XF Imaging and Normalization Technical Overview

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Data Quality Management using Brightfield Images with the Seahorse XF Imaging and Normalization System

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Agilent Seahorse XF Analyzers measure the rates of cellular metabolism in specialized microplates. As in most other cell-based assays, XF assays are dependent on the amount of sample in the well. XF assays require additional consideration with respect to the location of the sample within the well. To accurately report the total cellular function of all cells in the well, it is important to ensure an even distribution of cells when seeding and working with XF microplates. Indeed, the most common cause of data variability between wells on the same microplate (having the same number of cells per well) is variability in cell distribution. This Technical Overview describes tools designed to help manage and document this variability using the Agilent Seahorse XF Imaging and Normalization System. Use of these tools allows users to make clear interpretations of their data and ensures accurate analysis.

Cell preparation errors can be identified by microscopic observation. However, review of XF24 and XF96 microplates through manual observation has some limitations. For example, it is unfeasible to thoroughly review every well of higher well count microplates, temperature is uncontrolled during this time, and there is no documentation of any findings. Instead, users tend to spot-check several wells, conclude that the cells look okay, and continue with their experiment. Thus, concatenating data across multiple experiments without proper documentation of sample quality makes data trend analysis and data organization difficult and time-consuming. The Agilent Seahorse XF Imaging and Normalization System provides a brightfield scanning function to capture and review individual well images using the XF Imaging and Cell Counting Software. By integrating the images seamlessly within the assay result file in Wave, individual images can be reviewed and flagged if desired. These new image documentation and flagging tools improve cell culture condition evaluation to ensure that XF data are of the highest quality and makes interpreting results easier and more reproducible.

Improving XF data quality

All cell-based assays have variability which arises from two sources; noise introduced by the measurement method and variability in the biological sample being measured. It is important to consider both factors when analyzing any dataset – uncovering the sources of error in an assay allows the researcher to both account for the error and improve assay design to minimize it in future assays. Exclusion of a data point from an analysis requires a valid reason independent from the true biological variation in a sample. However, in the case that these statistical outliers are the result of variations in sample prep, it can be acceptable to remove these samples from an analysis. Figure 1 demonstrates the statistical variability in Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) in A549 lung cancer cells assayed on the same XF96 microplate. The raw data exhibit a large spread in both OCR and ECAR. Though all wells were seeded with 1 x 10^5 cells/well, two wells lost cells in the center portion during the medium replacement step (Figure 1A). Those two wells were flagged (yellow triangles at the upper right corner) after image data review either within the XF Imaging and Cell Counting software or in Wave software after the images have been imported. Normalization to cell count can account for some but not all of this variability (Figure 1B). Because the technical error due to cell scraping was documented with brightfield images, these wells were removed from the analysis. The coefficient of variation (%CV) of each rate improved (graphs at right), and the assay reported the true biological variability of the samples more accurately. As a result, interpretability of this data set also improved.

