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Revealing Cellular Metabolic Phenotype and Function Using Agilent XF Substrate Oxidation Stress Tests

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Abstract

Agilent Seahorse XF technology measures energy metabolism in live cells in real time, providing critical functional information that relates directly to cellular health and fitness. The Agilent Seahorse XF Substrate Oxidation Stress Test provides key metrics to facilitate investigation of specific mitochondrial substrates that are relevant or required for cellular phenotype and function. This study discusses an overview of this assay strategy, examples of resulting data, and ways to best apply these methods in the context of cancer and immunological cell metabolism and its relevance to drug discovery.

Introduction

Metabolism is now recognized as a critical factor in many important cell functions in normal and disease states.[1,2] Determining substrate requirements in controlled experiments can inform across a range of research areas, including drug target identification and validation in cancer, immune dysfunction, cardiovascular disease, neurodegenerative disease, and stem cell disease models.[3,4,5]

Agilent Seahorse XF technology is a label-free, integrated platform that seamlessly combines the XF Analyzer, sensor cartridge, assay kits, and software to deliver functional cellular metabolic data with real-time kinetics. Built on this platform, Agilent offers a variety of XF assay kits that provide a full spectrum of information, from broad assessment of cellular function down to specific details of metabolic mechanisms, which are outlined in Figure S1.

A suite of optimized assays for measuring cellular substrate oxidation, the XF Substrate Oxidation Stress Tests, has been developed. These assays interrogate three primary substrates that drive the mitochondrial activities: long-chain fatty acids (LCFAs), glucose/pyruvate, and/or glutamine (Figure 1). By leveraging comprehensive mitochondrial assessment enabled by the XF Cell Mito Stress Test with inhibitors of specific substrate oxidation pathways, cells are interrogated not only under lower substrate demand (i.e. basal respiration), but also under conditions of higher substrate demand (i.e. maximal respiration) where critical substrate dependence/reliance is more often revealed (Figure 2). These kits facilitate the convenient investigation of specific substrate oxidation processes, and the central role they play in the fundamental cellular functions of activation, proliferation, and differentiation. Additionally, they can be used to characterize responses to genetic manipulations, pharmaceutical interventions, or specific disease-relevant microenvironments with respect to oxidation of specific substrates.
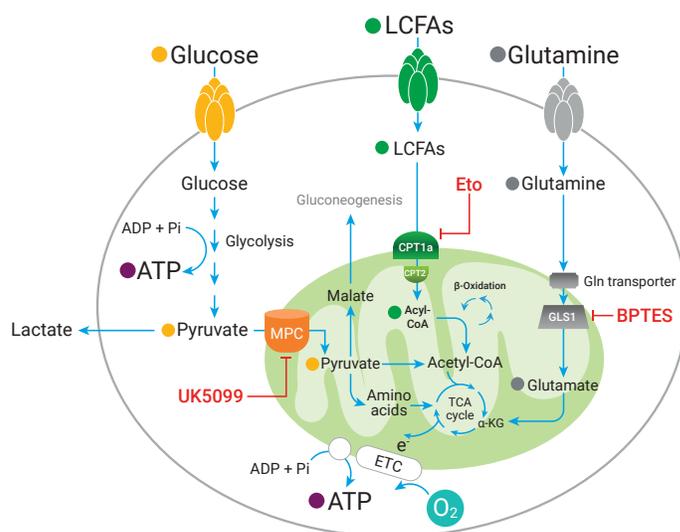
Figure 1. Primary metabolic pathways, including glycolysis, the TCA cycle, electron transport (ETC), and oxidative phosphorylation (OXPHOS). Glucose/pyruvate, glutamine, and long-chain fatty acid oxidation are highlighted. Red lines/text denote relevant inhibitors of glucose/pyruvate, LCFA, and glutamine metabolism, which in turn specifically limits oxidation of that respective substrate.
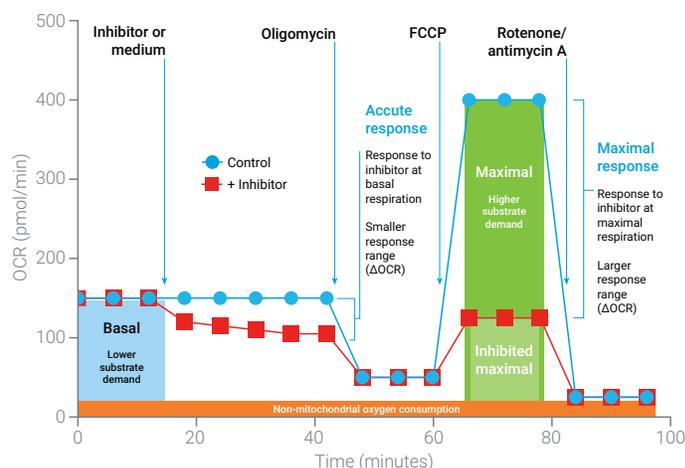
Figure 2. Agilent Seahorse XF Substrate Oxidation Stress Test profile of the respiration parameters critical for substrate demand. Sequential compound injections measure basal respiration, acute response to inhibitor (etomoxir or UK5099 or BPTES), and maximal respiration in the absence and presence of inhibitor. Note that while minimal changes may be measured under basal conditions, i.e. the acute response, much larger responses are often revealed under conditions of high substrate demand (e.g. FCCP), thus revealing differences in the ability of the cells to oxidize the substrate in question.

