Application Note

Cell Metabolism

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Accurate Assessment of Metabolic Reprogramming during Adipocyte Differentiation

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Abstract

Mitochondria play a critical role in cellular function. They are responsible not only for sustaining cellular bioenergetic demands but also have a critical role sensing and responding to environmental signals. Indeed, mitochondrial dysfunction is linked to various diseases including several neurodegenerative diseases as well as chronic illnesses including diabetes, cardiovascular, muscular, and kidney disorders. In adipocytes, mitochondrial dysfunction is associated with metabolic disorders such as obesity and type 2 diabetes, impairing lipid metabolism, and increasing oxidative stress, which further disrupts systemic metabolism. During adipocyte differentiation, mitochondrial activity adjusts to meet evolving metabolic demands. While Agilent Seahorse XF analyzers are commonly used for in vitro mitochondrial function assessments, monitoring metabolic phenotypes in mature adipocytes is challenging due to the significant increase in metabolic rates during differentiation. In this application note, we describe an optimized workflow for accurate metabolic profiling during in vitro differentiation, which combines the standard Agilent Seahorse XFe24/XF Flex V7 PS culture microplate and the Agilent Seahorse XFe24/XF Flex V28 culture microplate with the Agilent Seahorse XF Flex analyzer. This combination of microplates expands the detection range of the instrument and captures a broad range of metabolic activities across several differentiation stages with great precision and accuracy.

Introduction

Adipocyte mitochondrial function is crucial for energy homeostasis, lipid metabolism, and insulin sensitivity. Dysfunctional mitochondria can lead to abnormal lipid accumulation, insulin resistance, and increased oxidative stress, contributing to obesity and type 2 diabetes.[1,2] Agilent Seahorse XF Analyzers are widely used for in vitro and ex vivo assessments of mitochondrial function. However, continuous and quantitative monitoring of mitochondrial function and the metabolic bioenergetic phenotype in mature adipocytes is challenging due to the significantly increased metabolic rate upon adipocyte differentiation.

3T3-L1 fibroblast cells can differentiate into mature adipocytes in culture when treated with a specific chemical cocktail, presenting a valuable tool for studying adipogenesis, adipocytes metabolism, and mechanisms associated with related diseases. During 3T3-L1 differentiation to mature adipocytes, cultured cells are allowed to reach confluency for two days before differentiation induction. In the first two days of differentiation, cells undergo one to two rounds of proliferation, reaching very high cell confluency compared to most other adherent cell types. Additionally, PGC-1α expression dramatically increases as 3T3-L1 adipocytes mature, reaching levels comparable to that in brown adipose tissue[3], further enhancing mitochondrial respiration. These factors collectively result in cells with very high respiratory rates (high oxygen consumption rate per well). Due to the high respiratory rates of these cells, hypoxia is induced in the microchamber during Seahorse XF rate measurements when cells are cultured in the standard Agilent Seahorse XFe24/XF Flex V7 PS culture microplate (XF V7) (p/n 100777-004). This induced hypoxia often leads to underestimation of the oxygen consumption rate (OCR) and insufficient oxygen concentration recovery between measurements.

The Agilent Seahorse XFe24/XF Flex V28 PS culture microplate (XF V28) (p/n 100882-004) is an alternative 24-well microplate, which features a larger microchamber (22 µL in XF V28 versus 5.65 µL in XF V7) to accommodate high respiring cells while maintaining the same well surface area as the standard XF V7 microplate. When the XF V28 microplate is used on an Agilent Seahorse XF Flex analyzer, a measurement program tailored for high respiring samples is enabled. The larger volume delivers a larger reservoir of oxygen to be depleted, and the enhanced mixing program minimizes microchamber hypoxia, thereby improving overall accuracy for high respiring cells.

