Normalization of Agilent Seahorse XF Data by *In-situ* Cell Counting Using a BioTek Cytation 5

Application Note

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Abstract

The Agilent Seahorse XF analyzer is a platform that measures cellular metabolic activity in multiwell microplates. Data normalization between different experimental conditions is commonly achieved using either protein assay or cell counting. We evaluated an XF data normalization protocol combining the *in situ* nuclear staining capability of the XF analyzer with BioTek Instrument's Cytation 5 system, which is able to count cellular objects *in situ*. Once the XF analysis is completed, an injection of membrane-permeable Hoechst 33342 from one of the remaining injection ports of the XF cartridge stains nuclei *in situ*. The fluorescently labeled nuclei can be imaged and counted on the Cytation 5 with no additional sample processing. In our trials using three morphologically distinct cell lines, we performed normalization of the basal oxygen consumption rates (OCR) and extracellular acidification rates (ECAR). The outcomes were highly comparable to protein assay. The workflow combining the post-XF analysis *in situ* nuclear staining and intact cell counting by Cytation 5 is a faster, more reliable data normalization procedure, and is also highly expandable for further downstream analysis.

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Introduction

Demand for performing quantitative measurements of cellular energy metabolism levels is increasing in many biomedical research areas such as metabolic disease, cancer, immunology, and stem cell. The real-time measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using the Agilent Seahorse XF analyzer are widely accepted as the industry standard for obtaining *in vitro* cellular metabolic parameters. XF analyzers report OCR and ECAR for any given sample in the microplate well, as such data normalization is required when there is any significant variation in the cell number between wells or between experimental batches.

Data normalization based on protein amount is a widely used method mainly because it is available in most biological laboratories, and it provides a universal unit of protein quantity representing cell amount per well of most cell types. However, it requires additional procedures including washing, lysing, and transferring the sample, thereby increasing risk of sample loss especially for cells that are weakly adherent. *In situ* cell counting for performing XF data normalization has several advantages compared to other methods. First, it reduces sample processing steps and lessens the risk of sample loss. Second, it does not require a standard reference sample or curve (occupying additional wells in a multiwell plate format) every time one performs the assay. Third, because it is not an end-point measurement compared with other methods, downstream cellular analysis after cell counting is also possible. For example, protein assay is still feasible even after cell counting. Finally, the nuclear staining can be automatically performed by using the drug injection ports and functionality native to the Agilent Seahorse XF analyzer, resulting in an easier, faster workflow.

BioTek Cytation 5 is a multichannel cell imaging platform capable of automated cell counting from fluorescently labeled nuclear images. Total cell counts in each well can be obtained either from a whole well stitched image, or from extrapolating a single center image. By exploiting the extrapolation protocol, the cell amount in each well can be acquired in a shorter time period, and data can be normalized seamlessly. This application note validates the cell count-based XF data normalization protocol combining the *in situ* nuclear staining capability of the XF analyzer with the rapid cell counting function of Cytation 5 using three morphologically distinct cell lines.

Results and Discussion

Normalization of XF data from a dynamically proliferating cell type

C2C12 cells are known to proliferate rapidly, and the cell number increases by ~4 fold within a day in log phase, according to our measurements (Figure 1A). Therefore, the cell amount as well as the metabolic rates can change, even in a short period of time. As expected, the total OCR and ECAR were significantly increased within 6 hours by approximately 20 % (Figure 1B and C, left columns). The cell number increases correspond to the metabolic rate increases according to *in situ* cell counting. The total OCR and ECAR were normalized by the cell numbers, and proliferation-caused increases in OCR and ECAR disappeared (Figure 1B and C, right columns). Interestingly, both normalized metabolic rates per 1,000 cells slightly decreased by the additional 6 hour culture in the same condition.

