Mitochondrial Physiology Network 21.17(02):1-15 (2016)
Version 02: 2018-02-19 ©2018 Oroboros
Updates: http://wiki.oroboros.at/index.php/MiPNet21.17_BloodCells

O2k-Protocols: Isolation of blood cells for HRFR

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1. Introduction

Respiratory assessment of human health and disease is often performed with isolated mitochondria, tissue homogenate or permeabilized fibres 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 far less invasive sampling procedure and can be at least temporarily stored after collection for later use in respirometric measurements. Blood cells obtained by venipuncture are then usually separated to obtain either platelets (PLT) or a mixed population of immune cells subsumed as peripheral blood mononuclear cell (PBMC), 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 FluoRespirometry (HRFR) are described, important aspects of the isolation procedure are highlighted, and the protocols for the respiratory characterization of platelets and PBMC are presented.

2. Isolation procedures for platelets and PBMC

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 PBMC for respirometric measurements. An overview on a selection of published methods is presented in Appendix 1, 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 [5].
In the isolation procedures, we compared using 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 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.

Sample preparation

The following describes the method used for isolation of PBMC from the 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) using 21 G needle. Tubes are transported to the lab at RT in thermo-insulating containers, protected from light. Blood is mixed gently by slow reverting tube for 6-10 times and counted on Sysmex XN-350 haematology analyser.

Normal ranges to be expected:

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

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

- 15 mL Ficoll-Paque™ PLUS is added into a 50 mL Leucosep tube and centrifuged 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 on 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 PBMC.
4. Carefully collect the layer of PBMC-PLT (~ 5 - 10 mL) with a Pasteur pipette and transfer it into a new sterile 50 mL Falcon tube.
   Add DPBS to 25 mL line and centrifuge at 120 *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 high contamination with PLT: N_{PLT}/N_{PBMC} ~ 20. Centrifugation at 120 *g* for 10 min gives ratio N_{PLT}/N_{PBMC} ~ 7 and ~ 97% of PBMC in the sediment).
5. Transfer the supernatant (supernatant 1) into a new 50 mL Falcon tube, add 5 mL of clear plasma from point 3 and 10% of the 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 (dilution 10x) on the Sysmex XN-350 haematology analyser.
   - Remove 2x 20 µL and 1x 30 µL in Eppendorf tubes for protein concentration, LDH and CS activity determination respectively, store at -80 °C (the samples for LDH determination should be immediately put into -80°C).

Calculate volume of cell suspension to add 4 x 10^6 PBMC into the 2 mL O2k chamber.

Continuation from step 5 with isolation of PLT:

8. Centrifuge supernatant 1 with 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 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.
- Remove 2x 20 µL and 1x 30 µL in Eppendorf tubes for protein concentration, LDH and CS activity determination respectively, store at -80 °C (-80°C is absolutely necessary for LDH determination).

Calculate volume of cell suspension to add 200 x 10^6 PLT into 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 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 PMBC (steps 6-10) or leave PLT for centrifugation after isolation of PBMC *(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 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.
- Remove 2x 20 µL and 1x 30 µL in Eppendorf tubes for protein concentration, LDH and CS activity determination respectively, store in -80°C (-80°C is absolutely necessary for LDH determination).

Calculate volume of cell suspension to add 200 - 300 x 10^6 PLT into 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 rest of the 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 PBMC (~ 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.
- Remove 2x 20 µL and 1x 30 µL in Eppendorf tubes for protein concentration, LDH and CS activity determination respectively, store in -80°C (-80°C is absolutely necessary for LDH determination).

Calculate volume of cell suspension to add 4 x 10^6 PBMC into the 2 mL O2k chamber.