In this application note, we developed an optimized workflow for the accurate determination of the metabolic profile of adipocytes during different stages of in vitro differentiation using the Seahorse XF Flex analyzer. The combined use of the standard XF V7 microplate with the XF V28 microplate, along with the optimized assay protocols and improved assay performance of the Seahorse XF Flex analyzer, results in an extended detection range. This extended detection range delivers accurate characterization of adipocyte metabolic phenotype from early differentiation stages to fully mature cells.

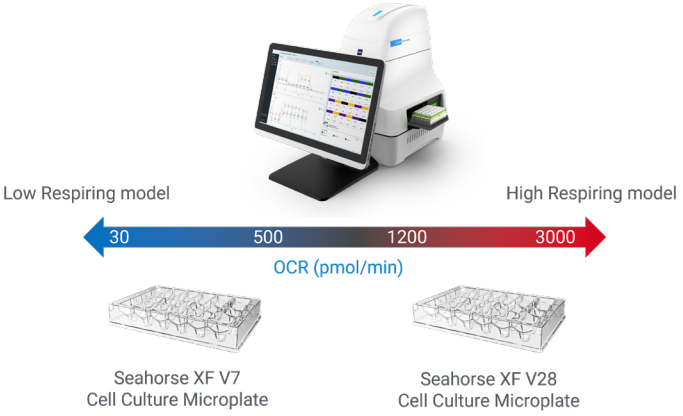

Figure 1. Schematic diagram of the detection range obtained in the Seahorse XF Flex analyzer by combining the use of the XF V7 and XF V28 microplates.

Experimental

Cell culture and adipogenic differentiation of 3T3-L1 fibroblasts

3T3-L1 fibroblasts were purchased from ATCC (CL-173). For maintenance, cells were cultured in DMEM (Gibco, 11995) with 10% Calf Bovine Serum (ATCC, 30-2030) and subcultured upon reaching 80% confluency. Before seeding, standard XF V7 microplates (p/n 100777-004) or XF V28 microplates (p/n 100882-004) were coated overnight with 100 µL per well of 0.2% gelatin (Sigma, G1393). For preconfluent conditions, 10,000 cells per well were seeded, and assays were conducted on the next day. For assays at day 0 (d0) and later differentiation stages, 20,000 cells per well were seeded, typically reaching confluence within 2 days.

