chen, H., and Polster, B.M. (2011). Adaptation of microplate-based respirometry for hippocampal slices and analysis of respiratory capacity. J. Neurosci. Res. *89*, 1979–1988.

Underwood, E., Redell, J.B., Zhao, J., Moore, A.N., and Dash, P.K. (2020). A method for assessing tissue respiration in anatomically defined brain regions. Sci. Rep. *10*.