Experimental

All cell lines were maintained according to manufacturer recommendations. For XF assays, A549 cells were seeded in XF96 cell culture microplates at a density of 1.5×10^4 cells per well in DMEM F12 (Corning 10-090-CV) supplemented with 2 mM Glutamax and 10% serum. C2C12 cells were seeded in XF96 cell culture microplates at a density of 1.2×10^4 cells per well, in DMEM high glucose (Gibco 11960) supplemented with 4 mM glutamine, 1 mM pyruvate, and 10% serum. HepG2 cells were seeded in XF96 cell culture microplates at a density of 2.0×10^4 cells per well and cultured in DMEM low glucose (Gibco 11885) supplemented with 2 mM Glutamax and 10% serum. All cells were incubated overnight at 37 °C, 5% CO_2. The following day, cells were washed twice with Substrate Oxidation Assay Media (XF DMEM pH 7.4 plus 10 mM XF Glucose, 1 mM XF Pyruvate, and 2 mM XF Glutamine) and incubated at 37 °C, no CO_2, for 60 minutes. Cell plates were then transferred to XFe96 analyzers for assay performance, using either etomoxir (4 µM), UK5099 (2 µM), or BPTES (3 µM) in Port A, followed by sequential injection of oligomycin (1.5 µM), FCCP (1.5 µM for all three cell types), and rotenone/antimycin A (0.5 µM each)(final concentrations). Cells were then counted (for normalization) using a Cytation 5 instrument.

All XF assays were performed as described in the XF Substrate Oxidation User Guide, including compound dilutions and sensor cartridge preparation.

Agilent Seahorse Analytics is a new, web-based software platform that provides a simple, streamlined data analysis workflow for the XF Substrate Oxidation Stress Test assay. Seahorse Analytics was used to calculate key parameters of the XF Substrate Oxidation Stress Test—basal respiration, acute response, and maximal respiration. Steps to perform data analysis using Seahorse Analytics are described in the XF Substrate Oxidation Stress Test User Guide.

% Basal OCR after inhibitor was added and % maximal OCR in the presence of inhibitor (Figure 7) for each cell type were calculated as follows and graphed using Microsoft Excel:

- **% Basal OCR after inhibitor** = [(Basal OCR + Acute Response OCR)/Basal OCR] × 100%

- **% Maximal OCR in presence of inhibitor** = [(Maximal Control OCR − Maximal Inhibited OCR)/Maximal Control OCR] × 100%

Materials

Material	Vendor
A549 Cell Line	ATCC
C2C12 Cell Line	ATCC
HepG2 Cell Line	ATCC
DMEM/F12 Medium	Corning
DMEM High Glucose Medium	Gibco
DMEM Low Glucose Medium	Gibco
Glutamax	Gibco
Serum	Hyclone
XF96 Tissue Culture Plates	Agilent
XFe96 Sensor Cartridges	Agilent
XF Calibrant	Agilent
XF DMEM pH 7.4 Assay Media	Agilent
XF Glucose	Agilent
XF Pyruvate	Agilent
XF Glutamine	Agilent
XF Long Chain Fatty Acid Oxidation Stress Test Kit	Agilent
XF Glucose/Pyruvate Oxidation Stress Test Kit	Agilent
XF Glutamine Oxidation Stress Test Kit	Agilent
Seahorse XFe96 Analyzer	Agilent
Cytation 5	BioTek, part of Agilent

XF Substrate Oxidation Stress Test: experimental strategy and assay design

The XF Substrate Oxidation Stress Tests combine the XF Cell Mito Stress Test (MST) with the following substrate pathway-specific inhibitors (Figure 1):

- Etomoxir (Eto) for inhibition of LCFAs through inhibition of carnitine palmitoyl transferase 1a (CPT1a).[6]

- UK5099 for inhibition of glucose or pyruvate through inhibition of the mitochondrial pyruvate carrier (MPC).[7]

- BPTES for inhibition of glutamine through glutaminase 1 (GLS-1).[8] Note that BPTES does not inhibit glutaminase 2 (GLS2).

The MST, a powerful and well accepted tool for the interrogation of mitochondrial function, in conjunction with these inhibitors, can be used to reveal dependence on a specific metabolic substrate. Basal and maximal respiration rates are key metrics of mitochondrial function reported by the MST. In the context of substrate oxidation, the basal, and in particular, the maximal respiration rates are largely impacted by cells' capacity to transport and oxidize available substrates.[9] This method is ideally suited to the assessment of cellular substrate demand under basal conditions,

and in response to elevated substrate demand (maximal respiration). Figure 2 outlines the kinetic profile of a standard substrate oxidation assay and relevant assay parameters.

For performance of a substrate oxidation stress test, basal respiration is established, followed by injection of the relevant pathway inhibitor. The acute response to the inhibitor is followed over several measurement cycles (typically six), then the standard sequence of oligomycin, FCCP, and rotenone/antimycin A injections are performed.

Measurements are highly informative, as the design described provides data on basal respiration and the impact of pathway inhibition under conditions of basal substrate demand, while also characterizing the impact of pathway inhibitions on maximal respiration, reflecting the cells' sensitivity to impairment of a specific metabolic pathway under conditions of high substrate demand.

Each kit is focused on testing a single substrate using the optimized (final) concentration of relevant inhibitor:

- Etomoxir (4 µM) to inhibit oxidation of LCFAs
- UK5099 (2 µM) to inhibit the oxidation of glucose or pyruvate
- BPTES (3 µM) to inhibit oxidation of glutamine (final concentrations)

The standard substrate oxidation stress test assays are designed to be performed under conditions of saturating substrates with respect to glucose (10 mM), pyruvate (1 mM), and glutamine (2 mM) in the assay media. The source

of long-chain fatty acids is any endogenous stores of lipid/LCFAs in the cell, and thus is cell type dependent. Figure 3 outlines the standard methods and assay conditions for each of the XF Substrate Oxidation Stress Tests. Note that, with the exception of the identity of the inhibitor used, the methods are identical.

Responses to an inhibitor (Figure 2) suggest that the cell has a demand or reliance for that particular substrate under the experimental conditions established and is further addressed in the discussion section below, but in general, this standard substrate oxidation assay is designed to be used when asking the following types of questions:

- Does the cell have a demand for a particular substrate or substrates?
- Is the cell highly reliant on a specific substrate, or can other substrates satisfy cellular demands?
- How is mitochondrial substrate demand or reliance affected if an intervention, such as a genetic manipulation or drug exposure, is applied to the cell?

