Application Note

Normalization

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XF Data Normalization by the Agilent Seahorse XF Imaging and Normalization System

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Abstract

Data normalization is required for any experiment to reach a valid conclusion, particularly when variation in sample quantity is involved. For real-time cellular metabolic analysis using Agilent Seahorse XF technology, the cell quantity per well is a commonly accepted reference value for data normalization. It can be assessed directly by cell counting or indirectly by measuring biomolecules such as total protein or genomic DNA. The Agilent Seahorse XF Imaging and Normalization system integrates XF technology with a Cytation 1/5 Cell Imaging Multi-Mode reader (BioTek Instruments, Inc). In this configuration, the Cytation 1/5 is controlled by the new Agilent Seahorse XF Imaging and Cell Counting software, which provides a simplified, automated, and validated solution for microscopic image-based cell counting. This turnkey solution includes 1) *in-situ* administration of a cell membrane-permeable nuclear staining compound (i.e. Hoechst 33342), 2) automated imaging and cell counting optimized for XF assays, and 3) synchronization of images and cell counts into XF result files in Wave software. This solution provides an efficient and reliable method of normalization with a seamless and rapid workflow for XF Analyzer users.

Introduction

Cellular metabolic functions are measured as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) and further converted into values like proton efflux rate (PER) and ATP production rate by the Agilent Seahorse XF technology. Similar to other biological experiments, those data should be normalized to derive a valid conclusion if there is any variation in the cell quantity between wells and experiments. Variation in cell quantity can have many different causes including, but not limited to, differences in the proliferation or death rate. Technical errors during sample preparation can also be a possible cause of variation. Regardless of the cause, cell quantity per well should be the primary choice of denominator to normalize XF analysis data with any cell amount variation.

The cell quantity per well can be counted directly or indirectly by measuring cellular components such as total protein and genomic DNA. For most users, the latter approach has been preferred to direct cell counting, which requires time and expertise. The Agilent Seahorse XF Imaging and Normalization system provides an XF data normalization solution using an automated imaging and cell counting workflow. Here, the cell number per well is counted from microscopic images captured by the Cytation 1/5. For the identification of individual cells, cell nuclei are stained with Hoechst 33342, a cell-permeable DNA-binding fluorescent probe detected with a DAPI filter cube in the Cytation 1/5. The cell counts from the images are calculated and reported by the Agilent Seahorse XF Imaging and Cell Counting software. Subsequently, this data is imported into the Wave software to normalize XF data.

The XF Imaging and Normalization system includes several unique features optimized for XF assays. First, each XF plate is registered by scanning its unique bar code, and subsequently, all image data, as well as the final cell counts, are automatically synchronized to the corresponding XF analysis file. All the information is documented in the Wave software for further review. Second, brightfield images can be obtained in the same workflow, and these images of the cell seeding condition in each well can be compared for data quality management. Third, as a default workflow, cell membrane-permeable nuclear staining dye Hoechst 33342 is included in the last injection of any XF analysis. This not only simplifies the procedure but also reduces the chance of technical error during manual administration of the dye. The cells are stained during the last set of mix and measurement steps in the XF assay. These are then imaged in the Cytation 1/5 without any further washing or fixation step upon the completion of the XF assay. This workflow can be applied for most cell types cultured *in vitro*. Finally, this procedure is performed on living cells, thus any additional analysis is still possible. For example, the recommended XF Imaging and Normalization system workflow can be followed by protein assay or immunofluorescence staining to get additional or complimentary data. These tailored features, in combination with brightfield imaging, facilitate improved data interpretation for XF Analyzer users.

Experimental

Normalization validation

RAW264.7, HT29 and SKOV3 cells were cultured in high glucose DMEM (Gibco, 11960-44) base growth medium supplemented with 10 % FBS (HyClone, SH30070.03), 2 mM L-glutamine (Corning, 25-005-CI), and 1 mM sodium pyruvate (Corning, 25-000-CI). A549 cells were cultured in DMEM:F12 (Corning, 10-090-CV) base growth medium supplemented with 10 % FBS and 2 mM GlutaMAX (Gibco, 35050-061). MCF7 cells were cultured in RPMI-1640 (Gibco, 21870) with 10 % FBS and 1 mM sodium pyruvate. HepG2 cells were cultured in low glucose DMEM (Gibco, 11885-084) supplemented with 10 % FBS, 2 mM GlutaMAX, and 1 mM pyruvate. For XF96, cells were plated at five different cell densities that cover ± 50 % range of cell density recommended; 1 x 10^4 cells/well for HepG2, 2 x 10^4 cells/well for A549, HT29, MCF7 and SKOV3, and 3 x 10^4 cells/well for RAW264.7. For XF24, they were plated at three different cell densities ranging from ± 25 %; 2 x 10^4 cells/well for HepG2 and 4 x 10^4 cells/well for A549 and MCF7.

Cells were cultured overnight and the metabolic phenotype measured by following the standard Agilent Seahorse XF Cell Energy Phenotype Test protocol[1] except for a modification: 20 µM Hoechst 33342 (Thermo, 62249) was added to the oligomycin/FCCP mix. Cells were counted by BioTek Cytation 1/5 using the XF Cell Imaging and Counting software[2] and data was normalized in Wave software. The data changes upon normalization were compared using the Agilent Seahorse XF Cell Energy Phenotype Report Generator[3].

