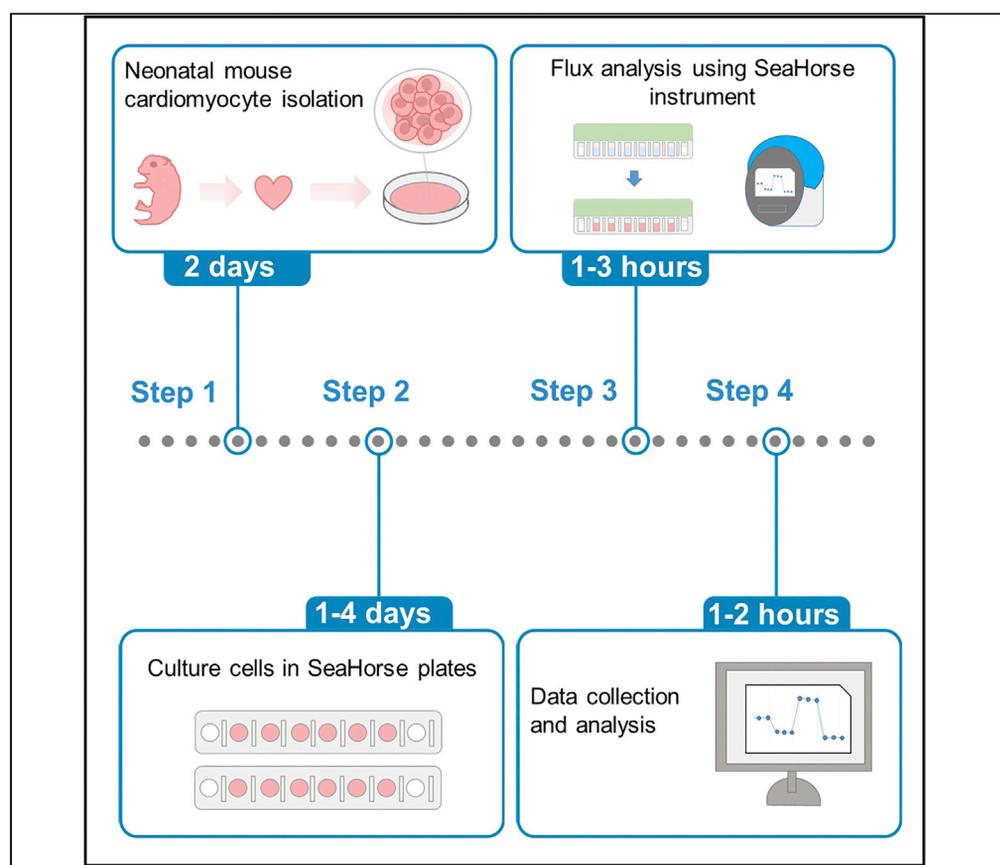


## Protocol

# Evaluation of long-chain fatty acid respiration in neonatal mouse cardiomyocytes using Seahorse instrument



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### Highlights

Isolation and culture of neonatal mouse cardiomyocytes

Measurement of long-chain fatty acid oxidation using Seahorse Flux Analyzer

Guidance on quantification of the generated Seahorse dataset

Metabolic switches play a critical role in the pathophysiology of cardiac diseases, including heart failure. Here, we describe an assay for long-chain fatty acid oxidation in neonatal mouse cardiomyocytes by using a Seahorse Flux Analyzer (Agilent). This protocol is a simplified but robust adaptation of the standard protocol that enables metabolic measurements in cells isolated from transgenic mouse models, which can be timesaving and informative. Cell isolation and culture represent a critical point that may require bench optimization.

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

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## Protocol

## Evaluation of long-chain fatty acid respiration in neonatal mouse cardiomyocytes using SeaHorse instrument

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## SUMMARY

Metabolic switches play a critical role in the pathophysiology of cardiac diseases, including heart failure. Here, we describe an assay for long-chain fatty acid oxidation in neonatal mouse cardiomyocytes by using a SeaHorse Flux Analyzer (Agilent). This protocol is a simplified but robust adaptation of the standard protocol that enables metabolic measurements in cells isolated from transgenic mouse models, which can be timesaving and informative. Cell isolation and culture represent a critical point that may require bench optimization. For complete details on the use and execution of this protocol, please refer to Angelini et al. (2021).

## BEFORE YOU BEGIN

This protocol as described below is especially designed to measure long-chain fatty acid (LCFA)  $\beta$ -oxidation in neonatal mouse cardiomyocytes (NMCMs). At Part 2, NMCMs obtained from Cre/loxP transgenic mouse can be cultured with 4OH-tamoxifen to induce gene knock-out before assessing respiration. Alternatively, we have also successfully used this protocol with neonatal rat cardiomyocytes that were pre-incubated with drugs (DMOG, etomoxir) or transfected with lentiviral particles before the assay. The protocol is designed to work with XFp 96 (Mini) SeaHorse Flux Analyzer instrument, but it may be applied to a SeaHorse XFe96 Analyzer. However, the use of the XFe24 instrument may require further adaptations that are not fully covered by this protocol.

The protocol also provides an informative section dedicated to the isolation and the correct handling of the neonatal mouse cardiomyocytes, since these cells are more fragile than neonatal rat cardiomyocytes.

## Institutional permissions

The proper preparation of cells from neonatal rodent pups requires ethical approval and permission from the experimenter's institution. These experiments, as shown in this protocol, were conducted as approved by the Baylor College of Medicine Institutional Animal Care and Use Committee, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.



## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Modified IMEM (Improved Minimum Essential Medium), with L-glutamine, without gentamicin sulfate	Thermo Fisher Scientific	Cat#A1048901
Horse Serum, heat inactivated, New Zealand origin	Thermo Fisher Scientific	Cat#26050070
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat#15140122
HBSS, calcium, magnesium	Thermo Fisher Scientific	Cat#24020117
Bovine Serum Albumin Fraction V, heat shock, fatty acid free (FA-free BSA)	Millipore Sigma	Cat#3117057001
L-Carnitine	Millipore Sigma	Cat#11242008001
Oligomycin	Millipore Sigma	Cat#495455-10MG
Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)	Millipore Sigma	Cat#C2920-10MG
Rotenone	Millipore Sigma	Cat#557368-1GM
Antimycin A	Millipore Sigma	Cat#A8674-25MG
Etomoxir	Millipore Sigma	Cat#236020
4-hydroxytamoxifen	Millipore Sigma	Cat#H6278-10MG
Sodium Palmitate	Millipore Sigma	Cat#P9767-5G
Dimethyl Sulfoxide - CAS 67-68-5 – Calbiochem	Millipore Sigma	Cat#317275-100ML
Poly-D-lysine	Millipore Sigma	Cat#P8920
Krebs-Henseleit Buffer Modified	Millipore Sigma	Cat#K3753-10X1L
HEPES	Thermo Fisher Scientific	Cat#J63218.AE
CaCl <sub>2</sub>	Millipore Sigma	Cat#1023780500
NaHCO <sub>3</sub>	Millipore Sigma	Cat#S5761-500G
Trypsin EDTA 0.05%	Thermo Fisher Scientific	Cat# 25300054
<b>Critical commercial assays</b>		
Worthington Neonatal Biochemical Isolation System kit	Worthington Biochemical Corporation	Cat#LK003300
Seahorse XF Calibrant Solution 500 mL	Agilent	Cat#100840-000
Quick Start™ Bradford Protein Assay Kit 1	Bio-Rad	Cat#5000201
Seahorse XF HS Mini Analyzer  Seahorse XFp FluxPak	Agilent	Cat#103022-100
<b>Experimental models: Cell lines</b>		
Neonatal Mouse Cardiomyocytes - Passage 0. Cardiomyocytes from male and female P1 mouse pup hearts.	<a href="#">Angelini et al. (2021)</a>	<a href="https://www.cell.com/cell-reports/fulltext/S2211-1247(21)01221-3">https://www.cell.com/cell-reports/fulltext/S2211-1247(21)01221-3</a>
<b>Experimental models: Organisms/strains</b>		
B6.FVB (129)-Tg (Myh6-cre/Esr1)1Jmk/J 6–12 months breeders	Jackson Laboratory	Cat#005657
<b>Software and algorithms</b>		
Wave (version 2.6.153)	Agilent	<a href="https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897">https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897</a>
Excel (Microsoft Office Suite)	Microsoft	<a href="https://www.microsoft.com/en-us/microsoft-365/excel">https://www.microsoft.com/en-us/microsoft-365/excel</a>
<b>Other</b>		
Seahorse XF HS Mini Analyzer	Agilent	<a href="https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-analyzers/seahorse-xf-hs-mini-analyzer-770502">https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-analyzers/seahorse-xf-hs-mini-analyzer-770502</a>
<b>Deposited data</b>		
Supplemental Material ( <a href="#">Methods Video S1</a> )	This paper	Mendeley data <a href="https://doi.org/10.17632/fdfjvh9brm.1">https://doi.org/10.17632/fdfjvh9brm.1</a>

### MATERIALS AND EQUIPMENT

⌚ Timing: 1 h

#### Stock solution Palmitate-BSA conjugated

Reagent	Weight	Resuspend in	Stock concentration
Free-fatty acid BSA	1.5 g	20 mL H <sub>2</sub> O	7.5%
Palmitate	27.8 mg	18.5 mL BSA 7.5%	

Stock solution can be kept up to six months to one year once stored at  $-20^{\circ}\text{C}$ . We highly recommend to aliquot, in order to avoid freeze-thaw cycles and multiple-users contamination. Once stored for a long time without use (more than a month), the Palmitate-BSA should need to be pre-warmed again ( $+42^{\circ}\text{C}$ ) before use.

