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Isolation of blood cells for HRR

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1. Introduction

Respiratory assessment of human health and disease can be performed with isolated mitochondria, tissue homogenate or permeabilized fibres prepared from tissue biopsies. The collection of tissue biopsies is invasive, limiting its applicability for large-scale screening. An alternative is the use of blood cells, which can be obtained in a far less invasive sampling procedure. Blood cells obtained by venipuncture are usually separated to obtain platelets (PLT) or a mixed population of immune cells subsumed as peripheral blood mononuclear cell (PBMCs), both of which have been successfully applied to characterize respiratory phenotypes of human diseases. A promising field of innovation is the extended storage or even cryopreservation of blood cells for later use in respirometry.

In the present study, isolation methods are described to obtain blood cells for high-resolution respirometry (HRR), methodological details of the isolation procedure are highlighted, and the protocols for the respiratory characterization of platelets and PBMCs are presented.

2. Isolation procedures for platelets and PBMCs

Isolation protocols described here are based on published methods used by different groups and optimized for obtaining maximum yield, purity and quality of PLT and PBMCs for respirometric measurements. An overview on selected published methods is presented in Appendix 1, showing the diversity of conditions relating to the media chosen for the separation and resuspension of cells, conditions of centrifugation (temperature, speed, breaks), and storage conditions of isolated cells prior to the experimental assay. It is important to keep the cells in sterile conditions and at constant temperature, to prevent activation and a change of phenotype of the cells [5].

No differences in respiratory performance was found in cells isolated in RPMI with BSA, RPMI and DPBS. We decided to use DPBS for suspension of cells, which is advantageous for later quantification of protein content, in addition to quantification of cell count, the mitochondrial matrix marker citrate synthase activity, and the cytosolic marker lactate dehydrogenase activity.

3. Chemicals and tubes

- Ficoll-Paque™ PLUS density gradient centrifugation medium: density 1.077, GE Healthcare.
- DPBS BE17-512F, Lonza.
- RPMI 1640 without L-glutamine BE12-167F, Lonza.
- Sterile centrifugation tubes: 50 ml Leucosep™, Greiner Bio-one.
- 50 ml Falcon tubes; 14 ml round-bottom Falcon tubes.

4. PBMCs and platelets

Isolation of PBMCs from whole blood is performed at room temperature (RT) with isolation media kept at RT. Instructions by the manufacturer are followed with slight modifications.

Sample preparation

1. 15 ml Ficoll-Paque™ PLUS density gradient centrifugation medium are added into a 50 ml Leucosep tube and centrifuged at 1,000 *g* for 1 min at RT using a swinging bucket rotor (intermediate acceleration, 6 of 9, low brakes, 2 of 9).
2. Two 9 ml samples of whole blood are collected in VACUETTE® K3EDTA (tri-potassium ethylenediaminetetraacetic acid) tubes and transported to the lab at RT in thermo-insulating containers, protected from light. A small subsample is removed and counted using the Sysmex XN-350 automated blood cell counter.

Reference ranges to be expected for normal samples:

Lymphocytes: 1-4 · 10^6 cells/ml
Monocytes: 0.1-0.5 · 10^6 cells/ml
Platelets: 150-300 · 10^6 cells/ml

1st method: Isolation procedure for PBMCs

1. Gently pour the blood to the Leucosep™ tube and add the same volume of DPBS.
2. Centrifuge at 1000 *g* for 10 min at RT, with brakes off [intermediate acceleration 6, brakes 0].
3. Collect 10 - 15 ml of clear plasma from the top of the tube into a new tube for later use, leaving another 10 - 15 ml above the layer of PBMCs.
4. Carefully collect the layer of PBMCs (~ 5 - 10 ml) with a Pasteur pipette and transfer to a new 50 ml Falcon tube.
5. Add 25 ml of DPBS and centrifuge at 110 *g* for 10 min at RT [fast acceleration, 9, intermediate brake, 6].
   (Note: The manufacturer instruction at this step is 250 *g* for 10 min, but this gives relatively high contamination with PLT: PLT/PBMCs ~ 20. Centrifugation at 110 *g* for 10 min gives a ratio PLT/PBMCs ~ 6 and the yield of PBMCs in the sediment ~ 88%).
6. Transfer the supernatant (supernatant 1) into a new 50 ml Falcon tube, add all clear plasma from point 3 and the rest of plasma after collection of PBMCs, add 10% of the volume of a 100 mM EGTA solution (10 mM EGTA final concentration) to prevent platelet activation and aggregation. This suspension could be used further for separation of platelets – continuing at point 9 below. (*Note:* the quality of this PLT preparation needs to be tested).
7. Resuspend the pellet gently in ~ 2 ml DPBS, add DPBS up to 25 ml, and centrifuge again at 110 *g* for 10 min at RT [fast acceleration 9, brake 6].
8. Discard the supernatant and gently resuspend the pellet containing PBMCs with 0.5 ml DPBS.

   Count and freeze subsamples:

   - Dilute 10 µl of cell suspension in 90 µl PBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex counter.
   - Remove 2 x 20 µl and 1 x 30 µl in Eppendorf tubes for protein concentration, LDH and CS activity determination respectively, freeze in -20 °C.

   Calculate volume of cell suspension to add 4 6 · 10^6 PBMCs into the 2 ml O2k chamber.

9. Centrifuge supernatant 1 with plasma and 10 mM EGTA from step 6 at 1000 *g* for 10 min at RT [fast acceleration 9, brake 2].
10. Gently resuspend the pellet in 5 ml DPBS, 10 mM EGTA, centrifuge for 5 min at RT [fast acceleration 9, brake 2].
11. Discard supernatant and resuspend the pellet containing PLT in 0.5 ml DPBS, 10 mM EGTA.

Count and freeze subsamples:

- Dilute 10 µl of cell suspension in 90 µl PBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex counter.
- Remove 2 x 20 µl and 1 x 30 µl in Eppendorf tubes for protein concentration, LDH and CS activity determination respectively, freeze in -20 °C.

