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Blood cells

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O2k-Protocols: Isolation of peripheral blood mononuclear cells and platelets from human blood for HRR

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1. Introduction

Assessment of human mitochondrial respiratory function is often performed with isolated mitochondria, tissue homogenate or permeabilized fibers prepared from tissue biopsies. However, the collection of tissue biopsies is invasive and experimentally cumbersome, limiting its applicability. An alternative is the use of blood cells, which can be obtained in a less invasive sampling procedure and can be temporarily stored after collection for later use in respirometric measurements. Blood cells obtained by venipuncture are then usually separated to platelets (PLTs) and a mixed population of immune cells subsumed as peripheral blood mononuclear cells (PBMCs), both of which have been successfully applied to characterize respiratory phenotypes of human diseases.
In the present study, isolation methods to obtain blood cells for high-resolution respirometry (HRR) are described, important aspects of the isolation procedure are highlighted, and 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 PLTs and PBMCs for respirometric measurements. An overview on a selection of published methods is presented in Supplement A, showing the diversity of conditions relating to the media chosen for the separation and resuspension of cells, the exact conditions of centrifugation as to speed and temperature, and the storage conditions of isolated cells prior to experimentation. It is important to keep the cells in sterile conditions and at constant temperature to prevent activation of the cells and changing their phenotype [1].
In the isolation procedures, we compared the use of RPMI+BSA, RPMI and DPBS for washing steps. Since we did not find differences in respiration of cells isolated with these media, we decided to use DPBS in our isolation protocols. Resuspension of cells in DPBS is advantageous for later quantification of respirometric measurements per protein content in addition to quantification per cell count, mitochondrial marker citrate synthase activity, and cytosolic marker lactate dehydrogenase activity.

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™ tubes (Greiner Bio-one); 50 mL Falcon tubes; 14 mL round-bottom Falcon tubes, EGTA 100 mM stock solution.

Sample preparation

The following describes the method used for isolation of PBMCs from whole blood. It is based on the use of Leucosep™ tubes (Greiner Bio-One) and Ficoll-Paque™ PLUS density gradient centrifugation medium following the instructions by the manufacturer with slight modifications. All isolation media are kept at room temperature (RT) and all the procedures are performed at RT.

Collection of blood:

Two 9 mL samples of whole blood are collected in VACUETTE® K3EDTA (tri-potassium ethylenediaminetetraacetic acid) tubes using a thick needle (gauge 21) to prevent haemolysis. Tubes are transported to the lab at RT in thermo-insulating containers, protected from light. One hour after blood collection, blood is gently mixed by slowly reverting the tube for 6-10 times and counted on a Sysmex XN-350 haematology analyser. Normal ranges of cell counts to be expected:

Platelets: 150 - 400 x 10^6 cells/mL
Lymphocytes: 0.95 - 4.4 x 10^6 cells/mL
Monocytes: 0.08 - 1 x 10^6 cells/mL

1st method: Isolation procedure for PBMCs and PLTs from 1 blood sample: focus on PBMCs.

- Add 15 mL Ficoll-Paque™ PLUS into a 50 mL Leucosep tube and centrifuge at 1000 *g* for 1 min at RT using a swinging bucket rotor [intermediate acceleration, 6 of 9, low brakes, 2 of 9].

1. Gently pour the blood onto the top of the polyethylene barrier of the Leucosep™ tube with Ficoll-Paque™ PLUS and add the same volume of DPBS.
2. Centrifuge at 1000 *g* for 10 min at RT, with brake 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 PBMC-PLT layer ("buffy coat", ~ 5 - 10 mL) with a Pasteur pipette and transfer it into a new sterile 50 mL Falcon tube. Add DPBS up to a total volume of 25 mL and centrifuge at 120 *g* for 10 min at RT [fast acceleration, 9, intermediate brake, 6]. (Note: The manufacturer instruction at this step reads 250 *g* for 10 min, but this gives high contamination with PLTs: PLT count to PBMC count ratio (N_{PLT}/N_{PBMC}) ~ 20. Centrifugation at 120 *g* for 10 min yields a ratio N_{PLT}/N_{PBMC} ~ 7 and ~ 97% of PBMCs in the sediment).
5. Transfer the supernatant (supernatant 1) into a new 50 mL Falcon tube, add 5 mL of clear plasma collected at step 3 and 10% of the total volume of 100 mM EGTA solution (10 mM EGTA final concentration) to prevent platelet activation and aggregation. This suspension is used further for separation of platelets – continuing at step 8 below.
6. Resuspend the pellet gently in ~ 2 mL DPBS, add DPBS up to 25 mL, and centrifuge again at 120 *g* for 10 min at RT [fast acceleration 9, brake 6].
7. Discard the supernatant and gently resuspend the pelleted PBMC fraction with 0.5 mL DPBS.