- Warming up water bath.
  - Prepare a water bath at  $42^{\circ}\text{C}$ .
  - Prepare a boiling water bath at nearly  $100^{\circ}\text{C}$ .
- BSA solution.
  - Prepare a solution of 7.5% free-fatty acid BSA in 20 mL H<sub>2</sub>O.
  - Pre-warm at  $42^{\circ}\text{C}$ .
- Palmitate conjugation – Palmitate 5 mM.
  - Weigh 27.8 mg of sodium palmitate and put it into a 50 mL tube (closed but loose cap).
  - Place the tube in the boiling water bath, until fatty acids are melted (no longer than few minutes).
  - Let it cool to  $70^{\circ}\text{C}$ . Inner temperature of the tube can be monitored using a thermometer.
  - Pour 18.7 mL of warm 7.5% BSA solution into the warm fatty acid.
  - Heat at  $42^{\circ}\text{C}$  for 30 min, increase to  $47^{\circ}\text{C}$  if needed to help the solubilization.

For a better time convenience, we highly recommend to aliquot the solution. This may also prevent freeze-thaw cycles and avoid multiple-users contamination.

**Alternatives:** Palmitate-BSA conjugate and BSA can be obtained as a ready-to-use working solution from Agilent (Seahorse XF Palmitate-BSA FAO substrate – Cat#102720-100).

#### KHB solution

Reagent	Weight	Resuspend in	Stock concentration
Krebs- Buffer- Modified (powder)	4.8 g		1x
CaCl <sub>2</sub>	0.01865 g		4.1 mM
NaHCO <sub>3</sub>	1.05 g		176 mM
H <sub>2</sub> O		480 mL (then complete to 500 mL)	

Adjust the pH to 7.3–7.4 (when the solution is at  $37^{\circ}\text{C}$ ).

KHB can be filtered and stored at  $4^{\circ}\text{C}$  up to one year. White precipitate can form with time, depending on freezer storage conditions. If so, preparation of a new buffer is recommended. Make sure to verify the accuracy of the pH before starting any new set of experiments.

**Note:** For CaCl<sub>2</sub> and NaHCO<sub>3</sub>, always add powder into the solvent, do not directly pour liquid directly on the powder.

If extracellular acidification measurement is intended to be performed using this protocol, the KHB solution should be prepared using 1.2 mM NaH<sub>2</sub>PO<sub>4</sub> instead of NaHCO<sub>3</sub>.

#### Stock solution Carnitine

Reagent	Weight	Resuspend in	Stock concentration
L-Carnitine	0.0098 g	1 mL H <sub>2</sub> O	50 mM

Stock solution can be kept up to six months to one year once stored at  $-20^{\circ}\text{C}$ . We highly recommend to aliquot this stock, in order to avoid freeze-thaw cycles and multiple-users contamination.

#### FAO medium

Reagent	Volume	Diluted in	Concentration
L-Carnitine 50 mM	100 $\mu\text{L}$		0.5 mM
HEPES 0.5 M	100 $\mu\text{L}$		50 mM
KHB		9.8 mL	

The FAO medium (containing L-Carnitine and HEPES) is a ready-to-use working solution. It is stable only for few hours and should be kept on ice between the run of Seahorse plates, and pre-warm right before use.

#### Stock solution Etomoxir

Reagent	Weight	Resuspend in	Stock concentration
Etomoxir	0.005 g	418 $\mu\text{L}$ DMSO	30 mM

Stock solution can be kept up to one year once stored at  $-20^{\circ}\text{C}$ . We highly recommend to aliquot these stocks, in order to avoid freeze-thaw cycles and multiple-users contamination.

#### Stock solution 4OH-Tamoxifen

Reagent	Weight	Resuspend in	Stock concentration
4-hydroxytamoxifen	0.010 g	1 mL ethanol	25 mM

Stock solution can be kept up to one year once stored at  $-80^{\circ}\text{C}$ . We highly recommend to aliquot these stocks, in order to avoid freeze-thaw cycles and multiple-users contamination.

**Alternatives:** FAO medium could be replaced by the Seahorse XF Base Medium, without phenol red (Agilent Cat#103335-100).

#### Stock solution Seahorse

Reagent	Weight	Resuspend in	Stock concentration
Oligomycin	0.0039 g	1 mL DMSO	5 mM
FCCP	0.00762 g	500 $\mu\text{L}$ DMSO	15 mM
Rotenone	0.019711 g	1 mL DMSO	50 mM

Stock solutions can be kept up to one year once stored at  $-20^{\circ}\text{C}$ . We highly recommend to aliquot these stocks, in order to avoid freeze-thaw cycles and multiple-users contamination.

**△ CRITICAL:** Due to their cytotoxicity, handle oligomycin, FCCP, Rotenone and Antimycin with care, preferably under the hood.

**Alternatives:** Seahorse ready-to-use solutions can be directly obtained from Agilent. Seahorse Cell Mito Stress Kit (working solution for Oligomycin, FCCP, Rotenone, Antimycin-A. Cat#103010-100); and Seahorse XF Palmitate Oxidation Stress Test Kit and FAO Substrate (Palmitate, free-fatty acid BSA, L-Carnitine. Cat#103693-100).

#### Working solution BSA-conjugated Palmitate

Reagent	Volume	Resuspend in	Stock concentration
Palmitate 10 mM	100 $\mu\text{L}$		1 mM
BSA 1 mM	200 $\mu\text{L}$		0.2 mM
FAO medium		1 mL	

Working solution can be kept for few hours at  $20^{\circ}\text{C}$ . It should not be reused afterwards nor frozen for later usage.

### Intermediate dilution Oligomycin

Reagent	Volume	Resuspend in	Stock concentration
Oligomycin	100 $\mu$ L		0.1 mM
FAO medium		1 mL	

Intermediate dilution can be kept for few hours at 4°C (ice). It should not be reused afterwards nor frozen for later usage.

### Working solution Oligomycin

Reagent	Volume	Resuspend in	Stock concentration
Oligomycin- 0.1 mM Dilution	48 $\mu$ L		16 $\mu$ M
FAO medium		252 $\mu$ L	

Working solution should not be reused.

### Intermediate dilution FCCP

Reagent	Volume	Resuspend in	Stock concentration
FCCP 15 mM	24 $\mu$ L		0.4 mM
FAO medium		900 $\mu$ L	

Intermediate dilution can be kept for few hours at 4°C (ice). It should not be reused afterwards nor frozen for later usage.

### Working solution FCCP

Reagent	Volume	Resuspend in	Stock concentration
FCCP- 0.4 mM dilution	200 $\mu$ L		80 $\mu$ M
FAO medium		800 $\mu$ L	

Working solution should not be reused.

### Intermediate dilution Rotenone-I

Reagent	Volume	Resuspend in	Stock concentration
Rotenone - stock	10 $\mu$ L		5 mM
FAO medium		990 $\mu$ L	

Intermediate dilution can be kept for few hours at 4°C (ice). It should not be reused afterwards nor frozen for later usage.

### Intermediate dilution Rotenone-II

Reagent	Volume	Resuspend in	Stock concentration
Rotenone – 5 mM dilution	10 $\mu$ L		0.5 mM
FAO medium		90 $\mu$ L	

Intermediate dilution should not be reused afterwards nor frozen for later usage.

### Working solution Rotenone/Antimycin

Reagent	Volume	Resuspend in	Stock concentration
Rotenone – 0.5 mM dilution	60 $\mu$ L		100 $\mu$ M
FAO medium		240 $\mu$ L	

Working solution can be kept for few hours at 4°C (ice). It should not be reused afterwards nor frozen for later usage.

## STEP-BY-STEP METHOD DETAILS

### Part 1. Neonatal mouse cardiomyocyte isolation

© Timing: Day 1: 1 h; Day 2: 2–3 h

This step is critical, as cell quality may influence the quality of the results with SeaHorse. The protocol is adapted from Neonatal mouse cardiomyocyte kit from Worthington (#LK003300). To our experience, it is important to isolate the cells from P0-P1 mouse pups.

1. Collection of mouse neonatal hearts.

a. Optional: Genotyping.

If the experiment involves transgenic mouse models, genotyping should be preferably performed before the sample collection, so genotype-matched hearts can be pooled during the isolation process.

- i. To limit any heat/cold stress and distress during the time of genotyping, the pups can be left in their bedding and tagged appropriately, according to your protocol approval. We usually used a non-toxic marker (100% ink-based Pentel) and gently label the upper part of their tail or their back.
- ii. Collect a 1–2 mm piece of tail, and proceed to the DNA isolation and genotyping, according to your manufacturers' protocol.

b. Euthanasia.