All XF analysis was performed by using cells in Agilent Seahorse XF base medium without phenol red (Agilent Technologies, 103335-100) supplemented with 10 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5 mM HEPES, pH 7.4 (Agilent Technologies, 103337-100).

Macrophage activation

RAW264.7 cells were plated at 2.5 x 10^4 cells/well and cultured overnight. Cells were further cultured for 16 h in the absence or presence of 100 ng/ml LPS with or without 20 ng/ml IFNγ, and the metabolic rates were analyzed by the Agilent Seahorse XF Cell Mito Stress Test, the Agilent Seahorse XF Glycolytic Rate Assay, or the Agilent Seahorse XF Real-Time ATP Rate Assay Kit according to the user manual[1,4]. The last injection solution in each assay, rotenone/antimycin A or 2DG, includes 20 µM Hoechst 33342 for nuclear staining.

XF imaging and normalization

Brightfield and fluorescently labeled nuclear images were collected by the XF Cell Imaging and Counting software and the analyzed results were incorporated to XF analysis data in the Wave software as schematically described in Figure 7. Brightfield images were captured as the quality reference during the outgassing step while the cartridge was being calibrated[2]. Cell number per well was measured by counting fluorescently labeled nuclei from images captured by Cytation 1/5. As described above, Hoechst 33342 was included in the last injection in the XF analysis protocol, with a rotenone/antimycin A injection in the XF Cell Mito Stress Test, 2DG injection in the XF Glycolysis Stress Test and the XF Glycolytic Rate Assay, and oligomycin/FCCP injection in the XF Cell Energy Phenotype Test.

Data presentation

XF data presented in this Application Note were prepared by using the GraphPad Prism software (GraphPad Software, Inc; Figure 1, 4, 5, and 6) or by the XF Cell Energy Phenotype Test Report Generator (Figure 2 and 3) after a data export using the Wave software.

Results and Discussion

Data normalization using cell counts from images

As a validation model for data normalization, we plated various cell types at three to five different densities by assuming the two basal metabolic rates, OCR and ECAR, proportionally correlate to the cell quantity per well. This assumption is true only when cells don't have any biological response to the cell density change. Among the cell lines tested, most of the basal metabolic rates showed a good linear correlation to the cell density within the range of the standard plating density commonly recommended for XF analysis, with a few exceptions (data not shown).

Figure 1 shows an example of how this data normalization solution works for the XF cell energy phenotype test[1]. RAW264.7 macrophages were plated at four different cell densities one day prior to the assay and their metabolic phenotypes were analyzed by an Agilent Seahorse XFe96 Analyzer. The only difference from the standard XF Cell Energy Phenotype Test was to include cell-permeable Hoechst 33342 dye along with the oligomycin/FCCP compounds in the same injection port (arrow). As shown in Figure 1A, OCR and ECAR increase proportionally to the seeding density. Upon completion of the XF analysis, fluorescence images were captured and counted by the XF Imaging and Cell Counting software as shown in Figure 1B. Figure 1C shows the data after normalization by dividing the metabolic rates by the cell numbers counted by the software. Both OCR and ECAR kinetic traces superimpose after applying the cell count normalization factor, particularly for the basal rates before the cells were stressed by oligomycin and FCCP. Notably, this data convergence by normalization happens only when there is no biological change occurring as a result of density differences. Therefore, data normalization can identify true biological change related to cell density difference. As shown in Figure 1C, the FCCP effect on OCR varied depending on cell density while the ECAR elevation by oligomycin was not affected by cell density even though the basal value of both rates converged well.

