User Guide

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Methods for Reducing Cell Growth Edge Effects in Agilent Seahorse XF Cell Culture Microplates

Introduction

Edge effect, defined as cultured cells growing or behaving differently in edge wells compared to inner wells, is a common and well-recognized problem in multi-well microplates[1]. Though cells are seeded at the same time and density, the cells in the wells on the outer edge of the microplate are distributed and grow differently, often at a decreased rate, when compared to cells seeded in the interior wells of the microplate. In data generated on the Agilent Seahorse XF Analyzers, this is often manifested as reduced absolute rates and/or increased error compared with wells/groups on the interior section of the plate. This document examines and quantifies some of the major sources of edge effects and demonstrates practical methods for reducing or eliminating this source of variability for your XF and other cell-based assays.

[1] *Lundholt BK, Scudder KM, Pagliaro L. A simple technique for reducing edge effect in cell-based assays. J Biomol Screen. 2003 Oct;8(5):566-7*

Procedures to minimize cell growth edge effect (Figure 1)

1. Seed cells according to established protocols: Seeding Adherent Cells in Agilent Seahorse XF96 Tissue Culture Microplates
2. After the cells are seeded, allow the plate to rest in the tissue culture hood at room temperature for 60 minutes. Note that cell plates may be gently moved to the rear portion of the biosafety cabinet for the rest step if desired.
3. After the resting period, transfer the cells to a properly controlled* tissue culture incubator. Placing the plates to the rear of the incubator can help to limit fluctuations in temperature when the incubator is accessed.

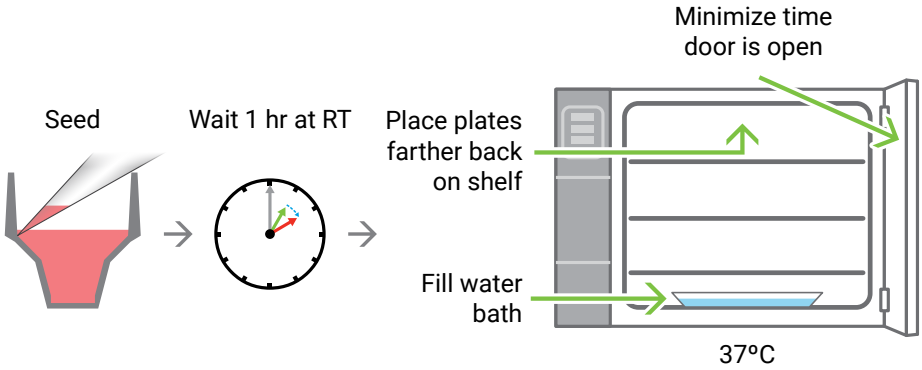


Figure 1. Procedures to minimize edge effect in XF tissue culture microplates.

Impact of Immediate Temperature Changes on Cell Distribution and Growth

Background

Typically, cells are seeded at room temperature (~25°C) and then transferred immediately into a tissue culture incubator, usually at 37°C. When the cell plate is transferred, the environmental conditions of the edge wells change more rapidly than the environmental conditions of the interior wells with respect to temperature, humidity and $\%CO_2$. These differences (especially shifts in temperature) drive the edge effect phenomenon with respect to cell growth.

Investigation using XF96 Tissue Culture Microplates suggests that cell growth edge effects are caused when the plate experiences a rapid change in temperature (e.g. 25°C > 37°C) before the cells have had adequate time to settle to the bottom of the well and begin adhesion to the cell plate. Figure 2 illustrates significant differences in cellular respiration between edge wells and interior wells.

This effect can be largely avoided by allowing the cells to rest at the same temperature at which they were seeded (e.g. ambient room temperature/25°C) before they are transferred the tissue culture incubator, allowing adequate time for the cells to settle and attach to the well surface. This is shown in Figure 3. Note the respiration rates of the cells in the edge and interior wells are nearly identical. These results are similar to those obtained in Ref. 1

**A properly controlled incubator is one in which the temperature, % humidity and % CO_2 are maintained according to the model's manual, doors are kept closed when the incubator is not being accessed and time doors are open is minimal.*