- i. Anesthetize the pups according to your protocol approval (Figure 1A). Be aware that P0-P1 pups are more resistant to inhalation method (isoflurane) but more sensitive to temperature stress.
- ii. Sterilize the abdomen with antiseptic solution.
- iii. Euthanize by decapitation using sharp scissors.

c. Heart collection.

- i. Surgically collect the heart while still beating (Figures 1B and 1C).
- ii. Hearts are immediately placed in ice-cold CMF Hank's Balanced Salt Solution (HBSS), pH 7.4 in a small petri dish put on an ice bed.
- iii. Once cleared from extra-cardiac tissues and blood, cut each heart in 2–3 longitudinal pieces only, and transfer the sections into 1.5 mL Eppendorf tubes (labeled accordingly) containing 450  $\mu$ L of HBSS (Figures 1D–1F). Count 3 mouse pup hearts for 450  $\mu$ L of HBSS.

d. Keep the tubes on ice until the end of the collection.

**Note:** You can use lamp-equipped magnifying glasses for assistance during the dissection process.

2. Cardiomyocyte isolation – Day 1.

**△ CRITICAL:** This step is critical, as cell quality may influence the quality of the results with SeaHorse. The protocol is adapted from Neonatal mouse cardiomyocyte kit from Worthington. For an alternative method isolation method that may not rely on commercial kit, the experiments may refer to this article (Ehler et al., 2013).

**Alternatives:** Pierce™ Primary Cardiomyocyte Isolation Kit (Cat#88281 from Thermo Fisher Scientific).

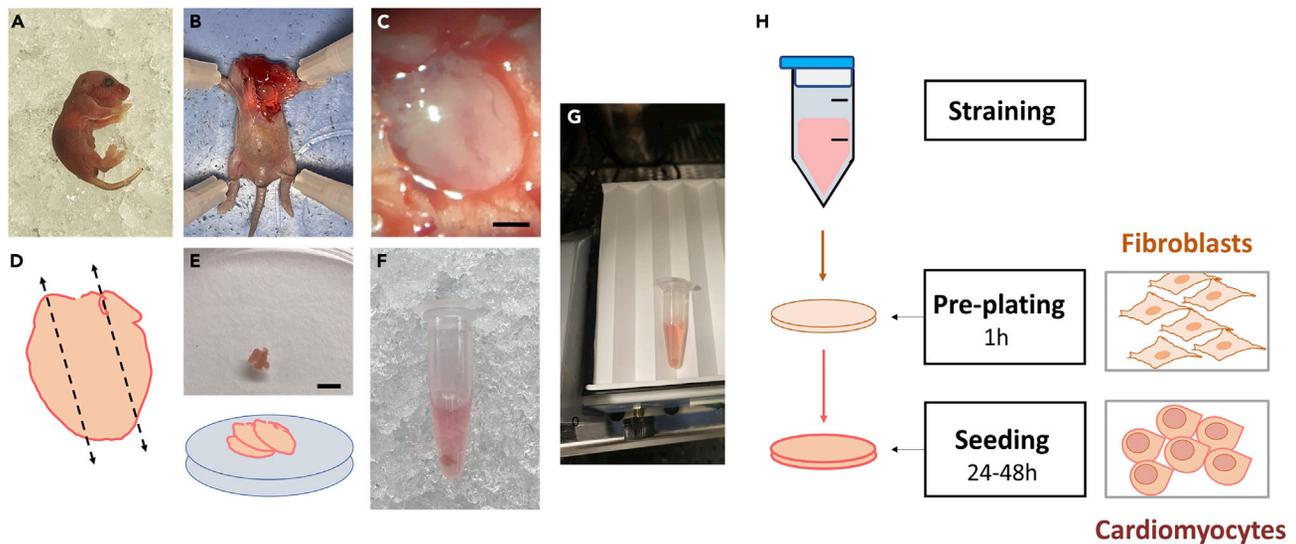
**Alternatives:** Neonatal Cardiomyocyte Isolation Kit, mouse (Cat#130-100-825 from Miltenyi Biotec).

To our experience, it is important to isolate the cells from P0-P1 mouse pups.

- a. If using the Worthington kit: Resuspend the trypsin vial in 2 mL of ice-cold sterile HBSS to obtain a 500  $\mu$ g/mL solution.
- b. Put the tubes on a plate (lid of a petri dish, for instance), and incubate for at least 10–12 h at 4°C (fridge).

3. Cardiomyocyte isolation – Day 2.

- a. Under a sterile hood, transfer the tissue and solution into a 10 mL tube. Use a wide-mouth (3 mm opening) pipet to avoid damaging the cells.
- b. Add 100  $\mu$ L of Trypsin inhibitor per 500  $\mu$ L of tissue-containing solution.



**Figure 1. Isolation of neonatal mouse cardiomyocytes**

(A–H) Set of photography and scheme depicting the first steps of the neonatal mouse cardiomyocyte isolation, from mouse pup anesthesia (A), heart harvesting (B and C as a focus of B; scale bar: 1 mm); heart slicing and collection (D–F; scale bar E.: 5 mm), rotating steps (G) and final steps (from straining to seeding) (H).

- c. Incubate for 5 min at 37°C.
- d. Resuspend collagenase in 5 mL of pre-warm Leibovitz L-15 medium. Add 250  $\mu$ L per 500  $\mu$ L of tissue-containing solution.
- e. Incubate at 37°C under slow rotation (2–4 rpm) for 45 min to one hour (Figure 1G). We usually put the 1.5 mL tubes into a 50 mL Corning tube during the rotation process, but this is optional.
- f. Prepare a complete medium containing Leibovitz L-15 medium with 10% horse serum 5% fetal bovine serum, 1% penicillin-streptomycin. Pre-warm at 37°C in a water bath. The L15 containing medium is stable up to 3–4 months at 4°C (fridge).
- g. Under the sterile hood, triturate the cells at least 10 times with a wide mouth pipet (or regular 10 mL pipet). Avoid air bubbles and high speed pipetting during the process, both of these would damage the cells. Just slowly aspirate the medium until 2/3 of the pipet tip or 10 mL pipet, and gently release it back into the tube.
- h. Pre-wash a 40  $\mu$ m strainer with 1 mL of L15 culture medium, then strain the cells, wash the strainer with 1 mL of medium. Repeat the operation a second time. Rinse the strainer with 2 mL of medium (Figure 1H, top panel).
- i. Pre-plating: Put the cell-containing medium in non-coated 35 mm<sup>2</sup> dishes. Leave the cells undisturbed for 1 h in the incubator at 37°C (Figure 1H, middle panel).

**△ CRITICAL:** This step enables the removal of most of the fibroblasts that are fast adhering compared with cardiomyocytes.

- j. Gently collect the medium with cells in suspension, do not wash too roughly or this will detach the fibroblasts. Then spin the cells at 150 g for 5 min at 21°C.
- k. Resuspend the cell pellet in appropriate volume of L-15 complete medium. Depending on the experimenter, the equivalent of 3–4 mouse pup heart can be seeded onto a 35 mm diameter dish (Figure 1H, bottom panel).
- l. Leave the seeded cells undisturbed between 24 and 48 h.

**Note:** Adhesion can be improved by pre-coating the plate with Poly-Lysine (Millipore Sigma, Cat# P8920).

At the end of the seeding time, the cardiomyocytes should be fully attached, and they may start beating (See [Methods Video S1](#) “Beating Neonatal Mouse cardiomyocytes”).

## Part 2. Cell culture on SeaHorse plate

⌚ **Timing:** 1–4 days

24–48 h after their seeding, the cardiomyocytes can be trypsinized and split into new plates. Seeding medium would be then replaced by culture medium. Cells isolated from transgenic mouse models can be treated with 4OH-tamoxifen for 4 consecutive days before assessment of respiration.

4. Transfer the cells in SeaHorse 8-well plates.

**Note:** Make sure the SeaHorse plates and sensors match with your SeaHorse Flux Analyzer instrument. The XFp Analyzer is now replaced by the XF HS Mini Flux Analyzer (See the link below for more information: [Agilent](#)).

a. Remove the seeding medium, gently wash the cells with sterile D-PBS, and apply a volume of Trypsin EDTA 0.05% (Gibco Cat# 25300054) according to the size of the dish (1 mL for a 35 mm diameter dish).

For proceeding the trypsinization from a different surface area, the experimenter should refer to “Useful Numbers for Cell Culture” from Thermo Fisher Scientific.

b. Incubate for 5 min at 37°C.

c. Collect the cells, spin at 150 g for 5 min at room temperature (19°C–21°C).

d. Discard the supernatant and resuspend the cells in 1 mL of complete culture medium (DMEM – Horse Serum 5% - Fetal Bovine Serum 10% - Penicillin/Streptomycin 1%).

e. Cell counting:

i. Prepare a 1:10 dilution in a 1.5 mL Eppendorf tube.

ii. Load 10  $\mu$ L of the 1:10 dilution in a counting chamber of a hemacytometer or a slide of an automatic cell counter.

iii. Estimate the cell concentration of your undiluted cell suspension.

f. Estimate the volume you need to pipet to load 25,000 cells in 100  $\mu$ L of medium. Prepare the dilution of your cell suspension accordingly.

