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Isolation and cryopreservation of human peripheral blood mononuclear cells for high-resolution respirometry

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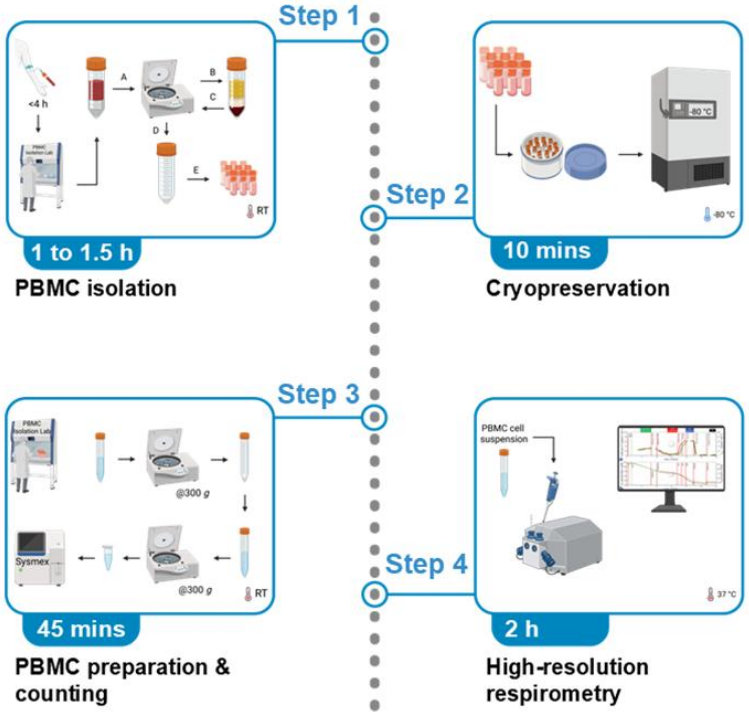
Summary

Ensuring high-quality preparations of peripheral blood mononuclear cells (PBMCs) is essential for obtaining consistent results matching the accuracy of high-resolution respirometry. Here, we present an advanced protocol to isolate and cryopreserve human PBMCs. We describe steps to maintain viability of cryopreserved cells and minimize platelet contamination through optimized centrifugation. This protocol provides a valuable reference for optimized PBMC isolation and cryopreservation in mitochondrial research and diagnostic applications.



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This PBMC isolation and cryopreservation protocol is a modified and optimized version of a widely applied procedure [1–4], and is tested by high-resolution respirometry with reference to freshly prepared cells.

Before you begin

PBMCs represent a heterogeneous population of immune cells (white blood cells; mainly lymphocytes) that play a critical role in the body's defense mechanisms. Due to their ready accessibility from human blood sample and the possibility to improve their resistance to cryopreservation, PBMCs have become a valuable subject of research and are considered as biomarkers in disease diagnosis and monitoring of treatment outcomes [2,5–12].

A considerable number of studies have investigated the mitochondrial respiratory function of PBMCs, yielding heterogeneous outcomes that largely reflect differences in sample handling, storage, and cryopreservation procedures [1,8,13–20]. Respiratory function of PBMCs declines after 24-hour storage at room temperature (RT; 20 to 25 °C) and deteriorates when stored in cold buffers [13–16,21,22] . Cryopreservation at -80 °C for several weeks or months preserves viability and mitochondrial respiratory capacity in PBMCs [5,7], but may lead to a pronounced decline [8]. Cryopreservation in liquid nitrogen (-196 °C) is claimed to be superior including a 24 h recovery incubation at 37 °C, which deviates from the respirometric measurements on fresh reference cells [23].Some respirometric studies use PBMCs cryopreserved at -80 °C for several months without comparison of fresh versus cryopreserved cells [1–4,6].

This manuscript outlines an optimized protocol for the isolation, cryopreservation, and thawing of human PBMCs for evaluation of mitochondrial function by high-resolution respirometry (HRR). Standardized preanalytical preparation of PBMCs is essential for obtaining reproducible measurements of mitochondrial respiration. This protocol is designed to maximize PBMC recovery, preserve cellular viability and mitochondrial integrity, and minimize platelet contamination in the final cell suspension.

Blood samples were taken from volunteers at the Department of Neurology at the Medical University of Innsbruck. All participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics committee of the Medical University of Innsbruck (EK 1354/2023).

Note: Any laboratory handling blood for cell isolation must be authorized with Biosafety Level 2 clearance and equipped with Laminar Flow cabinets. Human samples carry infectious risk, so strict adherence to institutional biosafety regulations is mandatory. This includes the proper use of personal protective equipment, decontamination of surfaces, and appropriate waste disposal procedures.

Preparation of tubes for PBMC isolation

Timing: 5 min

1. Preparation of Leucosep™ tubes:
    a. Gently invert the Ficoll-Paque™ PLUS bottle 5 times to ensure thorough mixing. Using a sterile serological pipette, fill each empty Leucosep™ tube with 15 mL Ficoll-Paque™ PLUS density gradient medium at RT (20 to 25 °C), following the manufacturer's instructions.
    Note: Tubes may be prefilled and stored at RT protected from light.
    b. Close the tubes and centrifuge at 1000 *g* for 30 s at RT. Then the density gradient medium should be located beneath the porous barrier disc.

Note: The centrifugation time was reported at 60 s previously in our lab (https://wiki.oroboros.at/index.php/MiPNet21.17_BloodCellsIsolation; retrieved 2026-03-20). However, 30 s is sufficient [14,24].

Optional: Leucosep™ tubes can be substituted by alternative tubes, if necessary (Troubleshooting 1).

Collection of human blood

Timing: 10 min

1. **Collection of human peripheral blood:**
    a. Collect 9 to 18 mL of peripheral blood from donors using pre-labelled 9-mL S-Monovette® EDTA K3E (tri-potassium ethylenediaminetetraacetic acid), in accordance with standard venous blood collection guidelines [25].
    Note: EDTA tubes yield superior recovery of lymphocytes in comparison with other tube types [21].
    Critical: PBMC isolation must be performed within **2 h** or maximally **4 h** of blood collection to ensure optimal results. Delays beyond this time window lead to a loss of mitochondrial function and cell viability [13–15,22] (Troubleshooting 2 and 3).
    Critical: Do not store blood samples at 4 °C or on ice, nor above 37 °C. Instead, maintain them at RT, to preserve mitochondrial function [22] (Troubleshooting 4,8 and 9).
    b. Immediately after collection, gently invert tubes 3 to 5 times to prevent clot formation.
    c. Store tubes at RT in a transport container designated for biological or clinical samples until further processing.

Note: Controlling the status of blood donors (*e.g.,* fasted or rested) depends on the scientific questions of the study. It may affect the mitochondrial function of the PBMCs.

Note: The timing stated for this section does not account for any pre-collection standards, such as a 15-minute resting period of participants.

Note: Storing blood for longer than 4 hours may reduce cell viability and alter blood cell populations. If it is necessary to store the blood for more than 4 hours, store in the dark at RT to minimize any deleterious effects [16,22].

Preparation of reagents and equipment for PBMC isolation

Timing: 1 to 2 h

1. **Prepare all solutions and media as described in 'Key resources' and 'Materials and equipment' sections under sterile conditions**:
    a. Label all required tubes and vials to prevent mix-ups.
    b. Allow all reagents for PBMC isolation to equilibrate at RT.
    c. Set the centrifuge to RT.
    d. Fill the cryo-freezing container with isopropanol and keep it at RT to ensure a slow freezing process.

Note: The isopropanol should be replaced after five uses.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human blood	Medical University of Innsbruck	N/A
Chemicals, peptides, and recombinant proteins		
FicoII-Paque™ PLUS density gradient centrifugation medium, sterile	Millipore Sigma	GE17-1440-02
Phosphate-buffered saline (PBS), sterile, no magnesium, no calcium	PAN Biotech	P04-36500
Dimethylsulfoxide (DMSO)	Sigma Aldrich	67-68-5
Gibco™ RPMI 1640 medium	PAN Biotech	P04-16500
Fetal Bovine Serum (FBS)	PAN Biotech	P30-3306
Isopropanol absolute	Applichem	67-63-0
Ethanol absolute 100 %	Bartelt	CL00.0505.5000
MiR05-Kit	Oroboros Instruments	60101-01
Bovine serum albumin (BSA)	Sigma Aldrich	9048-46-8
Acridine Orange/Propidium Iodide Stain	Logos biosystem	F23011
Sysmex XN-350 reagents		
XN-L CHECK L1	Sysmex	213570
XN-L CHECK L2	Sysmex	213571
XN-L CHECK L3	Sysmex	213572
LYSERCELL WDF	Sysmex	AZ124801
CELLPACK DCL	Sysmex	CU228496
SULFOLYSER	Sysmex	5433514
CELLCLEAN	Sysmex	83401621
FLUOROCELL WDF	Sysmex	AA325279
Software and algorithms		
DatLab 7 to 8.3	Oroboros Instruments	N/A

Other		
BD Vacutainer® EDTA blood collection tube	Pipette	N/A
Leucosep™ tube	Greiner Bio-One	227290
LUNA-FL Automated Fluorescence Cell Counter	Logos biosystem	
LUNA™ Cell Counting Slide	Logos biosystem	L12001
Sysmex XN-350 Hematology Analyzer	Sysmex	
Biological transport box	Merck	HS120052
ThawSTAR CFT2 Transporter	Stemcell	100-0642
Cryobox	N/A	N/A
15-mL polypropylene centrifuge tube	Eppendorf	0030122151
50-mL polypropylene centrifuge tube	Eppendorf	0030122178
0.6-mL microcentrifuge tube	Merck	T5149
2.0-mL Cryomaster® cryovial	Cryomaster	4-6106
Freezer, -80 °C	N/A	N/A
Freezer, -20 °C	N/A	N/A
Fridge, 4 °C	N/A	N/A
Mr. Frosty™ Freezing Container	ThermoFisher Scientific	5100-0001
Filter tips, 10 µL, sterile	AL-Labortechnik	4-0201
Filter tips, 20 µL, sterile	AL-Labortechnik	4-0201
Filter tips, 100 µL, sterile	AL-Labortechnik	4-0200
Filter tips, 200 µL, sterile	AL-Labortechnik	4-0197
Filter tips, 1 mL, sterile	AL-Labortechnik	4-0203
Tips, 5 mL, sterile	Ratiolab	2400650
Water bath	N/A	N/A
Centrifuge-swing out rotor	Heraus Megafuge 16R Thermo Scientific	75004271
Serological pipette, accu-jet® ,10 mL	Brand	612-6824
Serological pipette, accu-jet®, 25 mL	Brand	612-6824
All-in-One Vortex	Biozym Scientific GmbH	553030
Micropipette 10 µL	Sartorius	LH-728674
Micropipette 20 µL	Sartorius	LH-728674
Micropipette 100 µL	Sartorius	LH-728674
Micropipette 200 µL	Sartorius	LH-728674
Micropipette 500 µL	Sartorius	LH-728674
Micropipette 1 mL	Sartorius	LH-728674
Oroboros O2k	Oroboros Instruments	10104-03
Hamilton syringe, 10 µL	Oroboros Instruments	51010-01
Hamilton syringe, 25 µL	Oroboros Instruments	51025-01
Hamilton syringe, 100 µL	Oroboros Instruments	51100-01
Syringe rack	Oroboros Instruments	51610-01
ISS-Integrated Suction System	Oroboros Instruments	20810-02

Materials and equipment setup

Cryopreservation medium

Reagent	**Final concentration**	**Volume**
FBS	90 % (v/v)	90 mL
DMSO	10 % (v/v)	10 mL
Total	n/a	**100 mL**

Prepare aliquots (10 mL) and store at -20 °C for up to 1 year.

Note: Before preparing the cryopreservation medium, inactivate fetal bovine serum (FBS) by heating to 50 °C for 20 min.

Critical: Use the same lot number of FBS for all samples in the same study (order accordingly) to avoid differences between batches that may affect the results [18].

MiR05-Kit – Mitochondrial respiration medium

Reagent	Final concentration	Mass/volume
EGTA*	0.5 mM	0.045 g
$MgCl_2$*	3 mM	0.071 g
Lactobionic acid*	60 mM	5.375 g
Taurine*	20 mM	0.625 g
KH_2PO_4*	10 mM	0.340 g
HEPES*	20 mM	1.191 g
D-Sucrose*	110 mM	9.413 g
BSA	1 g/L	0.250 g
H_2O	n/a	up to 250 mL
Total	**n/a**	**250 mL**

*Chemical provided in 1 vial of MiR05-Kit.
Adjust pH to 7.1 with a 5 M KOH solution, prepare aliquots (45 mL) and store at -20 °C for up to 51 months [26].

Critical: Follow manufacturer's instructions to prepare MiR05-Kit solution.

Step-by-step method details

Isolation of PBMC

Timing: 1 to 2 h

This section describes PBMC isolation from whole blood using density gradient centrifugation (Figure 1). Before starting the procedure ensure that all tubes are labelled, required equipment is prepared, and the centrifuge is set correctly.

1. **Isolation of the PBMC layer from whole blood:**
    a. Under sterile conditions, invert the whole blood collection tubes 5 times to resuspend the cells and decant the blood carefully into the prepared Leucosep™ tube (Figure 2A) to prevent any mixing. Centrifuge at 1000 g for 10 min at RT, ensuring that the centrifuge brake is deactivated (Troubleshooting 2).