Calculate volume of cell suspension to add 200 - 300 x 10^6 PLT into the 2 ml O2k chamber.

2nd method: Isolation procedure for PLT and PBMCs

In this protocol (steps 2 to 5), 14 ml round-bottom Falcon tubes are used.

1. Centrifuge whole blood at 200 *g* for 10 min at RT [acceleration 9, no brakes].
2. Pipette platelets rich plasma (PRP) into a new tube, leaving 2-4 mm layer above the rest of the blood. Add 10% of a 100 mM EGTA solution into PRP to avoid platelet activation and aggregation during centrifugation, mix gently. Proceed with steps 3-5 simultaneously with further isolation of PMBCs (steps 6-10) or leave PLT for centrifugation after isolation of PBMCs *(Note: the time effect has to be tested).*
3. Centrifuge PRP at 1000 *g* for 10 min at RT [intermediate acceleration 6, low brakes 2] (Note: some PLTs may still remain in plasma, but this setting gives a good yield of good quality PLTs for 4 chambers of the O2k.)
4. Gently resuspend the sediment in 4 ml DPBS, 10 mM EGTA, centrifuge at 1000 *g* for 5 min at RT [acceleration 6, brakes 2].
5. Gently resuspend in 0.5 ml of DPBS, 10 mM EGTA.

Count and freeze subsamples:

- Dilute 10 µl of cell suspension in 90 µl PBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex counter.
- Remove 2 x 20 µl and 1 x 30 µl in Eppendorf tubes for protein concentration, LDH and CS activity determination respectively, freeze in -20°C.

Calculate volume of cell suspension to add 200 - 300 x 10^6 PLT into O2k chamber.

6. Collect the buffy coat (the rest of plasma + layer of the blood bellow the plasma ~ 3 ml) with a Pasteur pipette and transfer it into a new tube, add the same volume of DPBS and mix gently. For maximum yield all the rest of blood can be taken and diluted 1+1 with DPBS (Note: this will increase the number of tubes per blood sample necessary for the next step of isolation).

7. Layer this mixture gently on the top of Ficoll-Paque 1.077 density medium (4 ml Ficoll-Paque + 6 ml of mixture).
8. Centrifuge at 1000 *g* for 10 min at RT [acceleration 9, brake 0].
9. Carefully collect the layer of PBMCs (~ 2 ml) with a Pasteur pipette and transfer it to a new 14 ml tube, add 2 volumes of DPBS.
10. Centrifuge at 350 *g* for 5 min acceleration 9, brake 6 and resusped the pellet containing PBMCs with 0.5 ml DPBS.

    Count and freeze subsamples:

    - Dilute 10 µl of cell suspension into 90 µl PBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex counter.
    - Remove 2 x 20 µl and 1 x 30 µl in Eppendorf tubes for protein concentration, LDH and CS activity determination, respectively, freeze in -20 °C.

    Calculate volume of cell suspension to add 4 - 6 x 10^6 PBMCs into the 2 ml O2k chamber.

5. Instrumental setup

Setup the OROBOROS O2k following standard procedures [16]. For each blood sample up to 4 instruments (2 for platelets and 2 for PBMCs) with 2 chambers each were run in parallel. Chambers were filled with 2.2 ml of either MiR05Cr or RPMI, as indicated below for each experiment. Media were equilibrated at 37 °C before closing the stoppers and thereby adjusting the final chamber volume to 2 ml. Before adding cells, stoppers were lifted, the necessary volume to be added from the cell stock was removed and replaced with cell suspension before closing the chamber again.

6. SUIT protocols for intact and permeabilized blood cells

Four different substrate-uncoupler-inhibitor-titration (SUIT) protocols were run in parallel, one in each chamber, to simultaneously characterize intact and permeabilized platelets or PBMCs. These SUIT protocols were based on extensive preliminary experiments aimed at developing a set of SUIT reference protocols fit to provide a basis for a comprehensive and comparative evaluation of mitochondrial respiration in a broad array of experimental systems [14, 15].

SUIT protocols for intact cells: RPMI-1640 without L-glutamine

A: Routine + EtOH + U + Rot + S10 + Ama

B: Routine + Omy + U + Rot + S10 + Ama

EtOH	ethanol – a solvent for Omy, added as control to Omy titration
Omy	oligomycin, 2.5 µM
U	uncoupler CCCP, added in steps from 1 up to 4 µM
Rot	rotenone, 0.5 µM
S10	succinate, 10 mM
Ama	antimycin A, 2.5 µM

By comparing the coupling control protocol in O2k chamber B with the simplified protocol in chamber A (without Omy), the effect of Omy on ETS can be studied. By addition of 10 mM S after Rot, the intactness is tested of the cell membrane.

SUIT protocols for permeabilized cells: MiR05Cr+Ctl

For respiration of permeabilized cells we used harmonized SUIT reference protocols RP1 and RP2 [14, 15]. Concentrations of some chemicals were optimized for respiration of blood cells, the full list of used chemicals for both protocols is shown below.

A: RP1: Routine+PM+Dig+D+c+U+G+S50+Oct+Rot+Gp+Ama+reox +AscTm+Azd

B: RP2: Routine+Dig+D+Oct+M0.05+M0.1+M2+c+P+G+S50+Gp+U +Rot+Ama+reox+AscTm+Azd

Chemical	concentration in O2k chamber
Ctl	Catalase 280 U/ml
P	Pyruvate, 5 mM
M0.05	Malate, 0.05 mM
M0.1	Malate, 0.1 mM
M	Malate, 2 mM
Dig	Digitonin, 8 µg/10^6 PBMCs and 20 µg/10^8 PLT
D	ADP, 1 mM
c	Cytochrome c, 10 µM
U	Uncoupler CCCP, added in steps from 1 up to 4 µM
G	Glutamate, 10 mM
S50	Succinate, 50 mM
Oct	Octanoyl carnitine, 2 mM
Rot	Rotenone, 1 µM
Gp	Glycerophosphate, 10 mM
Ama	Antimycin A, 2.5 µM

AscTm Ascorbate, 2 mM, TMPD (N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride), 0.5 mM
Azd Azide, 100 mM

7. Quantity, purity and quality of blood cells isolated

Applying the above described isolation methods for PBMCs, the typical gain of cells amounted to:

- PBMC cell count: 25 ± 2.3 million cells (obtained from 16-18 ml whole blood)
- PLT/PBMCs as assessed with the Sysmex cell counter: 6.5 ± 0.8 (range 1.8 – 12.2 depending on the ratio PLT/PBMCs in the whole blood, see Graph 1). The PLT/PBMCs ratio in preparation of PBMCs obtained by the 2nd method was similar (5.6 ± 1.8).
- Viability as assessed by Trypan blue exclusion with the Countess II cell counter: 86 ± 1.8 %.