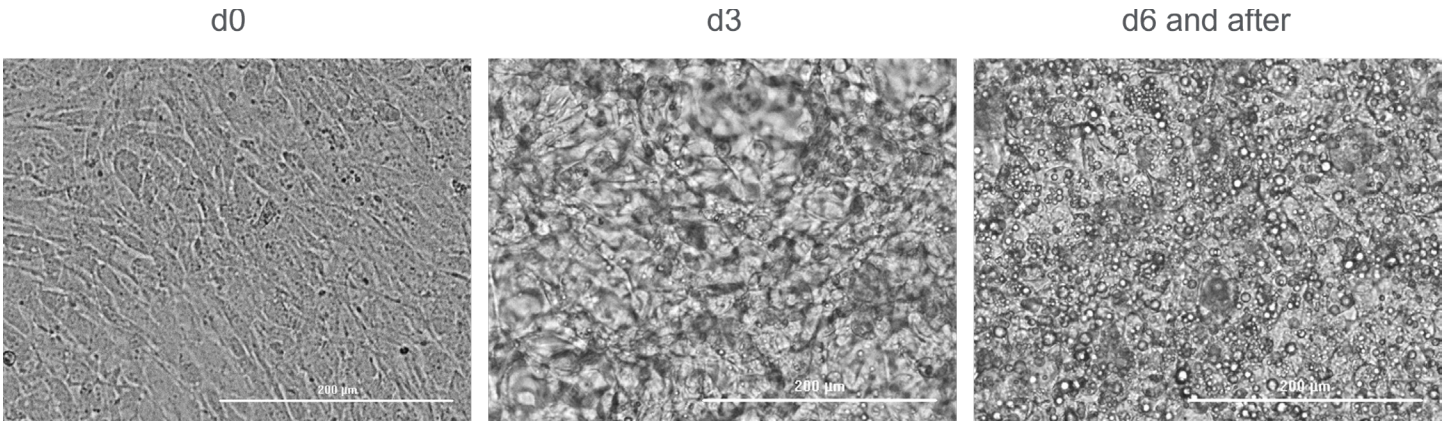

Figure 2. Phase contrast images (10× magnification) of 3T3-L1 cells before differentiation (d0), during differentiation (d3) and as mature adipocytes (d6 and after) acquired using the Agilent BioTek Cytation 5 cell imaging multimode reader. Scale bar = 200 µm.

Differentiation was induced two days postconfluence (d0) using DMEM supplemented with 10% FBS (HyClone, SH30070.03), 0.5 mM IBMX (Sigma, I5879), 5 µM Rosiglitazone (Sigma, R2408), 1 µM dexamethasone (Sigma, D4902), and 200 nM insulin (Sigma, I9278). Two days after induction, on day 2 (d2), differentiation media was replaced with DMEM containing 10% FBS and 200 nM insulin. From day 4 onward, cells were maintained in differentiation media consisting of DMEM with 10% FBS and 20 nM insulin, with media refreshed every 1 to 2 days.

Seahorse XF assays

All experiments in XF V7 microplates were performed using the default protocol conditions of the assay template (3 min mix, 2 min wait, and 3 min measurement). A default protocol was also used for the XF V28 microplate on the Agilent Seahorse XFe24 analyzer (3 min mix, 2 min wait, 3 min measure). For experiments performed in XF V28 microplates on the Seahorse XF Flex analyzer, protocol conditions were adjusted to maximize O_2 recovery in high respiring samples (3 min mix, 0 min wait, and 4 min measurement), except after addition of FCCP and before addition of rotenone/antimycin A, where measurements were performed using 3 min mix, 0 min wait, and 2.5 min measurement (Figure 3).

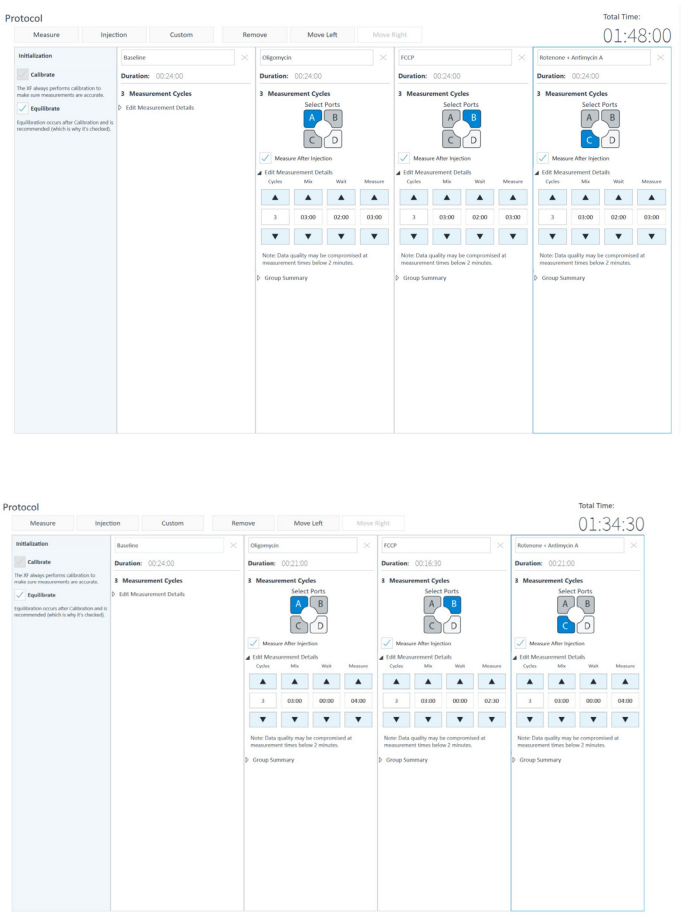

Figure 3. Seahorse XF Cell Mito Stress Test protocol conditions used for XF V7 (A) and XF V28 – Seahorse XF Flex (B) assays.

