Technical Overview

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Methods and strategies for normalizing XF metabolic data to cellular parameters

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Importance of Data Normalization

Normalization of functional biological data is a key component in the workflow for performing and/or subsequent analysis of raw data to ensure accurate and consistent interpretation of results. XF metabolic assays are no different in this aspect, and some form of normalization is required for most experiments performed. Whether comparing different cell types, genetic modifications or compound treatments, the data must be normalized to a common shared parameter for correct comparison. Normalization of XF assays can be applied on several levels, including cell number, genomic DNA, and total cellular protein. This document focuses primarily on methods that use cell number (or a surrogate for cell number) to normalize XF rate data (OCR, ECAR, PER).

Factors Affecting Cell Density and Cell Proliferation Rates

When preparing for an XF assay, a variety of factors can affect the cell density (number of cells per well), including: proliferation rate, degree of cell differentiation, rate of cell death and plating, or cell adherence efficiency.

Proliferation rates are critical as most anchorage-dependent cells require at least an overnight culture prior to an XF assay, and cell number can change during this culture period. It is especially important to know the proliferation rate when interventions (e.g. genetic modifications, chronic drug treatments, etc.) are being introduced, as these often result in changes in cell growth rates and thus must be taken into consideration when analyzing and interpreting XF data.

Understanding the growth rate of the cell type of interest may be determined empirically by charting cell number vs. time. An ideal strategy is to plate the proper number of cells per well by considering any differences in doubling times among experimental groups; thus minimizing variations in the cell number across groups at the time of the XF assay. Any variations in final cell count which cannot be controlled can be normalized by measuring cell number or cellular contents per well. Another important consideration is keeping the culture time between cell seeding and the XF assay constant if similar types of XF assays are to be performed over a span of days or weeks.

Methods of Normalization

Total Cellular Protein

Normalizing to total cellular protein is relatively quick and inexpensive, and can be used with almost any standard microplate reader. Cells are lysed, and typically a portion of the well content is used for quantitation via Bradford or BCA protein detection reagents. It is recommended to always perform a standard protein concentration curve to ensure accurate quantitation and allow absolute comparison of data from assay to assay. Figure 1 shows raw OCR and ECAR data that has been normalized to total cellular protein.

While straightforward, this method makes the implicit assumption that any intervention made to the cells does not alter total cellular protein content significantly. This normalization method can become problematic if treatment of the cells causes shifts in mitochondrial biogenesis, which can alter the protein content of the cell, and true differences in activity can be concealed [1]. Assessing mitochondrial biogenesis is discussed in more detail below. Normalization using total protein is also not applicable if there are there significant variations in the amount of extracellular matrix protein present among different experimental groups or if plates are coated with protein containing cellular adherents (e.g. collagen, laminin, Matrigel®).

Nuclear DNA

In cases where total protein or cell counting may not be relevant or feasible, nuclear DNA content per well may be used to normalize XF rate data [2]. This method is based on the assumption that, unlike certain instances with total cellular protein described above, nuclear DNA correlates linearly with cell number. Various fluorescence or colorimetric dyes that incorporate into dsDNA are typically used to quantitate nuclear DNA. References [3] and [4] provide a thorough review of these methods and dyes, including exemplary data with PicoGreen and CyQuant reagents. As with a total protein assay, a standard curve using a reference dsDNA (e.g. Lambda DNA) is recommended to ensure accurate quantitation and allow absolute comparison of data sets.