By comparing the ratio of PLT to PBMC in isolated PBMC fraction and the whole blood sample we found a strong positive correlation between these two parameters (Fig. 1). This result indicates that the purity level of PBMC fraction obtained by isolation methods is influenced by the PLT to PBMC ratio in the donor's blood.

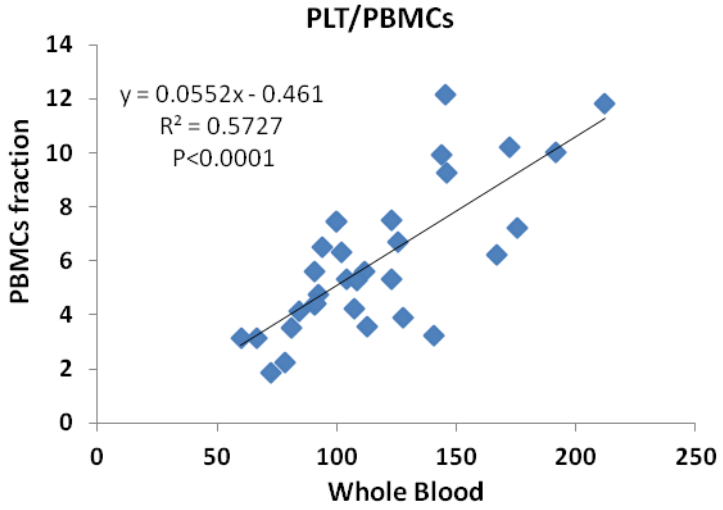

Figure 1. PLT to PBMC ratio in the isolated PBMC fraction as a function of PLT to PBMC ratio in whole blood. The ratio PLT/PBMC in PBMC fraction correlates closely with PLT/PBMC in the whole blood (P<0.0001; Pearson test). N=32 blood samples.

8. Respiratory characteristics of intact platelets and PBMCs

Figure 2 shows examples of measurements and their evaluation on intact PLTs isolated from platelet-rich plasma as described above and examined with a modified Coupling Control Protocol using MiR05Cr.

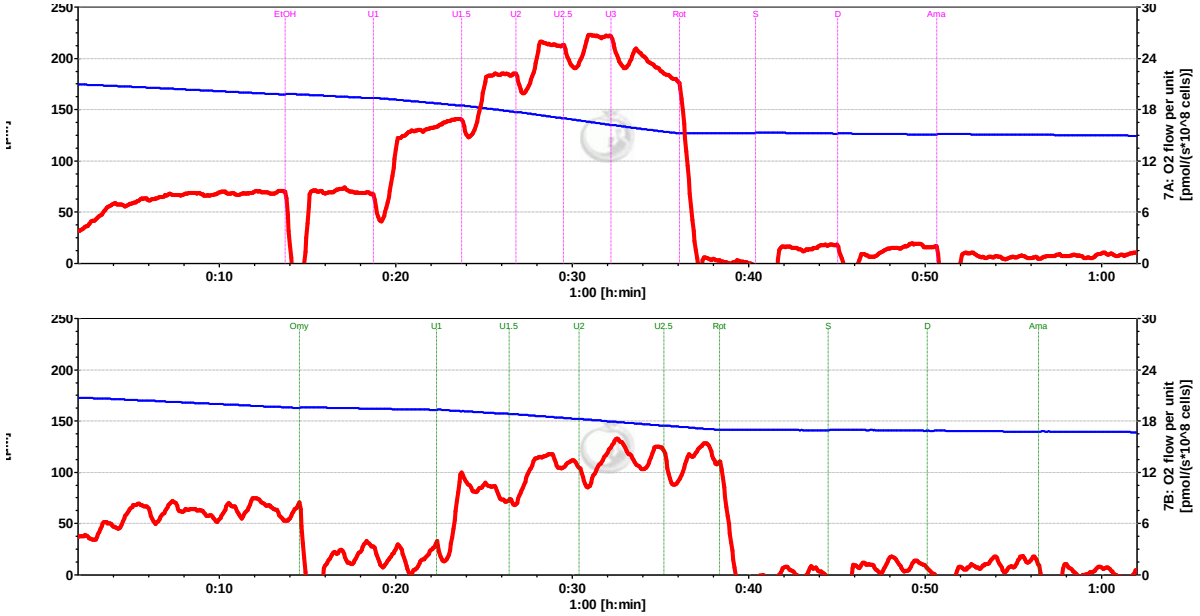

Figure 2A. Respiration of PLT in a coupling control protocol in MiR05Cr. Blue trace: oxygen concentration (left Y-axis [µM]). Red trace: oxygen flux per chamber volume (right Y-axis [pmol·s^{-1}·10^{-8}cells]). 2016-04-20 P7-02.DLD