The Agilent Seahorse XF Cell Mito Stress Test (p/n 103015-100) was performed on preconfluent and postconfluent (d0) pre-adipocytes, as well as on days 3, 6, and 9 postdifferentiation. FCCP concentration was optimized for each differentiation stage according to recommendations in the user guide. The final reagent concentrations used in the assays were: 2.5 µM oligomycin (Oligo), 2 µM FCCP, and 1 µM rotenone/antimycin A (R/A), except for preconfluent 3T3-L1 cells, where 1.5 µM FCCP was used.

The Agilent Seahorse XF Substrate Oxidation Stress Tests (p/n 103672-100, 103673-100, 103674-100) were conducted on preconfluent, d0 pre-adipocytes, and day 6 (d6) mature adipocytes, following the kits' user guide. The concentrations of oligomycin, FCCP, and rotenone/antimycin A used were the same as those previously optimized in the Seahorse XF Cell Mito Stress Test assays.

For preconfluent 3T3-L1 cells, normalization was performed using cell counts based on Hoechst nuclear staining. At advanced stages of differentiation, nuclear staining became less reliable due to cell stacking and multilayer formation; therefore, protein concentration was used for data normalization.

Results and discussion

Advantages and limitations of the XF V7 microplate

The XF V7 microplate features a transient microchamber volume of 5.65 µL during rate measurements, making it highly sensitive and well-suited to study respiration of most cell types including 3T3-L1 pre-adipocytes. This is evident in the changes in O_2 levels observed during assays (Figure 4A), where we note relatively constant slopes and moderate O_2 depletion during each measurement. Effective recovery of O_2 concentration in the medium is also observed after the mixing period, both at basal measurements and after FCCP injection, resulting in an accurate canonical OCR kinetic graph for an XF Cell Mito Stress Test (Figure 4B).

However, when similar experiments are conducted with mature adipocytes (day 6) using V7 plates, or during the adipogenic maturation process when metabolic activity is significantly elevated, the initial O_2 levels in the extracellular medium are markedly lower (around 75 mmHg versus expected ~ 150 mmHg). Almost complete O_2 depletion occurs during measurements, even at the basal conditions (Figure 4C). Furthermore, hypoxic conditions worsen following FCCP injection due to increased OCR from mitochondrial uncoupling. This hypoxia leads to incomplete recovery of O_2 levels between measurements, resulting in a false negative spare respiratory capacity (SRC) (Figure 4C and 4D) and inaccurate OCR determination. These findings highlight the critical importance of monitoring O_2 levels during XF assays to identify high-respiring cell models that may require optimization of assay conditions for accurate metabolic analysis.