Note: Do not dilute the blood sample with phosphate-buffered saline (PBS), to avoid dilution of plasma compounds, particularly if the plasma is required for subsequent studies.

b. Carefully remove the Leucosep™ tube from the centrifuge without disturbing the separated layers and place it under the laminar flow cabinet (Figure 2B).

c. Using a 10-mL serological pipette, collect the plasma layer (5 to 10 mL, depending on the whole blood volume) into a 15-mL polypropylene centrifuge tube (Figure 3A). Avoid disturbing the PBMC layer.
Note: The plasma can be aliquoted and stored at -80 °C for future studies.

d. Carefully extract the "white" PBMC layer (buffy coat) using a 1-mL micropipette (Figure 3B).

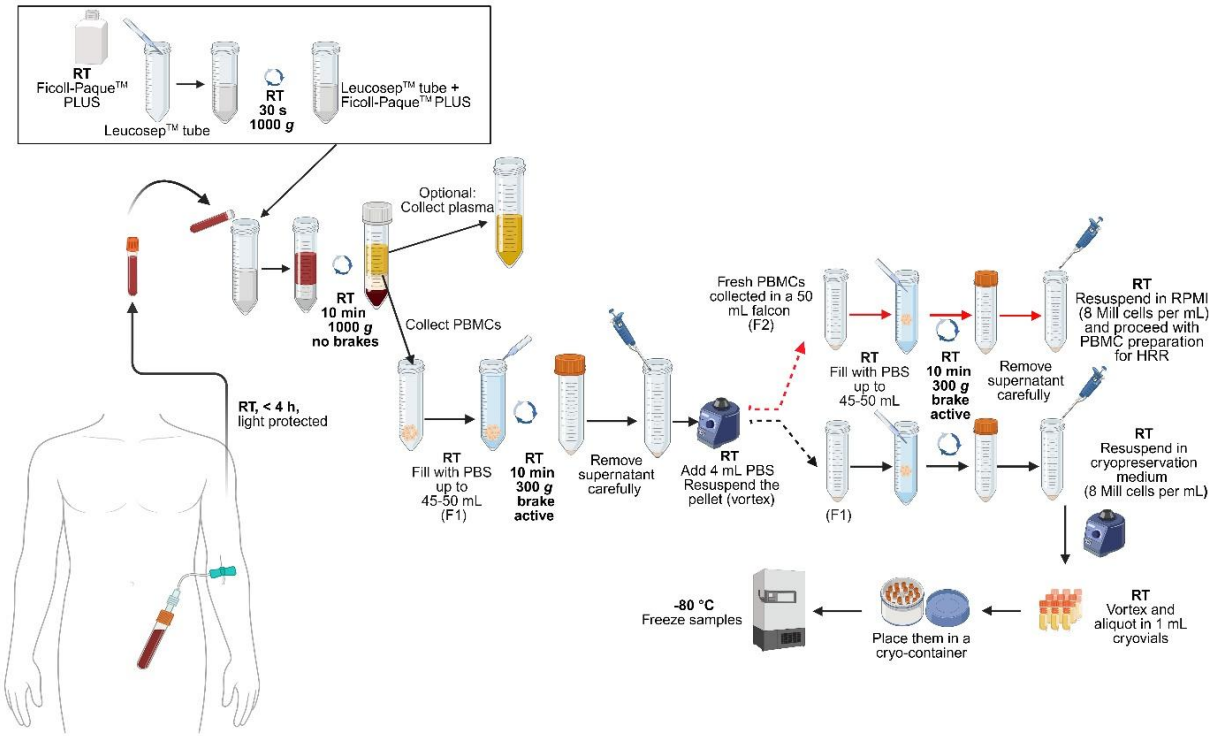

Figure 1: Workflow of PBMC isolation and cryopreservation. RT, room temperature (20 to 25 °C). F1 and F2, separate 50-mL polypropylene centrifuge tubes. PBS, phosphate-buffered saline. RPMI, RPMI-1640-medium. HRR, high-resolution respirometry. Cryopreservation medium, see Materials and equipment setup.

Critical: This step is crucial for optimal PBMC isolation and requires sufficient training. Minimize disturbance of the separation medium and limit micropipette movements to avoid contamination.

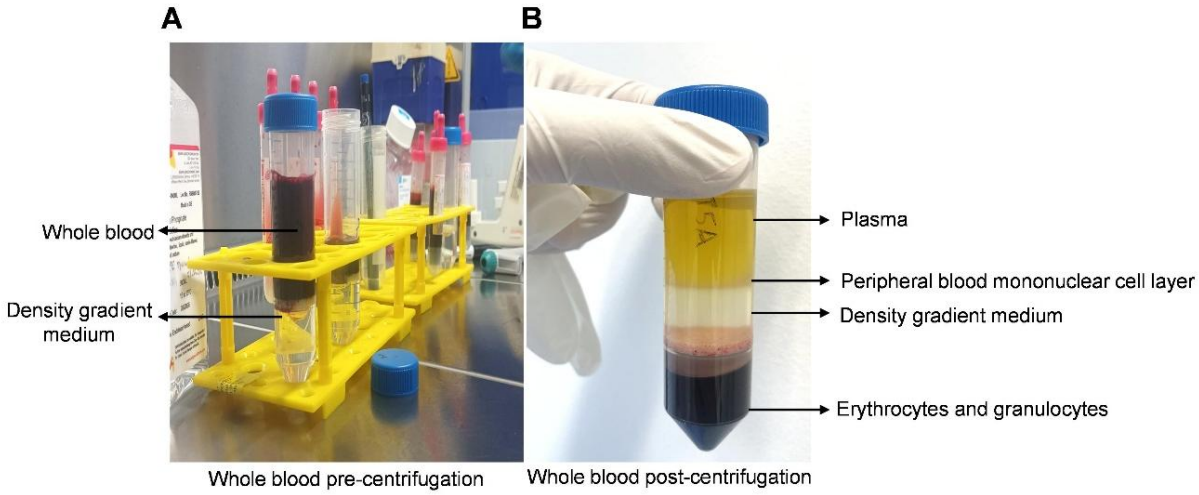

Figure 2: Separation of whole blood components using density gradient medium. A. Whole blood atop the density gradient medium in a Leucosep™ tube prior to centrifugation, related to step 1b. **B.** Separated blood components after centrifugation: plasma, PBMC layer, density gradient medium, separation filter, and erythrocytes and granulocytes, related to step 1c.

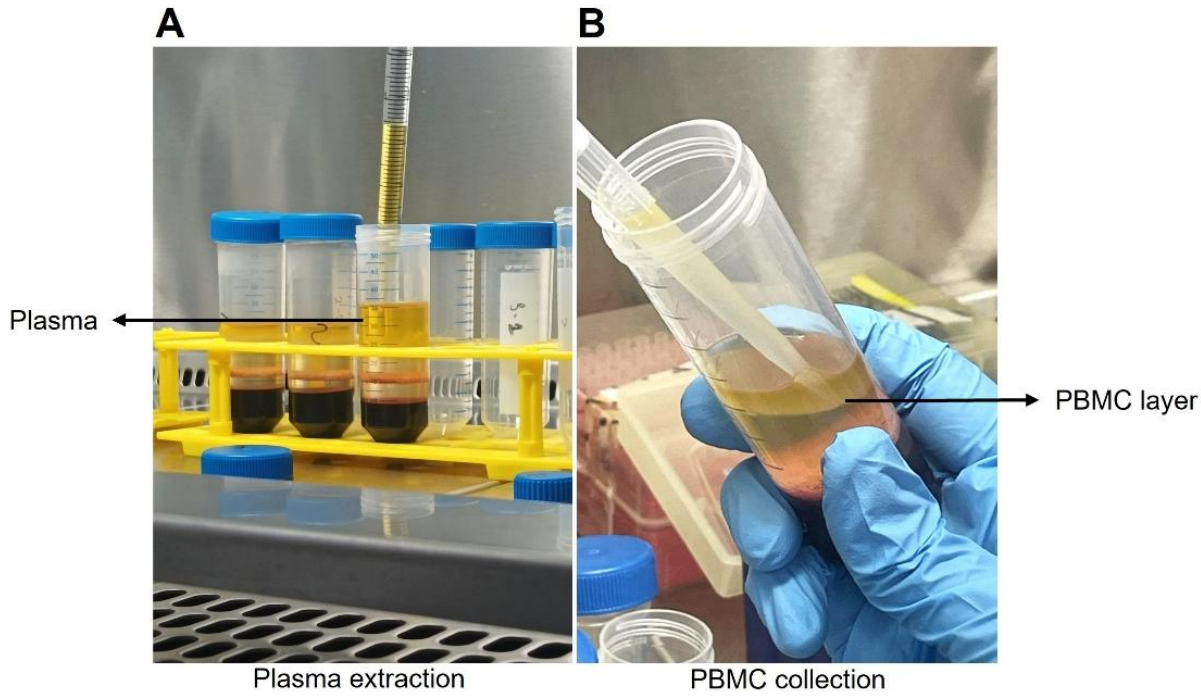

Figure 3: Collection of plasma and PBMC fraction after centrifugation. A. Plasma extraction. **B.** PBMC extraction after plasma collection. Related to step 1d.

2. **Purify the PBMC suspension:**

   a. Transfer the harvested cells into a 50-mL polypropylene centrifuge tube (F1) and adjust the volume to 50 mL with sterile PBS at RT using a 25-mL serological pipette.

b. Centrifuge the F1 tube at 300 *g* for 10 min at RT with the brake reactivated.
Note: The volume of PBS used has been optimized to minimize platelet contamination [14] (**Troubleshooting 4**).
Note: Centrifugation speeds of 200 to 300 *g* do not impact mitochondrial function, but lower speeds (e.g., 150 *g*) may reduce PBMCs yields (**Troubleshooting 2**).
Critical: Ensure that the brake is activated during this centrifugation step.

c. After centrifugation, carefully remove the supernatant from F1 with a 10-mL serological pipette or a laboratory suction pump (Integrated Suction System ISS; Oroboros Instruments, Innsbruck, Austria) until approximately 1 mL remains. Use a 1-mL micropipette to remove the remaining supernatant. Avoid disrupting the pellet.
Critical: Perform this step promptly after centrifugation to prevent resuspension of cells into the supernatant which can lead to decreased cellular yield (**Troubleshooting 2**).

d. Resuspend the pellet in 4 mL sterile PBS at RT using a 1-mL micropipette at least 10 times until the pellet is disrupted.
Optional: Vortex the cell suspension at 2000 to 2200 rpm for 15 s, then transfer 20 µL to a 0.5-mL microcentrifuge tube for PBMC counting and cell viability assessment (see section **PBMC counting**).
Optional: To prepare both fresh and cryopreserved PBMCs from the same blood sample, transfer the necessary number of fresh PBMCs into a separate 50-mL polypropylene centrifuge tube (F2). The remaining sample will be cryopreserved (F1).
Critical: Vortex the PBMC suspension at 2000 to 2200 rpm for 5 s before pipetting or aliquoting into separate tubes to prevent precipitation and ensure consistent cell concentrations between aliquots.

e. Add sterile PBS at RT to adjust the final volume to 50 mL using a 25-mL serological pipette.

f. Centrifuge the tubes at 300 *g* for 10 min at RT with the brake activated.

3. Freshly isolated PBMC suspension:

a. After centrifugation, carefully discard the supernatant from the F2 tube with a 10-mL serological pipette or an ISS until approximately 1 mL remains. Use a 1-mL micropipette to remove the remaining supernatant. Avoid disrupting the pellet.

b. Resuspend the PBMC pellet in 1 mL of RPMI by pipetting gently up and down at least 10 time with a 1-mL micropipette until the pellet is disrupted, avoid harsh pipetting that causes formation of foam in the cell suspension.
Optional: The fresh PBMC suspension can be resuspended directly in PBS if the cells are going to be processed for HRR measurement immediately.

Note: For measurements of freshly isolated PBMCs, keep the PBMC suspension at RT until further assessment with the Oroboros Bioenergetics Platform. For HRR measurements with healthy participants, 1.5 to 3 million cells per mL is recommended.

PBMC cryopreservation

Timing: 5 to 10 min

1. **Cryopreserve the PBMC suspension:**

    a. After centrifugation, carefully discard the supernatant from the F1 tube using a 10-mL serological pipette or an ISS until approximately 1 mL remains. Use a 1-mL micropipette to remove the remaining supernatant. Avoid disrupting the pellet.
    b. Resuspend the PBMC pellet in 1 mL cryopreservation medium at 37 °C by pipetting gently up and down at least 10 times with a 1-mL micropipette until the pellet is disrupted.
    c. After resuspension, add the appropriate volume of cryopreservation medium to achieve the desired cell concentration in the F1 tube.
    Note: For cryopreservation, it is recommended to use not more than 10 million cells per mL [16,18].
    d. Aliquot the PBMC suspension into cryovials.
    Critical: Vortex the PBMC suspension at 2000 to 2200 rpm for 5 s before pipetting or aliquoting into each cryotube to prevent precipitation and ensure consistency of cell concentration across aliquots.
    e. Place the PBMC cryovials in a cryo-container filled with isopropanol and store at -80 °C overnight.
    f. The following day, transfer the cryovials from the cryo-container into a standard freezer storage box.

    Pause point: Mitochondrial function was maintained in cryopreserved cells for 1 and 2 weeks at -80 °C compared to fresh PBMCs (see Expected Outcomes section).

Preparation of isolated PBMCs for HRR experiment

Timing: 40 to 50 min

This section describes the protocol for processing cryopreserved or freshly isolated PBMCs for HRR including cell counting and viability measurements using the LUNA-FL counter and the hematology analyzer Sysmex XN-350.

Note: Alternatively, PBMCs may be counted manually (**Troubleshooting 6**).

Note: Switch on the Sysmex XN-350 at least 20 min before counting. The device takes around 15 min before it is ready to use. This time is not considered in the final timing.