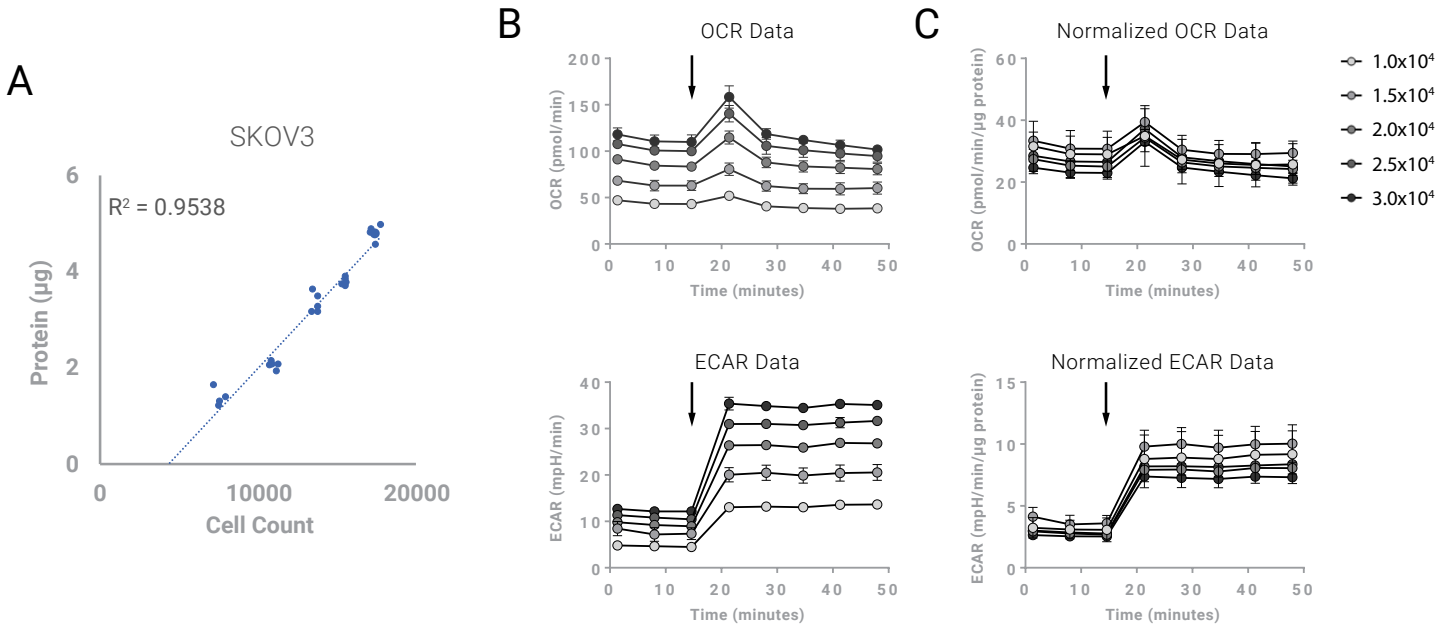


Figure 1. Example of XF data normalization using total cellular protein from SKOV3 cells. Cells were plated at 1x10⁴, 1.5x10⁴, 2x10⁴, 2.5x10⁴, 3x10⁴ cells per well in XF96 tissue culture microplates (n=6), cultured for 24 hours, followed by assessment of basal and stressed OCR and ECAR (stress induced by 1.0 µM oligomycin + 0.5 µM FCCP, final, arrows). A) Correlation of cell number counted using Cytation 1 vs. total cellular protein values shows a linear relationship. B) Raw OCR and ECAR values for basal and stressed rates at different plating densities. C) OCR and ECAR values for basal and stressed rates at different plating densities normalized to total cellular protein. (Mean ± SD, n=6)

Counting of Cells

The most robust normalization method for XF metabolic rate data involves counting of cells in each well of the microplate via direct imaging of the cells or imaging stained nuclei. Both imaging methods rely on dedicated high-throughput, automated imaging instruments. A number of imaging systems may be used for counting cells directly post XF assay, including the BioTek Instruments' Cytation 1, which may be used for both direct cell counting and counting nuclear stained cells.