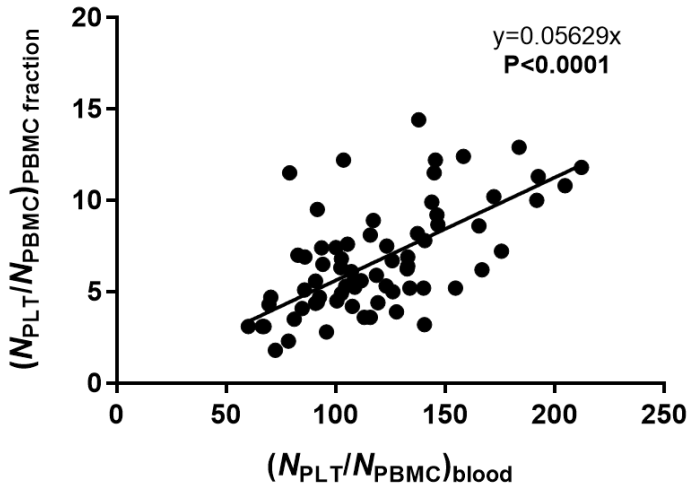
Quantity, purity and quality of isolated fractions of PBMC and PLT

Applying the above described isolation methods for PBMC, the typical yield of cells amounted to:

- PBMC cell yield: 25 ± 2.3 million cells (obtained from 16-18 mL whole blood, recovery ~ 65 %).

- N_{PLT}/N_{PBMC} as assessed with the Sysmex cell counter: 6.5 ± 0.8 (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 preparation of PBMC 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 %.
- Viability assessed with Luna™ automated cell counter using acridine orange: ~ 96 %.

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 (see Graph 1). This result indicates that the purity level of PBMC fraction obtained by described isolation methods is influenced by ratio N_{PLT}/N_{PBMC} in the donor's blood.



Graph 1. N_{PLT}/N_{PBMC} in PBMC fraction isolated by 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 PBMC and PLT

Instrumental setup

Setup of the O2k followed standard procedures as described in detail elsewhere [16]. For each blood sample up to 4 instruments (2 for PLT and 2 for PBMC) with 2 chambers each were run in parallel. Chambers were filled with 2.2 mL of MiR05Cr (for protocols with permeabilized cells), or RPMI (for protocol with intact PBMC), or M199 (for protocol with intact PLT). Media were equilibrated to 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.

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 platelets or PBMC. 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:

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

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

B: cells + 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 coupling control protocol in chamber B with the simplified protocol in chamber A which omits Omy we wanted to systematically 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 [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: cells+PM+Dig+D+c+U+G+S10+Rot+Gp+Ama+AscTm+Azd

B: RP2: cells+Dig+D+M0.1+Oct+M2+c+P+G+S10+Gp+U+Rot+Ama+ +AscTm+Azd

Chemical concentration in O2k chamber:

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, 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, 200 mM