   Count and freeze subsamples:

   - Dilute 10 µL of cell suspension into 90 µL DPBS in an Eppendorf tube for counting (10x dilution) on the Sysmex XN-350 haematology analyser.
   - Transfer two 20 µL and one (1) 30 µL aliquots of the stock suspension into Eppendorf tubes for determination of protein concentration, LDH and CS activity, respectively, store at -80 °C (the samples for LDH determination should be immediately put into -80°C).

Calculate the volume of cell suspension required to have 4 x 10^6 PBMC in the 2 mL O2k chamber.

Continuation from step 5 with isolation of PLT:

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

Count and freeze subsamples:

- Dilute 10 µL of cell suspension into 90 µL DPBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex XN-350 haematology analyser.
- Transfer two 20 µL and one 30 µL aliquots of the stock suspension into Eppendorf tubes for determination of protein concentration, LDH and CS activity, respectively, store at -80 °C (-80°C is a must for LDH samples).

Calculate the volume of cell suspension required to have 200 x 10^6 PLT in the 2 mL O2k chamber.

2nd method: Isolation procedure for PLT and PBMC from 1 blood sample: focus on PLT.

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 platelet rich plasma (PRP) into a new tube, leaving a 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 PMBC (steps 6-10) or leave PLT for centrifugation after isolation of PBMC *(Note: the time effect must be tested).*
3. Centrifuge PRP at 1000 *g* for 10 min at RT [intermediate acceleration 6, low brakes 2] (Note: some PLT remain in plasma, but this setting gives a good yield of good quality PLT 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 pelleted PLT fraction in 0.5 mL of DPBS, 10 mM EGTA.

Count and freeze subsamples:

- Dilute 10 µL of cell suspension into 90 µL DPBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex XN-350 haematology analyser.
- Transfer two 20 µL and one 30 µL aliquots of the stock suspension in Eppendorf tubes for determination of protein concentration, LDH and CS activity, respectively, store at -80°C (-80°C storage is a must for LDH determination).

Calculate the volume of cell suspension required to have 200 - 300 x 10^6 PLT in the 2 mL O2k chamber.

Continuation from step 2 with isolation of PBMC:

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 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 resuspend pelleted PBMC fraction with 0.5 mL DPBS.

Count and freeze subsamples:

- Dilute 10 µL of cell suspension into 90 µL DPBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex XN-350 haematology analyser.
- Transfer two 20 µL and one 30 µL aliquots of stock suspension in Eppendorf tubes for determination of protein concentration, LDH and CS activity, respectively, store at -80°C (-80°C storage is a must for LDH determination).

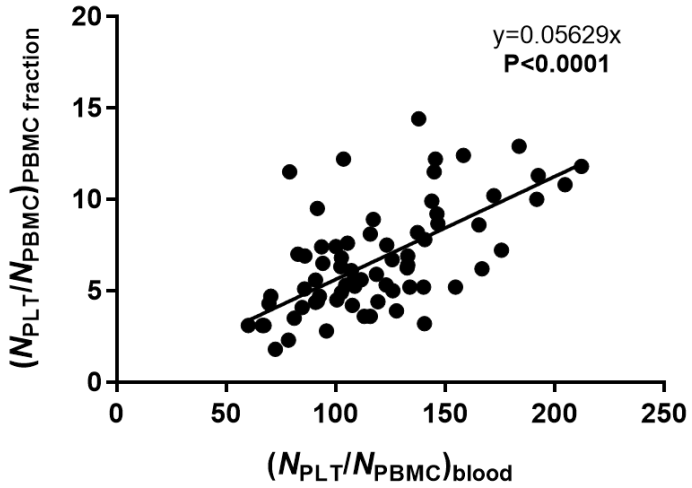
Calculate the volume of cell suspension required to have 4 x 10^6 PBMCs in the 2 mL O2k chamber.

Quantity, purity and quality of isolated fractions of PBMCs and PLTs

Applying the above described isolation methods for PBMCs, the typical characteristics of the PBMC fraction (expressed in medians) are:

- PBMC yield: 25.3 million cells (obtained from 16-18 mL of whole blood, recovery ~ 67 %).
- N_{PLT}/N_{PBMC} as assessed with the Sysmex cell counter: 6.3, N=74 (range 1.8 – 12.2 depending on the ratio N_{PLT}/N_{PBMC} in the whole blood, see Graph 1). The ratio N_{PLT}/N_{PBMC} in the PBMC fraction obtained by the 2nd method is similar (4.7, N=5).
- Viability as assessed by trypan blue exclusion with the Countess II cell counter: 83 %, N=52.
- Viability assessed with Luna™ automated cell counter using acridine orange staining: ~ 96 %, N>20.