Imaging and quantifying cell number using a cell permeable nuclear stain has advantages over direct cell imaging in that the workflow is simpler (e.g. no requirement to fix cells) and can be automated with no need to prepare reference samples. Because direct counting of cells is mediated by microscopic image capture followed by image analysis, it is best applied when cells are well-dispersed and show clear defined morphology (e.g. A549 or SKOV3). This method is better for less-well dispersed cells, or those with clustered morphology (e.g. MCF7). In addition, the non-destructive nature of this protocol makes it compatible with other downstream analyses, such as measuring total protein, PCR or immunostaining. Note that cell permeable nuclear dyes may be injected directly onto the cells *in situ* via an injection port on the XF cartridge, or can be applied post XF assay if all four injection ports are used.

Figure 2 shows an example of *in situ* nuclear staining and segmentation using the Cytation 1, then used for normalization of XF Cell Energy Phenotype Test data using SKOV3 cells. Figure 3 shows further examples of normalization using three different cell types varying in morphology and nuclear size.

More detailed aspects of these imaging/normalization methods, including workflows and comparative examples, may be found at: "Normalization of Agilent Seahorse XF Data by In-situ Cell Counting Using a BioTek Cytation 5" (http://www.agilent.com/cs/library/applications/5991-7908EN.pdf).