Like most XF assays, the substrate oxidation stress tests are typically performed subsequent to a pretreatment condition, or intervention, as designed by the researcher. This is shown as either a chronic intervention to the cells (e.g. a genetic manipulation or long-term drug exposure), hours to days upstream of the XF assay, or as an acute intervention (e.g. drug exposure) just prior to the XF assay (Figure 3). In some cases, both chronic and acute interventions may be
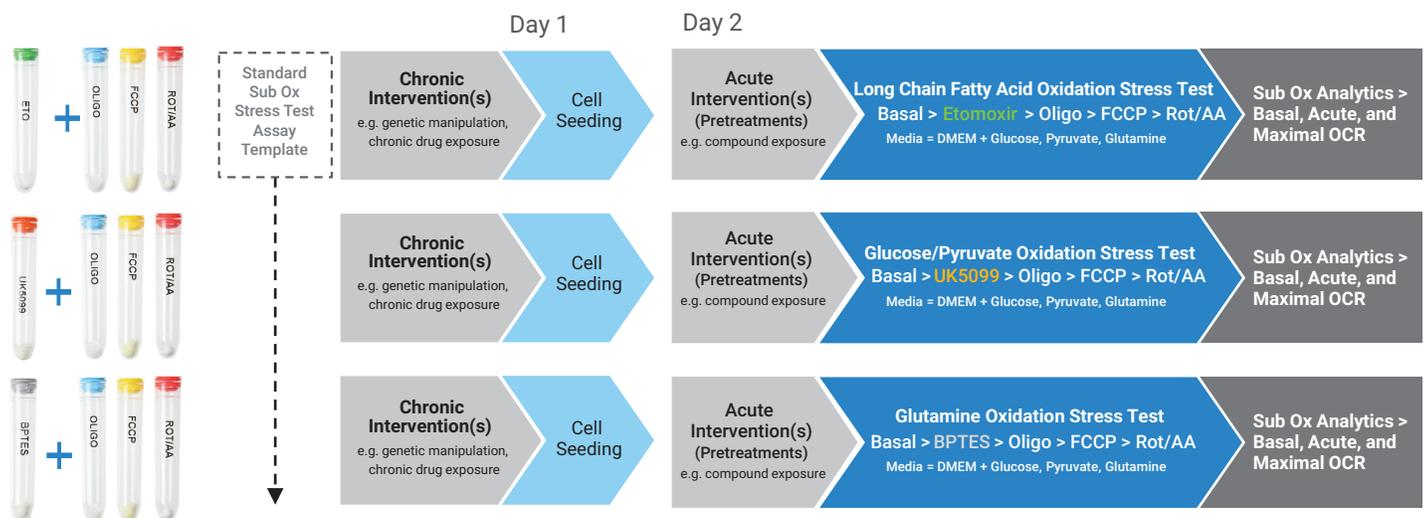
Figure 3. Use of Agilent Seahorse XF Substrate Oxidation Stress Tests to investigate mitochondrial substrate demand or reliance. Each kit is focused on testing a single substrate using the relevant inhibitor: etomoxir to inhibit oxidation of LCFAs, UK5099 to inhibit the oxidation of glucose or pyruvate, and BPTES to inhibit oxidation of glutamine. Chronic or acute interventions (genetic manipulations/drug exposures) may be performed upstream of the assay to understand the effects of these modulations on the oxidation of specific mitochondrial substrates. See text for further information.

used (e.g. rescue of genetic dysfunction through compound exposure). The kits may be used individually (i.e. focusing on one specific substrate) for investigating how a series of interventions or compounds may affect oxidation of that particular substrate; or as a complementary suite (i.e. focusing on two or more substrates) to elucidate overall effects of a given intervention with respect to substrate oxidation and mitochondrial function. The XF Substrate Oxidation Stress Tests User Guide offers several assay template design suggestions based on various experimental scenarios, and Figure 4 illustrates a simple assay design

Figure 4. Agilent XFe96 assay template for substrate oxidation stress test using multiple kits/inhibitors. Group designations and plate layout to test one cell type with three different inhibitors, including a control group (assay media injected from port A). This template was replicated three times, changing only the cell type used (A549, C2C12, or HepG2). All other assay template information and XF instrument commands were the same across assays.

template used to generate the data presented.

Note that a separate, advanced protocol and dedicated kit for the specific investigation of long-chain fatty acid oxidation (LCFAO) are also available. The kit provides additional components including the XF Palmitate-BSA substrate; the assay protocol is described in full in the XF Substrate Oxidation Stress Tests User Guide.

Results and discussion

Application of the XF Substrate Oxidation Stress Tests
Using one kit each of the three substrate oxidation stress tests (glucose/pyruvate, long-chain fatty acid, and glutamine), three standard cell lines were examined using the standard assays and templates previously described (Figures 2 to 4). Complete details of cell culture, cell seeding, and XF assay preparation are described in the Experimental section of this document and in the XF Substrate Oxidation Stress Tests User Guide.