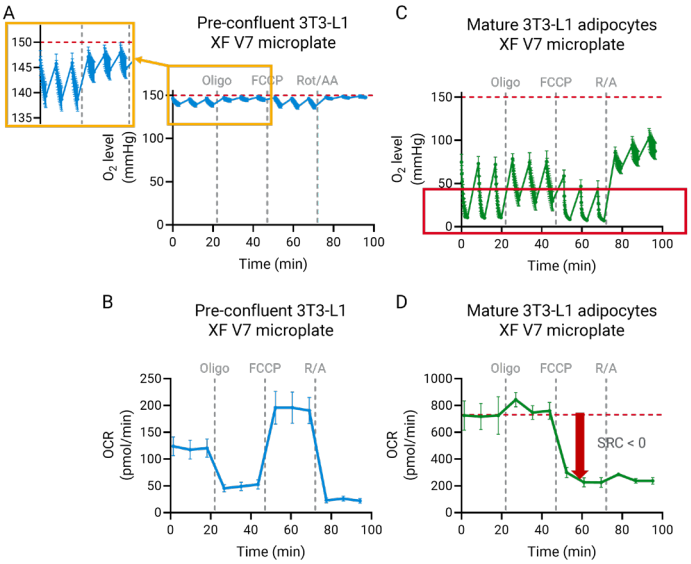

Figure 4. Seahorse XF Cell Mito Stress test comparison between preconfluent 3T3-L1 fibroblasts (A, B) and mature adipocytes (C, D) using the XF V7 microplate and the Seahorse XF Flex analyzer. Mature adipocytes' high respiration results in severe oxygen depletion during measurements (red box) and underestimation of maximal respiration, resulting in false negative spare respiratory capacity calculations (red arrow).

Improved OCR measurements for high-respiring cells using the XF V28 microplate and the Seahorse XF Flex analyzer

To optimize bioenergetic measurements in high-respiring cell models, we performed parallel studies of mature adipocytes (d6) differentiated in the XF V28 microplates using the Seahorse XF Flex analyzer. As shown in Figure 5A, the larger microchamber volume of the XF V28 microplate helps maintain oxygen levels within the recommended range during measurements (O_2 > 50 mmHg), effectively preventing the hypoxic conditions observed when using the XF V7 plate (Figure 4C). In addition, the modified mixing program for the XF V28 microplate, incorporated within the Seahorse XF Flex analyzer, improved re-oxygenation in the microchamber between measurements (Figure 5A). This prevents excessive O_2 depletion and generates a reliable XF Cell Mito Stress Test kinetic graph (Figure 5B), including the expected OCR increase following FCCP injection and a positive spare respiratory capacity (SRC >0).

In contrast, when the same cells cultured in XF V28 microplates were analyzed in the Seahorse XFe24 analyzer, using the default mixing program and instrument protocol (Figure 5C), insufficient oxygen recovery is observed due to suboptimal mixing parameters. This results in inaccurate characterization of mitochondrial function (Figure 5D).

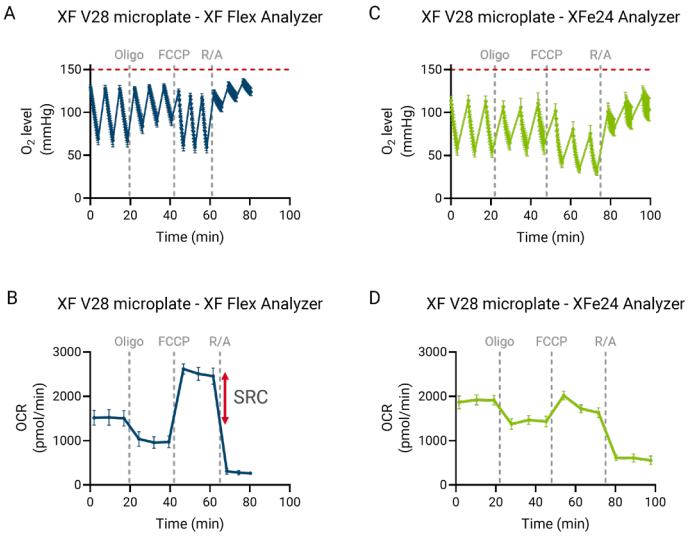

Figure 5. Comparison of Seahorse XF Cell Mito Stress Test results in mature 3T3-L1 adipocytes (day 6) using the Seahorse XF Flex analyzer (A and B) and the Seahorse XFe24 analyzer (C and D) with the XF V28 microplate. The optimized mixing program and protocol for the XF V28 microplate, implemented in the Seahorse XF Flex analyzer, enables accurate determination of maximal respiration and yields a positive spare respiratory capacity (SRC; red arrow).