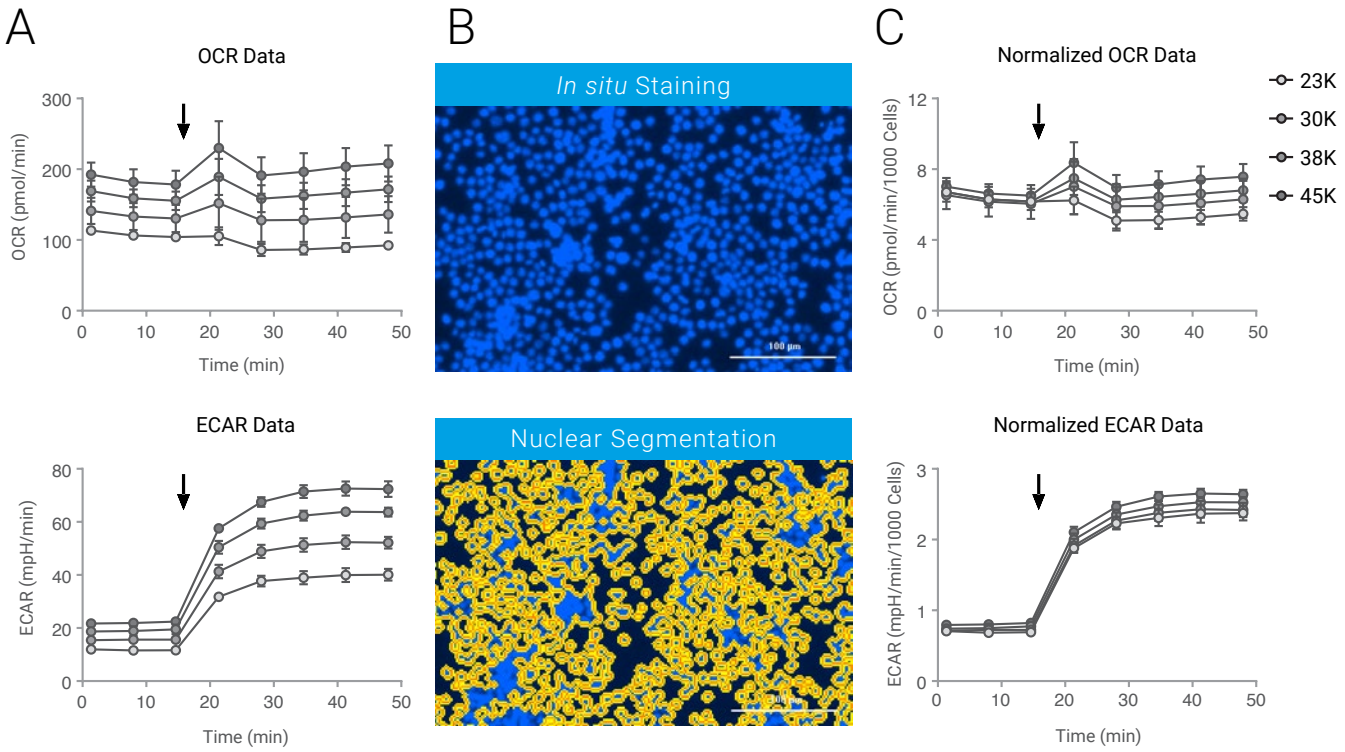

Figure 1. Normalization of the XF Cell Energy Phenotype Test data using the XF Imaging and Normalization system. RAW264.7 cells were seeded at various cell densities, as indicated, a day prior to the assay. Hoechst 33342 (final 2 µM) was co-injected (arrow) with a oligomycin/FCCP mixture in the XF Cell Energy Phenotype Assay; A) data before normalization; B) a representative image acquired and the corresponding nuclear segmentation result; C) normalization results. Data shown are mean ± SD, n = 6 technical replicates. Cells growing in clumps are also identified and counted.