Figure 5 (panels A, B, and C) shows the kinetic OCR traces for A549, C2C12, and HepG2 cells, respectively. The substrate oxidation stress test parameters of basal respiration, acute response, and maximal respiration are shown for HepG2 cells
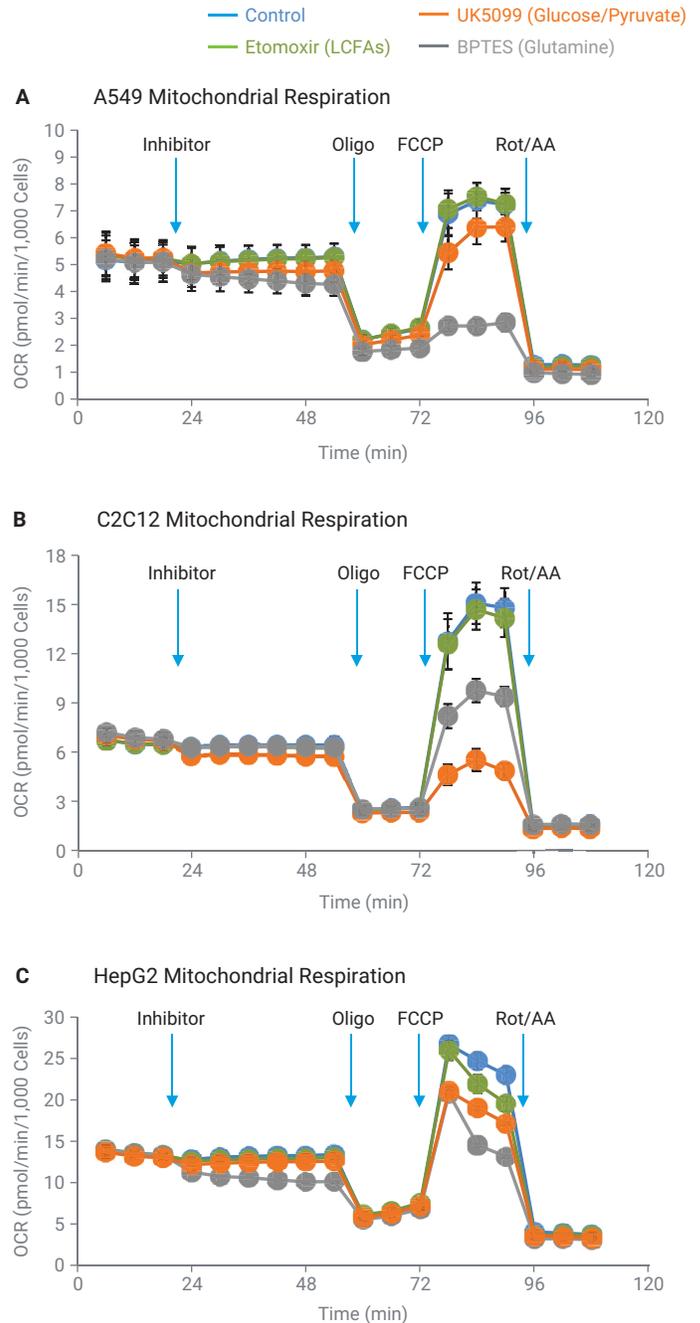
Figure 5. Comparing substrate oxidation among A549, C2C12, and HepG2 cells using XF Glucose Pyruvate, Long Chain Fatty Acid, and Glutamine Oxidation Stress Test kits. A549 (A), C2C12 (B) and HepG2 (C) cells were seeded in Agilent XF96 Cell Culture Plates and grown overnight. Cells were subject to XF substrates oxidation stress tests using injections of assay media (control), etomoxir (LCFA oxidation), UK5099 (glucose/pyruvate oxidation), or BPTES (glutamine oxidation), followed by common sequential injections of oligomycin, FCCP, and rotenone/antimycin A. XF Assay Media = XF DMEM, pH 7.4 + 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine. Each cell type was assayed on three individual days (i.e. n = 3), with resulting data being compiled and processed through Agilent Seahorse Analytics. Error is reported as ±SEM.

in Figure 6.

Inspection of the basal respiration parameter for each cell type shows the expected results, with all conditions showing identical basal OCRs before inhibitors are added. Upon injection of inhibitor, each cell type displays a detectable, but relatively small, acute response with respect to respiration under basal assay conditions. However, under conditions of maximal respiration (i.e. under conditions of higher substrate demand by the mitochondria), responses to inhibitors are significantly increased, and different responses to different inhibitors become apparent across the three cell types tested.

For example, in A549 cells (Figure 5A), a large response to BPTES is observed, but a much smaller response was seen with UK5099, and no response was seen with etomoxir. This suggests that A549 cells are significantly reliant on glutamine oxidation under conditions of high substrate demand. In comparison, C2C12 cells show significant responses to UK5099 and BPTES (Figure 5B), indicating that both of these substrates are required by the cell under maximal substrate demand conditions. Conversely, HepG2 cells display a more balanced dependence on glucose/pyruvate, LCFAs, and glutamine under conditions of maximal respiration (Figure 5C), as there are significant responses to UK5099, Etomoxir, and BPTES, respectively. Figure 7 summarizes the % basal OCR after inhibitors are added and the % maximal OCR in the presence of inhibitor across the three cell types. Note that changes in maximal respiration are shown to be the most informative parameter when assessing mitochondrial substrate reliance due to the conditions of increased
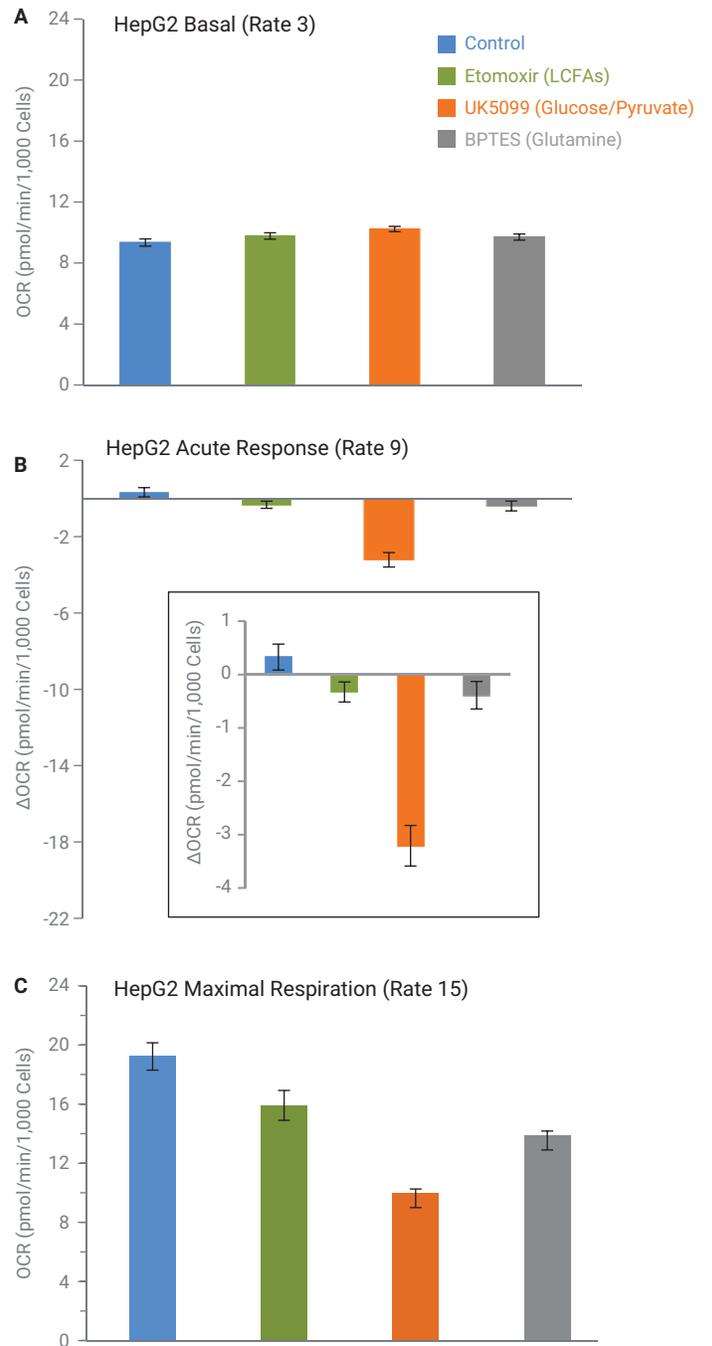
Figure 6. Example of substrate oxidation stress test parameters of basal respiration, acute response, and maximal respiration for HepG2 cells. (A) Basal respiration rates. (B) Acute response; note this value is expressed as a change (Δ) in OCR relative to the basal rate. (C). Maximal respiration rates in the presence of individual inhibitors. Graphs and values are derived from the kinetic trace shown in Figure 5 using Agilent Seahorse Analytics. The same parameters were calculated for A549 and C2C12 cells (data not shown).

