New tools bring greater understanding to CELLULAR METABOLISM research

The links between mitochondrial dysfunction and various diseases and pathological conditions are being increasingly revealed, such that mitochondrial analyses have gained renewed interest from both the academic research and drug discovery labs. This revival of interest in cellular metabolism assays is being aided by new analysis tools that enable the characterisation of metabolic phenotypes, their interdependency with specific signalling pathways and their reprogramming during disease states. New analysis tools based on biomarker assays, fluorogenic dyes suitable for imaging and oxygen biosensing probes are proving particularly useful in this respect. Innovations in the analysis of cellular bioenergetics are expected to aid the collection of functional data needed to drive transformational change in research and impact drug discovery over the coming years.

By Dr John Comley

Mitochondrial dysfunction and dysregulated cellular metabolism are now known to play a significant role in many pathological conditions and diseases, and is also a major mechanism of drug-induced toxicity. Oxygen consumption is regarded as one of the most informative and direct measures of mitochondrial function. Traditionally, the standard 'Clark' electrode method has been used to manually measure oxygen consumption and investigate mitochondrial respiration. However, it is both time and labour-intensive, requires large amounts of cellular material, and can only test one sample at a time. Other technologies such as ELISAs, Western blots and metabolic flux analysis using 13C-labelled substrates have been used to detect changes in some metabolites and metabolism-associated proteins, and molecular biology approaches can identify changes in gene expression. However, newer technologies (ie those based on oxygen biosensing probes) now exist to enable scientists to measure the functional outcome of the gene and protein changes in the metabolic pathways at higher-throughput using smaller sample volumes. Some of these technologies can provide precise, label-free, non-destructive measurements on multiple samples simultaneously. These technologies are now increasingly driving the identification and

Figure 1: Extent to which respondents' current research involves cellular metabolism

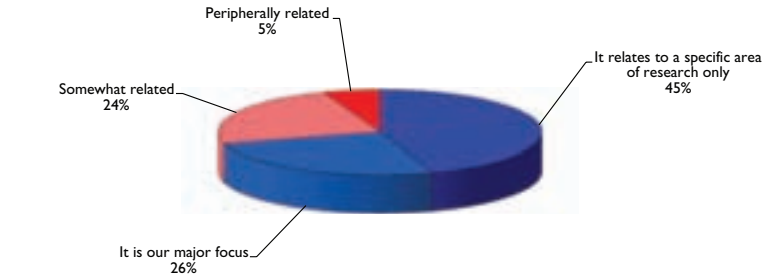


Figure 2: Importance of cellular metabolism in the research areas

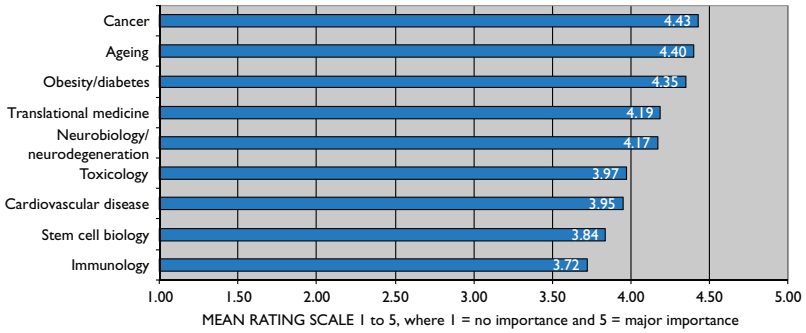
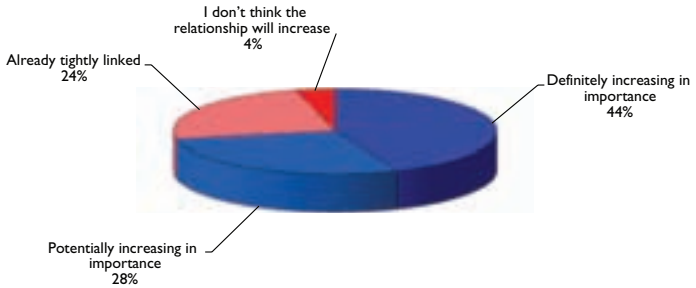


Figure 3: Future relationship between cellular metabolism and respondents' research area



recognition that there are distinct metabolic phenotypes known to be associated with states of cellular activation, growth, stress and disease. The ability of cells to switch between mitochondrial respiration and aerobic glycolysis, while critical for normal activation of some cell types (eg T-cells), is associated with disease in other cells (eg Warburg metabolism is defined by an increased utilisation of glucose via glycolysis as a cellular resource, and is a common phenotype of cancerous cells).

Scientists are now investigating these newer metabolic analysis technologies to characterise metabolic phenotypes, their interdependency with specific signalling pathways and their reprogramming during disease states. The metabolic properties of cancer cells have proven to be remarkably different from those of normal cells and dysregulated cellular metabolism is linked to drug resistance in cancer therapy. In addition to cancer, metabolic analysis has enabled new discoveries and opportunities in a number of other fields including immunology, neurodegeneration, obesity/diabetes, toxicity and stem cells. Innovations in the analysis of cellular bioenergetics are expected to aid the collection of functional data needed to drive transformational change in research and impact drug discovery over the coming years.

In March 2014, HTStec undertook a market survey on cellular metabolism. The aims of this survey were to: 1) assess current opinion about the relevance of cellular metabolism research; 2) to document knowledge of, interest in and goals for performing cellular metabolic assays; 3) to confirm current practices and preferences in use of cellular metabolism assays; and 4) to assess interest and feedback on a new approach for measuring cellular metabolism.

This article highlights selected findings from that market survey and reviews some technologies and tools available to study metabolic analysis.

Cellular metabolism research in context

The extent to which survey respondents' research involves cellular metabolism was as follows: 45% it is related to a specific area of research only; 26% it is our major focus; 24% somewhat related; and the remaining 5% were peripherally related (**Figure 1**).