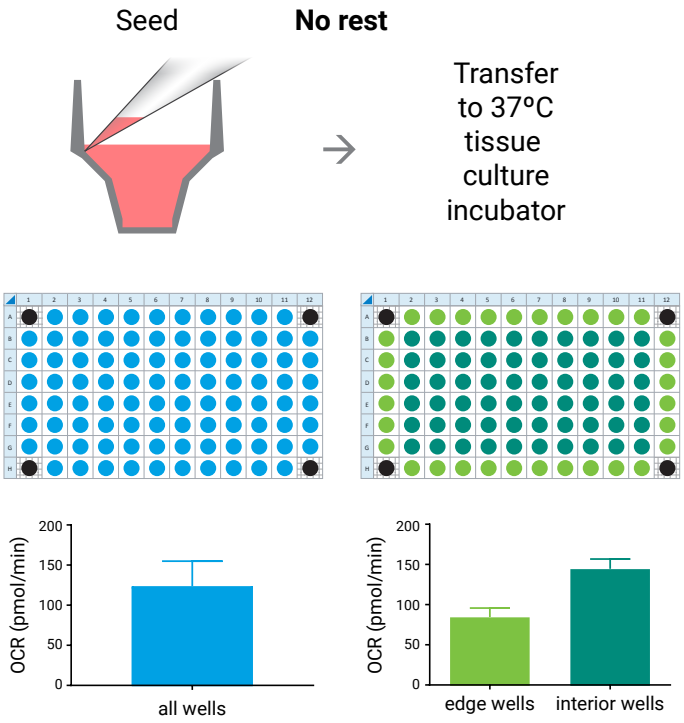


Figure 2. Effects of No Rest: Edge wells show significant differences in respiration rate when compared to interior wells. C2C12 cells were seeded at 1 X 10^4 cells per well in XF96 tissue culture microplates and transferred immediately to a 37°C tissue culture incubator and cultured for 24 hr. OCR was measured and data is divided into 2 groups: edge wells and interior wells.

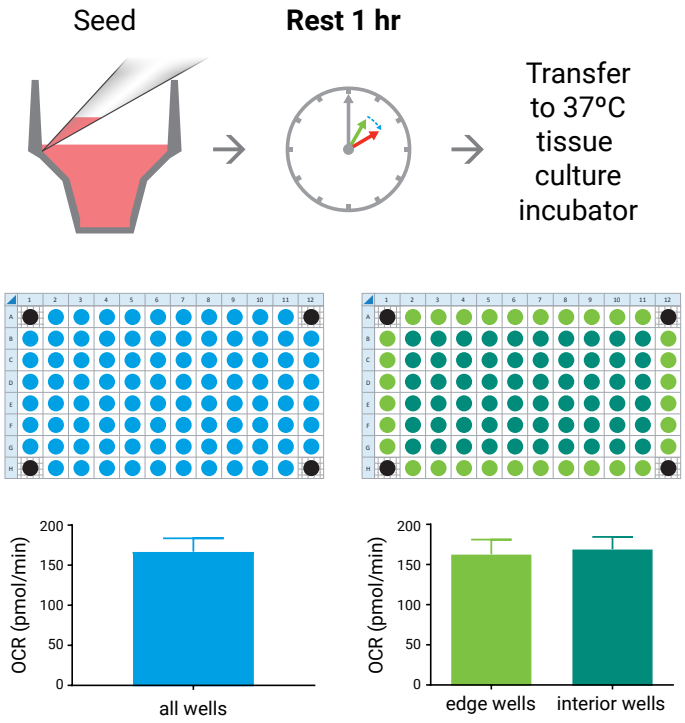


Figure 3. Effects of Rest: Edge wells show identical respiration rates when compared to interior wells. C2C12 cells were seeded at 1 X 10^4 cells per well in XF96 tissue culture microplates, allowed to rest for 1 hr at ambient conditions, then transferred to a 37°C tissue culture incubator and cultured for 24 hr. OCR was measured and data is divided into 2 groups: edge wells and interior wells.

Cell Seeding and Growth - Conditions Tested (Figure 4):

1. **Rest** - cells seeded at room temperature, allowed to rest 1hr, then transferred to 37°C tissue culture incubator.
2. **No Rest** – cells seeded at room temperature and transferred to 37°C tissue culture incubator.

The impact of temperature on cell seeding and edge effects was visualized using the Biotek Cytation 1 and analyzed for cell count. The entire area of the well was imaged to demonstrate the importance of even cell distribution in XF Tissue Culture Plates. XF assays were then performed to assess OCR and ECAR. Note that while the examples below focus on OCR, matching results were obtained for ECAR data with respect to decreases in cell growth edge effects when the cell plate is allowed to rest before transfer to a tissue culture incubator.

