OROBOROS O2k-Protocols

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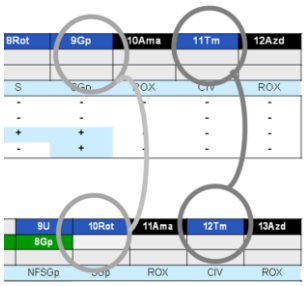
Updates: http://wiki.oroboros.at/index.php/MiPNet21.06_SUIT-RP

SUIT reference protocol for OXPHOS analysis by high-resolution respirometry



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Contents

Summary: We developed a substrate-uncoupler-inhibitor titration (SUIT) protocol with the aim to provide a common reference for comparison of respiratory control in mt-preparations obtained from a large variety of species, tissues and cell types. A SUIT reference protocol (SUIT-RP) is required for establishing a database on comparative mitochondrial physiology. The SUIT-RP is applied in the MitoFit proficiency test with HEK 293T cells. It includes a large number of chemicals used in various specific SUIT protocols, subjecting these to quality control in the MitoFit proficiency test.

The SUIT-RP consists of two harmonized SUIT protocols. SUIT-RP1 starts with linear coupling control, *L* – *P* - *E*, with the type N substrates pyruvate and malate (N-pathway to Q: CI-linked), thus separating coupling control and the subsequent linear sequence of pathway control in the ETS state (Figure 1). SUIT-RP2 has a focus on OXPHOS capacity of fatty acid oxidation (FAO_P; F-pathway) compared to OXPHOS capacity with combined NF-type substrates (NF-pathways to Q: $CI\&FAO_P$). RP2 adds a sequence of pathway control steps to measure maximum OXPHOS and ETS capacity with a NFSGp substrate combination to activate pathways converging at the Q-junction through Complex I (CI), electron-transferring flavoprotein complex (CETF), Complex II (CII), and glycerophosphate dehydrogenase complex (CGpDH) ($PGMSOctGp_E$; NFSGp–pathways; Figure 2). Finally, RP1 and RP2 provide information on the activity of the single enzyme step of Complex IV (CIV) downstream of Q. These SUIT-RP are harmonized (Figure 3) such that they can be statistically evaluated as repeat measurements of cross-linked respiratory states, while additional information is obtained when the two protocols are conducted in parallel. Therefore, RP1 and RP2 are complementary with their focus on specific respiratory coupling and pathway control aspects, extending previous strategies for respirometric OXPHOS analysis.

SUIT reference protocols

RP1: 1PM 2D 3c (3NADH) 4U 5G 6S 7Oct 8Rot 9Gp 10Ama 11Tm 12Azd
RP2: 1D 2Oct 3M 4c (4NADH) 5P 6G 7S 8Gp 9U 10Rot 11Ama 12Tm 13Azd

1. SUIT RP1: N(*L-P-E*) coupling control

PM +mt: NFSGpTm_1PM 2D 3c 4U 5G 6S 7Oct 8Rot 9Gp 10Ama 11Tm 12Azd

	4U	5G	6S	7Oct	8Rot	9Gp	10Ama	11Tm	12Azd
E									
P	2D 3c								
L	1PM								
	N	N	NS	NFS	S	SGp	ROX	CIV	ROX
CI	+	+	+	+	-	-	-	-	-
CETF	-	-	-	+	-	-	-	-	-
CII	-	-	+	+	+	+	-	-	-
CGpDH	-	-	-	-	-	+	-	-	-

Figure 1. RP1: N(*L-P-E*) coupling control.

SUIT protocol category: NFSGpCIV

SUIT protocol subcategory: N+NS+NFS+S+SGp+CIV

SUIT protocol acronym:
NFSGpTm_1PM 2D 3c 4U 5G 6S 7Oct 8Rot 9Gp 10Ama 11Tm 12Azd

RP1 spotlights

- N or CI-linked linear coupling control: *L* – *P* - *E*, thus separating coupling control (*L-P-E*) and pathway control (in the ETS state).

- Oct is added in the ETS state to avoid uncoupler effect with FA.
- N_E (4U and 5G), NS_E (6S), NFS_E (7Oct), S_E (8Rot) and SGp_E (9Gp) are measured. If $NFS_E \approx NS_E$, then this sequence allows calculation of the additivitiy index of N- and S- linked ETS capacity (related to supercomplex-channeling). This criterium is tested in step 7Oct, evaluating the effect of Oct on NS_E (6S).
- To compare RP1 with RP2: harmonization between protocols in states SGp_E, (RP1:9Gp, RP2:10Rot), and CIV_E (RP1: 11Tm, RP2: 12Tm).
- If Oct is without effect on NS_E (N=PGM; expected in many types of mt), then additional harmonization between protocols is obtained in states PM_P (RP1:2D) = $PMOct_P$ (RP2:5P).
- Harmonization with many previous SUIT protocols up to step 6Rot.

Limitations

- $NFSGp_E$ is not obtained (substrate combination for maximum ETS capacity), in favour of measuring S_E (8Rot). This reference state has to be calculated using the NFS_E/$NFSGp_E$ (9Gp/10Rot; N=PGM) ratio between RP1 and RP2.

RP1mt mitochondrial preparation: isolated mitochondria (imt), tissue homogenate (thom), and permeabilized fibers (pfi). See Supplement.