The importance of cellular metabolism in the research areas was rated most important with respect to the cancer research area. This was closely followed in importance by ageing, obesity/diabetes and then translational medicine. Rated least important from a cellular metabolism perspective was the immunology area. However, all research

areas rated cellular metabolism of moderate to high importance (**Figure 2**).

The future relationship between cellular metabolism and survey respondents' research area was broken down as follows: 44% definitely increasing in importance; 28% potentially increasing in importance; 24% already tightly linked; and the remaining 4% I don't think the relationship will increase. The results suggest cellular metabolism studies will feature highly in respondents' research areas in the future (**Figure 3**).

Main drivers to perform cell metabolism assays

Most (51%) survey respondents selected to determine the metabolic phenotype of cells and/or detect switches in metabolic pathway usage as their main scientific driver to perform cell metabolism assays. This was very closely followed by to correlate energy metabolism with proliferation, differentiation, survival, etc (50% selecting) and then to correlate gene/protein expression with metabolic function (46% selecting). The least important drivers were to reveal metabolic dependencies of cells on substrates such as glucose, glutamine and fatty acids (19% selecting) and to determine mitochondrial toxicity of a compound of interest (17% selecting) (**Figure 4**).

Assays, techniques and instrumentation used to investigate cellular metabolism research

The assays and techniques survey respondents made greatest current use of to investigate cellular metabolism in their research areas were protein expression and gene expression assays (61% and 56% using respectively). These assays were followed by redox assays (39% using) ROS production or oxidative stress (39% using) and then ATP production (27% using). The assays/techniques least currently used to investigate cellular metabolism were metabolic flux assay (mass spectrometry-based) (15% using), extracellular acidification (13% using) and mitochondrial mass or biogenesis (only 8% using) (**Figure 5**).

The instrumentation currently most used by survey respondents for metabolism assays was microplate reader (absorbance, fluorescence, luminescence) (82% using). This was followed by PCR/qPCR/RT-PCR (66% using); Western blotting device (65% using); and then fluorescence microscope (56% using). The Clark-type electrode/respirometer was currently used by 14%. The extracellular flux analyser (ie an oxygen and proton biosensing probe-based instrument from

Figure 4: Main scientific drivers to perform cell metabolism assays

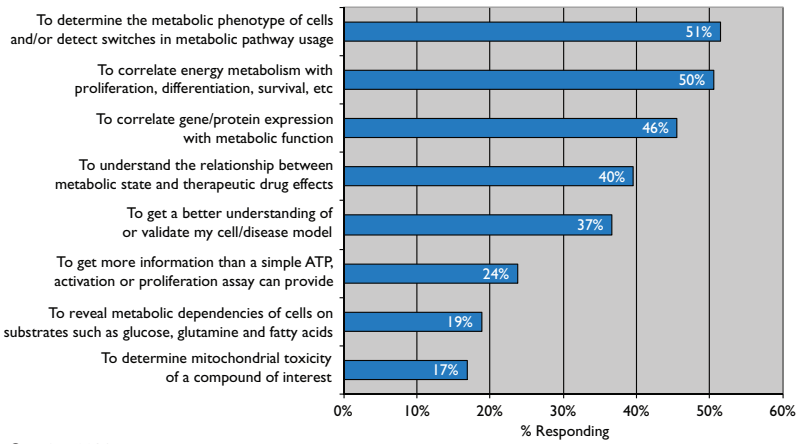


Figure 5: Assays currently used to investigate cellular metabolism in respondents' research

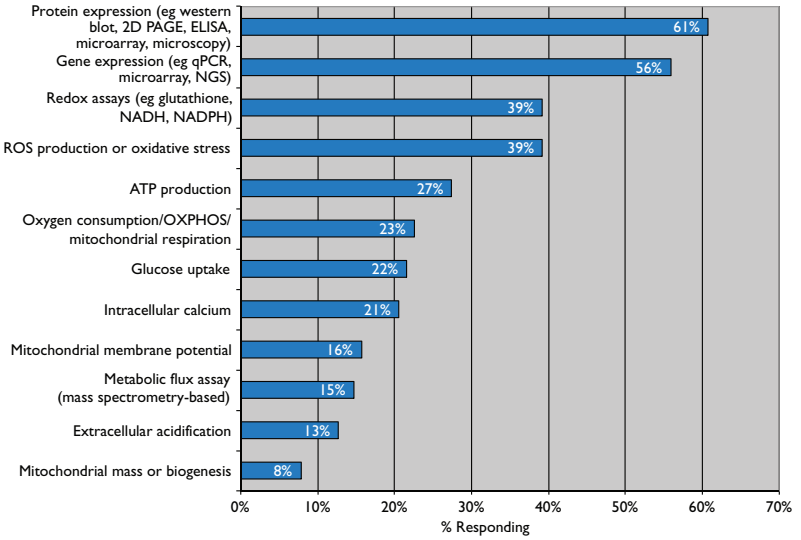


Figure 6: Instrumentation currently most used for cellular metabolism assays

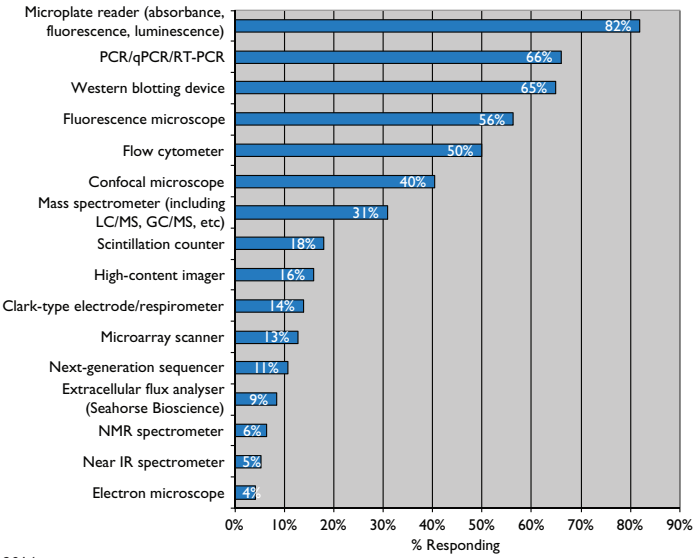


Figure 7: Specific assay types frequently encountered in cell metabolism research