3.1. Respiratory characteristics of intact platelets and PBMC

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

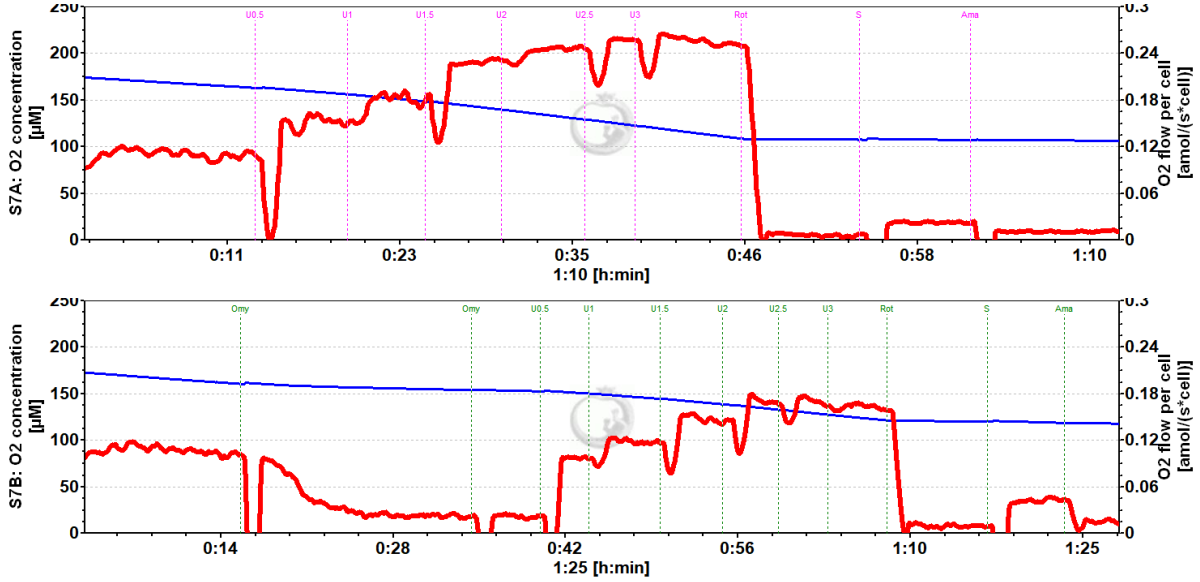


Fig. 1. Respiration of intact PLT in cell culture medium M199 using Coupling Control Protocol. The Blue trace denotes oxygen concentration (left Y-axis [µM]), the red trace oxygen flux per chamber volume (right Y-axis [amol·s^{-1}·cell^{-1}]). Experiment: 2016-10-21 PS7-02.DLD; 250 million PLT were added in each chamber.

Figure 2 shows example 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.

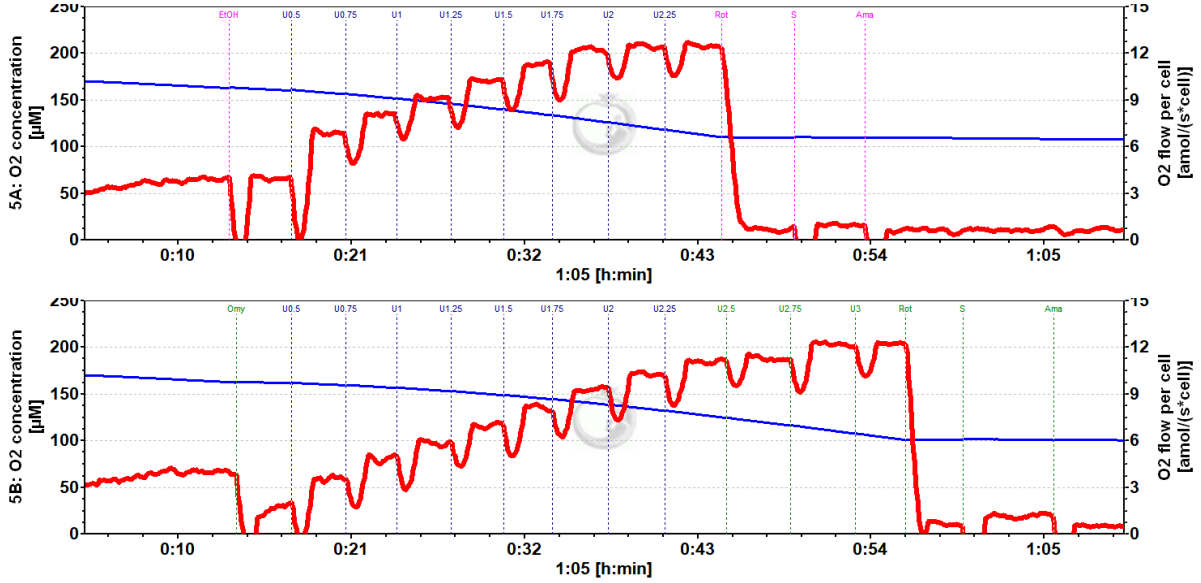


Fig. 2. Respiration of intact PBMC examined using a Coupling Control Protocol determined in RPMI. Further details as in Legend to Fig. 1. Experiment: 2016-06-01 P5-03.DLD; 6 million PBMC were added in each chamber.

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 much lower than ET-pathway capacity measured without previous Omy titration (see Fig 1). 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 PLT and PBMC

Permeabilized platelets and PBMC 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 [14, 15].

