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Review: Quantifying Mitochondrial Dysfunction in Complex Diseases of Aging

Martin P. Horan, Nicolas Pichaud, and J. William O. Ballard

Faculty of Sciences, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia.

Address correspondence to J. William O. Ballard, PhD, Faculty of Sciences, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia. Email: w.ballard@unsw.edu.au

There is accumulating evidence that mitochondrial respiratory malfunction is associated with aging-associated complex diseases. However, progress in our understanding of these diseases has been hampered by the sensitivity and throughput of systems employed to quantify dysfunction and inherent limitations of the biological systems studied. In this review, we describe and contrast two methodologies that have been developed for measuring mitochondrial function to address the need for improved sensitivity and increased throughput. We then consider the utility of each methodology in studying three biological systems: isolated mitochondria, cultured cells, and cell fibers and tissues. Finally, we discuss the application of each methodology in the study of mitochondrial dysfunction in Alzheimer's disease, type 2 diabetes mellitus, and aging-associated autophagy impairment and mitochondrial malfunction. We conclude that the methodologies are complementary, and researchers may need to examine multiple biological systems to unravel complex diseases of aging.

Key Words: Mitochondrial dysfunction—Aging.

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OLD age is associated with impaired mitochondrial function, and this has been proposed as a proximal mechanism for aging. In 1980, Miquel and colleagues (1) first proposed the mitochondrial theory of aging and suggested that mitochondria were key sources for the production and targets of free radicals and that these are associated with aging and a number of age-related diseases (1). From that time onward, much evidence implicating mitochondria in the aging process has emerged (2,3,4,5,6,7).

Mitochondrial respiratory malfunction is also intimately associated with aging-associated complex diseases. But not everyone agrees that mitochondria are central to the development of these complex diseases. Complex diseases are just that, complex, and the mechanistic role of mitochondrial dysfunction in the causality of these diseases is highly debated. Likely, however, the study of cellular metabolism and specifically the functional properties of mitochondria will provide a greater understanding of the underlying metabolic malfunctions associated with the progression of many complex diseases. The frequency of mitochondrial DNA mutations in disease may be as high as 1/5,000 (8,9), and the involvement of mildly deleterious mitochondrial mutations in complex diseases of aging are almost certainly much higher.

In this review, we describe two new methodologies and contrast the utility of each system in studying respiration in isolated mitochondria, in cultured cells, and in cell fibers and tissues. We then discuss the application of each methodology in the study of mitochondrial malfunction in two common aging-associated complex diseases: Alzheimer's disease and type 2 diabetes mellitus. We finally discuss the technology and its role in elucidating mitochondrial malfunction in aging-associated autophagy impairment. We conclude that the methodologies and biological systems have unique strengths and weaknesses that have the potential to complement each other for the evaluation of respiratory malfunction in complex disorders of aging.

Description of the Methodologies for Assaying Mitochondrial Function

Mitochondrial respiration occurs on the inner mitochondrial membrane and is achieved by the flow of electrons through the electron transport system, which consists of four complexes (complex I, II, III, and IV) with a further complex (complex V) serving as a site for ATP synthesis (ATP synthase; Figure 1). Impairment of any complex disrupts electron flow and may cause mitochondrial respiratory dysfunction (10,11). Analysis of mitochondrial metabolic dysfunction has, to date, predominantly relied upon the classical oxygen electrode (12). This system has been in use over the past 50 years and has led to an intimate understanding of mitochondrial respiratory function. Although the oxygen electrode remains an inexpensive and useful tool for the evaluation of mitochondrial respiration, it nonetheless has notable limitations with regard to stability of the signal, instrumental background noise, resolution, and throughput (13).

There are a multitude of biochemical assays that are specific for the determination of mitochondrial malfunction. These include assays for cytochrome *c* oxidase and succinate

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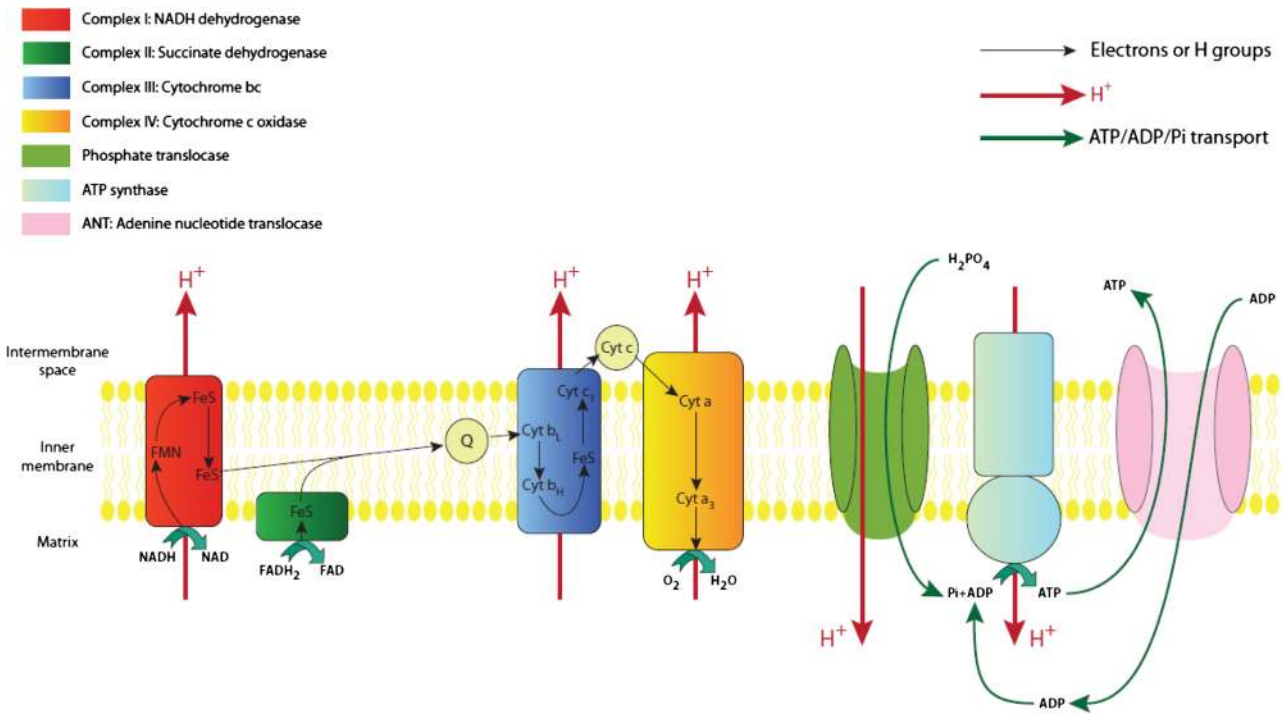

