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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 (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 FluoRespirometry (HRFR) are described, important aspects 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 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, so as to not inadvertently activate the cells and change their phenotype [5].
In the isolation procedures, we compared using RPMI+BSA, RPMI and DPBS. Since we did not find differences in respiration of cells isolated with these media, we decided to use DPBS in our protocols. Resuspendation 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.

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™ 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 PBMCs 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.

Preparation:

1. 15 ml Ficoll-Paque™ PLUS are added into a 50 ml Leucosep tube and centrifuged at 1000 x 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.

   Normal ranges to be expected:
   Platelets: 150-300 x 10^3 cells/µl

Lymphocytes: 1-4 x 10^3 cells/µl
Monocytes: 0.1-0.5 x 10^3 cells/µl

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 x 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 it into a new 50 ml Falcon tube.
5. Add 25 ml of DPBS and centrifuge at 110 x g for 10 min at RT [fast acceleration, 9, intermediate brake, 6].
   (Note: The manufacturer instruction at this step is 250 x g for 10 min, but this gives relatively high contamination with PLT: PLT/PBMCs ~ 20. Centrifugation at 110 x g for 10 min gives 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 x 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 into 90 µl PBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex counter.
   - Remove 2x 20 µl and 1x 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.

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 into 90 µl PBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex counter.
    - Remove 2x 20 µl and 1x 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 x 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 into 90 µl PBS in an Eppendorf tube for counting (dilution 10x) on the Sysmex counter.
- Remove 2x 20 µl and 1x 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 x 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 x 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 2x 20 µl and 1x 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.

4. Instrumental setup

Setup of the Oroboros 2k followed standard procedures as described in detail elsewhere [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 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.

5. 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 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. 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

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: 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

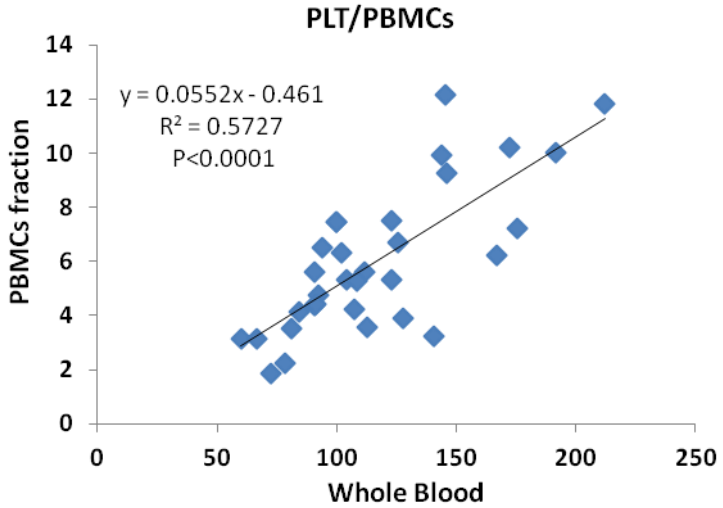
AscTm ascorbate, 2 mM, TMPD (N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride), 0.5 mM
Azd azide, 100 mM

6. 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 PBMCs in isolated PBMCs 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 PBMCs fraction obtained by described isolation methods is influenced by PLT to PBMCs ratio existing in the donor's blood.



Graph 1. PLT to PBMCs ratio in PBMCs fraction isolated by described methods as a function of PLT to PBMCs ratio in the whole blood. The ratio PLT/PBMCs in PBMCs fraction correlates closely with PLT/PBMCs in the whole blood ($P<0.0001$ by Pearson test). The values are from 32 blood samples.

7. Respiratory characteristics of intact platelets and PBMCs

Figures 1 and 2 show 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.