In situ cell counting to normalize XF data difference caused by cell amount variation

A distinct advantage of the XF analyzer is its capability of delivering metabolic modulators or inhibitors through the injection ports of the sensor cartridges. The nuclei of a sample can be labeled *in situ* using the injection capability and delivering cell permeable dye such as Hoechst 33342 to the XF analyzer. As exemplified in Figure 2, an Agilent Seahorse XF Cell Energy Phenotype Test result from A549 cells plated at various cell densities was successfully normalized by *in situ* cell counts.

The Hoechst 33342 injection (red arrow) was followed by an oligomycin/FCCP injection (green arrow) for the nuclear staining *in situ*. After the analysis and nuclear staining, the XFp microplate was transferred to the Cytation 5, and the nuclear images were captured. As exemplified in the lower panel image, the individual nuclei were identified and counted by BioTek Gen5 software.

The raw value increase of OCR and ECAR were highly correlated to the seeding density increase before normalization (Figure 2A and B). In contrast, the variation in OCR and ECAR was significantly reduced after normalizing the data (Figure 2D). There was no significant difference in either OCR or ECAR by the Hoechst 33342 injection. *In situ* staining is also possible by co-injection of Hoechst 33342 with an oligomycin/FCCP mix (data not shown) instead of serial injections.

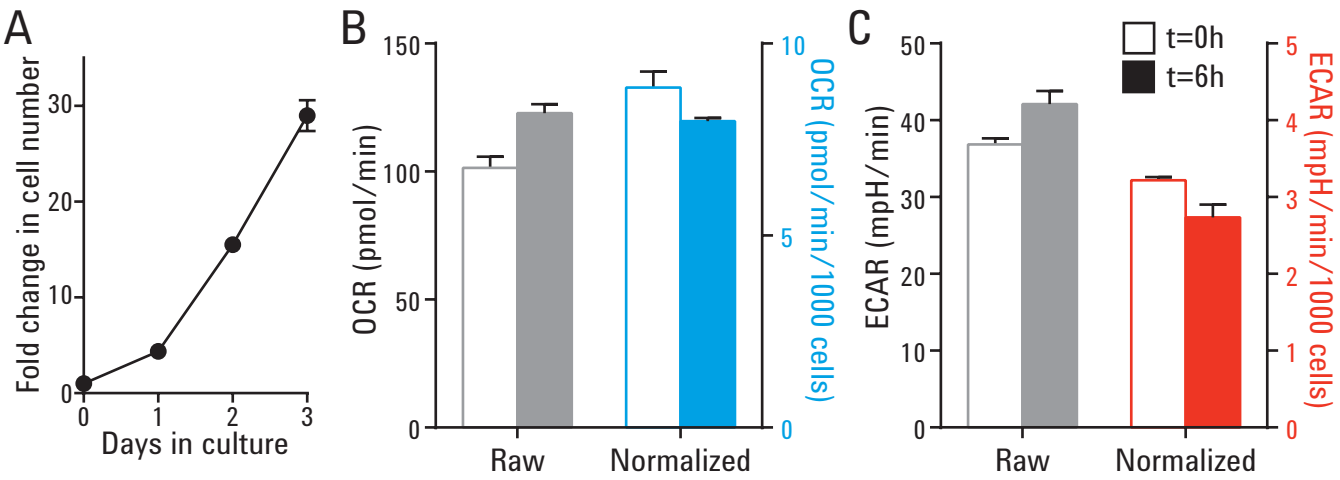

Figure 1. Normalization of XF data difference caused by rapid cell proliferation. A) Growth rate of C2C12 cell line. B) and C) Comparisons of OCR (B) and ECAR (C) of C2C12 measured at two different time points 6 hours apart. Open bars represent t = 0 hours, and filled bars represent t = 6 hours. Each data point was normalized by cell number counted (right axis), and compared with the data before normalization (left axis).