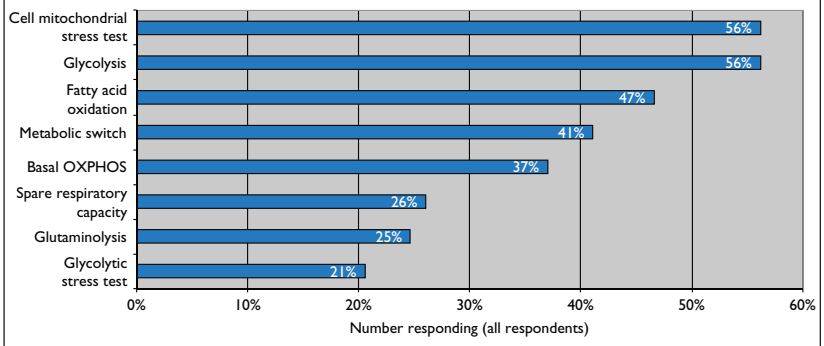
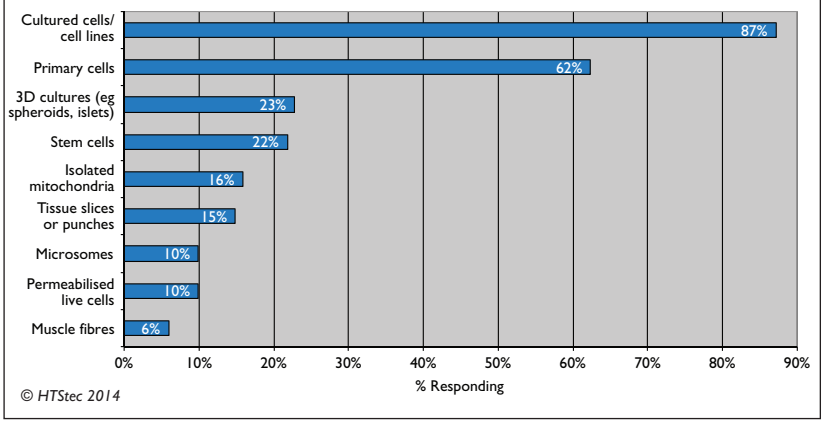


Figure 8: Biological materials measured for energy metabolism by respondents



Seahorse Bioscience) was used by 9% of respondents today. Least used was the electron microscope with only 4% using (**Figure 6**).

Of a list of specific assay types used in cell metabolism research, glycolysis and cell mitochondrial stress test were the most frequently encountered or relevant by survey respondents (56% responding to both). They were followed by fatty acid oxidation (47% responding) and metabolic switch (41% responding). Least encountered were glycolytic stress test (21% responding). Glycolysis or cell mitochondrial stress test was the most encountered specific assays in all survey groups (**Figure 7**).

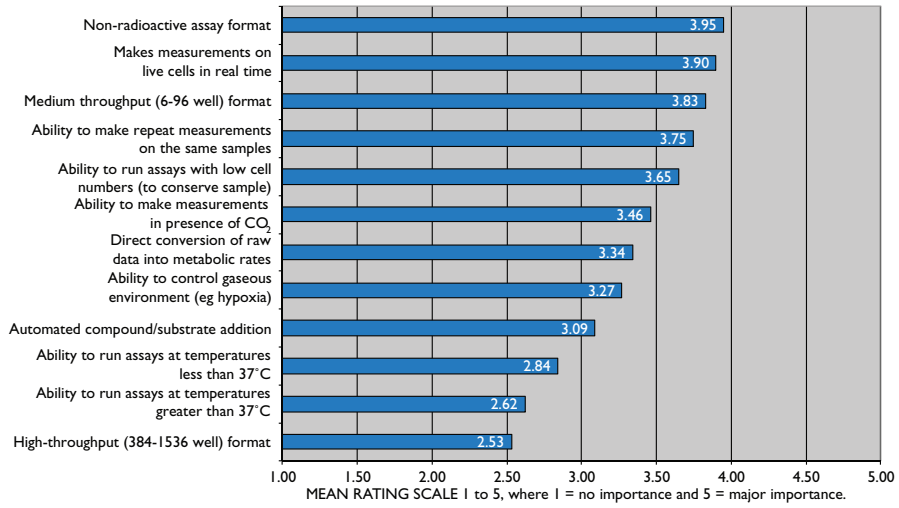
Future cellular metabolism assay requirements

The biological materials survey respondents were most measuring or want to measure energy metabolism in were cultured cells/cell lines (87% measuring). This was followed by primary cells (62% measuring), and then 3D cultures (eg spheroids, islets) (23% using) and stem cells (22% using). The biological material least measured for energy metabolism was muscle fibres (only 6% measuring) (**Figure 8**).

Survey respondents rated non-radioactive assay format as the most important feature when considering the adoption of a new cell metabolism assay platform. This was closely followed by makes measurements on live cells in real time; medium throughput (6-96 well) format; and then ability to make repeat measurements on the same samples. Rated least important were ability to run assays at temperatures greater than 37°C and high-throughput (384-1536 well) format (**Figure 9**).

Survey respondents ranked generates functional data on living cells as the main driver (influence) for considering switching to a new cellular metabolism platform based on a biosensing probe, such as Seahorse Bioscience XF Analyzers. This was followed by facilitates access to important metabolic measurements/readouts; allows more assays to be done in the same amount of time; and then more economical than my current method. Ranked of lesser influence in switching to a new cellular metabolism platform was easier to use than my current method. Ranked of least influence was it will help get my work published in top-tier cell metabolism journals (**Figure 10**).