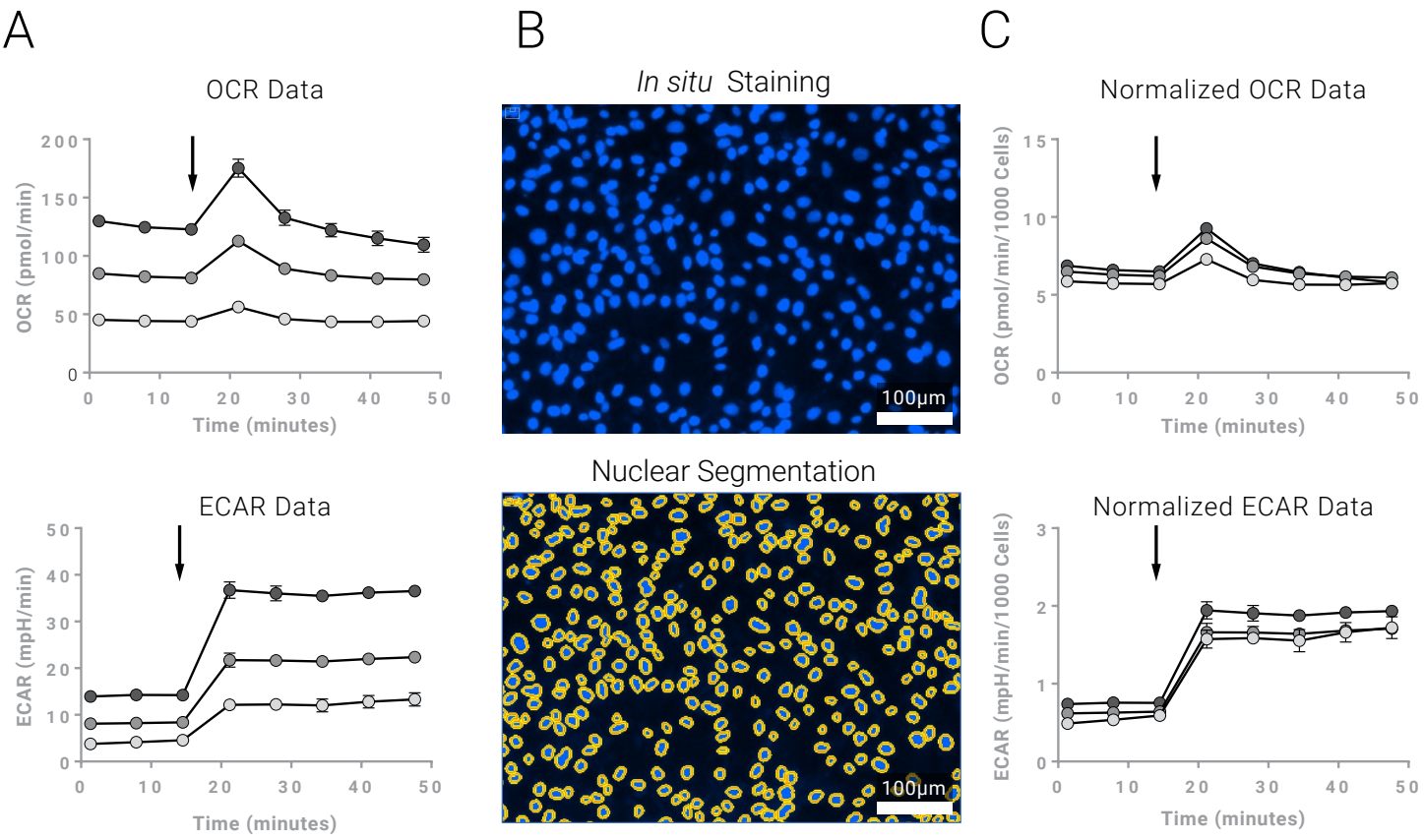


Figure 2. Example of XF data normalization using *in situ* nuclear staining and *in situ* cell counting. SKOV3 cells were plated at $1x10^4$, $2x10^4$, $3x10^4$ cells per well, cultured 24 h, subject to the XF Cell Energy Phenotype Test and followed by image analysis. A) Raw OCR and ECAR change with injection (arrows) of oligomycin + FCCP (1.0 µM and 0.5 µM final, respectively), including 20 µM Hoechst 33342 (2 µM final). B) Representative images of nuclei fluorescently labeled by Hoechst 33342 (upper panel) and nuclei identified and outlined using the Cytation 1 (lower panel). C) OCR and ECAR normalized by *in situ* nuclear staining cell counts (Mean ± SD, n=4).