Step	State	Comment
PM		CI-linked substrates are added to the medium before mt (mtprep). The state without added substrates is not well defined, slightly higher than ROX due to the presence of some endogenous substrates (shown by a slight decline of respiration and mt-membrane potential upon inhibition by Rot; Krumschnabel et al 2014).
+mt		Incubation up to 20 min to allow stabilization of flux when using high oxygen or during slow exhaustion of endogenous substrates, to obtain N_L.
1PM	PM_L	N-linked LEAK state.
2D	PM_P	OXPHOS coupling efficiency (*P-L* or $\approx P$ control factor), $j_{\approx P}$ = $\approx P/P$ = $(P\text{-}L)/P$ = $1\text{-}L/P$, is measured in the N-linked pathway state (with a possible contribution by partially activating CII-linked respiration; Sumbalova et al 2016a), with defined coupling sites (CI, CIII, CIV) and at high flux compared to FAO (human skeletal muscle: compare with F_P and N_P; Gnaiger et al 2015).

3c	PMc_P	Quality control test early in the SUIT protocol. In pathological states with *c* release, early addition of *c* provides information on the FCF_c = 1-N/Nc, and separates the FCF_c from other injuries in the subsequent respiratory states. All subsequent states contain *c*, which is not explicitely written in the following substrate states.
3NADH		NADH is titrated only in case of a high cytochrome *c* control factor, FCF_c>0.1, to check for the presence of inverted mitochondrial particles and permeability of the inner mt-membrane. If FCF_c<0.1, then the high intactness of the outer mt-membrane provides an indication for intactness of the inner mt-membrane, and thus NADH does not have to be added.
4U	PM_E	CCCP is titrated stepwise to maximum flux, to evaluate limitation of OXPHOS by the phosphorylation system, expressed as the apparent excess *E-P* capacity factor (*E-P* coupling control factor), j_{ExP} = (*E-P*)/*E* = 1-*P*/*E*. If j_{ExP}>0, then the ETS coupling efficiency rather than the OXPHOS coupling efficiency is the proper expression of coupling, $j_{\approx E}$ = ≈*E*/*E* = (*E*-*L*)/*E* = 1-*L*/*E*.
5G	PGM_E	FCR_G = 1-PM/PGM, reveals an additive effect of convergent electron flux through NADH (N-linked), with a possible contribution by partially activating S-linked respiration.
6S	$PGMS_E$	FCF_S = 1-N/NS. It may be important to check if the uncoupler concentration titrated in the PM substrate state is also sufficient for this substrate state.
7Oct	$PGMSOct_E$	FCF_F = 1-NS/NFS. This *FCF* is low or zero in many mt-types. Then also state PM_P is identical to $PMOct_P$ in RP2, and may thus further link the two protocols in the SUIT reference assay for statistical analysis (protocol harmonization). Inhibition is observed at higher FA concentrations.
8Rot	S_E	FCF_{NF} = 1-S/NFS. Rot inhibits CI and FAO simultaneously. In some cases it takes very long, until a steady state is reached after inhibition by Rot. Addition of Gp before Rot would not allow a valid estimation of S-linked capacity (compare RP2).
9Gp	SGp_E	Gp-linked capacity is not measured isolated from S in the SUIT reference protocol. FCF_{Gp} = 1-S/SGp. This late addition of Gp is a compromise

		for evaluation of the Gp-linked capacity. Malonic acid does not effectively inhibit CII at S_{50} (competitive inhibition at 50 mM succinate). Little is known about the diagnostic value of this Gp-flux control factor. The substrate Gp is expensive.
10Ama	ROX	Inhibition may take very long, particularly in human muscle fibres (Pesta et al 2011; Lemieux et al 2011). This may make ROX correction questionable, particularly if ROX is high in comparison with the initial LEAK state, N_L.
11Tm	CIV_E	Ascorbate (As) is added before TMPD (Tm). $Tm_{0.5}$ is not saturating CIV, and thus represents a compromise, to prevent a too high chemical O_2 background. Apparent CIV activity may thus be lower than ETS capacity determined in the same run. Inhibitor-threshold titrations would be required.
12Azd	ROX	Cyanide is avoided due to the presence of P, but very high Azd concentrations are required. The oxygen dependence of the chemical O_2 is evaluated by a reoxygenation soon after titration of Azd, and can be semi-automatically performed by using the DatLab background calibration function (Slope).

RP1pce (permeabilized cells). See Supplement.

Step	State	Comment
mt	*R*	In experiments with intact cells (ce), ROUTINE respiration (*R*) is measured initially, based on endogenous substrates.
PM		CI-linked substrates are added to the medium after mt (ce).
Dig	PM_L	Digitonin permeabilizes the plasma membrane. CI-linked LEAK state of permeabilized cells (pce).