1. **Thawing of cryopreserved PBMCs (Figure 4A):**

    a. Set a water bath at 37 °C.
    b. Pre-warm two 15-mL polypropylene centrifuge tubes containing 9 mL PBS per cryovial of PBMC sample (1 mL) at 37 °C.
    Note: If you are processing more than 1 mL of PBMC suspension, increase the volume of PBS accordingly to maintain the appropriate PBS/PBMC ratio (10:1; PBS:PBMC suspension) for washing the PBMC sample.
    c. Transport the PBMC cryovials from the -80 °C freezer to the water bath in a ThawSTAR CFT2 Transporter or on dry ice (Figure 4B).
    d. Partially thaw cryopreserved PBMCs by promptly inserting the cryovial(s) into a 37 °C water bath for 1.5 min (**Troubleshooting 8**, Figure 4C).
    Note: Slow thawing may negatively impact mitochondrial function and cell viability [18,19,22] (**Troubleshooting 8** and **9**) .
    Critical: If the sample is fully thawed, reduce the thawing time to avoid damage due to prolonged exposure to DMSO [19].

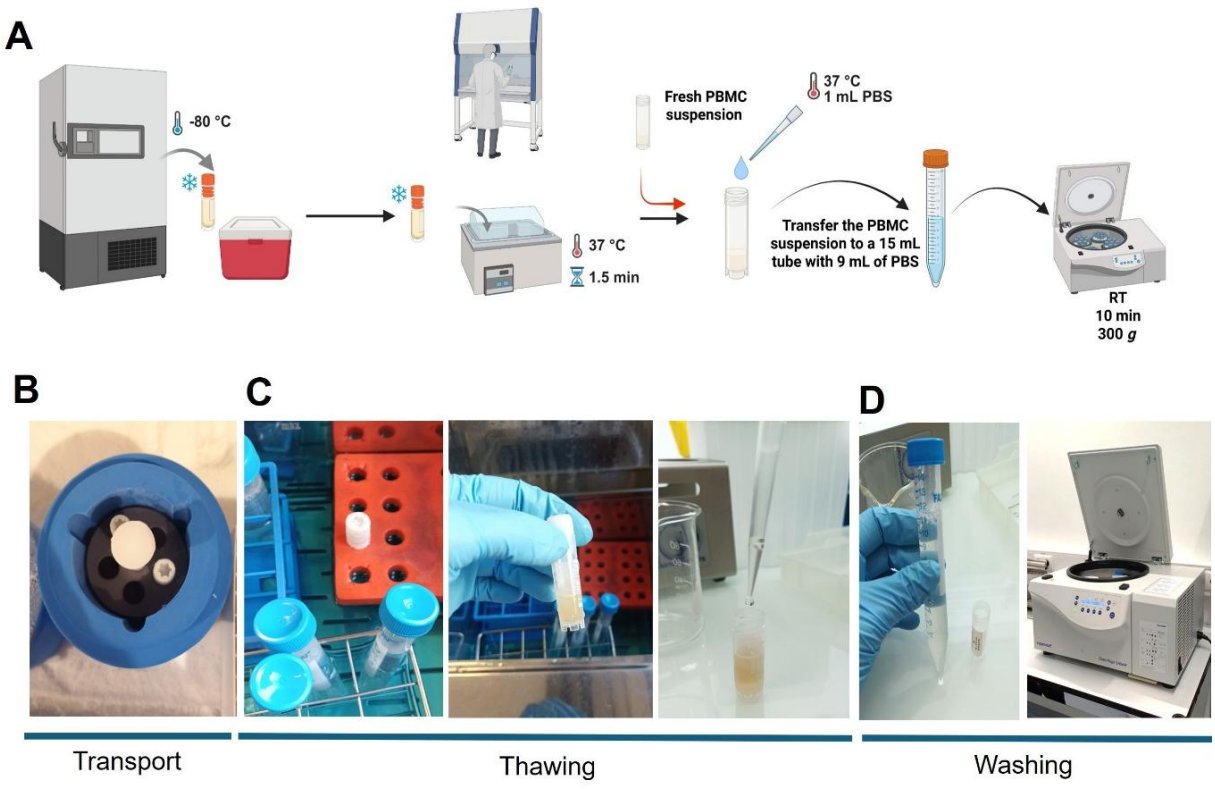

Figure 4: Steps in PBMC sample preparation for high-resolution respirometry. A. Diagrammatic representation of the PBMC sample preparation process. **B.** Transport of cryopreserved PBMCs, related to step 1c. **C.** Thawing of PBMCs, related to step 1d. **D.** Washing steps in PBS at 37 °C, related to steps 2a-f.

2. Purification of PBMC suspension (fresh or cryopreserved):

Note: Fresh and cryopreserved PBMCs must undergo similar processing steps to ensure comparability of procedures. Centrifugations decrease platelet contamination, which affects total oxygen consumption (**Troubleshooting 4**).

a. Add 1 mL PBS with a 1-mL micropipette at 37 °C drop by drop to the PBMC vial. Mix the sample by pipetting gently up and down at least 3 times.
   Note: For cryopreserved PBMCs, ensure the sample is fully thawed afterwards (Figure 4C).
b. Transfer the PBMC suspension (2 mL) into the 15-mL tube containing the remaining PBS (Figure 4D).
c. Centrifuge the tube(s) at 300 *g* for 10 min at RT.
d. Remove approximately 9 mL supernatant using a 10-mL serological pipette or an ISS and use a 1-mL micropipette to carefully remove the remaining liquid, avoiding disturbance of the pellet.
   Critical: Promptly discard the supernatant after centrifugation to prevent loss of PBMCs during washing steps (**Troubleshooting 2**).
e. Resuspend the pellet with 1 mL PBS at 37 °C from the second 15-mL polypropylene centrifuge tube by pipetting gently up and down at least 10 times with a 1-mL micropipette until the pellet is disrupted. Once the pellet is fully resuspended (homogeneous suspension), add the remaining PBS.
f. Wash the PBMC suspension again, repeating steps c and d from this section (**Troubleshooting 4**).
   Note: Previous studies report that the use of a solution containing PBS+FBS can be utilized to stabilize the PBMC suspension. However, the use of different FBS batches might result in unpredictable differences in mitochondrial function of the PBMCs, increasing the variability of results [18,20]. Thus, the use of PBS without FBS supplementation is recommended for these washing steps.
   Note: A 24-h or overnight resting period after thawing has been reported in previous studies. This may lead to selective loss of cell population subsets, reduced viability, phenotypic changes, and alterations in functional capacity or inflammatory marker expression, resulting in measurements that no longer accurately represent the *in vivo* physiological state [20,23].
g. Resuspend the pellet in 0.56 mL MiR05 by pipetting with a 1-mL micropipette at least 10 times until the pellet is disrupted for cell counting and viability determination as described in **steps 3 and 4.**
   Critical: Resuspending in 0.56 mL MiR05 is recommended to stay within the detection limit of the Sysmex XN-350 (3000 lymphocytes per µL). However, if the cell count is higher, the resuspension volume may be increased.

3. PBMC counting with Sysmex XN-350:

The Sysmex XN-350 hematology analyzer is a flow cytometer used to measure whole blood and purified blood cell suspensions, providing detailed information on the number and type of blood cells in the sample.

Critical: Ensure that instrument's quality control (QC) and routine cleaning have been performed within the last 7 days, following the manufacturer's instructions.

a. Vortex the PBMC suspension at 2000 to 2200 rpm for 5 s to create a homogenous mixture, then transfer 60 µL of the cell suspension into an appropriate tube, as specified by the manufacturer.
b. Set up the Sysmex XN-350 for measurement.
    i. On the main menu, click "Mode" and select "Whole blood" (Figure 5A).
    Note: "Whole Blood" mode is required, and 25 µL cell suspension is used in this mode.
    Critical: Ensure that the aspiration mode is deactivated.
    ii. Select "Manual" and input the sample ID information.
c. Vortex the sample at 2000 to 2200 rpm for 5 s before measurement. Insert the aspiration needle all the way to the bottom of the sample tube, and then press the start switch (Figure 5B).
    Note: When measuring isolated PBMCs, a data error message will appear since the cell count falls outside the standard range. This is expected as isolated PBMCs do not represent whole blood samples (Figure 5C).
d. Measure each sample in duplicate. Use the average for subsequent steps.
e. Analyze the PBMC count:
    i. In the Main screen appears the number of white blood cells (WBC), platelets, as well as the number of lymphocytes, monocytes, neutrophils, eosinophils and basophiles and corresponding percentages (Figure 6A).
    ii. In the Graph screen, the scattergram can be analyzed to evaluate the cell counts (Figure 6B).
    Note: Cell size may be affected, which could lead to incomplete cell counting **(Troubleshooting 5).** If all cells are counted, the PBMC count consists of lymphocytes and monocytes.
    iii. To analyze the total number of white cells, click on "Service" and then on "Switch" (Figure 6C). The total white blood cell differential (WDF) number is now shown in the table and should be considered for the counting analysis. The WDF analysis detects the white blood cells without differentiating between cell populations.

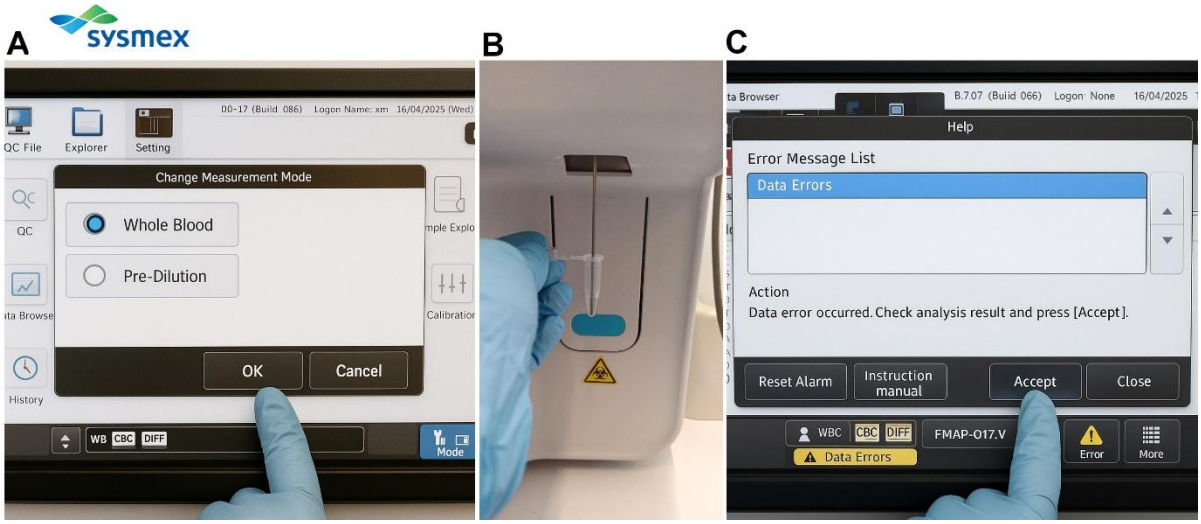

Figure 5: PBMC counting with the Sysmex XN-350. A. Measurement mode screen to select whole blood mode. **B.** Aspiration of the sample by the Sysmex XN-350. **C.** Data error message on the main screen - accept**.**

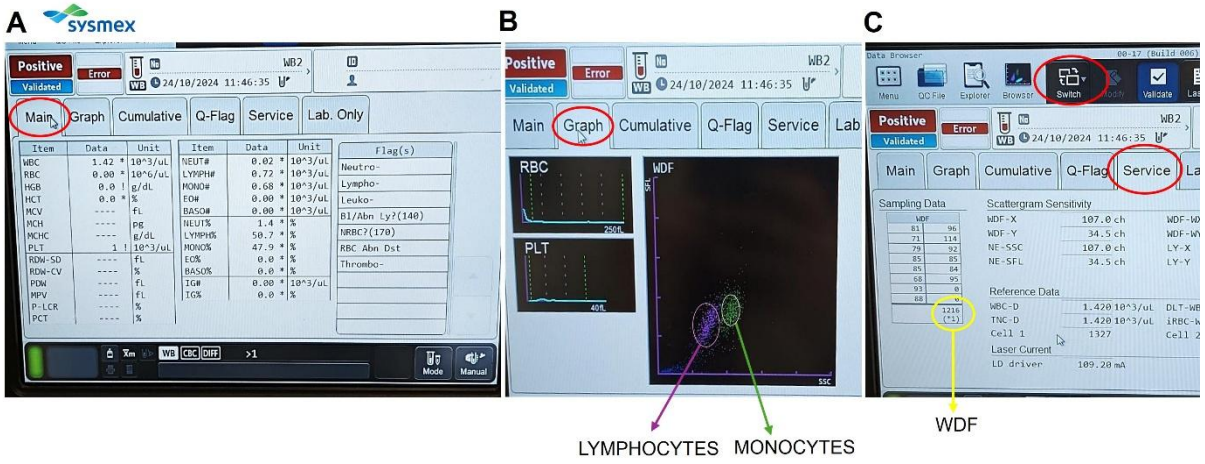

Figure 6: PBMC count with the Sysmex XN-350. A. Main data screen of the Sysmex XN-350 displaying the count of white blood cells (WBC), platelets (PLT), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), and basophils (BASO) and cell population percentages (%). **B.** Representative scattergram illustrating the separation of different WBC populations. Monocytes are shown in green and lymphocytes in purple. **C.** Service screen showing the total white blood cell differential (WDF).

4. Evaluation of PBMC viability and cell count with the LUNA-FL counter

The LUNA-FL counter is used to determine both the number and viability of cells. While other stains can be used, Acridine Orange/Propidium Iodide (AO/PI) is most reliable for assessing PBMC viability [27].