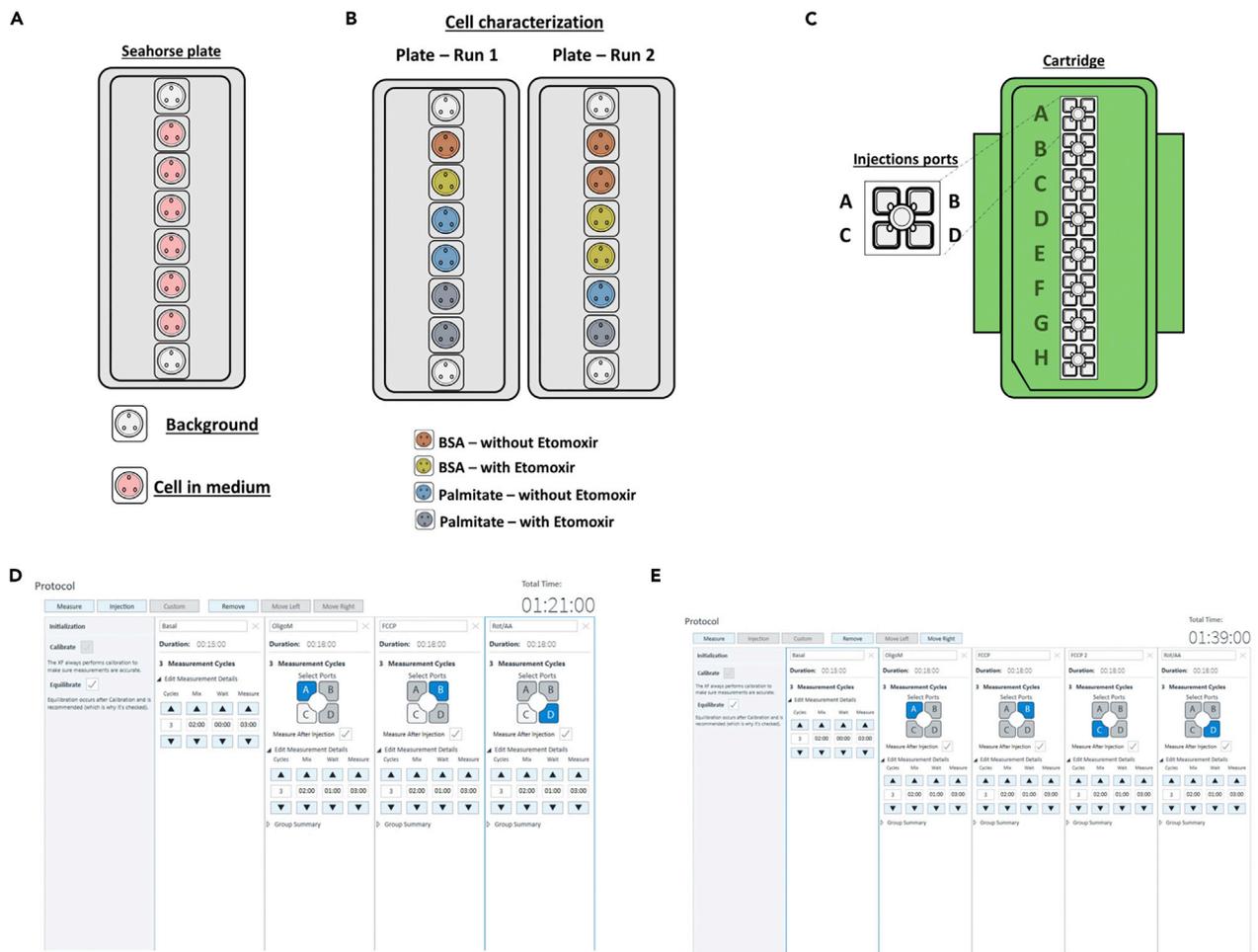
**Note:** Please note that the complete estimation of cell lipid metabolism should include BSA-incubated and Palmitate-incubated wells, in presence or absence of etomoxir. This may enable to decipher exogenous to endogenous fatty acid oxidation (as discussed below). Consequently, any tested condition (Pharmacological drug incubation, or tamoxifen-induced gene disruption, for instance) may multiply the number of plates required per assay exponentially ([Figure 2B](#) shows an example of plate setup).

g. Pipet the volume according to your calculation, and load 6 well of an 8-well plates. Adjust the volume in the wells to 250  $\mu$ L–300  $\mu$ L.

**Note:** One or two wells should be kept as a cell-free background per plate ([Figure 2A](#)).

h. Place your plates in an incubator at 37°C/5% CO<sub>2</sub>, and leave them seeding for additional 24 h, or at least 12 h.

**Optional:** To our experience, if cells are freshly isolated and healthy, and the SeaHorse miniplates being used, re-coating of the SeaHorse miniplates is not necessary. However, if preferred, the experimenters could eventually pre-coat the plates with poly-lysine or collagen



**Figure 2. Neonatal mouse cardiomyocyte in a Seahorse miniplate**

- (A) Schematic representation of Seahorse miniplate preparation for a LCFA oxidation assay.  
 (B) Example of plate groups that can be assigned for the assay.  
 (C) Schematic representation of a cartridge (Left: injection ports, Right: utility plate for sensors hydration and calibration).  
 (D) Screenshot from the Wave software representing the optimal protocol for LCFAO measurement (D). OligoM depicts “Oligomycin”; and Rot/AA “Rotenone/Antimycin A”.  
 (E) For optimization purpose, a second injection could be added to the program, as shown in (E).

for at least 1 h before seeding the cells. Previously, we had performed assay with or without pre-coating and we noticed no difference in term of OCR.

**Note:** Cell morphology can be difficult to clearly assess on Seahorse plate. The NMCMs might appear more round-shaped, but they should be beating as a syncytial at 1–2 days after seeding (Methods Video S1). Depending on the genes targeted by tamoxifen-induced disruption, treatment with tamoxifen may later affect their beating rate or synchronization.

#### 5. Cell culture maintenance.

The cells can be kept in culture for up to one week. Medium should be changed every two days. Our maintenance medium usually contains DMEM – Horse Serum 5% - Fetal Bovine Serum 10% - Penicillin/Streptomycin 1%. Other formulation may be possible, but maintenance medium should always contain glucose, serum and antibiotics.

#### 6. Optional: treatment with tamoxifen.

- a. Dilute 4OH-tamoxifen at 100 nM in complete maintenance medium.
- b. Gently wash the cells with D-PBS and replace medium by 200  $\mu$ L of fresh medium containing tamoxifen (Day 0 of treatment).
- c. Check the viability of the cells after 24 h (Day 1 post-tamoxifen).

**Note:** Do not increase 4OH-tamoxifen concentration to speed up or strengthen the process, since the reagent is highly cytotoxic for neonatal cardiomyocytes at higher dose.

- d. Replace the medium by fresh medium containing 100 nM of 4OH-tamoxifen at Day 2 or Day 3, and every 2–3 days, if longer incubation is needed.
- e. Gene disruption is usually completed between Day 3 and Day 4.

**Note:** Before running any Seahorse plate experiment with a new transgenic mouse line, it is highly recommended to first ascertain the efficiency of the gene disruption in NMCMs, as well as the absence of tamoxifen cytotoxicity.

Knock-out validation methods could include: DNA isolation and genotyping of the “floxed” alleles of your transgene of interest; RNA isolation and RT-qPCR for the mRNA coding the protein of interest, or Western blot for the protein of interest. The optimal methods may depend on 1- the yield of DNA, RNA or proteins that you could extract from your treated cell preparation; 2- the abundance of the mRNA or the protein coded by your gene of interest.

Changes in beating rate could be also noticed in the plate when metabolism or Ca<sup>2+</sup> cycling are impacted. This observation remains empirical and may be difficult to evaluate with additional electrophysiological approach on a Seahorse plate.

**⏸ Pause point:** Tamoxifen can be replaced at Day 2 instead of Day 3 with no consequence on cell viability. Depending on the efficiency of the gene disruption, the cells can be ready to run between Day 3 (12 h after medium change at Day 3) and Day 5.

### Part 3. LCFA $\beta$ -oxidation using Seahorse

⌚ Timing: 1–3 h

The day before the experiment:

7. 12 h before the experiment :
  - a. Replace medium by substrate-limited medium: MEM + with 1% serum (fetal bovine or horse), 1% antibiotics, 1% Glutamine, 1% L-Carnitine. Incubate the cells in their regular incubator (18.6% O<sub>2</sub>, 5%CO<sub>2</sub> at 37°C).

This starvation step promotes the consumption of endogenous substrates or exogenous metabolite byproducts (glutamine, glucose or pyruvate oxidation) that may interfere with the assay (Zeidler et al., 2017).

- b. Prepare cartridge hydration following the manufacturer’s recommendation (Agilent – Hydrating a XFp Sensor Cart). See in [Figure 2C](#) a schematic representation of a cartridge.

The recommendations have changed since we started working with the Seahorse instrument, suggesting that the Seahorse Calibrant Buffer composition might have slightly changed. The new protocol includes a hydration step and a calibration step. To perform the hydration, first, an aliquot of Seahorse Calibrant Buffer (5 mL enough for 3 cartridges) is placed in a non-CO<sub>2</sub> 37°C and left for at least 12 h. Then, under a sterile cell culture hood, remove the top (green) part of the cartridge and place it upside down on your sterile bench. Add 200  $\mu$ L of sterile distilled water into each wells of the cartridge utility plate (clear, [Figure 2C](#) right panel). In order to prevent

evaporation, add 400  $\mu\text{L}$  of sterile water in the compartments (moats) on each side of the utility plate (Figure 2C, right panel).