2. SUIT RP2: F-N and NFSGp pathway control

SUIT protocol category: NFSGpCIV

SUIT protocol subcategory: F+NF+NFS+NFSGp+SGp+CIV

SUIT protocol acronym:
NFSGpTm_1D 2Oct 3M 4c 5P 6G 7S 8Gp 9U 10Rot 11Ama 12Tm 13Azd

D+mt: NFSGpTm_1D 2Oct 3M 4c 5P 6G 7S 8Gp 9U 10Rot 11Ama 12Tm 13Azd

E						9U	10Rot	11Ama	12Tm	13Azd
P	1D	2Oct3M4c	5P	6G	7S	8Gp				
L										
	ROX	F	NF	NF	NFS	NFSGp	SGp	ROX	CIV	ROX
CI	-	-	+	+	+	+	-	-	-	-
CETF	-	+	+	+	+	+	-	-	-	-
CII	-	-	-	-	+	+	+	-	-	-
CGpDH	-	-	-	-	-	+	+	-	-	-

Figure 2. RP2: F-N and NFSGp pathway control.

RP2 spotlights

- Depletion of endogenous substrates with D (1D; State 2).
- F_P (3M) compared to NF_P (5P).
- The full set of pathways converging at Q, NFSGp, is covered (8Gp and 9U in coupling states *P* and *E*), and thus the maximum apparent excess *E-P* capacity factor, j_{ExP} = 1-*P*/*E*, can be calculated.
- Harmonization between protocols RP1 and RP2 in states SGp_E, (RP1:9Gp, RP2:10Rot), and CIV_E (RP1: 11Tm, RP2: 12Tm).
- Harmonization with many previous protocols up to S.
- *P*/*E* (8Gp/9U) at high ETS capacity compared to RP1.

Limitations

- S_E is not obtained (but it is obtained in RP1).

RP2mt Mitochondrial preparation: isolated mitochondria (imt), tissue homogenate (thom) and permeabilized fibers (pfi). See Supplement.

Step	State	Comment
+D		In experiments with mt-preparations (mtprep), ADP is added to the medium before the mt.
+mt		D accelerates the depletion of endogenous substrates.
1D	ROX	Substrate depleted ROX state (State 2; Chance, Williams 1955).
2Oct	Oct	Oct alone does not establish an ETS (and OXPHOS) competent substrate state in many mt-types, since M is required to form oxaloacetate and prevent accumulation of acetyl-Co A by the citrate synthase reaction.
	Oct_P	Stimulation of OXPHOS by Oct alone in the presence of D indicates an obscure mechanism of anaplerosis or the presence of N-substrates in the medium.
3M	$OctM_P$	M is titrated stepwise: M.05; M.1; M2. Note that M alone can support OXPHOS if mt-malic enzyme is active, and thus FAO may be overestimated.

4c		See RP1.
4NADH		See RP1.
5P	$PMOct_P$	M_2 is required to reduce flux through CII (minimize inhibition by malonate), such that N-linked OXPHOS capacity can be estimated without high scope of compensation by S-linked respiration (Sumbalova et al 2016a). GM_P includes a higher share of S-linked respiration in comparison with PM_P. FCF_N = 1-F/NF, important information on training status or cardiac failure (Pesta et al 2011; Lemieux et al, 2011).
6G	$PGMOct_P$	The state NF_P is obtained.
7S	$PGMSOct_P$	The state NFS_P is obtained.
8Gp	$PGMSOctGp_P$	RP2 focuses on maximum *P,* evaluating additivity of NFSGp in OXPHOS state. FCF_{Gp} = 1- NFS/NFSGp.
9U	$PGMSOctGp_E$	CCCP is titrated in the NFSGp state with high ETS capacity, to evaluate limitation of OXPHOS by the phosphorylation system. The apparent excess *E-P* capacity factor (*E-P* coupling control factor), j_{ExP} = (*E-P*)/*E* = 1-*P*/*E*, is measured in the state of maximum ETS capacity.
10Rot	SGp_E	This state is not a generally valid estimate of S_E (compare RP1 where S_E is obtained). The state SGp_E is identical in RP1 and RP2, and may thus link the two protocols in the SUIT reference assay for statistical analysis (protocol harmonization).
11Ama	ROX	See RP1.
12Tm	CIV_E	See RP1.
13Azd	ROX	See RP1.

RP2pce Permeabilized cells (pce). See Supplement.

Step	State	Comment
mt	*R*	See RP1pce.
0Dig		See RP1pce.
1D	ROX	See RP1pce.

3. Harmonization between RP1 and RP2

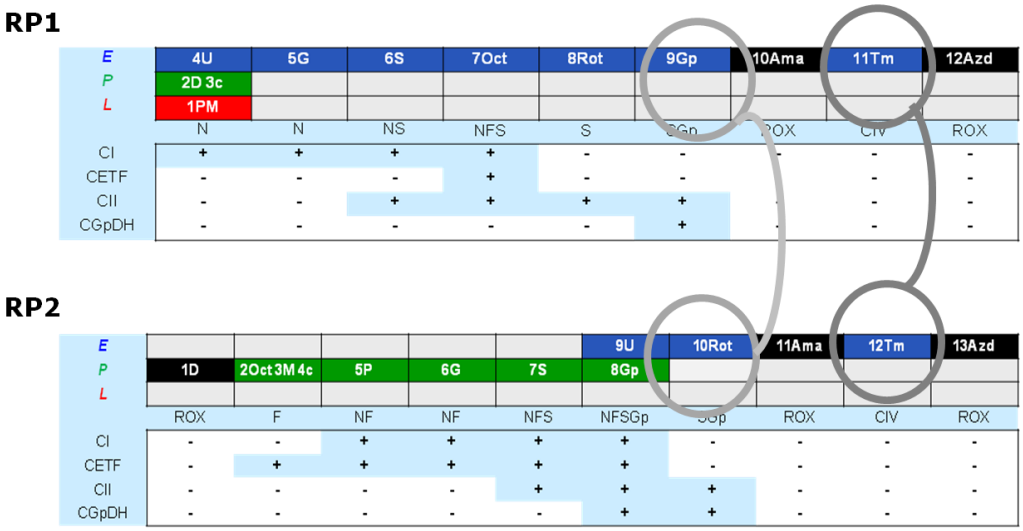

Figure 3. Harmonization of RP1 and RP2.