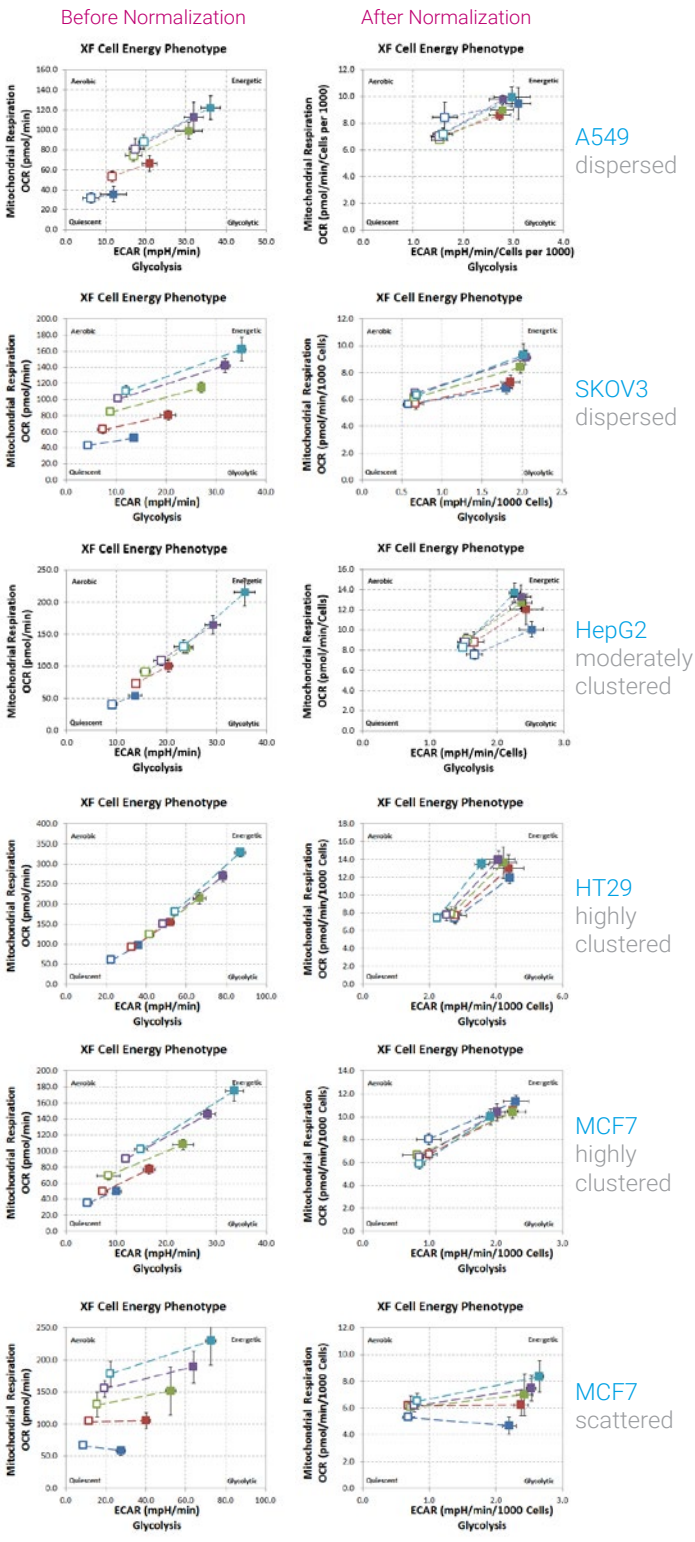

Figure 2. Normalization of the XF Cell Energy Phenotype Test data from multiple cancer cell lines on Agilent Seahorse XF96 Microplates. Cell energy phenotypes of six different cell lines seeded at five different cell densities were obtained by using the XF Cell Energy Phenotype Report Generator. The phenotype diagrams before (left column) and after (right column) normalization are compared for each cell line.

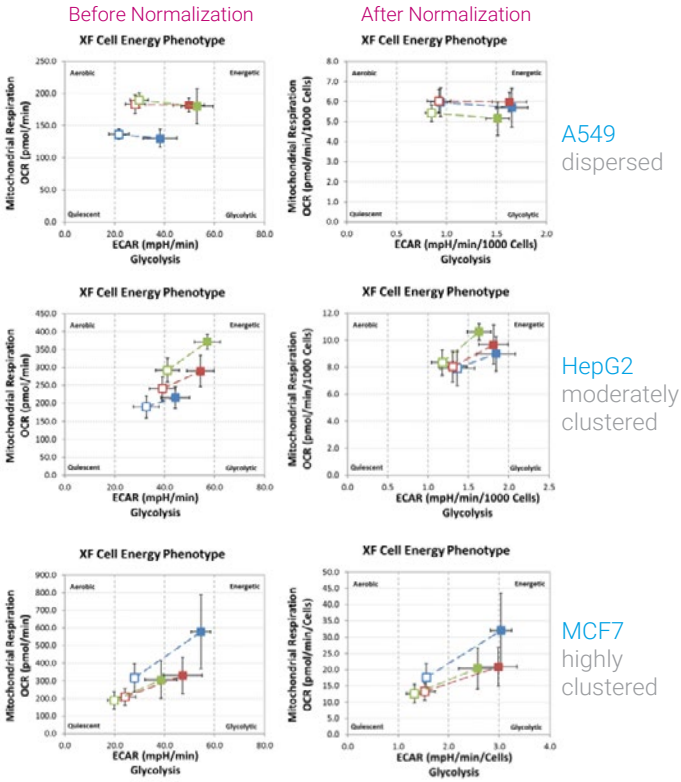

Figure 3. Normalization of the XF Cell Energy Phenotype Test data from multiple cancer cell lines on XF24 plates. Cell energy phenotypes of three different cell lines seeded at three different cell densities were obtained by using XF Cell Energy Phenotype Report Generator. The phenotype diagrams before (left column) and after (right column) normalization are compared for each cell line. Data shown are mean ± SD, n = 6 technical replicates.

This protocol and workflow was further validated by using different cell types exhibiting varied morphological characteristics *in vitro*. Up to 16 cell lines including RAW264.7 with various nuclear sizes and distribution were seeded at five different cell densities and the metabolic phenotypes were examined by employing the XF Cell Energy Phenotype Test. As described above, Hoechst 33342 was included in the oligomycin/FCCP mixture. The data were normalized by the cell numbers calculated by the XF Imaging and Cell Counting software. Figure 2 shows a summary comparing the data from six representative cell lines before and after applying normalization. As shown, except for a few extreme cases, all basal metabolic rates converge well after applying the cell count normalization factor. A similar outcome was obtained from cells seeded on Agilent Seahorse XF24 Microplates at three different densities (Figure 3).