By comparing the ratios of the PLT to PBMC count in the isolated PBMC fraction, $(N_{PLT}/N_{PBMC})_{PBMC\ fraction}$ and in the whole blood sample, $(N_{PLT}/N_{PBMC})_{blood}$ we found a positive correlation between these two parameters (see Graph 1). This result shows that the purity level of the PBMC fraction obtained with the described isolation methods depends on the N_{PLT}/N_{PBMC} ratio in the whole blood.



Graph 1. N_{PLT}/N_{PBMC} in the PBMC fraction isolated with the described methods as a function of N_{PLT}/N_{PBMC} in the whole blood. (Close correlation, P<0.0001 by Pearson test). The values are from 72 blood samples.

3. Respirometric analysis of mitochondrial function in PBMCs and PLTs

Instrumental setup

Setup of the O2k followed standard procedures as described in detail elsewhere [2]. For each blood sample up to 4 instruments (2 for PLTs and 2 for PBMCs) with 2 chambers each were run in parallel. Chambers were filled with 2.2 mL of MiR05Cr (for protocols with permeabilized cells) [3], or RPMI (for protocols with intact PBMCs), or M199 (for protocols with intact PLTs). Media were equilibrated to 37°C before full insertion of the stoppers and thereby adjusting the final chamber volume to 2 mL. Before adding cells, stoppers were lifted, the required volume to be added from the cell

stock was removed and replaced with cell suspension and stoppers were fully inserted into the chambers again.

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 the respiration in intact and permeabilized PBMCs or platelets. 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 [4, 5, 6].

SUIT protocols for intact cells:

Medium: RPMI-1640 without L-glutamine for PBMC, M199 for PLT

For respiration of intact cells, we used a coupling control protocol CCP(S) [4]. In chamber A oligomycin was replaced by the solvent ethanol.

A: ce1;ce2EtOH;ce3U;ce4Rot;ce5S10;ce6Ama
B: ce1;ce2Omy;ce3U;ce4Rot;ce5S10;ce6Ama

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 chamber B with the simplified protocol in chamber A which omits Omy, we wanted to study the effect of Omy on ET-pathway capacity. By addition of 10 mM S after Rot we aimed to test the intactness of the cell membrane.

SUIT protocols for permeabilized cells: MiR05Cr+Ctl = MiR06Cr

For respiration of permeabilized cells we used harmonized SUIT reference protocols RP1 and RP2, described in detail elsewhere [5, 6]. 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: ce1;1PM;1Dig;2D;2c;3U;4G;5S10;6Rot;7Gp;8Ama;9AsTm;10Azd
B: RP2: ce1;1Dig;1D;2M0.1;3Oct;4M2;4c;5P;6G;7S10;8Gp;9U;10Rot;
11Ama;12AsTm;13Azd

The final concentration of the chemicals in the O2k chamber:

Cr	creatine 20 mM
Ctl	catalase 280 IU/mL

P	pyruvate, 5 mM
M0.1	malate, 0.1 mM
M	malate, 2 mM
Dig	digitonin, 8 µg/10^6 PBMC 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
S10	succinate, 10 mM
Oct	octanoyl carnitine, 0.5 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, 200 mM

3.1. Respiratory characteristics of intact PBMCs and platelets

Figure 1 shows examples of measurements on intact PBMC isolated by protocol 1 described above and examined with a modified Coupling Control Protocol using RPMI as a respiration medium.