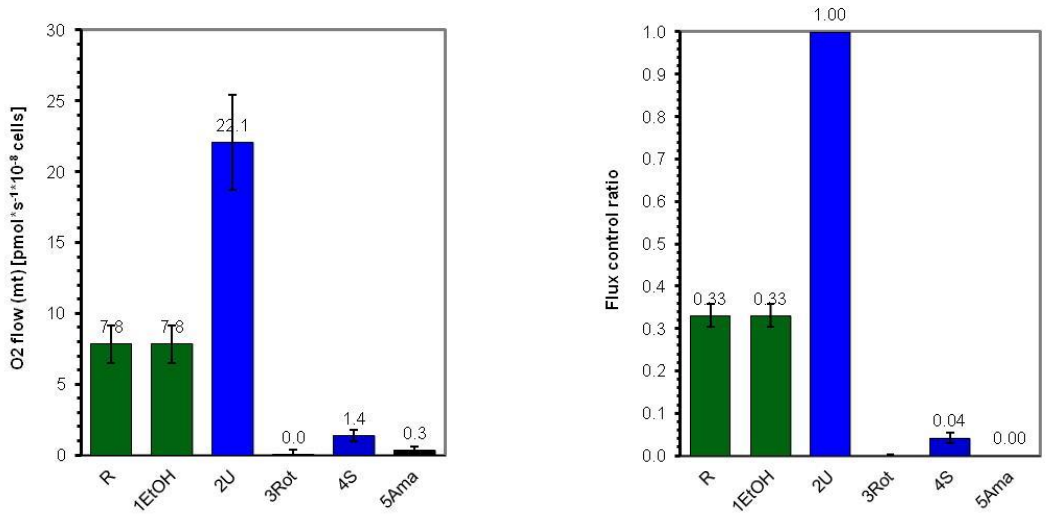

Figure 2B. Respiration of human PLT in MiR05Cr. Left panel: Flow per Mill cells. Right panel: Flux control ratios normalizing ROX-corrected flux to ETS capacity. Means ± SEM of 5 experiments. Succinate was added to test the intactness of the cell membrane.

Figures 3 to 5 show examples of measurements and their evaluation on intact PBMCs isolated by protocol 1 described above, in a modified coupling control protocol in MiR05Cr+Ctl and RPMI.

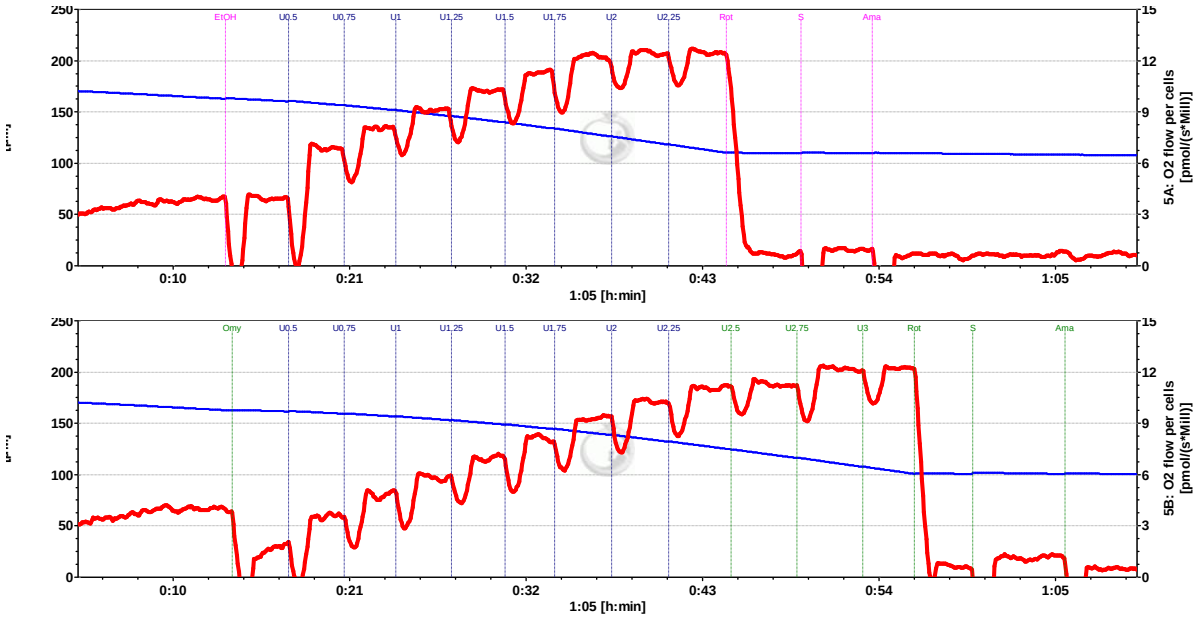

Figure 3. Respiration of intact PBMCs in a coupling control protocol in RPMI. Details as in Fig. 1. 2016-06-01 P5-03.DLD

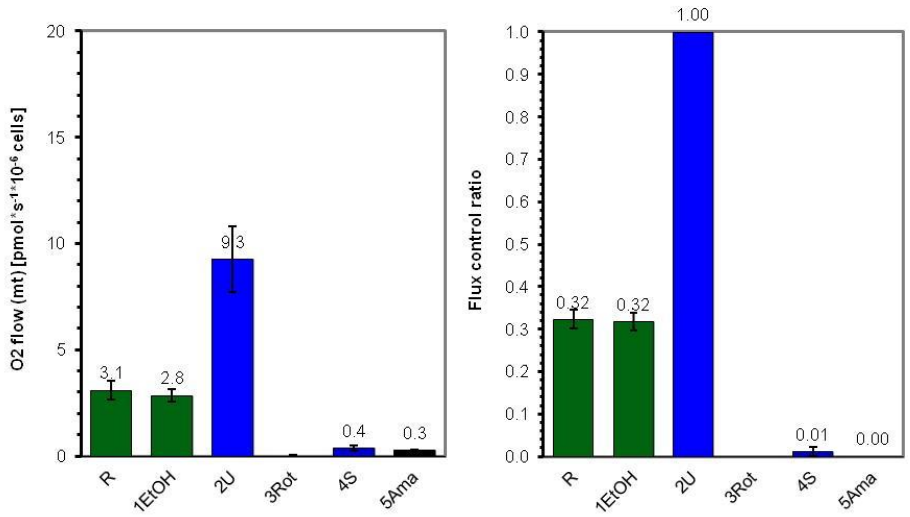

Figure 4. Respiration of intact PBMCs in MiR05Cr. Means ± SEM; *N*=5.