A   % Basal OCR after inhibitor added

A549   C2C12   HepG2

B   % Maximal OCR in presence of inhibitor

A549   C2C12   HepG2
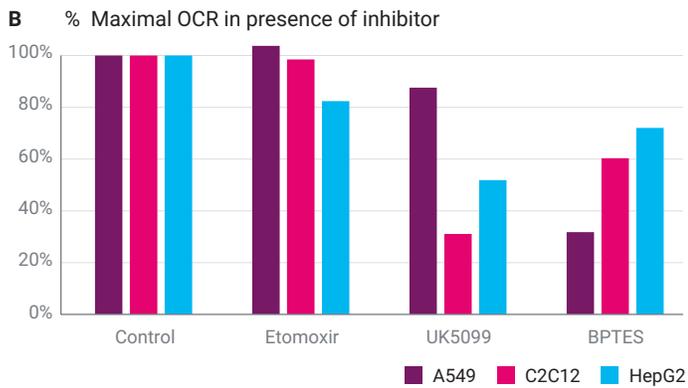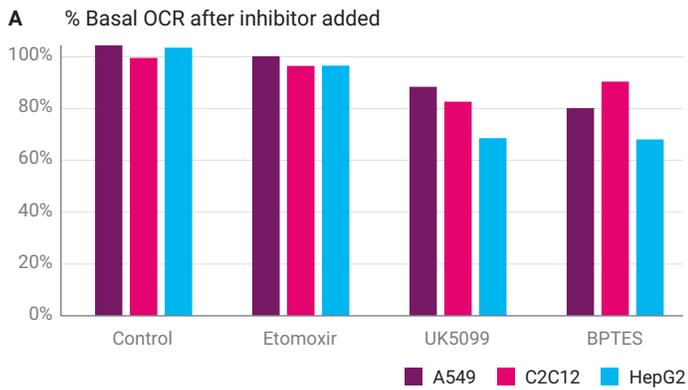
C

% Basal OCR	LCFAs (Etomoxir)	Glucose/Pyruvate (UK5099)	Glutamine (BPTES)
A549	100%	88%	80%
C2C12	96%	83%	90%
HepG2	97%	69%	68%

D

% Maximal OCR	LCFAs (Etomoxir)	Glucose/Pyruvate (UK5099)	Glutamine (BPTES)
A549	104%	88%	32%
C2C12	97%	31%	60%
HepG2	82%	52%	72%

Figure 7. Comparative substrate oxidation in A549, C2C12, and HepG2 cells. (A) % Basal OCR after inhibitor added and (B) % maximal OCR in presence of inhibitor from data in Figure 5 with respect to each inhibitor. Tables C and D summarize data from panels A and B, respectively. See Experimental section for calculation of % values. Note the increased sensitivity to inhibitors under maximal respiration (increased substrate demand) compared to acute responses under basal respiration conditions, as well as the highly differential responses to inhibitors based on cell type.

substrate demand.

Relevance of substrate oxidation to cancer and immunological cell metabolism, drug discovery, and suggested applications

Understanding substrate dependencies is critical for advancing therapeutic opportunities across a range of diseases. Indeed, investigation of the genes, proteins, and pathways that modulate metabolism is a promising avenue for developing novel targets for a broad range of ailments (Figure 8). Emerging evidence demonstrates that metabolites themselves, including the oxidation of specific mitochondrial substrates, can drive cell function and phenotype with respect to proliferation, differentiation, and dysfunction/disease.[5]