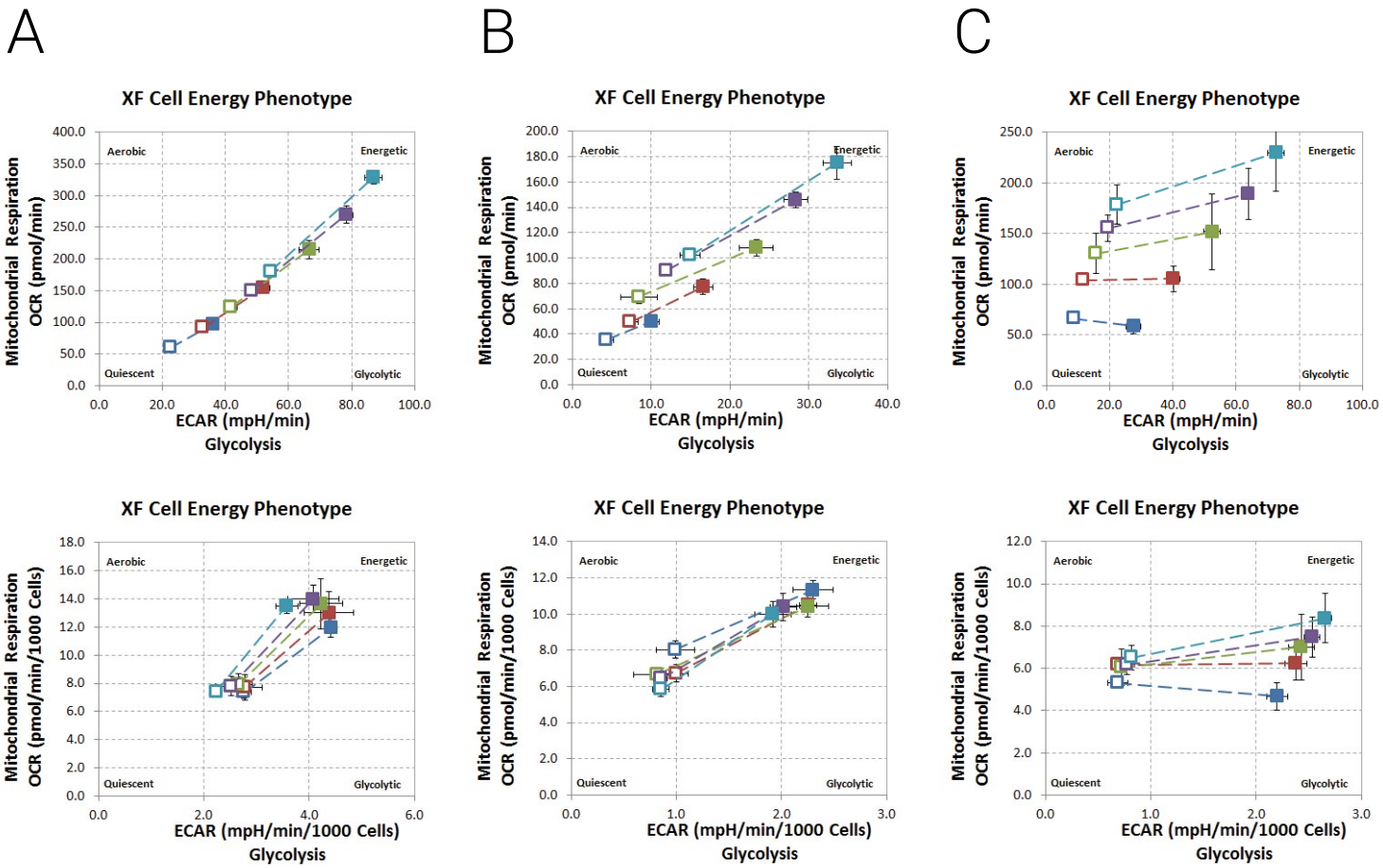


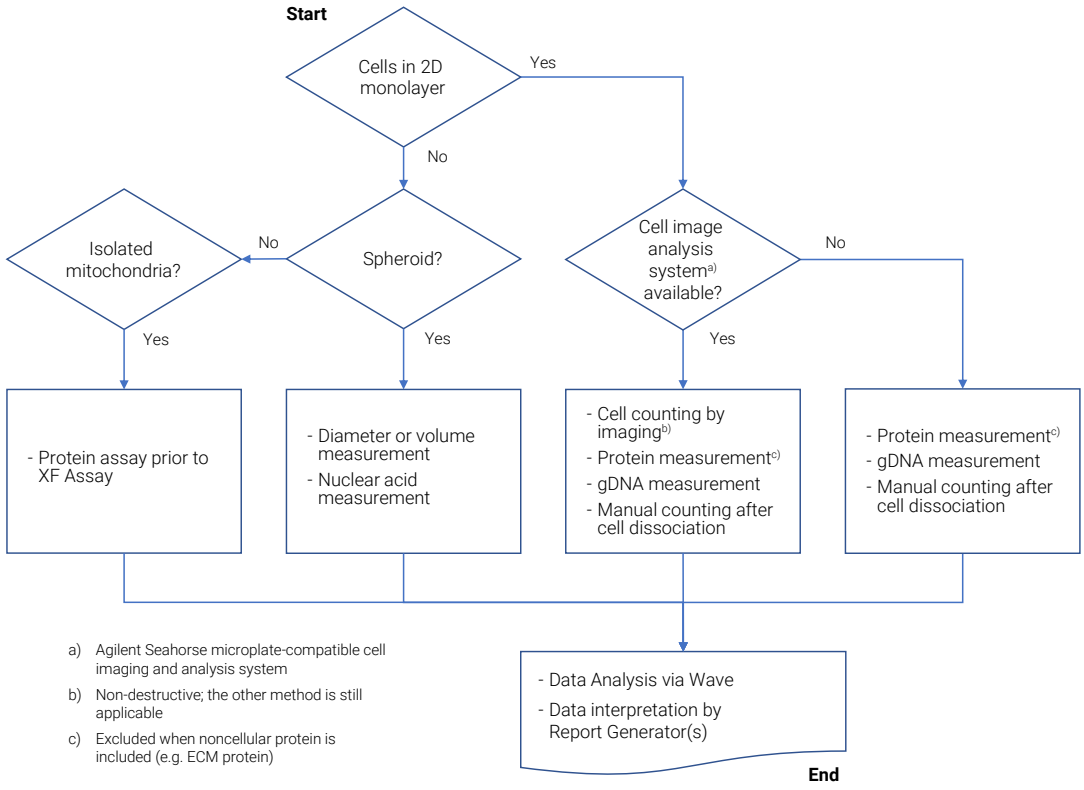
Figure 3. Example of XF data normalization using *in situ* nuclear staining of HT-29 (A), MCF7 (B), and RAW264.7 (C) using the Cytation 1. Cells were seeded at $1x10^4$, $1.5x10^4$, $2x10^4$, $2.5x10^4$, $3x10^4$ cells per well for HT-29 and MCF7, and at $1.5x10^4$, $2.8x10^4$, $3.0x10^4$, $3.8x10^4$, $4.5x10^4$ cells per well for RAW264.7 in XF96 tissue culture microplates and cultured for 24h. XF Cell Energy Phenotype Test Kit performed with injection of oligomycin + FCCP (1.0 µM and 0.5 µM final, respectively) including 20 µM Hoechst 33342 (2.0 µM final). XF Energy Maps generated by Seahorse XF Cell Energy Phenotype Report Generator are compared before (upper panels) and after (lower panels) normalization (Mean ± SD, n=6).

