

Quantitative Analysis of Neutrophil Activation Using Agilent Seahorse XF Technology

Authors

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Abstract

Immune cells have evolved complex mechanisms to detect and respond to infection. Neutrophils are the initial immune defense recruited to site of infection and injury, they have several mechanisms to eliminate infections among them production of reactive oxygen species (ROS), phagocytosis and release neutrophil extracellular traps (NET).

In this application note, we present an optimized workflow using the Agilent Seahorse XF analyzer that allows detection and quantification of neutrophil activation by measuring oxygen consumption rate (OCR). Using the XF analyzer the amount of oxygen consumed by phorbol 12-myristate 13-acetate (PMA)-activated neutrophils is monitored in real time as a measure of neutrophil activation. Neutrophil mitochondria do not consume a significant amount of oxygen, but to ensure that mitochondrial OCR is excluded, the assay is performed in the presence of inhibitors of mitochondrial function. This analytical method is non-invasive and highly sensitive allowing for real-time and label-free kinetic measurements of multiple parameters of response (intensity, duration, peak) unlike established methods of quantifying ROS. These results highlight the utility of the XF neutrophil activation assay to enable studies of innate immune responses to modulators, drug treatments, and micro-environment cues.

Introduction

Neutrophils are phagocytic cells that represent the main anti-microbial defense of the innate immune response. Upon appropriate stimulation, the membrane-associated NADPH oxidase (NOX2) of neutrophils is activated resulting in a powerful oxidative burst, during which a large amount of oxygen is consumed to generate superoxide and concomitant other reactive oxygen species (ROS). Generation of ROS is critical for effective antimicrobial immunity, but also plays a significant role during inflammatory responses^{1,2}.

The requirement of ROS production was demonstrated pharmacologically with the use of inhibitors and in subjects with chronic granulomatous disease (CGD), whom have mutations in the subunits of NADPH oxidase complex (NOX2). CGD subjects cannot produce ROS or NETs and suffer life threatening recurrent infections^{3,4}.

Most current available assays to detect and quantify oxidative burst are indirect and based on fluorescent/luminescent detection of ROS derived from superoxide anion formation. Despite good sensitivity, these methods are not specific, prone to artifacts, sensitive to compartmentalization of the probe or do not allow proper understanding of the duration and inactivation phase of the response since the oxidized probe irreversibly accumulates during the assay^{5,6,7}. Measurement of oxygen consumption rate (OCR) is a direct method to quantify neutrophil activation through the measure of the activity of the key enzyme NOX2. This method is not compromised by confounding effects of ROS secondary or competing reactions or probe accessibility⁸.

Agilent Seahorse XF technology allows the quantitative measure of neutrophil activation monitoring the rapid changes in non-mitochondrial oxygen consumption rate (OCR) non-invasively and in real time^{9,10,11,12,13}. In addition, XF technology allows the simultaneous measurement of proton efflux rate (PER) an indicator of glycolysis¹⁴. Activated neutrophils rely on glycolysis to meet their rapidly changing cellular energetic and metabolic demands¹⁵. Glucose metabolism is also important to sustain the pentose phosphate pathway that generates NADPH, one of the substrates of NOX2 enzyme and necessary for NET release¹⁶.

In this application note, we present examples monitoring neutrophil activation by quantifying OCR and comparing to established ROS and NET quantitation methods. The simultaneous measure of glycolysis via PER provides valuable metabolic information not possible with other methods. These examples can be adapted for kinetic analysis of oxidative burst in other phagocytic cells.

Results and Discussion

Oxygen consumption is an early measure of neutrophil activation

Human peripheral blood neutrophils (huPBN) were isolated from fresh whole blood and plated at 4×10^4 cells per well in Cell-Tak™ coated XFe96 cell culture microplates. Changes in OCR were measured in response to activation by PMA, a potent activator of NOX2 enzyme, in the presence of mitochondrial inhibitors rotenone and antimycin A (Rot/AA). Addition of mitochondrial inhibitors in the assay before neutrophil activation ensures any oxygen consumption from mitochondria respiration is excluded. Figure 1 shows the rapid rise in OCR observed when neutrophils are stimulated with PMA, reaching a peak of OCR within 30 min, and declining after 60 min to slowly reach the basal values between 90-120 min after stimulation. The profile of the curve demonstrates the dynamics of oxidative burst that includes rapid assembly of the NADPH oxidase enzyme complex and then disassembly^{1,10,13}.

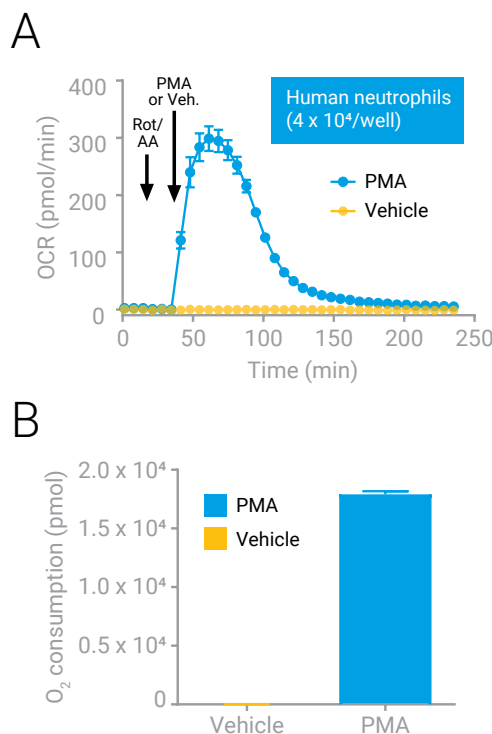


Figure 1. Oxygen consumption is an early measure of neutrophil activation A) XF Neutrophil Activation Assay kinetic trace of oxygen consumption rate (OCR) in the presence of Rot/AA to block mitochondrial function. An initial injection of inhibitors Rot/AA at 0.5 μ M each to discard mitochondrial contribution to OCR, followed 24 min later with the injection of activator PMA (100 ng/ml) or vehicle control (black arrows). B) O₂ consumption is calculated from the area under the curve (AUC) of the complete kinetic range between activation and return to basal rates. The data shown are the mean and \pm SD, n=6 technical replicates.