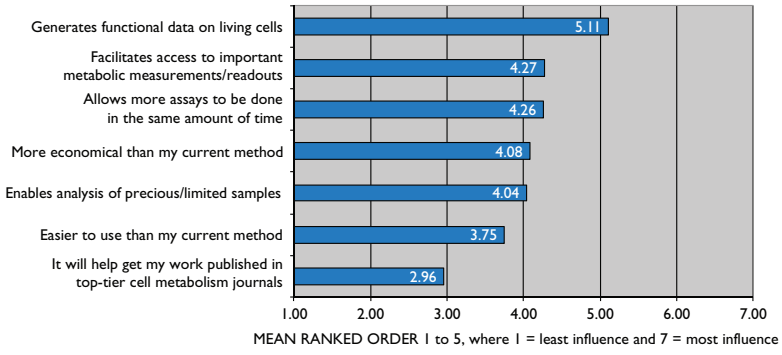
Figure 9: Importance of features when considering the adoption of a new cell metabolism platform



Latest vendor offerings supporting cellular metabolism research

Deregulated metabolism is a recognised hallmark of cancer. For example, normal glucose oxidation in the cytoplasm followed by ATP generation in mitochondria can be replaced by more extensive glycolysis in the cytoplasm without mitochondrial involvement. Cancer cells may benefit by generating ATP rapidly by glycolysis and redirecting energy-rich intermediates to the biosynthesis of the lipids, proteins and nucleic acids needed for accelerated growth and cell division. As a consequence of these changes, the mitochondrion and the cell become less sensitive to apoptosis. Therapies that return a cancer cell's metabolism to a more normal state have the potential to limit its capacity to grow and divide while also restoring sensitivity to apoptosis. Whether you are looking at Lactate Dehydrogenase activity or want to detect the concentration of Acetyl-CoA, **Abcam** (www.abcam.com) offers a range of products to help measure these metabolic changes. These metabolism kits utilise the naturally occuring pathways and a specifically designed probe to produce a byproduct that can be measured by spectrometry and quantify your metabolite of interest. The ATP Assay kit range is able to detect from 1pM to 1µM of active ATP in a variety of sample types in less than one hour. These kits provide flexible tools for studying cancer cell metabolism pathways for basic research and therapy development (**Figure 11**).

Figure 10: Main drivers to consider switching to a new cellular metabolism platform



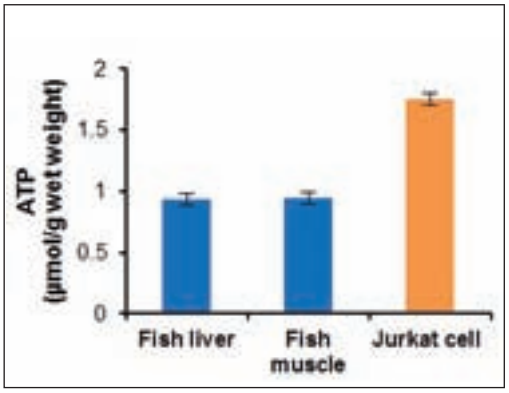
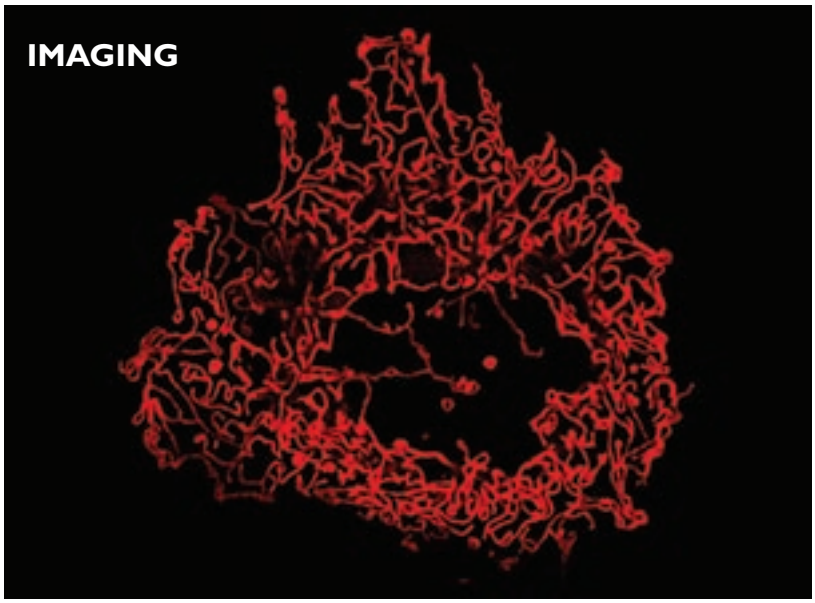


Figure 11
Quantitation of ATP in fish liver (2.5µl of 10 times diluted sample), fish muscle (5µl of 10 times diluted sample) and Jurkat cell lysate (5ul) using Abcam fluorometric ATP Assay Kit. Samples were spiked with known amounts of ATP (300pmol)



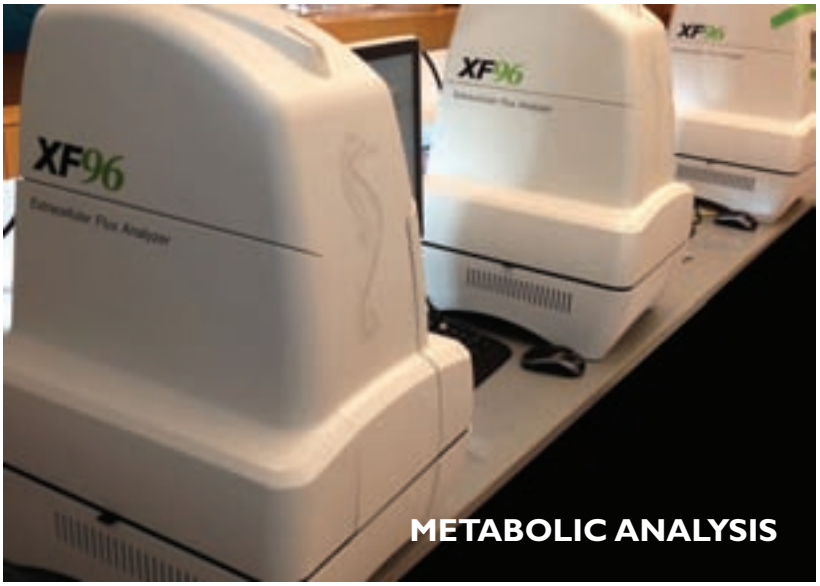


Figure 12
BioEnergetics' scientific approach combines both mitochondrial respirometry and high content imaging to create a cost-effective strategy to accurately determine the efficacy/liability of lead compounds and explore their underlying mechanism of action