Overall, using the XF V28 microplate with the Seahorse XF Flex analyzer enables accurate measurement of metabolic profiles in highly active cell types. Further studies across various cell types indicate that the optimal OCR measurement range for the XF V7 microplate is approximately 30–1,200 pmol/min, while the XF V28 microplate can reliably measure OCRs up to 3,000 pmol/min. However, the XF V28 microplate is not recommended for cells with basal OCRs below 500 pmol/min, due to insufficient sensitivity and increased noise leading to greater data variability.

Table 1. Recommended OCR and O_2 level in XF V7 and XF V28 microplates.

	XF V7	XF V28
Recommended OCR	30–1,200 (pmol/min)	500–3,000 (pmol/min)
Recommended O_2 level	165–50 mmHg	165–50 mmHg

Combining XF V7 and XF V28 microplate assays for accurate metabolic profiling across adipocyte differentiation stages

As previously discussed, mitotic clonal expansion is critical for adipogenesis in 3T3-L1 cells and occurs during the first two days of differentiation. From day three onward, cell proliferation ceases while differentiation into mature adipocytes continues. This is accompanied by an increase in cell mass and a marked rise in PGC1α expression[3], resulting in a significant elevation in oxygen consumption rate per well and substantial changes in mitochondrial activity.

Accurate characterization of these bioenergetic shifts during adipocyte differentiation requires data from both XF V7 and XF V28 microplates, selected based on the OCR range and differentiation stage. Our comparative analysis across differentiation timepoints shows that pre-adipocytes are best assessed using the XF V7 plate, while from day three onward, the XF V28 microplate provides more reliable measurements (Figure 6A–D). FCCP titration was also optimized at each stage to ensure accurate determination of maximal respiration and spare respiratory capacity (SRC).

As shown in Figure 6A–D, both basal and maximal OCR increase as differentiation progresses. However, when normalized to protein content, ATP-coupled respiration and SRC are significantly lower in mature adipocytes (days six and nine) compared to day three (Figure 6E–F). This decrease is attributed to the continued rise in total protein as cells mature, despite a stable cell number.[4]

These findings highlight mitochondrial adaptations during adipogenesis and underscore the value of combining XF V7 and XF V28 microplates to accurately monitor dynamic metabolic changes across differentiation stages.