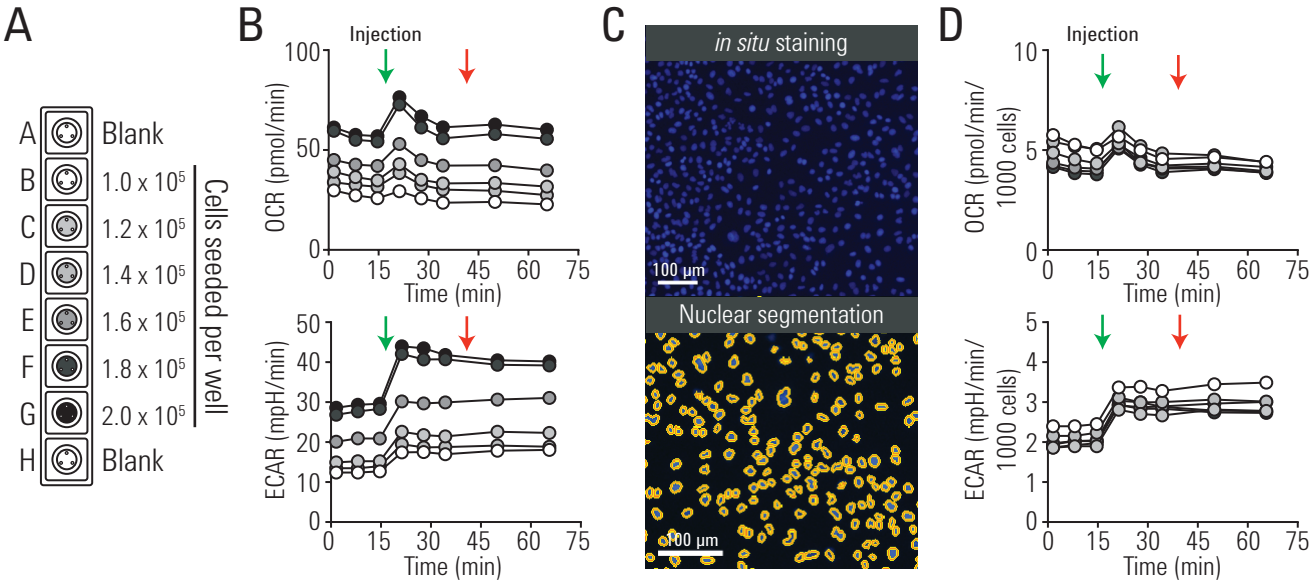

Figure 2. Example of XF data normalization using *in situ* nuclear staining and *in situ* cell counting. A) Seeding density variation of A549 on an XFp plate. B) Raw OCR and ECAR change with serial injections of oligomycin/FCCP (green arrow) and Hoechst 33342 (red arrow) before normalization. C) Representative images of nuclei fluorescently labeled by Hoechst 33342 injection (upper panel) and nuclei identified and outlined by Gen5 software (lower panel). D) OCR and ECAR normalized by *in situ* cell counts.

Partial counting and whole-well counting

The total cell number in each well was estimated by extrapolating the counts from a single center image in this normalization. The dimension center image captured by Cytation 5 was 2.876 mm^2, while the area covered by cells in each well was 10.67 mm^2, approximately 3.71 fold. To validate whether cell counts estimated from a single image can represent the total cell count in each well, we compared the partial counts with the counts from the stitched whole-well image in Gen5 software. All three cell lines we tested showed linear correlation between the partial and the whole-well counts (Figure 3).