Mitochondrial dynamics, mitophagy and metabolism are tightly balanced processes that contribute to mitochondrial quality control. Impairment in any of these fundamental mitochondrial functions can cause cellular dysfunction and disease. As mitochondria emerge as targets for drug discovery, the field is only beginning to unravel the complexity of mitochondrial bioenergetics in intact cells and tissues. Depending on cell type, mitochondrial function can vary drastically based on differences in energy demand and nutrient availability, which are specific to the physiological requirements of the cell. This becomes clear when assessing mitochondrial respiration of cardiomyocytes versus cancer cells. Cardiomyocytes, which display high energy demand and require efficient nutrient use to support continuous contraction, depend on mitochondrial oxidative phosphorylation to meet energetic needs. Conversely, cancer cells display enhanced glycolysis and reduced oxidative phosphorylation (Warburg effect), which supports increased cell proliferation by consuming nutrients faster but utilising them less efficiently. Until recently, the lack of higher throughput assays has made mitochondrial drug discovery experimentally demanding and not always feasible. By combining new ways to measure mitochondrial respiration (eg Seahorse Bioscience), along with faster methods to conduct content imaging, it has allowed for a cost-effective strategy to accurately and rapidly determine the efficacy/liability of lead compounds and explore underlying mechanisms of action. However, understanding all the concepts of mitochondrial physiology can be challenging and time-consuming. Successful mitochondrial research requires that the right questions are asked, appropriate experiments designed and results correctly interpreted. Fortunately, **BioEnergetics** (www.bioenergetics-cro.com) provides the mitochondrial expertise needed to successfully take on the challenges of mitochondrial drug discovery (**Figure 12**).

In essence, metabolism is a series of biochemical reactions catalysed by different enzymes at each step with an ultimate goal of either breaking down a complex biological molecule or synthesising another. The study of these reactions and any aberrations therein lies at the heart of metabolic disease-related research. **BioVision** (www.biovision.com/metabolism-assays-1128) scientists have developed an extensive repertoire of high-throughput-suitable, quantitative cell-based assays (Enzymatic or ELISA) for a wide selection of pathway intermediates spanning across every major metabolic process in a cell. These assays span carbohydrate, lipid and protein metabolism, cofactors/coenzymes, ions and other markers involved in not just normal physiology but in diabetes/obesity, ageing, neurological and cardiovascular diseases, cancer, inflammation and more. These assays are simple (add-and-read, 96-well format), flexible (colorimetric, fluorometric or luminescent) and versatile (compatible with many sample types such as cells, tissues, culture media, food, serum, plasma, urine from different species). The Picoprobe™ assays constitute the most sensitive line on the market and can reliably detect ~20 picomoles of a particular analyte. No special training/disposal is required and stability of one year

makes the kits easy to use and store. For more than a decade, scientists across disciplines have used these assays to efficiently generate reproducible data for investigative or clinical research – be it for discovering new potential drug targets, screening enzyme inhibitors, measuring serum components/disease markers, preclinical drug metabolism or studying enzyme structure-function relationships (**Figure 13**).

Hansatech Instruments (www.hansatech-instruments.com) has been manufacturing scientific equipment for measurement of cellular respiration and photosynthesis for almost 40 years. The latest product is the Oxygraph Plus system which combines a high precision control and data acquisition unit with a sample chamber and a Clark type polarographic oxygen sensor. The floor-mounted sensor allows ease of access to the sample during measurement without perturbation of the signal. It is therefore ideal for conducting measurements of respiration and making chemical additions during the protocol. The sample volume is adjustable in the range 200µl-2.5ml. The Oxygraph can be used for a broad range of applications from studies of mitochondria and cellular respiration to measurements of isolated chloroplast suspensions in photosynthesis research applications with up to 100% oxygen concentration. The Oxygraph control unit also offers the ability to simultaneously measure a pH or ion-selective electrode signal alongside the oxygen measurement. The control unit connects to a PC via a USB port and comprehensive Windows control and data acquisition software is provided to acquire data directly to the PC (**Figure 14**).