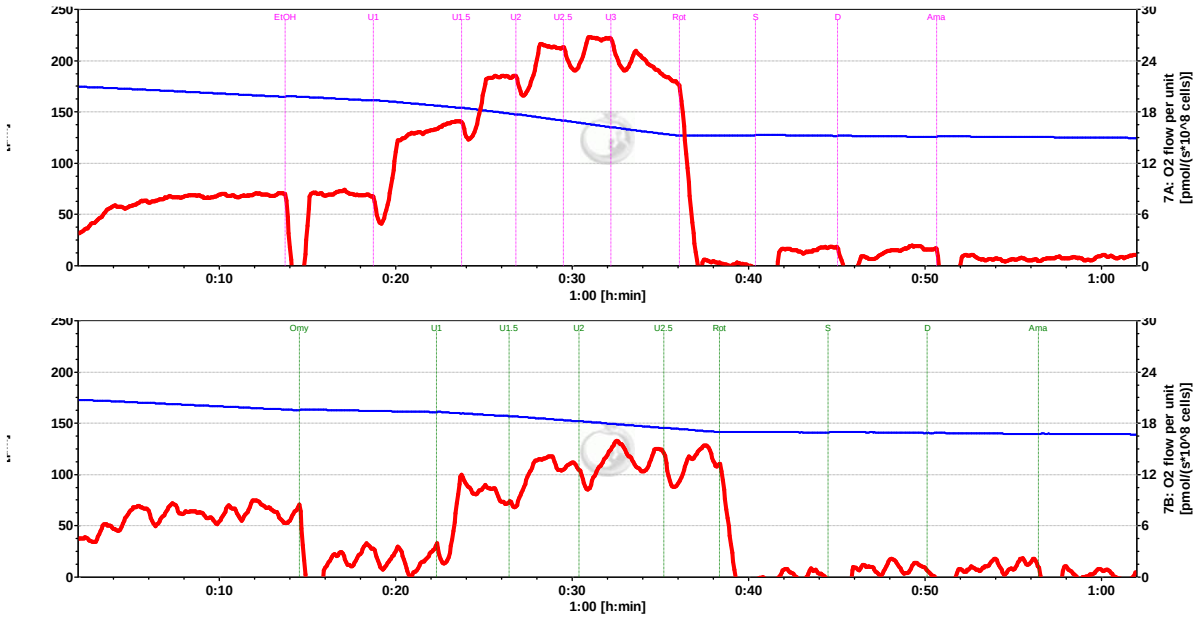


Fig. 1. Respiration of PLTs examined using a Coupling Control Protocol determined in MiR05Cr. The Blue trace denotes oxygen concentration (left Y-axis [µM]), the red trace oxygen flux per chamber volume (right Y-axis [pmol·s^{-1}·10^{-8}cells]). Experiment: 2016-04-20 P7-02.DLD

