Human blood cells: isolation and HRR

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Blood cells as a material for examination of mitochondrial function

- **Blood** – easily accesible
- For diagnostic purposes – replace biopsies

- Many studies show possibility to use for respirometric studies

PLT

Monocytes

Lymphocytes } **PBMCs** – *Peripheral Blood Mononuclear Cells*

- **Selection of the cell type from blood cells** which would reflect bioenergetics of a target organ in various pathophysiological conditions could be crucial for the translational research

Focus on methodological aspects of the work with human blood cells

- Methods for isolation of **PBMCs** and **PLT** from the same blood sample
- Methods for cell counting
- Protocols for respiration of intact and permeabilized cells
- Normalization of respirometric measurements

- Results from **MitoFit study on PBMCs**

Basic information

- Blood is **collected in K_3EDTA tubes** and transported to the lab at **RT** in thermo-insulating containers, protected from light

- **15-18 ml of blood** is optimal for isolation of **PBMCs** and **PLT** for **4 O2k chambers for each cell type**

- Blood cells in whole blood are counted on **Sysmex XN-350** hematology analyser

- Isolation procedure starts 1 h after blood taking - takes 1 h

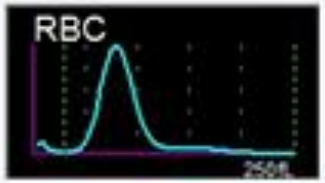
Sysmex XN-350 hematology analyzer

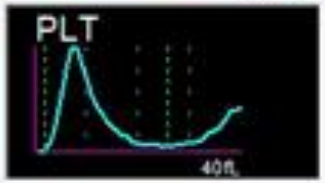
Main | Graph | Q-Flag | Service

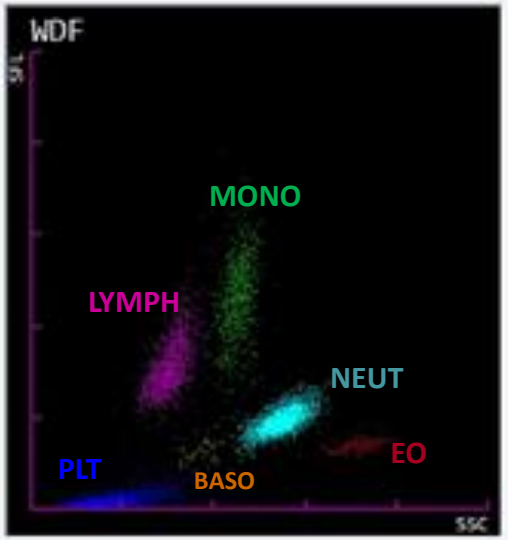
Item	Data		Unit
WBC	6.79		10^3/uL
RBC	6.23	+	10^6/uL
HGB	16.5		g/dL
HCT	49.1		%
MCV	78.8	-	fL
MCH	26.5		pg
MCHC	33.6		g/dL
PLT	267		10^3/uL
RDW-SD	48.8		fL
RDW-CV	18.0	+	%
PDW	12.1		fL
MPV	10.0		fL
P-LCR	25.7		%
PCT	0.27		%

Item	Data		Unit
NEUT#	4.15		10^3/uL
LYMPH#	1.53		10^3/uL
MONO#	0.86	+	10^3/uL
EO#	0.19		10^3/uL
BASO#	0.06		10^3/uL
NEUT%	61.1		%
LYMPH%	22.5		%
MONO%	12.7		%
EO%	2.8		%
BASO%	0.9		%
IG#	0.01		10^3/uL
IG%	0.1		%

Main | Graph | Q-Flag | Service





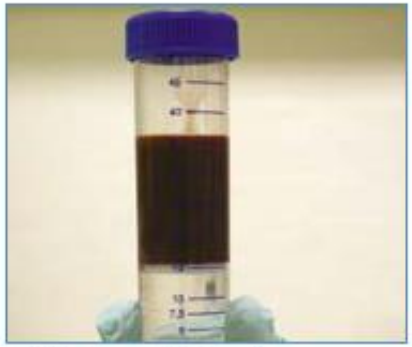


Cell counting in whole blood

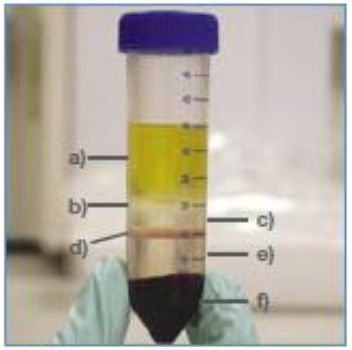
Total number of **PLT** and cells
in **different populations of WBC**

Method 1: isolation of PBMCs

1) Filling with sample material

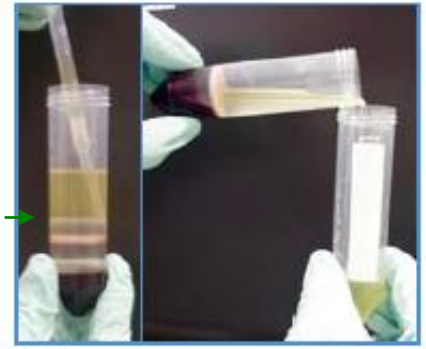


2) Before centrifugation



3) After centrifugation

PBMCs →



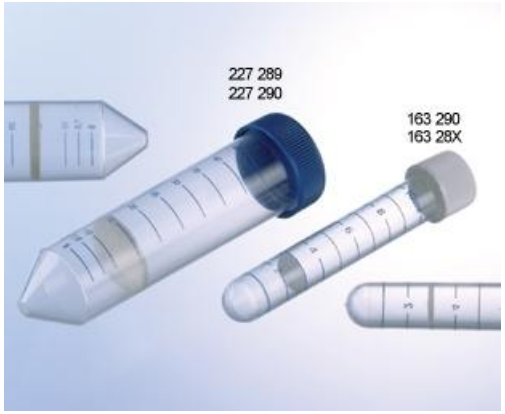
4) Harvest by means of a Pasteur pipette or by decanting into another centrifugation tube

- 50 ml Leucosep tube with 15 ml Ficol-Paque™ PLUS density 1.077 gradient centrifugation medium
- 18 ml whole blood in K_3EDTA tubes
- Dilution 1:1 with DPBS
- Centrifugation **1000xg for 10** min, RT, acceleration (6 of 9), no brakes
- Collection of layer of PBMCs 5-10 ml, add 25 ml DPBS, centrifugation **120xg** for 10 min, acc. 9, brakes 6 of 9
- Washing with 25 ml DPBS, centrifugation 120xg for 10 min, acc. 9, brakes 6
- Resuspension in **0.5 ml DPBS**

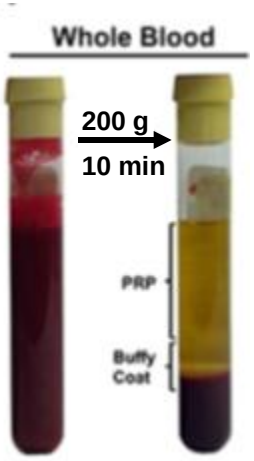
Whole isolation procedure: 1 h

Manufacturer instruction: **250xg → PLT/PBMCs ~ 20**

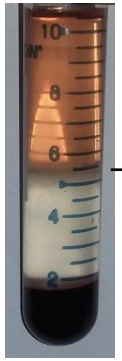
120xg ~6



Method 2: isolation of PBMCs and PLT



Buffy coat diluted 1:1 DPBS after centrifugation 1000xg 10 min



PBMCs layer

Ficol-Paque™ PLUS

Red blood cells

Centrifuge whole blood at **200xg for 10** min, acc. 9, no brakes!

PLT:

- Transfer **PRP** into 14 ml round-bottom Falcon tube add 10% of volume 100 mM EGTA
- centrifuge at **1000xg for 10 min**, acc. 9, brakes 1
- gently resuspend the sediment in 4 ml DPBS,10 mM EGTA
- centrifuge at **1000xg for 5 min**, acc. 9, brakes 1
- Resuspend in **0.5 ml DPBS,10 mM EGTA**

PBMCs:

Prepare: 2x 14 ml round-bottom Falcon tubes with **4 ml Ficol-Paque™** PLUS density 1.077 gradient centrifugation medium

- Transfer **buffy coat** into a new tube, dilute 1:1 with DPBS
- Layer carefully 6 ml of buffy coat diluted with DPBS on the top of Ficol-Paque™ PLUS
- Centrifuge **1000xg for 10 min**, RT, acceleration (6 of 9), no brakes
- Collect the layer of PBMCs ~ 2 ml, add 2 volumes of DPBS
- centrifuge at **350xg for 5 min**, acc. 9, brakes 6 of 9
- Resuspend in **0.5 ml DPBS**

Sysmex XN-350 hematology analyzer

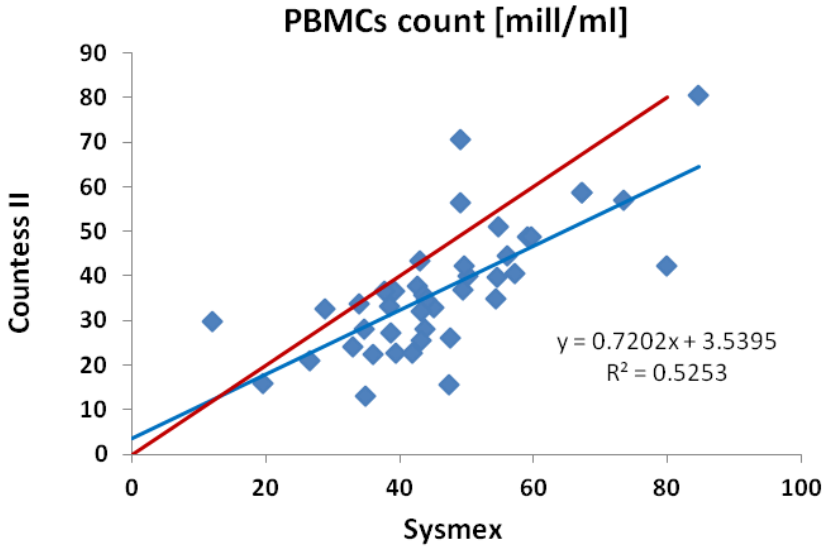
Countess II cell counter

Viability test in PBMCs
by Trypan blue, ***cell size*** *and* ***morphology***

Cell counting in PBMCs and PLT preparations

dilution 10x in DPBS

The control for **purity of preparation**



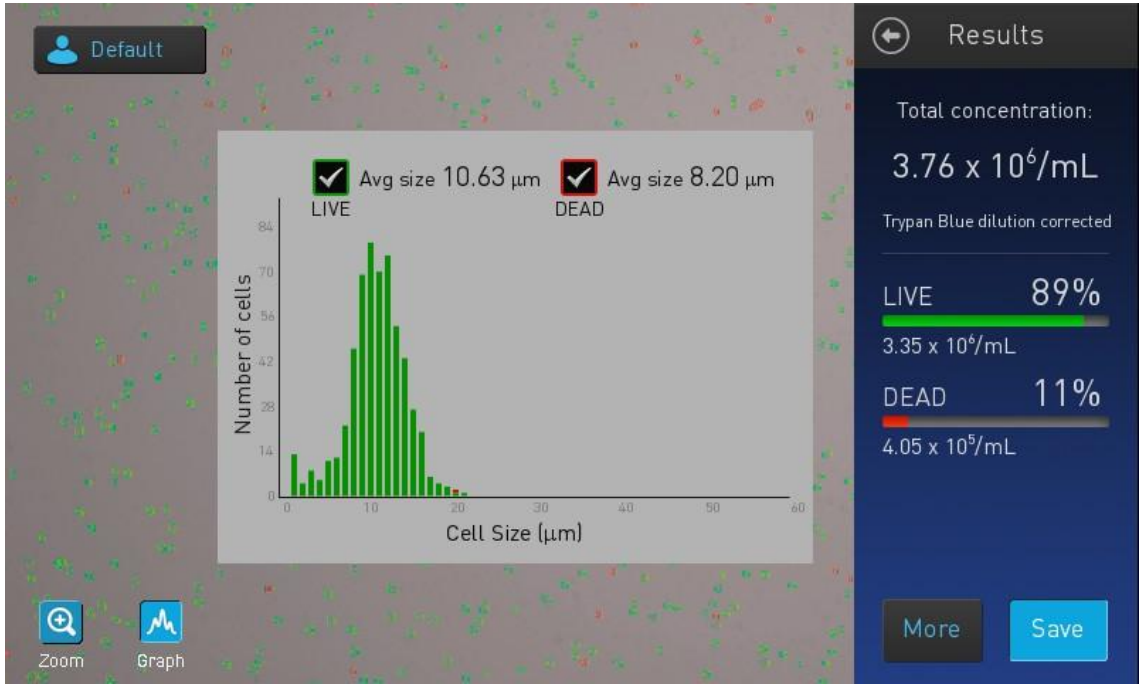
Countess II cell counter

Morphology

Cell count

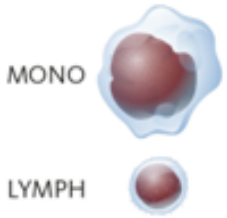
Viability

Cell size



Characteristics of PBMCs preparation:

Cell count: **47.4 ± 2.8 x 10^6 cells/ml**
Viability: **86 ± 1.8%**
Cell size: **8.79 ± 0.56 µM**



~ 20 %

~ 80 %

protein: 82.8 ± 3.1 µg/10^6 cells

Purity: **PLT/PBMCs ~ 6 => ~ 21% of protein PLT**

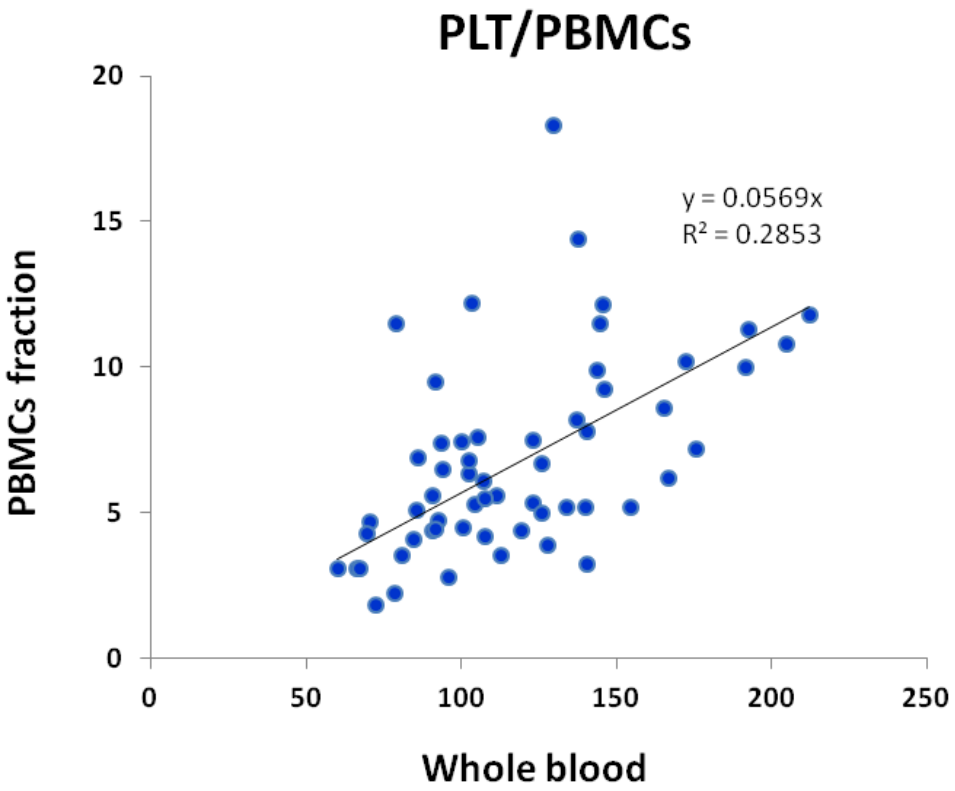
Characteristics of PLT preparation from PRP:

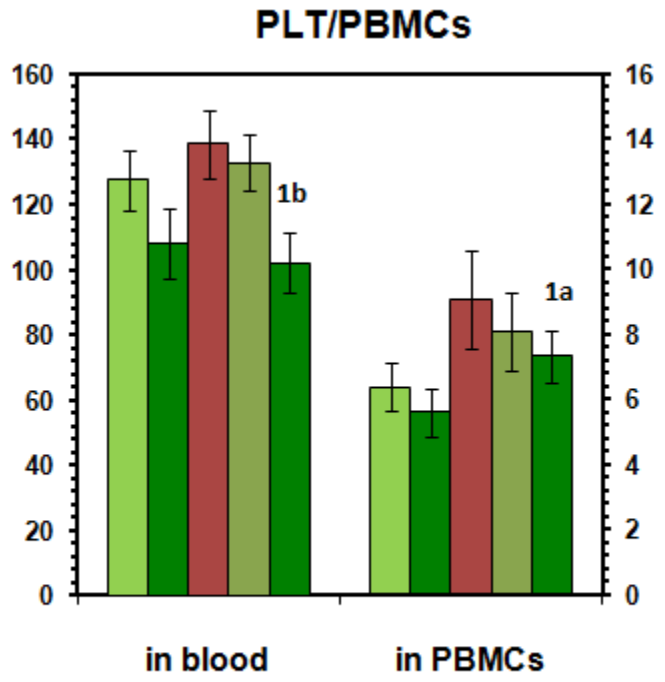
Cell count: **2574 ± 330 x 10^6 cells/ml**

protein: 2.91 ± 0.23 µg/10^6 cells

Purity: **PBMCs/PLT** = 0.036% ~ **1% protein PBMCs** in PLT

Contamination of PBMCs with PLT





PBMCs fraction/Whole blood = 0.052 +- 0.003

Freezing subsample of suspension for **later analysis**:

- **proteins**
- **CS** – mitochondrial marker
- **LHD** – marker of cytoplasma **-80°C**

Oxygraph-2k: **4-6 mill PBMCs/**chamber
200-300 mill PLT/chamber

Lifting the stoppers and adding calculated volume of cell suspension (80-150 µl) to O2k chamber

Protocols:

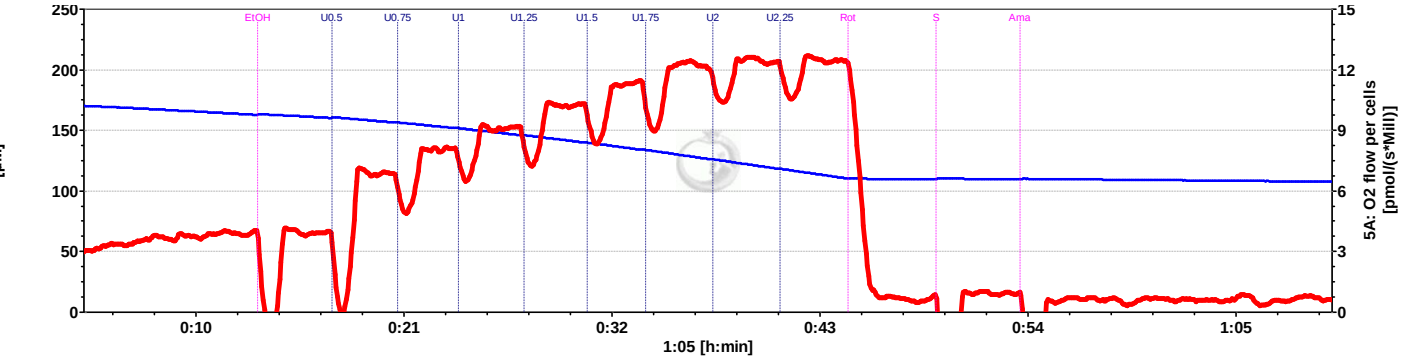
- **intact cells: CCP** in **RPMI** without L-glutamine
- **permeabilized cells: RP1** and **RP2** in **MiR06Cr+Ctl**

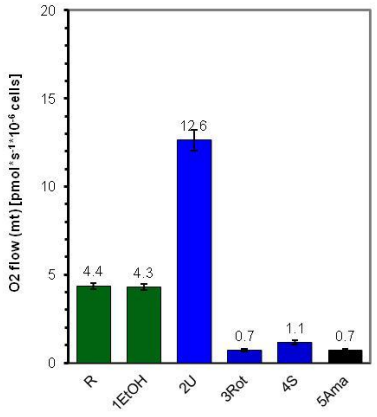
Respiration of intact PBMCs

Coupling Control Protocol in RPMI without L-glutamine

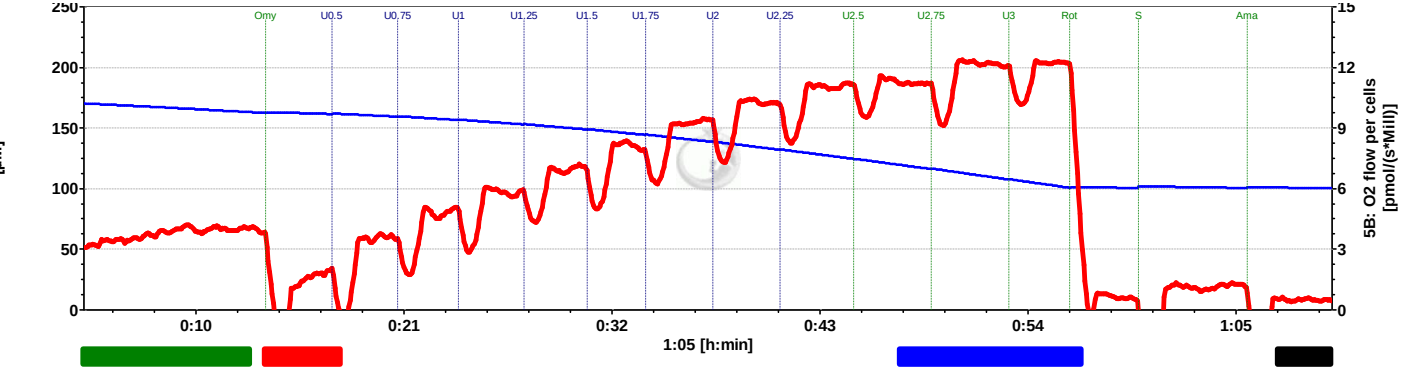
CCP_RP1 **Cells + EtOH + CCCP + Rot + S + Ama**

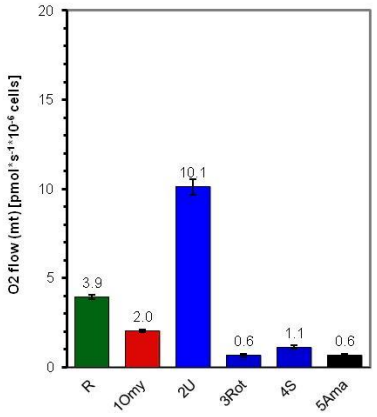
N = 15





CCP_RP2 **Cells + Omy + CCCP + Rot + S + Ama**

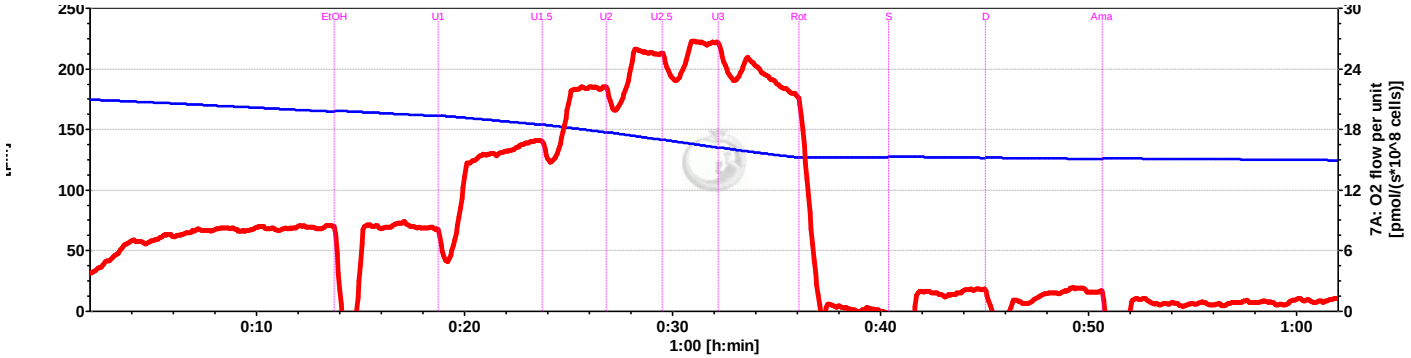




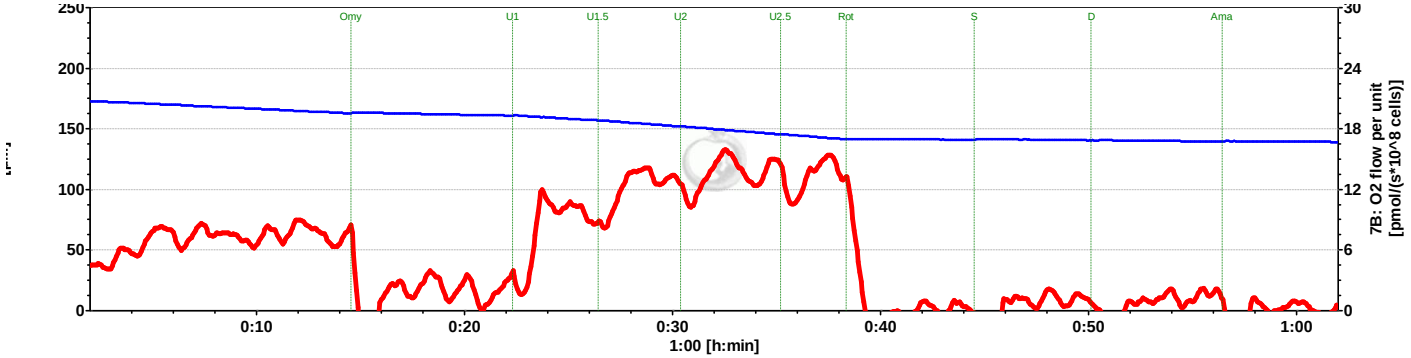
Respiration of intact PLT

Coupling Control Protocol in MiR05

CCP_RP1 **Cells + EtOH + CCCP + Rot + S + Ama**

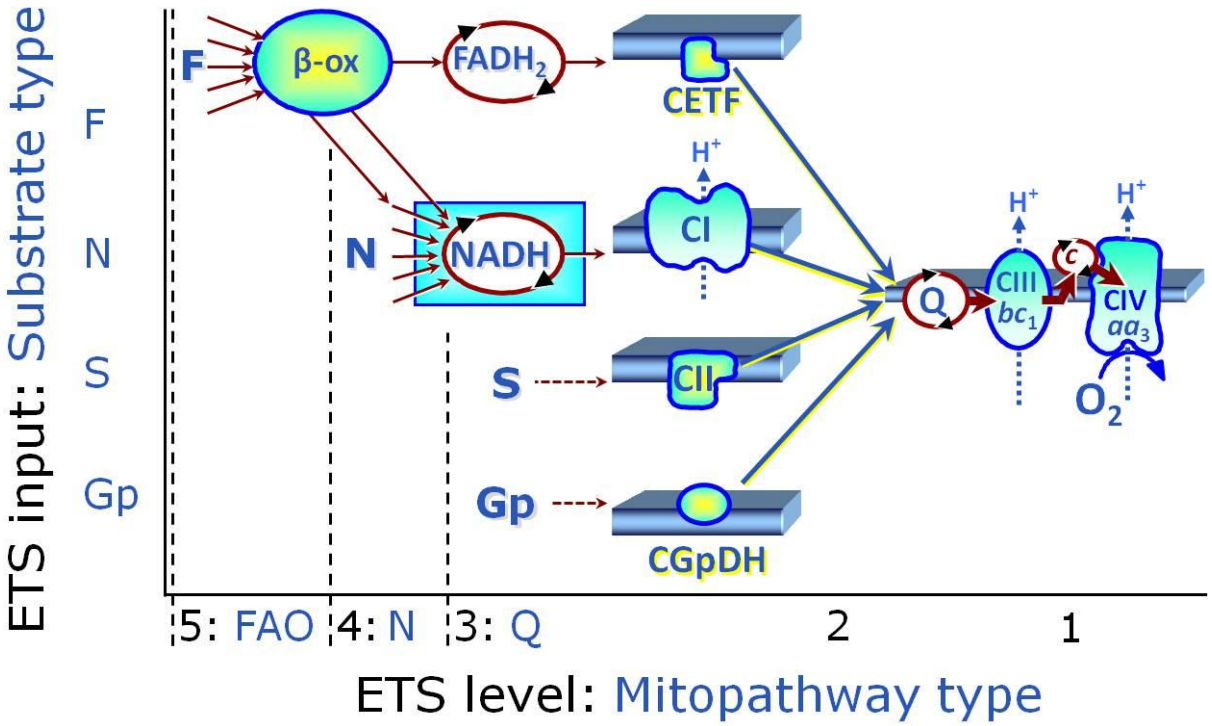


CCP_RP2 **Cells + Omy + CCCP + Rot + S + Ama**



- ETS after Omy is frequently much lower than without Omy
- **Respiratory rates of intact cells depend on medium applied**