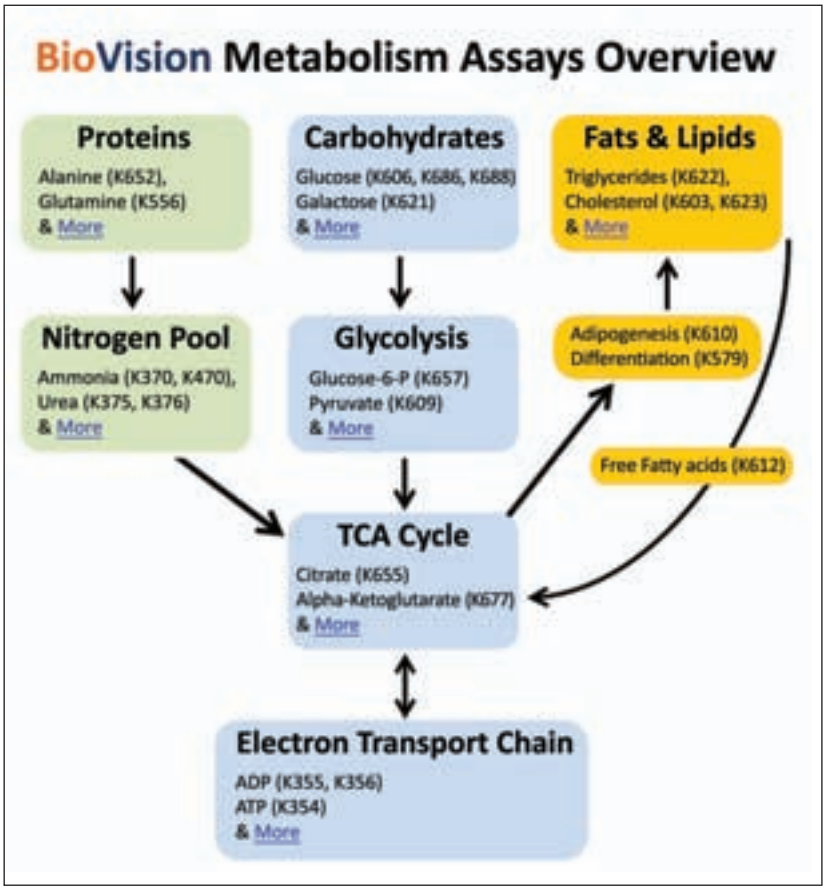


Figure 13: A schematic highlighting some of the key categories from BioVision's extensive metabolism assay portfolio

Mitochondria are potent producers of cellular superoxide, a major cause of the cellular oxidative damage. Mitochondrial superoxide is generated as a by-product of oxidative phosphorylation. In an otherwise tightly coupled electron transport chain, approximately 1-3% of mitochondrial oxygen consumed is incompletely reduced; these 'leaky' electrons can quickly interact with molecular oxygen to form superoxide anion, the predominant reactive oxygen species in mitochondria. Increases in cellular superoxide production and the resulting cellular oxidative damage have been implicated in cardiovascular diseases, including hypertension, atherosclerosis and diabetes-associated vascular injuries, as well as in neurodegenerative diseases such as Parkinson disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS). The production of superoxide by mitochondria can be visualised in fluorescence microscopy and live-cell

