

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 muscle fibers prepared from biopsies. The collection of tissue biopsies is invasive, requiring specific ethical approval. An alternative is the use of blood cells, which can be obtained in a less invasive sampling procedure by venipuncture. Cells are then usually separated to platelets (PLT) and a mixed population of immune cells subsumed as peripheral blood mononuclear cells (PBMC), both of which have been successfully applied to characterize respiratory phenotypes of human diseases. Isolated blood cells can be stored temporarily after collection for later use in respirometric measurements.

We describe isolation methods to obtain blood cells for high-resolution respirometry (HRR) and present protocols for the respiratory characterization of platelets and PBMC.

2. Isolation procedures for platelets and PBMC

Isolation protocols described here are based on published methods and are optimized for obtaining maximum yield and purity. The quality of PLT and PBMC was assessed by mitochondrial respiratory function. An overview of some published methods is presented in Supplement A, illustrating the diversity of conditions relating to the media chosen for separation and resuspension of cells, the exact conditions of centrifugation in terms of 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].

We compared the use of RPMI+BSA, RPMI and DPBS for washing steps in the isolation procedure. 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 determination of protein content, cell count, mitochondrial marker citrate synthase activity, and cytosolic marker lactate dehydrogenase activity, which are important for normalization of respiratory rate [2].

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 method used for isolation of PBMC from whole blood is based on the use of Leucosep™ tubes (Greiner Bio-One) and Ficoll-Paque™ PLUS density gradient centrifugation medium following the instructions of the manufacturer with slight modifications. Isolation media are kept at room temperature (RT) and all 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 hemolysis. Tubes are transported to the lab at RT in thermo-insulated containers, protected from light. One hour after blood collection, blood is gently mixed by slowly inverting the tube 6-10 times. Cells are counted on a Sysmex XN-350 hematology analyzer. Normal ranges of cell count expressed in units of mega x [Mx] per milliliter [mL] ($10^6 \times \text{mL}^{-1}$ [2]) are:

Platelets:	150-400 Mx·mL ⁻¹
Lymphocytes:	0.95-4.4 Mx·mL ⁻¹
Monocytes:	0.08-1.0 Mx·mL ⁻¹

Method 1: Isolation of PBMC and PLT from the same blood sample: focus on PBMC

1. 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).
2. 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.
3. Centrifuge at 1000 *g* for 10 min at RT, with brake off (intermediate acceleration 6, brakes 0).
4. 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.
5. Carefully collect the PBMC-PLT layer ("buffy coat", ~5 to 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 PLT: PLT count to PBMC count ratio ($N_{\text{PLT}}/N_{\text{PBMC}}$) ~20. Centrifugation at 120 *g* for 10 min yields a ratio $N_{\text{PLT}}/N_{\text{PBMC}}$ ~7 and ~97 % of PBMC in the sediment.
6. Transfer the supernatant (supernatant 1) into a new 50 mL Falcon tube, add 5 mL of clear plasma collected at step 4 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 9 below.
7. 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).
8. 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 in 90 μL DPBS in an Eppendorf tube for counting (10x dilution) on the Sysmex XN-350 hematology analyzer.
- 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 (the samples for LDH determination should be immediately put into -80°C).

Calculate the volume of cell suspension required to have 4 Mx PBMC in the 2 mL O2k chamber.

Continuation from step 6 with isolation of PLT:

9. Centrifuge supernatant 1 with the clear 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 at 1000 *g* for 5 min at RT (fast acceleration 9, brake 2).
11. Discard supernatant and resuspend the pelleted PLT fraction in 0.5 mL DPBS, 10 mM EGTA.

Count and freeze subsamples:

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

Calculate the volume of cell suspension required to have a PLT count concentration of 100 Mx/mL in the 0.5-mL or 2-mL O2k chamber.

Method 2: Isolation of PLT and PBMC from a single blood sample: focus on PLT

In steps 2 to 5 round-bottom Falcon tubes of 14 mL 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 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: 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 sufficient yield of 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 μ L of cell suspension into 90 μ L DPBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex XN-350 hematology analyzer.
- Transfer two 20 μ L and one 30 μ L aliquots of the stock suspension to Eppendorf tubes for determination of protein concentration, LDH

and CS activity, respectively, store at -80°C as required for LDH determination.