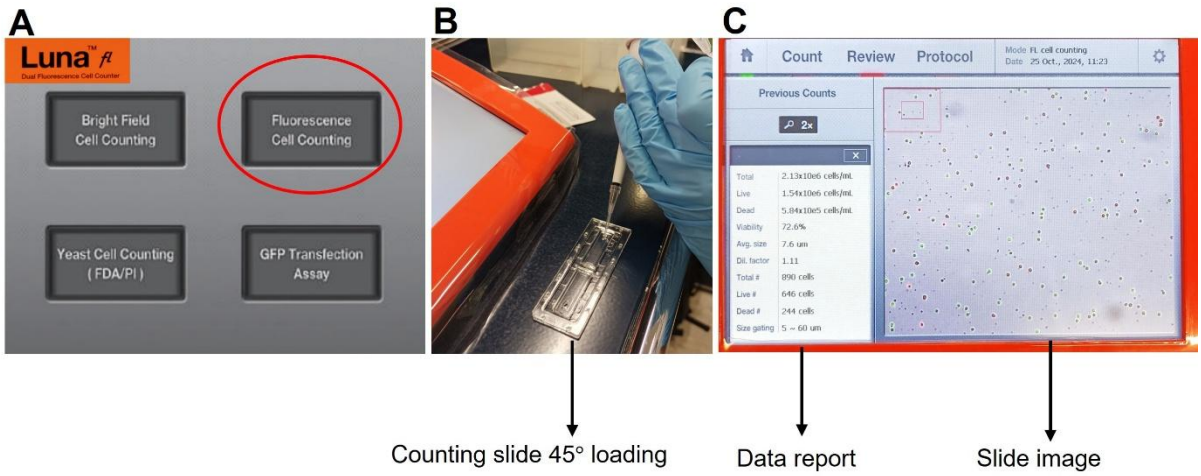

Figure 7: PBMC count and viability with the LUNA-FL counter. A. Main screen for measurement mode selection. **B.** Loading the counter slide with the PBMC suspension and dyes AO/PI**. C**. Representative data report and slide image.

Note: While PBMCs can be counted with the LUNA-FL counter and cell viability can be assessed, this method does not identify specific cell populations and platelet contamination cannot be determined.

a. Set the LUNA-FL counter into the Fluorescence Cell Counting mode (Figure 7A), with the following specifications for PBMCs:
   Cell concentration range: $5 \cdot 10^4$ to $1 \cdot 10^7$ x/mL
   Cell size range: 5 to 60 µM
   Light exposure: Green 3, Red 5
   Dilution factor for the AO/PI addition: 1.11
   Note: light exposure might be adjusted if there is very low or very high intensity due to reasons such as AO/PI lot, expiration date, storage time, etc.

b. Vortex the PBMC suspension at 2000 to 2200 rpm for 5 s and pipette 18 µL into a black microcentrifuge tube.

c. Add 2 µL of the AO/PI solution and mix by pipetting up and down 3 times with a 20-µL micropipette.
   Note: Both AO and PI are photosensitive. To prevent degradation or photobleaching of these dyes, prepare and store as 20 µL aliquots under dark or low light conditions.
   Critical: AO and PI fluorescence decreases over time, so promptly handle and count the cells to minimize fluorescence-related errors (**Troubleshooting 5**).

d. Vortex the microtube tube at 2000 to 2200 rpm for 5 s and load 10 µL of the mixture into the chamber of a LUNA™ Cell Counting Slide (Figure 7B).
   Critical: Be careful not to under- or over-load the chamber and avoid bubble formation (**Troubleshooting 5**).

e. Once the sample is loaded, proceed with the measurement following the manufacturer's instructions (Figure 7C).

High-resolution respirometry experiment

High-resolution respirometry (HRR) is a technique for accurate measurement of oxygen concentration and flux using the Oroboros Bioenergetics Platform. This technique allows detailed respirometric analysis of various biological samples, including isolated mitochondria, permeabilized cells and tissues, and living cells. Precision OXPHOS analysis by HRR provides in-depth insights into mitochondrial function and cellular bioenergetics under physiological and pathological conditions [28].

CRITICAL: Before preparing the PBMCs for HRR, ensure that the Oroboros is calibrated and the instrumental background correction is performed to guarantee accurate resolution of O_2 flux [29,30].

Timing: 30 to 150 min

Note: Experimental timing depends on the SUIT protocol selected for HRR.

1. **Addition of PBMCs to the Oroboros chamber:**

   a. Start the DatLab software on the PC connected to the Oroboros and select the SUIT protocol for respirometric analysis. Press “Start measurement”.
   b. Remove any remaining medium from the Oroboros chambers for complete volume replacement and stop the stirrers [30].
   c. Vortex the PBMC suspension at 2000 to 2200 rpm for 5 s and promptly add an excess volume (0.6 mL) of PBMC suspension to the 0.5-mL chamber (**Troubleshooting 10**).
   Note: The Oroboros chamber can be calibrated at experimental volumes of 2.0 or 0.5 mL using the classic-volume chamber or small-volume chamber, respectively. PBMCs present low respiratory fluxes compared to other cell lines and types, and as such, an experimental volume of 0.5 mL is advantageous to reduce the amount of sample needed [30].
   Note: The PBMC sample can be added either by partial or full replacement of the aqueous medium in the chamber[30]. For partial volume replacement, remove the same volume of liquid from the chamber as the volume of PBMC suspension to be added, using the same micropipette (**Troubleshooting 10**). For full volume replacement, resuspend the PBMC solution with the necessary medium to achieve the desired cell concentration in the chamber. Remove the medium completely from the Oroboros chamber, then add the PBMC suspension to the chamber.

d. Switch on the stirrers and insert the stoppers into the chamber without fully closing them. Allow the cell suspension to equilibrate for 2 min.
Note: In case of bubble(s) sticking to the stirrer or stopper, stop the stirrer intermittently to free the bubbles.

2. **SUIT protocol experiment:**

a. Slowly close the Oroboros chamber to avoid bubbles.
b. Add experimental information including cell concentration, into the "Experiment" window in the DatLab software under the "Sample and medium" section. In this way, the measured O_2 flux (per volume) can be expressed accurately per unit of biological sample as specific flow or O_2 flow per cell [amol·s^{1}·x^{-1}].
Note: Sample information can be edited after the experiment, during data analysis.
c. In case of performing standard SUIT protocols, instructions are provided by the manufacturer in the DatLab software.
d. After completing the SUIT protocol, clean the Oroboros chamber thoroughly according to the manufacturer's instructions.

Note: The sample can be recovered from the chamber and used for other analyses (e.g., citrate synthase activity, protein, telomere length, genomics) after the HRR measurement.

Expected outcomes

Successful cell isolation and cryopreservation should yield more than 2 million PBMCs per mL of whole blood. The PBMC population should constitute >85 % lymphocytes, with a viability >85 % and low platelet contamination (PLT/PBMC count ratio <3), both before and after cryopreservation and cell thawing (Figure 8). Fresh and cryopreserved PBMCs should exhibit comparable HRR profiles with routine respiration >5 pmol·s^{-1}·mL^{-1}, and ET capacity reduction <10 % after 2 weeks of cryopreservation (Figure 9). Respiratory quality control, assessed by the respirometric cell viability index, should correlate with the optical cell viability quantification method (Figure 8B). The cytochrome *c* control efficiency is expected to remain similar between fresh and cryopreserved cells, indicating that the freezing procedure preserves the integrity of the mitochondrial outer membrane (Figure 10A). In PBMCs, cytochrome *c* control efficiency exceeding 10 % is not associated with changes in electron transfer (ET) capacity, either before or after permeabilization (Figure 10B–C). The lack of correlation suggests that even elevated cytochrome *c* control efficiencies do not impair mitochondrial respiratory capacity, consistent with some observations reported in other cell types or tissues [31,32].

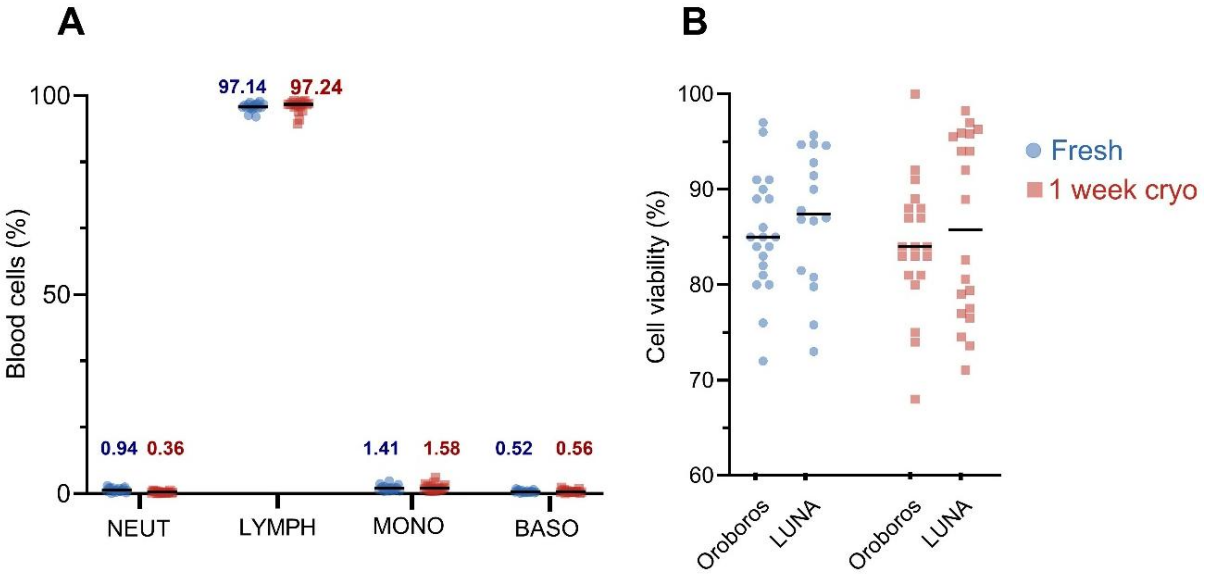

Figure 8: Percentage of different cell types and cell viability in freshly isolated and cryopreserved PBMC samples from the LUNA-FL counter and Oroboros. A. Percentage of lymphocytes, monocytes, neutrophils and basophils from the same PBMC samples, either freshly isolated or after one week of cryopreservation. **B.** Cell viability determined by the LUNA-FL counter and calculated from high-resolution respirometry results obtained with the Oroboros, in freshly isolated and cryopreserved samples. *N* = 20. Two-way ANOVA with Šídák's multiple comparisons test was used to determine *p*-values (shown when $p \leq 0.05$). Data (DOI:10.5281/zenodo.15780720).

Limitations

This protocol is optimized for human blood samples and has been successfully utilized in several studies. For its use with other organisms (especially non-mammalian), evaluation and additional optimization are required.

Specific characteristics of the blood samples may lead to some limitations. Donors' lifestyle or use of medication can affect mitochondrial function of PBMCs. Environmental conditions (e.g., exposure to toxins, different seasons or diurnal factors) and the donor's status before the blood draw can lead to variable results within the same individual [33,34]. Additionally, blood from donors with a lower white cell count (leukopenia) may result in a lower number of isolated PBMCs. The blood volume used in the isolation procedure should be adjusted accordingly.

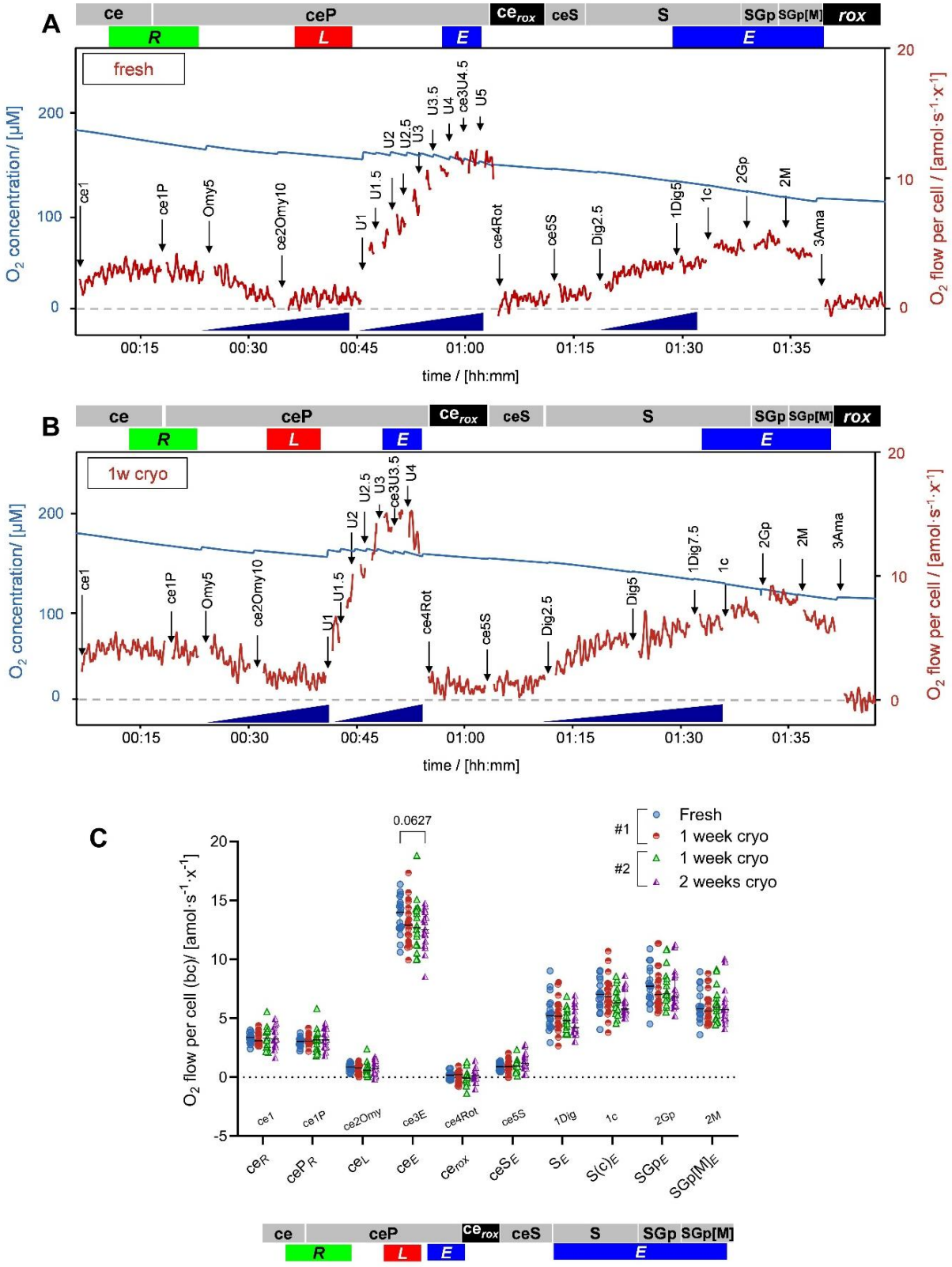

Figure 9: Mitochondrial function in freshly isolated and cryopreserved PBMCs assessed by high-resolution respirometry. Freshly isolated and 1-week cryopreserved PBMCs were analyzed using the Oroboros 0.5 mL chamber (*N* = 20) with the protocol SUIT-003 O2 ce-pce D115. **A & B.** Representative traces of O_2 flow