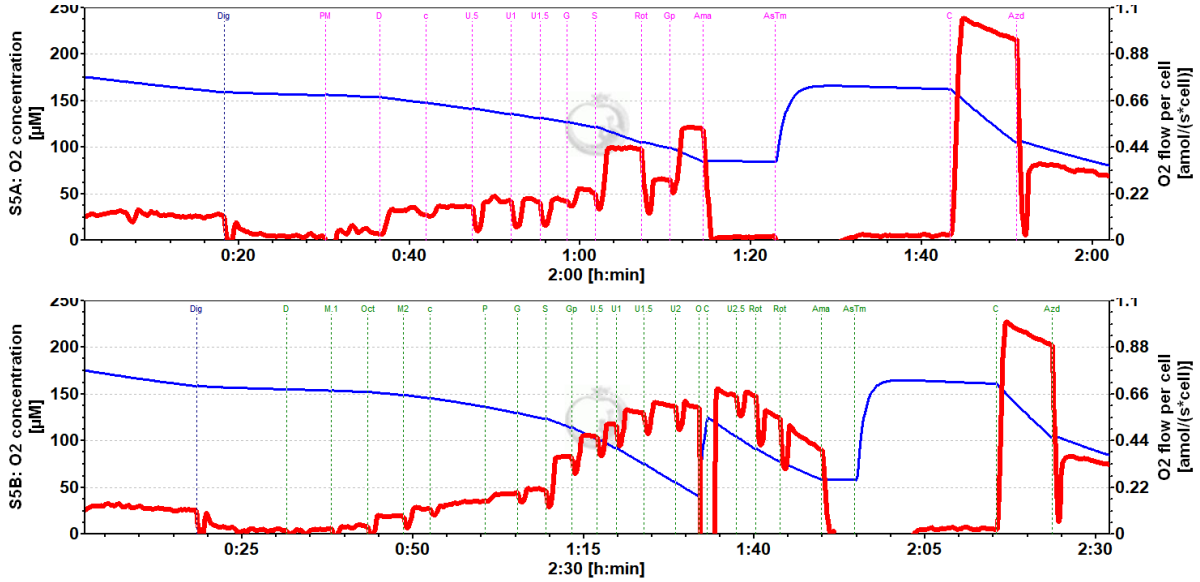


Fig. 3. Respiration of platelets examined with SUIT reference protocols RP1 in upper panel, and RP2 in lower panel. Blue and red traces denote oxygen concentration [µM] and oxygen fluxes [amol·s^{-1}·cell^{-1}], respectively. 250 million of PLT was added to each chamber. Experiment: 2016-10-19 P5-02.DLD.