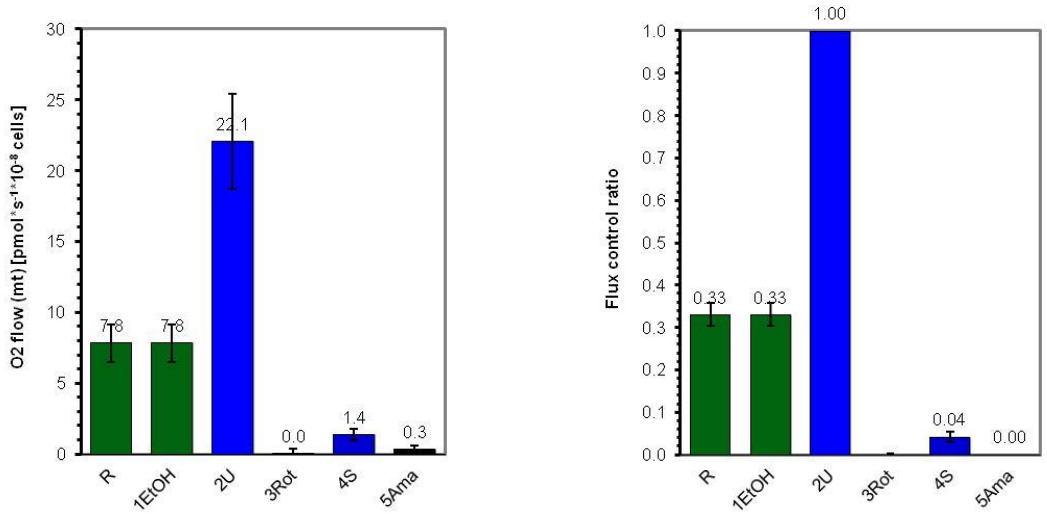


Fig. 2. Respiration rates of human PLTs examined in MiR05Cr. The left panel denotes cell number specific fluxes, the right panel Flux Control Ratios obtained by normalizing ROX-corrected fluxes to ET capacity. Data are means ± SEM of 5 experiments. Succinate was added to test the intactness of the cell membrane.

Figures 3, 4 and 5 show example of measurements and their evaluation on intact PBMCs isolated by protocol 1 described above and examined

with a modified Coupling Control Protocol using MiR05Cr+Ctl and RPMI as a respiration medium.

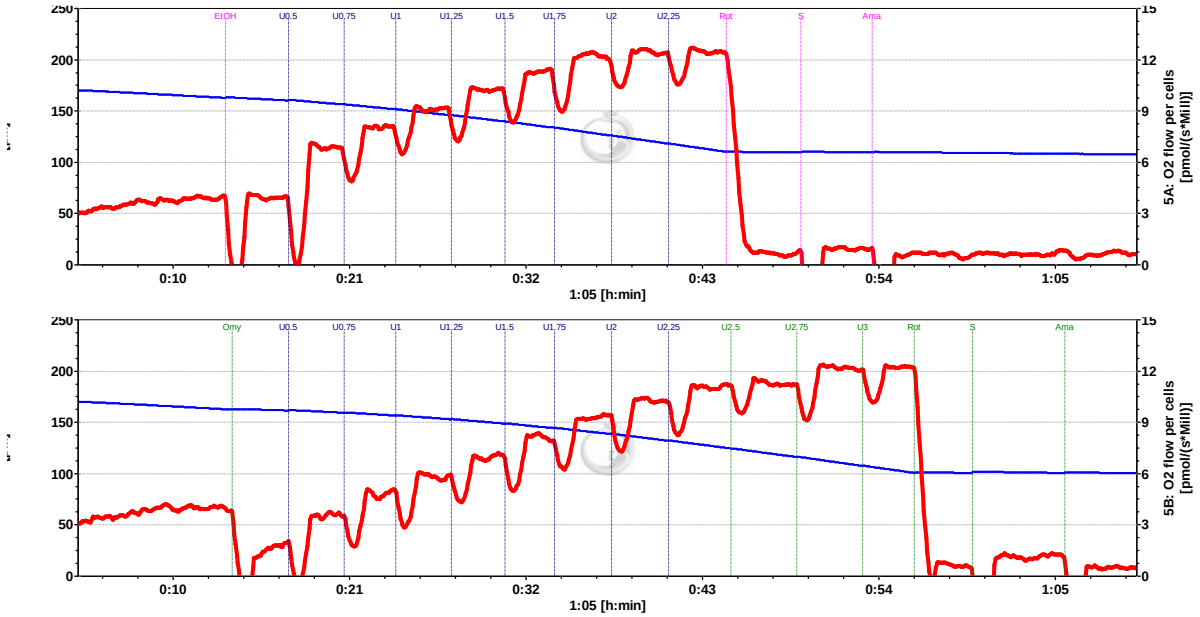


Fig. 3. Respiration of intact PBMCs examined using a Coupling Control Protocol determined in RPMI. Further details as in Legend to Fig. 1. Experiment: 2016-06-01 P5-03.DLD