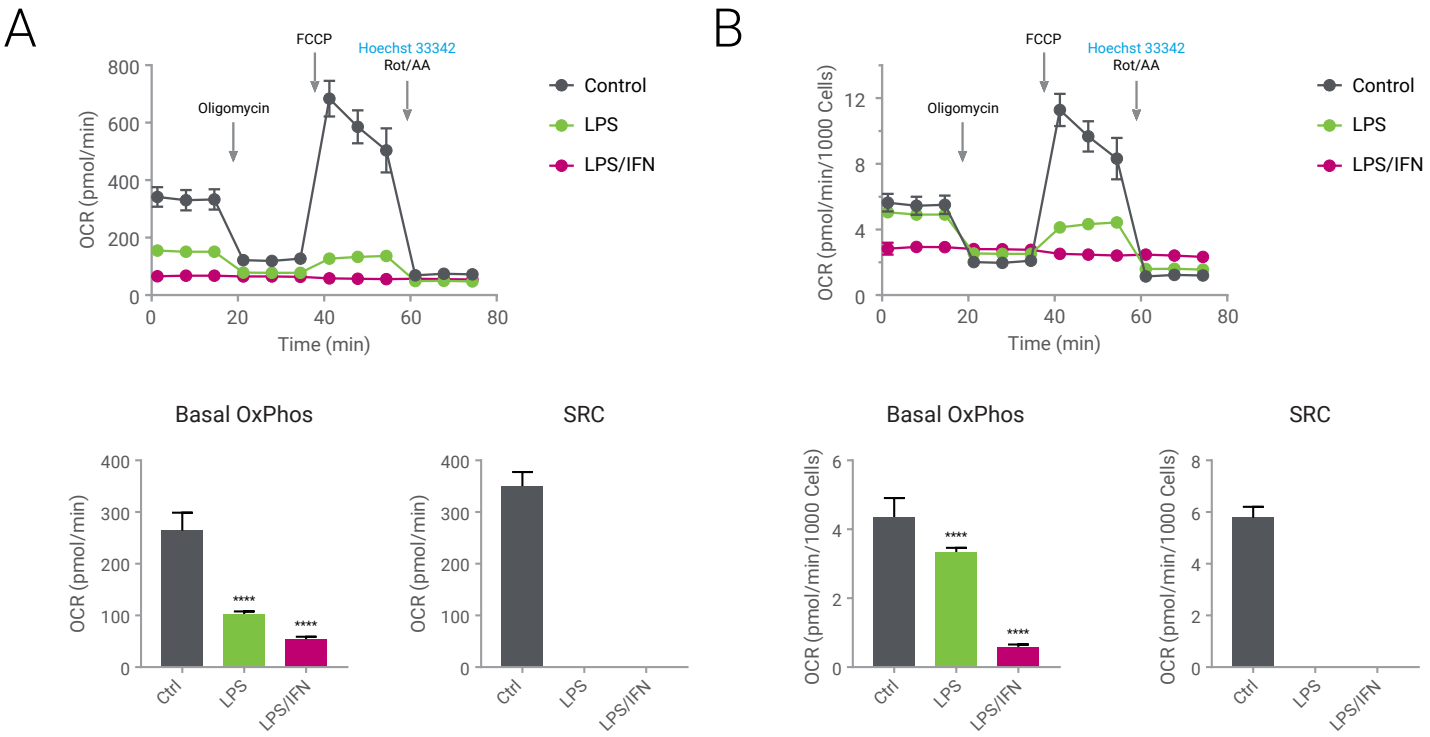

Figure 4. Data normalization effect on the XF Cell Mito Stress Test results. RAW264.7 cells were cultured overnight in the presence of 100 ng/ml LPS alone or together with 20 ng/ml IFNγ and mitochondrial function was analyzed by the XF Cell Mito Stress Test. A) before normalization; B) after normalization. Data shown are mean ± SD, n = 5 technical replicates (ns, not significant; *, p < 0.05; **, p < 0.005; ***, p < 0.0005; ****, p < 0.00005).