4. Reference protocols in mitochondrial models

4.1. SUIT-RP in HEK 293T cells

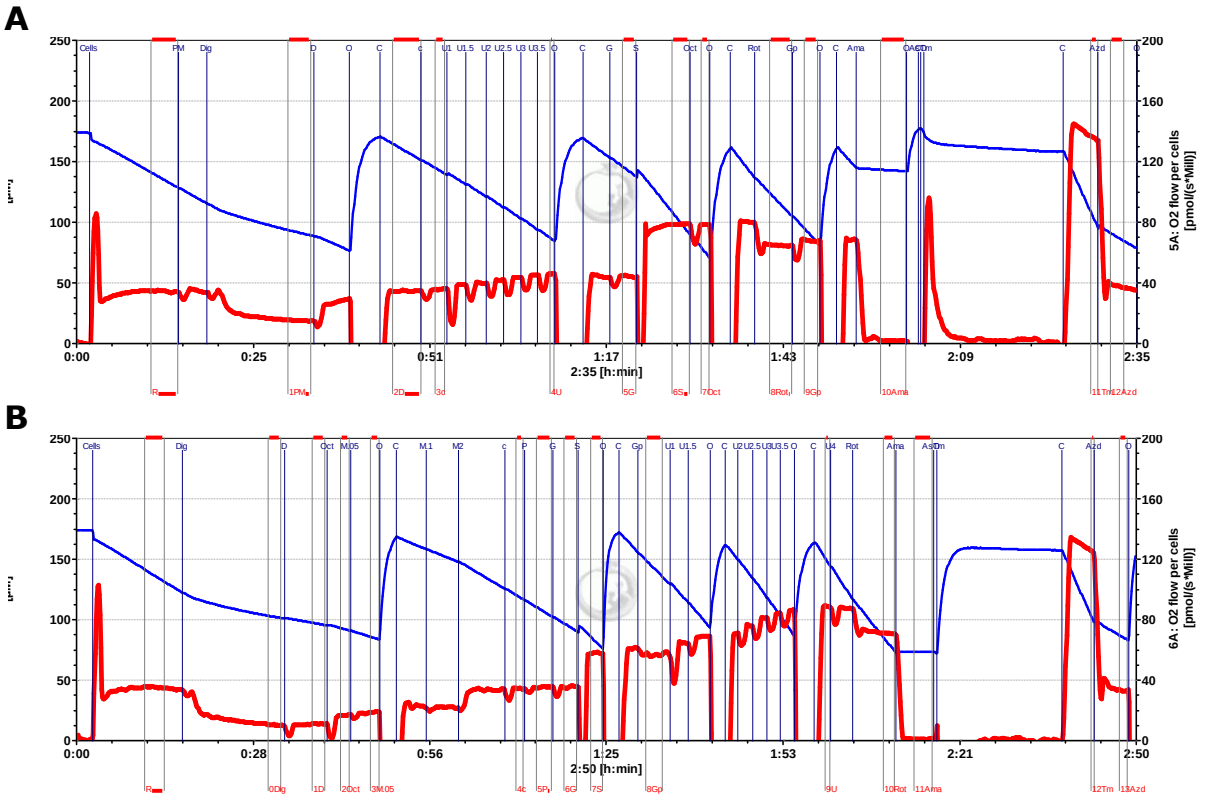

Figure 4. RP1 and RP2 in permeabilized HEK 293T cells. **(A)** RP1 and **(B)** RP2 performed in parallel in MiR06Cr at 37 °C and normoxic conditions. Oxygen concentration ([µM] blue line) and oxygen flow per cells [pmol·s^{-1}·Mill^{-1}] (red line).