Oxygen consumption is a linear and sensitive measure of neutrophil activation

The linearity range of the XF analyzer detection method was determined using huPBN plated at multiple cell densities (5×10^3 to 4×10^4 cells/well) on Cell-Tak™ coated XF96 cell culture microplates and the method was validated by comparison to established methods for quantifying production of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). Figure 2B shows a schematic representation of oxidative burst reactions and the assays frequently used to measure neutrophil activation. During activation assays neutrophils were activated with PMA in real time using the automatic injection port system of XF analyzer, or activated and then monitored using kinetic spectrophotometric reading at 550 nm of cytochrome c reduction or by fluorescence detection using Amplex Red assay. The maximum peak of OCR on the XF analyzer was directly proportional to the number of plated cells. To better quantify the activation response, the total area under the curve (AUC) was calculated representing the total O_2 consumed during the activation response. Figure 2C shows that the total O_2 consumption during activation response is proportional to the cell number illustrating the linear response of this assay (Figure 2C). Similar responses were obtained when compared with other methods for oxidative burst

detection, but the cytochrome c assay had a less sensitive range of detection (Figure 2D) and Amplex Red assay had a smaller dynamic range of detection (Figure 2E).

The XF analyzer generates a microchamber between sensor and cell monolayer during each individual measurement, increasing the sensitivity of the oxygen consumption rate measurements during assays. Between measurements, a mixing period allows re-oxygenation of the extracellular medium assuring that cells are not exposed to low levels of O_2 for long periods or avoiding underestimation of oxidative burst due to depletion of oxygen in the well. Neutrophils have a strong increase of OCR during oxidative burst. If an OCR value of 600 pmol/min or higher is obtained during the assay using the XFe96/XF96 or XFp analyzers, oxygen level in the microchamber may be too close to 0 (this can be observed by displaying the oxygen level data in Agilent Seahorse XF Wave software). Approaching the limits of the dynamic range of the instrument can cause an underestimation of the response. In this case, cell number or activation reagent concentration should be adjusted to ensure the assay is run within the linear dynamic range of XF analyzer. In general, when using huPBN, assays were run plating 4×10^4 cells per well of a XF96 cell culture microplate and neutrophils were activated with 100 ng/ml PMA. The specific cell number optimum may vary with the cell source and strength of activator used.