Calculate the volume of cell suspension required to have a PLT count concentration of 100-150 Mx/mL in the 0.5 or 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 below 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 the remaining blood can be 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 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 hematology analyzer.
- Transfer two (2) 20 μL and one (1) 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 Mx PBMC in the 2 mL O2k chamber.

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

Based on the isolation methods described above for PBMC, the typical characteristics of the PBMC fraction (expressed as medians) are:

- PBMC yield: 25.3 million cells (obtained from 16-18 mL of whole blood, recovery $\sim 67\%$).
- $N_{\text{PLT}}/N_{\text{PBMC}}$ as assessed with the Sysmex cell counter: 6.3, $N=74$ (range 1.8–12.2 depending on the ratio $N_{\text{PLT}}/N_{\text{PBMC}}$ in the whole blood, see Graph 1). The ratio $N_{\text{PLT}}/N_{\text{PBMC}}$ 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 LunaTM automated cell counter using acridine orange staining: $\sim 96\%$, $N>20$.

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

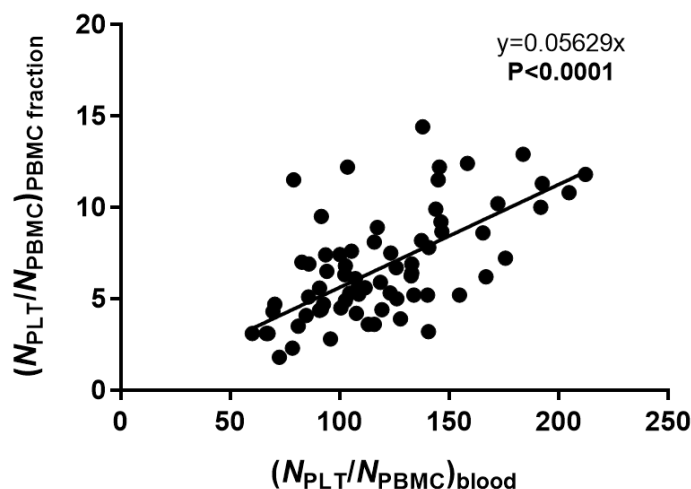


Figure 1. $N_{\text{PLT}}/N_{\text{PBMC}}$ in the PBMC fraction isolated with the described methods as a function of $N_{\text{PLT}}/N_{\text{PBMC}}$ in the whole blood. (Close correlation, $P<0.0001$ by Pearson test). The values are from 72 blood samples.

3. Respirometric analysis of mitochondrial function in PBMC and PLT

Instrumental setup

Setup of the O2k followed standard procedures [3]. For each blood sample up to four instruments (two for PLT and two for PBMC) with two chambers each were run in parallel. Chambers were filled with 2.2 mL of MiR05Cr (for protocols with permeabilized cells) [4], or RPMI (for protocols with living PBMC), or M199 (for protocols with living PLT). 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 living and permeabilized blood cells

Four substrate-uncoupler-inhibitor-titration (SUIT) protocols were run in parallel, one in each chamber, to simultaneously characterize the respiration in living and permeabilized PBMC 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 [3, 5, 6].

SUIT protocols for living cells:

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

For respiration of living cells, we used a coupling control protocol CCP(S) [7]. In chamber A oligomycin was replaced by the vehicle 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 μ M and 10 nM*
U	uncoupler CCCP, added in steps from 1 up to 4 μ M
Rot	rotenone, 0.5 μ M
S10	succinate, 10 mM
Ama	antimycin A, 2.5 μ M

* Oligomycin has frequently been shown to exert an inhibitory effect on ET-capacity. For measurement of LEAK respiration in many mt-preparations and cell types, using a lower oligomycin concentration minimizes or prevents inhibition of ET respiration. Therefore, we recommend to test and replace in your specific model the previously reported oligomycin concentration (2.5 μ M) for a lower standard oligomycin concentration (5-10 nM).

By comparing the coupling control protocol in chamber B with the simplified protocol in chamber A which omits Omy, we studied the effect of Omy on ET-pathway capacity. With the addition of 10 mM S after Rot we aimed to test the plasma membrane barrier function.