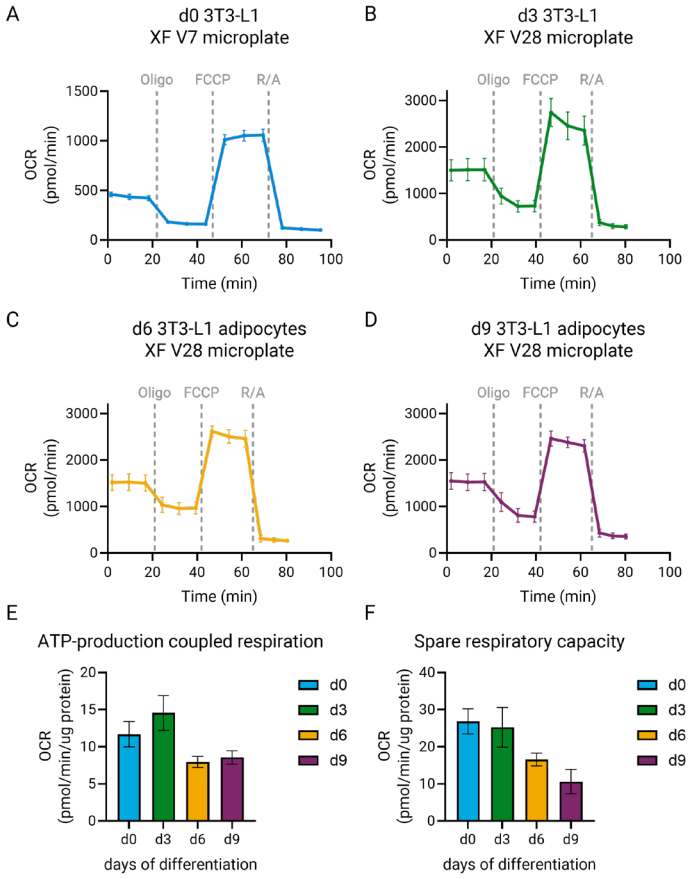

Figure 6. Seahorse XF Cell Mito Stress Test analysis of 3T3-L1 cells during differentiation using a combination of XF V7 and XF V28 microplates on the Seahorse XF Flex analyzer. (A–D) OCR kinetic profiles of d0 pre-adipocytes (A), d3 differentiating cells (B), and mature adipocytes at d6 (C) and d9 (D). (E) ATP-production coupled respiration and (F) spare respiratory capacity, both normalized to total protein content, across different stages of differentiation.

Substrate Oxidation Stress Tests reveal differential fuel dependence in pre- and mature 3T3-L1 adipocytes

Glucose, fatty acids, and amino acids are the primary fuels utilized by various cell types to support mitochondrial respiration. To evaluate the relative dependence on each substrate, we performed substrate oxidation stress tests using specific metabolic inhibitors: UK5099 (a mitochondrial pyruvate carrier inhibitor), Etomoxir (a CPT1α inhibitor blocking long-chain fatty acid oxidation), and BPTES (a glutaminase inhibitor targeting glutamine oxidation). These inhibitors were administered before the Seahorse XF Mitochondrial Stress Test reagents, following the XF Substrate Oxidation Stress Test user guide.

We found that preconfluent 3T3-L1 fibroblasts are sensitive to all three inhibitors and showed comparable reductions in maximal respiration when glucose, fatty acid, or glutamine metabolism was inhibited (Figure 7A, E). In contrast, d0 pre-adipocytes showed greater dependence on glucose and fatty acid oxidation, with a milder response to glutamine inhibition (Figure 7B, F). Mature adipocytes, on the other hand, displayed a distinct metabolic pattern: exposure to UK5099 and BPTES caused a rapid, transient decline in basal respiration, accompanied by a compensatory increase in glycolytic activity, as shown in OCR and ECAR kinetics (Figure 7C–D). However, these cells quickly adapted, resulting in no significant change in maximal respiration despite the presence of the inhibitors (Figure 7G).

In summary, assaying pre-adipocytes using the V7 plate and mature adipocytes using the V28 plate provides accurate measurements of maximal respiration, generating a positive spare respiratory capacity that facilitates precise detection of fuel dependence at different stages of adipocyte differentiation.