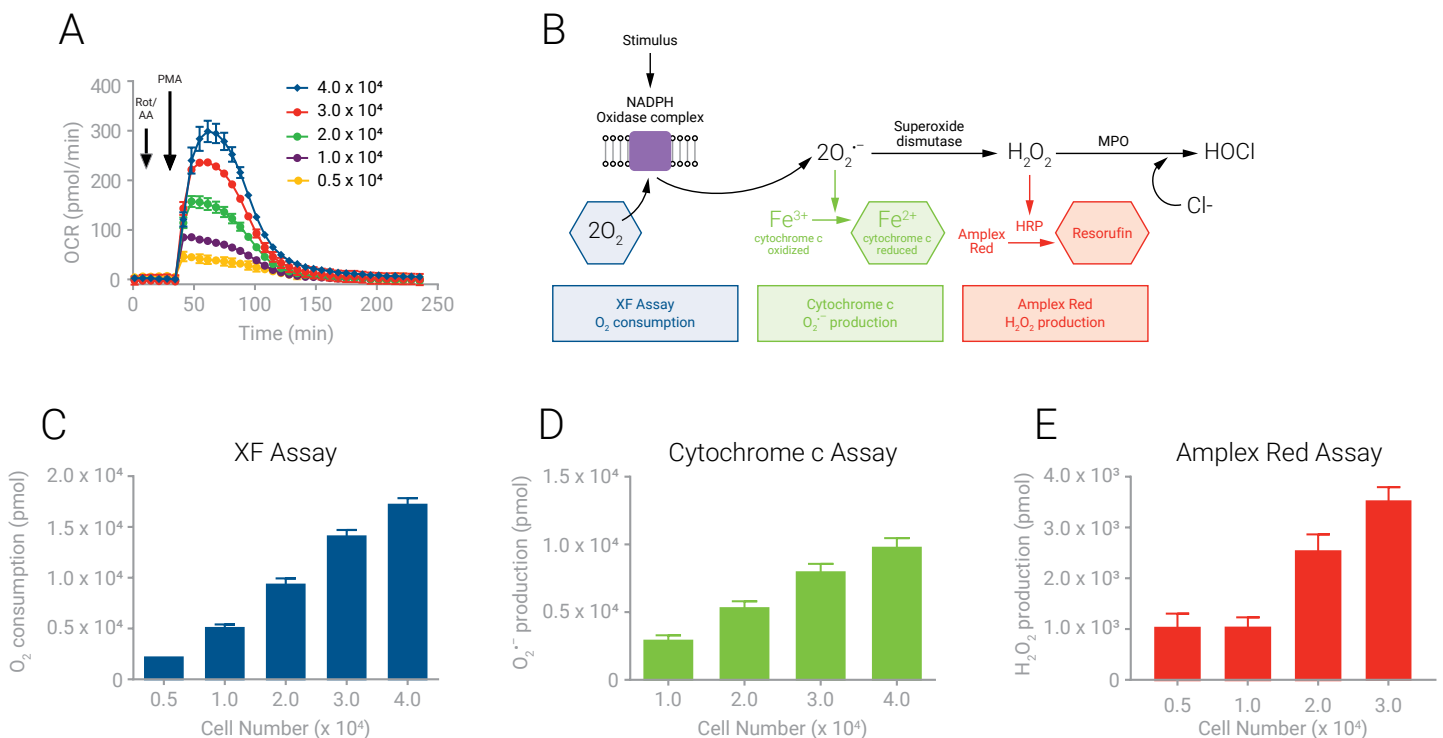


Figure 2. Oxygen consumption is linear and sensitive with respect to cell number A) XF neutrophil activation kinetic trace of OCR with varying cell number. B) Scheme of oxidative burst noting assays used to measure neutrophil activation. C) O_2 consumption calculated from AUC of XF kinetic trace. D) Quantitation of superoxide production by cytochrome c assay. E) Quantitation of hydrogen peroxide production by Amplex Red assay. The data shown are the mean and + SD, n=4 technical replicates.

Oxygen consumption is a direct and specific measurement of neutrophil activation

The specificity and functional relevance of the activation-associated elevation in OCR was validated using two NOX inhibitors: diphenyleneiodonium chloride (DPI) (Sigma, D2926) and VAS2870 (Sigma, SML0273). As shown in Figure 3A, DPI or VAS2870, Rot/AA, and PMA (black arrows) were sequentially administered, and OCR was monitored. The immediate increase in OCR and O_2 consumption (AUC) with PMA treatment was completely blocked (Figure 3A and 3B) when cells were pretreated with the NOX2 inhibitors, as was superoxide and hydrogen peroxide production (Figure 3C and 3D). The inhibition in oxidative burst also correlates with inhibition of downstream neutrophil extracellular trap formation (Figure 3E and 3F). These data demonstrate the known link between O_2 consumption, ROS production and downstream NET production^{4, 15, 16}.

Parameters of oxygen consumption are indicative of activation kinetics

The dynamic and contextual activation data obtained using the XF analyzer are demonstrated in Figure 4. HuPBN were plated at 4×10^4 per well of XF96 cell culture microplates and activated with varying doses of PMA in the presence of Rot/AA. The real-time OCR data obtained with varying doses of PMA are shown in Figure 4A. The multiple parameters of neutrophil activation can be evaluated using XF technology (Figure 4B), such as total O_2 consumption, EC50 of total O_2 consumption, maximum OCR, the time to maximum OCR and duration of the response of the OCR burst (Figure 4C-4F). These contextual data may lead to a better understanding of the mechanism and time-course of neutrophil activation, and contribute to the development of novel agents that maintain activation at a level that allows suppression of infection while preventing inflammatory responses.

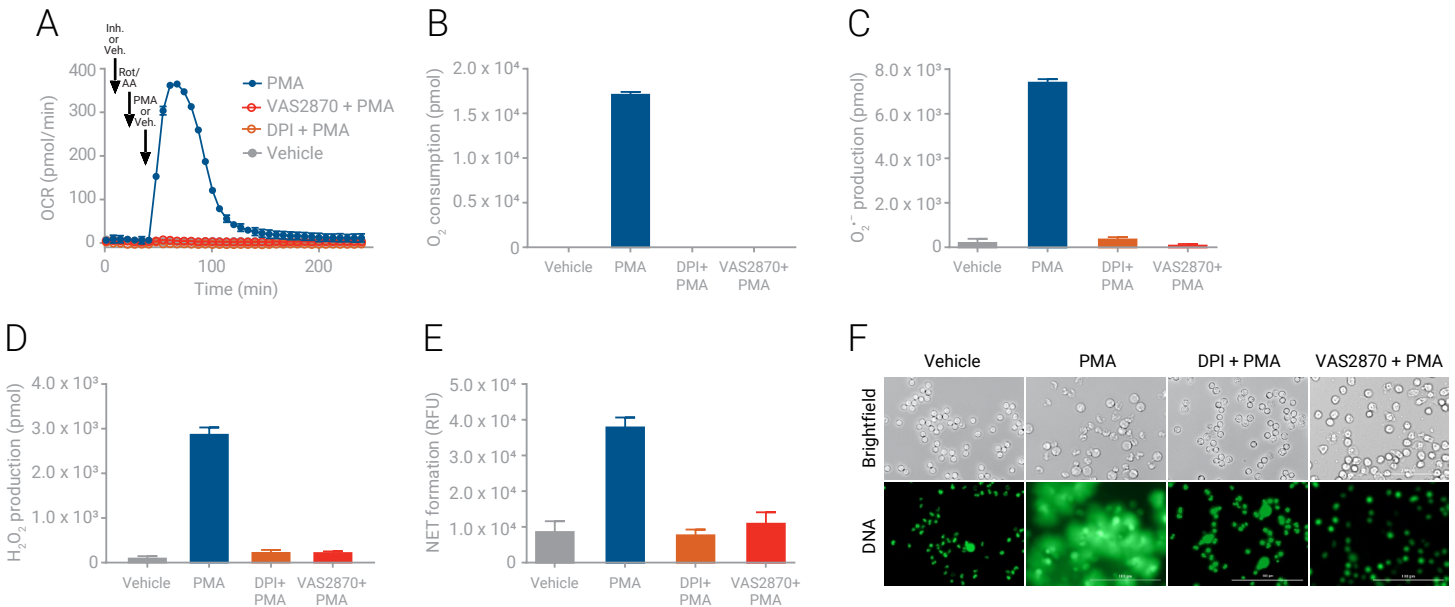


Figure 3. Oxygen consumption is a specific measure of neutrophil activation A) XF Neutrophil Activation kinetic trace of OCR with NOX inhibitors using 4×10^4 cells per well, an initial injection of inhibitors 10 μ M DPI or 5 μ M VAS2870 final each, followed 6 min later by injection of 0.5 μ M Rot/AA. After 24 min PMA (100 ng/ml) or vehicle control are injected (black arrows). B) O_2 consumption calculated from AUC of XF kinetic trace. C) Quantitation of superoxide production by cytochrome c assay. D) Quantitation of hydrogen peroxide production by Amplex Red assay. E) Quantitation of NET DNA released 4 h post activation using PicoGreen dye (480 nm ex/520 nm em), F) Cell images of NET formation 4 h post activation PicoGreen DNA stained (469 nm ex/520 nm em) and bright field 20x. The data shown are the mean and + SD, n=4 technical replicates.