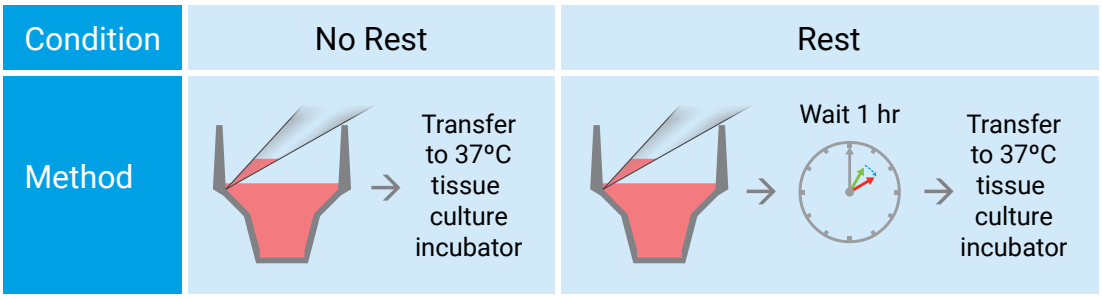
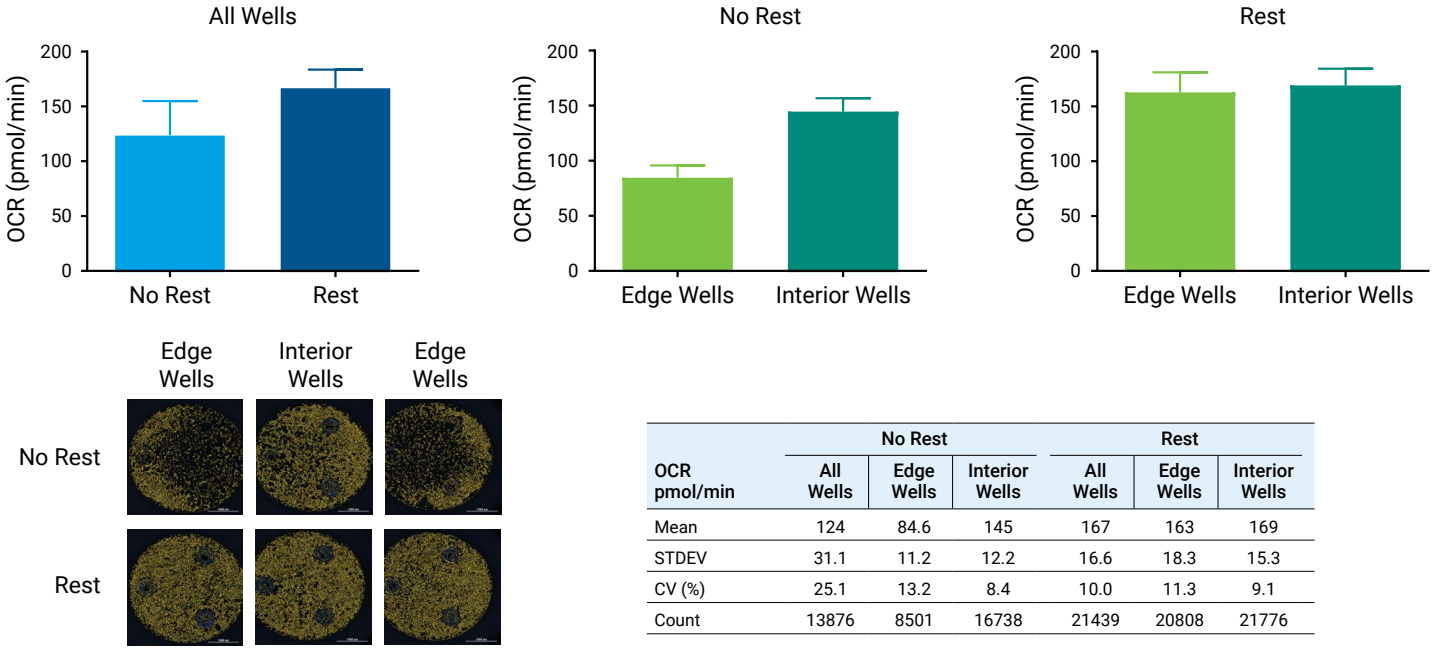


Figure 4. Conditions and Methods tested to reduce edge effect.

Examples

Example 1: XFe96. C2C12 cells seeded at 1.0 x 10^4 cells/well, 24 hr. incubation.



OCR pmol/min	No Rest			Rest		
	All Wells	Edge Wells	Interior Wells	All Wells	Edge Wells	Interior Wells
Mean	124	84.6	145	167	163	169
STDEV	31.1	11.2	12.2	16.6	18.3	15.3
CV (%)	25.1	13.2	8.4	10.0	11.3	9.1
Count	13876	8501	16738	21439	20808	21776

Figure 5. Effects of No Rest and Rest Methods on C2C12 cell monolayers and respiration rates. OCR was measured for No rest and Rest conditions. For All Wells, data is divided into 2 conditions: no rest and Rest. Note the increased CV value for All Wells, No rest. For No rest vs. Rest, data is divided into 2 conditions: Edge wells and Interior wells. Note the edge wells and interior wells show nearly identical respiration rates under rest conditions, resulting in lower average coefficients of variation (CVs) when all wells (i.e. edge + interior) are in a single group.