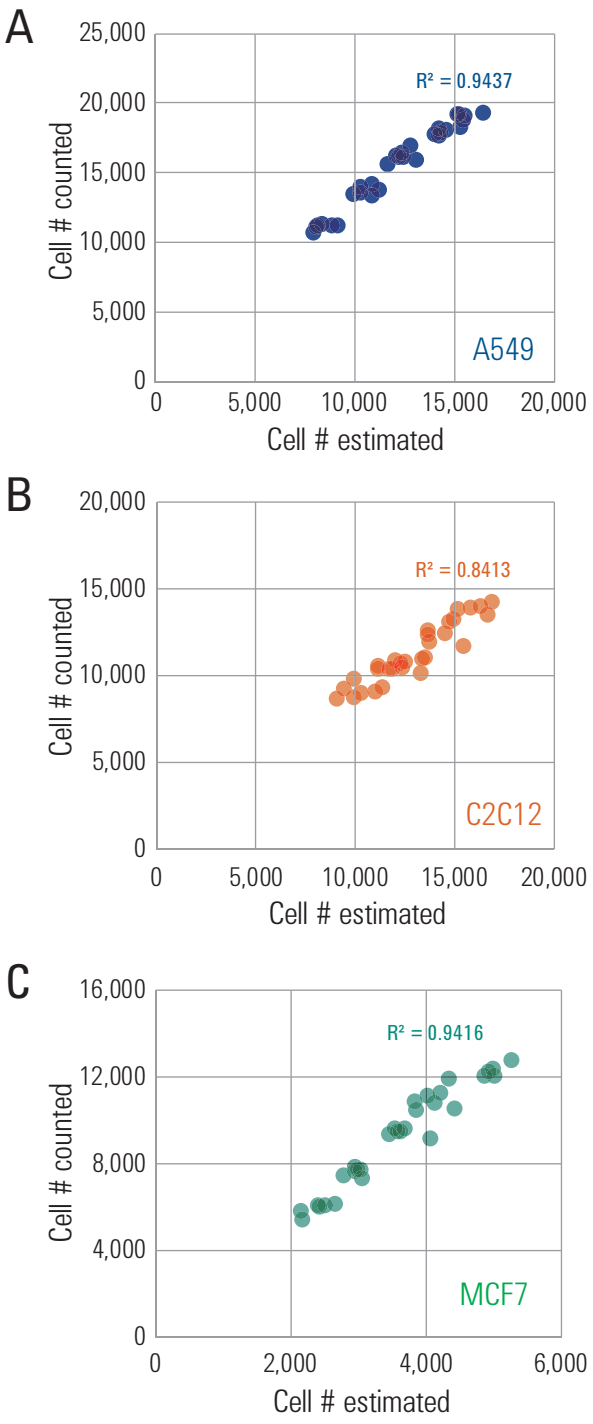

Figure 3. Comparison of the whole-well cell counts with an estimated cell number from the partial counting. A) A549, B) C2C12, C) MCF7.

Cell number assessment using fluorescence imaging and data normalization

To validate normalization efficacy, we normalized the basal OCR and ECAR of three cell types seeded at various densities. Since the cells were still intact after counting, we also measured the protein amount, as described in the Materials and Methods section, and compared the normalization outcomes (Figure 4). The difference in OCR and ECAR caused by varying the plating density were corrected by calculating the cell number as well as by protein assay, as expected. Notably, for all three cell lines, cell counts were closely correlated to both OCR and ECAR, and fit better than protein assay. In addition to the normalization of data variation caused by seeding density difference, normalization revealed that the MCF7 cell line is more oxidative and less glycolytic compared to the other two cell types.

Discussion

The metabolic rate difference caused by cell amount variation can be eliminated by cell count-based normalization according to the data obtained from their cell lines. Figure 1 shows that data normalization is highly required when the cell amount is variable or dynamically changing. It can be more critical when we compare the metabolic phenotypes between cell types that have proliferation rate or morphology differences. As exemplified in Figure 4, the less glycolytic and more oxidative nature of MCF7 cells cannot be assessed without normalization.