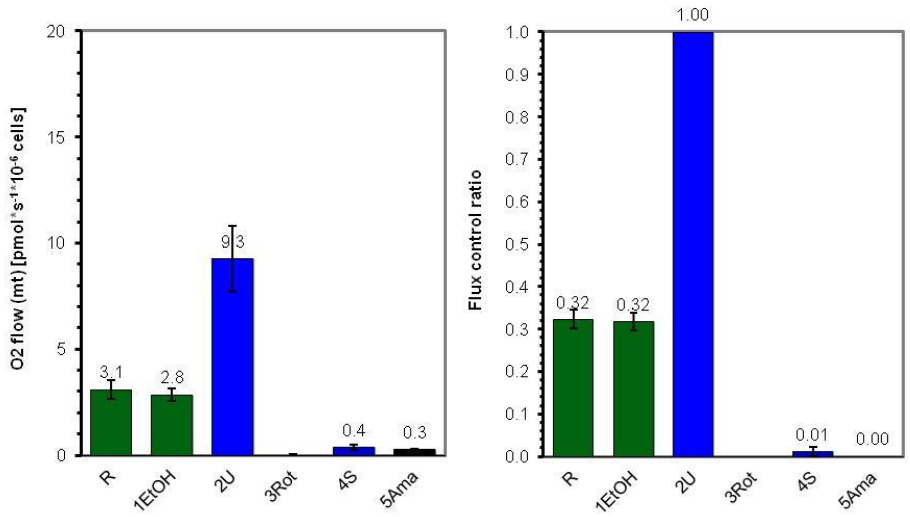


Fig. 4. Respiration rates of intact PBMCs examined in MiR05Cr. Data are means ± SEM of 5 measurements.