For more information on how to image and normalize using cell counts and the BioTek Cytation 1, please refer to the Seahorse XF Imaging and Normalization System.

C2C12 cells were seeded at 1 x 10^4 cells/well in replicate XF96 tissue culture microplates. One plate was immediately transferred to a tissue culture incubator at 37°C (no rest). The other was left to rest for 1 hour at ambient temperature. After the 1 hour rest at ambient conditions, this cell plate was then transferred to the same tissue culture incubator. Cells were cultured for an additional 24 hours before imaging wells with the Biotek Cytation 1 and measuring basal respiration rates (OCR) in an XFe96 Analyzer. Data is summarized in Figure 5.

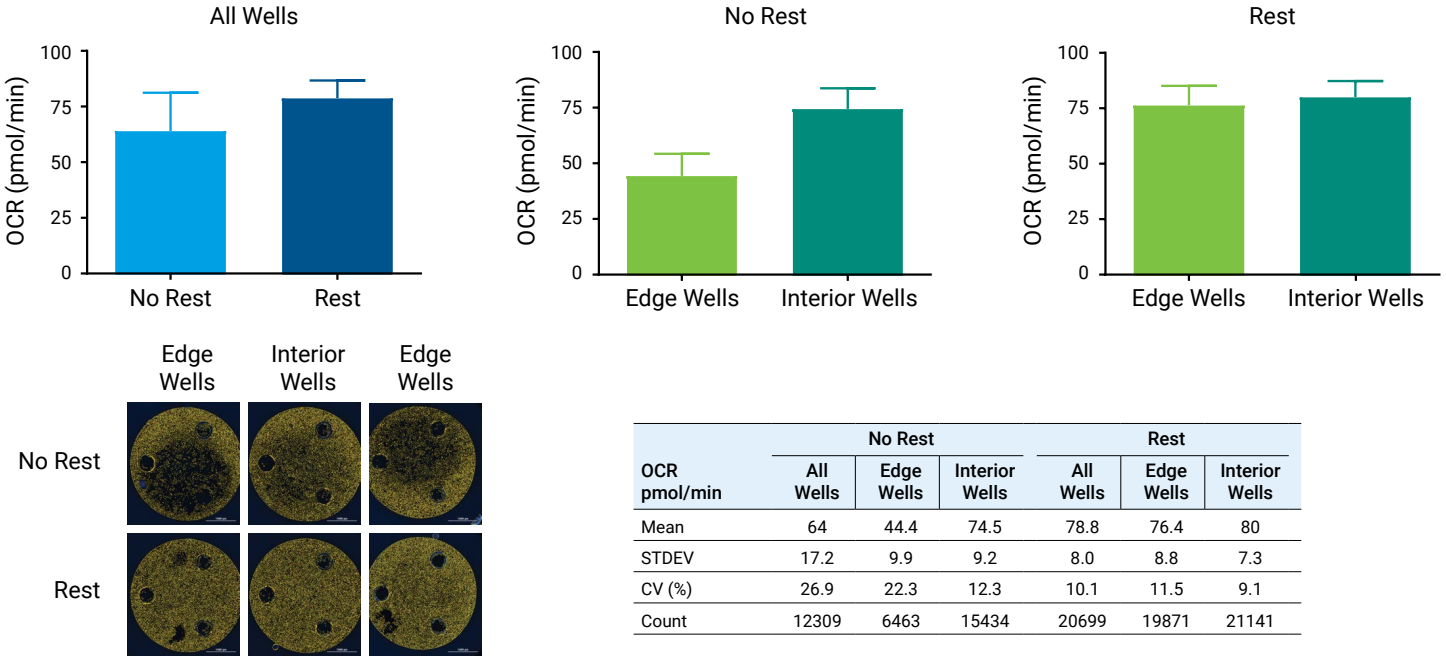
Results indicate that the cell plate allowed to rest for 1 hour before transfer to a tissue culture incubator shows consistent cell distribution in wells that are both in the interior and edges of the plate. In contrast, the plate transferred immediately to 37°C shows reduced cell density and/or inconsistent monolayers in the center of all the wells, especially in the edge wells. This inconsistency resulted in significant differences in OCR values between edge and inner wells, and substantially increased coefficients of variation (CVs) when edge and interior wells are grouped together (Figure 5 - All Wells graph and table, 25% CV v. 10% CV).