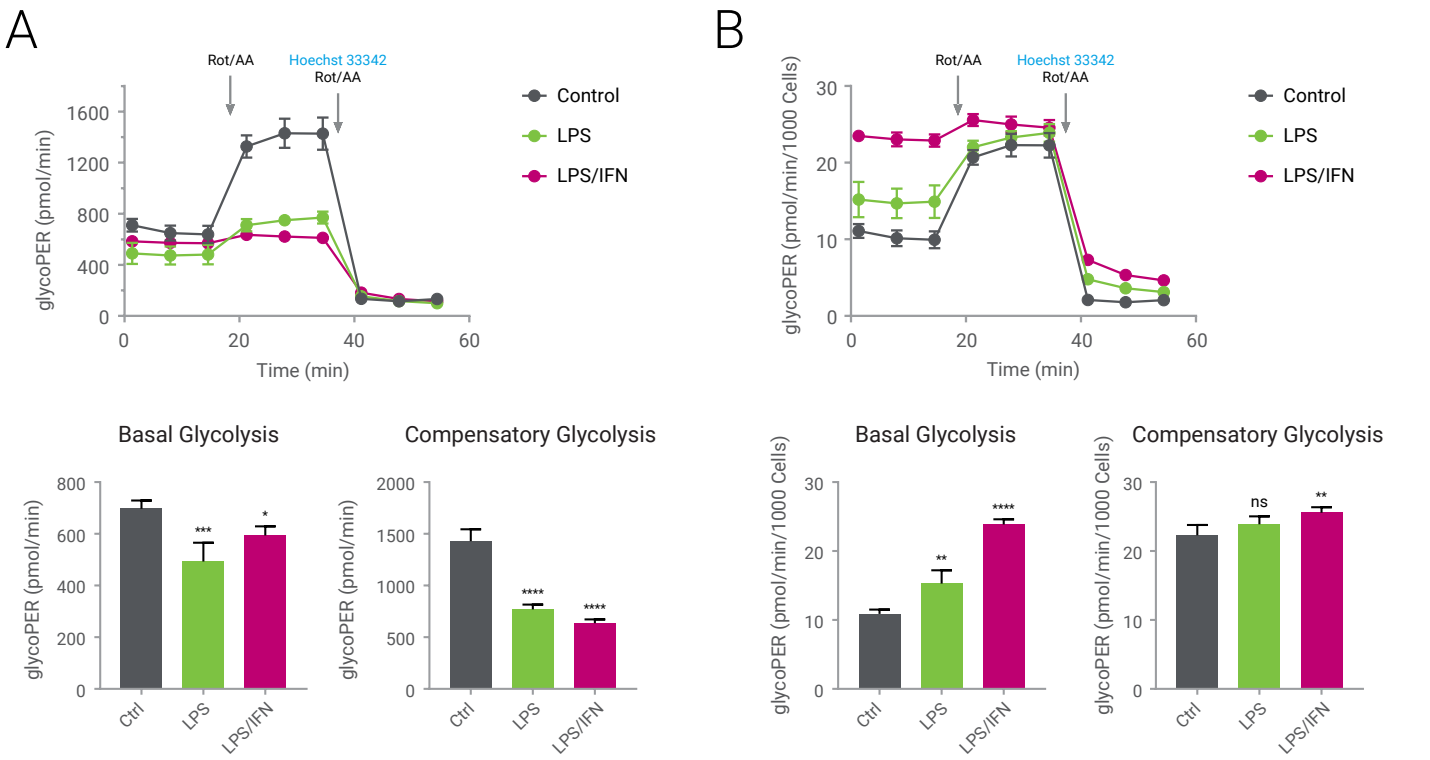

Figure 5. Data normalization effect on the XF Glycolytic Rate Assay results. RAW264.7 cells were cultured overnight in the presence of 100 ng/ml LPS alone or together with 20 ng/ml IFNγ and glycolytic activity changes upon macrophage activation were analyzed by the XF Glycolytic Rate Assay. The overall glycoPER kinetic data as well as the basal and compensatory glycolytic rates were compared; A) before normalization; B) after normalization. Data shown are mean ± SD, n = 5 technical replicates (ns, not significant; *, p < 0.05; **, p < 0.005; ***, p < 0.0005; ****, p < 0.00005).

Data normalization leads to correct data interpretation

A macrophage is a type of immune cell well known to exhibit metabolic plasticity and a dynamic metabolic phenotype switch that is associated with activation. RAW264.7 murine macrophage is widely used as a model for *in vitro* proinflammatory activation by pathogenic stimuli. *In vitro* exposure to bacterial lipopolysaccharide (LPS) and/or cytokine interferon-γ (IFNγ) triggers an activation-associated metabolic change of RAW264.7 macrophages from an oxidative to a glycolytic phenotype. This is demonstrated by an increase in ECAR (or PER) and decrease in OCR[5]. The metabolic rates, however, should be normalized for quantitative comparison because macrophage activation is tightly associated with proliferation rate change, with proliferation severely reduced upon activation.

Figure 4 is an example of how data normalization affects the data interpretation of the XF Cell Mito stress test results[1]. Figure 4A is the data summary before normalization, and both basal mitochondrial respiration and spare respiratory capacity (SRC) were severely suppressed either by LPS alone or LPS/IFNγ costimulation. However, the data interpretation turns out to be different when the data is normalized by cell number. Although LPS alone suppressed mitochondrial respiration, the effect was rather specific to the SRC, while a significant amount of basal respiration was maintained. In contrast, LPS/IFNγ costimulation suppressed mitochondrial function completely, including the basal respiration (Figure 4B).

Interpretation of the XF Glycolytic Rate Assay[1] results was more dramatically affected by data normalization (Figure 5). Without normalization, the glycolytic rates of macrophages appeared to be downregulated slightly for the basal measurement and more severely for the compensatory activity upon activation (Figure 5A). By considering the XF Cell Mito Stress Test results shown in Figure 4A, the overall metabolic suppression seemed to happen without any significant phenotype switching. However, according to the normalized data shown in Figure 5B, the basal glycolysis was significantly up-regulated by LPS stimulation and IFNγ costimulation maximized the basal glycolytic rate. Interestingly, the change in compensatory glycolysis was minimal even with complete suppression of mitochondrial respiration when treated with LPS/IFNγ (Figure 4).

Metabolic switching can be more precisely characterized by comparing the contribution of mitochondrial respiration and glycolysis on ATP production. Figure 6 is an Agilent Seahorse XF Real-Time ATP Rate Assay[1,4] result showing that LPS stimulation-made macrophages relied more on glycolytic ATP production, while LPS/IFNγ co-stimulation-made macrophages were completely dependent on glycolysis. Without normalization, the total ATP production rates seemed to be suppressed upon activation. However, the data revealed that total ATP production rates per cell were kept stable, and it became evident that the cells exhibited an extremely glycolytic phenotype due to the mitochondrial downregulation.