per cell [amol·s^{-1}·x^{-1}], corrected for instrumental background O_2 flux, in freshly isolated (**A**) or 1-week cryopreserved (**B**) PBMCs. Titrations are indicated by arrows; titration spikes were eliminated. O_2 concentration was maintained above 100 µM throughout the experiment. The sequence of respiratory states, characterized by titrations and corresponding rates: **ce1**, routine respiration *R* of living cells. **ce1P**, pyruvate 5 mM as external substrate. **ce2Omy10**, Omy titrations in 5 nM steps; leak respiration *L*. **ce3U4.5** (panel A) and **ce3U3.5** (panel B), uncoupler titrations to optimum CCCP concentration of 4.5 µM and 3.5 µM, respectively; ET capacity *E*. **ce4Rot**, rotenone 0.5 µM inhibiting Complex I; residual oxygen consumption *rox*. **ce5S**, succinate 10 mM; stimulating respiration in plasma membrane-permeable cells. **1Dig5** (panel A) and **1Dig7.5** (panel B), digitonin titrations in 5 mg·mL^{-1} steps to optimum 5 or 7.5 mg·mL^{-1} concentration, respectively, for complete plasma membrane permeabilization; succinate-pathway ET capacity S_E. **1c**, cytochrome *c* 10 µM; test of mitochondrial outer membrane integrity. **2Gp**, glycerophosphate 10 mM; SGp_E. **2M**, malate 2 mM; SGp[M]$_E$. **3Ama**, antimycin A 2.5 µM inhibiting Complex III; *rox*. **C.** O_2 flow per cell [10^6 x·mL^{-1}] of (#1) freshly isolated and one week cryopreserved cells, or (#2) one- and two-weeks cryopreserved cells. PBMCs from the same donors were collected and isolated on different dates in #1 and #2 (*N* = 20 per date). Individual data points with medians of biological replicates shown as horizontal lines. Two-way ANOVA with Šídák's multiple comparisons test was used to determine *p*-values (shown when $p \leq 0.1$). Data (DOI:10.5281/zenodo.15780720): 2025-02-06-J-010B_03, 2025-02-13-J-010B_03.

Mitochondrial function in PBMCs may reflect systemic mitochondrial functional fitness. This is suggested in studies of aging, fatigue, Covid-19, cognitive performance and related pathologies[2,5,35–42], hypoxia [43], ovarian cancer [44], or exercise and cardiovascular fitness [33,45–50], although mitochondrial performance differs in PBMCs, platelets, and different organs [28,50–53]. In other cases, however, respiratory parameters of the peripheral PBMCs do not reflect cardiovascular fitness, metabolic states of central organs, or pathological indications [6,40,51,52,54–56].

Further research is required to evaluate if this represents a feature of cell-specific expression of mitochondrial traits, if variations of PBMC isolation and cryopreservation protocols may explain some controversial results [57], if diurnal and gender differences play a role [35,53,58,59], or if the fundamental cellular coupling control protocol (mitochondrial stress test) lacks resolution of mitochondrial fitness. Extended respirometric protocols with permeabilized cells reveal additional – and possibly decisive – details on mitochondrial energy metabolism in precision OXPHOS analysis [28].

PBMCs are a heterogeneous population of cells, each with distinct bioenergetic profiles [60]. Changes in the percentage of these cell types – due to technical issues during the isolation process or to intrinsic differences in the donors' blood – may result in variability between donors [53]. Acute exercise and cardiorespiratory fitness are well

correlated with age-associated changes and respiratory performance of CD4⁺ cells [61,62]. This must be considered when interpreting the data. The use of only one cell type should be considered in cases where the PBMC population varies significantly between donors, or within the same donor under different conditions or time points.

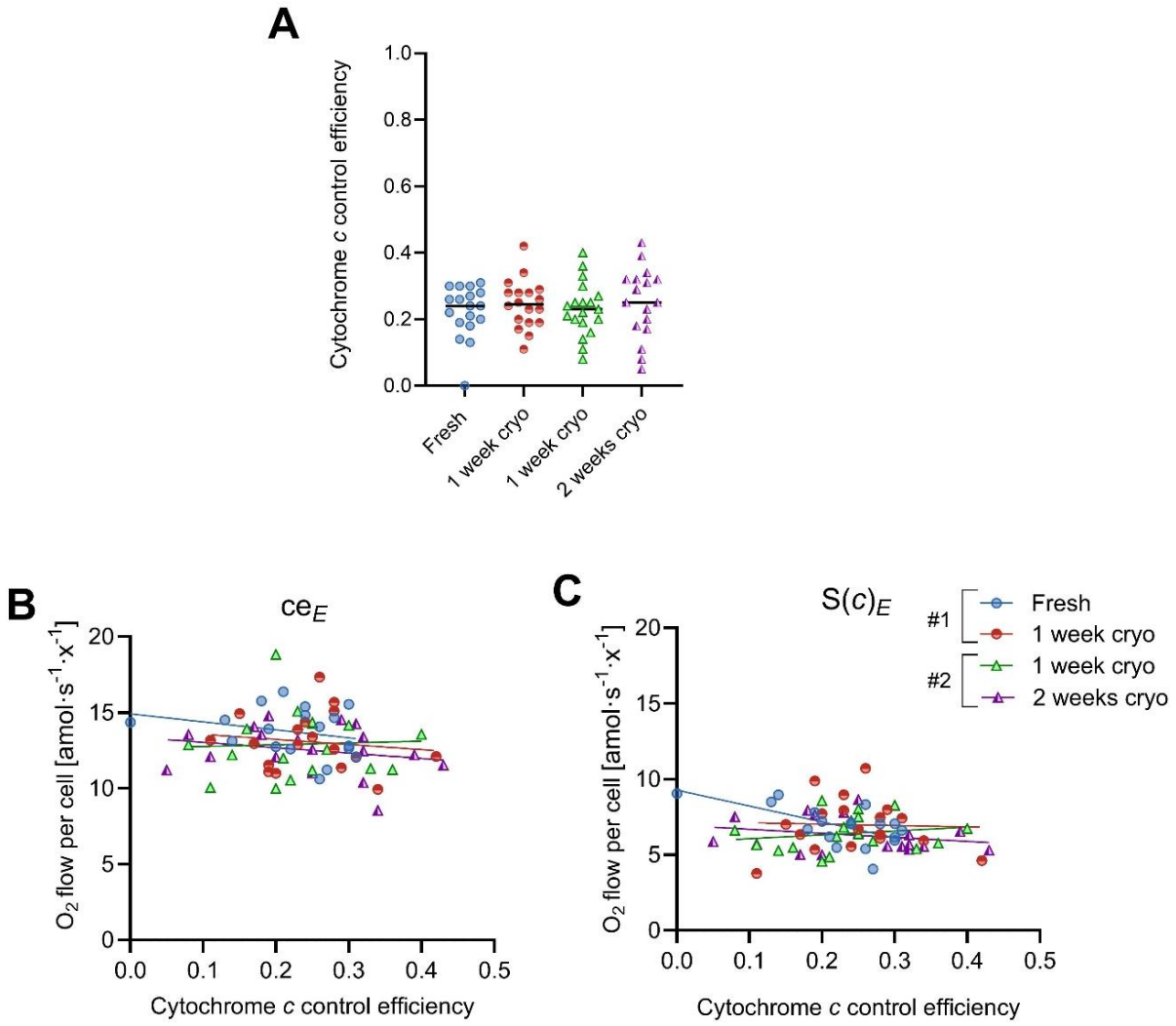

Figure 10: Cytochrome *c* control efficiency in fresh and cryopreserved PBMCs and correlation with electron transfer capacity. Cytochrome *c* control efficiency is calculated, in a given coupling and pathway state (CHNO), as the increase of respiratory capacity after addition of cytochrome *c* normalized by *c*-stimulated respiration ($J_{CHNO(c)} - J_{CHNO}$)/$J_{CHNO(c)}$ [27,30]. **A.** Cytochrome *c* control efficiency in fresh, 1 and 2 weeks cryopreserved PBMCs. Biological replicates are shown as individual points, with the medians as lines. Two-way ANOVA with Šídák's multiple comparisons test was used to determine *p*-values (shown when $p \leq 0.1$). **B-C.** Correlation between cytochrome *c* control efficiency and ET capacity (O_2 flux per cell [amol·s^{-1}·x^{-1}]). **(B)** ET capacity in living cells (ce_E) and **(C)** S-pathway ET capacity ($S(c)_E$) in permeabilized cells. PBMCs from the same donors were collected and isolated on different dates in #1 and #2 (N = 20 per date). Individual data points and linear regression fits shown as lines for each group.

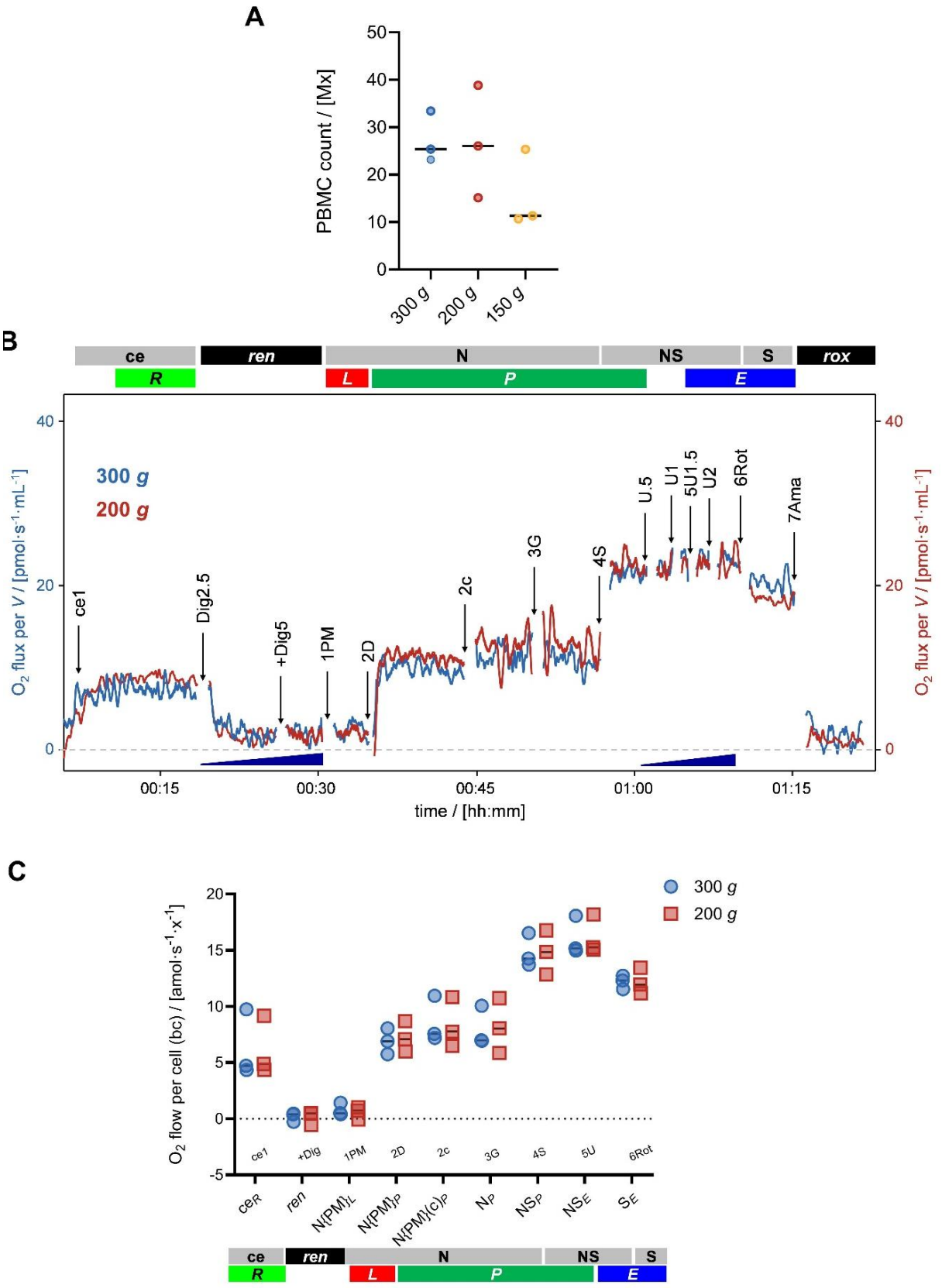

Figure 11: Effect of centrifugation speed during PBMC isolation on mitochondrial function. A. Total PBMC count (Mx = 10^6 x) obtained from 9 mL blood with centrifugation speeds 150, 200 and 300 *g* (*N* = 3 for each condition). **B-C.**