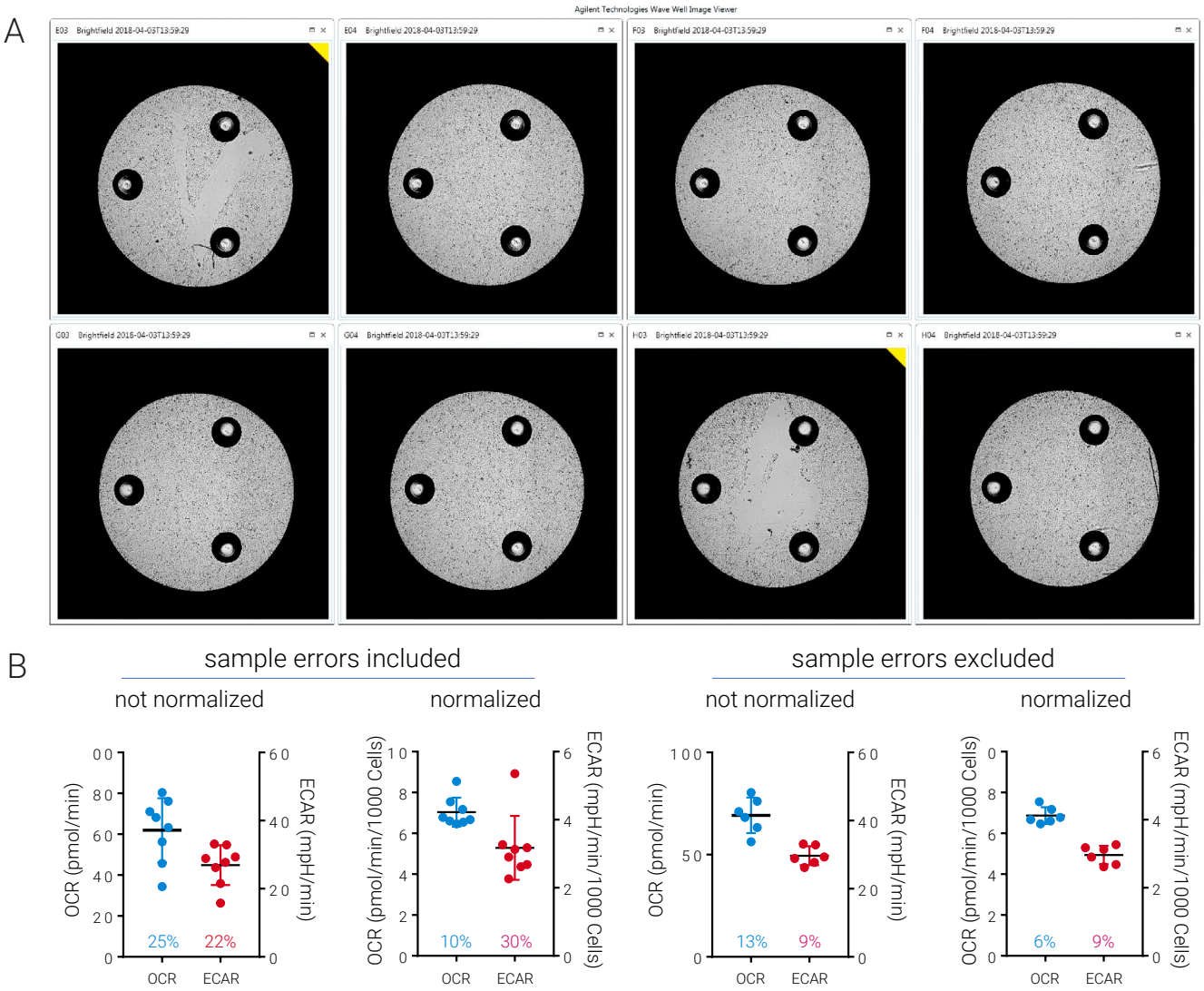

Figure 1. Error identification and documentation for XF analysis using brightfield images. A) Whole well brightfield images of A549 cells are compared by using the "well comparison" function in Wave software. Wells experiencing cell loss are marked by yellow flags on the upper right corner. B) Basal OCR and ECAR comparison before and after outlier exclusion. The % values on each graph are the corresponding %CVs.

A standardized workflow using the XF Imaging and Normalization System

Agilent's XF Imaging and Cell Counting software can obtain brightfield images from individual wells acquired with the integrated Cytation 1 multi-mode reader (BioTek Instruments, Inc.). As shown in Figure 2, the XF96- or XF24-well plate is registered by scanning the bar code, and after image acquisition the images are exported to the corresponding Wave file upon completion of XF analysis.

There are three major advantages of using the automated functionality of the XF Imaging and Normalization System in addition to the data quality improvement. First, all samples need CO_2-free incubation for one hour prior to XF analysis while a cartridge is being calibrated. Both CO_2 outgassing and brightfield imaging can be performed simultaneously using the Cytation 1's precisely controlled environmental chamber with temperature control. Thus, the brightfield imaging can be included in the CO_2 outgassing step. These two parallel processes of calibration and pre-incubation can be coordinated seamlessly by using a single controller. Second, imaging data can be seamlessly linked to the assay result file in Wave. Any well that has suspect cell seeding conditions which could potentially affect the functional measurements can be flagged and these well or data flags are visible at all times during data review in Wave software. By documenting the image information in Wave, images can be reviewed at any time after XF analysis. Finally, the brightfield image information is also linked to the corresponding cell counts which are also obtained by the XF Imaging and Normalization System.