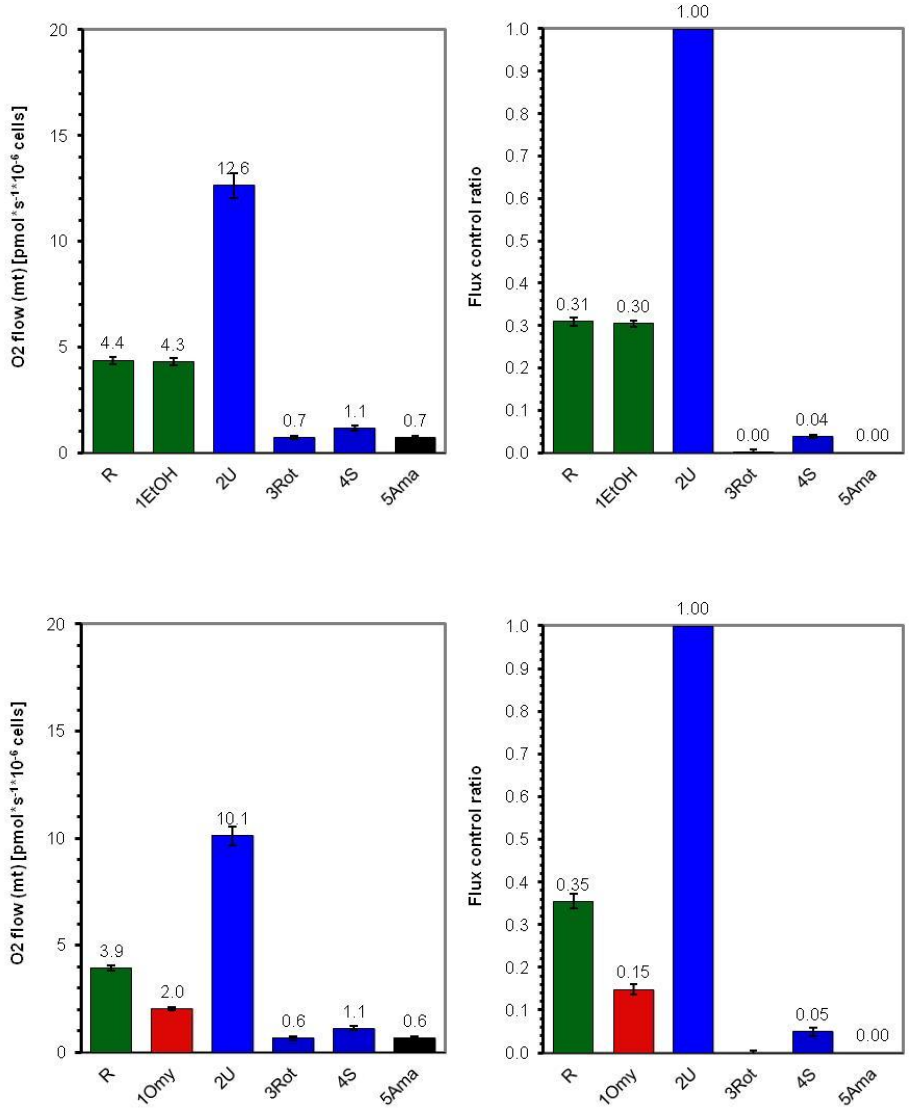

Figure 5. Respiration of intact PBMCs in RPMI. Means ± SEM; *N*=16.

Respiration of intact cells depended on the respiration medium. RPMI supported higher ROUTINE respiration than MiR05Cr. While RPMI contains many fuel substrates, cells respiring in MiR05Cr are entirely dependent on endogenous substrates. Many cell culture media contain non-physiological concentration of glucose and L-glutamine to support cell growth and these substrates may also fuel or inhibit (Crabtree effect) respiration. We used RPMI without L-glutamine in the present experiments. Selection of an optimal medium composition for evaluation of ROUTINE respiration of intact cells must be carefully considered.

ETS capacity after Omy is frequently much lower than ETS measured without previous Omy titration (Fig 1). In Fig. 5, ETS capacity measured after Omy accounted for only 80% of ETS measured in the absence of Omy. The effect of Omy on ETS capacity depends stress conditions and respiration medium, resulting in different relative Omy effects on ETS capacity in the same cell type.

9. Respiratory characteristics of permeabilized platelets and PBMCs

Permeabilized platelets and PBMCs were examined using two different SUIT reference protocols, RP1 and RP2 each with a slightly different focus, but containing cross-linked respiratory states. Together, these protocols allow for a comprehensive assessment of mitochondrial respiratory capacities [14, 15].