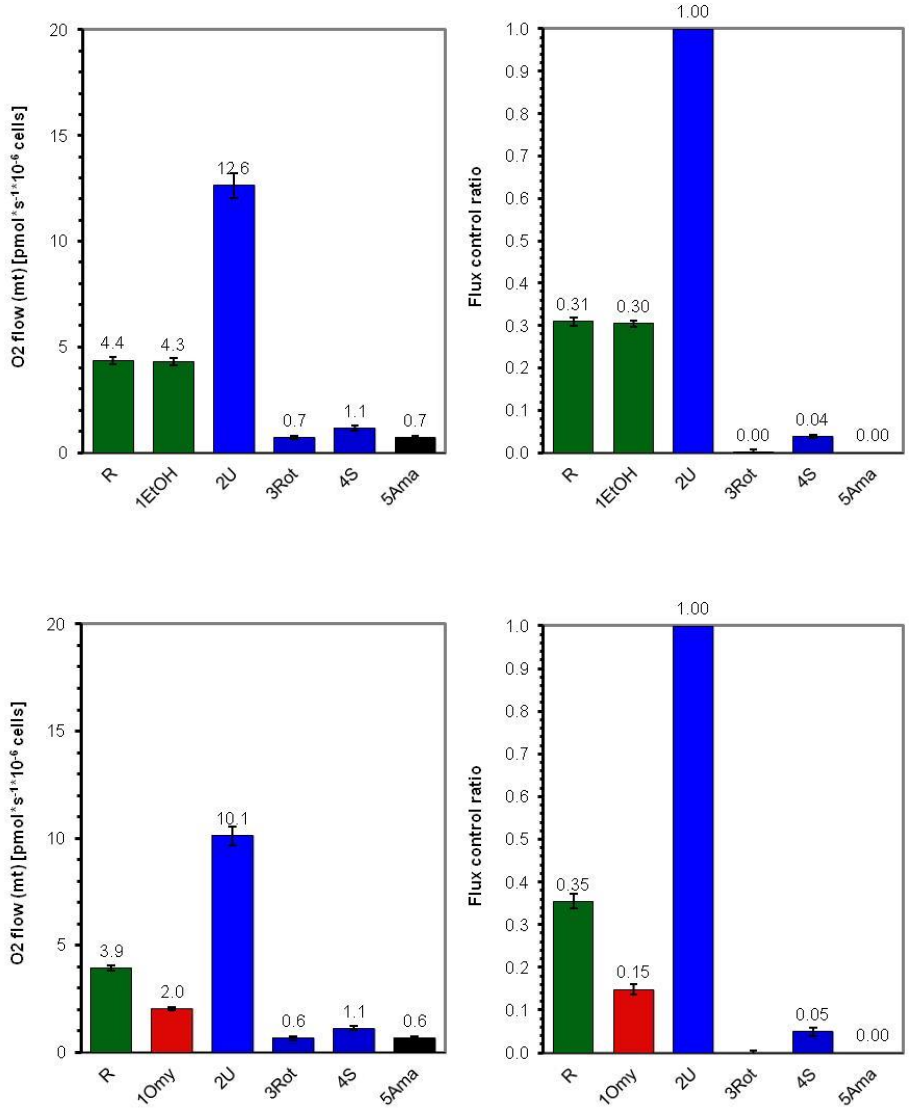


Fig. 5. Respiration rates of intact PBMCs examined in RPMI. Data are means ± SEM 16 experiments.

The respiratory rates of intact cells depended on the respiration medium used, with RPMI consistently supporting slightly higher rates than MiR05Cr. While RPMI contains many substrates, providing external support for respiration, cells in MiR05Cr 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. We used RPMI without L-glutamine in the present experiments, but the medium contained other constituents supporting mitochondrial respiration. Selection of optimal medium composition for evaluation of respiration of intact cells is thus clearly not a trivial task and may pose a real challenge.

ET-pathway respiration after Omy is frequently much lower than ET-pathway measured without previous Omy titration (see Fig 1). In line, in the experiments depicted in Fig. 5 the ET capacity measured after Omy accounted for only 80% of ET-pathway measured in the absence of Omy. This effect of Omy on ET capacity can depend on the respiration medium used, producing different relative Omy effects on ET capacity in the same cell type.

8. 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].

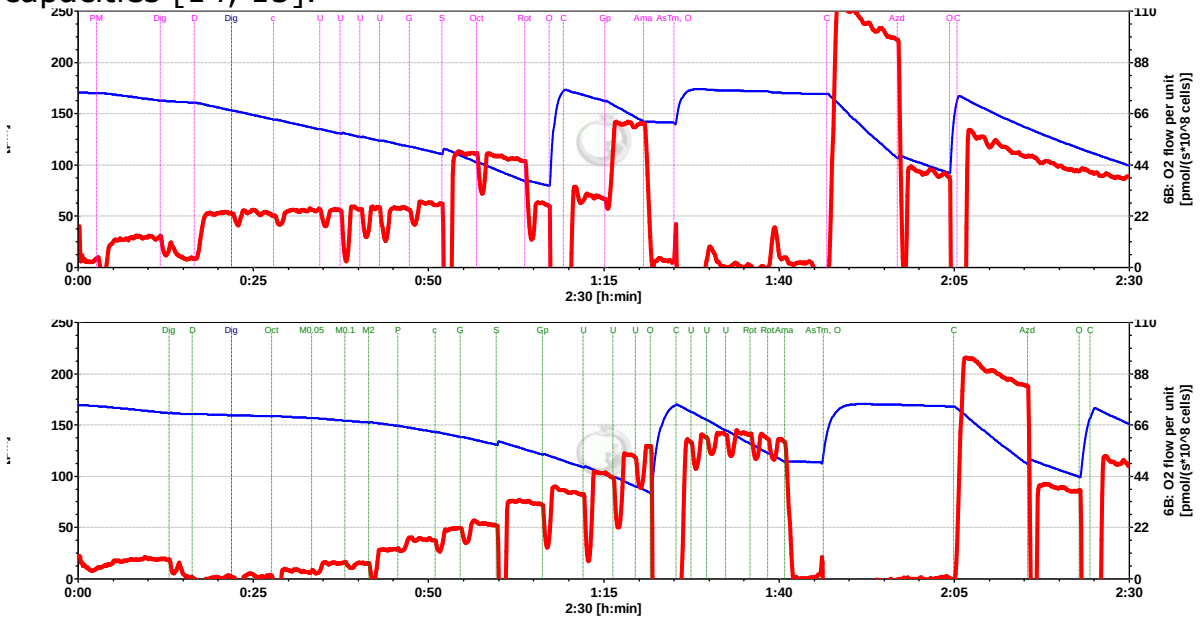


Fig. 6. Respiration of platelets examined with SUIT reference protocols RP1 in upper panel, and RP2 in lower panel. Blue and red traces denote oxygen concentration and oxygen fluxes, respectively. Experiment: 2016-04-19 P6-01.DLD.