SUIT protocols for permeabilized cells: MiR05Cr+Ctl = MiR06Cr

For respiration of permeabilized cells we used harmonized SUIT reference protocols SUIT-001 O2 ce-pce D004 (RP1 for blood cells) and SUIT-002 O2 ce-pce D007a (RP2 for blood cells) [3, 7]. Concentrations of some chemicals were optimized for respiration of blood cells. The list of used chemicals for both protocols is shown below.

A:D004_ce1;1PM;1Dig;2D;2c;3U;4G;5S10;6Rot;7Gp;8Ama;9AsTm;
10Azd

B: D007a_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 $\mu\text{g}/10^6$ PBMC and 20 $\mu\text{g}/10^8$ 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-phenylenediamine dihydrochloride), 0.5 mM
Azd	azide, 200 mM

3.1. Respiratory characteristics of living PBMC and platelets

Figure 2 shows examples of measurements on living PBMC isolated by protocol 1 and examined with a modified Coupling Control Protocol using RPMI as a respiration medium.

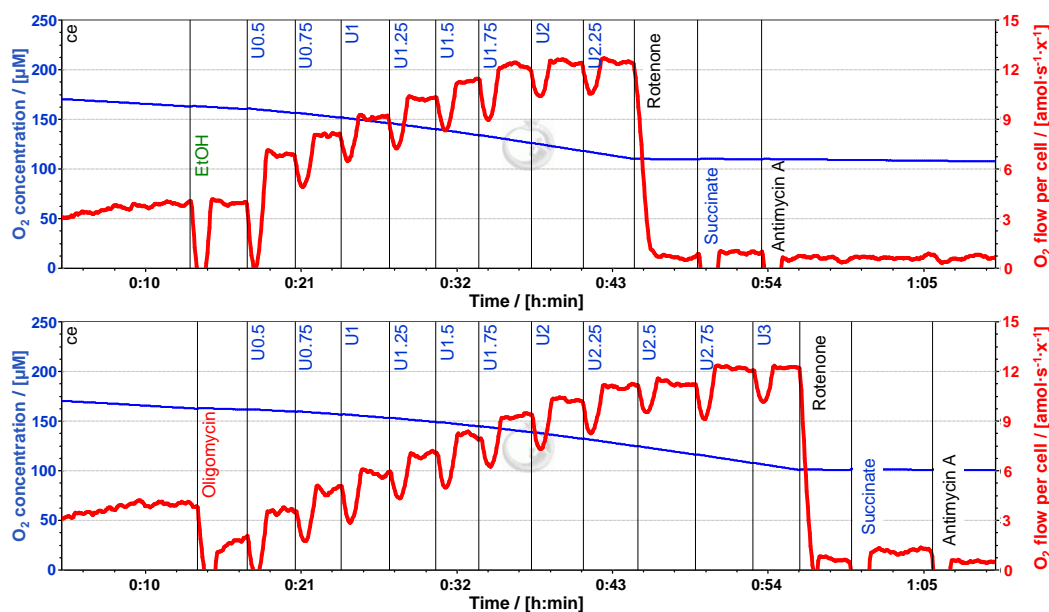


Figure 2. Respiration of living PBMC examined using a Coupling Control Protocol in RPMI. The blue trace denotes oxygen concentration (left Y-axis [μM]), the red trace oxygen flux per cell (right Y-axis [$\text{amol}\cdot\text{s}^{-1}\cdot\text{x}^{-1}$]). Experiment: 2016-06-01 P5-03.DLD; 6 Mx PBMC were added to each chamber.

Figure 3 shows examples of measurements on living PLT isolated by protocol 1 and examined with a modified Coupling Control Protocol in cell culture medium M199.