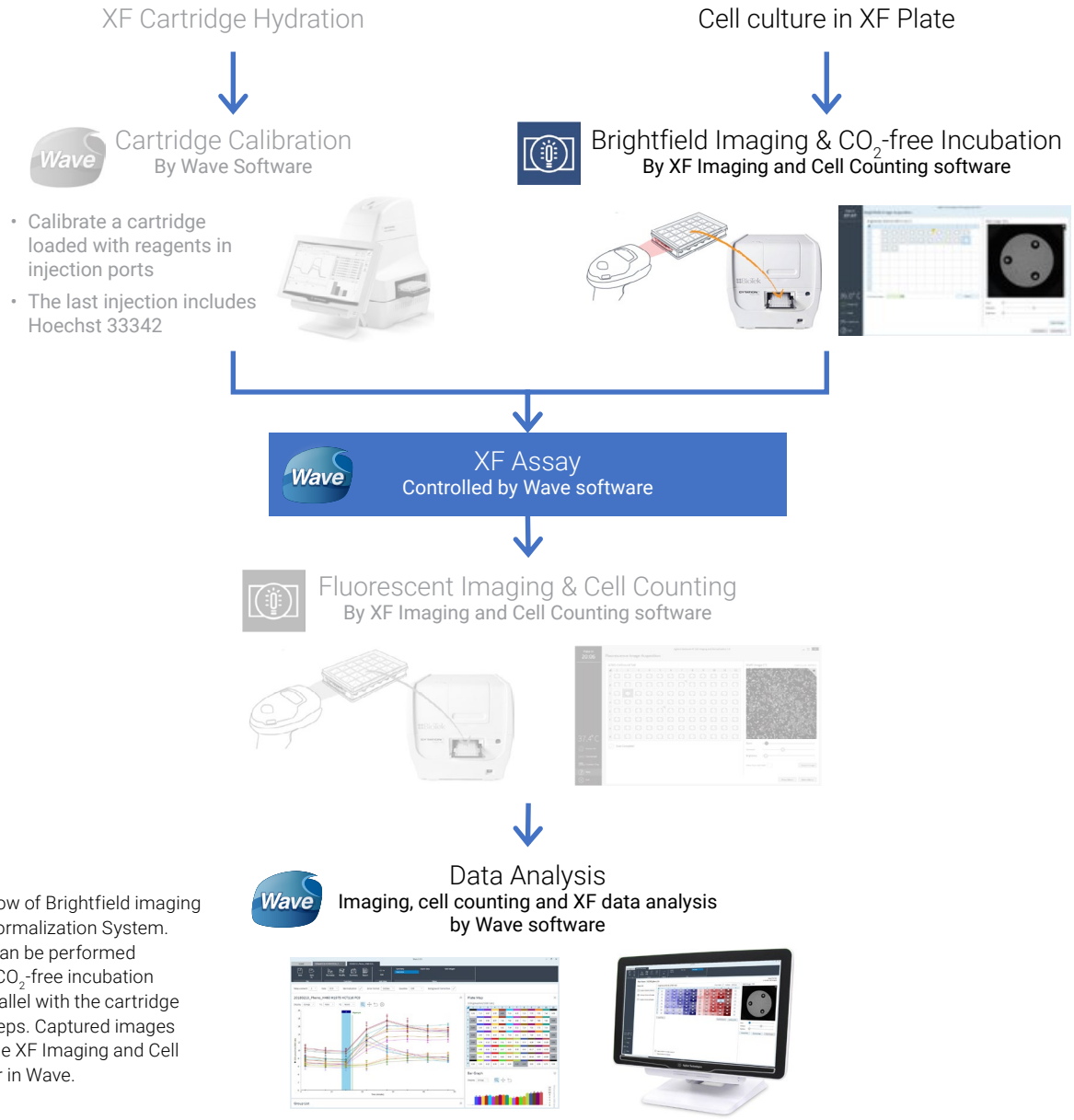

Figure 2. The workflow of Brightfield imaging in XF Imaging and Normalization System. Brightfield imaging can be performed during the period of CO_2-free incubation for outgassing in parallel with the cartridge sensor calibration steps. Captured images can be reviewed in the XF Imaging and Cell Counting software or in Wave.

Determining cell seeding errors using brightfield images

As described above, the ideal condition for XF analysis is even cellular distribution at sub-confluent density. High data variability often correlates with uneven cell distribution. Beyond the example of cell loss or absence given above, variability in cell distribution due to seeding is also a concern. Figure 3 shows four different errors in cell seeding which can add variability to a dataset and create difficulty in downstream analysis.

Edge effects are common in higher well-count plates where cells settle differently in outer wells due to convection of the media as it warms.[1] An example of this in an XF96 microplate is shown in Figure 3A. In this image, cells are clustered around the outer edge of the well and are thus unevenly distributed within the well. This effect is decreased when microplates are allowed to rest on the tissue culture biosafety cabinet deck for one hour prior to transferring to the cell culture incubator.[2, 3] In Figure 3B, there is large scale cell loss in one portion of the well. This can be a result of either overly aggressive pipetting when seeding or while washing the cells during the change to assay media. This may be of concern with cell lines that are loosely adherent (e.g. HEK293 cells). In all instances, gently pipetting cell suspension or media will prevent this from occurring. Additional examples of cell scraping are shown in Figures 3C. The example showed in Figure 3D is a result from excessive aspiration and delayed media change which causes cells to dry up partially. In general these errors are preventable, and having documentation of sample quality allows researchers to determine whether to move forward with analysis of a particular well or group.