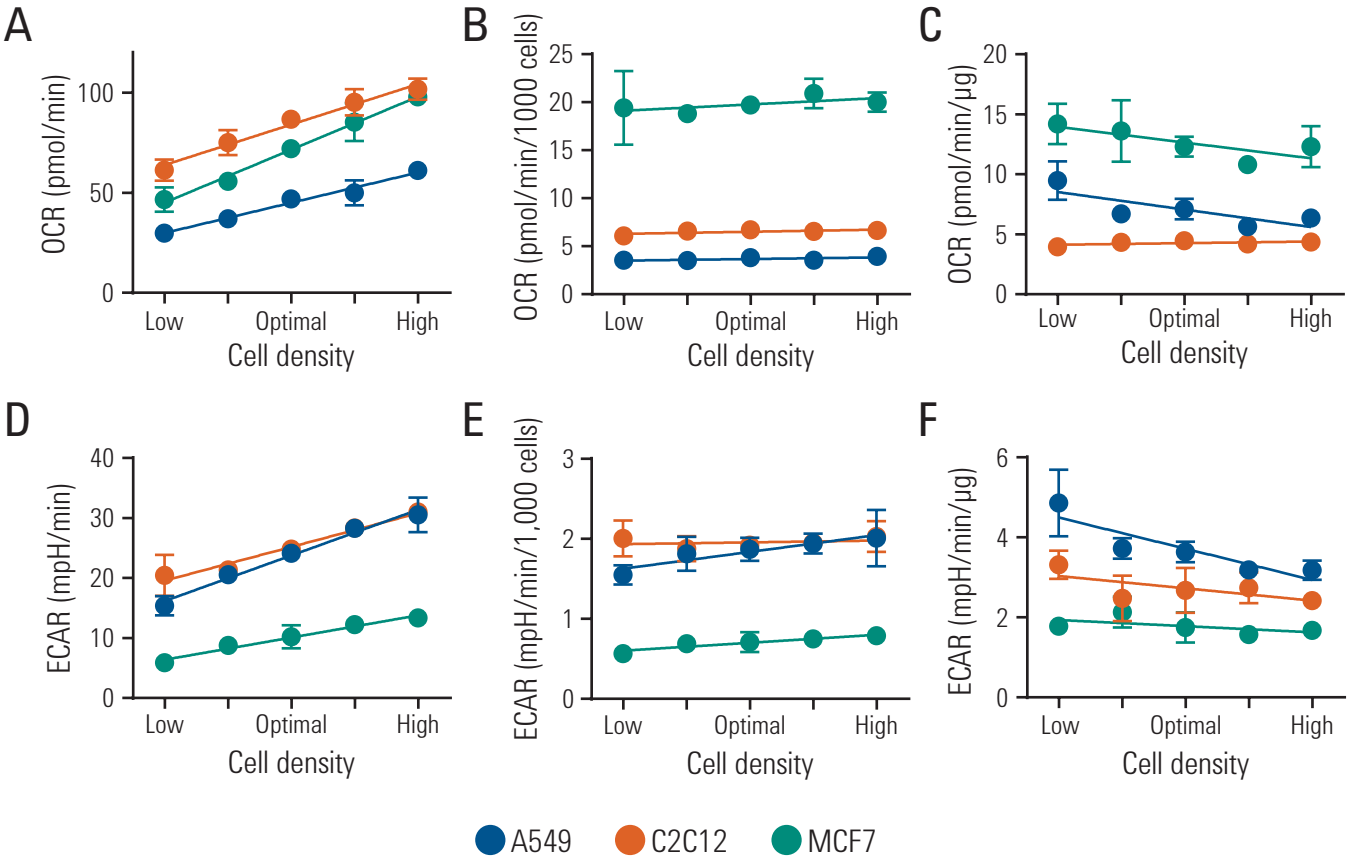

Figure 4. Normalization of basal OCR and ECAR of A549, C2C12, and MCF7 cells using cell counts or protein amount. A) OCR before normalization. B) OCR normalized by cell counts. C) OCR normalized by protein amount. D) ECAR before normalization. E) ECAR normalized by cell counts. F) ECAR normalized by protein amount. Seeding density variation; A549, 15,000 ±5,000 cells/well; C2C12, 12,000 ±4,000 cells/well; MCF7, 25,000 ±10,000 cells/well; (n >5).