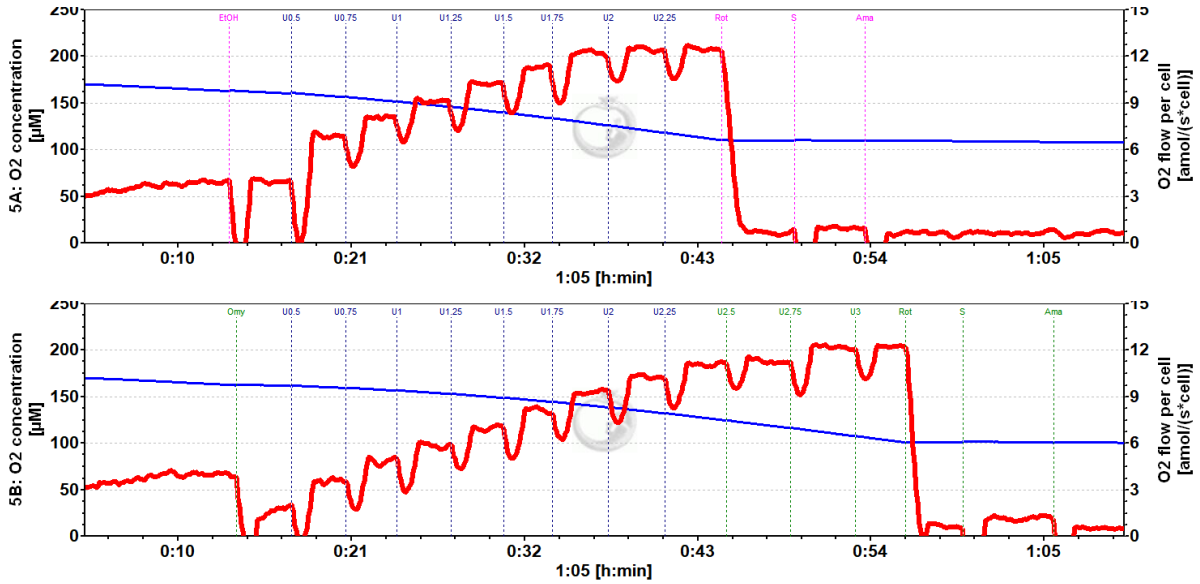


Fig. 1. Respiration of intact PBMCs examined using a Coupling Control Protocol in RPMI. The Blue trace denotes oxygen concentration (left Y-axis [µM]), the red trace oxygen flux per cell (right Y-axis [amol·s^{-1}·$cell^{-1}$]). Experiment: 2016-06-01 P5-03.DLD; 6 million PBMC were added to each chamber.

Figure 2 shows examples of measurements on intact PLT isolated by the 1st isolation method described above and examined with a modified Coupling Control Protocol in cell culture medium M199.

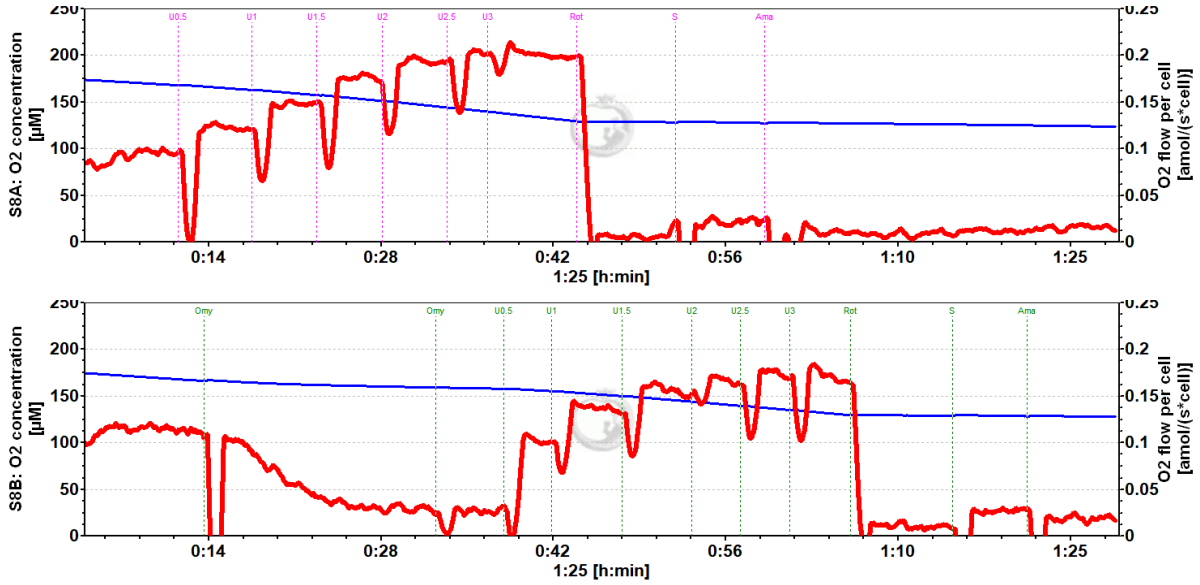


Fig. 2. Respiration of intact PLT in cell culture medium M199 using a coupling control protocol. Further details as in Legend to Fig. 1. Experiment: 2016-10-21 PS8-02.DLD; 220 million PLT were added to each chamber.