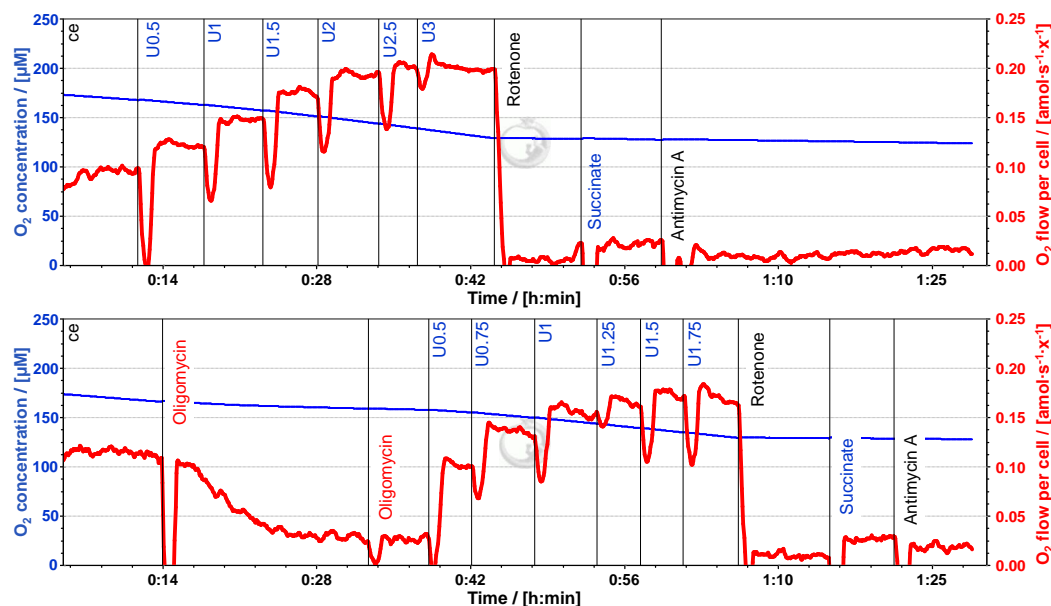


Figure 3. Respiration of living PLT in cell culture medium M199 using a coupling control protocol. Further details as in Legend to Fig. 2. Experiment: 2016-10-21 PS8-02.DLD; 220 Mx PLT were added to each chamber.

Oxygen flow of living cells depended on the respiration medium, 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 endogenous substrates. In general, media for cell culture usually contain non-physiological concentration of glucose and L-glutamine to support cell growth and these substrates may also fuel respiration. Selection of optimal medium composition for evaluation of respiration of living 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 (Figure 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 PLT and PBMC

Permeabilized PBMC 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 [3, 5].