Both protein assay and *in situ* cell counting appeared very effective to normalize the data. However, *in situ* cell counting is a more flexible approach because viable samples are still available after the counting, as shown in the workflow (Figure 5). As described above, by administrating Hoechst 33342 through the injection ports in the Seahorse XF analyzer, we can exclude any additional sample processing to count cells. It also reduces the chance of contamination from foreign objects, which can interfere with nuclear imaging and the resultant image analysis processing steps.

Although Cytation 5 is capable of counting the cell number within a whole well using the Montage function in Gen5 software, our findings recommend center image-based extrapolation. While the single image-based cell count can be representative of the whole-well cell count per well, the cell count from the well corners can be inaccurate particularly if cells have severe edge effects such as MCF7. Furthermore, the contribution of cells at the edge on OCR and ECAR readings is minimal, as the rate calculation is optimized for cells that are evenly distributed within the central region of each well. Single image-based cell counting also has a significant advantage in reducing the assay time and minimizing damage to cells by UV excitation during imaging.

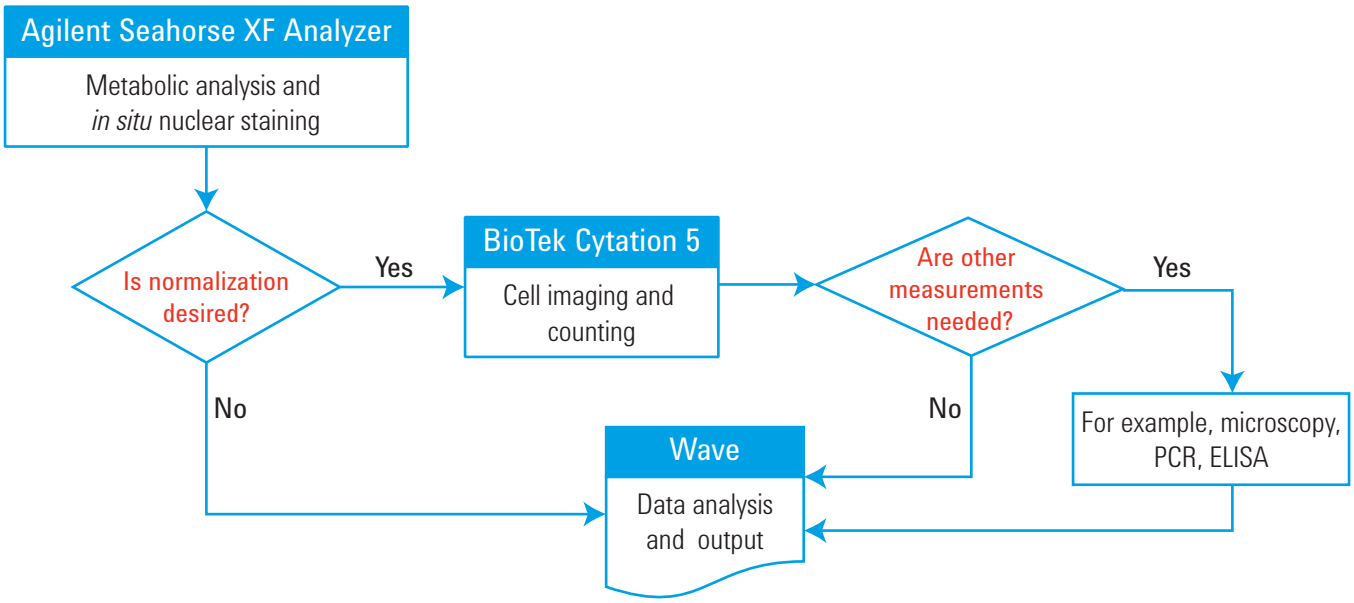

Figure 5. Scheme of XF data normalization workflow using *in situ* cell staining and counting methods combining the Agilent Seahorse XF analyzer and a Cytation 5.