The respiratory rates of intact cells depended on the respiration medium used, with RPMI consistently supporting slightly higher rates than MiR06Cr. While RPMI contains many substrates, providing external support for respiration, cells in MiR06Cr are entirely dependent on internally provided substrates. In general, media for cell culture usually contain non-physiological concentration of glucose and L-glutamine to support cell growth and these same substrates may also fuel respiration. Selection of optimal medium composition for evaluation of respiration of intact cells is clearly not a trivial task and may pose a real challenge.
ET-pathway capacity after Omy is frequently lower than ET-pathway capacity measured without previous Omy titration (see Fig 2). This effect of Omy on ET-pathway capacity can depend on the respiration medium used, producing different relative Omy effects on ET-pathway capacity in the same cell type.

3.2. Respiratory characteristics of permeabilized PLTs and PBMCs

Permeabilized PBMCs and platelets were examined in mitochondrial respiration medium MiR06Cr 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 [5, 6].

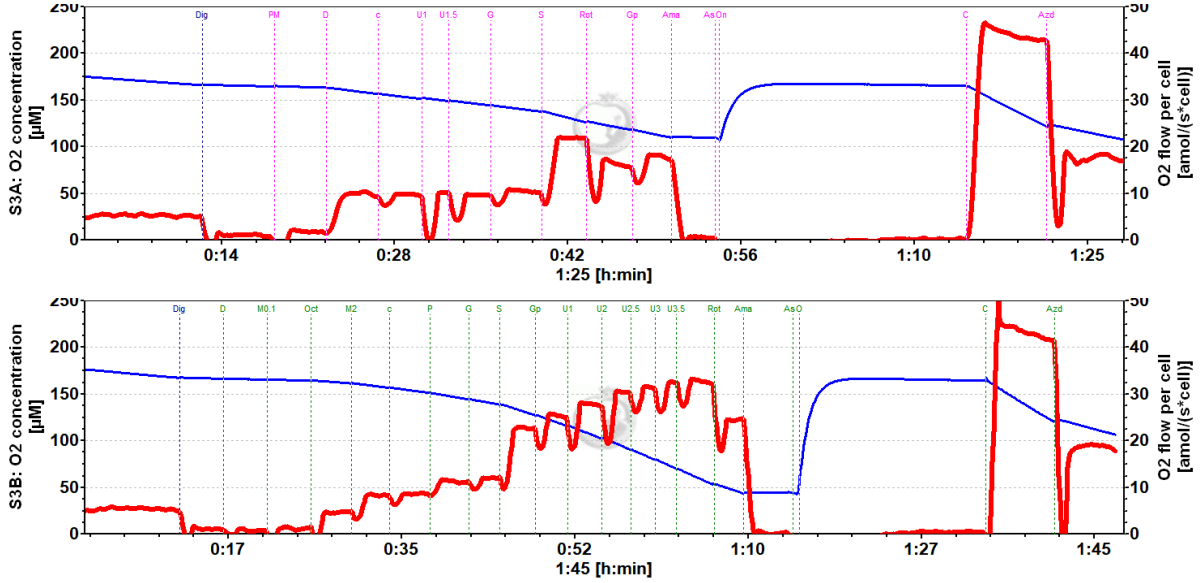


Fig. 3. Respiration of PBMCs examined with SUIT reference protocols RP1 (upper panel) and RP2 (lower panel). Blue and red traces denote oxygen concentration [µM] and oxygen fluxes [amol·s^{-1}·cell^{-1}], respectively. Experiment: 2016-10-12 PS3-02.DLD; 5 million PBMCs were added to each chamber.