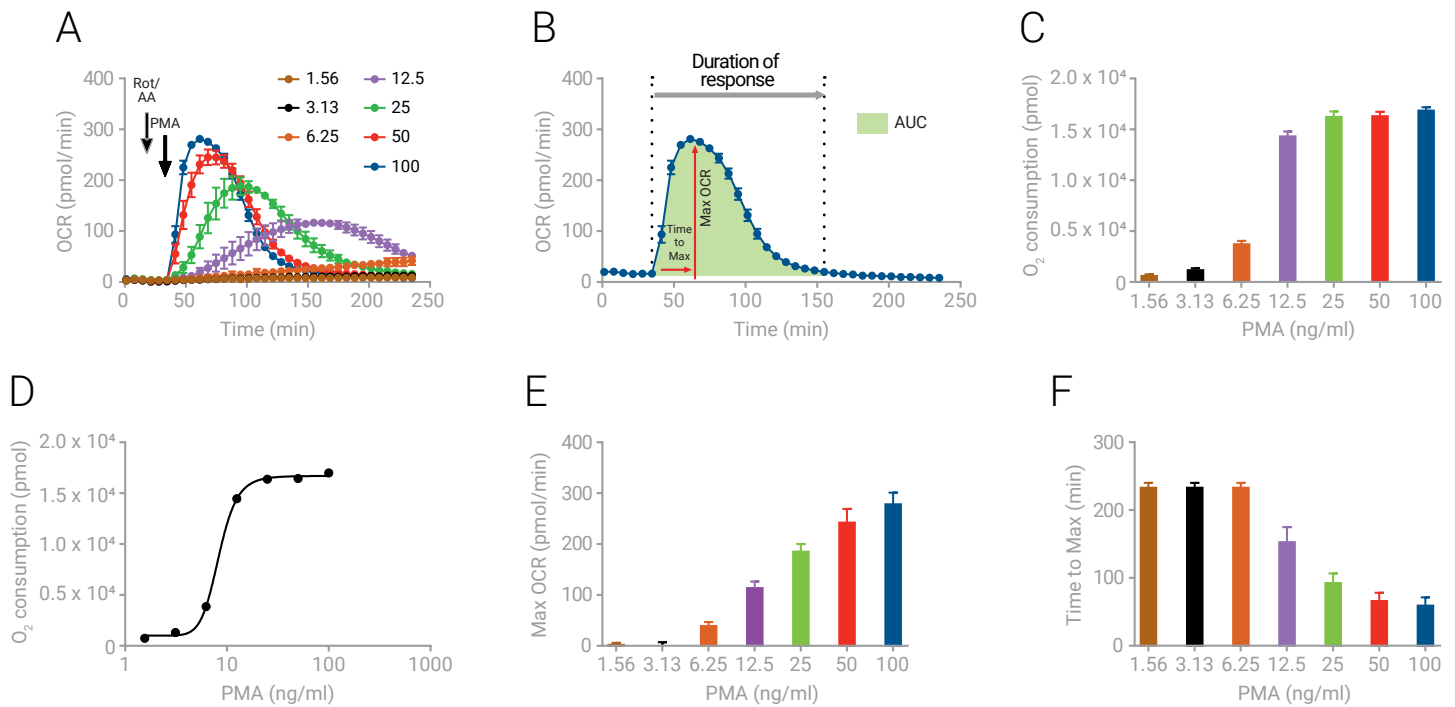


Figure 4. Parameters of oxygen consumption rate are indicative of activation kinetics A) XF neutrophil activation kinetic trace of OCR with dose of PMA in 4×10^4 cells per well. B) Schematic of XF activation parameters. C) O₂ consumption calculated from AUC of XF kinetic trace. D) EC₅₀ of PMA from XF O₂ consumption AUC. E) XF maximal OCR. F) Time to reach maximal OCR of XF kinetic trace. The data shown are the mean and + SD, n=4 technical replicates.

Oxidative Burst in neutrophils requires glycolysis

The increase in OCR or oxidative burst after activation with PMA is associated with a simultaneous increase of proton efflux rate (PER) indicative of the dependence of neutrophils on glycolysis during activation¹⁴. In addition, no significant difference was observed in O₂ consumption or proton efflux when neutrophils were stimulated in the presence or absence of mitochondrial inhibitors Rot/AA (data not shown). These data support the importance of glycolysis but not mitochondrial respiration for neutrophil activation. The specificity and functional relevance of the associated activation with glycolysis to meet energy demand of oxidative burst was validated using the inhibitor 2-Deoxy-D-glucose (2-DG) (Agilent, 103344-100). As shown in Figure 5A and 5D, 2-DG (or vehicle) Rot/AA and PMA (or vehicle) (black arrows) were serially administered, and OCR and PER were monitored.