Additional examples of reduced edge effects using the prescribed rest period after cell seeding.

Additional examples of seeding X96, XF24 and XFp plates under No rest and Rest conditions are provided below.

- For XF96 Tissue Culture Microplate examples, the results observed with C2C12 cells are essentially reproduced for A549, HepG2 and HEK293 cells, suggesting that the Rest method employed is suitable for many cell types and results in more consistent XF data among edge and interior wells (Figures 6-8).
- For XFp Tissue Culture Miniplates, while there are no true edge wells, the rest method still resulted in higher average OCR values and lower CVs across wells, indicating this is a valuable method for increasing well to well consistency in XFp tissue culture miniplates (Figure 9).
- For XF24 Tissue Culture Microplates, the rest method resulted in higher average OCR values and more consistent cell monolayers (cell imaging), indicating this is a valuable method for increasing well to well consistency in XF24 tissue culture microplates (Figure 10).
- For ECAR data, the improvements observed for OCR with respect to cell growth edge effects are essentially mirrored for ECAR data in all examples shown below. This, again, suggests that the Rest method employed results in more consistent XF data between edge and interior wells.

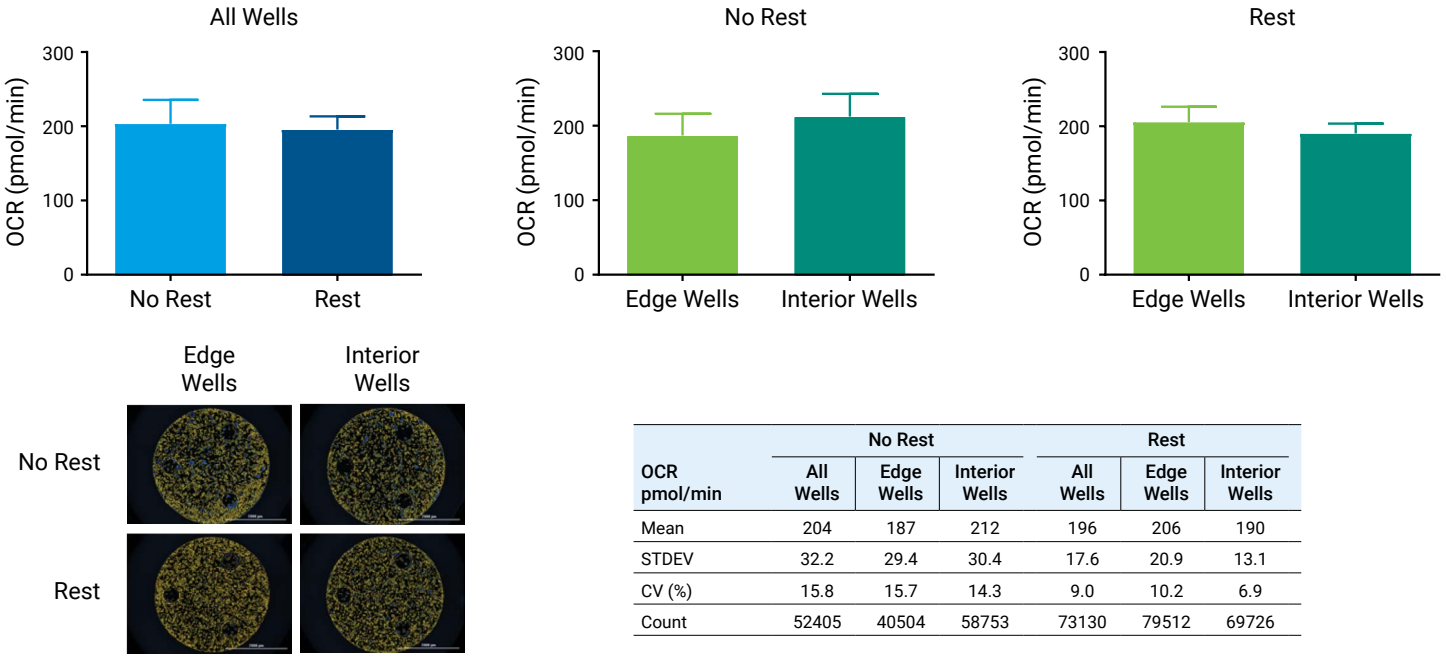
Example 2: XFe96. A549 cells seeded at 1.5 x 10^4 cells/well, 24 hr. incubation.