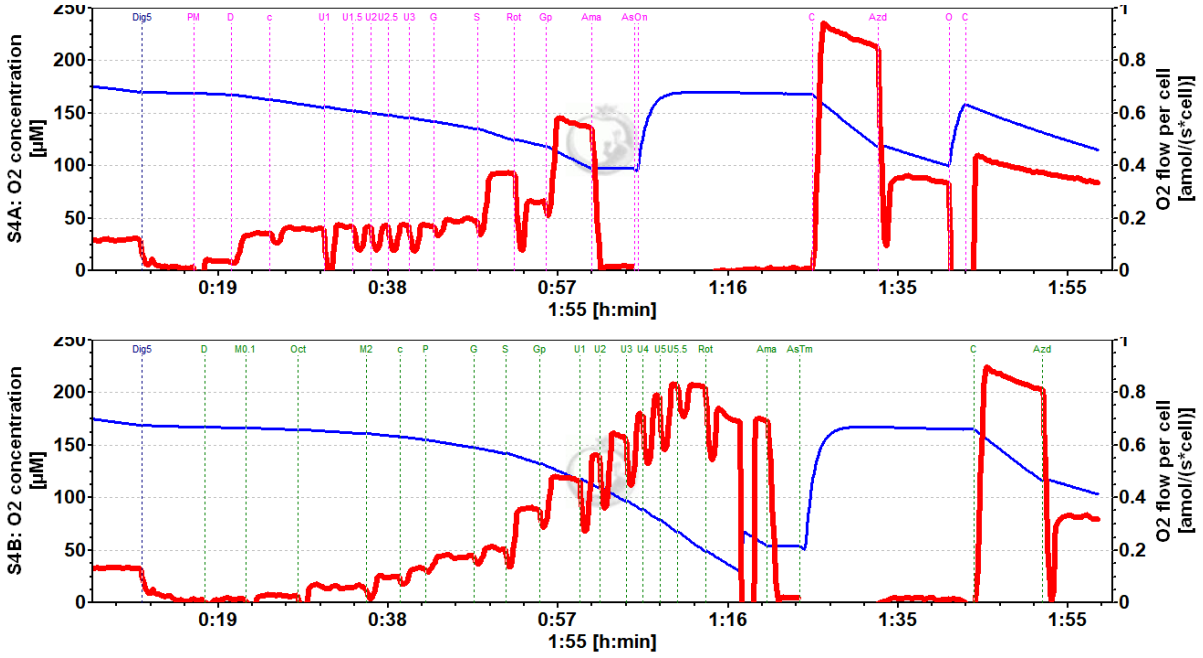


Fig. 4. Respiration of platelets examined with SUIT reference protocols RP1 (upper panel) and RP2 (lower panel). Details as in Legend to Fig. 3. 250 million PLTs were added to each chamber. Experiment: 2016-10-18 PS4-02.DLD.