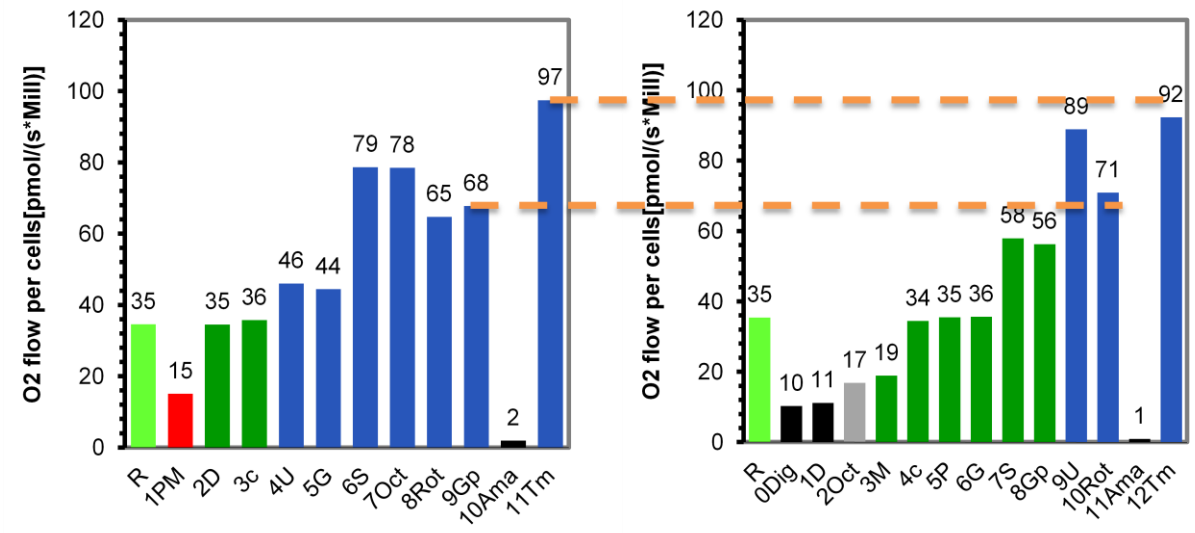

Figure 5. Harmonization between RP1 and RP2 in permeabilized HEK 293T cells.

4.2. SUIT-RP in brain tissue homogenate

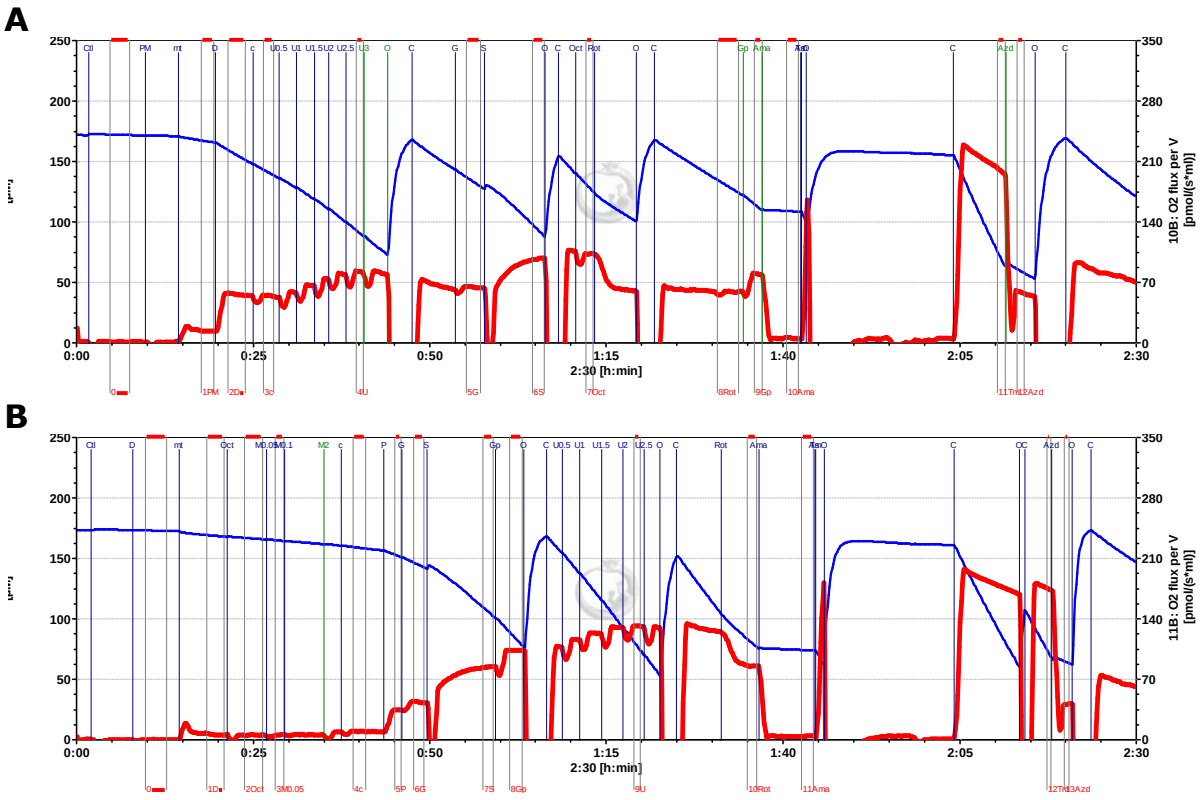

Figure 6. HRR traces obtained in brain tissue homogenate (thom) from mouse after ischemic damage. **(A)** RP1 and **(B)** RP2 carried out in parallel in MiR06Cr at 37 °C and normoxia. Oxygen concentration ([µM] blue line) and oxygen flux per tissue mass [pmol·s^{-1}·mg^{-1}] (red line).