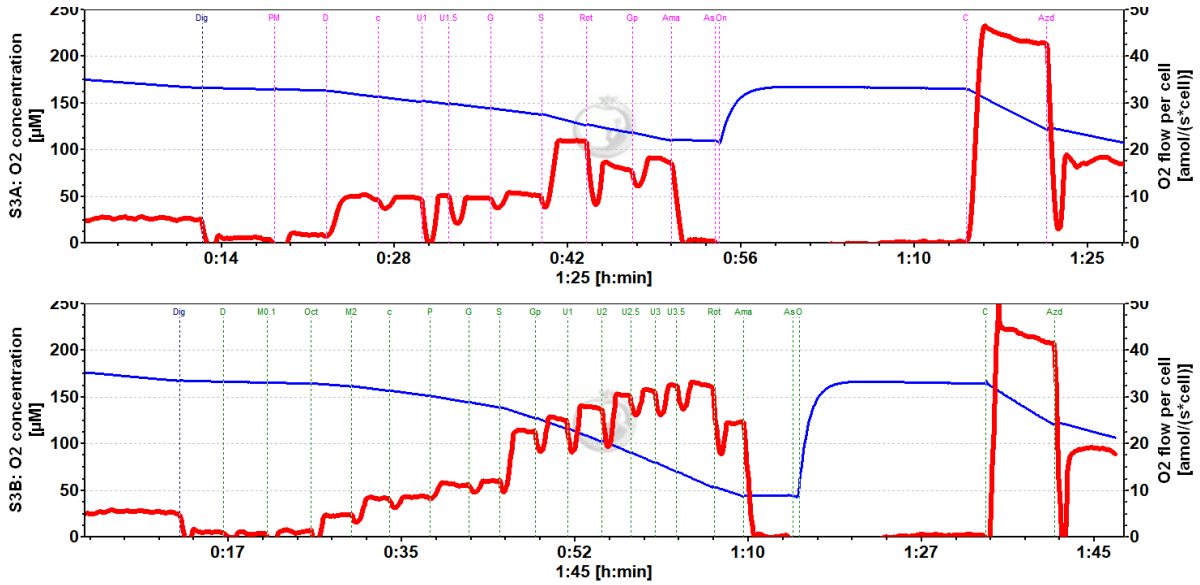


Fig. 4. Respiration of PBMC examined with SUIT reference protocols RP1 in upper panel and RP2 in lower panel. Details as in Legend to Fig. 3. Experiment: 2016-10-12 P3-02.DLD; 5 million of PBMC were added to each chamber.