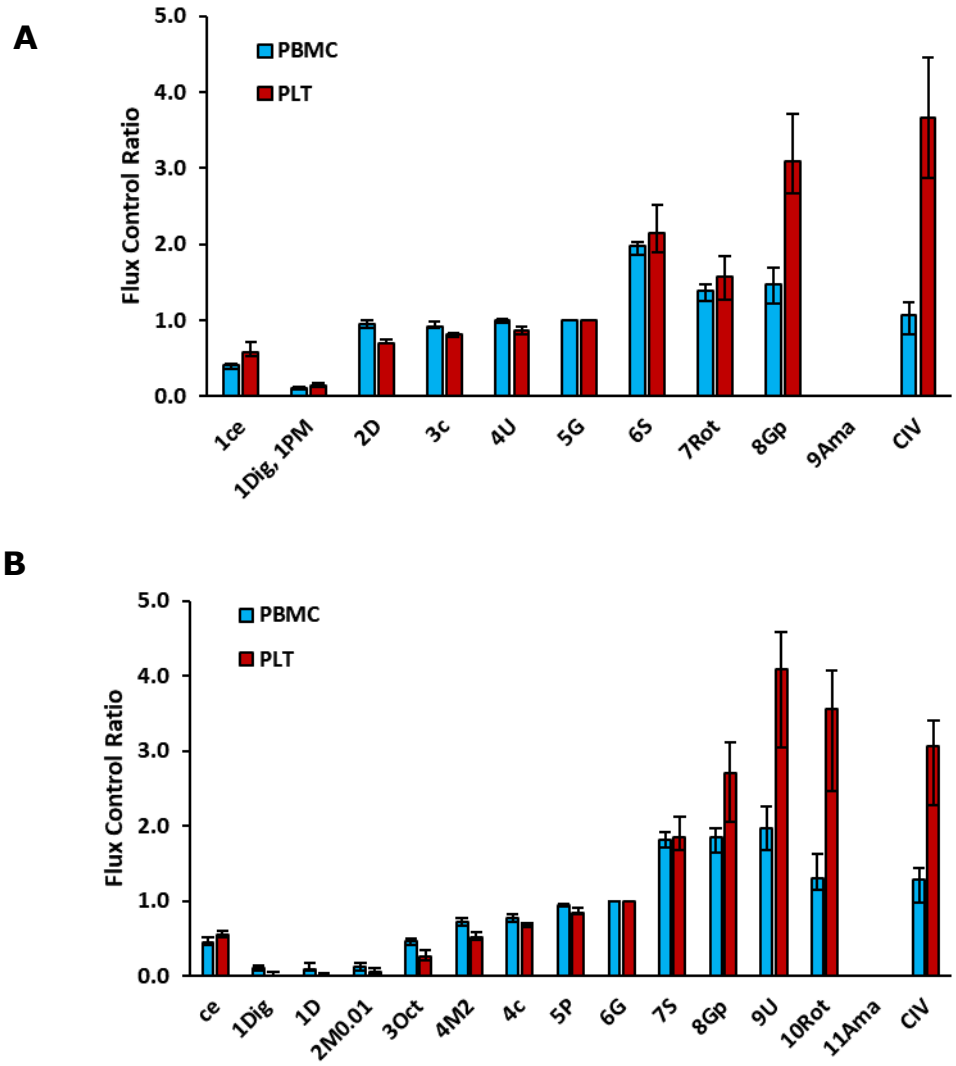


Fig. 5. Comparison of relative O_2 fluxes in PBMCs and PLTs examined by SUIT reference protocols RP1 (A) and RP2 (B). Flux Control Ratios were calculated by normalizing to ET-pathway capacity in the presence of N-linked substrates (5G) in RP1 and to OXPHOS capacity in the presence of N-linked substrates and Oct (6G) in RP2. Data are medians with interquartile range of 29 samples for PBMCs and of 18 samples for PLTs. O_2 fluxes were corrected for the contribution from contaminating cells.

The graphs above show that the respiratory signature of PBMCs and PLTs is different. In RP1 and RP2 the response to Gp is significantly higher in PLTs than in PBMCs. The phosphorylation system highly limits respiration of PLTs with substrates of FNSGp(PGM) as evident in RP2 from the increased respiratory rates after addition of uncoupler in these cells; in comparison, in PBMCs the effect of uncoupler at this substrate combination is very low. These differences between PBMCs and PLTs in step changes in the protocols RP1 and RP2 are expressed in Fig.6 as Flux Control Factors. Based on different responses of PLTs to Gp and uncoupler in RP2, the contamination of PBMC fraction by PLTs could be clearly recognized.

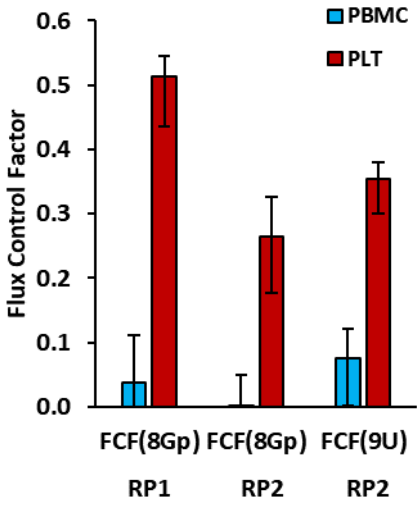


Fig. 6. Step changes from SUIT reference protocols RP1 and RP2 which were significantly different for PBMCs and PLTs, expressed as Flux Control Factors. Data are medians with interquartile range of 29 samples for PBMCs and of 18 samples for PLTs. O_2 fluxes were corrected for the contribution from contaminating cells.

The contamination of the PBMC fraction with PLTs must always be considered when working with PBMCs isolated by these two methods (the median ratio PLT to PBMC ~ 6.3), as it could significantly affect the respiratory rates and the cell mass in the chamber. E.g., at N_{PLT}/N_{PBMC} of 7, PLTs account for ~14% of total respiration [7] and ~22% of total protein content (the amount of protein per 10^6 cells was determined as 2.46 μg for PLTs, N=15 and 66.6 μg for PBMCs, N=41).

4. Conclusions

Respiratory rates of intact blood cells depend on the respiratory medium applied. Oligomycin may negatively affect the values of the measured ET-pathway capacity. PBMCs and PLTs have different respiratory patterns as recognized by two harmonized SUIT reference protocols. The ratio of PLT to PBMC count in the PBMC fraction closely correlates with the ratio of PLT to PBMC count in the whole blood. Although with the described methods for isolation of PBMCs the ratio of PLT count to PBMC count in the PBMC fraction (median value 6.3) is only 5.4% of the ratio in whole blood (median value 117.2), this contamination by PLTs can significantly contribute to the respiration of the PBMC fraction. Therefore, the currently described isolation methods should be improved for better purity of the PBMC fraction. In respirometric studies on blood cells the purity of cell preparation should always be emphasised and documented.