- c. Place the green part of the cartridge back onto the utility plate, so the sensors are fully submerged.
- d. Incubate the cartridges at 37°C in a Non-CO<sub>2</sub> incubator. The cartridges can be wrapped with aluminum foil or parafilm to prevent any overnight evaporation.

**Note:** Once rehydrated, the cartridges can be short-term stored at 4°C up to 24–48 h before being used for any assay. They need to be kept wrapped in parafilm and aluminum foil to avoid evaporation and contamination. The leftover of Calibrant buffer should be discarded.

8. At least 3–6 h before the experiment: Turn on the Seahorse Flux Analyzer to make sure the instrument is set at 37°C at the time of experiment.
9. Prepare the program using the Wave software (Figure 2D) and save the file into the same USB that will be used for data collection.

**Note:** For the first tests of optimization, to our experience, adjustment of substrate, inhibitors and especially FCCP uncoupling concentration might be needed (as discussed in the [expected outcomes](#) and [troubleshooting](#) sections). If so, add FCCP working solution into the injection port C and add another injection and measurement in your program accordingly (Figure 2E).

**△ CRITICAL:** This is important to keep the wait time at minimum while assessing respiration in neonatal cardiomyocytes.

**Day of experiment:** Before you start, make sure to prepare a layout of your miniplate(s) that clearly indicates the treatment of your cells.

10. Prepare the FAO medium (up to 10–20 mL), and filter it with a 0.20  $\mu\text{m}$  filter gauge.

The FAO medium is a working solution that should not be stored or reused.

11. Under a sterile hood, wash the wells of the plate (containing cells) twice with 200–300  $\mu\text{L}$  of FAO medium.

Make sure the original culture medium is fully removed and washed out, as volume leftover may affect reagent concentration and cell response. It can also introduce well to well variation.

12. Add 130  $\mu\text{L}$  of FAO medium in each well and incubate the plate in a Non-CO<sub>2</sub> 37°C incubator for 60 min.
13. At the same time, remove the cartridge from the Non-CO<sub>2</sub> incubator. Remove the water from the wells of the utility plate and replaced it by the pre-warm aliquoted Seahorse Calibrant Buffer. Place the cartridge back to the Non-CO<sub>2</sub> incubator and incubate for 45–60 min.
14. Prepare the Seahorse reagent working solutions.
  - a. If using Agilent Mito Stress Kit, follow the manufacturer instructions to resuspend the pouches (Agilent Mito Stress Kit). The pouches are single-used and should not be stored.
  - b. If preparing solutions from powder-made stock, follow the preparation steps, as mentioned in [materials and equipment](#).
  - c. BSA-conjugated Palmitate: Pre-warm Palmitate and BSA stock solution at 52°C. Mix 100  $\mu\text{L}$  of Palmitate 10 mM + 200  $\mu\text{L}$  of 1 mM BSA in room temperature FAO medium (final volume = 1 mL) immediately.
  - d. Fatty-acid free BSA: Mix 200  $\mu\text{L}$  of 1 mM BSA stock with 800  $\mu\text{L}$  of FAO medium.
  - e. Oligomycin: Intermediate Dilution at 1:100 in FAO medium.

**Note:** The dilution is stable for few hours after preparation. Can be used for the preparation of working solution of several runs.

Working solution: 48  $\mu\text{L}$  of 1:100 dilution + 252  $\mu\text{L}$  of FAO medium.

Load 25  $\mu\text{L}$ /well (1  $\mu\text{M}$  / per well).

f. FCCP: Intermediate Dilution: 24  $\mu\text{L}$  of stock solution in 900  $\mu\text{L}$  of FAO medium.

Working solution: 200  $\mu\text{L}$  of dilution + 800  $\mu\text{L}$  of FAO medium.

Load 25  $\mu\text{L}$ /well (16  $\mu\text{M}$  / per well).

g. Rotenone: Intermediate Dilution #1): 10  $\mu\text{L}$  of stock solution in 990  $\mu\text{L}$  of FAO medium Intermediate Dilution#2): 1:100 of Dilution #1 in FAO medium.

Working solution: 60  $\mu\text{L}$  of Intermediate Dilution#2 + 240  $\mu\text{L}$  of FAO medium.

Load 25  $\mu\text{L}$ /well (0.5  $\mu\text{M}$  / per well).

**Note:** The stock solutions can be stored at  $-20^{\circ}\text{C}$  after resuspension. The working solution should not be reused from one experiment to another. However, while kept on ice, the intermediate dilutions can be reused for few hours.

15. Load the injection ports of a pre-equilibrated cartridge (still on the top of the utility plate, the sensors submerged in calibration medium) with oligomycin, FCCP and Rotenone/Antimycin A, in A to D according to your injection program (Figure 2C, left panel). With our method, the injection volume is equal to 25  $\mu\text{L}$  per port.

(If using the ready-to-use solutions from Agilent, the experimenters should refer to the manufacturer recommendation and protocol: Agilent Mito Stress Kit).

16. Bring the cartridge to the Seahorse Flux Analyzer. Start the program. Launch the calibration step, following the instrument instruction.

This calibration step should approximatively take 15–20 min, depending on the instrument.

17. Pre-treatment with Etomoxir needs to be performed in order to ascertain the impact of endogenous fuel source. Add 20  $\mu\text{L}$  of Etomoxir in the wells dedicated to Etomoxir-treatment at the beginning of the calibration time.

a. Etomoxir : 5  $\mu\text{L}$  of stock Etomoxir (at 30 mM) in 745  $\mu\text{L}$  of FAO medium. 20  $\mu\text{L}$  per well. (40  $\mu\text{M}$  per well).

18. Once the Seahorse instrument is ready and calibration is complete, add 25  $\mu\text{L}$  of working solution of BSA-conjugated-Palmitate (and/or Fatty acid-free BSA) in the wells.

19. Start the program by loading the cell plate into the instrument.

The program should be fully complete within 1 h and half.

20. At the end of the experiment, collect your dataset by exporting the file with a USB key.

The instrument is ready to start the second run, if needed.

If so, a new cartridge can be loaded for calibration.

21. Get your plate back from the Seahorse instrument.

Remove FAO medium and gently wash twice with PBS.

22. Add 10–20  $\mu\text{L}$  of cell lysis buffer (regular lab-made buffer or ready-to-use RIPA buffer). You should obtain enough material to estimate the protein concentration via a Bradford or BCA assay (see details in [quantification and statistical analysis](#) section below).

**Optional:** Depending on the relative protein abundance of the gene of interest, target gene disruption could be directly verified by running a Western blot from 5–10  $\mu\text{L}$  of this cell lysate (see more information below in the [quantification and statistical analysis](#) paragraph). If intended to do so, add protease/phosphatase inhibitor cocktail to the cell lysis buffer and quickly collect the cells while putting the plate on an ice-bed right after the end of the Seahorse experiment.

### EXPECTED OUTCOMES

#### Description of the expected OCR diagram

At the end of the experiment, you should obtain a stereotypical diagram representing the oxygen consumption rate (OCR) per minute, and this OCR should be affected by the drugs injected through the cartridge ([Figure 3A](#), top panel). The instrument also measures the extracellular acidification rate (ECAR, change in pH) ([Figure 3A](#), bottom panel) and the OCR/ECAR as an indicator of the cell energetic rate (not shown). The OCR usually dramatically changes in response to the combination of drugs (oligomycin, FCCP and Rotenone/Antimycin A), substrates in the assay medium (BSA versus Palmitate) and also in response to pre-incubation with etomoxir (see an example in [Figure 3B](#)).

In [Figure 3A](#), you can see an example of results for the LCFA oxidation assay for NMCs in response to Oligomycin, suboptimal versus optimal dose of FCCP and Rotenone/Antimycin A (and schematic representation in [Figure 3C](#)).

A reasonable average basal OCR for NMCs is between 100–200  $\text{pmol O}_2/\text{min}^{-1}$ , which is a little bit above Agilent recommendation but the averaged expectation for highly oxidative cells such as neonatal cardiomyocytes, as previously reported by our studies and others ([Angelini et al., 2021](#); [Chandra et al., 2018](#); [Powers et al., 2013](#)).

In response to Oligomycin, OCR should significantly decrease ([Figures 3A and 3C](#)), and ECAR should increase, because of the inhibition of the Complex V of the mitochondria respiratory chain ([Figure 3A](#) bottom panel).

(NB: Note that this increase in ECAR should be only an indicator of the complex inhibition, but it should not be used for any quantitative interpretation).