OCR pmol/min	No Rest			Rest		
	All Wells	Edge Wells	Interior Wells	All Wells	Edge Wells	Interior Wells
Mean	64	44.4	74.5	78.8	76.4	80
STDEV	17.2	9.9	9.2	8.0	8.8	7.3
CV (%)	26.9	22.3	12.3	10.1	11.5	9.1
Count	12309	6463	15434	20699	19871	21141

Figure 6. Effects of No Rest and Rest Methods on A549 cell monolayers and respiration rates. A549 cells were seeded at 2 x 10^4 cells/well in replicate XF96 tissue culture microplates. One plate was immediately transferred to a tissue culture incubator at 37°C (no rest). The other was left to rest for 1 hour at ambient temperature. After the 1 hour rest at ambient conditions, this cell plate was then transferred to the same tissue culture incubator. Cells were cultured for an additional 24 hours before imaging wells with the Biotek Cytation 1 and measuring basal respiration rates (OCR) in an XFe96 Analyzer. OCR was measured and data is divided into 2 groups: edge wells and interior wells. Note the edge wells and interior wells show nearly identical respiration rates under rest conditions, resulting in lower average coefficients of variation (CVs) when all wells (i.e. edge + interior) are in a single group.

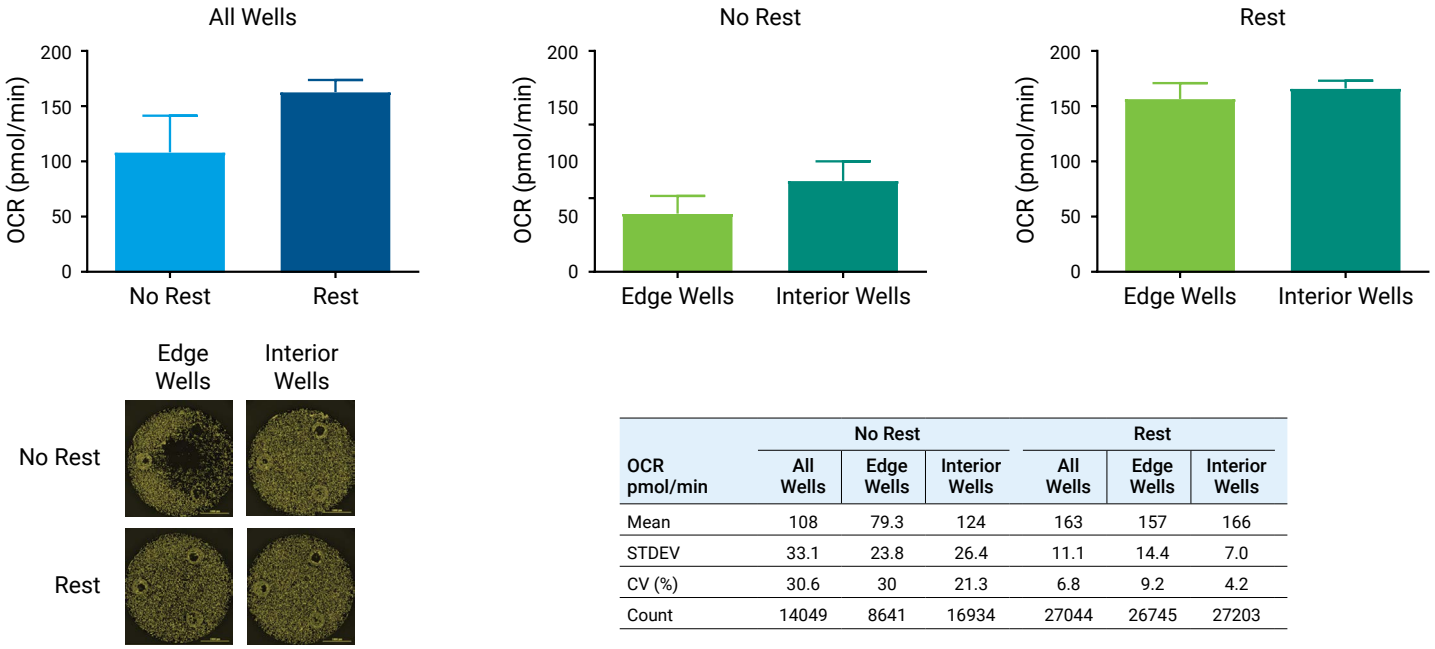
Example 3: XFe96. HepG2 cells seeded at 2 x 10^4 cells/well, 24 hr. incubation.