5. References

1. Chacko BK, Kramer PA, Ravi S, Johnson MS, Hardy RW, Ballingern SW, Darley-Usmar VM. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. Lab Invest. 2013; 93(6): 690–700.
2. http://wiki.oroboros.at/images/0/04/MiPNet20.04_Checklist.pdf
3. http://www.bioblast.at/index.php/MiR05Cr
4. http://www.bioblast.at/index.php/Ce1;ce2Omy;ce3U;ce4Rot;ce5S;ce6Ama
5. http://www.bioblast.at/index.php/Harmonized_SUIT_protocols
6. http://www.bioblast.at/index.php/MiPNet21.06_SUIT_reference_protocol
7. http://www.bioblast.at/index.php/Sumbalova_2017_MiP2017_WG4
8. Tsai HH, Chang SC, Chou CH, Weng TP, Hsu CC, Wang JS (2016). Exercise Training Alleviates Hypoxia-induced Mitochondrial Dysfunction in the Lymphocytes of Sedentary Males. Sci Rep. 12;6:35170. doi: 10.1038/srep35170.
9. Weyrich AS, Elstad MR, McEver RP, McIntyre TM, Moore KL, Morrissey JH, Prescott SM, Zimmerman GA. Activated platelets signal chemokine synthesis by human monocytes. J. Clin. Investigation. 1996;97(6):1525-34.
10. Kramer PA, Chacko BK, Ravi S, Johnson MS, Mitchell T, Darley-Usmar VM. 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).
11. Bynum JA, Adam Meledeo M, Getz TM, Rodriguez AC, Aden JK, Cap AP, Pidcoke HF. Bioenergetic profiling of platelet mitochondria during storage: 4°C storage extends platelet mitochondrial function and viability. Transfusion. 2016;56 Suppl 1:S76-84.
12. Ehinger JK, Morota S, Hansson MJ, Paul G, Elmér E. Mitochondrial dysfunction in blood cells from amyotrophic lateral sclerosis patients. J Neurol. 2015;262(6):1493-503.
13. Sjövall F, Ehinger JK, Marelsson SE, Morota S, Frostner EA, Uchino H, Lundgren J, Arnbjörnsson E, Hansson MJ, Fellman V, Elmér E. Mitochondrial respiration in human viable platelets-methodology and influence of gender, age and storage. Mitochondrion. 2013 Jan;13(1):7-14. 1.
14. Pecina P, Houšťková H, Mráček T, Pecinová A, Nůsková H, Tesařová M, Hansíková H, Janota J, Zeman J, Houštěk J. Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry. BBA Clin. 2014 Oct 1;2:62-71.
15. Leuner K, Schulz K, Schütt T, Pantel J, Prvulovic D, Rhein V, Savaskan E,Czech C, Eckert A, Müller WE. Peripheral mitochondrial dysfunction in Alzheimer's disease: focus on lymphocytes. Mol Neurobiol. 2012;46(1):194-204.

http://bioblast.at/index.php/O2k-Protocols

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MitoFit mitochondria fit&well

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Tirol Standortagentur

Supplement A

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

Full blood centrifugation	PRP[1] -> PLT centrifugation	PLT resuspension	Buffy coat centrifugation	PBMC centrifugation	PBMC resuspension	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, **brakes 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 µL 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 µL PBS, count for 4 chambers transport on ice **intact cells:** RPMI+FCS, **permeabilized:** MiR05	[2a,8]
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,9]
16 mL of blood 500 *g* 10 min acc 9, no brakes	1000 *g* 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]
18 mL blood EDTA tubes Transport at RT			Dilute 1:1 with RPMI, pour on Leucosep tube prepared with Ficoll-Pague 1.077 g/mL Centrifuge 800 g 10 min, acc 6, no brakes	Collect PBMCs, dilute with RPMI to 30 mL, centrifuge 100 g 10 min, acc 9 brake 6 Resuspend the pellet in 25 mL of RMPI and centrifuge again	R7509 RPMI-1640 Medium Modified Resuspend in 5 mL MiR05 (permeabilized cells) or RPMI (intact cells)	[5a]

500 *g* 15 min, acc 5-6, **no brakes**	1500 *g* 8 min, acc 9, brakes 6	Wash with sterile PBS+1 µg/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 s** Resuspend in 80 µL RPMI+BSA, add 20 µL antiCD15-labelled magnetic beads, separate by magnetic activated cell sorting (MACS) separator	[10]
	1000 *g* 10 min RT	Resuspend in MiR05 Respiration intact MiR05				[11]
20 mL bood K2EDTA tubes (Vacuette, Greiner Bio-One, Austria)		200*10 ^{6} cells Dig: 1 µg/10^{6} cells			3.5-5*10^{6} cells Dig: 6 µg/10^{6} cells MiR05 – permeabilized cells Plasma – intact cells	[12]
21 mL blood K2EDTA tubes (Vacuette Austria) 300 *g* 15 min RT	4600 *g* 5 min RT	Resuspend in plasma Dig: 1 µg/10 ^{6} platelets				[13]
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 µg/mg prot KCl medium for respiration	[14]
			400 *g* 30 min Ficoll-Hypaque (Biochrom KG)			[15]

[1]PRP platelet rich plasma

References for Supplement A: personal communication

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