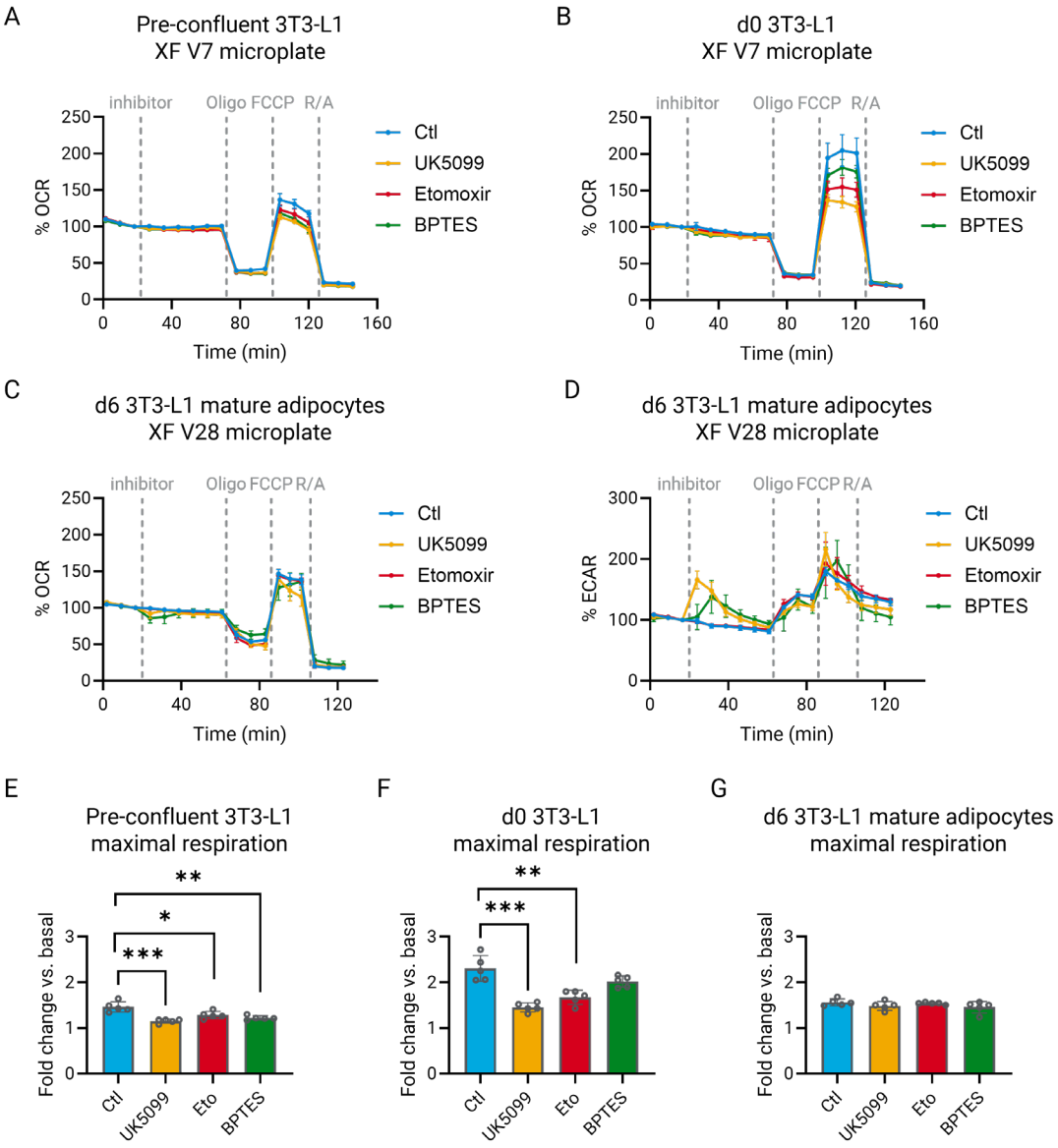

Figure 7. Seahorse XF Substrate Oxidation Stress test in pre- and mature 3T3-L1 adipocytes. OCR kinetic trace baselined to measurement 3 for preconfluent 3T3-L1 fibroblasts (A), d0 pre-adipocytes (B) and d6 3T3-L1 adipocytes (C). (D) ECAR kinetic trace baselined to measurement 3 of mature d6 3T3-L1adipocytes. (E-G) Maximal respiration, relative to basal, in preconfluent 3T3-L1 fibroblasts, d0 L1 pre-adipocytes and d6 3T3-L1 adipocytes. Representative data of 2 independent experiments. Data represents mean ± SD of 5 technical replicates. *P<0.05, **P<0.01, ***p<0.001.

Conclusion

Metabolic reprogramming during cell differentiation plays a crucial role in determining lineage commitment, offering valuable insights to optimize differentiation protocols. It also helps enhance our understanding of genetic perturbations that affect the cellular function of differentiated cells, guiding therapeutic development. Due to the drastic changes in bioenergetic metabolism during differentiation, it is important to establish appropriate assay conditions that enable accurate determinations of mitochondrial activity at different stages along the process. The Agilent Seahorse XF Flex analyzer provides a workflow for accurate assessment of mitochondrial function during the whole course of adipocyte differentiation. This workflow reduces the need to adjust differentiation protocols or time frames to accommodate precise metabolic measurements and delivers new insights into cell metabolism and its modulation.

References

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Products used in this application

Agilent products

Agilent Seahorse XF Flex Analyzer

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