Materials and Methods

Materials

Three cell lines with distinct morphological and geographical characteristics were obtained from ATCC and analyzed: a) A549, evenly dispersed with epithelial morphology, b) C2C12, evenly dispersed with fibroblastic irregular morphology, and c) MCF7, highly clustered with epithelial morphology. C2C12 myoblast cells were cultured in high glucose DMEM (Gibco, 11960-044) supplemented with 10 % FBS (HyClone, SH30070.03), 2 mM L-glutamine (Corning, 25-005-CI), 1 mM sodium pyruvate (Corning, 25-000-CI) at 37 °C under 10 % CO_2 atmosphere. A549 and MCF7 cells were maintained at 37 °C under 5 % CO_2. A549 cells were cultured in 50:50 DMEM/F12 (Corning, 10-090-CV) with 10 % FBS and 2 mM L-glutamine, and MCF7 cells were cultured in RPMI-1640 (Gibco, 21870-076) supplemented with 10 % FBS, 2 mM L-glutamine, 1 mM sodium pyruvate.

For XF analysis, we used an Seahorse XF Cell Energy Phenotype Test Kit (p/n 103325-100) and an Agilent Seahorse XF Basal DMEM (p/n 102353-100) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM glucose (Corning, 25-037-CI), and 5 mM HEPES (Sigma, H0887).

Cell nuclei were stained using Hoechst 33342 (Thermo Scientific, 62249). Protein Assay Dye Reagent Concentrate (BioRad, 500-0006) was used to make Bradford Assay Buffer by diluting with distilled water to a 3:10 ratio.

Growth rate measurement

C2C12 cells were plated on multiple 6-well plates at 5×10^4 cells/well. Cells in selected wells (n = 3) were detached with trypsin and EDTA, and counted every 24 hours.

Agilent Seahorse XF analysis and *in situ* nuclear staining

Basal and maximum OCR and ECAR of three cell lines were measured using an XF Cell Energy Phenotype Test Kit with slight modifications: a) 20 µg/mL Hoechst 33342 (2 µg/mL) was included in the oligomycin/FCCP mix or injected separately to stain nuclei in the XF analyzer, and b) 5 mM HEPES was included in assay medium, the Seahorse Basal DMEM. Briefly, cells were plated on XFe96 or XFp plates at various cell densities one day prior to the analysis. To compare the OCR and ECAR variation caused by C2C12 cell proliferation within 6 hours, cells were plated into two XFp culture plates at the same time, and the metabolic rates were measured on the same instrument 6 hours apart.

BioTek Cytation 5 imaging and cell counting

Cell images were captured using a 4x lens with a DAPI filter immediately following XF analysis. Hoechst-stained fluorescent nuclear images were captured using the autofocus capability in Gen5 software as single center images or stitched whole-well images. The nuclear number was counted using the Cell Analysis function in the Gen5 software program, and data were exported to normalize XF data. The whole-well cell number was extrapolated by multiplying the central partial counts by a factor of ~3.71, based on the ratio of well dimension to the image size.

Protein assay

After imaging, cells were carefully washed once with PBS and lysed in 20 µL/well of Lysis Buffer containing 10 mM Tris-Cl (pH 7.4) and 0.1 % Triton X-100. The cell lysate was diluted in Bradford Assay Buffer (1/20), and the absorbance at 595 nm was compared with BSA standard to calculate the protein amount in each well.

Conclusion

In situ cell counting by BioTek Cytation 5 using cells stained as part of the XF assay workflow inside of an Agilent Seahorse XF analyzer can be used to normalize cellular metabolic rates, and is applicable for various cell types. While the normalization outcome was comparable to protein quantitation, the *in situ* cell counting protocol made the assay workflow faster, and was amenable to performing downstream assays, resulting in experimental flexibility.

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Published in the USA, March 22, 2017
5991-7908EN

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