OCR pmol/min	No Rest			Rest		
	All Wells	Edge Wells	Interior Wells	All Wells	Edge Wells	Interior Wells
Mean	204	187	212	196	206	190
STDEV	32.2	29.4	30.4	17.6	20.9	13.1
CV (%)	15.8	15.7	14.3	9.0	10.2	6.9
Count	52405	40504	58753	73130	79512	69726

Figure 7. Effects of No Rest and Rest Methods on HepG2 cell monolayers and respiration rates. HepG2 cells were seeded at 2 x 10^4 cells/well in replicate XF96 tissue culture microplates. One plate was immediately transferred to a tissue culture incubator at 37°C (no rest). The other was left to rest for 1 hour at ambient temperature. After the 1 hour rest at ambient conditions, this cell plate was then transferred to the same tissue culture incubator. Cells were cultured for an additional 24 hours before imaging wells with the Biotek Cytation 1 and measuring basal respiration rates (OCR) in an XFe96 Analyzer. OCR was measured and data is divided into 2 groups: edge wells and interior wells. Note the edge wells and interior wells show nearly identical respiration rates under both rest and immediate transfer conditions, indicating that this cell type may be less susceptible to the plating edge effect.

Example 4: XFe96. HEK293 cells seeded at 2 x 10^4 cells/well, 24 hr. incubation.

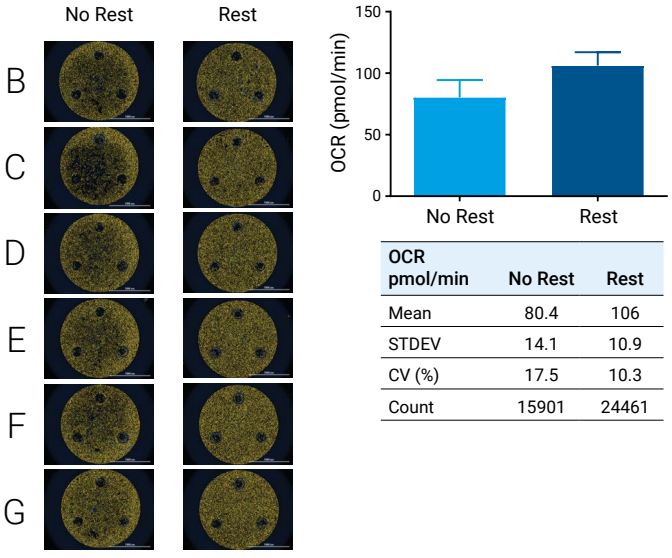


OCR pmol/min	No Rest			Rest		
	All Wells	Edge Wells	Interior Wells	All Wells	Edge Wells	Interior Wells
Mean	108	79.3	124	163	157	166
STDEV	33.1	23.8	26.4	11.1	14.4	7.0
CV (%)	30.6	30	21.3	6.8	9.2	4.2
Count	14049	8641	16934	27044	26745	27203

Figure 8. Effects of No Rest and Rest Methods on HEK293 cell monolayers and respiration rates. HEK293 cells were seeded at 2 x 10^4 cells/well in replicate XF96 tissue culture microplates. One plate was immediately transferred to a tissue culture incubator at 37°C (no rest). The other was left to rest for 1 hour at ambient temperature. After the 1 hour rest at ambient conditions, this cell plate was then transferred to the same tissue culture incubator. Cells were cultured for an additional 24 hours before imaging wells with the Biotek Cytation 1 and measuring basal respiration rates (OCR) in an XFe96 Analyzer. OCR was measured and data is divided into 2 groups: edge wells and interior wells. Note that for this cell type, resting the plated cells improved cell activity overall, as well as reducing the edge effect.

Example 5: Effects of No Rest v. Rest for XFp Tissue Culture Miniplates.

A549 cells seeded at 1.5 x 10^4 cells/well, 24 hr. incubation.

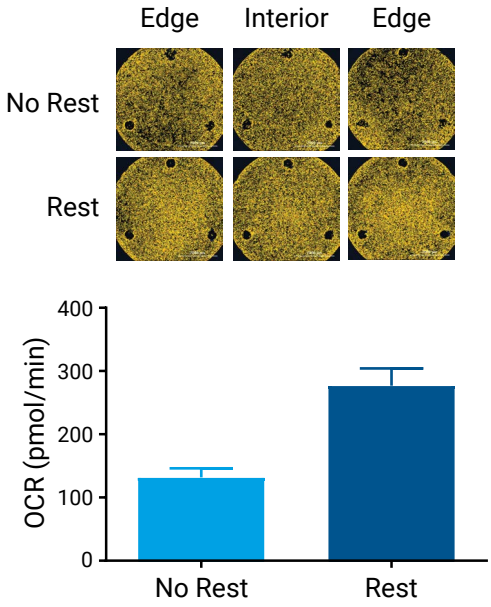


OCR pmol/min	No Rest	Rest
Mean	80.4	106
STDEV	14.1	10.9
CV (%)	17.5	10.3
Count	15901	24461