Respiration of permeabilized PBMCs

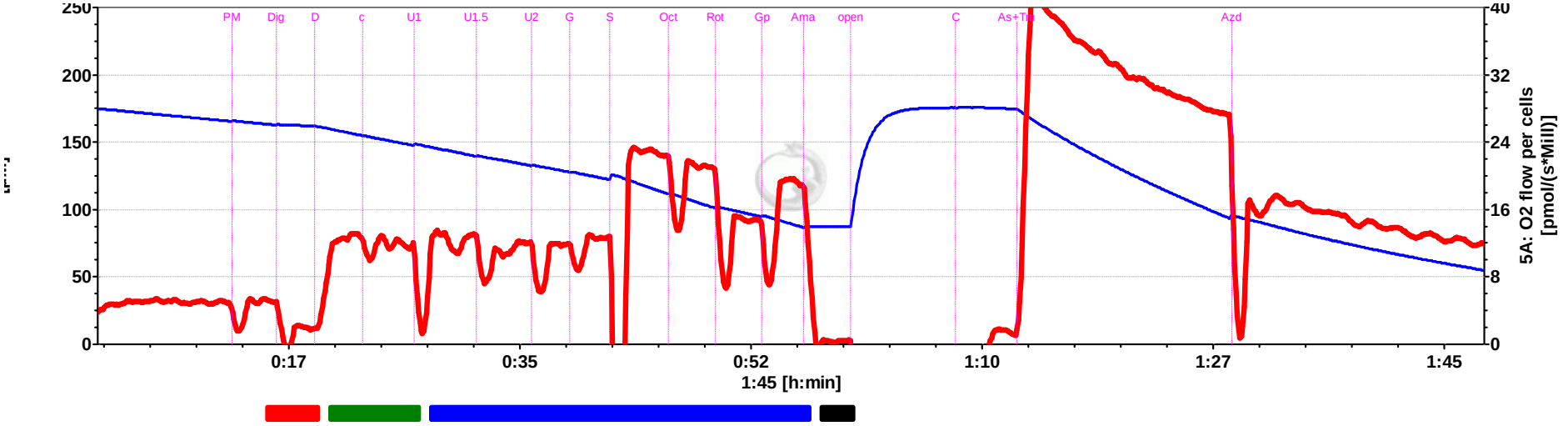


Aim: maximum information about **mitochondrial respiratory system**

Respiration of permeabilized PBMCs

2016-06-02 P5-02.DLD

RP1

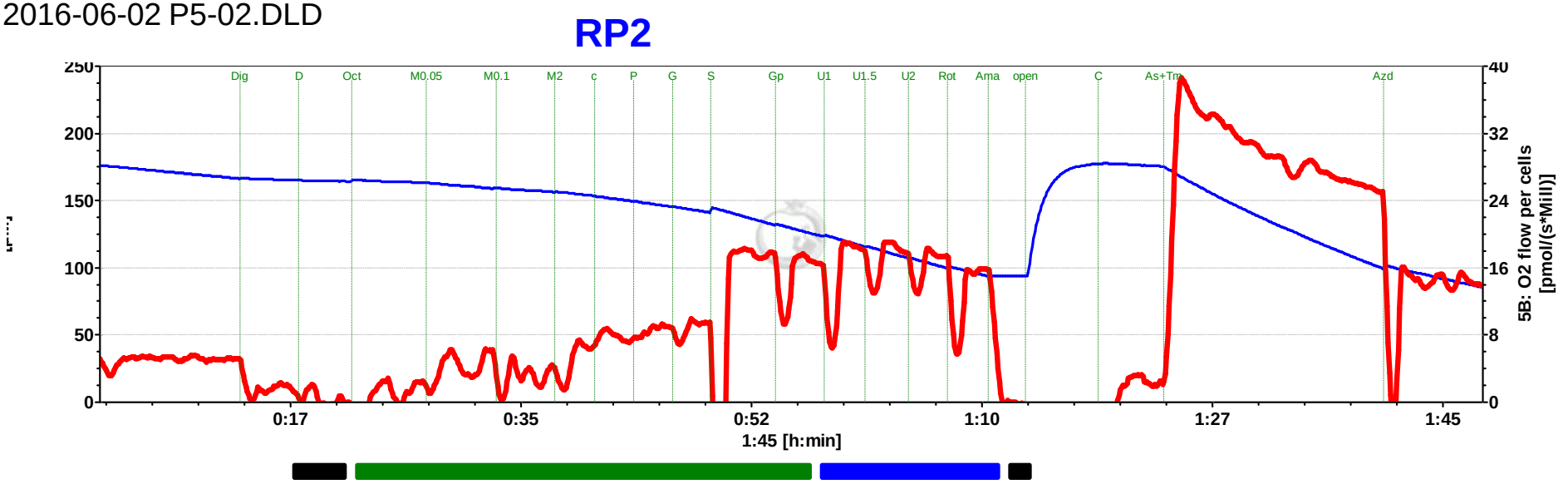


PM +mt: NFSGpTm_1PM 2D 3c 4U 5G 6S 7Oct 8Rot 9Gp 10Ama 11Tm 12Azd

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

Respiration of permeabilized PBMCs

2016-06-02 P5-02.DLD



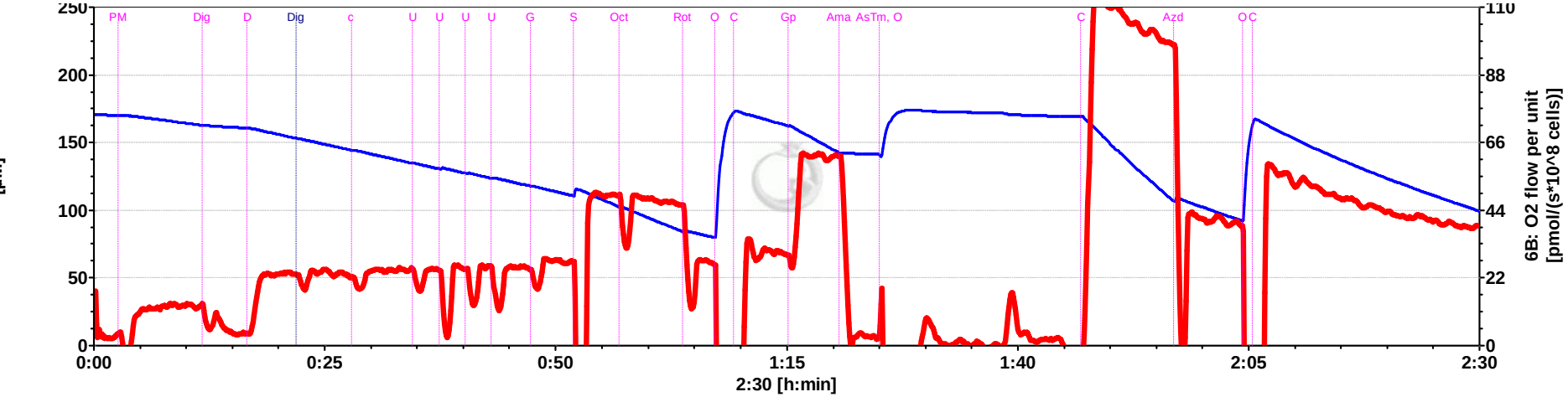
D+mt: NFSGpTm_1D 2Oct 3M 4c 5P 6G 7S 8Gp 9U 10Rot 11Ama 12Tm 13Azd

E						9U	10Rot	11Ama	12Tm	13Azd
P	1D	2Oct3M+c	5P	6G	7S	8Gp				
L										
	ROX	F	NF	NF	NFS	NFSGp	SGp	ROX	Tm	ROX
CI	-	-	+	+	+	+	-	-	-	-
CETF	-	+	+	+	+	+	-	-	-	-
CII	-	-	-	-	+	+	+	-	-	-
GpDH	-	-	-	-	-	+	+	-	-	-

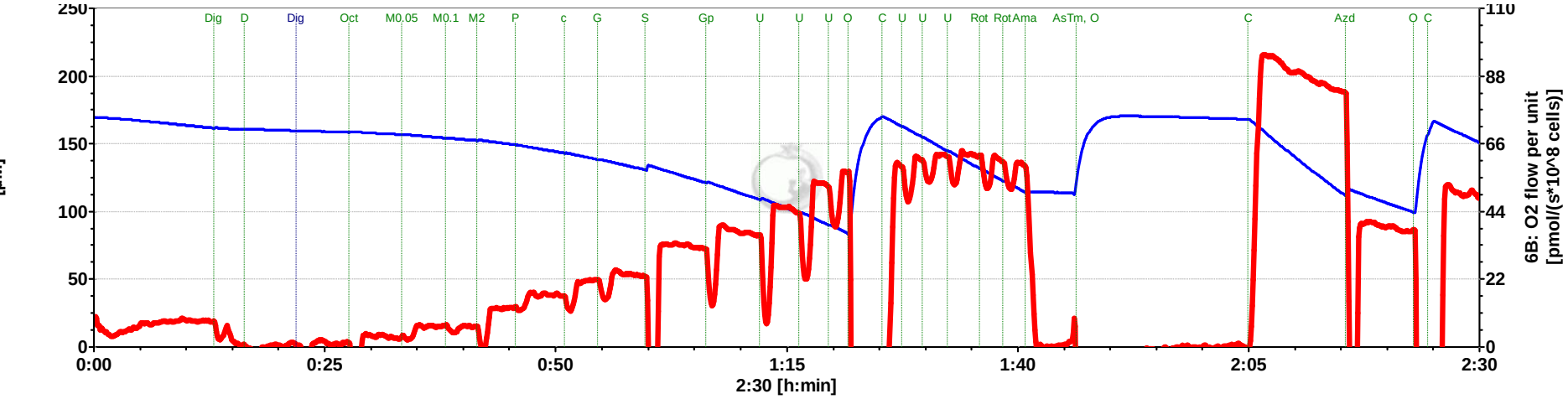
Respiration of permeabilized PLT

2016-04-19 P6-01.DLD

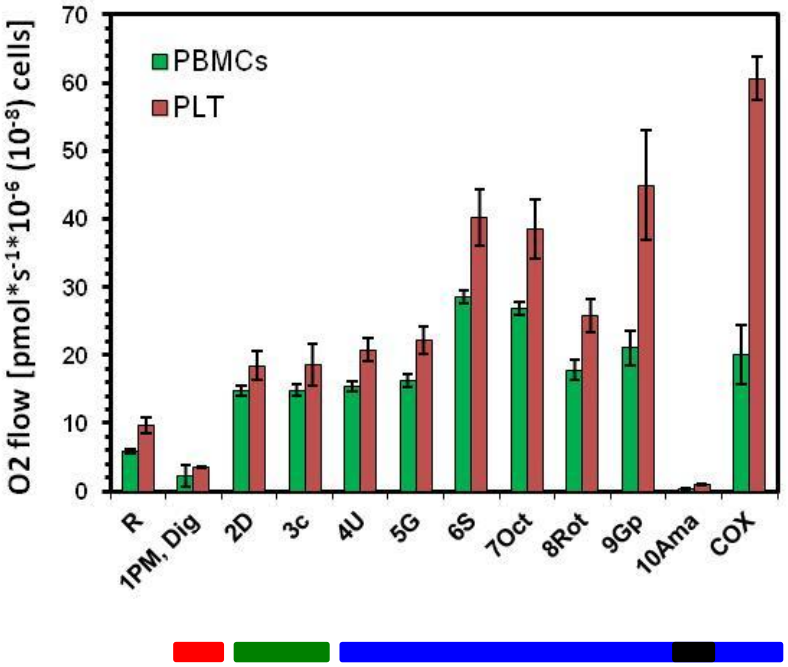
RP1

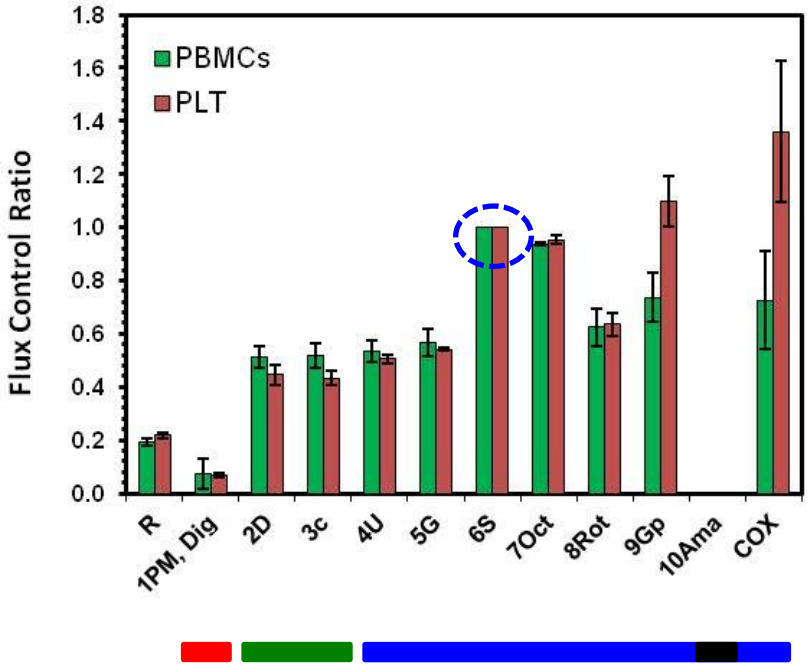


RP2

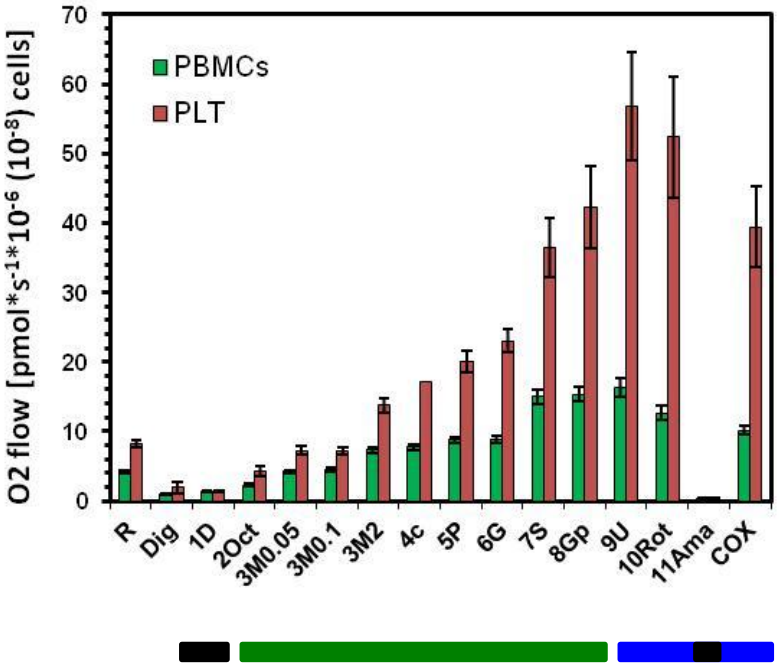


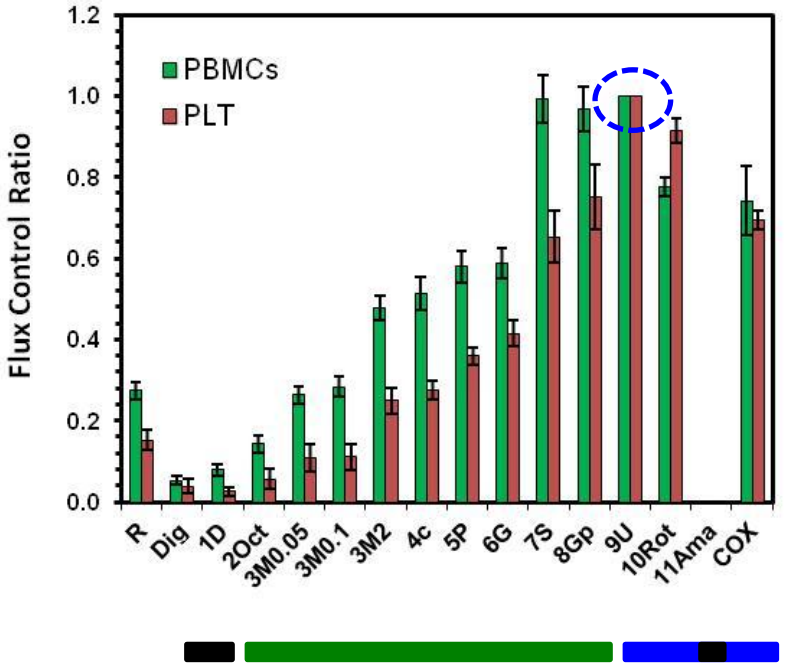
RP1


O2 flow [pmol*s-1*10-6 (10-8) cells]
70
60
50
40
30
20
10
0
PBMCs
PLT
R
1PM, Dig
2D
3c
4U
5G
6S
7Oct
8Rot
9Gp
10Ama
COX