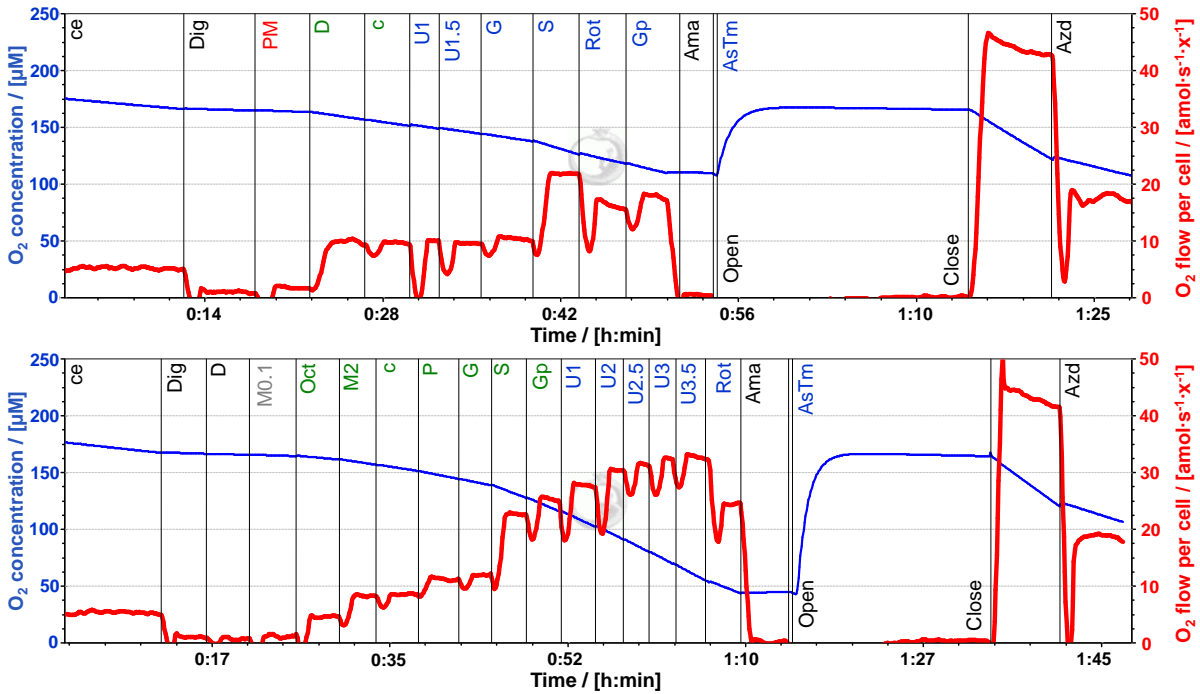


Figure 4. Respiration of PBMC examined with SUIT reference protocols RP1 (upper panel) and RP2 (lower panel). Blue and red traces denote oxygen concentration μM and oxygen fluxes $[\text{amol}\cdot\text{s}^{-1}\cdot\text{x}^{-1}]$, respectively. Experiment: 2016-10-12 PS3-02.DLD; 5 Mx PBMC were added to each chamber.

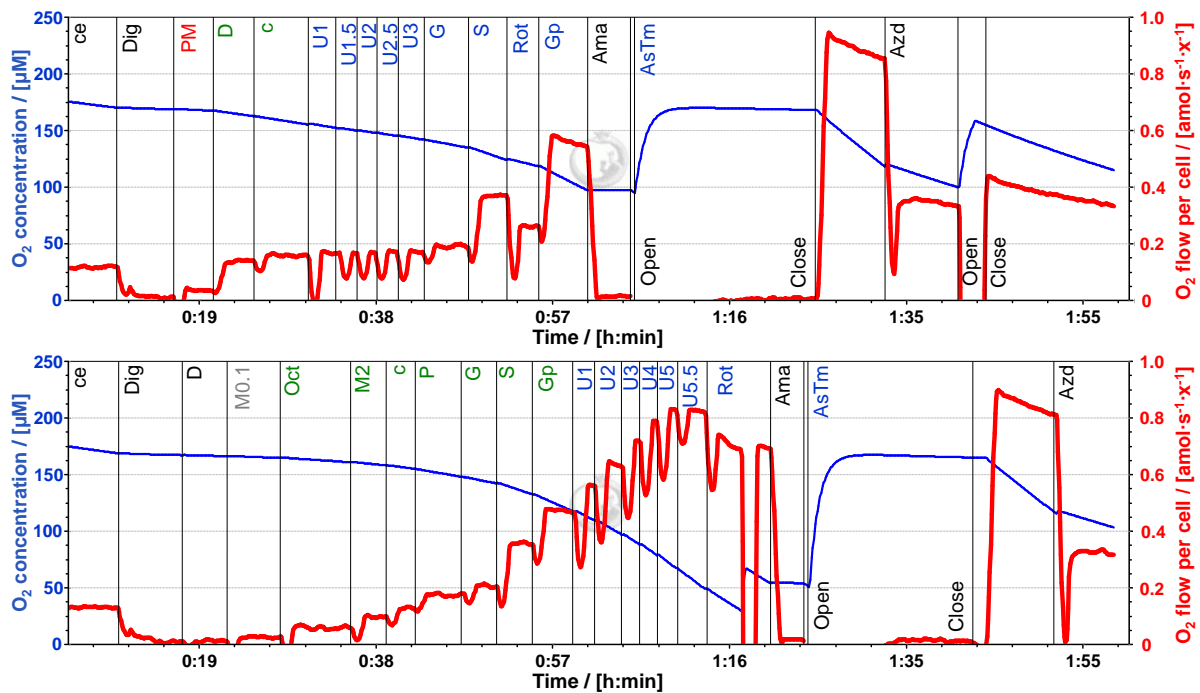


Figure 5. Respiration of platelets examined with SUIT reference protocols RP1 (upper panel) and RP2 (lower panel). Details as in Legend to Fig. 3. 250 Mx PLT were added to each chamber. Experiment: 2016-10-18 PS4-02.DLD.