Cancer cell proliferation is driven by substrate availability. Substrates not only fuel cancer cells to malignancy and proliferation, but the availability of specific substrates is a key characteristic of the tumor microenvironment to influence, among other things, the efficacy of chemotherapeutic treatments.[10] With respect to drug development, the need to better understand substrate requirements in cancer cell metabolism is critically important. Therapeutic strategies for cancer treatment involve targeting of several metabolic pathways including glycolysis, the TCA cycle, electron transport (ET), and oxidative phosphorylation (OXPHOS), as well as pyruvate, glutamine, and fatty acid oxidation (Figure 1). For example, while glutamine oxidation was shown to be minimal in murine p53 KrasG12D lung tumors,[11] multiple *in vitro* studies indicate a major role in glutamine oxidation, including PDAC cells, suggesting that respiration has an anabolic role in that it stimulates aspartate biosynthesis in proliferating cancer cells.[12]

It has been demonstrated that one of the most abundantly used bioenergetic pathways in prostate cancer cells is enhanced uptake of palmitate and the oxidation of fatty acids, providing an alternate route for meeting the bioenergetics needs of tumor cells and further, new evidence shows that pancreatic cancer cells are especially dependent on mitochondrial oxidative phosphorylation under low nutrient conditions, and that mitochondrial metabolism represents a key metabolic vulnerability.[12]
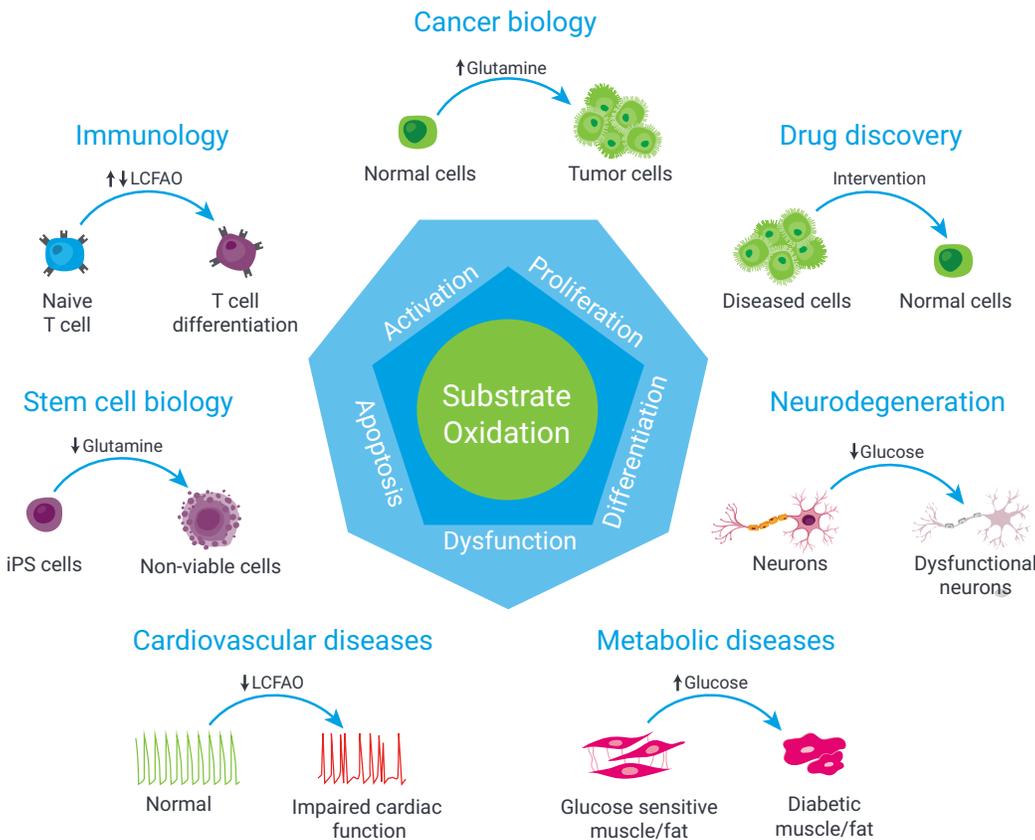
Figure 8. Examples of mitochondrial substrate oxidation driving cell phenotype and (dys)function. Agilent Seahorse XF Substrate Oxidation Stress Test Assays facilitate investigation and understanding of which specific mitochondrial substrates are relevant or required for a specific cellular phenotype and function.

With respect to experimental design, Figure 9A outlines one possible scenario for testing multiple genetic variants, natural or engineered, of a cancer type to understand if the cells have similar or unique substrate oxidation "signatures" and are these substrates relevant for cell proliferation or transformation. This information can also point to metabolic liabilities, facilitating therapy discovery.

Cellular energy metabolism is also understood to influence many immune cell pathways as an indicator and controller of immune cell function and lineage commitment. Interestingly, the metabolites themselves can directly influence immune cell differentiation and function.[5] Consequently, altering the metabolic machinery and generation of metabolites can regulate immune function. For example, glutamine metabolism plays a critical role in M2 macrophage polarization by fueling the TCA cycle, and promotes the generation of stable phenotypes found in trained immunity,[13] while positive central memory T cells were found to import glucose and glycerol to synthesize fatty acids and triglycerides, which are then hydrolyzed through lipolysis to fuel fatty acid oxidation (FAO).[14]

Regarding immune cell function and substrate oxidation assays, Figure 9B illustrates how the XF Substrate Oxidation Stress Tests may be used with various types of immune cells (naïve, activated, memory, etc.) to investigate substrate

dependence or reliance on one or more substrates for correct cell differentiation or function, and potentially, how immune cell fate might be manipulated or controlled through oxidation of specific substrates.