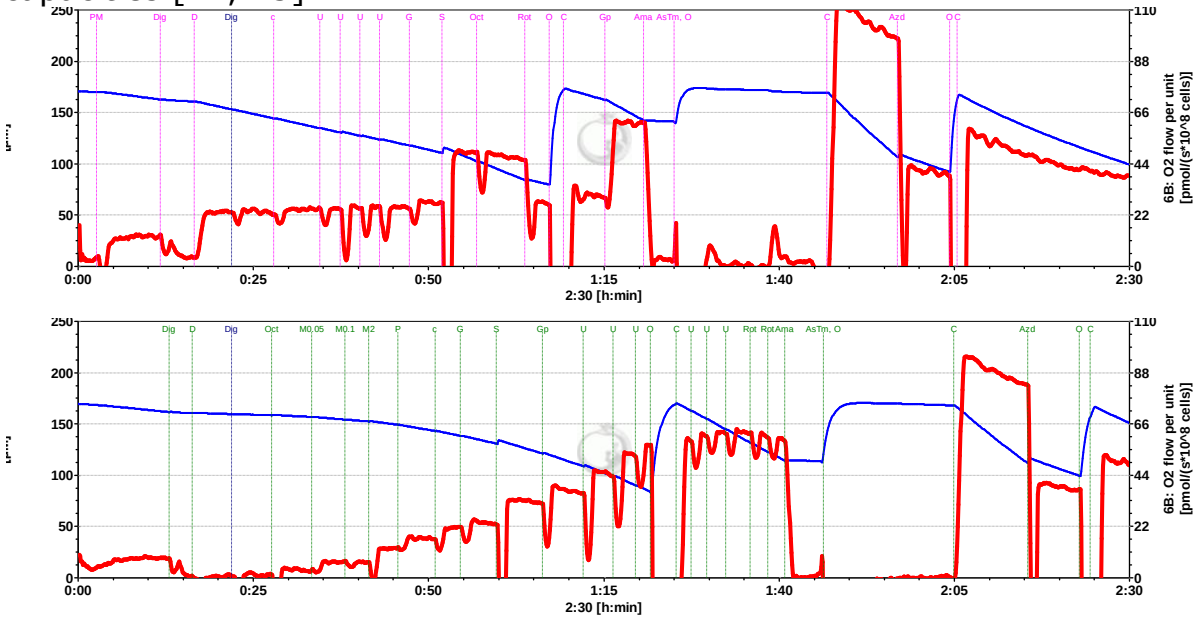

Figure 6. Respiration of platelets in SUIT reference protocol RP1 (upper panel) and RP2 (lower panel). Blue and red traces denote oxygen concentration and oxygen flow, respectively. 2016-04-19 P6-01.DLD.

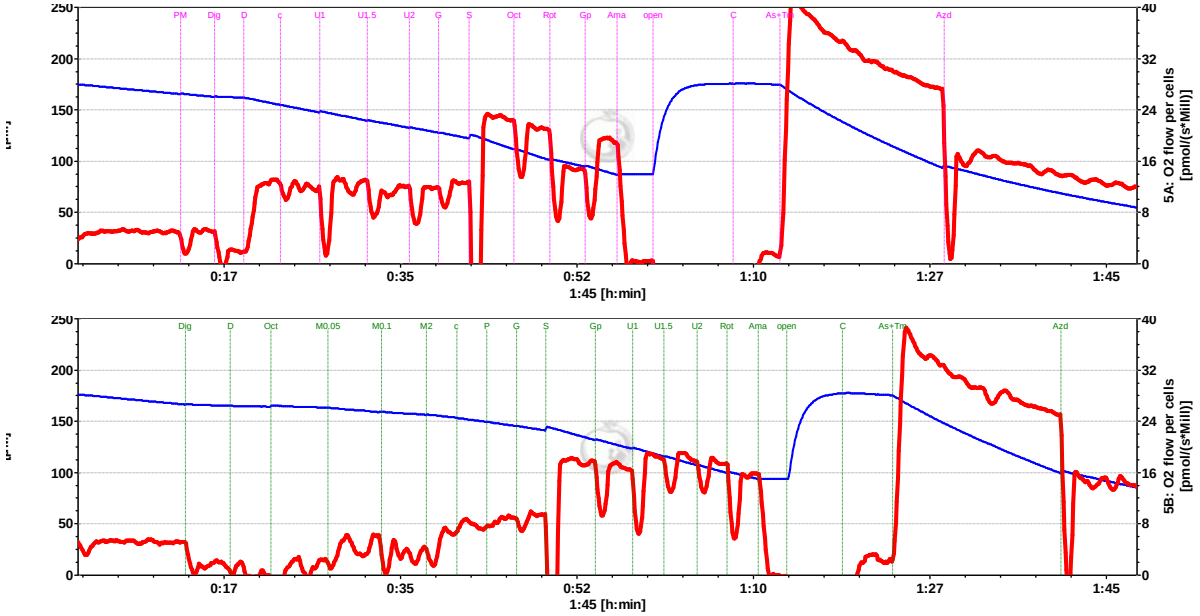

Figure 7. Respiration of PBMCs in SUIT reference protocol RP1 (upper panel) and RP2 (lower panel). Details in Fig. 1. 2016-06-02 P5-02.DLD.