Figure 9. Effects of No Rest and Rest Methods on cell monolayer and respiration rates in the XFp. A549 cells were seeded at 1 x 10^4 cells/well in replicate XFp tissue culture miniplates. One plate was immediately transferred to a tissue culture incubator at 37°C (no rest). The other was left to rest for 1 hour at ambient temperature. After the 1 hour rest at ambient conditions, this cell plate was then transferred to the same tissue culture incubator. Cells were cultured for an additional 24 hours before imaging wells and measuring basal respiration rates (OCR) in an XFp Analyzer. OCR was measured and data is divided into 2 groups: No rest and Rest. Note the Rest method shows higher OCR values and lower average coefficients of variation (CVs) due to more consistent cell monolayers/seeding density across wells as judged by imaging.

Example 6: Effects of No Rest v. Rest for XF 24 Cell Culture Microplates.

A549 cells seeded at 1.5 x 10^4 cells/well, 24 hr. incubation.



OCR pmol/min	No Rest	Rest
Mean	132	275
STDEV	14.3	28.0
CV (%)	10.8	10.2
Count	38452	48848

Figure 10. Effects of No Rest and Rest Methods on cell monolayer and respiration rates in the XFe24. A549 cells were seeded at 1 x 10^4 cells/well in replicate XF24 tissue culture miniplates. One plate was immediately transferred to a tissue culture incubator at 37°C (no rest). The other was left to rest for 1 hour at ambient temperature. After the 1 hour rest at ambient conditions, this cell plate was then transferred to the same tissue culture incubator. Cells were cultured for an additional 24 hours before imaging wells and measuring basal respiration rates (OCR) in an XFe24 Analyzer. OCR was measured and data is divided into 2 groups: No rest and Rest. Note the Rest method shows higher OCR values and more consistent cell monolayers/seeding density across wells as judged by imaging.

Potential Impact of Changes in Humidity

1. Typical fluctuations in the relative % humidity of the tissue culture incubator showed almost no impact on the consistency of cell adherence and growth when compared to effects of temperature change, as measured by imaging and XF analysis (data not shown). Consistency in cell growth is only affected when there is a gross lack of humidity in the incubator (e.g. an empty water pan).

   Note that all XF tissue culture microplate wells will experience a minor loss of media volume when used correctly (~5 µL and ~25-50 µL for XF96 and XFe24 tissue culture microplates, respectively). The edge wells, and in particular the corners, may lose slightly larger volumes. When using an incubator experiencing large amounts of use (i.e. frequently opening/closing the door), the effects of changes in humidity will likely be greater than an incubator that is opened/closed less frequently.

2. In the cell lines tested (A549, HepG2, C2C12, HEK293), normal fluctuations in relative humidity (e.g. due to door opening/closing) did not play a consistent role on edge effect.

3. Several tests were also conducted with either modifying incubator conditions or using products marketed to reduce variation in cell quality due to uneven evaporation. No significant improvement in data quality was noted in any of these conditions (data not shown). Data suggests that when cells are incubated in a properly humidified 37°C incubator, minor fluctuations in relative humidity had little or no impact on XF data quality. Only an un-humidified incubator resulted in sufficient evaporation to impact cell growth and XF data quality.

Summary and Further considerations

- While the Rest method shows significant reductions in edge effects and improving XF data quality, it should be noted that edge effects are not 100% eliminated and results may vary by cell type.
- Note that all of the above tests used a 24-hour cell culture period. Longer term culture is likely to magnify edge effects and thus all edge effects may not be avoidable. However this method is still recommended to ensure the cells are seeded and begin to grow as consistent as possible.
- Cells seeded in plates placed on a surface warmed to 37° and immediately transferred to 37°C tissue culture incubator (no rest) showed inconsistent seeding, as judged by well imaging and XF analysis (data not shown)
- Cells seeded on XF tissue culture microplates at room temperature, spun briefly in a centrifuge, then transferred to a 37°C tissue culture incubator showed inconsistent seeding, as judged by well imaging and XF analysis (data not shown)
- If it is an option for the tissue culture incubator, a set of inner doors that further subdivide the interior of the incubator may be of use. This may improve cell quality by reducing fluctuations in heat and humidity if the incubator is opened frequently and/or for cells that are sensitive to rapid/repeated changes in environmental conditions.

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