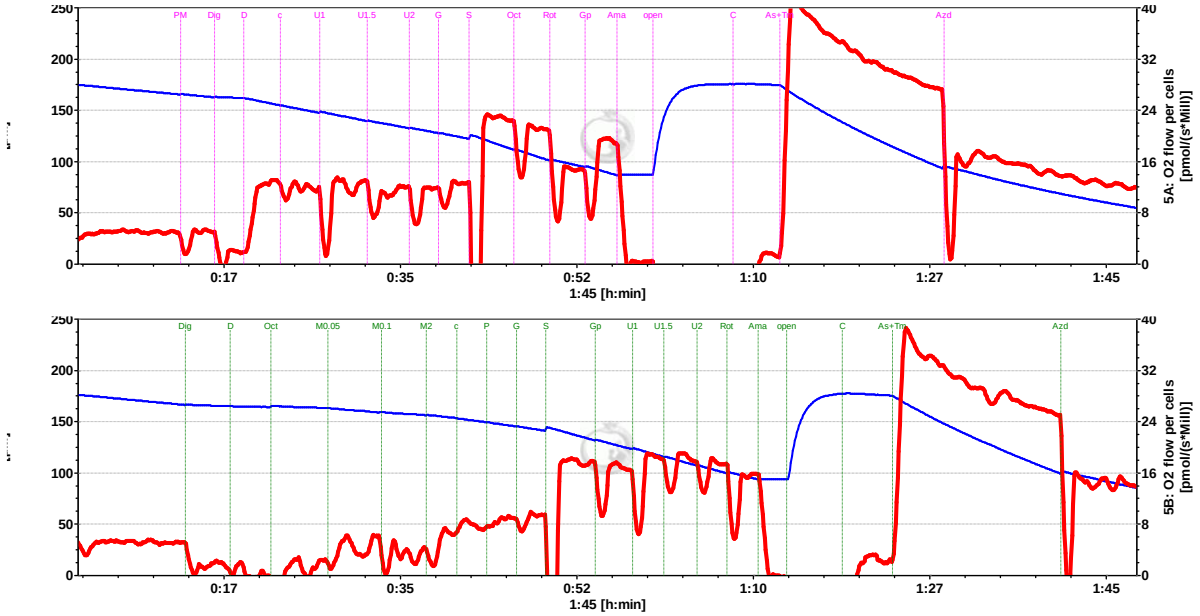


Fig. 7. Respiration of PBMCs examined with SUIT reference protocols RP1 in upper panel and RP2 lower panel. Details as in Legend to Fig. 1. Experiment: 2016-06-02 P5-02.DLD.