Flux Control Ratio
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PBMCs
PLT
R
1PM, Dig
2D
3c
4U
5G
6S
7Oct
8Rot
9Gp
10Ama
COX

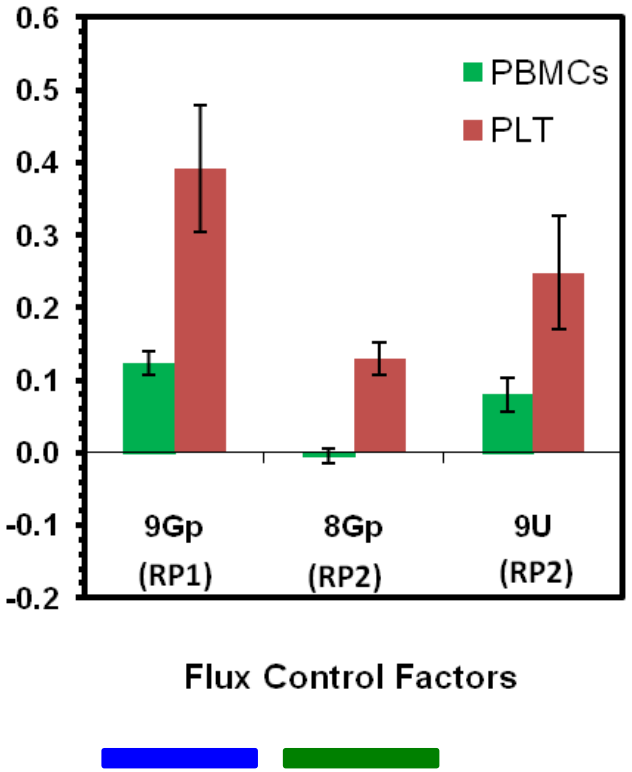
RP2





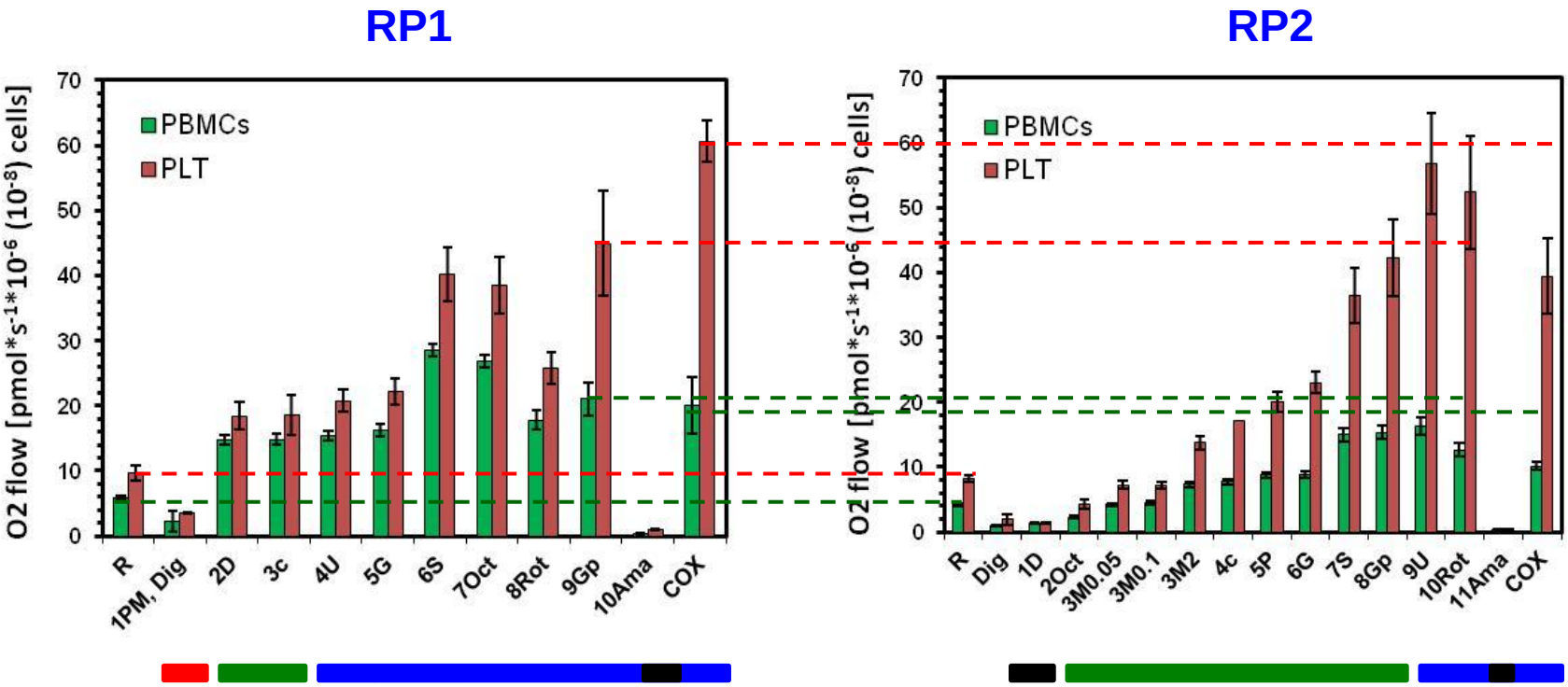
- The rate of PLT respiration per 10^8 cells is higher than the rate of PBMCs respiration per 10^6 cells
- contaminating PLT can significantly affect respiratory rates of PBMCs preparation (PLT/PBMCs ~ 6)

Step changes from RP1 and RP2 significantly different for PBMCs and PLT expressed as Flux Control Factors



- **PBMCs** and **PLT** have different respiratory patterns as recognized by two harmonized SUIT reference protocols.
- **Contamination of PBMCs with PLT** can significantly affect their apparent respiration and therefore purity of the cell preparation should be emphasized in the selection of isolation method.

Harmonization of reference protocols



cross-linked respiratory states don´t match in PBMCs or PLT

MitoFit project

- **Active group:**

  middle aged subjects 39-64 years old 5 females/10 males
  participated in a regular and supervised physical activity program over 6 years

- **Untrained group:**

  age matched (40-65 years old) 6 females/9 males , no chronic diseases, BMI < 30
  not performing regular exercise the last few years

Isolation of blood cells PBMCs and **PLT** from **18 ml of blood**

Oxygraph-2k

Respiration of intact cells: CCP1 and CCP2

Respiration of permeabilized cells: RP1 and RP2

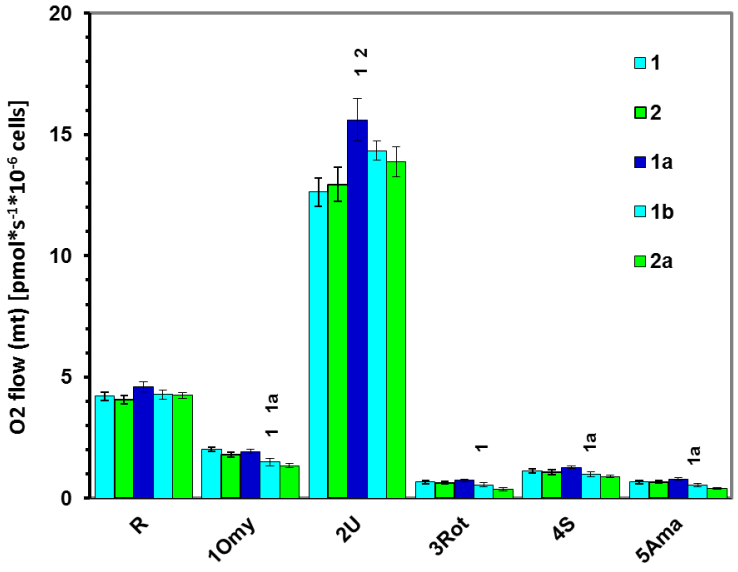
Design of MitoFit project

Group			
1	**untrained group**	**5-2016**	
2	**active group**	**5-2016**	
1a	**untrained group**	after 2 months of **supervised training**	**7-2016**
1b	**untrained group**	after next 2 months without training	**10-2016**
2b	**active group**	**10-2016**	

Physical parameters at the beginning of the project

	Untrained Group			Active Group		
	Females (N=6)	**Males (N=9)**	**Total (N=15)**	**Females (N=5)**	**Males (N=10)**	**Total (N=15)**
Age (years)	51 ±5	52 ±7	**52 ± 6**	55 ± 9	51 ± 7	**52 ± 8**
Hight (cm)	171 ± 4	177 ± 5	**175 ± 6**	168 ± 5	178 ± 6	**176 ± 8**
Weight (kg)	69.5 ±7.5	84.5 ± 10.1	**78.5 ± 11.7**	60.5 ± 2.6	80.4 ± 2.9	**73.8 ± 10.0**
BMI	23.8 ±2.9	26.9 ± 3.1	**25.7 ± 3.3**	21.5 ±1.6	25.0 ± 1.4	**23.8 ± 2.2**
dROMs	349 ± 39	286 ± 35	**313 ± 48**	339 ±52	279 ±40	**299 ± 52**
BAP	1932 ± 260	1949 ± 244	**1942 ± 241**	2185 ±70	1828 ±313	**1947 ± 308**
VO_2max (ml/min)	**2033 ±438**	**2796 ± 267**	**2497 ± 509***	**2180 ±174**	**3615 ±394**	**3137 ± 774**
VO_2max (ml/min/kg)	29.2 ±4.8	33.8 ± 6.9	**32.0 ± 6.4***	36.0 ± 2.4	45.1 ±5.7	**42.1 ± 6.5**
HR_max	172 ± 14	171 ± 10	**172 ± 11**	174 ± 9	176 ±9	**176 ± 9**
Watt_max	146 ± 38	198 ± 26	**177 ± 40***	165 ± 21	285 ± 26	**245 ± 63**
Watts/kg	2.1± 0.5	2.4 ± 0.6	**2.3 ± 0.6****	2.7 ± 0.3	3.6 ± 0.4	**3.3 ± 0.5**
Lactate_max	8.2 ± 2.1	8.7 ± 2.3	**8.5 ± 2.2***	10.6 ± 2.5	11.4 ± 2.2	**11.1 ± 2.3**
BORG_max	19.2 ± 1.3	17.9 ± 1.6	**18.4 ± 1.6**	16.6 ± 1.1	18.7 ± 1.3	**18.0 ± 1.6**

Respiration of intact PBMCs



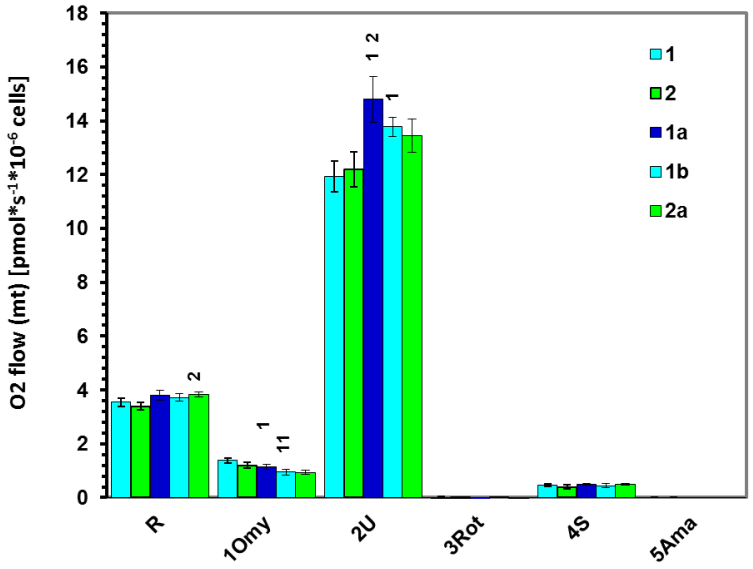
1	**Untrained**
2	**Active**
1a	**1** after 2 months of training
1b	**1a** after 2 months of break
2a	**2** after 5 months

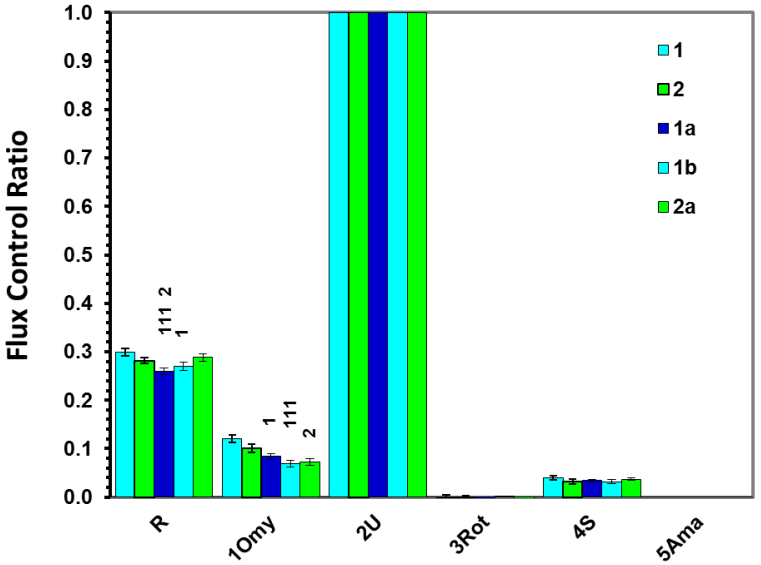
Beginning: no difference between 1 and 2