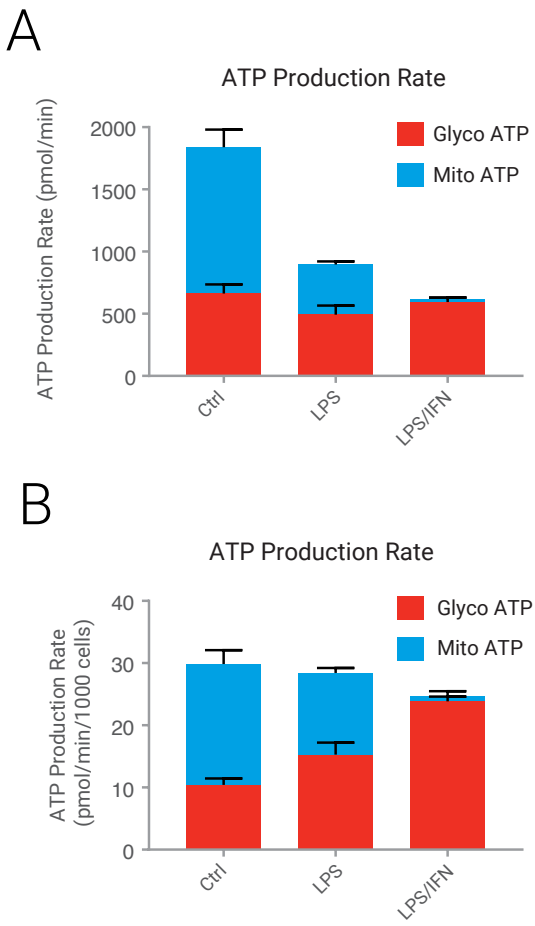

Figure 6. Data normalization effect on ATP production rate comparison. The contributions of mitochondrial respiration and glycolysis on ATP production were compared after overnight activation of RAW264.7 macrophage; A) before normalization; B) after normalization. Data shown are mean ± SD, n = 5 technical replicates.

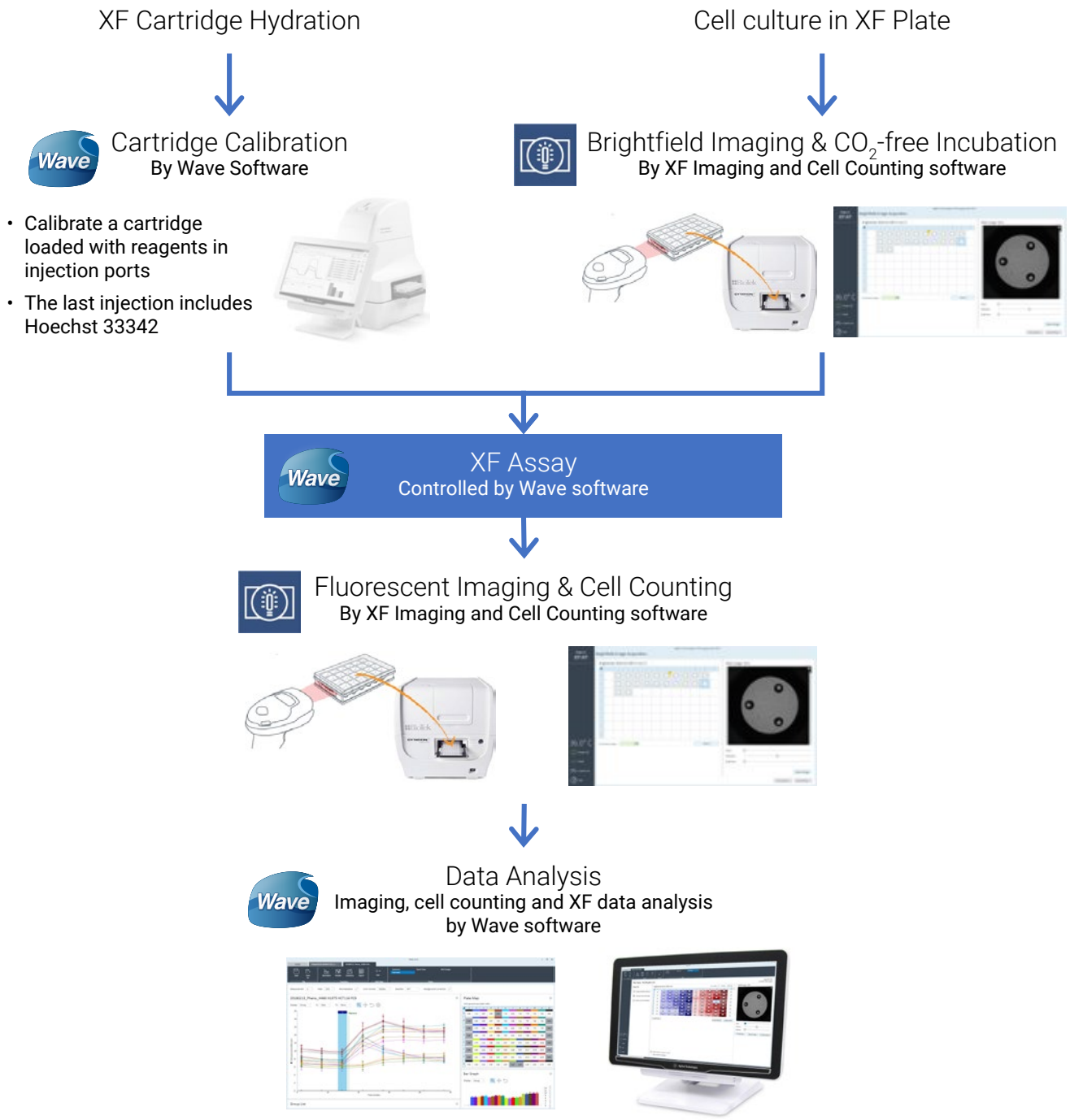

Figure 7. A typical workflow of the XF Imaging and Normalization system

Cell counting and data normalization workflow

Fluorescent staining of cell nuclei is a widely used method to identify individual cells, because it allows cells to be segmented into individual objects in a microscopic image for most two-dimensional cell cultures. Hoechst 33342 is the most commonly used membrane-permeable blue dye that can stain live cell nuclei, which can then be imaged and analyzed microscopically or counted by flow cytometry. In the recommended workflow, Hoechst 33342 is combined with the final reagent in an XF assay kits injection strategy. The dye is directly injected together with the kit reagent(s) for XF analysis (e.g. rotenone/antimycin A mix in the XF Cell Mito Stress Test and the XF Real-Time ATP Rate Assay, 2DG in the XF Glycolytic Rate Assay, etc.). The injected dye binds to nuclei during the last three to five measurement cycles. Because Hoechst 33342 labeled nuclei can be detected and identified without any additional washing step, cells can be imaged immediately by the Cytation 1/5 upon the completion of an XF assay. These cell numbers are calculated by the XF Imaging and Cell Counting software and used to transform XF data as accurate and reproducible normalization factors (Figure 7).