Respiration of PBMCs in the 0.5-mL Oroboros chamber using DatLab protocol SUIT-008 O2 ce-pce D025 (N = 3, n = 3) with PBMCs isolated from the same blood sample either at 300 (blue) or 200 *g* (red). **B**. Representative traces of O_2 flux per volume V [pmol·s^{-1}·mL^{-1}], corrected for instrumental background O_2 flux. Titrations are indicated by arrows. Titration spikes were eliminated. O_2 concentration was kept above 100 µM. Sequence of respiratory states in protocol SUIT-008, characterized by titrations and corresponding rates: **ce1**, routine respiration R of living cells. **+Dig5**, digitonin titrations until complete plasma membrane permeabilization at 5 mg·mL^{-1}, residual endogenous respiration *ren*. **1PM**, pyruvate 5 mM & malate 2 mM; N-pathway leak respiration N{PM}$_L$. **2D**, ADP 1 mM; N-pathway OXPHOS capacity N{PM}$_P$. **2c**, cytochrome *c* 10 µM; test of mitochondrial outer membrane integrity. **3G**, glutamate 10 mM; N$_P$, where N indicates the substrate combination PGM. **4S**, succinate 10 mM, NS-pathway OXPHOS capacity NS$_P$. **5U1.5**, uncoupler titrations to optimum CCCP concentration 1.5 µM; NS-linked ET capacity NS$_E$. **6Rot**, rotenone 0.5 µM inhibiting Complex I, succinate-pathway ET capacity S$_E$. **7Ama**, antimycin A 2.5 µM inhibiting Complex III; residual oxygen consumption *rox*. **C.** O_2 flow [amol·s^{-1}·x^{-1}] calculated as O_2 flux per V [pmol·s^{-1}·mL^{-1}] divided by total cell count concentration [10^{6}x·mL^{-1}]. Biological replicates are shown as individual points and medians as lines. No significant differences between both groups were found by two-way ANOVA with Šídák's multiple comparisons test in any of the respiratory states (p≥ 0.05). Data (DOI:10.5281/zenodo.15780720): 2024-09-23_X-0003_03.

Troubleshooting

Problem 1:

Lack of access to Leucosep™ tubes.

Potential solution:

Use of other tubes is possible. While Prefilled Leucosep™ tubes (Greiner Bio-One; 227288) are available and can reduce preparation times, they still require an initial centrifugation step to position the density gradient below the porous barrier disc. Alternatively, standard 50-mL polypropylene centrifuge tubes can be used with Ficoll-Paque™ PLUS, but this change requires extra care when layering blood above the density gradient medium, as there is no porous barrier disc to separate them. Alternative lymphocyte separation techniques can be utilized, but cell yield may differ from that obtained with the procedure described in this protocol [23].

Problem 2:

Low PBMC count after isolation (e.g. <0.5 million PBMCs per mL of blood).

Potential solution:

Freshly isolated blood should be used for this protocol. Higher PBMC yields are obtained when the blood is processed within 2 h of blood collection. All required

reagents and isolation processes must be held and conducted at RT. Below RT, the density of the Ficoll-Paque™ PLUS increases, which can lead to poor recovery or increased loss of PBMCs into the Ficoll-Paque layer. Ensure sufficient time to equilibrate the centrifuge and reagents to RT prior to isolation. A lower centrifugation speed (≃150 *g*) in step 2b of the section Isolation of peripheral blood mononuclear cells (PBMCs) leads to lower PBMC yield after isolation (Figure 11A). No differences were found at higher centrifugation speeds (200 vs 300 *g*) (Figure 11B to C). After centrifugation (steps 2c and 2f), promptly remove the supernatant to avoid any cell loss due to a partial resuspension of the cells. Vibration of the rotor or failure to turn the brake off during the initial centrifugation may disrupt the gradient, leading to poor differentiation of cell layers and loss of PBMCs. PBMC yields can differ between donors, especially in patients with blood disorders. Increasing the volume of blood collected should be considered in specific cases.

Problem 3:

Low viability of the PBMC suspension.

Potential solution:

Substantial delays (>4 h) in PBMC isolation can reduce cell viability (Figures 12, S1 and S2). Ideally, PBMC isolation from whole blood should be performed promptly after blood collection, and it is recommended to process the blood within 2 h or maximally 4 h [23,58]. Similarly, both the storage temperature of whole blood prior to processing and the temperature maintained during PBMC isolation can adversely affect cell viability. Temperatures between 20 to 37 °C have been shown to be optimal for preserving PBMC viability during these preanalytical steps [16,17].

Problem 4:

High red blood cell and/or platelet contamination.

Potential solution:

The use of Leucosep™ tubes with a porous barrier helps to reduce contamination from red blood cells and platelets. When adding whole blood onto the separation medium, decrease the ejection speed and tilt the tube to avoid mixing of the layers. This pre-mixing is a particular problem if alternative tubes are used that lack the porous barrier. The centrifuge and reagents must be at RT for optimal separation. Below RT, the density of the Ficoll-Paque™ PLUS increases, which can lead to poor PBMC recovery or increased contamination with red blood cells and neutrophils. Ensure sufficient time to equilibrate the temperature of the centrifuge and reagents. Washing the collected PBMC layer with 50 mL PBS decreases platelet contamination. Two washing steps during the preparation (steps 2c and 2f of the section Preparation of PBMCs for HRR experiment) of the PBMCs are essential to limit platelet contamination (Figure 13).

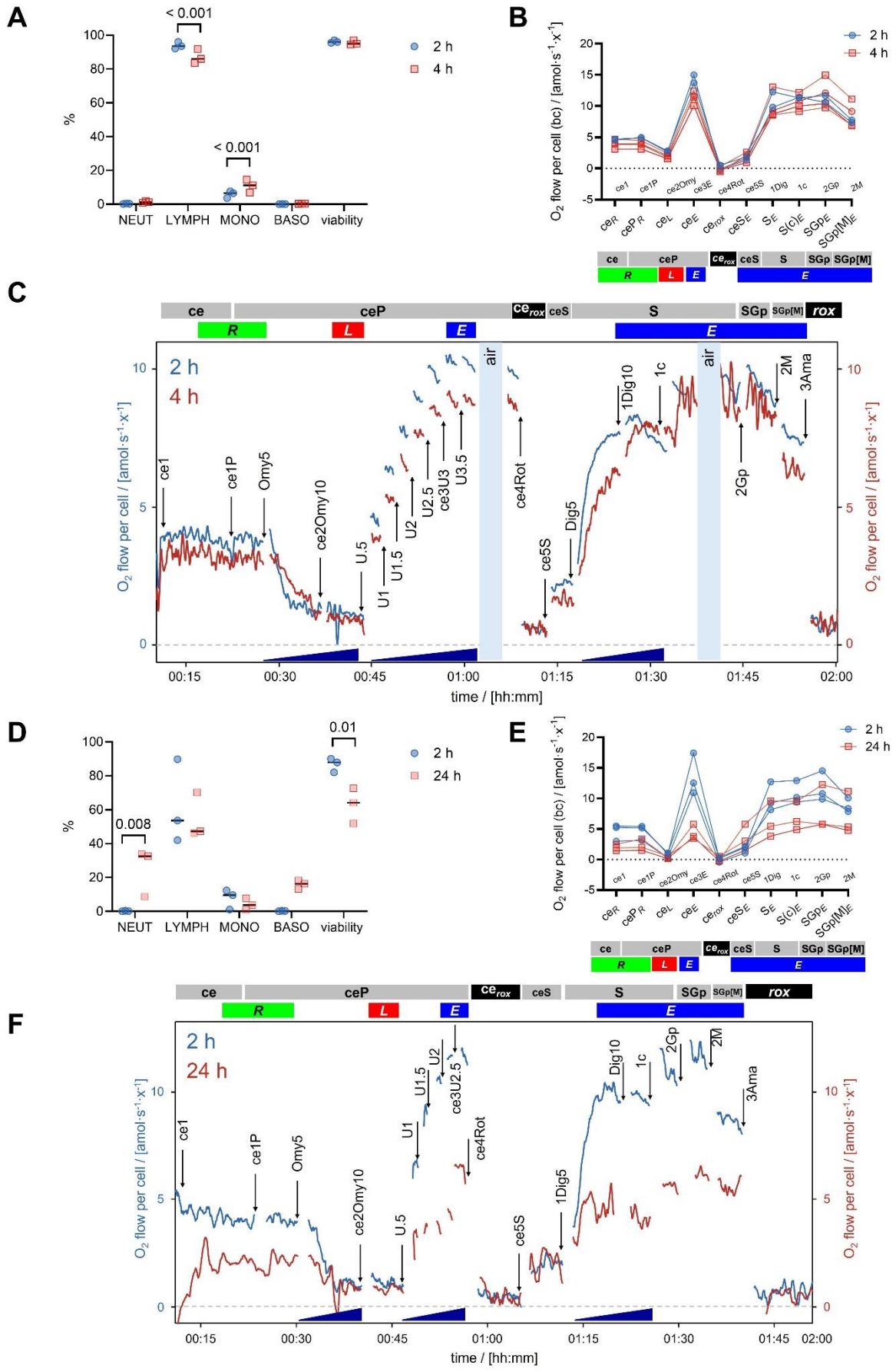

Figure 12: Effect of the duration between blood collection and PBMC isolation.

PBMC isolation was performed either 2 vs 4 h (**A-C**) or 2 vs 24 h (**D-F**) after blood collection of the same blood sample (*N* = 3 for each condition). **A**, **D.** PBMC population (neutrophils NEUT, lymphocytes LYMPH, monocytes MONO, basophils BASO) measured with Sysmex XN-350. Viability was analyzed with the LUNA-FL after PBMC isolation in fresh samples. Biological replicates are shown as individual points, with the medians shown as lines. Two-way ANOVA with Šídák's multiple comparisons test was used to determine *p*-values (shown when $p \leq 0.1$). **B-C** and **E-F.** Respiration of PBMCs (1 week cryopreservation) in the 0.5 mL Oroboros chamber using DatLab protocol SUIT-003 O_2 ce-pce D115. **B, E.** O_2 flow [amol·s^{-1}·x^{-1}], calculated as O_2 flux per *V* [pmol·s^{-1}·mL^{-1}] divided by total PBMC count concentration [10^{6}x·mL^{-1}]. **C, F.** Representative traces of O_2 flow per cell [amol·s^{-1}·x^{-1}], corrected for instrumental background O_2 flux. Titrations are indicated by arrows. Titration spikes were eliminated. O_2 concentration was kept above 100 µM. Sequence of respiratory states, characterized by titrations and corresponding rates: **ce1**, routine respiration *R* of living cells. **ce1P**, pyruvate 5 mM as external substrate. **ce2Omy10**, Omy titrations in 5 nM steps; leak respiration *L*. **ce3U3** (panel C) and **ce3U2** (panel F), uncoupler titrations to optimum CCCP concentration of 3 µM and 2 µM in panel C and F, respectively; ET capacity *E*. **ce4Rot**, rotenone 0.5 µM inhibiting Complex I; residual oxygen consumption *rox*. **ce5S**, succinate 10 mM; stimulating respiration in plasma membrane-permeable cells. **1Dig10** (panel C) and **1Dig5** (panel F), digitonin titrations in 5 mg·mL^{-1} steps to optimum 10 or 5 mg·mL^{-1} concentration in panel C or F, respectively, for complete plasma membrane permeabilization; succinate-pathway ET capacity S_E. **1c**, cytochrome *c* 10 µM; test of mitochondrial outer membrane integrity. **2Gp**, glycerophosphate 10 mM; SGp_E. **2M**, malate 2 mM; $SGp[M]_E$. **3Ama**, antimycin A 2.5 µM inhibiting Complex III; *rox.* Data (DOI:10.5281/zenodo.15780720): 2024-07-08_Q3-006_03 and 2025-01-20_J-009B_03.

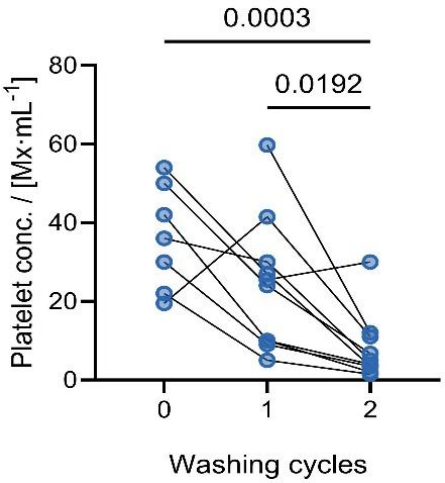

Figure 13: Evaluation of platelet contamination in freshly isolated PBMCs following sequential washing cycles. Platelet concentration (Mx = 10^6 x) was measured using the Sysmex XN-350 in freshly isolated PBMC suspensions after each washing cycle, as described in the protocol. Data are presented as individual points (*N* = 7). Two-way ANOVA with Šídák's multiple comparisons test was used to determine *p*-values (shown when $p \leq 0.05$)

Problem 5:

Low reproducibility between counting methods.