After training: ↑ ETS and **↓ *L*** vs **1**

After break: no differences between 1b and 2b

↓ *L* vs 1 and 2





[1]P<0.05 vs 1

[111]P<0.001 vs 1

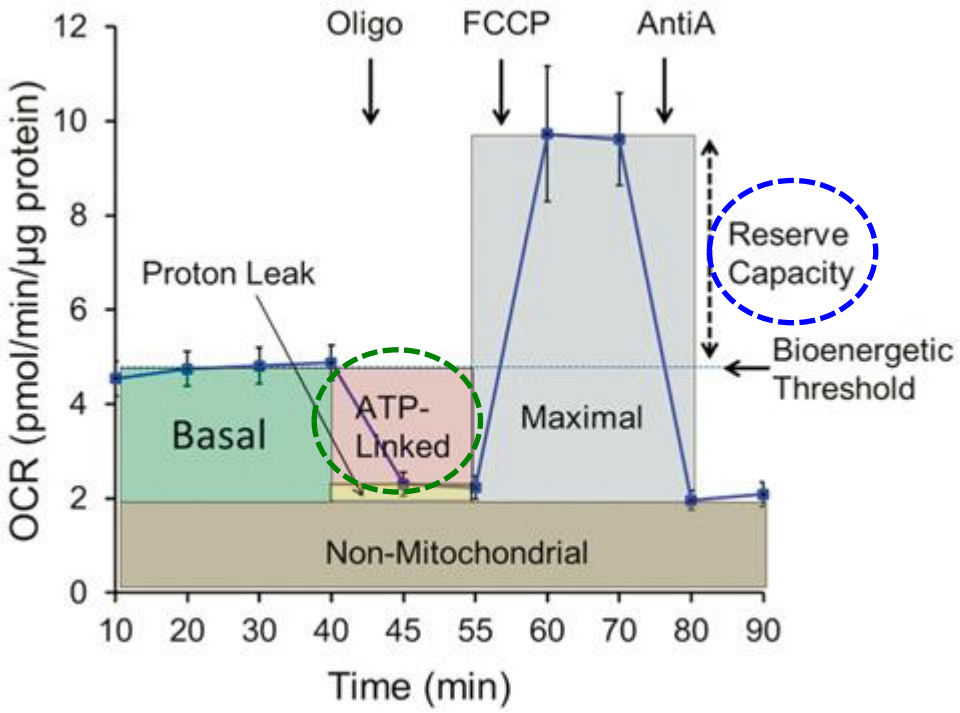
[1a]P<0.05 vs 1a

[2]P<0.05 vs 2

Calculated parameters in intact PBMCs

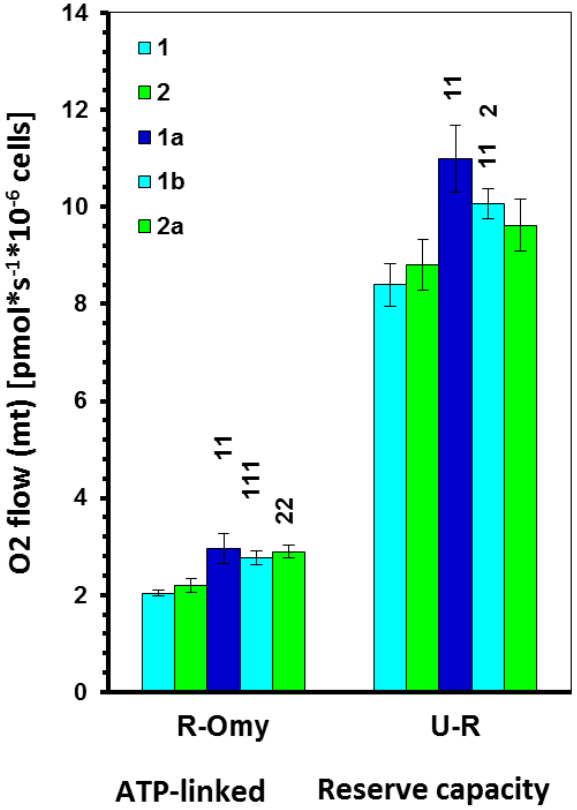
Coupling efficiency: (R-L)/R

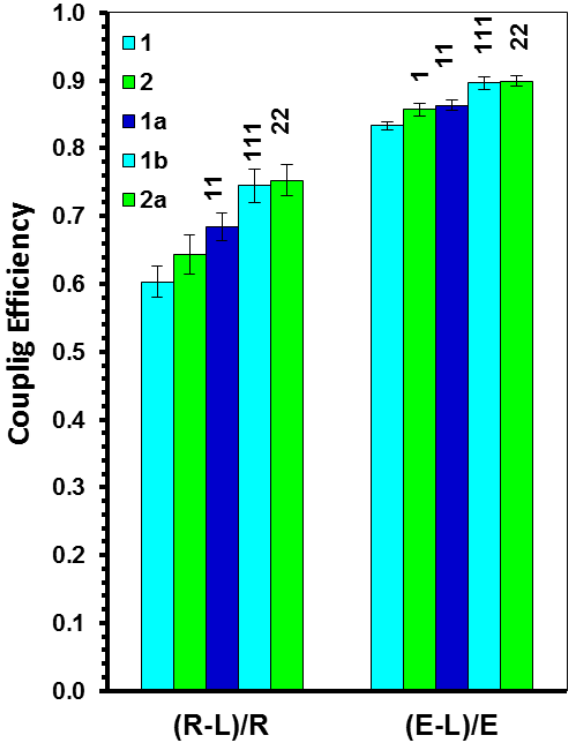
(E-L)/E



Chacko et al. *Clinical Science* 2014

Calculated parameters in intact PBMCs



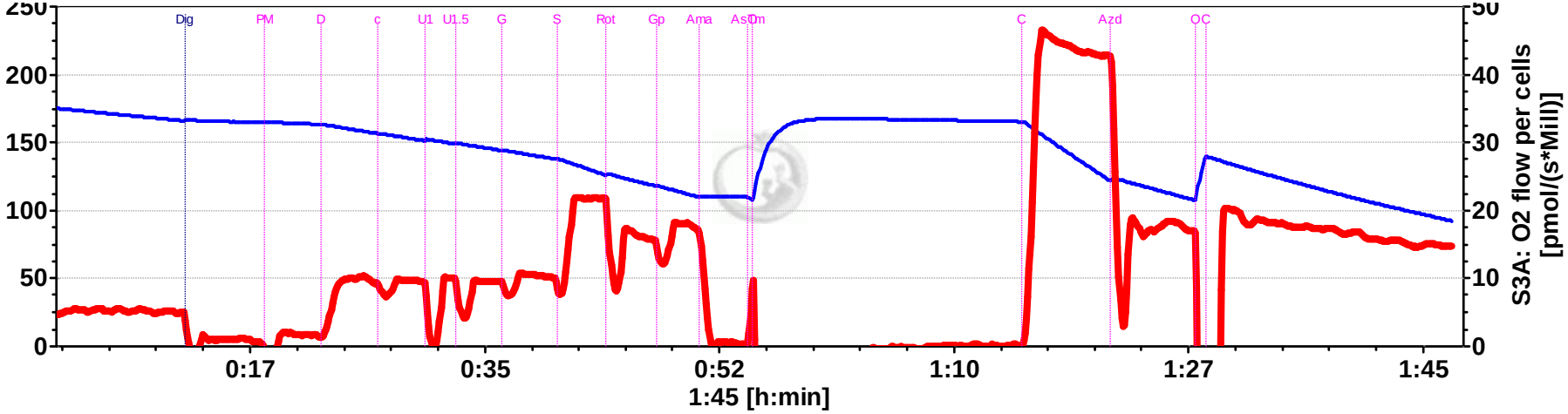


Increased coupling efficiency with training?
Improvements in handling of cells?
Seasonal changes?

^{11}P<0.01 vs 1
^{111}P<0.001 vs 1
^{2}P<0.05 vs 2
^{22}P<0.01 vs 2
^{222}P<0.01 vs 2

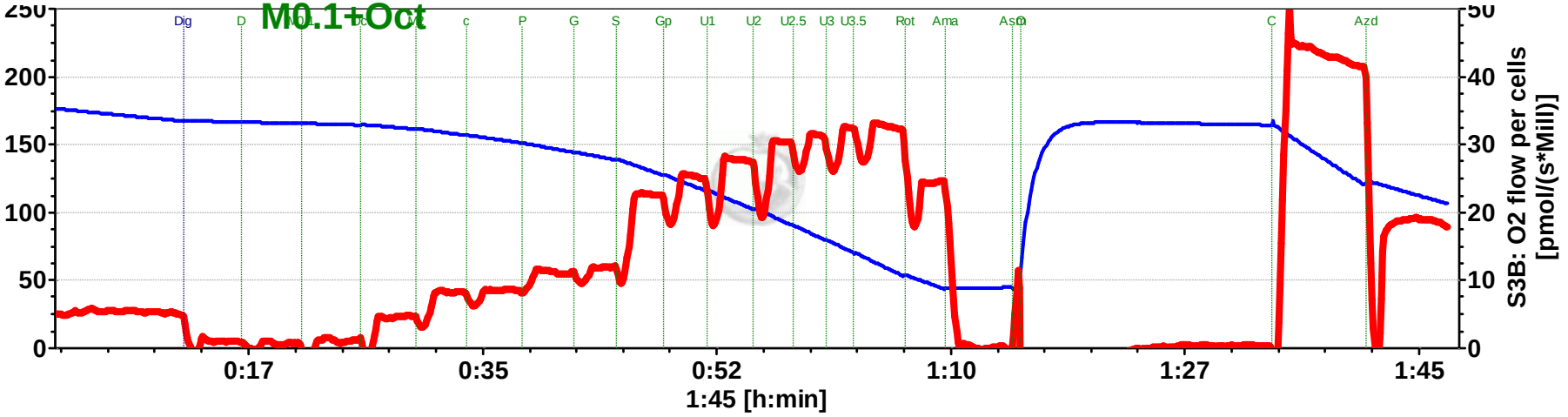
Improved reference protocols

R+Dig+PM2+D+c+Utitr+G+S10+~~Oct~~+Rot+Gp+Ama+AsTm- reox20 min-close 7min**+Azd**



R+Dig+D+Oct+M0.05+M0.1+M2+c+P+G+S10+Gp+Utitr+Rot+Ama+AsTm reox20 min-close 7min**+Azd**