Special Cases:

- Non-proliferative cells: including primary and/or post-mitotic cells that are cultured for a period of time, but do not replicate (e.g. cortical neurons, neonatal rat ventricular myocytes, brown adipocytes, differentiated iPSCs, etc.[5, 6]). Typically, cells are counted before seeding into XF Tissue Culture Microplates to provide an initial value. However, it is recommended to perform some relevant method of normalization post-XF assay to account for any potential loss of cells due to detachment or loss of viability over the time course of the culture.
- Acutely attached cells: some cells or XF applications require cells to be acutely adhered, usually via centrifugation, to the XF Tissue Culture Microplate, (e.g. the T cell Activation Assay http://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-00c1/t/page/fm/1). In these cases, quantitative cell counting before the assay is typically performed and a known number of cells is introduced into each well. Again, it can still be valuable to perform a post assay assessment of well content to account for any potential loss of cells due to detachment during the assay.
- Normalization of 3D samples, such as spheroids, may be based on size or volume of the sample. Spheroids are typically grown in a separate vessel, beginning with several hundred to several thousand cells. While more difficult to assess by total protein, nuclear DNA or cell count, using geometric parameters such as spheroid diameter, total spheroid volume may be calculated and used as a normalization parameter [7].
- Isolated Mitochondria or Synaptosomes: use of isolated mitochondria or synaptosomes in the XF instruments requires quantifying the sample protein content prior to the XF assay and seeding an optimized amount. In these case, post-assessment of the mitochondrial or synaptosomal protein, and thus normalization, is typically not required [8, 9].

Choosing the Most Relevant Normalization Method

The initial choice of normalization method often begins with the type of sample being analyzed. The scheme below illustrates a decision-making process for choosing an optimal method. The normalization techniques described here each have their respective advantages and disadvantages, and no single normalization method is universally applicable for every experimental design and subsequent analysis.