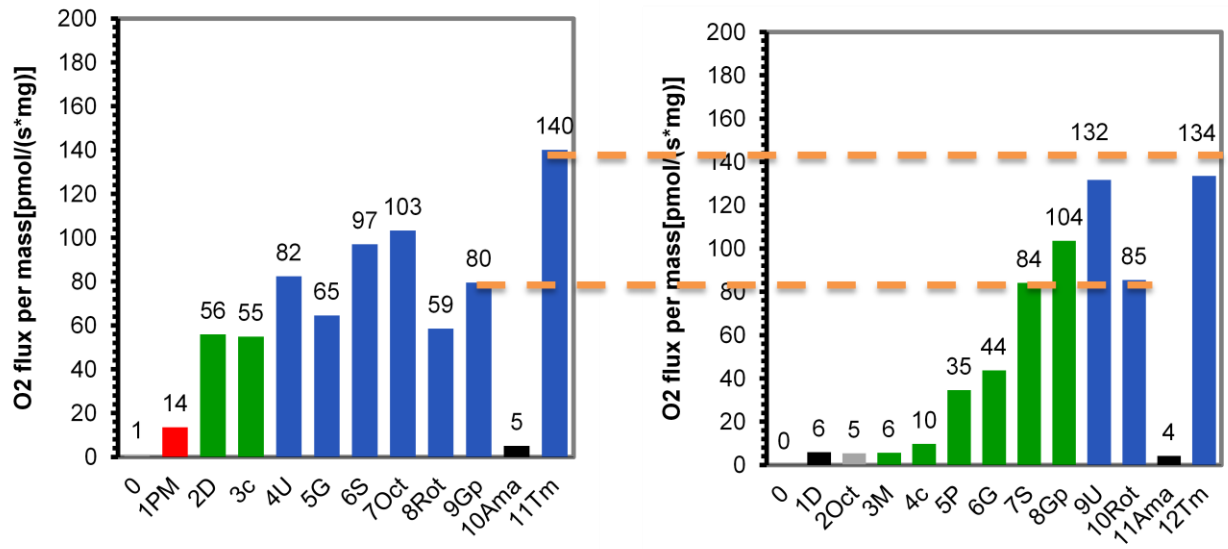

Figure 7. Harmonization between RP1 and RP2 in brain thom after ischemic damage.

5. Test experiments

Test experiments are required to finalize the RP2 for specific applications.

G 10 mM may not be saturating, and higher concentrations should be checked in a test experiment.

Gp Different sources of Gp are tested (sn-Glycerol 3-phosphate bis(cyclohexylammonium) salt, Sigma G7886 and sn-Glycerol 3-phosphate lithium salt, Sigma, 94124). Gp (sn-Glycerol 3-phosphate lithium salt, 94124 Sigma) is expensive. In preliminary experiments we did not find differences between both Gp types.

5.1. SUIT RP1

D D is tested to be saturating in NFSGp$_P$. 7.5 mM (in pfi) may not be saturating in all cases, and higher concentrations of ADP should be checked.

Depletion of endogenous substrates with D is possible.

Oct Oct.5 (0.5 mM) might be generally applicable, but in preliminary experiments a higher concentration (1 mM) should be evaluated to check for saturation of flux.

+S Step titration from S_{10} to S_{50} to test if S_{10} is saturating NS- and S-linked respiratory capacity. If fluxes with S_{50} > S_{10} in NS$_E$, then S_{50} is added immediately in the OXPHOS state. If S_{50} < S_{10}, then it is tested if S_{50} > S_{10} in S_E, in which case S_{50} is only added in S_E. If S_{10} is

saturating in all states, S_{50} may be tested only occasionally, to exclude a shift in the succinate kinetics (in pathologies, ageing, etc).

Dig — Optimal digitonin concentration has to be tested in different types of cells for permeabilization of the plasma membrane.
» http://wiki.oroboros.at/index.php/Digitonin

5.2. SUIT RP2

Oct — $Oct_{0.5}$ is tested to be saturating in OXPHOS and not inhibiting or uncoupling (titration of high Oct after M.05 or M.1).

M — $M_{0.1}$ is tested to be saturating FAO in OXPHOS without activating N-linked respiration beyond F-linked capacity (HEK: mtME). M should be titrated stepwise (M.05; M.1; M2) in the presence of D, to compare the malate kinetics of F_P and N_P.

6. Technical details

Temperature 37 °C.
Data recording interval: 2 s.
Effective chamber volume: 2 ml
Stirrer speed 750 rpm.

DatLab file — The default name of the DatLab file contains the date, Power-O2k number and serial experimental number for each day. Example: 2016-01-17 P1-02.DLD

Events — Set an 'Event' in DatLab at the time of titration. Use the abbreviated event name, and add information in the comment.

MiR05+CtlCr — Catalase (Ctl) is present in all cells, hence addition of Ctl is considered physiological, even if reoxygenations are not required with H_2O_2. MiR06=MiR05+Ctl

Creatine (Cr) is present in many vertebrate cells, and thus should be added generally. With Cr, lower ADP concentrations are saturating for OXPHOS. It may be argued that it should be replaced in invertebrates (*Drosophila, C. rabditis*).

MiR06Cr / O2 — Mitochondrial respiration medium, 2 ml in the O2k-chamber, plus 100 µl in the capillary of the stopper (more accurately: 88 µl without meniscus). Increase the oxygen concentration to ~450 µM for pfi experiments. Close the chamber.

mt — mt-preparation: isolated mitochondria (imt), permeabilized fibers (pfi), tissue homogenate (thom).

pce	Permeabilized cells.
D	If there is time available (20 min), this period may yield a single point for the instrumental high-O2k background. D may be added just before titrating mt (imt, thom) or before opening the chamber for addition of pfi.
pfi / O2	During addition of pfi, the O_2 concentration drops and should be increased immediately to ~450 µM before closing the O2k chamber.
U	'Slope smoothing' should be reduced to 20 (=20 data points used for calculation of the slope), to evaluate very quickly the stimulation of respiration and the need for additional titration steps of CCCP. If only FCCP (more expensive) is available, this can be used and be fully compared with CCCP titrations (a minimally higher CCCP than FCCP concentration may be required for maximum flux).
Cleaning	After the experiment clean the O2k chambers: 3x water, 1x liver homogenate (20 min), 3x water, 3x EtOH 70% (5 min), 1x EtOH 100% (15 min).