Potential solution:

The LUNA-FL and Sysmex XN-350 automated cell counters provide adequate and reproducible results when compared to each other (Figure 14). However, if discrepancies arise between the two methods, potential sources of error should be considered.

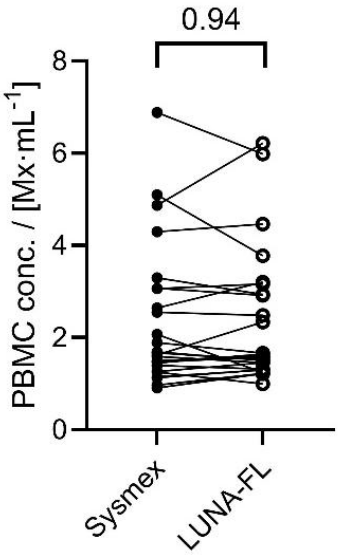

Figure 14: Comparison of automated PBMC counting methods. The same PBMC suspension was evaluated in parallel by two counting methods. PBMC concentration assessed by automated methods: Sysmex XN-350 vs LUNA-FL. *N* = 26. Paired t-test was used to determine *p*-value.

If the error originates from the LUNA-FL, ensure that the manufacturer's instructions are followed carefully. Do not over- or underload the counting chamber and avoid bubble formation. Staining with acridine orange and propidium iodide (AO/PI) is rapid and prolonged exposure to these dyes can lead to high background fluorescence or cytotoxicity which can produce inaccurate results. Keep the incubation time consistent between samples. Use the correct fluorescence intensity – following manufacturer's instructions – to avoid a bleaching phenomenon.

If the error originates from the Sysmex XN-350, check that the calibration and/or quality control values have been applied and use the correct settings, following manufacturer's instructions. Cell size may be affected by the resuspension media [63]. In the current protocol, resuspension of PBMCs in MiR05 is recommended because it ensures more consistent cell size and distribution and allows the instrument to accurately identify different cell populations (Figure 15). The Sysmex XN-350 may report a lower PBMC count due to factors such as the resuspension medium, pre-treatment, or manipulation of the cells [64]. This discrepancy can be detected in the white cell differential (WDF) scattergram generated by the instrument or by comparing WDF value with the value of total WBC count and/or the combined monocyte and lymphocyte counts (Figure 6). In such cases, cell concentration can still be estimated using the WDF channel, which reflects the number of cells in 0.83 µL of suspension. However, this estimation does not allow for differentiation of cell populations.

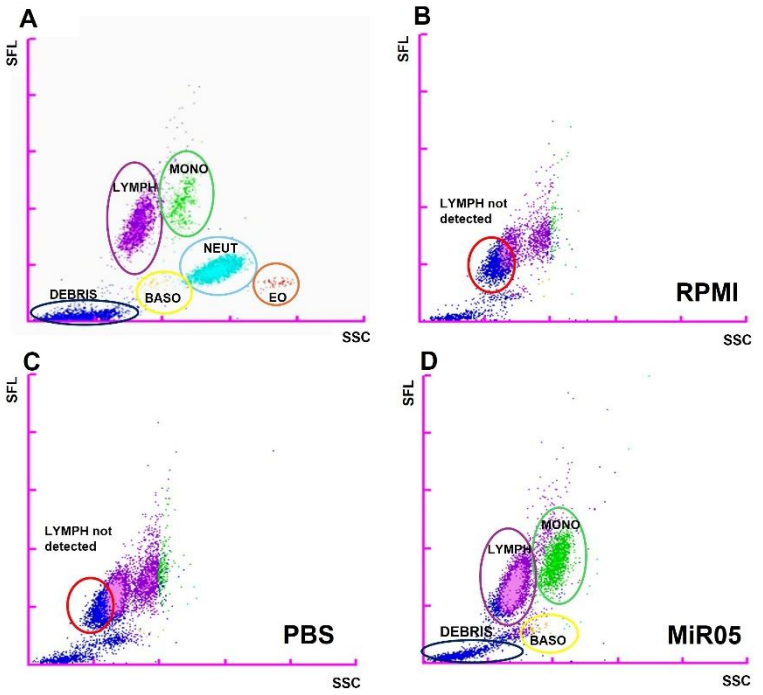

Figure 15: Characterization of PBMC suspension with the Sysmex XN-350. A. Representative example of scattergram reporting the ideal separation of the different white blood cell populations using human whole blood. Monocytes (MONO) are shown in green, lymphocytes (LYMPH) in purple, neutrophils (NEUT) in light blue, basophils (BASO) in yellow, eosinophils (EO) in orange. Debris (DEBRIS) is presented in dark blue. **B-D**. Effect of different media on PBMC separation and counting. **B**. RPMI. **C**. PBS, phosphate-buffered saline. **D**. MiR05, mitochondrial respiration medium.

Problem 6:

Inability to count PBMCs.

Potential solution:

Automated cell counters are recommended but manual counting using a Neubauer chamber or similar is possible. In this case, it is recommended that the same person performs the counting of all samples in the same study to avoid counter-associated variability. The estimated PBMC recovery range is 0.5 to 3 million PBMCs per mL of whole blood. To prepare the aliquots and to calculate the volume of resuspension medium, it is recommended to use an estimated PBMC count between 1 to 1.5 million per mL of whole blood when no counting method is available. Normalization by total protein is also a possibility [31]. Normalization by cell count or total protein must be distinguished from normalization by mitochondrial markers, e.g., citrate synthase activity [31] .

Problem 7:

Counting replicates have >10 % variability.

Potential solution:

This problem might indicate a non-homogeneous suspension during the counting process. PBMC suspensions must be vortexed for 5 s before counting, to avoid settling of PBMCs and ensure the homogeneity of the suspension.

Normalization by cell count is required for quantitative expression of respiratory data. Variability of cell counts introduces errors in cell-specific respiration. Any bias in cell counting may distort results obtained by high-resolution respirometry.

Problem 8:

Low cell recovery and/or viability after cryopreservation.

Potential solution:

Cryopreservation and thawing processes affect cell viability. To maximize cell recovery and viability, freezing should be performed slowly, while thawing should be done rapidly. Ensure the cryopreservation medium is correctly formulated and at RT before use. The use of pre-chilled freezing medium impairs the viability and functionality of the PBMCs, whereas using freezing medium used at initial RT results in optimal viability [19]. Work promptly, avoiding PBMCs lingering in the cryopreservation medium at RT during freezing or thawing. Prolonged exposure of PBMCs to DMSO after thawing can result in reduced cell recovery and viability [16,20,23]. Use Mr. Frosty™ cryopreservation container to freeze cells at a controlled rate of 1 °C per min and fill it with the required amount of isopropyl alcohol for optimal cryopreservation.

During the thawing and cell preparation process, partially thaw the cells in an incubator at 37 °C and promptly add pre-warmed PBS drop-by-drop (step 2a of Preparation of PBMCs for HRR experiment), to avoid osmotic shock. All solutions must be pre-warmed to 37 °C for the thawing and preparation process to ensure preservation of cell viability.

Problem 9:

Low respiratory fluxes.

Potential solution:

Mitochondrial respiratory function can be compromised when PBMC isolations are not performed promptly (<2 h of blood collection; Figures 12, S1 and S2). Decrease in mitochondrial respiratory function is not only due to a lower cell viability (Figure 16). During thawing, pre-warmed solutions at 37 °C are essential to maintain mitochondrial functionality. Pre-chilled solutions hamper mitochondrial functionality and should be avoided.

Problem 10:

Different rates of routine respiration in technical replicates.

Potential solution:

This problem may arise from the incorrect addition of PBMCs to the Oroboros chamber. PBMC suspensions must be vortexed for 5 s before pipetting cells into the chamber, to avoid PBMC settling and to ensure homogeneity of the suspension. It is recommended that the chamber stirring is off during the addition of PBMCs, to avoid

formation of bubbles or foam. If performing a partial media replacement for PBMC addition, ensure that the same micropipette tip is used for media removal and addition of PBMCs. Full media replacement is recommended to reduce errors in technical replicates.

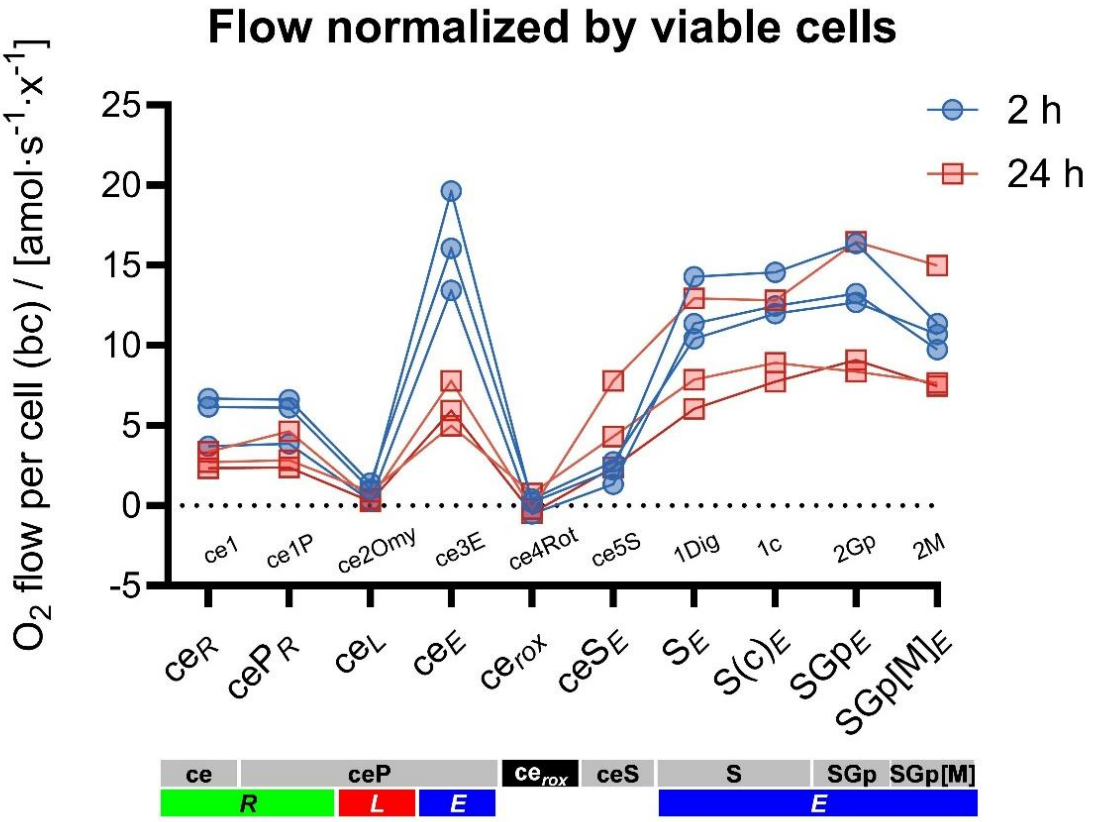

Figure 16: Differences in mitochondrial respiratory function of PBMCs after 24 h delay of isolation are not explained by cell viability differences. O_2 flow per cell [amol·s^{-1}·x^{-1}] from Figure 12E, with PBMCs isolated either at 2 or 24 h after blood collection (*N* =3 for each condition). Differences in mitochondrial respiratory rates are found after 24 h, either normalizing by total (Figure 12E) or viable PBMC count. Data (DOI:10.5281/zenodo.15780720): same as Figure 11E.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be provided by the lead contact, Alejandra Romero (alejandra.romero-martinez@vascage.at).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Jaime Willis (jaime.willis@oroboros.at)

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate code. Original data for figures in the publication is available Open Access at Zenodo repository: 10.5281/zenodo.15780720

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Author contributions

Conceptualization: A.R., J.R.W., E.G., A.T.G. Methodology: A.R., J.R.W., M.K., T.L., E.G., A.T.G., A.P. Investigation: A.R., J.R.W., T.L., A.T.G. Formal analysis: A.R., J.R.W., A.T.G. Visualization: A.R., J.R.W., A.T.G. Writing – original draft: A.R., J.R.W., A.T.G. Writing – review & editing: A.R., J.R.W., M.K., A.T.G., E.G.

Declaration of interests

J.R.W. is an employee of Oroboros Instruments. A.T.G was employed by Oroboros Instruments during the project. E.G. is the founder and CEO of Oroboros Instruments.

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Supplementary material

Figure 8 files: 2025-01-21_H0366_03, 2025-01-21_J-009B_03, 2025-01-23_J010B_03, 2025-01-23_X-0005_03, 2025-01-28_H-0366_03, 2025-01-28_J-009B_03, 2025-01-29_J-010B_03, 2025-01-29_J-009B_03, 2025-01-30_H-0366_03, 2025-01-30_J-009B_03, 2025-02-04_H-0366_03 , 2025-02-04_X-0003_03, 2025-02-05_H-0366_03, 2025-02-05_J-009B_03, 2025-02-06-J-010B_03, 2025-02-11-X-0003_03, 2025-02-11-J-011B_03, 2025-02-13-J-010B_03, 2025-02-18-J-010B_03, 2025-02-18-J-009B_03, 2025-02-25-J-009B_04, 2025-02-25-H-0366_03.