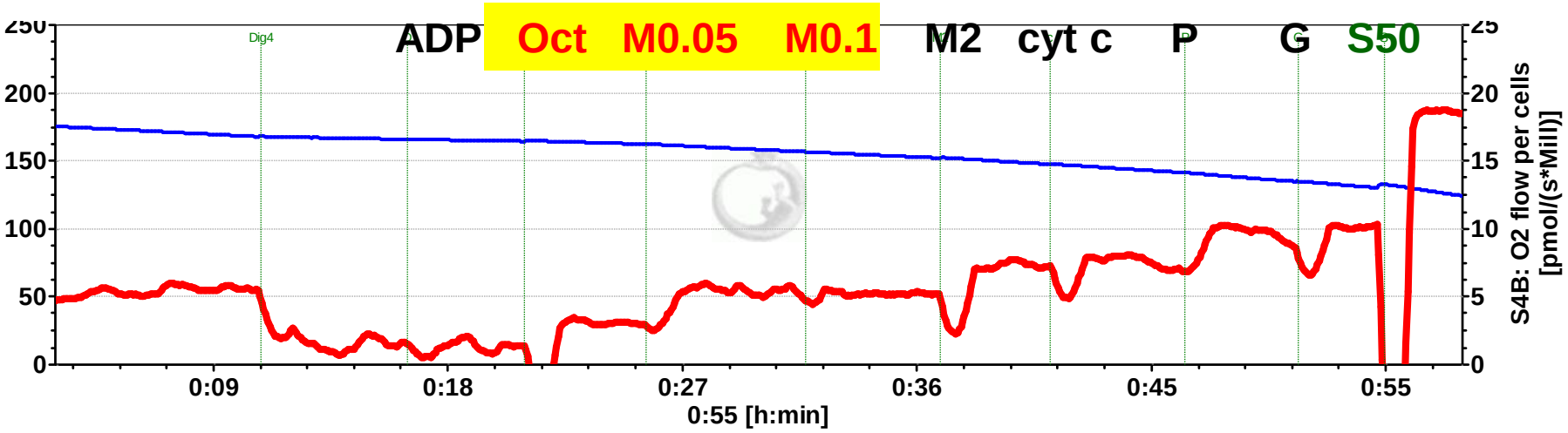
M0.1+Oct



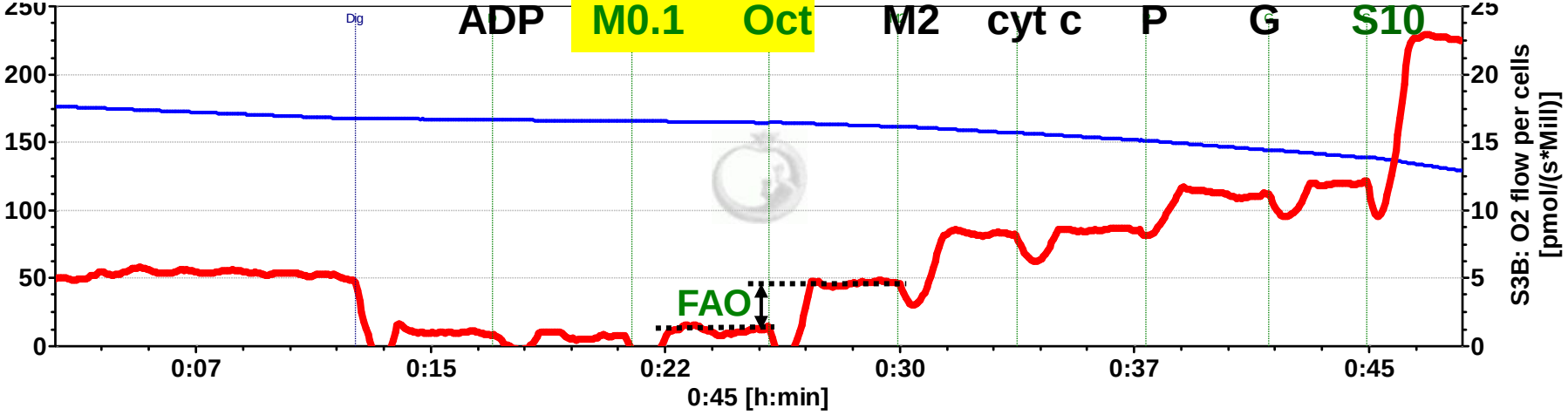
2016-10-12 PS3-02.DLD

Evaluation of FAO in PBMCs

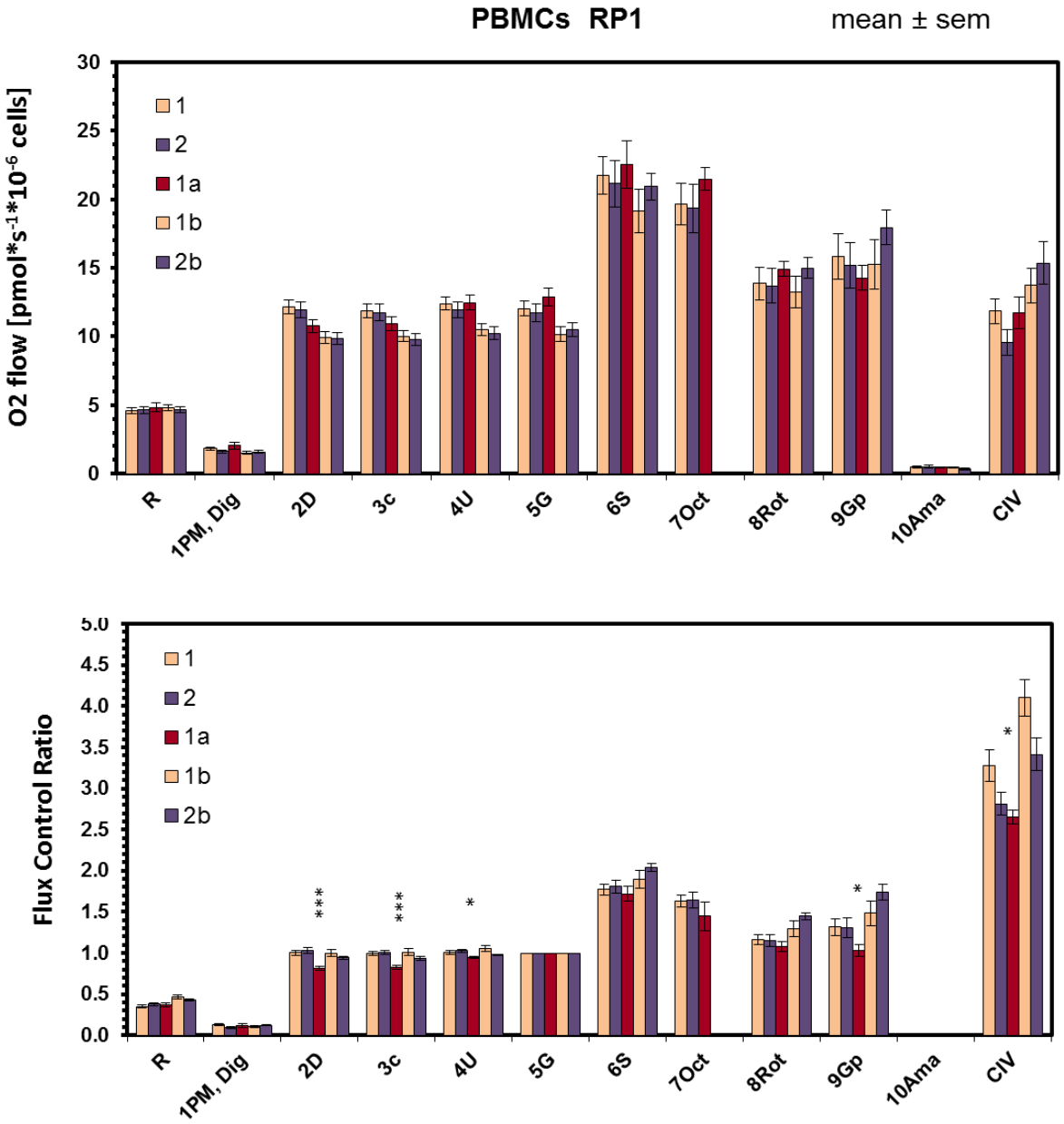
Original RP2



Improved RP2



Respiration of permeabilized PBMCs – RP1



1 **Untrained**

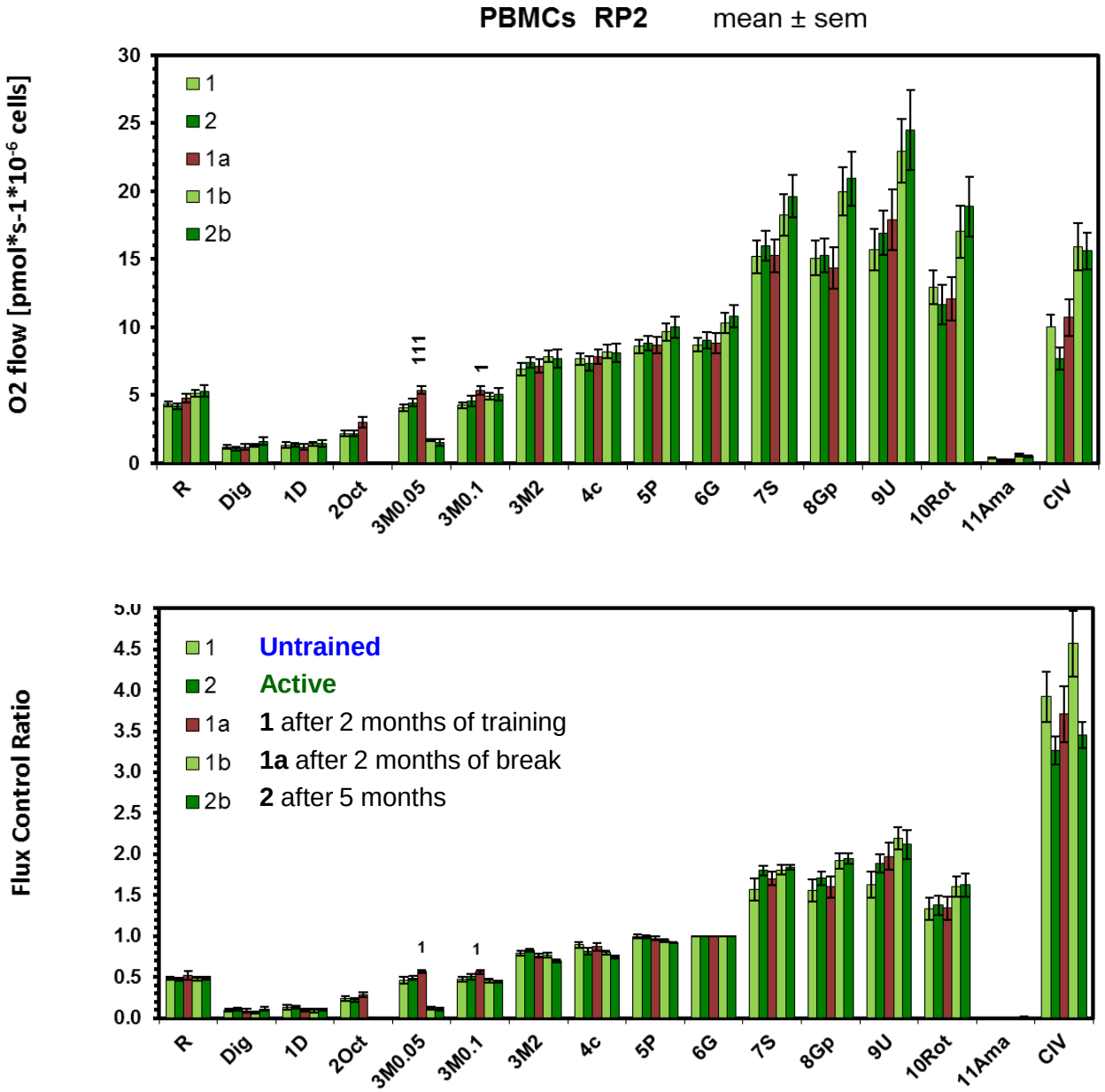
2 **Active**

1a **1** after 2 months of training

1b **1a** after 2 months of break

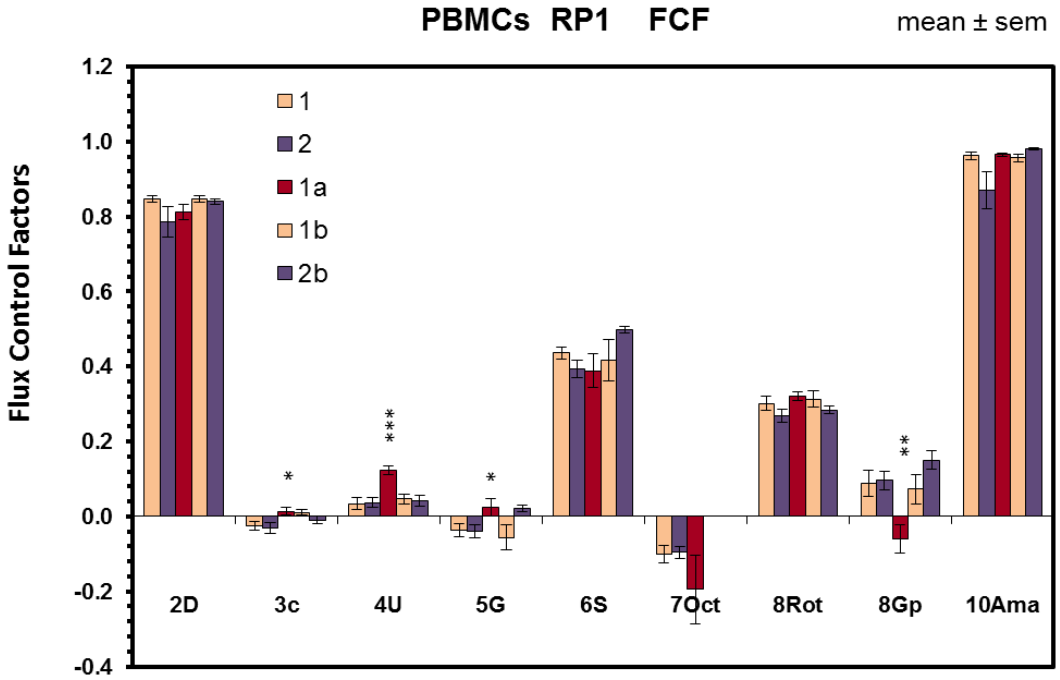
2b **2** after 5 months

Respiration of permeabilized PBMCs – RP2



[1]P<0.05 vs 1

[111]P<0.001 vs 1



1 **Untrained**

2 **Active**

1a **1** after 2 months of training

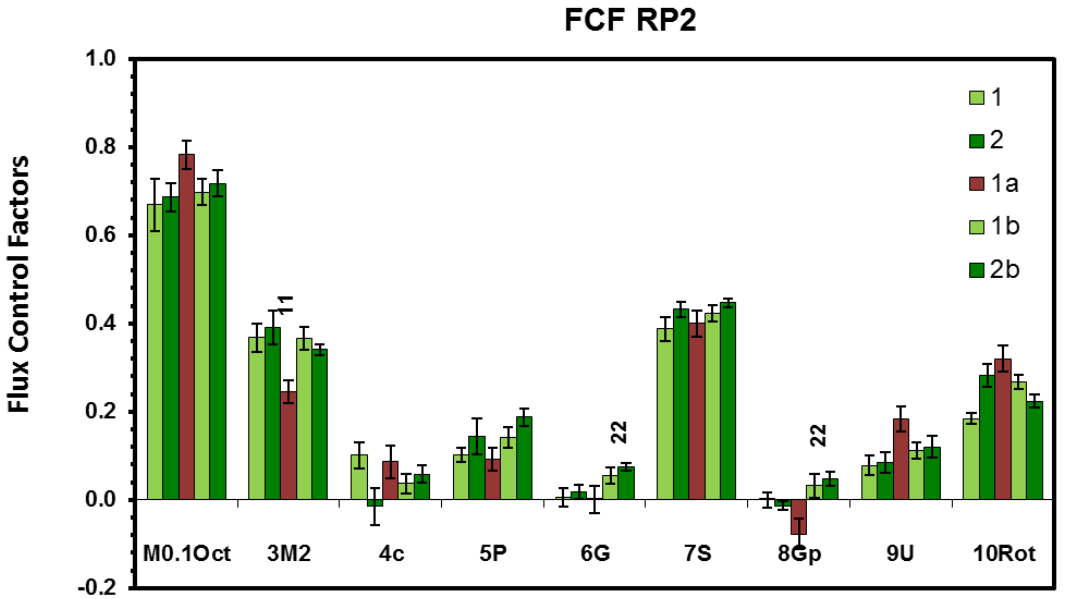
1b **1a** after 2 months of break

2b **2** after 5 months

*P<0.05 vs 1

**P<0.01 vs 1

***P<0.001 vs 1



11P<0.01 vs 1

22P<0.01 vs 2

Summary

Beginning: no difference between 1 and 2

Untrained after training: ↑ respiration with OctM0.1 ~ FAO

After break: no differences between 1b and 2b

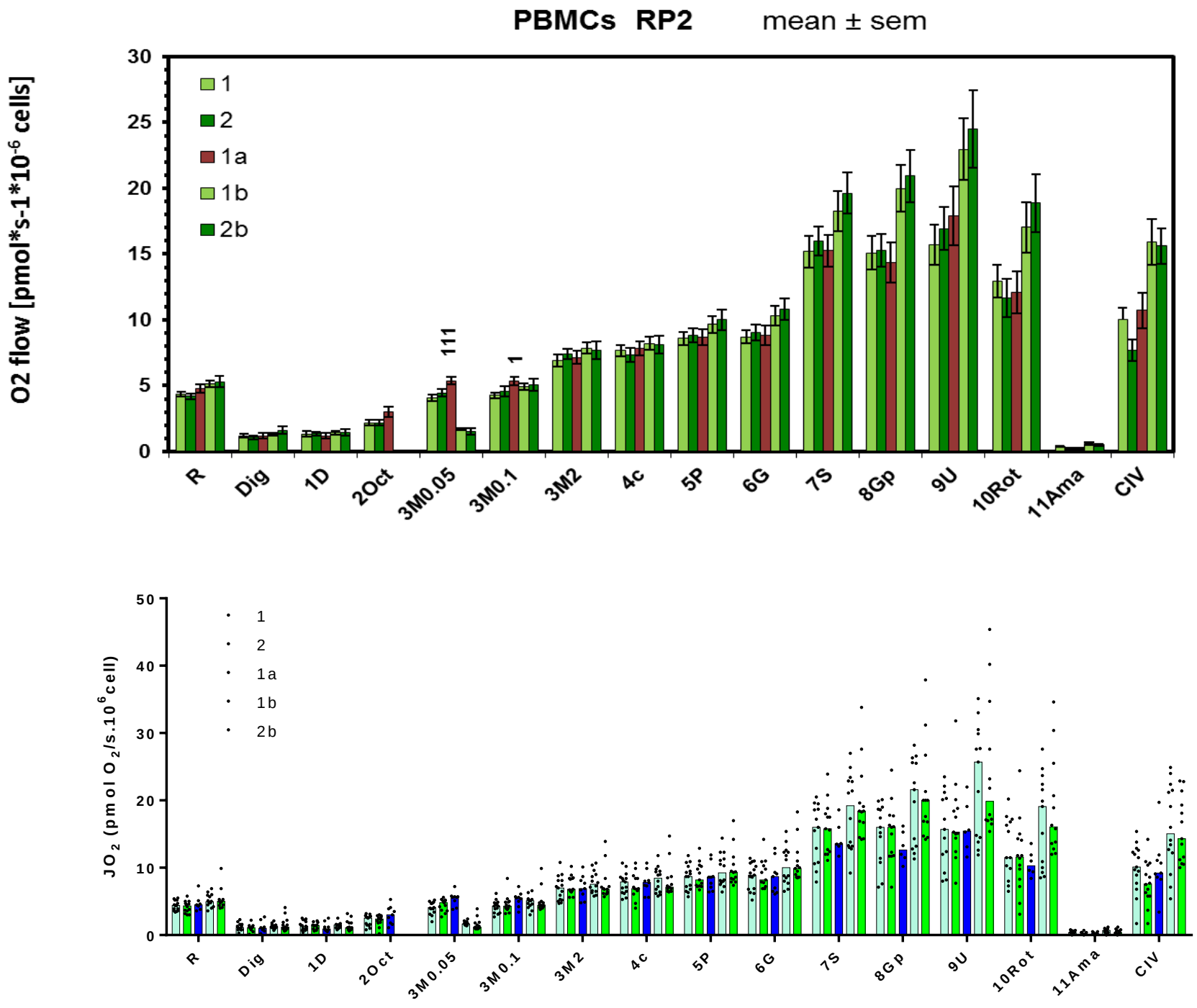
Conclusions

- No difference in mitochondrial respiration between active and untrained group at the same time of sampling
- Difference between 2 sampling times
- Effect of training on FAO ?