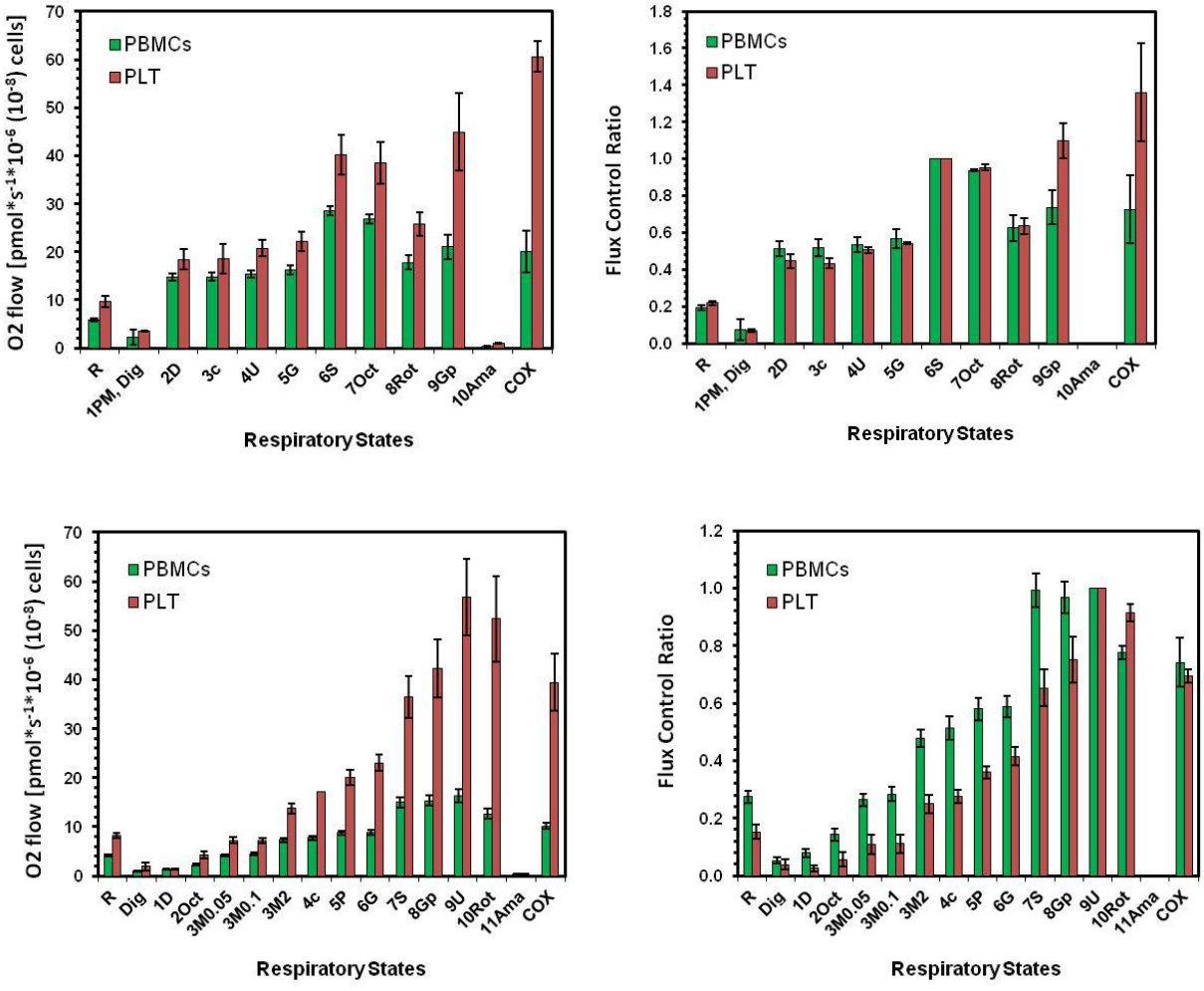


Fig. 8. Comparison of respiration rates and relative O_2 fluxes in PBMCs and PLT examined by SUIT reference protocols RP1 (upper graphs) and RP2 (lower graphs). Flux Control Ratios were calculated by normalizing to ET-pathway in the presence of CI&II-linked substrates in RP1 and to ET-pathway in the additional presence of Gp and Oct in RP2. Respiration rates are expressed per 10^6 PBMCs or 10^8 PLT. Data are means ± SEM of 2-4 experiments.

From the graphs above it could be seen that the respiratory signature of PLTs and PBMCs is different. The rate of PLT respiration per 10^8 cells is higher than the rate of PBMCs respiration per 10^6 cells, but reference to mitochondrial markers will be required to make more meaningful quantitative comparisons of total respiration. In RP1 the response to Gp is significantly higher in PLT than in PBMCs (FCF_{Gp} 0.39 in PLT vs 0.12 in PBMCs, see Fig. 9), in RP2 the phosphorylation system highly limits respiration of PLT with substrates of CETF&CI&II&GPDH as evident from the increased respiratory rates after addition of uncoupler in these cells; in comparison, in PBMCs the effect of uncoupler on respiration rate is very 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 rates observed as well as the cell mass in the chamber. E.g., 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).