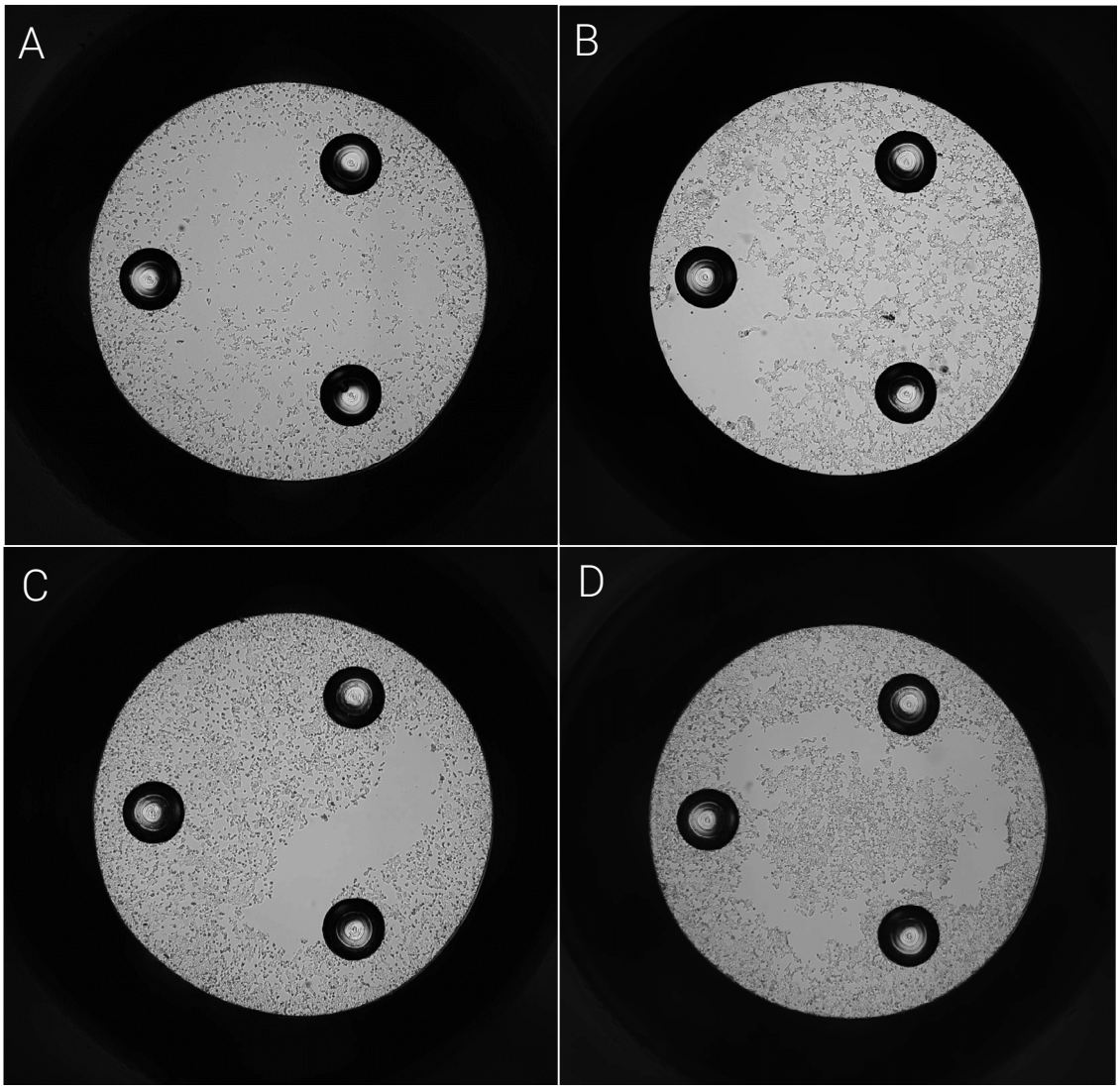

Figure 3. Examples of errors potentially increase data variability. A) Low cell density with severe edge effect in seeding. B) Corner cell loss C) Cell loss by scraping. D) Radial cell loss.

Summary

This Technical Overview focused on cell distribution within the microplate wells because it is a frequent source of error in XF assays often leading to data variability. Notably, other aspects of the sample such as cell adhesion status or morphological variation can be screened by using brightfield images. Automated brightfield image acquisition using the Seahorse XF Imaging and Normalization System enables (1) time and effort savings by reviewing sample quality before running an XF assay, (2) identification of sample errors before and after XF analysis not by metabolic rate variation but by sample condition, and (3) documentation of sample quality information which is seamlessly linked within the corresponding Wave assay result file. This quality control feature will help to increase the confidence of data interpretation and provides a new standard for optimizing XF data.

References

1. Lundholt, B.K., *et al.* A simple technique for reducing edge effect in cell-based assays. *J. Biomol. Screen.* **8**, 566-570 (2003).
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