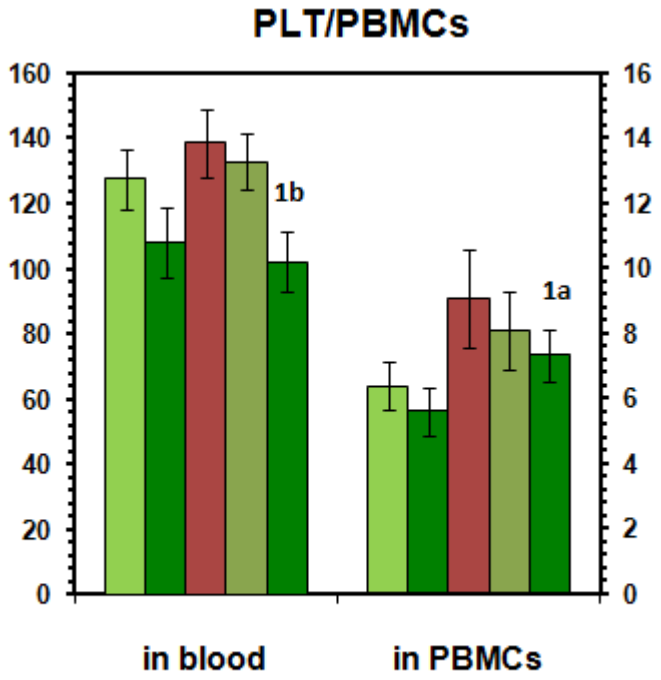
LFU: Institut für Sportwissenschaften, ISW (Univ.-Prof. DDr. Martin Burtscher, Verena Menz)

SME: Sporttherapie Mag. Huber GmbH, Innsbruck, STH (Mag. Reinhard Huber)

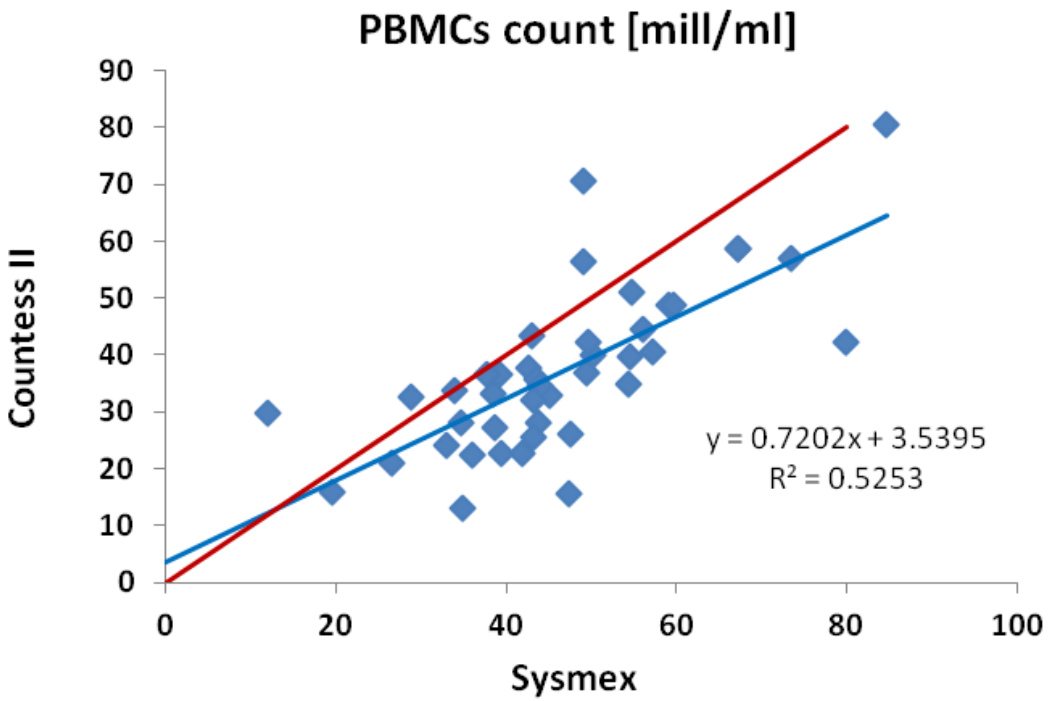
Thank you


PBMCs RP2
mean ± sem
O2 flow [pmol*s-1*10-6 cells]
1
2
1a
1b
2b
111
1
R
Dig
1D
2Oct
3M0.05
3M0.1
3M2
4c
5P
6G
7S
8Gp
9U
10Rot
11Ama
CIV
JO2 (pmol O2/s.10^6cell)

PLT/PBMCs

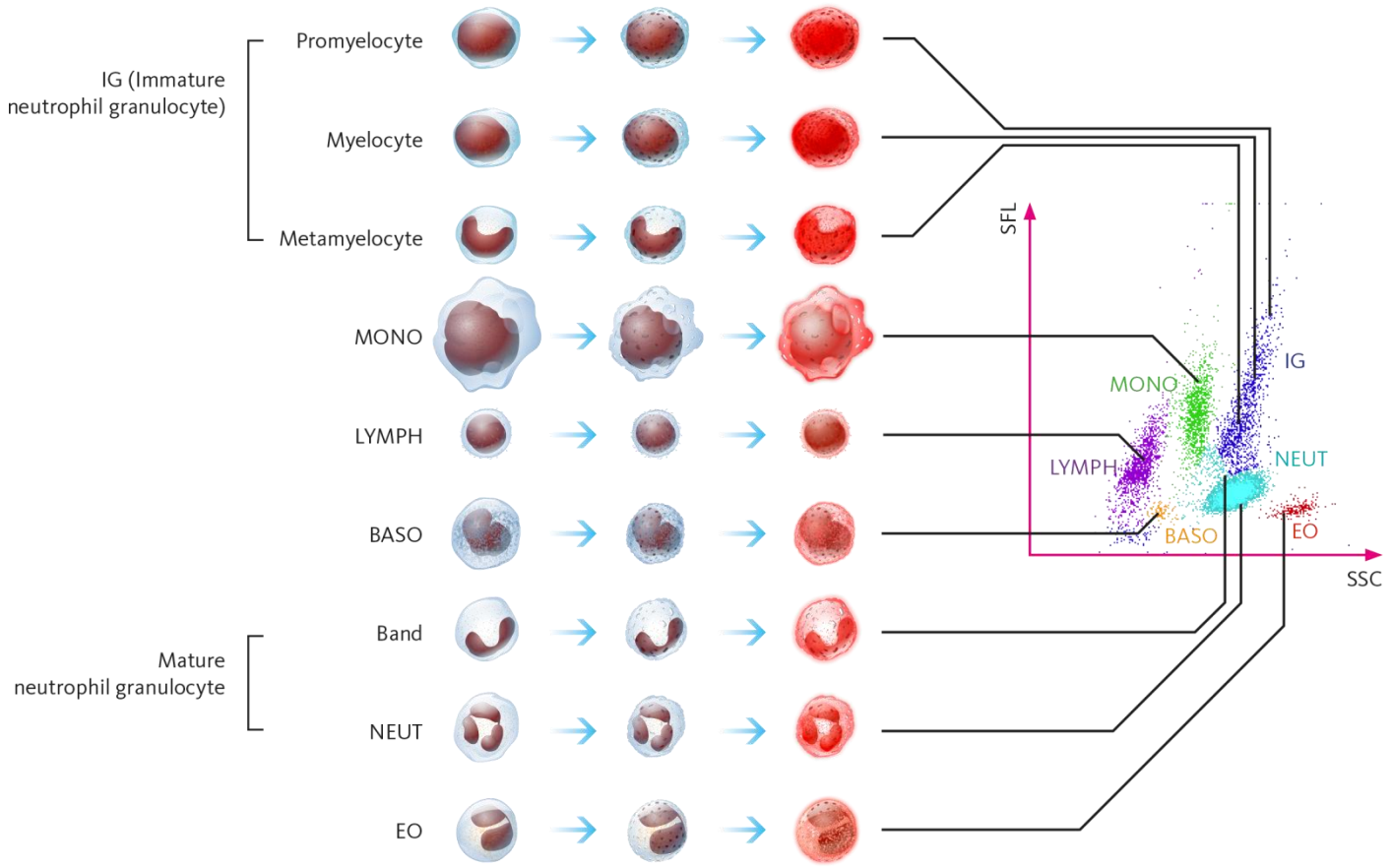

PLT/PBMCs
160
140
120
100
80
60
40
20
0
16
14
12
10
8
6
4
2
0
1b
1a
in blood
in PBMCs

Counting cells



WDF Scattergramm

The different fluorescent intensity correspond to the different cell complexity and internal cell structure and allows the recognition of the different leucocytes populations



Karabatsiakis_TP_2014

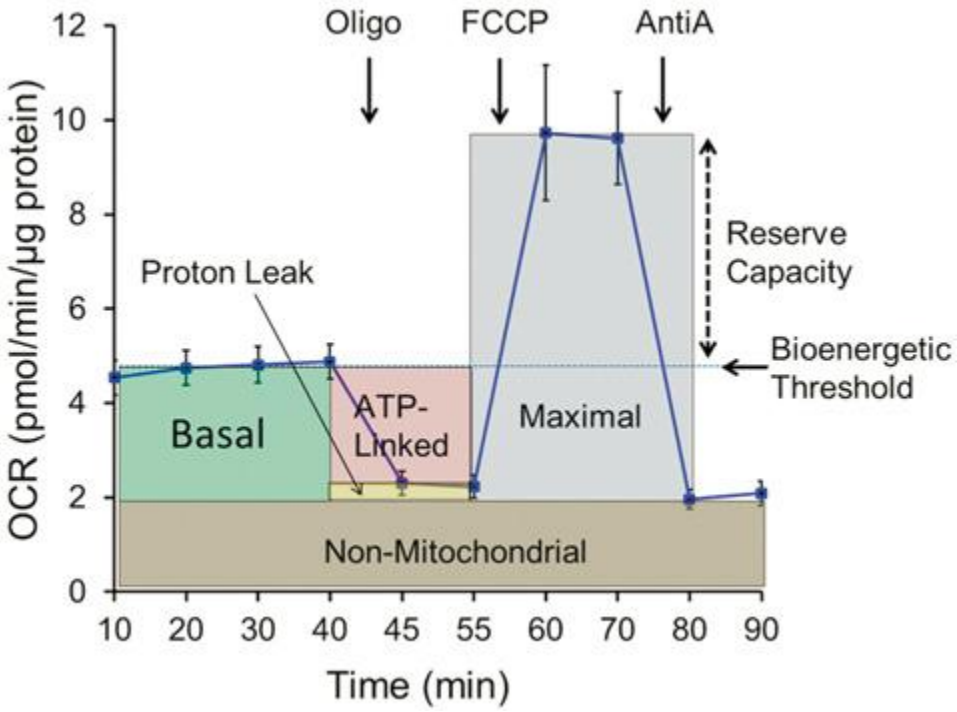
Respiration related to ATP turnover was calculated as the difference between routine and LEAK respiration (R − L) and spare respiratory capacity as the difference between maximal uncoupled respiration and routine respiration (E − R).42

Following the recommendations of the manufacturer (Oroboros Instruments), the following flux control ratios were calculated for further analyses: routine flux control ratio (routine respiration over uncoupled respiration, R/E) and coupling efficiency (ATP turnover over routine respiration, (R − L)/R).

Chacko_CS_2014

Bioenergetic Health Index:

$$\mathrm{BHI} = \log \frac{(\text{reserve capacity})^a \times (\text{ATP-linked})^b}{(\text{non-mitochondrial})^c \times (\text{proton leak})^d}$$



jove Journal of Visualized Experiments www.jove.com

Video Article

Bioenergetics and the Oxidative Burst: Protocols for the Isolation and Evaluation of Human Leukocytes and Platelets

Philip A. Kramer*[1], Balu K. Chacko*[1], Saranya Ravi[1], Michelle S. Johnson[1], Tanecia Mitchell[1], Victor M. Darley-Usmar*[1]

[1]UAB Mitochondrial Medicine Laboratory, Center for Free Radical Biology, Department of Pathology, University of Alabama at Birmingham

*These authors contributed equally

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URL: http://www.jove.com/video/51301
DOI: doi:10.3791/51301

Keywords: Immunology, Issue 85, bioenergetics, translational, mitochondria, oxidative stress, reserve capacity, leukocytes

Date Published: 3/27/2014

Citation: Kramer, P.A., Chacko, B.K., Ravi, S., Johnson, M.S., Mitchell, T., Darley-Usmar, V.M. Bioenergetics and the Oxidative Burst: Protocols for the Isolation and Evaluation of Human Leukocytes and Platelets. *J. Vis. Exp.* (85), e51301, doi:10.3791/51301 (2014).

Kramer et al., J. Vis. Exp. 85, e51301, 2014

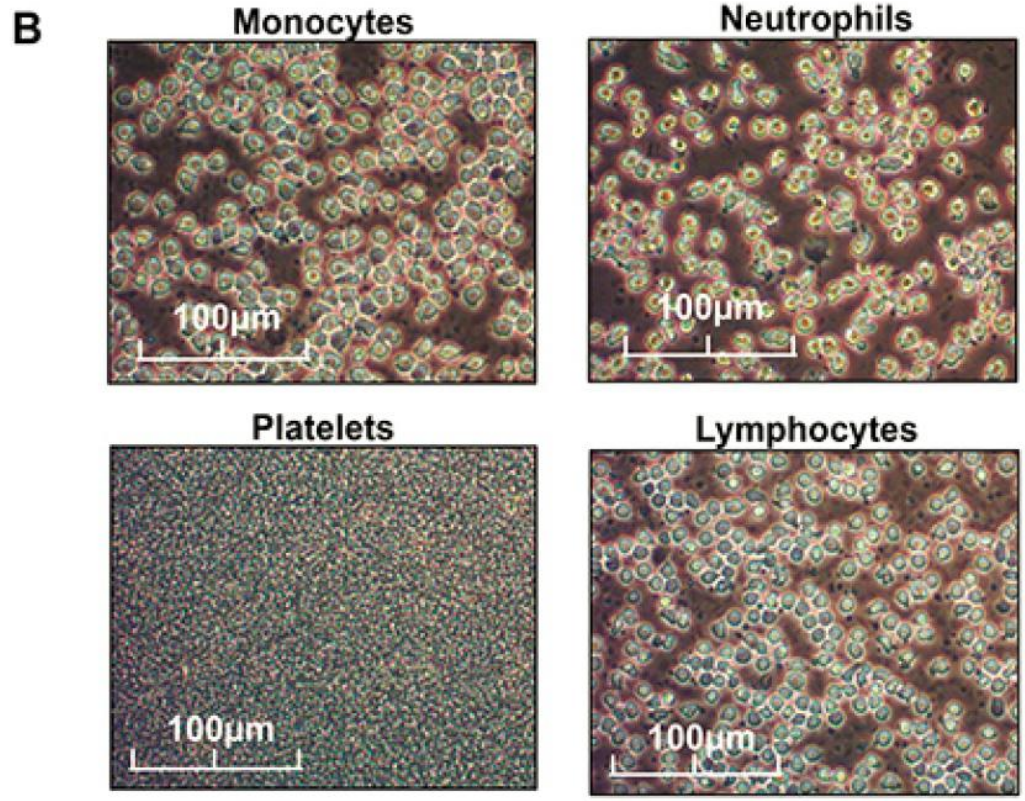
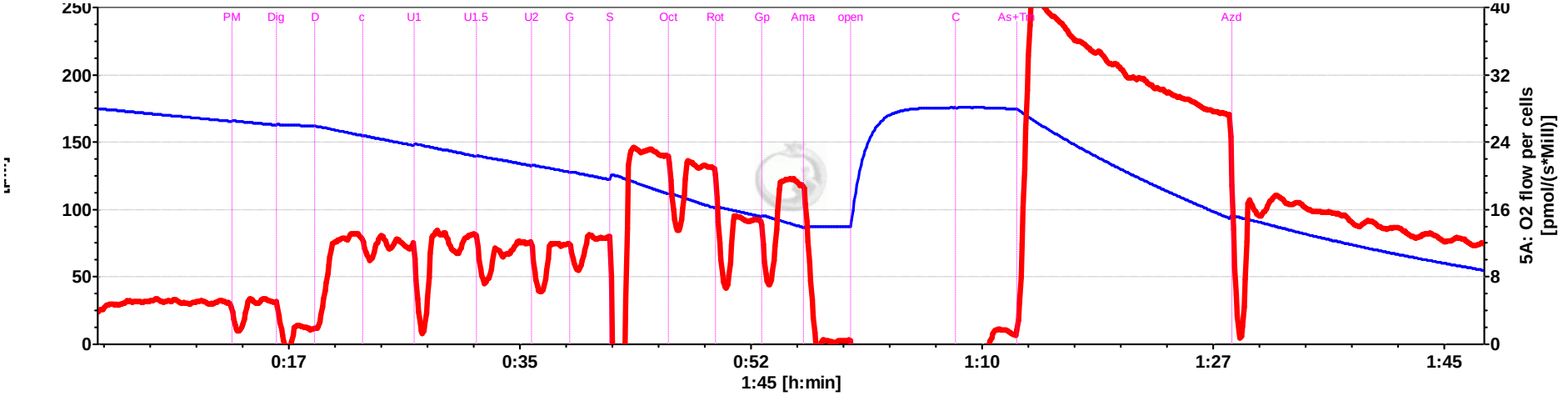


Figure 1. Isolation of Leukocytes and Platelets from Whole Blood. A) Freshly collected specimens are separated into their plasma and cellular components by centrifugation and purified by Ficoll density gradient, and Magnetic Activated Cell Sorting (MACS) by positive (monocyte and neutrophil) or negative selection (lymphocytes), while platelets are isolated by high-speed centrifugation of the platelet rich plasma. **B)** Images of the isolated cell populations once resuspended in XF DMEM and plated at indicated cell densities. Please click here to view a larger version of this figure.