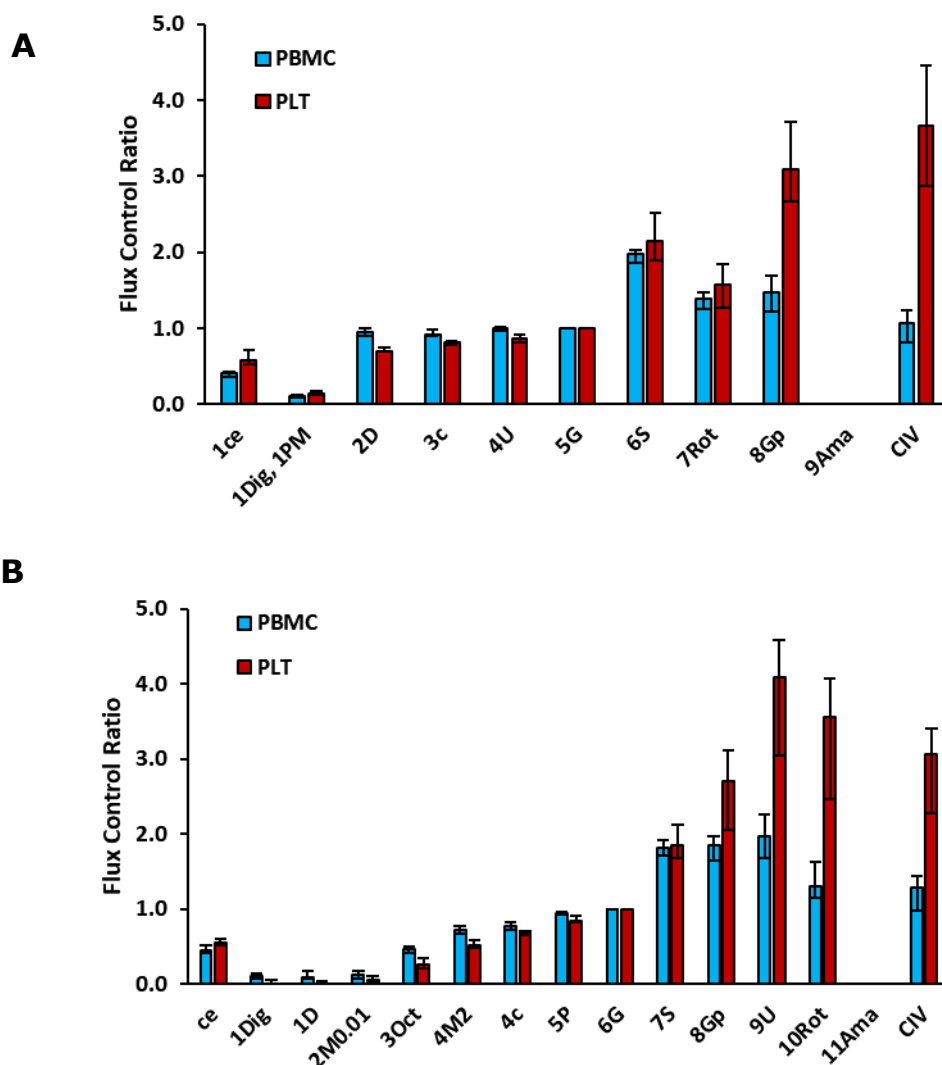


Figure 6. Comparison of relative O₂ fluxes in PBMC and PLT examined by SUIT reference protocols RP1 (**A**) and RP2 (**B**). Flux Control Ratios were calculated by normalizing to ET-pathway capacity in the presence of N-linked substrates (5G) in RP1 and to OXPHOS capacity in the presence of N-linked substrates and Oct (6G) in RP2. Data are medians with interquartile range of 29 samples for PBMC and of 18 samples for PLT. O₂ fluxes were corrected for the contribution from contaminating cells.