Figure 9 files: 2025-01-21-H0366, 2025-01-21-J-009B, 2025-01-23-J010B, 2025-01-23-X-0005, 2025-01-29-J-010B, 2025-01-29-J-009B, 2025-02-04-H-0366, 2025-02-04-X-0003, 2025-02-06-J-010B, 2025-02-18-J-010B, 2025-02-18-J-009B, 2025-01-28-H-0366, 2025-01-28-J-009B, 2025-01-30-H-0366, 2025-01-30-J-009B, 2025-02-05-H-0366, 2025-02-05-J-009B, 2025-02-11-X-0003, 2025-02-11-J-011B, 2025-02-13-J-010B, 2025-02-25-J-009B, 2025-02-25-H-0366, 2025-04-29_X-0005, 2025-04-29_X-0003, 2025-04-29_Q3-002, 2025-05-05_J-009B, 2025-05-05_Q3-002(A), 2025-05-06_X-0003, 2025-05-06_X-0005, 2025-05-06_Q-0003, 2025-05-07_J-009B, 2025-05-07_Q-0009, 2025-05-12_J-009B, 2025-05-12_J-010B, 2025-05-14_J-009B, 2025-05-14_Q-0009, 2025-05-15_J-001A, 2025-05-15_J-010B, 2025-05-21_Q-0003, 2025-05-22_Q-0003, 2025-05-22_J-011B, 2025-05-22_Q-0009, 2025-05-22_J-001A, 2025-05-27_Q3-002, 2025-05-27_Q-0009)

Figure 11 files: 2024-09-23_Q3-006_03, 2024-09-23_X-0003_03, 2024-09-23_XB-001_03, 2024-09-25_Q3-006_03, 2024-09-25_X-0003_03, 2024-09-25_XB-001_03, 2024-10-24_Q3-006_03, 2024-10-24_X-0003_03, 2024-10-24_XB-001_03.

Figure 12 files: 2024-07-08_H-0366_03, 2024-07-08_Q3-006_03, 2024-07-09_H-0366_03, 2025-01-20_J-009B_03, 2025-01-20_J-010B_03, 2025-02-26_H-0366_03.

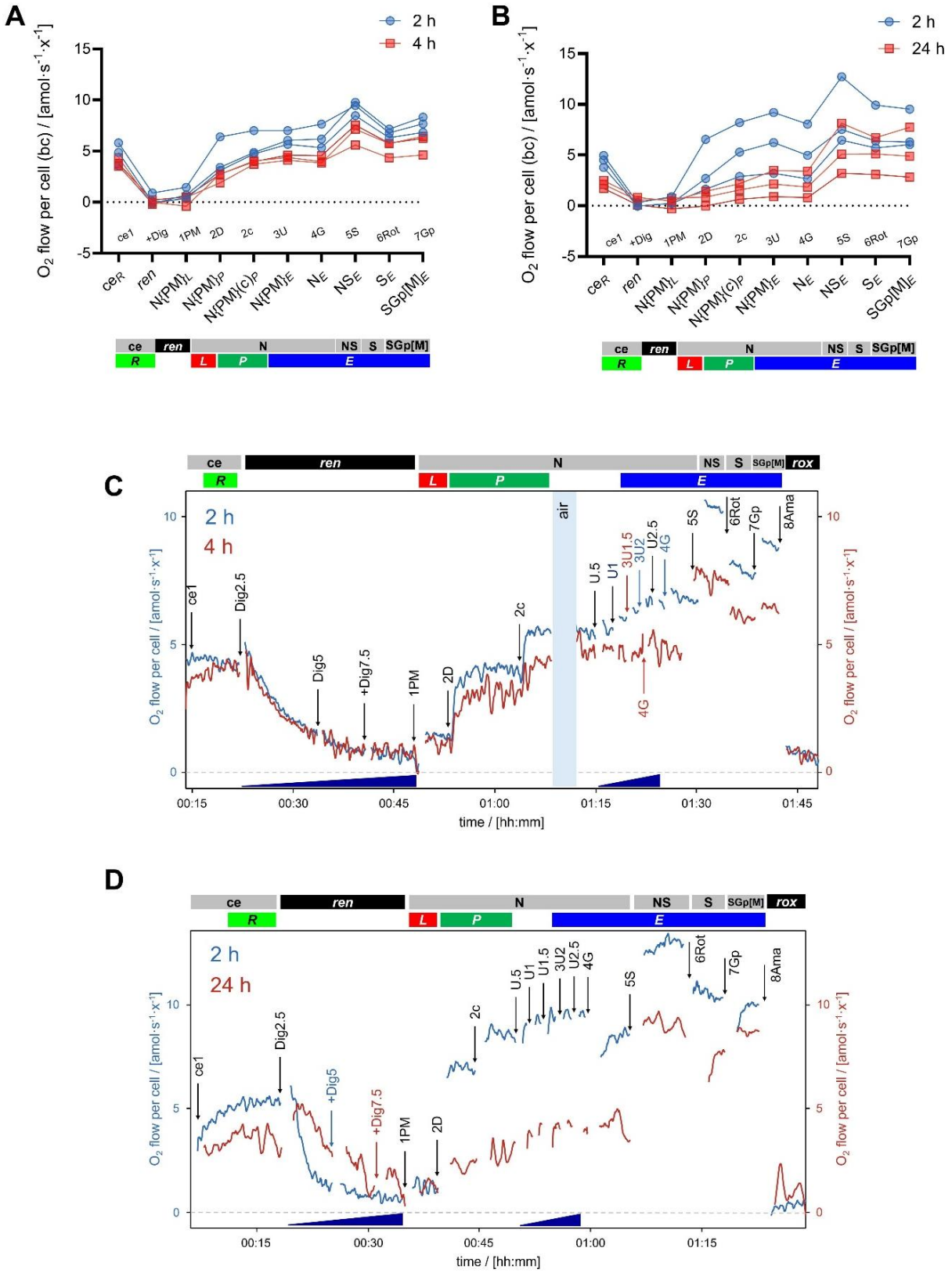

Figure S1: Effect of the delay of PBMC isolation following whole blood collection on respiration using reference protocol 1, RP1. PBMC isolation was performed either 2 or 4 h (**A, C**) or 2 or 24 h (**B, D**) after blood collection of the same blood sample (*N* = 3 for each condition).

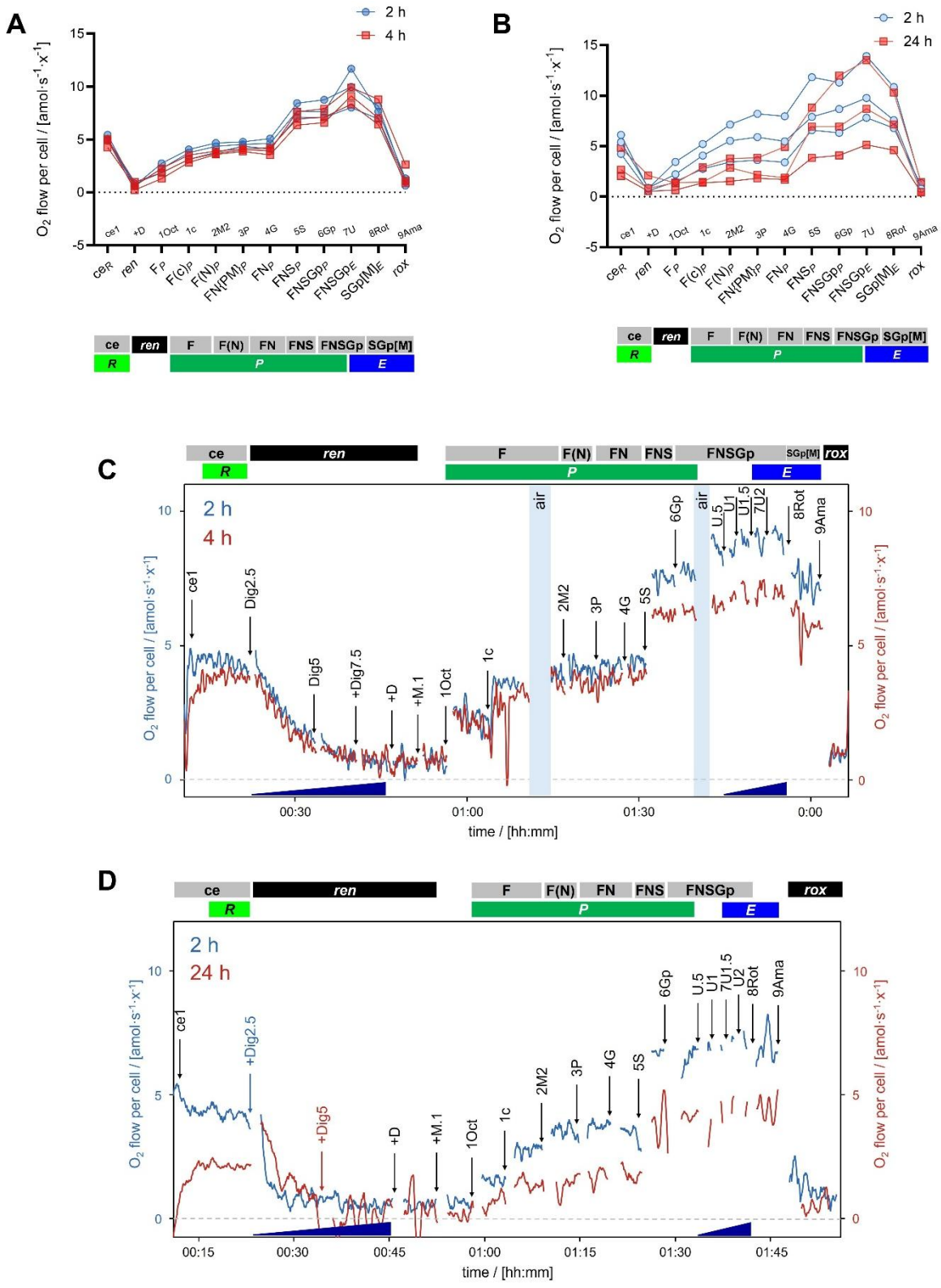

Figure S2: Effect of the delay of PBMC isolation following whole blood collection on respiration using reference protocol 2, RP2. PBMC isolation was performed either 2 or 4 h (**A, C**) or 2 or 24 h (**B, D**) after blood collection in the same blood sample (*N* = 3, for each of condition).

Figure S1: Respiration of PBMCs in the 0.5-mL Oroboros chamber using DatLab protocol SUIT-001 O2 ce-pce D004. **A-B.** O_2 flow per cell [amol·s^{-1}·x^{-1}]. **C-D.** Representative traces of O_2 flow per cell [amol·s^{-1}·x^{-1}], corrected for instrumental background O_2 flux. Titrations are indicated by arrows. Titration spikes were eliminated. Oxygen concentration was kept above 100 µM. Sequence of respiratory states, characterized by titrations and corresponding rates: **ce1**, routine respiration *R*. **+Dig7.5**, digitonin 7.5 mg·mL^{-1}, permeabilization of plasma membrane, residual endogenous respiration *ren*. **1PM**, pyruvate 5 mM & malate 2 mM; N-pathway leak respiration N{PM}$_L$. **2D**, ADP 1 mM; N-pathway OXPHOS capacity N{PM}$_P$. **2c**, cytochrome *c* 10 µM. **3U2**, uncoupler titrations to optimum CCCP concentration 2 µM, NADH-linked ET capacity N{PM}$_E$. **4G**, glutamate 10 mM; N$_E$, where N indicates the substrate combination PGM. **5S**, succinate 10 mM; NS-pathway ET capacity NS$_E$. 6Rot, rotenone 0.5 µM; S$_E$. 7Gp, glycerophosphate 10 mM; SGp*E*. **8Ama**, antimycin A 2.5 µM; *rox*. Data (DOI:10.5281/zenodo.15780720): 2024-07-08_J-010B_03 and 2025-01-20_X-0003_03.
Files: 2024-06-19_X-0003_03, 2024-07-08_J-010B_03, 2024-07-08_X-0003_03, 2025-01-20_H-0366_03, 2025-01-20_X-0003_03, 2025-02-26_J-010B_03.

Figure S2: Respiration of PBMCs in the 0.5-mL Oroboros chamber using DatLab protocol SUIT-002 O2 ce-pce D007a. **A-B.** O_2 flow per cell [amol·s^{-1}·x^{-1}] not baseline corrected (*rox* was higher than *ren* in some cases). **C-D.** Representative traces of O_2 flow per cell [amol·s^{-1}·x^{-1}], corrected for instrumental background O_2 flux. Titrations are indicated by arrows. Titration spikes were eliminated. Oxygen concentration was kept above 100 µM. Sequence of respiratory states, characterized by titrations and corresponding rates: **ce1**, *R*. **+Dig7.5** and **+Dig5**, digitonin 7.5 and 5 mg·mL^{-1} in panel C and D, respectively; *ren*. **+D**, stimulating *ren*. **+M.1**, **1Oct** and **1c**, malate 0.1 mM, octanoylcarnitine 0.5 mM and cytochrome *c*; F-pathway OXPHOS capacity F$_P$ = *J*(1Oct(*c*)) – *J*(+M.1). **2M2**, malate 2 mM supporting the anaplerotic N-pathway F(N)$_P$. **3P**, FN{PM}$_P$. **4G**, FN$_P$, where N indicates the substrate combination PGM. **5S**, FNS$_P$. **6Gp**, FNSGp$_P$. **7U2** and **7U1.5**, uncoupler titrations to optimum CCCP concentration 2 and 1.5 µM, respectively; FNSGp$_E$. **8Rot**, SGp$_E$. **9Ama**, *rox*. Data (DOI:10.5281/zenodo.15780720): 2024-07-08_X-0005_03 and 2025-01-20_X-0005_03.
Files: 2024-07-08_J-011B_03, 2024-07-08_X-0005_03, 2024-07-09_J-011B_03, 2025-01-20_Q3-002_03, 2025-01-20_X-0005_03, 2025-02-26_J-011B_03;2025-02-26_Q3-002_03