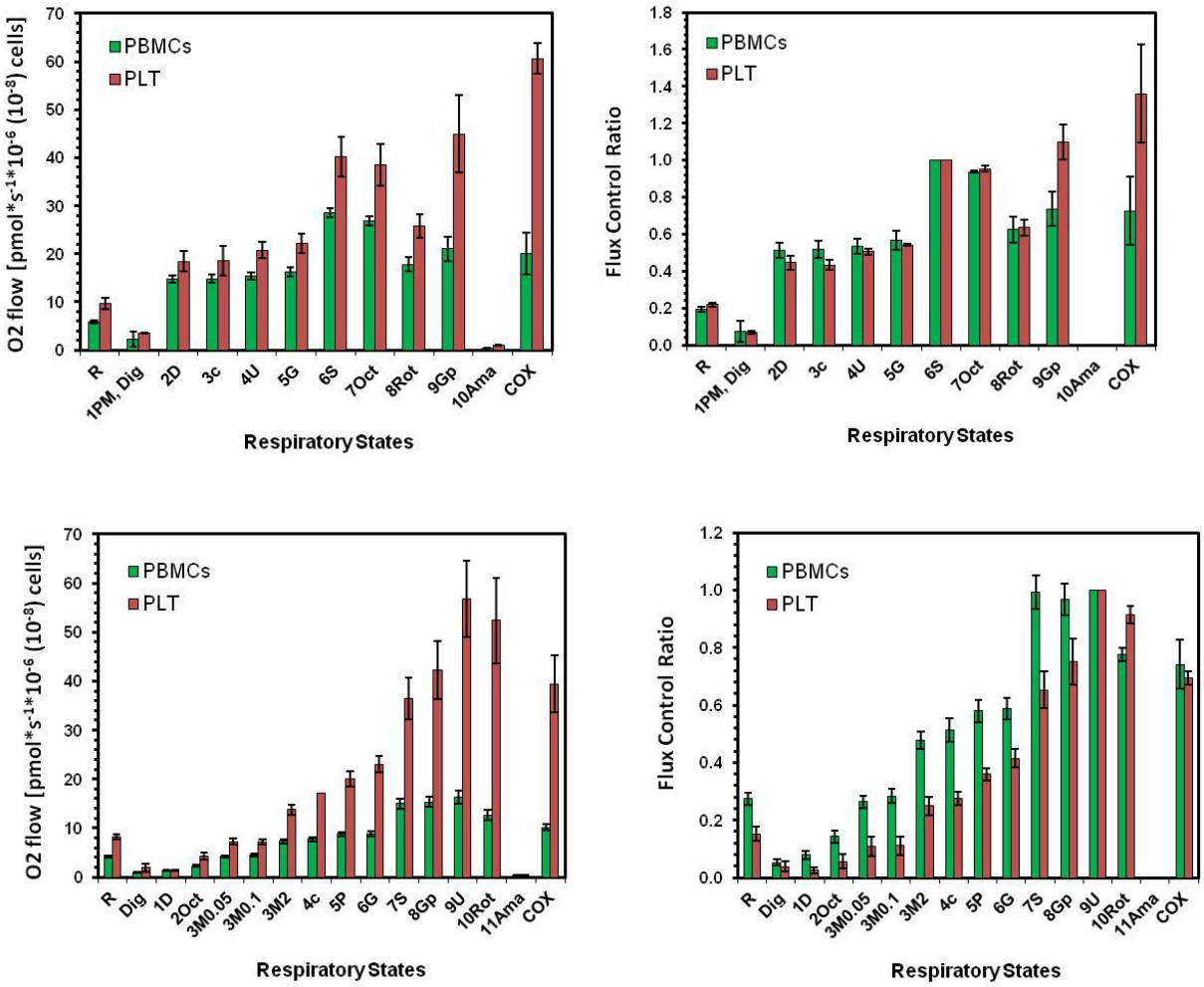

Figure 8. Comparison of respiration in PBMCs and PLT in SUIT reference protocol RP1 (upper panels) and RP2 (lower panels). Flux control ratios were calculated by normalizing to NS-ETS capacity in RP1 and to FNSGp-ETS in RP2. Respiration expressed per 10^6 PBMCs or 10^8 PLTs. Means ± SEM; *N*=2-4.

The above figures illustrate the differences in the respiratory signatures of PLTs and PBMCs. In RP1 the response to Gp is significantly higher in PLT than in PBMC (FCF_{Gp} 0.39 in PLT vs 0.12 in PBMCs, Fig. 9), in RP2 the phosphorylation system highly limits respiration of PLT in the FNSGp-pathway control state, shown by the stimulation of respiration by uncoupler titration. By comparison, in PBMCs the effect of uncoupler titration is low (FCF_U 0.25 in PLT vs 0.06 in PBMCs). Importantly, the small contamination with PLT must always be considered when working with PBMCs isolated by these two methods (the mean ratio PLT/PBMCs ~ 6), as it could significantly affect the respiratory patterns observed and the cell mass in the chamber. At a PLT/PBMC ratio of 6, PLTs may account for 21% of protein content (the amount of protein per 10^6 cells was 2.91±0.23 μg for PLT and 82.8±3.1 μg for PBMCs).

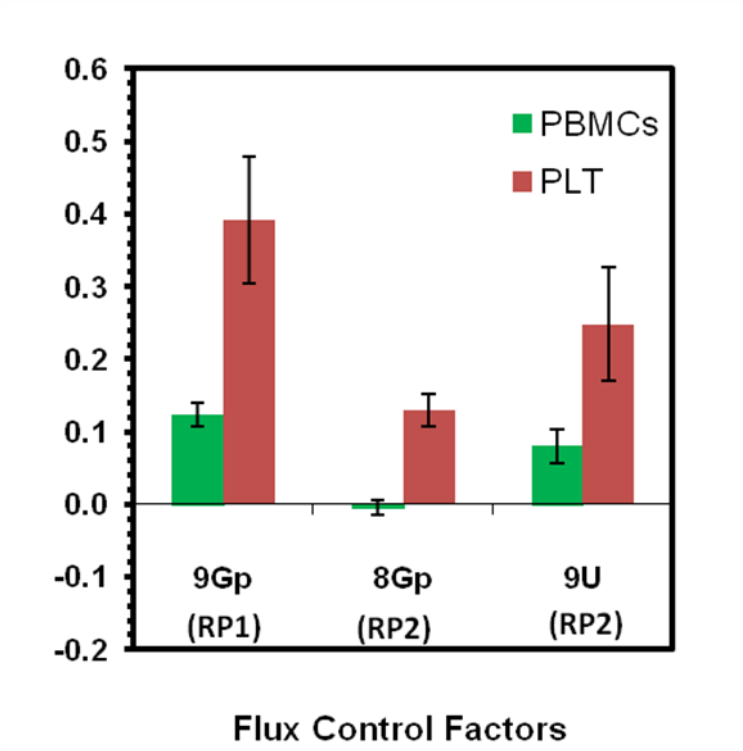

Figure 9. Step changes from SUIT reference protocols RP1 and RP2 which were significantly different for PBMC and PLT expressed as Flux control factors. Means ± SEM of 30 and 4 samples, respectively.

10. Conclusions

Respiration of intact blood cells depends on the respiratory medium applied. Different respiratory patterns of PBMC and PLT are revealed in the SUIT protocols RP1 and RP2. The PLT/PBMC ratio in the isolated PBMC fraction closely correlates with the PLT/PBMC ratio in whole blood. Contamination of PBMCs with platelets can significantly affect their apparent respiration. Therefore, purity of the cell preparation must be considered in the optimization of isolation methods.