In many cases, more than one normalization method can be applied. However, any method quantifying cell number based on cellular metabolism (e.g. MTT assay, total ATP level) is not recommended as XF assays are specifically designed to measure cellular metabolism, and thus a normalization technique independent of metabolic function should be applied. Table 1 below provides key advantages and disadvantages of the normalization methods presented above:

Normalization Method	Advantages	Disadvantages
Total Cellular Protein	- Inexpensive - Compatible with most plate readers	- Sample transfer can introduce error - Incompatible with ECM coated plates - Cells destroyed to obtain protein - Incompatible if there are changes in mitochondrial biogenesis
Genomic DNA	- Compatible with most fluorometric plate readers	- Sample transfer can introduce error - Incomp atile with multi-nucleated cells - Cells destroyed to obtain nuclear DNA
Cell Imaging	- Most direct method of obtaining cell and/or nuclei counts - No processing after XF assay - Cells remain viable for downstream applications - Compatible with ECM coated plates	- Requires dedicated cell counting instrumentation

Note that central to any normalization method used is the assumption that a linear relationship exists between cell number and signal being measured; the amount of analyte on a per cell basis remains unchanged. This assumption, though, is not always valid. For example, a cell that has increased metabolic activity via mitochondrial biogenesis will have a higher OCR on a per-cell basis, however, this difference in respiration may be underestimated or even concealed if total cellular protein was the normalization method applied. As stated above, if mitochondrial biogenesis is suspected, total cellular protein should not be used for XF assay data normalization, but rather genomic DNA, or preferably, cell number.

Cell Number vs. Cell Viability

Another important aspect to consider when normalizing XF data is the relationship between cell number and cell viability, i.e. what percentage of the cells in each well or treatment group are viable? This becomes especially important when orthogonal measurements of cell proliferation and/or cytotoxicity are used in conjunction with XF data. If measuring cell viability is required, it is critical to use a method that is not affected by acute treatments with any XF assay kit reagents, which can inhibit mitochondrial and/or glycolytic function. In particular, this includes viability assays dependent on cellular NAD(P)H oxidoreductases, such as MTT and MTS assays. Caution should also be exercised if measuring total cellular ATP levels as a proxy for cell viability/proliferation, as recent investigation has demonstrated discrepancies when correlating cellular ATP (and MTT) to absolute cell numbers [10]. Alternative viability assays, including the MultiTox-Fluor Cytotoxicity Assay, are compatible with XF assays reagents and may be used post-XF assay to obtain the ratio of live to dead cells. Note that cell viability is most often expressed as a relative ratio or percent, and thus the absolute number of cells must be measured for accurate normalization of XF data.

Additional Consideration for Normalization

As described above, there are cases in which certain methods of normalization should not be applied to XF data. These situations are often related to changes in mitochondrial number/mass per cell (i.e. mitochondrial biogenesis *v*. mitophagy), changes in expression of mtDNA encoded proteins and/or stoichiometry of mitochondrial electron transport and oxidative phosphorylation complexes (and even complex subunits) with respect to each other.

In these scenarios, total cellular protein should not be used for normalization, as important differences in cell biology could be masked. Use of cell counting and/or gDNA are applicable in these instances. If changes in mitochondrial number/mass are suspected, measuring relative changes in mtDNA or mtDNA : nDNA ratio via qRTPCR are applicable orthogonol verification methods [1, 11]. In these cases where mitochondrial mass/number changes, it is suggested to have a positive control of mitochondrial biogenesis (e.g. treatment of cells with AICAR, metformin, etc. [12]) to establish the dynamic range and sensitivity of cellular and mitochondrial responses. Detecting changes in relative amounts or stoichiometry of ETC/OxPhos complexes may be assessed by immunoblots of several electron transport chain proteins standardized to one or more cytoplasmic proteins [13, 14].

Apply Normalization in Wave and Using the Baseline Button:

The Wave software used to view XF data has a built in "Baseline" feature that transforms absolute XF rate data to a relative (%) scale. Most often, the baseline is set to the rate just prior to the first injection. Baselining data is most appropriate when attempting to minimize slight well to well differences in rate due to variations in cell seeding or proliferation, and is helpful to visualize changes in rates from acute treatments/injections.