O2k-cleaning SOP
» http://bioblast.at/index.php/MiPNet19.03_O2k-cleaning_and_ISS

Full version with references
» http://wiki.oroboros.at/index.php/MiPNet21.06_SUIT_reference_assay

PM +mt: NFSGpTm_1PM 2D 3c 4U 5G 6S 7Oct 8Rot 9Gp 10Ama 11Tm 12Azd

E	4U	5G	6S	7Oct	8Rot	9Gp	10Ama	11Tm	12Azd
P	2D 3c								
L	1PM								
	N	N	NS	NFS	S	SGp	ROX	CIV	ROX
CI	+	+	+	+	-	-	-	-	-
CETF	-	-	-	+	-	-	-	-	-
CII	-	-	+	+	+	+	-	-	-
CGpDH	-	-	-	-	-	+	-	-	-

O2k and DatLab file: **P**___ (A / B) 2016- Operator:
Sample type: Cohort: Sample code:
Sample.Subsample number: Unit: Concentration:
Medium:

Event	Mark name	State	Final conc. 2 ml O2k	Stock [mM]	Comment	Tit. [µl]	A	B
MiR								
O2			~200 µM		~450 µM O_2 for pfi			
P			5 mM	2000		5		
M	**0**		2 mM	400		10		
mt								
O2	**1PM**	PM_L			~450 µM O_2 for pfi			
D	**2D**	PM_P	2.5 mM	500	7.5 mM (30 µl) for pfi	10		
c	**3c**	PM_{Pc}	10 µM	4	NADH only if FCF_c >.1	5		
U	**4U**	PM_E	Δ0.5 µM	1	CCCP	Δ1		
G	**5G**	PGM_E	10 mM	2000		10		
S	**6S**	$PGMS_E$	50 mM	1000		100		
Oct	**7Oct**	$PGMSOct_E$	0.5 mM	100		10		
Rot	**8Rot**	S_E	0.5 µM	1		1		
Gp	**9Gp**	SGp_E	10 mM	1000		20		
Ama	**10Ama**	ROX	2.5 µM	5		1		
As			2 mM	800		5		
Tm			0.5 mM	200	~20 min open, C	5		
C	**11Tm**	Tm_E			~450 µM O_2 for pfi			
Azd	**12Azd**	ROX	≥100 mM	4000	~5 min	100		

D+mt: NFSGpTm_1D 2Oct 3M 4c 5P 6G 7S 8Gp 9U 10Rot 11Ama 12Tm 13Azd

E						9U	10Rot	11Ama	12Tm	13Azd
P	1D	2Oct 3M 4c	5P	6G	7S	8Gp				
L										
	ROX	F	NF	NF	NFS	NFSGp	SGp	ROX	CIV	ROX
CI	-	-	+	+	+	+	-	-	-	-
CETF	-	+	+	+	+	+	-	-	-	-
CII	-	-	-	-	+	+	+	-	-	-
CGpDH	-	-	-	-	-	+	+	-	-	-

O2k and DatLab file: P___ (A / B)	2016-	Operator:
Sample type:	Cohort:	Sample code:
Sample.Subsample number:	Unit:	Concentration:
Medium:		

Event	**Mark name**	**State**	**Final conc. 2 ml O2k**	**Stock [mM]**	**Comment**	**Tit. [µl]**	**A**	**B**
MiR								
O2			~200 µM		~450 µM for pfi			
D	**0**		2.5 mM	500	7.5 mM (30 µl) for pfi	10		
mt								
O2	**1D**	ROX	~200 µM		~450 µM for pfi			
Oct	**2Oct**	Oct	0.5 mM	100		10		
M.05	**3M.05**	Oct_P	0.05 mM	50		2		
M.1	**3M.1**	Oct_P	0.1 mM	50		2		
M2	**3M2**	Oct_P	2 mM	400		9.5		
c	**4c**	Oct_{Pc}	10 µM	4	NADH only if FCF_c >.1	5		
P	**5P**	$PMOct_P$	5 mM	2000		5		
G	**6G**	$PGMOct_P$	10 mM	2000		10		
S	**7S**	$PGMSOct_P$	50 mM	1000		100		
Gp	**8Gp**	$PGMSOctGp_P$	10 mM	1000		20		
U	**9U**	$PGMSOctGp_E$	Δ0.5 µM	1	CCCP	Δ1		
Rot	**10Rot**	SGp_E	0.5 µM	1		1		
Ama	**11Ama**	ROX	2.5 µM	5		1		
As			2 mM	800		5		
Tm			0.5 mM	200	~20 min open, C	5		
C	**12Tm**	Tm_E			~450 µM O_2 for pfi, C			
Azd	**13Azd**	ROX	≥100 mM	4000	~5 min	100		