The immediate increase in OCR (Figure 5B) or superoxide production (Figure 5C) with PMA treatment was blocked when glycolysis was inhibited by pre-treatment with 2-DG. There is a parallel immediate increase in PER with PMA treatment and this increase in PER is likewise inhibited with 2-DG (Figure 5D and 5E). PER is a measure of proton production and is indicative of glycolysis, but also other sources can contribute to an increase in PER like protons produced through the pentose phosphate pathway. However, measurement of lactate accumulation during the oxidative burst (Figure 5F) indicates that the majority of PER are produced from glycolysis and that oxidative burst relies on glycolysis. The inhibition of glycolysis also inhibited downstream neutrophil extracellular trap formation (Figure 5G and 5H) demonstrating the requirement of functioning glycolysis to meet energy and substrate demands of oxidative burst^{13,15,16}.

Future application of XF neutrophil activation assay

The interdependent operation of several cellular processes, the irreversible nature of phagocytosis and the participation of large quantities of reactive metabolites necessitate an extremely high level of cellular regulation of this phenotypic adaptation. XF real-time activation assay allows for the interrogation of the dynamic nature of these events. As seen in this assay, to meet the rapidly changing cellular energy and metabolite demand of activation, neutrophils increase the metabolic rate through glycolysis during the oxidative burst. The XF neutrophil activation assay provides a simple and direct way to examine modulators of oxidative burst (ROS production) and metabolic effects thru parallel measure of glycolysis and oxidative burst.

Conclusions

Neutrophil activation with PMA treatment causes an immediate increase in OCR, which can be monitored in real time using XF technology. This is indicative of the assembly and activation of NOX2 enzyme pathway and ROS production in activated neutrophils. There is a simultaneous increase in PER indicative of the increase in glycolysis to meet metabolic demands. Due to the noninvasive nature of the XF analyzer, downstream assays may be performed on the same cells such as PCR, Elisa and NET assays. This application is a specific and kinetic assay performed on live cells in real time providing temporal resolution of neutrophil activation not possible with other assays. This multidimensional measure of neutrophil activation may lead to further insight into the effect of modulators such drug treatments, microenvironment and disease progression during innate immune response.