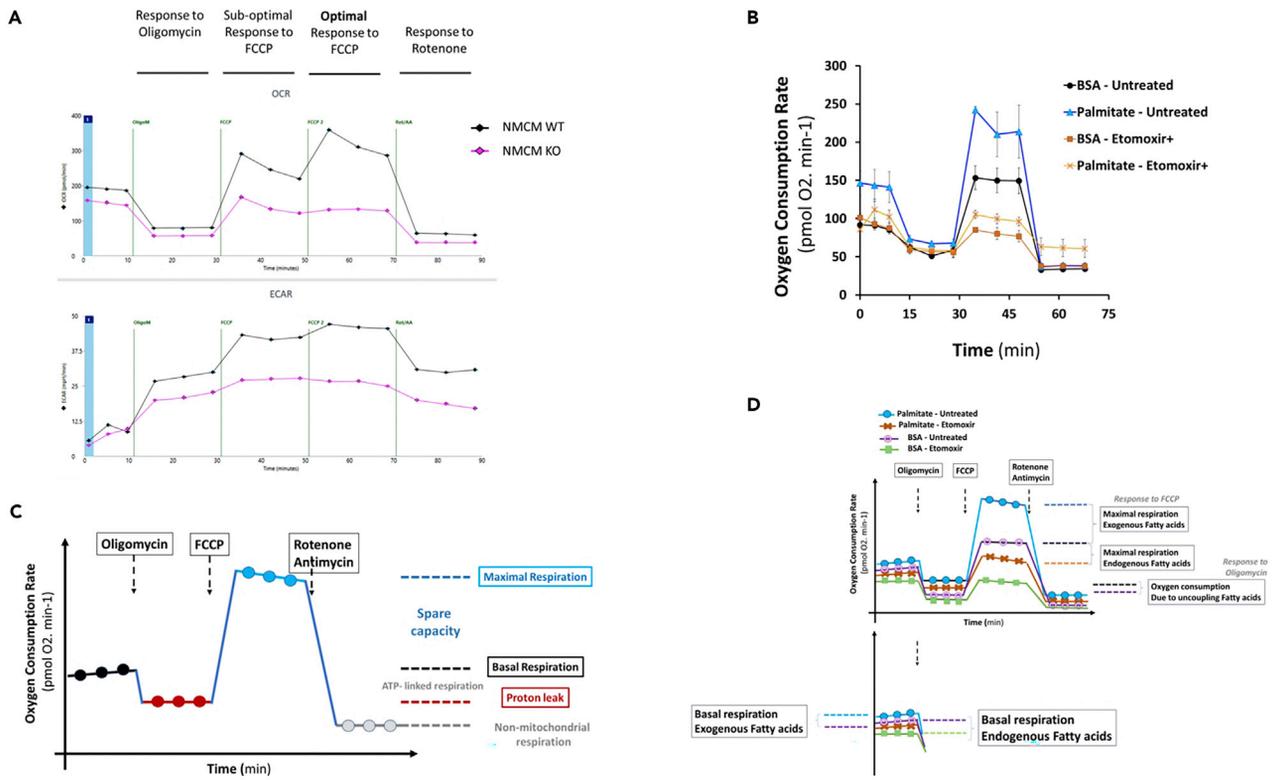
FCCP should lead to an increase in OCR and a consecutive acidification of the extracellular medium, due to the mitochondrial membrane uncoupling ([Figures 3A and 3C](#)).

The injection of Rotenone/Antimycin inhibits the complex I and the complex III respectively, leading to an immediate interruption of mitochondrial respiration ([Figures 3A and 3C](#)).

#### Interpretation of the diagram

The differences of OCR “plateau” before, after and in-between each drug injection provides some critical hints regarding the oxidative metabolism of the cells, as summarized on the right panel of [Figure 3C](#).

Briefly, the residual OCR after Rotenone/Antimycin provided the rate of non-mitochondrial respiration, i.e the oxygen consumption that is independent from fatty acid metabolism (cell enzymatic reactions). This non-mitochondrial respiration rate should be subtracted from any oxidation rate calculation below:



**Figure 3. Representative diagram of OCR during an assay of LC-FAO**

(A) Raw diagram as directly seen on the Wave software after a run performed in a Seahorse Flux Analyzer. For the purpose of the demonstration, the diagram has been annotated to more clearly identify the changes from basal values in response to Oligomycin, FCCP and Rotenone/Oligomycin (top panel). As discussed in the [troubleshooting](#) section, FCCP could require preparation/cell line optimization. In our test, the first injection of FCCP (4  $\mu$ M) was not enough to induce the optimal response of FCCP (obtained at the second injection, to reach a total concentration of 8  $\mu$ M). The pink line represents the data acquired for a well containing tamoxifen-treated PHD2/3 knocked-out cardiomyocytes (see [Angelini et al., 2021](#) for references). (C) Schematic analysis of the results. Each inhibitor is used to provide a specific asset of information regarding long-chain fatty acids oxidation. (B and D) (B) Example of OCR diagrams obtained in BSA/Palmitate-treated cardiac cells, without or in response to Etomoxir, and the schematic representation of the dataset in (D). Part of the dataset shown in the diagrams were obtained from experiments conducted for a previous study ([Angelini et al., 2021](#)), and presented with the permission of the co-authors.

The baseline OCR (before oligomycin) provided the *basal respiration* of the cell.

The difference between the basal OCR and the OCR after oligomycin represents the ATP-linked respiration.

The residual OCR after oligomycin represents the oxygen consumption due to interfering proton-leak.

The maximum OCR induced after FCCP represents the Maximal respiration capacity of the cells.

The difference of rate between FCCP-induced OCR and oligomycin-induced OCR enable the estimation of the Spare capacity (oxidative reserve capacity of the cells).

### Excepted outcome and interpretation in response to BSA or etomoxir

Another set of information can be generated while using BSA-only or etomoxir incubation (Figures 3B–3D). The OCR calculated in response to BSA enable to estimate the influence of endogenous fuel sources into the global oxygen consumption of the cells, hence providing the maximal inhibitory condition for the LCFA oxidation.

The pre-incubation of the cells with etomoxir disrupts the LCFA mitochondrial uptake and oxidation, through the inhibition of the Carnitine-Palmitoyl-O-Transferase 1B. Consequently, the combined usage of etomoxir and BSA provide a hint of the respective contribution of endogenous LCFA and additional fuel sources. We provided in [Figure 3B](#) an example of OCR calculation in response to palmitate vs BSA, without or with etomoxir. Interpretation of the diagram is also shown in [Figure 3D](#).

The difference in OCR between etomoxir-treated and untreated cells provided the rate of basal respiration for exogenous fatty acids (palmitate-incubated cells) and endogenous fatty acids (BSA-only-incubated cells), respectively ([Figure 3D](#), bottom panel).

The OCR difference between untreated-palmitate and untreated-BSA cells after oligomycin provides the oxygen consumption due to uncoupling fatty acids. The deviation of OCR after FCCP between Untreated-Palmitate OCR and Untreated-BSA OCR provides the Maximal oxidation due to exogenous fatty acids, while the difference between Untreated-BSA-OCR and Etomoxir-BSA-OCR generates the maximal rate of oxidation due to endogenous fatty acids.

However, to our experience, the reliance of the cardiomyocytes to fuel sources may be more extensively investigated through a three inhibitors combinatory approach using the MitoFuel Flex approach instead (Etomoxir for LCFA, UK5099 for Glucose/Pyruvate, BPTES for Glutamine), as we used in our previous study ([Angelini et al., 2021](#)).

### Excepted outcome in case of metabolic defects in the cells

In [Figure 3A](#), we provided an example of metabolic impairment in NMCMs. These cells were isolated from the conditional knock-out mouse we called “NMCM KO” in which tamoxifen triggers the disruption of Phd2 and Phd3 genes, encoding for the prolyl-4-hydroxylase domain proteins (PHD) 2 and 3, main dioxygen sensors of the cardiomyocytes. In our recent study ([Angelini et al., 2021](#)), we demonstrated that PHDs2/3 usually prolyl-hydroxylate CPT1B which favors CPT1B binding to VDAC1 and promotes LCFA mitochondrial uptake.

The loss of PHD2 and PHD3 in the NMCMs hence lead to the impaired LCFA oxidation, and this is what we could noticed while assaying the OCR in a SeaHorse Flux analyzer ([Figure 3A](#), pink line compared with the wildtype, blue line).

If ATP production is disrupted in your cells, the response to oligomycin can be less pronounced or milder than depicted in [Figure 3A](#).

But when the fatty acid  $\beta$ -oxidation is impaired (as it is the case in our NMCMs KO), the response to oligomycin and Rotenone is usually preserved, albeit the basal OCR can be lower. However, the response to FCCP is the most impaired ([Figure 3A](#). Pink line represents the OCR consumption of our PHDKO mouse cardiomyocytes ([Angelini et al., 2021](#))).

### Measurement and calculation

The dataset of the run is exported from the SeaHorse instrument via an USB flash disk. They are then opened into the Wave software (Agilent). The variations of OCR per each measurement time point can be observed. Groups can be assigned accordingly if your plate contains multiple groups of study. The newest version of the Wave software enables the printing of the complete summary, and an export of the dataset directly in Excel file.

## QUANTIFICATION AND STATISTICAL ANALYSIS

1. Protein quantification for normalization.

Use up to 5  $\mu\text{L}$  of cell lysate to perform the protein quantification method of your choice (Bradford or BCA), using a standard curve based on Bovine Serum Albumin. Usually 0–5  $\mu\text{g}\cdot\mu\text{L}^{-1}$  BSA graduation is enough for the quantification of the protein content of the wells of a Sea Horse plate. We provided in [Figure 3](#) an example of protein quantification. In this experiment, three plates of NMCMs were run the same day. The cell lysates of plate 1, 2 and 3 were collected and frozen immediately after each assay. They were stored less than 24 h at  $-20^{\circ}\text{C}$  and the Bradford assay was performed the following day.