11. References

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Supplement A

Table 1. Overview of methods for the isolation of platelets and PBMCs

Full blood centrifugation	PRP[1] -> PLTs centrifugation	PLT resuspendation	Buffy coat centrifugation	PBMCs centrifugation	PBMCs resuspendation	Ref.
18 ml blood EDTA tubes **Transport at RT** 200 g 10 min, **no brakes**	Add 10 % 100 mM EGTA 1000 g 10 min no brakes **Brakes 1**	Wash with sterile PBS (4 ml +0.4 ml EGTA) 1000 g 5 min no brakes Resupend pellet with 0.5 ml RPMI or PBS +10% EGTA **Transport at RT**	Dilute buffy coat 2x with RPMI or PBS layer on Ficoll (4 ml 1.077) + 6 ml of diluted buffy coats Centrifuge 1000 g 10 min, acc 6, **no brakes**	Collect PBMC (2 ml), wash with RPMI or PBS (+6 ml) Centrifuge 350 g 5 min, acc 9, b**rakes 6**	Resuspend in 0.5 mL RPMI or PBS Cell count for 4 chambers **Transport on ice**	[1a]
20 ml blood in EDTA tubes transport on ice 150 g 10min, **no brakes**	Add 10 % 100 mM EGTA, 750 g 5 min, no brakes	Resuspend in 200 ul PBS, count for 4 chambers transport at RT	Dilute rest with equal amount of **PBS or saline**, layer on 5 ml of Histopaque 1.077 in 15 ml round bottom tube (4 tubes per person) Centrifuge 800 g 15 min or 1000 g 10 min no brakes	Collect the layer with PBMCs and wash with PBS 350 g 5 min	Resuspend in 200 ul PBS, count for 4 chambers transport on ice **intact cells:** RPMI+FCS, **permeabilized:** MiR05	[2a]
20 ml bood In citrate dextrose tubes transport at RT 200 g 20 min, **no brakes**	700 g 20 min no brakes add PGE1 resuspend in PSG 700 g 20 min, **no brakes**, add PGE1	Resuspend in 2-4 ml **M199** – they can be activated, respiration intact in the same medium Cell count with hematocrit **Do not transport below 20°C (25-30 optimum)** **intact cells:** M199	Take buffy coats and layer on Ficoll-Hypaque the same volume in 15 ml tubers (2 tubes per person) Centrifuge 400 g 30 min, **no brakes**	Collect PBMCs Dilute 5x with RPMI 700 g 8 min, brake 6	Resuspend in 1 ml **RPMI** with 10 mM glucose, respiration intact in the same medium Cell count ~ 20 million for 4 chambers transport on ice **intact cells:** RPMI	[3a]
16 ml of blood 500 g 10 min acc 9, no brakes	1000g 10 min, acc 9, brakes 6	4.5 ml MiR05 or RPMI for intact transport at 36°C	Dilute with **RPMI**, pour on Leucosep tube with Ficoll-Pague 1.077 g/ml, fill up to 50 ml Centrifuge 1000 g 10 min, **no brakes**	Collect PBMCs, dilute with RPMI to 45 ml Centrifuge 200 g 10 min, acc 9 brake 6	4.5 ml MiR05 or RPMI for intact transport at 36°C	[4a]

500 g 15 min, acc 5-6, **no brakes**	1500g 8 min, acc 9, brakes 6	Wash with sterile PBS+1 ug/ml PGI2, repellet with 1 ml PBS+PGI2 1500 g 8-10 min, acc 9, brakes 6	Dilute 4x with basal RPMI, Layer on Ficoll density gradient (3 ml 1.077+3 ml 1.119) in 15 ml tube. Add 8 ml of diluted blood Centrifuge 700 g 30 min, acc 6, **no brakes**	Collect: Upper layer (MNCs) and Middle band (PMNs) separately Add 4 volumes of RPMI Centrifuge 700 g 15 min, RT, brake on	Resuspend in 1 ml RPMI+0.5% fatty acid free BSA in 1.5 ml tube **Centrifuge in picofuge for 30 sec** Resuspend in 80 ul RPMI+BSA, add 20 ul antiCD15-labelled magnetic beads, separate by magnetic activated cell sorting (MACS) separator	[7]
20 ml bood K2EDTA tubes (Vacuette, Greiner Bio-One, Austria)		200*10^6 cells Dig: 1 ug/10^6 cells			3,5-5*10^6 cells Dig: 6 ug/10^6 cells MiR05 – permeabilized cells Plasma – intact cells	[2]
EDTA			1-2 h after collection 4°C Ficoll-Paque PLUS (GE Healthcare Bio-Sciences) Blood layered on equal volume of Ficoll 800 g 20 min	1 ml of lymphocytes diluted by 15 ml of erythrocyte lysing buffer, 20 min on ice	Pellet by centrifugation at 800 g 20 min, resuspend in PBS with 1:500 protease inhibitor cocktail Sigma 0.6 mg prot /measurement Dig 50 ug/mg prot KCl medium for respiration	[9]
			400 g 30 min Ficoll-Hypaque (Biocrom KG)			[8]
			350 g 25 min	Add 3x volume HBBS 10 min 100g	R7509 RPMI-1640 Medium Modified	[5a]
21 ml blood K2EDTA tubes (Vacuette Austria) 300 g 15 min RT	4600 g 5 min RT	Resuspend in plasma Dig: 1 µg/1×10^6 platelets				[12]

40 ml blood K2EDTA tubes (Vacuette, Greiner Bio-One, Austria)				Mononuclear cells and granulocytes	cytokines TNFa, IL-1b, IL-6 CS, mtDNA, cyt c Dig: 6 µg/1×10^6 cells	[11]
	1000 g 10 min RT	Resuspend in MiR05 Respiration intact MiR05				[1]

[1]PRP platelet rich plasma

References extra for Appendix 1: personal communication

[1a] Zuzana Sumbalova and Luiz F Garcia-Souza - adapted from the protocols below
[2a] Shao Chang
[3a] Luiz Garcia
[4a] Florian Hoppel
[5a] Elisa Calabria