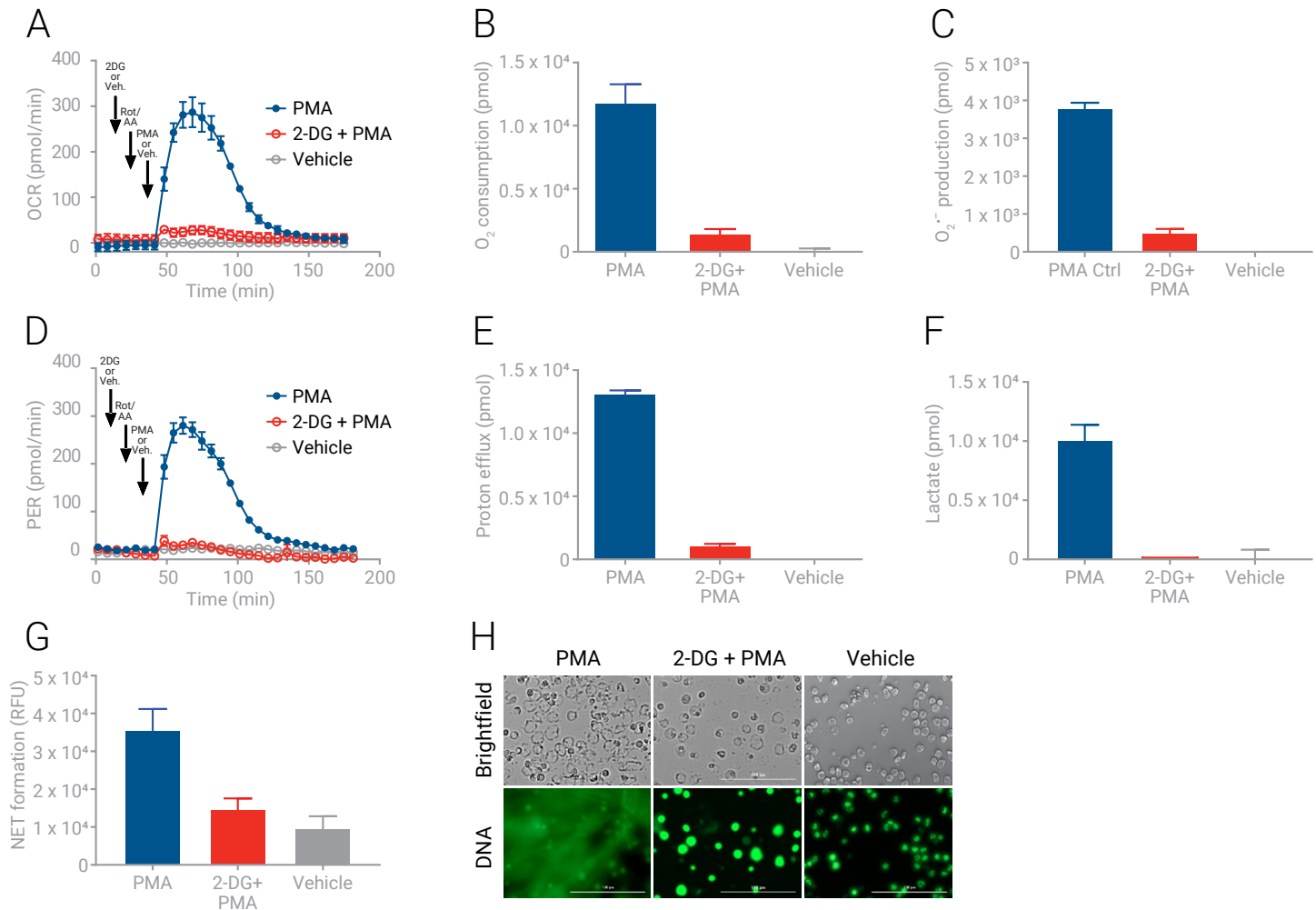


Figure 5. Oxidative Burst in neutrophils requires glycolysis A) XF Neutrophil Activation kinetic trace of OCR in the presence of 2-DG in 4×10^4 cells per well. An initial injection of inhibitors 50 mM 2-DG final, followed 6 min later by injection of Rot/AA at 0.5 μ M. After 24 min, PMA (100 ng/ml) or vehicle control are injected (black arrows). B) O_2 consumption calculated from AUC of XF kinetic trace. C) Quantitation of superoxide production by cytochrome c assay. D) XF kinetic trace PER. E) Proton efflux calculated from AUC of XF kinetic trace. F) Lactate accumulation with PMA activation. G) Quantitation of NET DNA released 4 h post activation using PicoGreen dye (480 nm ex/520 nm em). H) Cell images of NET formation 4 h post activation PicoGreen DNA stained (469 nm ex/520 nm em) and bright field 20x. The data shown are the mean and + SD, n=4 technical replicates.

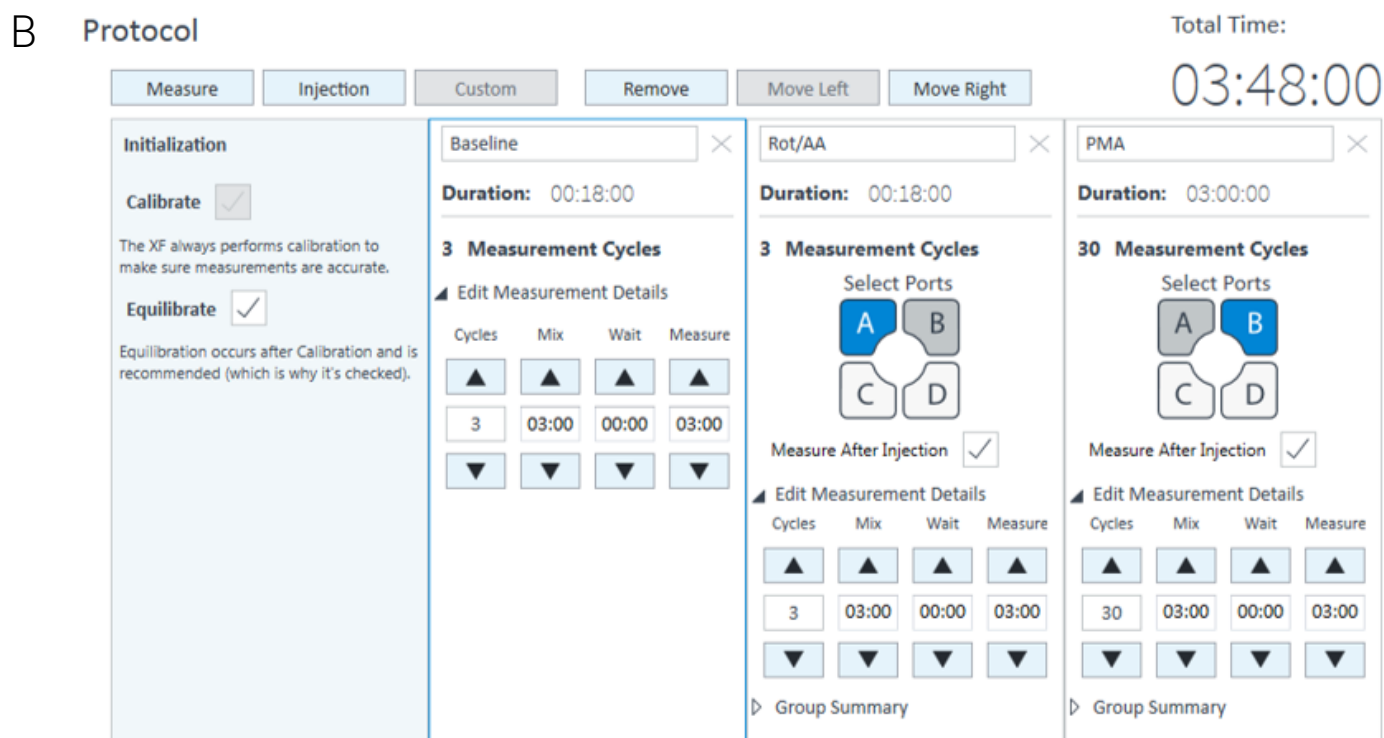
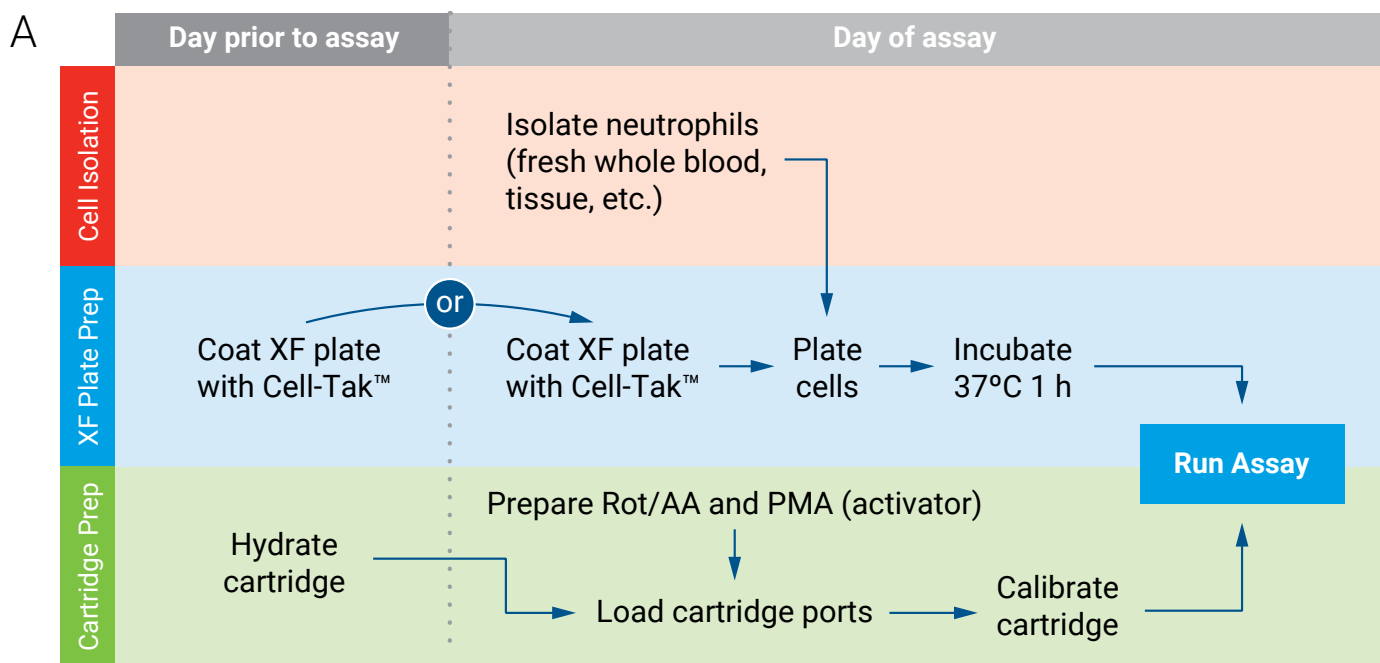