The Normalization function in the Wave software provides a simple method to apply normalization data to the measured rate data (OCR, ECAR, PER). To use the normalization function, an independent assessment of the plate wells for cell number, protein concentration, DNA content is required as discussed above.

To normalize data in Wave, three components are used:

- Normalization Values (required): The numeric data generated from the independent assessment of the well (cell count, protein concentration, DNA content).
- Normalization Unit (required): This alphanumeric field describes the units to which the data are to be normalized. It comprises the unit of measure of the normalization values (such as "cells", "mg", "ng", and so forth).
- Normalization Scale Factor: This number determines what value the rate data will be scaled to. Default is 1 and adjustment is optional.

Please see: https://www.agilent.com/cs/library/usermanuals/public/S7894-10000_Rev_B_Wave_2_4_User_Guide.pdf for further details and information on applying normalization values in Wave.

This feature should not be considered a substitute for normalization, however, as critical information may be lost upon transformation (Fig. 4). Consideration should be taken regarding data presentation and the ability to compare results among laboratories, thus reporting of absolute normalized values is encouraged. For these reasons, the Baseline feature should be used only for initial comparison of groups that have exact same conditions at start of the assay, and a relevant method of absolute normalization should be applied.

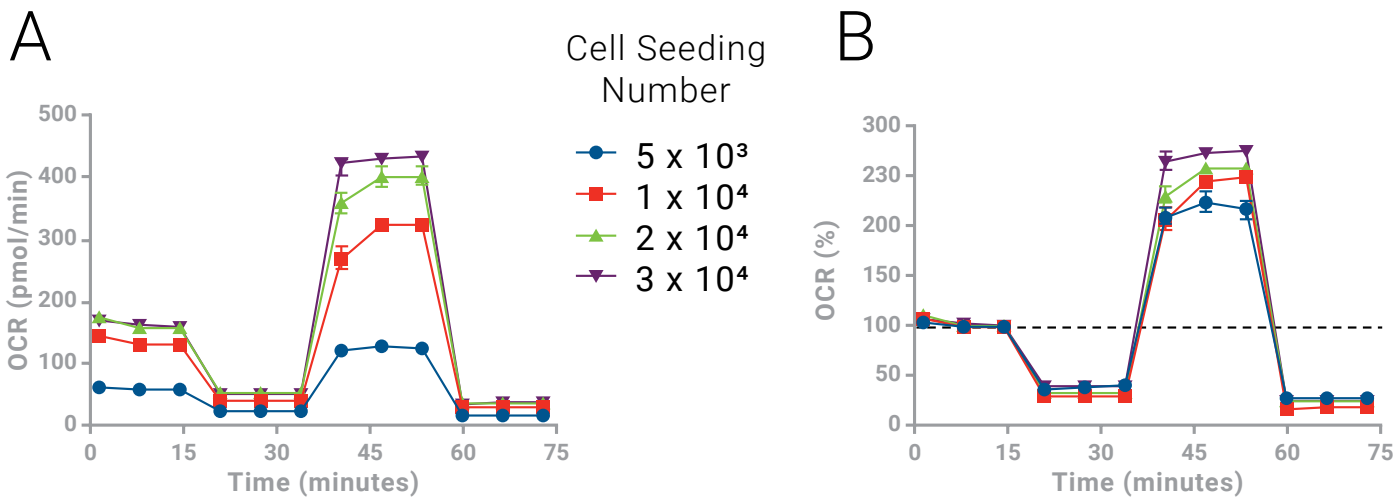


Figure 4. Absolute vs. Baselined OCR data: Panel A shows absolute OCR with a correlation of respiration rate to cell number. Panel B shows that for each cell density, responses to XF Cell Stress Test compounds are approximately equivalent, however information regarding differences in OCR with respect to seeding density is lost upon transformation with the Baseline feature.

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Printed in the USA, February 13, 2018
5991-8980EN

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