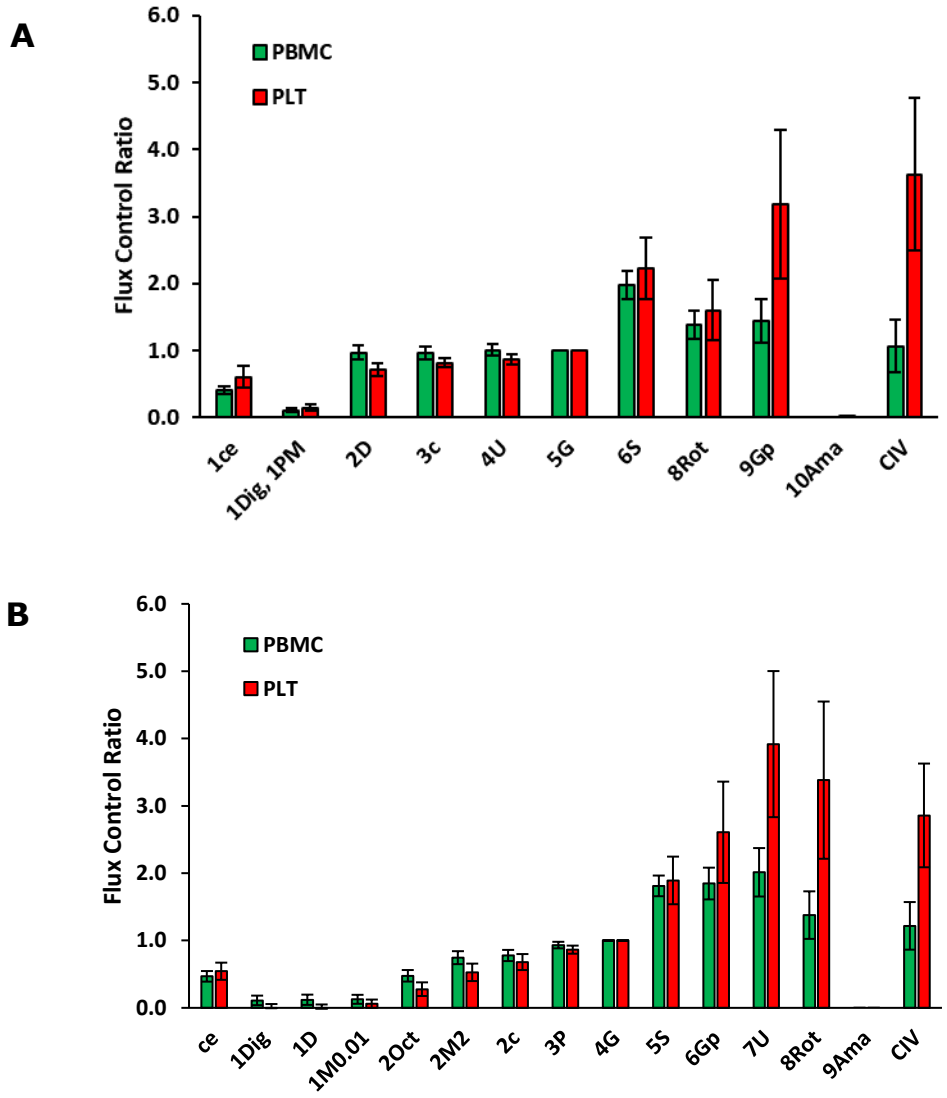


Fig. 5. Comparison of respiration relative O_2 fluxes in PBMC and PLT examined by SUIT reference protocols RP1 (A) and RP2 (B). Flux Control Ratios were calculated by normalizing to ET-pathway capacity in the presence of N-linked substrates in RP1 and to OXPHOS capacity in the presence of N-linked substrates and Oct in RP2. Data are means ± SD of 29 samples of PBMC and 18 samples of PLT. O_2 fluxes were corrected for contribution from contaminating cells.

From the graphs above it could be seen that the respiratory signature of PLT and PBMC is different. In RP1 the response to Gp is significantly higher in PLT than in PBMC (FCF_{Gp} 0.39 in PLT vs 0.12 in PBMC, see Fig. 6), in RP2 the phosphorylation system highly limits respiration of PLT with substrates of FNSGp(PGM) as evident from the increased respiratory rates after addition of uncoupler in these cells; in comparison, in PBMC the effect of uncoupler on respiration rate is very low (FCF_U 0.25 in PLT vs 0.06 in PBMC). Importantly, the small contamination with PLT must always be considered

when working with PBMC isolated by these two methods (the mean ratio PLT to PBMC ~ 7), as it could significantly affect the respiratory rates observed as well as the cell mass in the chamber. E.g., at N_{PLT}/N_{PBMC} of 7, PLT may account for 25% 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 PBMC).

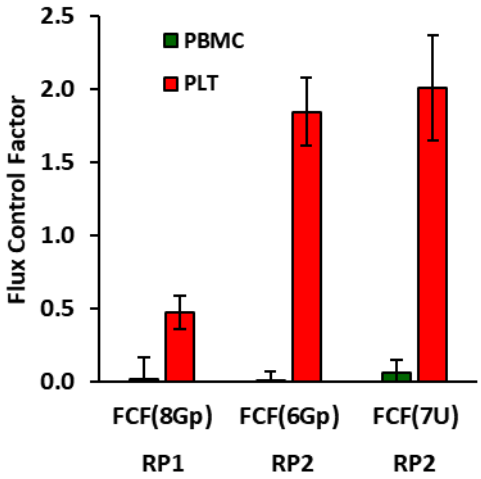


Fig. 6. Step changes from SUIT reference protocols RP1 and RP2 which were significantly different for PBMC and PLT expressed as Flux Control Factors. Data are means ± SD of 29 samples of PBMC and 18 samples of PLT. O_2 fluxes were corrected for contribution from contaminating cells.

4. Conclusions

Respiratory rates of intact blood cells depend on the respiratory medium applied. PBMC and PLT have different respiratory patterns as recognized by two harmonized SUIT reference protocols. The ratio of PLT to PBMC in PBMC fraction closely correlates with the ratio of PLT to PBMC in the whole blood. Contamination of PBMC with platelets can significantly affect their apparent respiration and therefore purity of the cell preparation should be emphasised in the selection of isolation method.

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Acknowledgements

The project MitoFit is funded by the Land Tirol within the program K-Regio of Standortagentur Tirol.

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Supplement A

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

Full blood centrifugation	PRP[1] -> PLT centrifugation	PLT resuspendation	Buffy coat centrifugation	PBMC centrifugation	PBMC 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, **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 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	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]

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	[7]
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	[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 µg/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 100 *g*	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/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/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-Chiang Chang
[3a] Luiz Garcia
[4a] Florian Hoppel
[5a] Elisa Calabria