Protein concentration provides a simple time-saving way to normalize the assays. It is also a duo-in-one method, since it can also provide enough material to run a Western blot later in the study.

The SeaHorse miniplates can be frozen and the protein concentration of every well can be estimated for all the plates at once in the days following the assay. The absorbance reading following the Bradford/BCA assay is performed on an unique 96-well plate using a plate reader, which limits the time and also the potential mistake margin from the experimenter bench side. The pipetting error can be easily monitored by running every sample at least in duplicate. This method also enable a pause time before the normalization of the assay. And the normalization can be also run at once for all the SeaHorse assays for a NMCM preparation.

However, this method only provides an overall estimation of the protein concentration without deciphering their origin (cardiomyocytes or contaminant fibroblasts) but no estimation regarding the effective number of cells in each well.

## 2. Normalization by cell number.

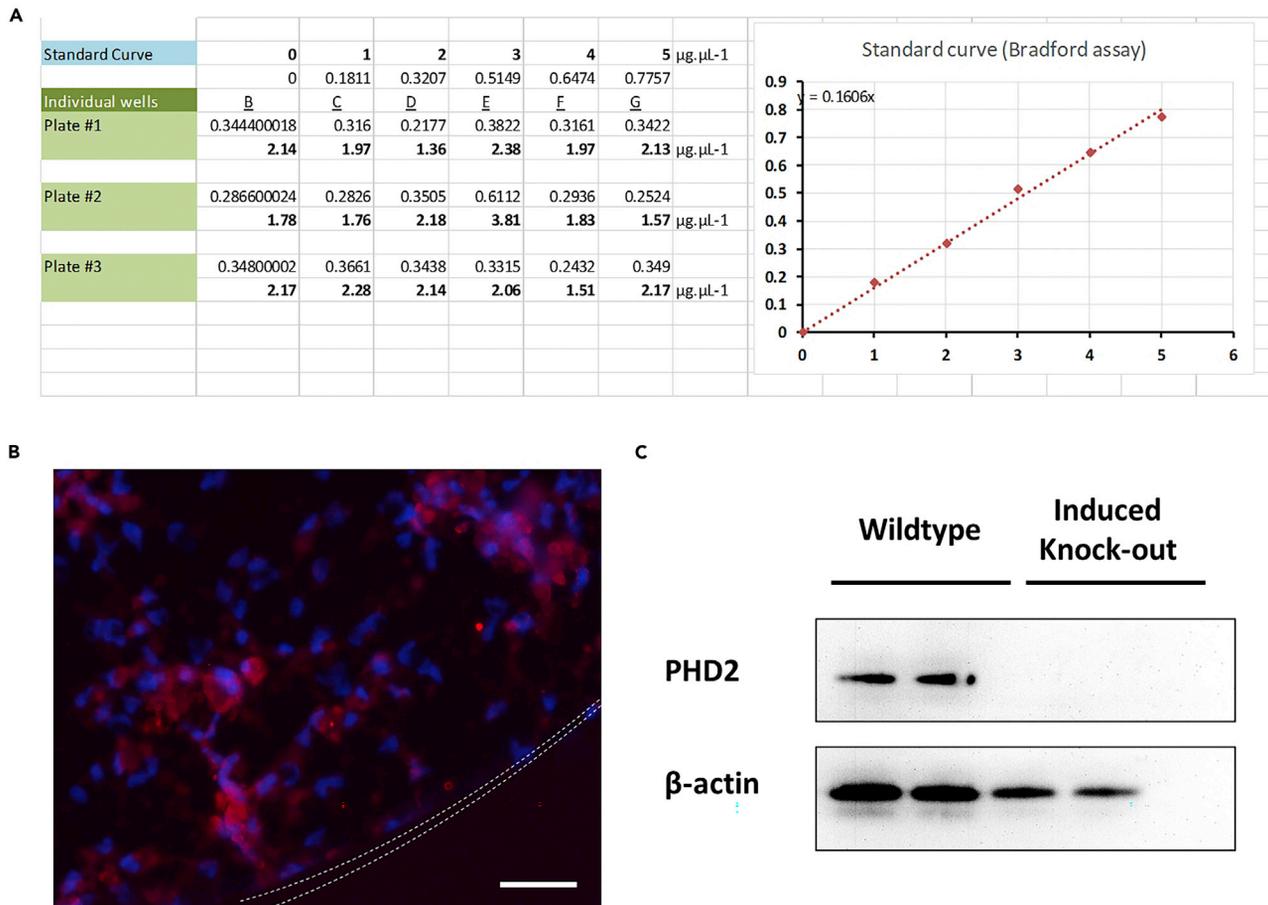
- a. DNA quantification: This method is based on the assumption that the content of DNA is linearly proportional to the number of cells, which might be the case in neonate mouse cardiomyocytes isolated from P0-P1 since they do not undergo diploidism at this stage of postnatal development ([Ikenishi et al., 2012](#)), and they are in post-mitotic state. Nuclear DNA quantification can be facilitated by the use of commercial kit as PicoGreen (Thermo Fisher Scientific P7589) in a regular plate reader. This method is also probably fast and time-saving, the authors have no expertise with these products. They might be recommended by Agilent for other formats that the SeaHorse miniplates (i-e: XFe96well or XF24 well plates), so the authors cannot guarantee that the assay is applicable for the SeaHorse miniplates.
- b. Cell counting: Ultimately, following the same concept that in 2a., the OCR can be also reported to the number of cells. Cell number can be estimated by counting the number of nuclei stained with DAPI or Hoescht at the end of the assay ([Figure 4B](#) is provided as an example of visualization of the cells stained with Dapi directly on a well of a SeaHorse miniplate). This method also provides an estimation of the number of cells that survived until the time of the assay, compared with the number of cells that were expected to be seeded on the wells at the end of the Part 2.

Both of these methods are recommended for normalization to cell numbers, but they provide a limited amount of additional information that could be extracted from the cells themselves compared to the total protein lysates.

However, this may be a good indirect way to ascertain the purity of the preparation. In fact, since the cardiomyocytes cannot proliferate, the number of nuclei should remain close to the number of cells seeded onto the plates at the beginning of the assay. A significantly higher (2–5 fold higher) should indicate a contamination of the assay by proliferating fibroblasts.

## 3. Mitochondrial content.

To our knowledge, it might be difficult to ascertain the mitochondrial content with such a small amount of cells per well. The experimenters should be able to verify the overall mitochondrial contents by Western blotting some abundant mitochondrial proteins (any TIMs, TOMs or CPT1B).



**Figure 4. Normalization method following a run of Seahorse**

(A) Screenshot from Excel representing the estimation of the protein concentration at the end of three separated runs of Seahorse (Plates # 1–3) by using Bradford method. On the left panel, plain text indicated the averaged absorbance (from duplicates), while the text in bold indicates the final estimated protein concentration, based on the equation of the Standard curve (shown on the right panel).

(B) Microscopic picture of NMCMs seeded on a well of a Seahorse Miniplate. Nuclei were counterstained with DAPI at the end of a Seahorse assay. The white dashed lines indicate the limit of the well (Scale bar: 100  $\mu\text{m}$ ).

(C) Representative Western blot performed with lysates from NMCMs isolated from wild-type (WT) or Cre-inducible Phd2/Phd3 knock out (KO) mouse pups. Cells were seeded on Seahorse miniplates and treated for 4 days with 4OH-tamoxifen, before the metabolic assay and the final sample collection. 5  $\mu\text{g}$  of total proteins were loaded in each lane.

Enzymatic activities for Citrate synthase or CPT1B could be also performed from NMCM lysates, but normalization through this approach may be challenging.

Following the normalization method used, OCR values can be reported as a rate to the protein concentration (OCR per  $\mu\text{g}$ ), or to the number of cells per well (OCR per 10,000 cells), or to the DNA if alternative methods were used. Regardless the methods, we highly encourage the experimenter to use the guidelines of the software that is more time-saving than our manual calculation method based on the old version of the software.

**Optional:** If using tamoxifen-inducible transgenic knockout NMCMs: If enough material has been collected following the Seahorse assay, a Western blot can be run to ascertain the decreased expression of the gene of interest.

In our case, prolyl-4-hydroxylase-domain-proteins 2/3 (PHDs) were successfully disrupted in the lysate of PHDHKO transgenic neonatal mouse cardiomyocytes after 4 days of pre-incubation with

4OH-tamoxifen (Figure 4C). Under the same conditions, the authors also successfully detected  $\beta$ -actin (used as a loading control in Figure 4C) and VDAC1 (date not shown).

## LIMITATIONS

To our knowledge, the method is reproducible and applicable to several cell types, including neonatal rat cardiomyocytes. However, multiple environmental parameters may affect the success rate of this experiment. The quality and the purity of the cells especially play a critical role in the outcome, and isolation of a healthy yield of NMCs may require some practice.