Figure 6. XF Neutrophil Activation assay design A) Workflow schematic describing necessary assay steps. B) An example of a real-time activation instrument protocol designed in Wave 2.4.

Material and Methods

Cells and materials

Human Neutrophils were isolated from fresh whole blood (All-Cells, WB010-ACD) by negative selection using immunomagnetic depletion with an additional erythrocyte depletion step following manufacturer recommendations (Miltenyi, 130-104-434 and 130-098-196). The resulting supernatant containing neutrophils was diluted by the addition of three volumes of XF RPMI Assay Media (RPMI Medium, pH 7.4 (Agilent Technologies, 103576-100), supplemented with 2 mM L-glutamine (Agilent, 103579-100) 1 mM pyruvate (Agilent, 103578-100), 10 mM glucose (Agilent, 103577-100). Centrifuge 500 x g for 10 min in 15 ml conical tubes. All supernatant was removed to avoid left overs of plasma. The cell pellet was resuspended in 1 ml of complete RPMI Assay media combined into one tube and centrifuged at 300 x g for 10 min. Resulting cell pellet was resuspended at $0.8\text{--}2 \times 10^6$ cells/ml in RPMI assay media depending on desired plating density. Figure 6A shows a schematic of XF assay workflow.

Real time metabolic assay design

Neutrophils were seeded on Cell-Tak™ (Corning, # 354240) coated XF96/XFp cell culture microplates/miniplates at a density of 4×10^4 cells per well corresponding to 50 μL of a 0.8×10^6 cells/ml cell suspension per well. 50 μL of RPMI assay media was added to background control wells (no cells). The cell culture microplate was placed in carrier tray and centrifuged for 1 min at $200 \times g$ to adhere the cells to the plate. An additional 130 μL of assay media was gently added (total volume = 180 μL) to each well and incubated at 37 °C without CO₂ for 45 min prior to XF assay. For further instruction on Cell-Tak™ coated XF96 microplates or XFp miniplates see Immobilization of Non-Adherent Cells with Cell-Tak™ for Assay on the Seahorse XFe /XF96 or XFp Analyzer. (<https://www.agilent.com/cs/library/technicaloverviews/public/5991-7153EN.pdf>)

For XFe96 instrument operation and guidelines, please refer to Basic Procedures to Run an XFe96 Assay ([https://www.agilent.com/en/products/cell-analysis-\(seahorse\)/basic-procedures-to-run-an-xf-assay](https://www.agilent.com/en/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay)). Figure 6A shows a schematic of assay workflow, be certain to hydrate cartridge the day prior to assay. Figure 6B shows an example of assay template design for 3 h analysis with rotenone and antimycin A (Rot/AA) (Agilent, component of 103344-100) mitochondrial inhibitor pretreatment prior to neutrophil activation.

Briefly, prepare compounds for assay in XF RPMI complete assay media at 10X the final desired concentration on cells. If using the Rot/AA component of the XF Glycolytic Rate Assay kit (Agilent, 103344-100), follow the instructions provided in the kit protocol for resuspending dried compound, then dilute

further to prepare 10X working stock of 5 μM for injection (final on cells 0.5 μM). For phorbol 12-myristate 13-acetate (PMA) dilute DMSO stock to 1 $\mu\text{g}/\text{ml}$ in XF RPMI assay media (final on cells 100 ng/ml). Remove a hydrated XF cartridge from the non-CO₂ incubator. For basic assay load each port of the cartridge as follows: 20 μL of Rot/AA 10X final is loaded into port A and 22 μL of PMA 10X final is loaded into port B. NOTE: Fill ports A and B of all wells, including those corresponding to the background wells, to ensure successful injections.

Once the ports are filled, transfer the cartridge and utility plate to the XF instrument and begin cartridge calibration using the assay template created as outlined below (Figure 6B). Once cartridge calibration is complete, follow the prompts in the Wave software to exchange the utility plate for the cell culture plate and initiate the XF assay. When the assay is complete, eject the cartridge/cell plate assembly and set aside for later analysis.