To this point, metabolic programming is emerging as a critical mechanism to alter immune cell activation, differentiation, and function.[15] The different metabolic requirements of the diverse cells that constitute an immune response provide a unique opportunity to separate phenotype and functions. One approach to cancer immunotherapy uses *ex vivo* culturing and genetic manipulation of autologous T cells, which are reinfused in adoptive transfer protocols, including chimeric antigen receptor (CAR)-T cell therapy. Here, T cells are genetically programmed to express chimeric proteins that connect highly specific antitumor antibody peptides with stimulatory pathways for T cell activation.[3] There has been recent investigation of targeting suppressive immune cells such as intra-tumoral Treg cells, myeloid-derived suppressor cells, and M2 macrophages. All of these subsets are uniquely interesting given their distinct metabolic profiles, which suggests that a relatively high degree of specificity in targeting through genetic manipulation or drug exposure is obtainable.[3]
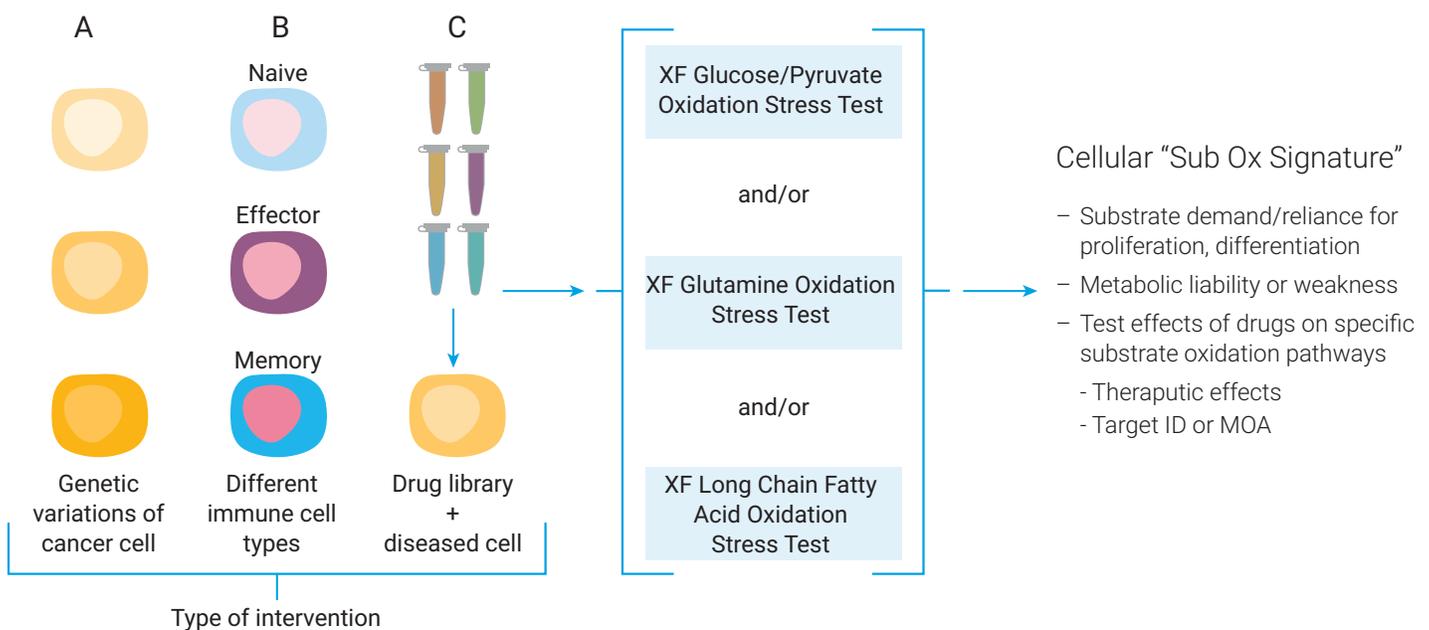
Figure 9. Examples of substrate oxidation stress test assay design. Potential experimental designs for applying the Agilent XF Substrate Oxidation Stress Tests to (A) cancer cell biology, (B) immune cell function, and (C) drug discovery areas of research. See Results and discussion for additional details.

Conclusion

Concerning experimental design for drug testing and drug development, these types of assays can be used to test the effects of drugs with respect to specific mitochondrial substrate oxidation pathways, either to understand potential therapeutic effects of compounds on dysfunction cells, or to facilitate drug target identification and elucidate mechanisms of action. Here, variables such as time of exposure to the drug (chronic or acute) and drug concentration may be investigated (Figure 9C).

This study describes the assay design and proof of concept for Agilent XF Substrate Oxidation Stress Tests, used to understand which substrates are relevant or required for specific cellular function or phenotype. These kits comprise a suite of optimized, convenient, rapid solutions for measuring cellular substrate oxidation by assessing three primary substrates that fuel mitochondria: long-chain fatty acids, glucose/pyruvate, and/or glutamine, and provides rich real-time multiparametric information not available from conventional endpoint substrate uptake or utilization assays. This assay design is recommended when asking if the cell has a demand for a particular substrate or substrates, or when investigating if an intervention applied to the cell is affecting the oxidation of a specific substrate by the mitochondria. This functional information facilitates investigation of how cells alter or shift oxidation of substrates to perform key cellular functions of activation, proliferation,