Figures 4 to 6 show that the respiratory signature of PBMC and PLT is different. In RP1 and RP2 the response to Gp is significantly higher in PLT than in PBMC. The phosphorylation system highly limits respiration of PLT with substrates of FNSGp(PGM) as evident in RP2 from the increased respiratory rates after addition of uncoupler; by comparison, in PBMC the effect of uncoupler at this substrate combination is very low. These differences between PBMC and PLT in step changes in the protocols RP1 and RP2 are expressed in Fig. 7 as Flux Control Factors *FCF*. Based on different responses of PLT to Gp and uncoupler in RP2, the contamination of PBMC fraction by PLT could be clearly recognized.

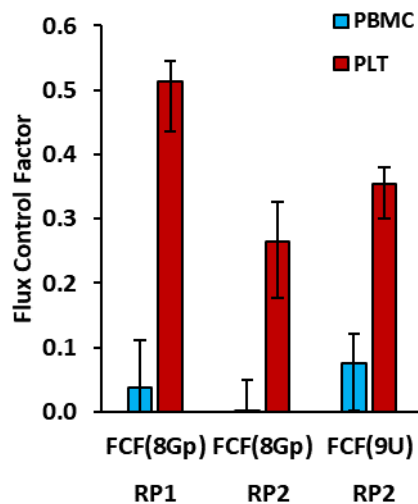


Figure 7. Step changes from SUIT reference protocols RP1 and RP2 which were significantly different for PBMC and PLT, expressed as Flux Control Factors. Data are medians with interquartile range of 29 samples for PBMC and of 18 samples for PLT. O₂ fluxes were corrected for the contribution from contaminating cells.

The contamination of the PBMC fraction with PLT must always be considered when working with PBMC 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. At $N_{\text{PLT}}/N_{\text{PBMC}}$ of 7, PLT account for $\sim 14\%$ of total respiration [5] and $\sim 22\%$ of total protein content (the amount of protein per 10^6 cells was determined as $2.46 \mu\text{g}/\text{Mx}$ for PLT, $N=15$; and $66.6 \mu\text{g}/\text{Mx}$ for PBMC, $N=41$).

4. Conclusions

Respiratory capacities of living blood cells depend on the respiratory medium applied. Oligomycin may negatively affect the ET-pathway capacity. PBMC and PLT 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 whole blood. Although with the described methods for isolation of PBMC the ratio of PLT count to PBMC count in PBMC fraction (median 6.3) was only 5.4 % of the ratio in whole blood (median 117.2), this contamination by PLT can significantly contribute to the respiration of 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

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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 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 Resuspend 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 PBMC and wash with PBS 350 g 5 min	Resuspend in 200 µL PBS, count for 4 chambers transport on ice living cells: RPMI+FCS, permeabilized: MiR05	[2a]
20 mL blood 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 living cells in the same medium Cell count with hematocrit Do not transport below 20°C (25-30 optimum) living 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 PBMC Dilute 5x with RPMI 700 g 8 min, brake 6	Resuspend in 1 mL RPMI with 10 mM glucose, respiration living cells in the same medium Cell count ~20 Mx for 4 chambers transport on ice living cells: RPMI	[3a,8]
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 living cells transport at 36°C	Dilute with RPMI , pour on Leucosep tube with Ficoll-Paque 1.077 g/mL, fill up to 50 mL Centrifuge 1000 g 10 min, no brakes	Collect PBMC, dilute with RPMI to 45 mL Centrifuge 200 g 10 min, acc 9 brake 6	4.5 mL MiR05 or RPMI for living cells 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-Paque 1.077 g/mL Centrifuge 800 g 10 min, acc 6, no brakes	Collect PBMC, dilute with RPMI to 30 mL, centrifuge 100 g 10 min, acc 9 brake 6 Resuspend the pellet in 25 mL of RPMI and centrifuge again	R7509 RPMI-1640 Medium Modified Resuspend in 5 mL MiR05 (permeabilized cells) or RPMI (living 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	[9]
	1000 g 10 min RT	Resuspend in MiR05 Respiration living cells MiR05				[10]
20 mL blood 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 – living cells	[11]
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]
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	[13]
			400 g 30 min Ficoll-Hypaque (Biochrom KG)			[14]

¹PRP platelet rich plasma

References extra 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