The basic instrument commands into the assay template file were as follows:

- i. Baseline measurement with 3 cycles; Mix 3 min, Wait 0 min, Measure 3 min
- ii. Inject Rot/AA- through Port A
- iii. 3 cycles; Mix 3 min, Wait 0 min, Measure 3 min
- iv. Inject PMA (activator) through Port B
- v. Measurement with 30 cycles; Mix 3 min, Wait 0 min, Measure 3 min

Total run time is 4 h. This allows for 3 h real time monitoring after activation and can be adjusted depending on the time window of interest.

Any inhibitor or modulator effect can be assessed by a slight modification in the instrument commands depending on the desired treatment time. In the example below 1 to 10 cycles is equivalent to 6 to 60 min treatment after inhibitor injection, then there is an additional Rot/AA injection and 3 cycles equal to 18 min prior to activation with PMA, so a total time of 24 to 78 min of treatment prior to activation.

- i. Baseline measurement with 3 cycles; Mix 3 min, Wait 0 min, Measure 3 min
- ii. Inject an inhibitor/modulator or assay medium through Port A
- iii. 1-10 cycles; Mix 3 min, Wait 0 min, Measure 3 min
- iv. Inject Rot/AA through Port B
- v. 3 cycles; Mix 3 min, Wait 0 min, Measure 3 min
- vi. Inject PMA (activator) through Port C
- vii. Measurement with 30 cycles; Mix 3 min, Wait 0 min, Measure 3 min

XF ECAR to PER transformation

PER is a new rate measurement calculated in Wave software (version 2.4 and higher). PER delivers an absolute and quantitative measure of extracellular acidification. Calculation of PER is only available in post-assay results. PER is not displayed while running an assay. ECAR is the default reported acidification data in Wave software. The three rate options displayed in the Rate Measurement drop-down menu are: OCR, ECAR, and PER. Details of PER calculations may be found in reference ¹⁴.

To accurately calculate and display PER after an XF assay is performed, the assay media (with corresponding buffer factor) must be assigned to all wells including background wells.

Wave will automatically display PER if the following conditions are true:

1. The Buffer Factor was automatically read from the sensor cartridge.
2. The assay template was designed with media = XF Glycolytic Rate Assay Medium (RPMI-based) in all Groups.
3. The assay was run using a supported plate type & cartridge: XFe96, XFe24, XFp, and XF96. The following are NOT Supported: sensor cartridges XF24 and XF24-3 and plate types Spheroid, Islet, PET and V28.

See Proton Efflux Rate Quick reference guide for more details http://www.agilent.com/cs/library/quickreference/public/Proton_Efflux_Rate_Quick_Reference_Guide_Final.pdf

Analysis

The Wave results file was saved to a shared folder on a local network or to a USB drive, and then opened on a PC or laptop using the Wave Desktop software. The data was exported from Wave Desktop software to GraphPad Prism software. The total O₂ consumption was calculated using area under the curve (AUC) analysis in GraphPad Prism version 7.00. (GraphPad Software, La Jolla California USA, www.graphpad.com). The AUC analysis was performed on OCR over the duration of response, which included OCR between the addition of the PMA stimulus and the time when oxidative burst is resolved (OCR returns to pre-stimulation levels). Therefore, the AUC analysis was performed on a subset of OCR measures, excluding values up to the rate just prior to PMA injection and any rate following the resolution to the basal rate. The AUC analysis in Graphpad Prism provides: AUC = Total O₂ consumed, Peak Y= Max OCR, Peak X-First X = time to reach MAX., Last X-First X= duration of response of oxidative burst.

Amplex Red Assay- Generation of Hydrogen Peroxide

The generation of hydrogen peroxide was quantified using Amplex Red Assay kit (Thermofisher, A22188). Neutrophils were incubated at 37°C with final 50 µM Amplex Red reagent, 10 U/ml HRP in PBS Mg²⁺ Ca²⁺ supplemented with 10 mM glucose. Fluorescence was then measured with a fluorescence microplate reader using excitation at 530 nm and fluorescence detection at 590 nm from 0 to 60 min reading every 10 min. The H₂O₂ concentration was determined using an H₂O₂ standard curve. Background fluorescence, determined for a no-H₂O₂ control reaction, has been subtracted from each value.

Cytochrome C Assay -Generation of Superoxide

The production of superoxide was quantified using the reduction of cytochrome c. Neutrophils were incubated with 50 µM horse heart cytochrome c (Sigma, C7752), 100U/ml catalase (Sigma, C1345) in PBS with Mg²⁺ Ca²⁺ with 10 mM glucose in the absence or presence of 30 U/ml SOD (CalBiochem, 574594) and activated with PMA. Reduction of cytochrome c was measured spectrophotometrically at 550 nm. Production of superoxide was calculated as the amount of reduced cytochrome c after PMA stimulation that is inhibited by SOD using the extinction coefficient of 21.1 mM⁻¹ cm⁻¹ at 550 nm ^{5,6}.

Monitor Neutrophil Extracellular Traps (NET)

NET formation was monitored using Quant-iT™ PicoGreen™ dsDNA reagent (Thermofisher, P7581). Neutrophils were seeded as above in Cell-Tak™ coated XF96/XFp microplate in assay media. Inhibitors were added and cells incubated at for 30 min at 37 °C. PMA was added and cells were incubated between 3 to 4h at 37 °C. 50 µL of PicoGreen reagent (diluted 1 to 20) was added to 100 µL of assay media on cells and incubated 10 min. The supernatant was read using a spectrofluorometric excitation at 480 nm and the fluorescence emission intensity was measured at 520 nm. For Imaging of NETs, cells were fixed in 4% formaldehyde and images taken at 20X, 469 nm ex/523 nm em using BioTek Cytation 5. These assays can be run on cells post XF assay.

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