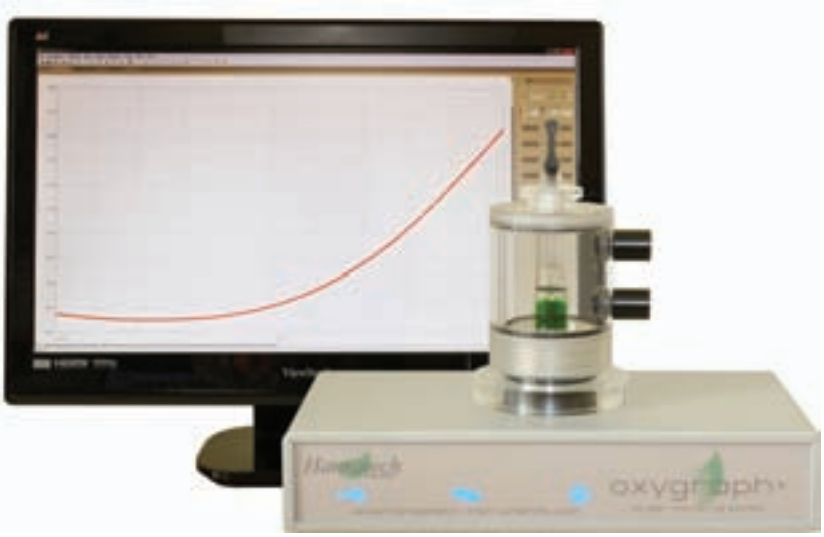


Figure 14: The Hanstech Instruments Oxygraph Plus oxygen electrode system

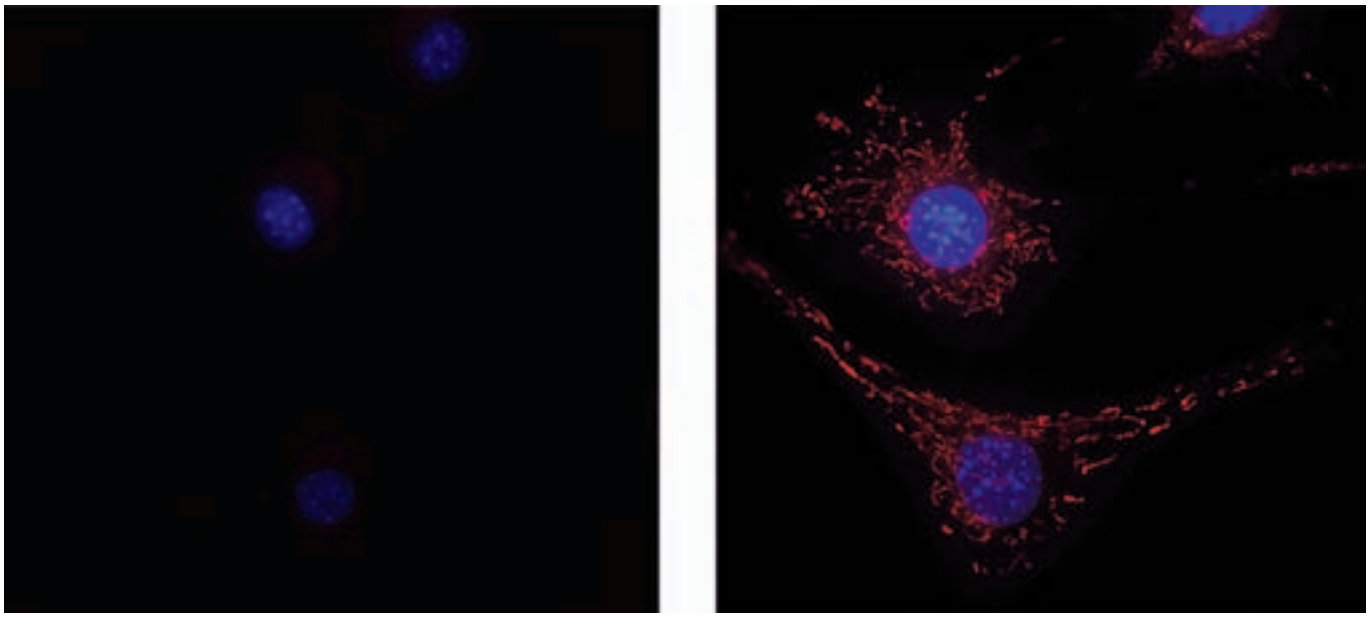
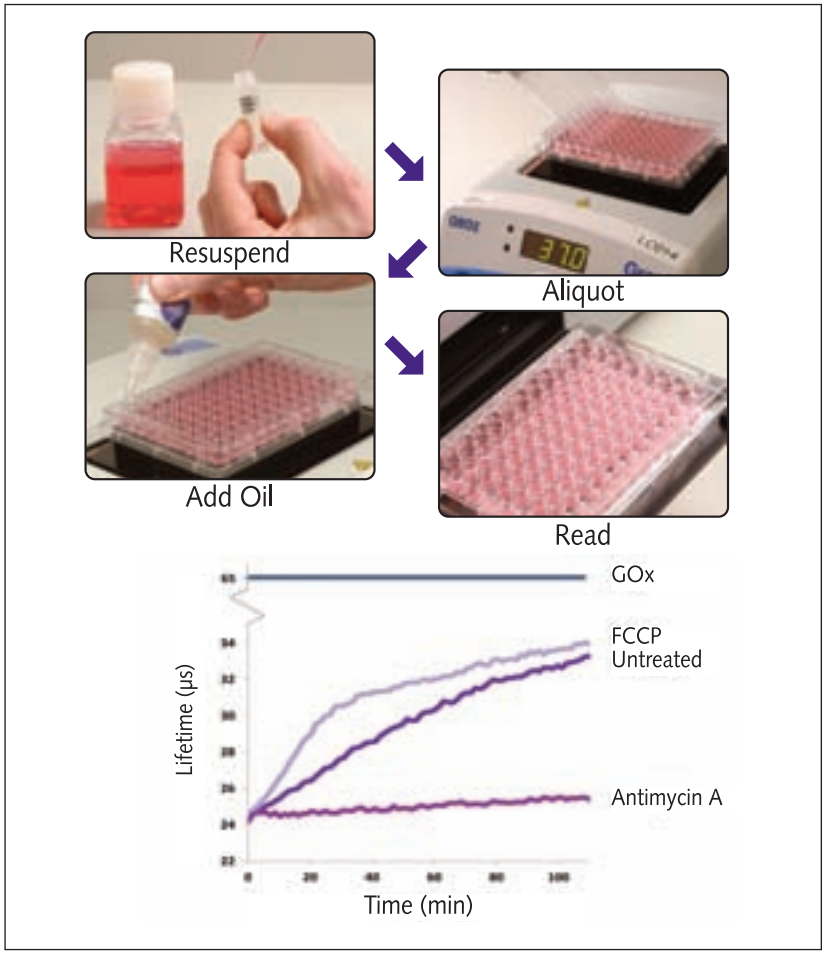


Figure 15: Detection of superoxide in live cells using MitoSOX™ Red superoxide indicator (M36008) from Life Technologies. Live NIH 3T3 cells were treated with FeTCPP, a superoxide scavenger (left) or untreated (right). Cells were then labelled with MitoSOX™ Red reagent, which fluoresces when oxidised by superoxide, and nuclei were stained with blue-fluorescent Hoechst 33342. The mitochondria of untreated cells exhibited red fluorescence, indicating the presence of superoxide, whereas the mitochondria of treated cells showed minimal fluorescence



imaging using the MitoSOX™ Red reagent (M36008) from **Molecular Probes® by Life Technologies™** (www.lifetechnologies.com); a novel fluorogenic dye highly selective for the detection of superoxide in the mitochondria. The MitoSOX™ Red reagent rapidly transverses cell membranes and is selectively targeted to the mitochondria. Once in the mitochondria, the reagent is oxidised by superoxide. MitoSOX™ Red is only oxidised by superoxide and not by other reactive oxygen or nitrogen species-generating systems. After oxidation, the MitoSOX™ Red reagent binds to nucleic acids and becomes highly fluorescent with a 590nm emission peak (**Figure 15**).

The MitoXpress® Xtra – Oxygen Consumption Assay from **Luxcel Biosciences** (www.luxcel.com) is a highly-flexible 96 or 384-well approach for real-time analysis of cellular respiration and mitochondrial function. The easy-to-use, mix-and-measure assay allows measurement of extracellular

Figure 16: Flow diagram showing preparation, use and analysis of the Luxcel MitoXpress® Xtra – Oxygen Consumption Assay (HS Method). Typical Lifetime profile of MitoXpress® Xtra for adherent cells, treated with different ETC compounds, including Antimycin A (inhibitor) and FCCP (uncoupler). The effect of Glucose Oxidase as a positive Signal Control is illustrated schematically

Figure 17
EMD Millipore's MAGPIX instrument and MILLIPLEX MAP OXPHOS kit boxes

oxygen consumption from whole cells (adherent and suspension), isolated mitochondria and a wide range of 3D cultures systems, all using standard fluorescence and TR-F plate readers. In this assay, the MitoXpress® Xtra probe is quenched by O_2 through molecular collision; the amount of fluorescence signal being inversely proportional to the amount of extracellular O_2 in the sample. Rates of oxygen consumption are calculated from the changes in fluorescence signal over time (or ratiometric Lifetime, when using dual-read TR-F). The reaction is non-destructive and fully reversible (neither MitoXpress® Xtra nor O_2 are consumed), facilitating measurement of time courses and drug treatments either increasing (eg FCCP) or decreasing (eg antimycin A) oxygen consumption. Luxcel's flexible plate reader format allows multiparametric or multiplex combination with Luxcel's other products, as well as combination with commonly available reagents to measure glycolysis, LDH, JC-1, ψ, ROS, and cellular ATP. For example, MitoXpress® Xtra in combination with Luxcel's pH-Xtra™ – Glycolysis Assay allows the simultaneous real-time measurement of mitochondrial respiration and glycolysis and analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states. Luxcel Biosciences has also partnered with **Cayman Chemical** (www.caymanchem.com) to develop and commercialise a range of combination kits to expand the range of available metabolism assays (**Figure 16**).