Considerations for accurate cell counting

Although the software provides an optimized cell counting solution for most cell types used for XF analysis by way of an automatic thresholding algorithm, there are several factors that should be considered to generate accurate cell counts for best normalization performance. It is critical to have detectable nuclear objects uniformly stained for better accuracy of cell counting. Reviewing the masked image in the XF Imaging and Cell Counting software is recommended. The masked image is an image layer that represents the algorithm's nuclei detection, which is superimposed on the raw fluorescence image and verifies that all valid objects are identified by the software. This review function is available after the completion of the postprocessing step of counting cells in the XF Imaging and Cell Counting software. If a significant number of cells are not identified in the masked images, users need to consider the options described in the following paragraphs to normalize the data.

First, some cell types are not suitable for *in situ* nuclear staining. For example, Hoechst 33342 can be exported by ABCG2 transporter, which is expressed in hematopoietic stem cells and results in poor retention of the dye[6]. For this kind of cell type, *in-situ* staining may not be applicable, and a staining after fixation may be preferred. The imaging and counting can still be performed in the XF Imaging and Cell Counting software and data can still be automatically linked to the corresponding XF data by utilizing the common bar code read in the database.

Second, inconsistency in nuclear staining intensity can also affect nuclear identification. Similar to other DNA-binding dyes, Hoechst 33342 has a higher affinity to condensed chromosomes, which are commonly found in apoptotic cells[7]. If the cell sample exhibits severe differences in fluorescence intensity between healthy cells and dying cells, the automatic thresholding algorithm may not work properly. Users may need to reanalyze the image data in the Gen5 software from BioTek Instruments and customize the cell counting parameters, including the threshold setting. The counting result obtained by Gen5 can be transferred manually to XF data in Wave.

Third, the cell seeding condition is also a critical factor affecting normalization performance. Cells should be seeded subconfluently with an even distribution for accurate normalization and optimized XF assay performance. In contrast to brightfield imaging, which provides whole-well images, the XF Imaging and Cell Counting software uses a single image capturing the center portion of each well. The nuclei are counted within this image and then the software calculates the total number of cells per well by extrapolation. The imaged center portion closely overlaps with the area where oxygen and pH are measured most effectively. Thus, similar to the OCR and ECAR calculation, automatic cell counting is performed under the assumption that cells are evenly distributed across the entirety of each well. The cell seeding condition can be reviewed by using brightfield imaging and any error-prone wells can be identified and removed if necessary[2].

Finally, any large foreign object in or under the well can affect the fluorescence images by scattering the excitation light. *In situ* staining can prevent the occurrence of foreign objects by eliminating exposure of the XF plate to an open environment during the staining. Also, any large object contamination can be reviewed by brightfield and fluorescence images in the XF Imaging and Cell Counting software as well as in the Wave software.

Protocol modification

The nuclear staining procedure can be modified depending on the user preference. Hoechst 33342 can be injected separately within the XF assay after the whole analysis, if there is an injection port available (e.g. Hoechst 33342 injection through port D after a standard running of the XF Cell Mito Stress Test). However, when an additional injection is added in the standard protocol, data compatibility needs to be verified if users want to run an XF Report Generator. For example, an additional injection of Hoechst 33342 after 2DG in the XF glycolytic rate assay is not compatible with the XF Glycolytic Rate Assay Report Generator, because it uses the last measurements as a reference value to assess the basal and compensatory glycolytic rates.

Conclusion

The XF Imaging and Normalization system provides an easy, rapid, and seamless workflow to reproducibly generate cell counts per well for performing normalization of XF data, by running XFe96 or XFe24 Analyzers with the Cytation 1/5 through a single XF 64-bit controller. Cell images and counts are obtained by the XF Imaging and Cell Counting software and the data automatically synchronizes and is documented within the Wave XF result file. This enables users to more successfully perform XF assays with data integrity assurance for achieving the most optimized and valid data interpretation.

References

1. http://www.agilent.com/chem/discoverxf
2. http://www.agilent.com/chem/normalization
3. https://www.agilent.com/en/support/cell-analysis/seahorse-xf-report-generators
4. http://www.agilent.com/chem/realtimeatp
5. http://www.agilent.com/chem/immunology
6. Scharenberg, C.W., Harkey, M.A. and Torok-Storb, B. (2002) The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood 99, 507-512.
7. Ellwart, J.W. and Dörmer, P. (1990) Vitality measurement using spectrum shift in Hoechst 33342 stained cells. Cytometry 11, 239-243.

www.agilent.com/chem/discoverxf

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