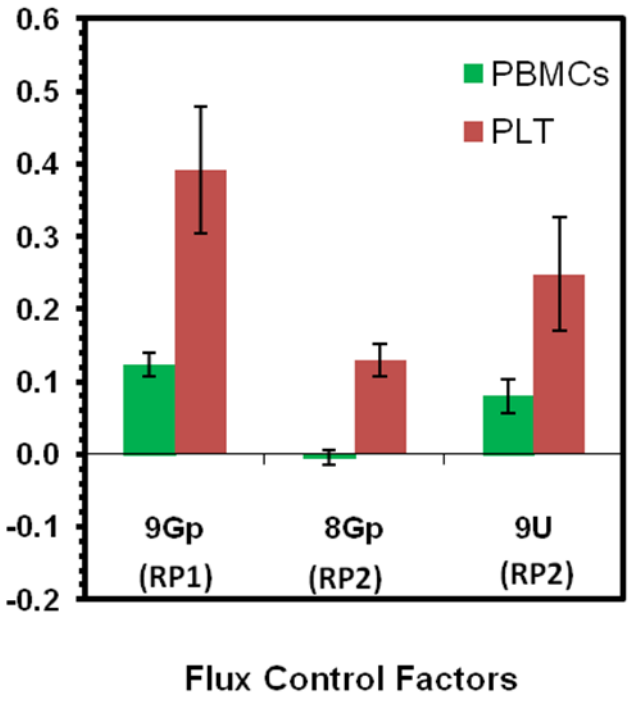


Fig. 9. Step changes from SUIT reference protocols RP1 and RP2 which were significantly different for PBMCs and PLT expressed as Flux Control Factors. Data are means ± SEM of 30 and 4 samples, respectively.

9. Conclusions

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

10. References

1. 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.
2. 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.
3. Fasching M, Fontana-Ayoub M, Gnaiger E (2014) Mitochondrial respiration medium - MiR06. Mitochondr Physiol Network 14.13(05):1-4. http://bioblast.at/index.php/MiPNet14.13_Medium-MiR06. – »Bioblast link«
4. Hroudová J, Fišar Z, Kitzlerová E, Zvěřová M, Raboch J. Mitochondrial respiration in blood platelets of depressive patients. Mitochondrion. 2013;13(6):795-800.
5. 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.
6. Karabatsiakis A, Böck C, Salinas-Manrique J, Kolassa S, Calzia E, Dietrich DE, Kolassa IT. Mitochondrial respiration in peripheral blood mononuclear cells correlates with depressive subsymptoms and severity of major depression. Transl Psychiatry. 2014;4:e397. doi: 10.1038/tp.2014.
7. 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).
8. 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.
9. 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.
10. Siewiera K, Kassassir H, Talar M, Wieteska L, Watala C. Higher mitochondrial potential and elevated mitochondrial respiration are associated with excessive activation of blood platelets in diabetic rats. Life Sci. 2016 Mar 1;148:293-304.
11. Sjövall F, Morota S, Persson J, Hansson MJ, Elmér E. Patients with sepsis exhibit increased mitochondrial respiratory capacity in peripheral blood immune cells. Crit Care. 2013 Jul 24;17(4):R152.
12. 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.

13. Ravi S, Chacko B, Sawada H, et al. Metabolic Plasticity in Resting and Thrombin Activated Platelets. Tan M, ed. PLoS ONE. 2015;10(4):e0123597. doi:10.1371/journal.pone.0123597.
14. http://www.bioblast.at/index.php/Harmonized_SUIT_protocols
15. http://www.bioblast.at/index.php/MiPNet21.06_SUIT_reference_protocol
16. http://wiki.oroboros.at/images/0/04/MiPNet20.04_Checklist.pdf

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

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MitoFit mitochondria fit&well

www.mitofit.org

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Tirol Standortagentur

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