The SeaHorse assay may require some bench/experimenter adaptation as well, and finding the optimal concentration of substrates and/or pharmacological drugs remains a key of a success for this assay and its accurate interpretation.

In addition, the tamoxifen-induced gene disruption may have a lesser impact, compared with what we observed in response to our genes of interest. The gene knockout may be fully achieved within 3–4 days but the protein can remain stable for one-two weeks, which could be thus difficult to investigate and clearly assess with primary neonatal mouse cardiomyocytes.

## TROUBLESHOOTING

### Problem 1

No cells, or few cells attached (Part 1. Neonatal mouse cardiomyocyte isolation, end of step 3).

Neonatal mouse cardiomyocytes are especially sensitive during the isolation step and the first day of seeding. Cells that are not attached within 24–48 h are more likely dead.

### Potential solution

- Check the cell culture for contamination, verify the setting of the incubators (temperature, CO<sub>2</sub>, humidity).
- Check the serum lot number and expiration date. Prepare and filter a new medium.
- Make sure to timely control the condition (time, concentration) of the digestion step with trypsin and collagenase. Do not try to increase time or increase the concentration of enzymes.
- You may try to pre-coat the dishes with laminin or collagen to facilitate the cell seeding.

### Problem 2

Low cell viability (Part 1. Neonatal mouse cardiomyocyte isolation, end of step 3).

After the seeding, the cells start detaching and dying.

### Potential solution

- Check the cell culture for contamination, verify the setting of the incubators (temperature, CO<sub>2</sub>, humidity).
- Try to prepare a fresh medium and filter it.

### Problem 3

Many cells, proliferating but not beating (Part 1. Neonatal mouse cardiomyocyte isolation, end of step 3).

If the cells are sprinkle-shaped, flat, proliferating and not beating, they are more likely fibroblasts. The pre-plating step usually prevents the contamination with fibroblasts, especially on P0-P1 neonatal mouse heart. But contamination remains possible.

### Potential solution

- Make sure to not over-wash the plate after the pre-plating. Otherwise, this may detach the fibroblasts and increase significantly their yield.
- Increase the time of pre-plating, or eventually proceed to a second pre-plating.

### Problem 4

Cardiomyocytes are not beating ([Part 1. Neonatal mouse cardiomyocyte isolation](#), end of step 3 and/or [Part 2. Cell culture on Seahorse plate](#), step 5).

Culture conditions are critical to promote cardiomyocyte beating.

### Potential solution

- Make sure the incubator settings are correct (temperature, CO<sub>2</sub>, humidity), and that the culture is free from contamination.
- Replace the medium regularly by fresh medium.
- If you isolate cells from a newly designed transgenic mouse, make sure to isolate some control cells from pure background first and aside, in order to decipher problems in culture conditions to potential cell phenotype.

In our experience, hypoxic-like conditions (loss of PHDs) affect cardiomyocyte beating.

### Problem 5

Low OCR with no changes in response to drug ([Part 3. LCFA  \$\beta\$ -oxidation using Seahorse](#), step 20).

The diagram remains at a very low OCR level (between 50–70 pmol O<sub>2</sub>.min<sup>-1</sup>), and the effects of the drugs is proportionally low (see example in [Figure 5A](#)).

### Potential solution

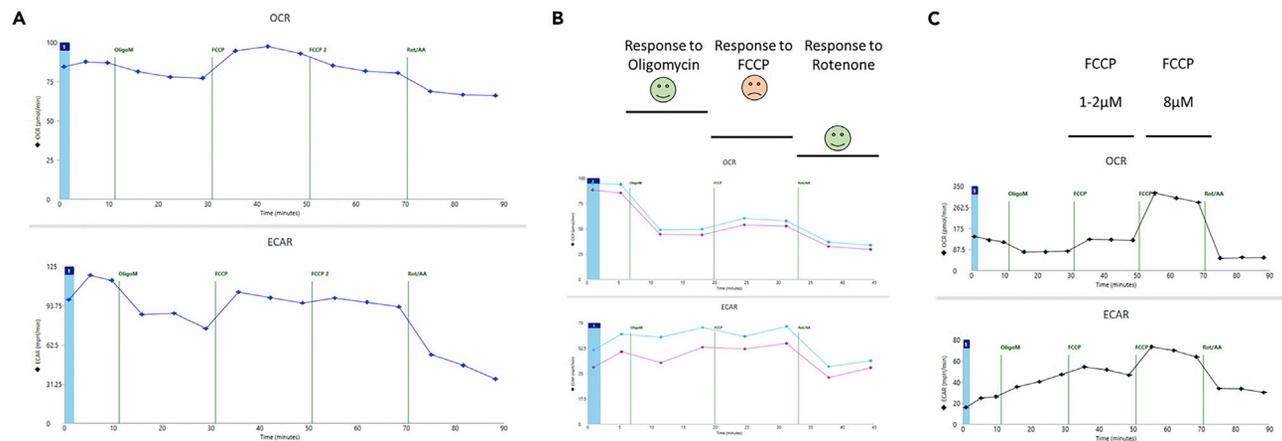
- Make sure the cells are alive, healthy and at the right density before starting the assay.
- Avoid touching the bottom of the wells with the tip of the pipet during the washing and medium change steps, as it could damage the integrity of your cells.
- If the cells were pre-incubated with any drug before the assay, verify that your stock solution is at the right concentration.
- Lastly, make sure that the cartridge was not reused, expired or damaged. Because unsealed/less efficiently sealed cartridge would cause leakage of the drugs; and leakage Rotenone solution would switch the cell metabolism in a non-respiratory state.

In the example ([Figure 5A](#)), it was an initial pipetting error that had led to a lower cell concentration in this specific well.

### Problem 6

No increase of OCR in response to FCCP ([Part 3. LCFA  \$\beta\$ -oxidation using Seahorse](#), step 20).

A 50% increase (compared to basal value) is already a fair good response to FCCP, but optimal uncoupling could reach up to 100% of the basal OCR value ([Figure 5B](#)). To our experience, the response to FCCP is the most sensitive to cell culture condition, but also to pH buffer. We provided an example of troubleshoot experiment in [Figure 5C](#), and optimization strategy as performed in neonatal rat cardiomyocytes.



**Figure 5. Troubleshooting of Seahorse assay**

(A) **Troubleshooting 5:** Low OCR and poor response to Mito Stress Test drugs. Diagram of OCR/ECAR representing a case of troubleshooting in neonatal mouse cardiomyocytes. B/C. **Troubleshooting 6:** Low response to FCCP.

(B) Case of troubleshooting.

(C) Optimization strategy of FCCP concentration (performed in neonatal rat cardiomyocytes).

### Potential solution

- It could be important to perform a pilot run with gradually increased concentrations of FCCP. The optimal concentration is obtained when OCR reaches a plateau (maximal respiration rate) at which non-additional increase of OCR could be obtained (Figure 5B).

As an example, we provide in Figures 5B and 5C the diagram representing our optimization strategy in NRCMs. As shown in Figure 5C, the first dose of FCCP was not enough to induce the full uncoupling of the mitochondrial membrane. A second injection of FCCP was followed by a significant and plateaued increase in OCR.

- If the experiment was previously working but no longer works, more likely that the stock of FCCP is too old or contaminated: When prepared from powder (as indicated above), FCCP stock looks bright yellow, but its color fades away when the compound starts getting oxidized and/damaged by freeze/thaw cycles.

### Problem 7

Irregular OCR and ECAR values (Part 3. LCFA  $\beta$ -oxidation using Seahorse, step 20).

- Fluctuating values for OCR or ECAR may indicate poor buffering capacities of the FAO medium. Outlying alterations of the pH values can be ascertained directly from the measurement performed per each well by the Seahorse instrument (record rawdata indeed include OCR, ECAR and pH). To prevent these artifacts, make sure the FAO medium is warm (37°C) while starting the assay. Verify that the stock KHB solution is still at 7.3–7.4 before running an experiment (measure pH at 37°C), because temperature (especially freezer long-term storage) may affect its pH.
- Verify that the incubator that is used for cartridge rehydration has no source of CO<sub>2</sub>, but a stable temperature. Low disturbance (open-close) should be maintained as much as possible during the whole time of calibration. Also, the time of cartridge degassing in the non-CO<sub>2</sub> incubator should not be significantly reduced.

To our experiences with the Seahorse Flux Analyzer, Problems 5 and 6 represent the main technical challenges of the method, while Problem 7 could occur occasionally, more likely due to the use of old

buffer or defective dehydration step. Many problems with unexpected data could be due to experimental mistake (loading the wrong compound/the wrong concentration).

If you encountered other problems that you cannot troubleshoot, feel free to contact the technical correspondent author for potential assistance.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Liang Xie ([liang.xie@bcm.edu](mailto:liang.xie@bcm.edu)).

#### Materials availability

This protocol did not generate new unique reagents.

#### Data and code availability

The dataset generated and the program can be provided upon request.

Supplemental movie is available as a Mendeley Data: <https://doi.org/10.17632/fdfjvh9brm.1>.

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101392>.

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### AUTHOR CONTRIBUTIONS

A.A. and L.X. wrote the manuscript. A.A. optimized the protocol and performed the experiments using the SeaHorse Flux Analyzer.

### DECLARATION OF INTERESTS

The authors have no conflict of interest.

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