Significant progress has been made in understanding the links between mitochondrial dysfunction and conditions such as diabetes, Parkinson's disease, Alzheimer's disease, cancer and ageing. In recent years, mitochondrial analyses have drawn renewed interest from both the research and drug development communities. Since the dominant function of mitochondria is the production of cellular energy through OXPHOS, the assessment of OXPHOS health and impairment has become the focal point of mitochondrial analysis. However, most traditional techniques for such analyses suffer from low throughput, time-consuming mitochondrial isolation steps and the non-physiological conditions associated with the detachment of

Figure 18
Seahorse Bioscience XFe96 Analyzer and XF Stress Test Kits

mitochondria from the cellular environment. In contrast to traditional techniques, **EMD Millipore**'s (www.millipore.com) MILLIPLEX® map OXPHOS Magnetic Bead Panels for human and rat/mouse conveniently and quantitatively detect multiple intact OXPHOS complexes from cell lysates or tissue extracts using Luminex xMAP® technology. These panels demonstrate the potential broad applications in the study of mitochondrial dysfunction-associated diseases in order to better understand disease mechanisms and to discover potential treatments. These panels can potentially be used in the drug development process to predict the mitochondrial impairment-linked drug-induced toxicity, correspondingly reducing late-stage drug attrition and improving drug safety. EMD Millipore has launched a series of multiplex panels to address cellular metabolism research questions, including the OXPHOS Panels, Oxidative Stress and Glycolysis Pathway Panels (**Figure 17**).

Seahorse Bioscience (www.seahorsebio.com) XF metabolic analysers have established standards for measuring cell metabolism by delivering meaningful new parameters for understanding the processes by which cells produce and consume energy. XF metabolic analysers simultaneously measure the two major energy pathways of the cell – mitochondrial respiration and glycolysis – in live cells using label-free, solid-state sensor cartridges in 8-, 24- and 96-well formats. XF metabolic analysers can be used with virtually every cell type, including primary cells, cell lines, suspension cells and spheroids, as well as islets, *C. elegans*, yeast, and isolated mitochondria. XF metabolic analysers utilise patented transient microchambers which enable sensitive, precise and non-destructive metabolic measurements in minutes; and can be programmed to sequentially add up to four compounds, facilitating selective perturbation of pathways of interest, agonist or antagonist response, or dose

response in a single sample. By measuring both pathways and using the injection ports, XF metabolic analysers make it easy to measure metabolic switching, reprogramming and phenotypes. Seahorse recently launched the XFe96 Spheroid Microplate. This microplate has a unique geometry that allows for measurements of individual spheroids for the study of three-dimensional cell culture, which more closely mimics *in vivo* conditions. Of particular importance for cancer researchers, the real-time metabolism of multicellular spheroids can now be analysed and used for characterising tumour metabolism and evaluating response to therapeutic agents (**Figure 18**).

Discussion

The tools and technologies supporting cellular metabolism research reported in this article broadly fall into four categories:

Traditional methods: These are based on polarography using a 'Clark' type oxygen electrode. Some enhancements to the standard offering are available, eg the ability to simultaneously measure a pH or ion-selective electrode signal alongside the oxygen (Hansatech Instruments) or microcathode electrode which do not require stirring (Warner Instruments).

Biomarkers assays: These represent the bulk of the commercial offerings and most are available as 96-well format assays kits or panels. They utilise a variety of standard assay techniques to probe the enzymes, byproducts and pathways involved in cellular metabolism. These range from cellular ELISA and enzymatic assays (Abcam, BioVision) to Luminex® xMAP® technology (EMD Millipore).

Fluorogenic dyes: These are highly selective for the detection of cellular superoxide, a major cause of cellular oxidative damage. Mitochondrial superoxide can be visualised in intact living cells by fluorescence microscopy or high content imaging (Life Technologies, Bioenergetics). Fluorescent intracellular oxygen imaging probes are also available (Luxcel Biosciences).

Biosensing technology: These use oxygen sensitive probes that react to oxygen changes in the surrounding solution/environment. Luxcel Biosciences assays are based on the ability of oxygen to quench the excited state of the MitoXpress® phosphorescent probe. As the test material respires, oxygen is depleted in from the solution, which is seen as an increase in probe phosphorescence signal. Changes in oxygen consumption, reflecting changes in mitochondrial activity, are seen as changes in MitoXpress® probe signal over time. Seahorse Bioscience ultises two different fluorophores embedded in polymer as the base of its sensing cartridge. Fibreoptics bundles excite the fluorophores and then read back the shift in fluorphore emissions due to changes in oxygen and protons. The actual measurement is accomplished by the instrument sensor cartridge being lowered 200um above the cells creating a patented transient microchamber, where changes in oxygen and protons can be detected almost immediately. The use of both these oxygen-sensing probes is proving particularly useful in the investigation of spheroids in cancer research.

In conclusion, significant progress has been made in the understanding of the links between mitochondrial dysfunction and various diseases and pathological conditions. An observation further enhanced by the recent realisation that many human diseases including cancer, obesity and diabetes share a common cause in metabolic imbalance. It therefore comes as no surprise that mitochondrial analyses have gained renewed interest from both the academic research and drug discovery labs. This revival of interest in cellular metabolism assays is being aided by new analysis tools that enable the characterisation of metabolic phenotypes, their interdependency with specific signaling pathways, and their reprogramming during disease states. However, this is a very diverse area and the usefulness of the tools reported will depend on the cell type under investigation, as well as the cellular microenvironment and proliferative status of the cell. A better understanding of cellular metabolism may ultimately lead to better treatments for human cancer. DDW

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