SUIT reference protocol RP1pce

2016-08-17

mt +PM +Dig: NFSGpTm_1PM 2D 3c 4U 5G 6S 7Oct 8Rot 9Gp 10Ama 11Tm 12Azd

E	4U	5G	6S	7Oct	8Rot	9Gp	10Ama	11Tm	12Azd
P	2D 3c								
L	1PM								
	N	N	NS	NFS	S	SGp	ROX	CIV	ROX
CI	+	+	+	+	-	-	-	-	-
CETF	-	-	-	+	-	-	-	-	-
CII	-	-	+	+	+	+	-	-	-
CGpDH	-	-	-	-	-	+	-	-	-

O2k and DatLab file: **P___** (A / B) 2016- Operator:
Sample type: Cohort: Sample code:
Sample.Subsample number: Unit: Concentration:
Medium:

Event	Mark name	State	Final conc. 2 ml O2k	Stock [mM]	Comment	Tit. [µl]	A	B
MiR								
O2			~200 µM					
mt	**R**	*R*						
P			5 mM	2000		5		
M			2 mM	400		10		
Dig	**1PM**	PM_L		8.1				
D	**2D**	PM_P	1 / 2.5 mM	500		4 /10		
c	**3c**	PM_{Pc}	10 µM	4	NADH only if FCF_c >.1	5		
U	**4U**	PM_E	Δ0.5 µM	1	CCCP	Δ1		
G	**5G**	PGM_E	10 mM	2000		10		
S	**6S**	$PGMS_E$	50 mM	1000		100		
Oct	**7Oct**	$PGMSOct_E$	0.5 mM	100		10		
Rot	**8Rot**	S_E	0.5 µM	1		1		
Gp	**9Gp**	SGp_E	10 mM	1000		20		
Ama	**10Ama**	ROX	2.5 µM	5		1		
As			2 mM	800		5		
Tm			0.5 mM	200	~20 min open, C	5		
C	**11Tm**	Tm_E						
Azd	**12Azd**	ROX	≥100 mM	4000	~5 min	100		

mt + Dig: NFSGpTm_1D 2Oct 3M 4c 5P 6G 7S 8Gp 9U 10Rot 11Ama 12Tm 13Azd

E						9U	10Rot	11Ama	12Tm	13Azd
P	1D	2Oct 3M 4c	5P	6G	7S	8Gp				
L										
	ROX	F	NF	NF	NFS	NFSGp	SGp	ROX	CIV	ROX
CI	-	-	+	+	+	+	-	-	-	-
CETF	-	+	+	+	+	+	-	-	-	-
CII	-	-	-	-	+	+	+	-	-	-
CGpDH	-	-	-	-	-	+	+	-	-	-

O2k and DatLab file: **P**___ (A / B) 2016- Operator:
Sample type: Cohort: Sample code:
Sample.Subsample number: Unit: Concentration:
Medium:

Event	Mark name	State	Final conc. 2 ml O2k	Stock [mM]	Comment	Tit. [µl]	A	B
MiR								
O2			~200 µM					
mt	**R**	*R*						
Dig	**0Dig**			8.1				
D	**1D**	ROX	1 / 2.5 mM	500		4 /10		
Oct	**2Oct**	Oct	0.5 mM	100		10		
M.05	**3M.05**	Oct_P	0.05 mM	50		2		
M.1	**3M.1**	Oct_P	0.1 mM	50		2		
M2	**3M2**	Oct_P	2 mM	400		9.5		
c	**4c**	Oct_{Pc}	10 µM	4	NADH only if FCF_c>.1	5		
P	**5P**	$PMOct_P$	5 mM	2000		5		
G	**6G**	$PGMOct_P$	10 mM	2000		10		
S	**7S**	$PGMSOct_P$	50 mM	1000		100		
Gp	**8Gp**	$PGMSOctGp_P$	10 mM	1000		20		
U	**9U**	$PGMSOctGp_E$	Δ0.5 µM	1	CCCP	Δ1		
Rot	**10Rot**	SGp_E	0.5 µM	1		1		
Ama	**11Ama**	ROX	2.5 µM	5		1		
As			2 mM	800		5		
Tm			0.5 mM	200	~20 min open, C	5		
C	**11Tm**	Tm_E						
Azd	**13Azd**	ROX	≥100 mM	4000	~10 min	100		

Supplement B

B1. Author contributions and acknowledgements

This communication is a pre-publication prepared by CD and EG. CD, ZS, HE, and GK performed test experiments. EG, CD, ZS, and GK contributed to the concept. GK co-wrote the manuscript. EG and CD edited the final version.

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http://www.mitofit.org/index.php/O2k-MitoFit

B2. General links

Introduction
» http://wiki.oroboros.at/index.php/Gnaiger_2014_MitoPathways

Respiratory substrate-coupling states
» http://www.bioblast.at/index.php/MitoPedia:_Respiratory_substrate-coupling_states

Table of titrations
» http://wiki.oroboros.at/index.php/MiPNet09.12_O2k-Titrations

Definition
» http://www.bioblast.at/index.php/Substrate-uncoupler-inhibitor_titration

Context
» http://www.mitofit.org/index.php/SUIT_protocol_library

Abbreviations
» http://www.bioblast.at/index.php/MitoPedia