Quantitative metabolic and energy phenotype
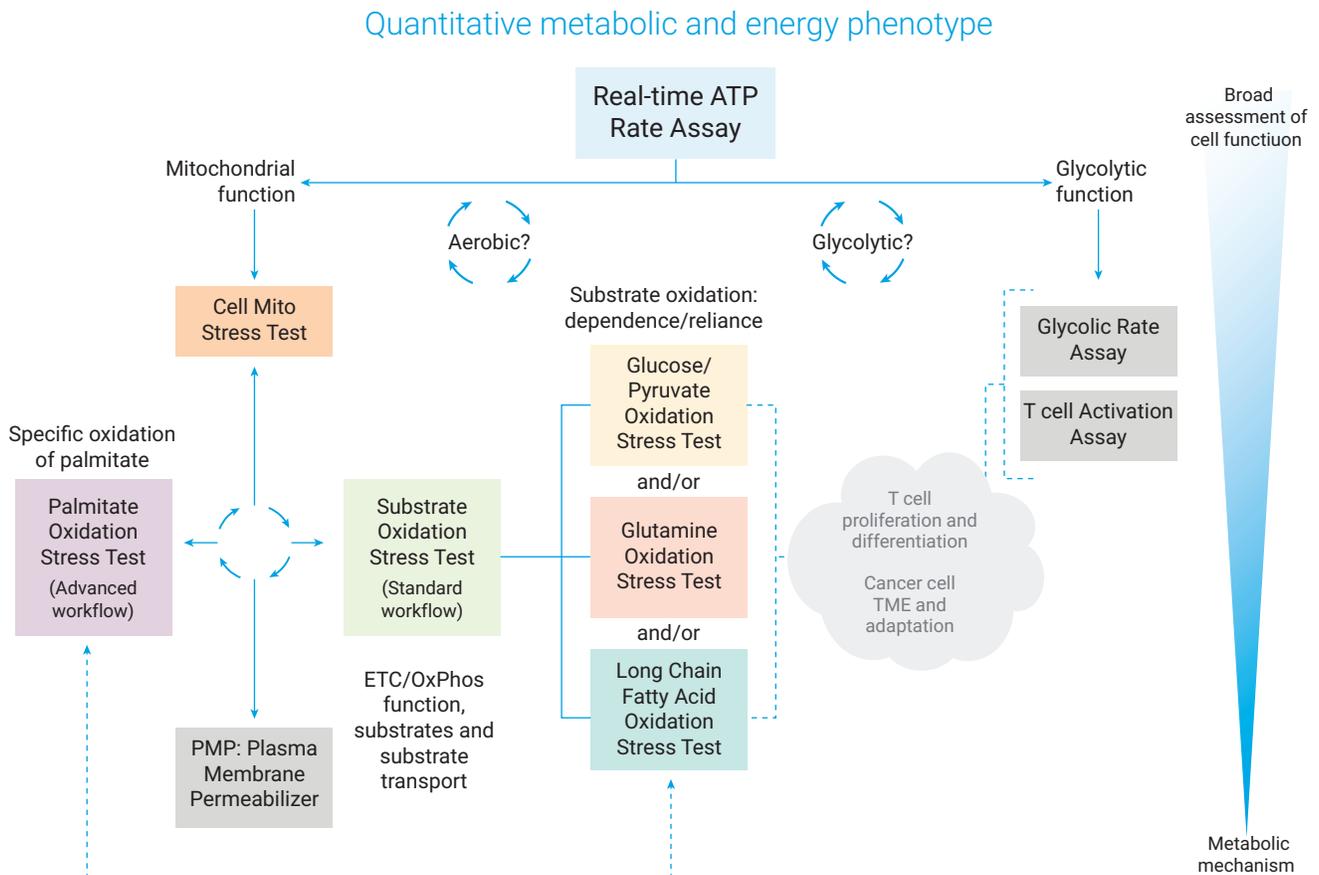
Figure S1. Agilent offers a variety of XF assay kits that provide a full spectrum of information, from broad assessment of cellular function to specific details of metabolic mechanism. Once a change in mitochondrial function has been identified, the next steps in the investigation are understanding what may be responsible for this change, including the effects of oxidation of glucose/pyruvate, glutamine, and long-chain fatty acids.

and differentiation.

References

1. Marx, V. How to Follow Metabolic Clues to Find Cancer's Achilles Heel. *Nat. Methods* **2019**, *16*, 221–24.

2. Vander Heiden, M. G.; DeBerardinis, R. J. Understanding the Intersections Between Metabolism and Cancer Biology. *Cell* **2017**, *168*, 657–69.

3. Beezhold, K.; Byersdorfer, C. A. Targeting Immuno-Metabolism to Improve Anti-Cancer Therapies. *Cancer Letters* **2018**, *414*, 127–35.

4. Corbet, C. Stem Cell Metabolism in Cancer and Healthy Tissues, Pyruvate in the Limelight. *Frontiers in Pharmacology* **2018**, *8*.

5. Patel, C. H. *et al.* Targeting Metabolism to Regulate Immune Responses in Autoimmunity and Cancer. *Nat. Rev. Drug Discov.* **2019**, *18*, 669–88.

6. Divakaruni, A. S. *et al.* Etomoxir Inhibits Macrophage Polarization by Disrupting CoA Homeostasis. *Cell Metab.* **2018**, *28*, 490–503.e7.

7. Hildyard, J. C. *et al.* Identification and Characterisation of a New Class of Highly Specific and Potent Inhibitors of the Mitochondrial Pyruvate Carrier. *Biochim Biophys Acta* **2005**, *1707*, 221–30.

8. Robinson, M. M. *et al.* Novel Mechanism of Inhibition of Rat Kidney-Type Glutaminase by Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES). *Biochemical Journal* **2007**, *406*, 407–14.

9. Divakaruni, A. S. *et al.* Analysis and Interpretation of Microplate-Based Oxygen Consumption and pH Data. *Methods Enzymol.* **2014**, *547*, 309–54.

10. Kalyanaraman, B. *et al.* a review of the Basics of Mitochondrial Bioenergetics, Metabolism, and Related Signaling Pathways in Cancer Cells, Therapeutic Targeting of Tumor Mitochondria with Lipophilic Cationic Compounds. *Redox Biology* **2018**, *14*, 316–27.

11. Kerr, E. M.; Martins, C. P. Metabolic Rewiring in Mutant Kras Lung Cancer. *Febs. J.* **2018**, *285*, 28–41.

12. Kalyanaraman, B. Teaching the Basics of Cancer Metabolism, Developing Antitumor Strategies by Exploiting the Differences Between Normal and Cancer Cell Metabolism. *Redox Biology* **2017**, *12*, 833–42.

13. Viola, A. *et al.* The Metabolic Signature of Macrophage Responses. *Frontiers in Immunology* **2019**, *10*.

14. Raud, B. *et al.* Fatty Acid Metabolism in CD8(+) T Cell Memory: Challenging Current Concepts. *Immunological Reviews* **2018**, *283*, 213–31.

15. Domínguez-Andrés, J.; Joosten, L. A. B.; Netea, M. G. Induction of Innate Immune Memory: The Role of Cellular Metabolism. *Current Opinion in Immunology* **2019**, *56*, 10–16.

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