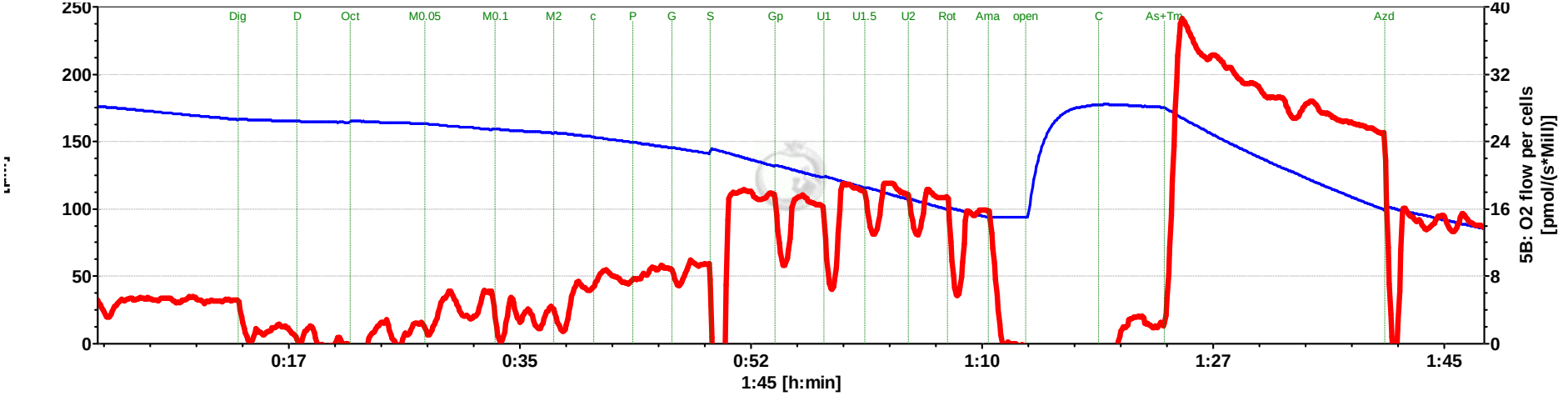
Respiration of permeabilized PBMCs

2016-06-02 P5-02.DLD

RP1



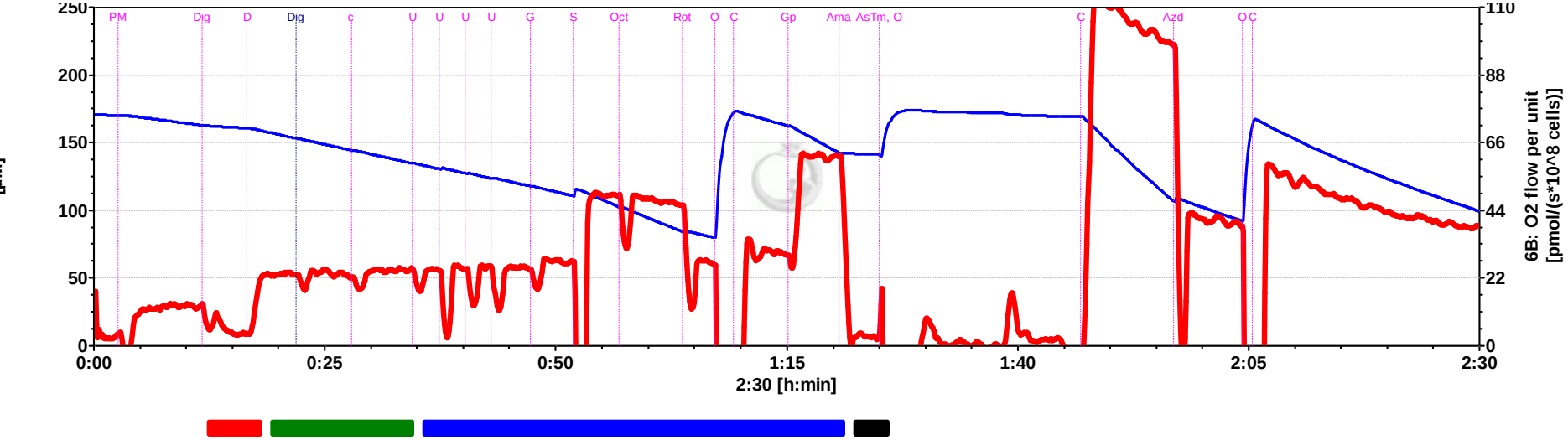
RP2



Respiration of permeabilized PLT

2016-04-19 P6-01.DLD

RP1



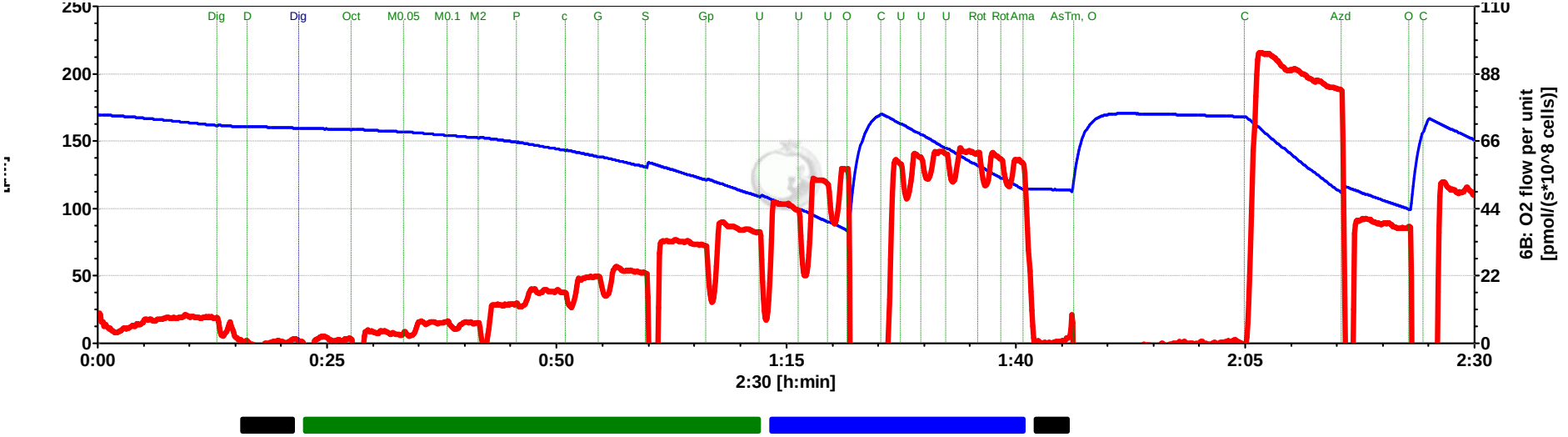
PM +mt: NFSGpTm_1PM 2D 3c 4U 5G 6S 7Oct 8Rot 9Gp 10Ama 11Tm 12Azd

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

Respiration of permeabilized PLT

2016-04-19 P6-01.DLD

RP2



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

E						9U	10Rot	11Ama	12Tm	13Azd
P	1D	2Oct3M+c	5P	6G	7S	8Gp				
L										
	ROX	F	NF	NF	NFS	NFSGp	SGp	ROX	Tm	ROX
CI	-	-	+	+	+	+	-	-	-	-
CETF	-	+	+	+	+	+	-	-	-	-
CII	-	-	-	-	+	+	+	-	-	-
GpDH	-	-	-	-	-	+	+	-	-	-

Calculated parameters in intact PBMCs

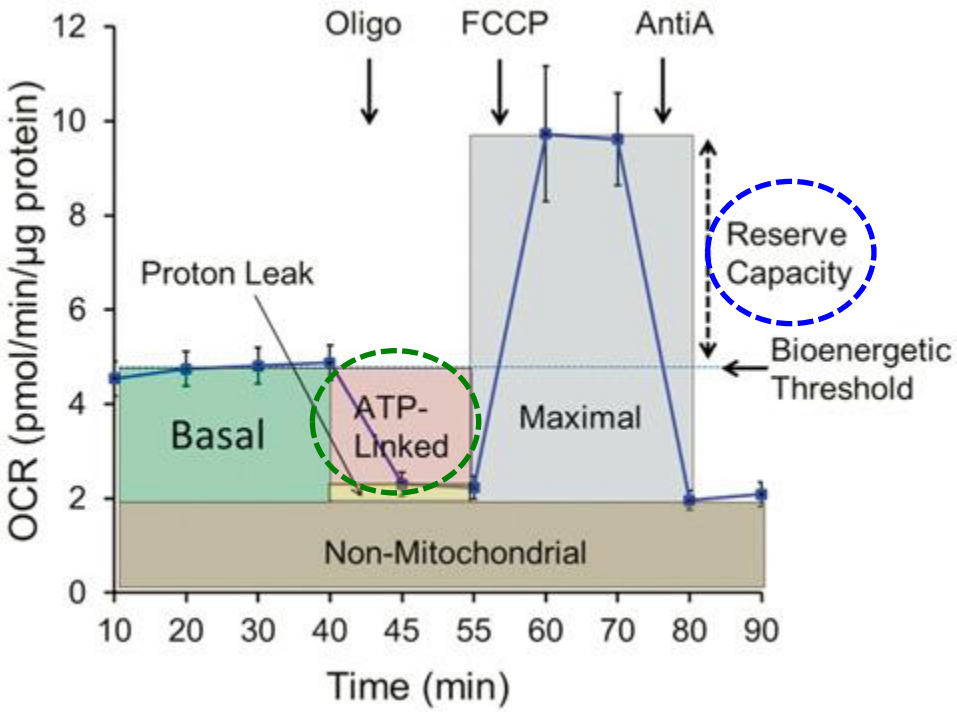
Coupling efficiency: (R-L)/R

(E-L)/E

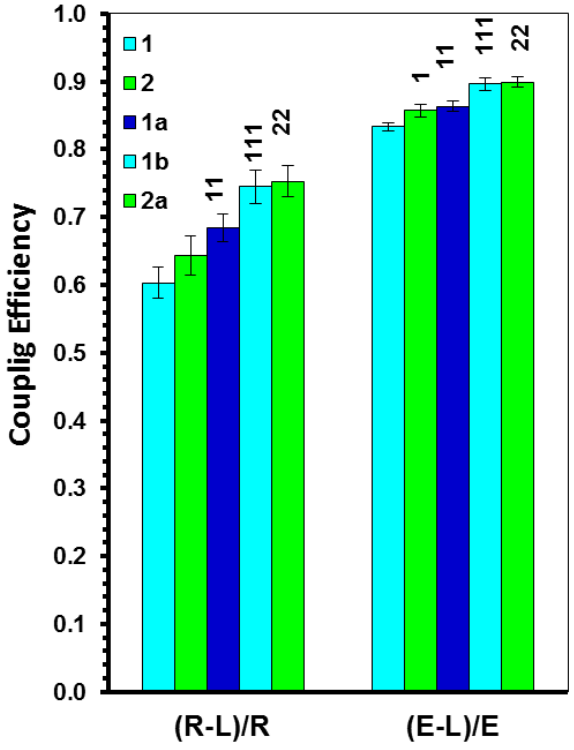
Chacko et al. *Clinical Science* 2014

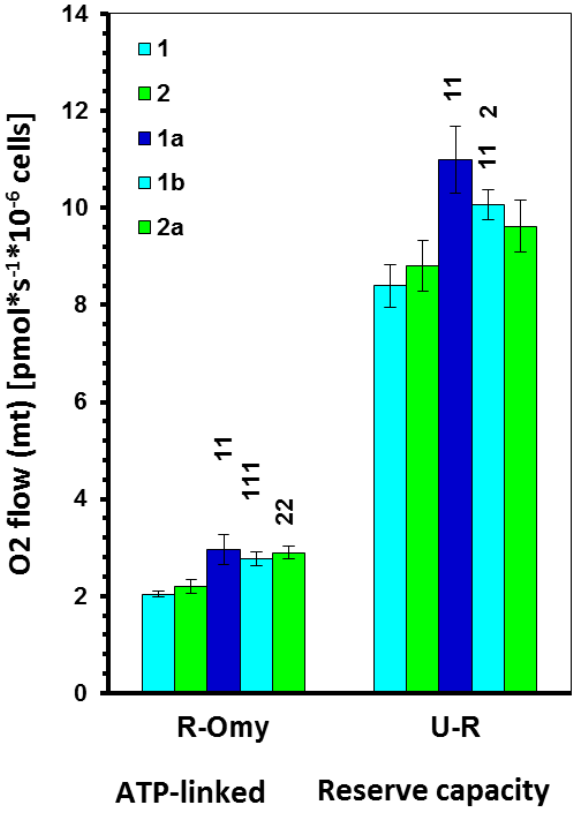
Bioenergetic Health Index:

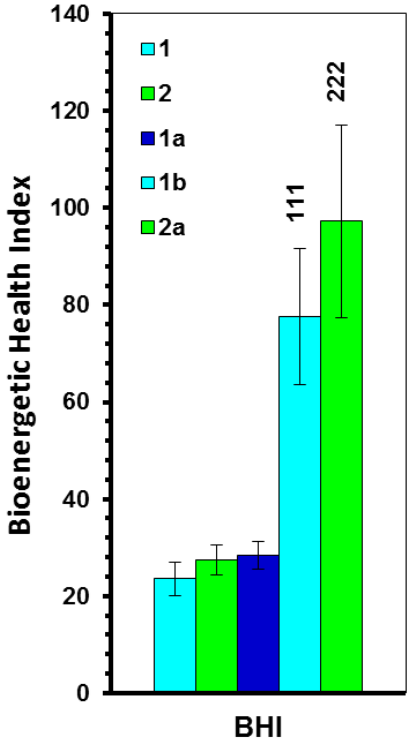
$$BHI = \log \frac{(\text{reserve capacity})^a \times (\text{ATP-linked})^b}{(\text{non-mitochondrial})^c \times (\text{proton leak})^d}$$



Calculated parameters in intact PBMCs







Increased coupling efficiency with training?
Improvements in handling of cells?
Seasonal changes?

^{11}P<0.01 vs 1
^{111}P<0.001 vs 1
^{2}P<0.05 vs 2
^{22}P<0.01 vs 2
^{222}P<0.01 vs 2