Figure 1. Oxidative phosphorylation in the mitochondrial inner membrane. Q = ubiquinone; Cyt = cytochrome; FMN = flavin mononucleotide; FeS = ironsulfur cluster.

dehydrogenase activity (14), membrane potential activity, Western blot, blue native polyacrylamide gel electrophoresis, reactive oxygen species assays, and ATP content assays (15). However, these assays are destructive to cells and tissues, and none can be performed in real time. Considering the need to more accurately assess mitochondrial oxidative phosphorylation function, two new systems have been developed that enable the detection of subtle changes in the respiration rate of mitochondria as a consequence of gene mutation, diet, and environmental insults. These systems are the high-resolution Oxygraph-2k (O2k, Oroboros Instruments, Austria) and the sensitive high-throughput Seahorse XF Extracellular Flux Analyzer (Seahorse XF, Seahorse Bioscience Inc.).

The specific measurement of mitochondrial bioenergetics can be performed with high accuracy using the O2k and Seahorse XF systems. Both can analyze, in real time, the mitochondrial respiratory function in the classic in vitro experiments on isolated mitochondria as well as in cultured cells and tissues. Wang and Moraes (16) found that by using three different human cancer cell lines (HeLa, 143B, and MDA-MB-231) and experimentally increasing mitochondrial biogenesis, both the O2k and Seahorse XF produced equivalent data when assaying for total respiration. Thus, the use of these systems in bioenergetic profiling of disease tissue is likely to offer novel insights into aging and complex disease development and progression. Furthermore, the application of these systems in the analysis of aging-associated complex diseases may potentially aid in the development of novel therapeutic intervention strategies.

The O2k and Seahorse XF have notable advantages and limitations for respiratory analysis (Table 1). Nonetheless, accurate and robust quantification of mitochondrial bioenergetic malfunction can be determined with high resolution and high sensitivity in both systems using either isolated mitochondria or intact cultured cells. Indeed, since the introduction of both these technologies, there has been an increase in the study of underlying respiratory malfunction in complex diseases (Table 2).

Oroboros O2k

The O2k was developed during the mid-1990s (50) and has been used extensively for respiratory analysis. The O2k is a development over the traditional oxygen electrode for measuring mitochondrial respiration (50). The system possesses two separate 2-mL chambers equipped with polarographic oxygen sensors that can measure in real time both the oxygen concentration (nanomoles/milliliter) and the oxygen consumption (picomoles/second/milliliter) within each chamber. The basic principle of the system is to measure the concentration and consumption of oxygen by injecting substrates directly to isolated mitochondria or cells that are suspended in solution within the chamber (50).

The high-resolution detection capability of the O2k is enhanced by a temperature controlling system allowing respiratory measurements to be made from 2°C to 45°C

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Table 1. Comparison of the Oroboros Oxygraph-2k (O2k) and the Seahorse XF Extracellular Flux Analyzer for Technical Specifications and Respiratory Specifics

Equipment Specifics	Oroboros O2k	Seahorse XF Extracellular Flux Analyzer
Cost of equipment*	$50k	$200k
Automated	No	Yes
Labor intensity	High (needs constant operator input)	Minimal (system is fully automated)
Internal calibration	No	Yes
Respiratory measurements	OXPHOS†	OXPHOS/glycolysis/CO_2
Assay format	2-Chamber based	24- or 96-Well plate based
High-resolution sensitivity	Yes (polarographic sensors)	Yes (fluorescence sensors)
High-throughput capability	No (two samples per run)	Yes (24–96 samples per run)
Time per sample assay	Slow—approximately 1 h for two samples	Fast—approximately 90 min for up to 96 samples
Controlled temperature range	Yes (4–45°C)	Yes, but ambient temperature needs to be 8°C below desired temperature
Background correction per assay	No (separate assay required per chamber)	Yes (background control wells on plate)
Tissue types analyzed	Mitochondria/cells/permeabilized fibers	Mitochondria/cells/pancreatic islets/muscle fibers/hippocampal slices
MMP‡	Yes	No
Substrates injected per assay	Unlimited	Limited to four
Injection of oxygen§	Yes	No
Cost per assay‖	Cheap—reagents/substrates required	Expensive—reagents/substrates plus new fluorescent plate for each assay

Notes: *Approximate U.S. dollars.
†Oxidative phosphorylation.
‡Mitochondrial membrane potential analysis.
§Analysis to determine if the respiration rate alters between high and low oxygen concentrations.
‖Cost is relative because the experimental design using the Oroboros O2k may prove to be equally or more expensive in the longer term if the cost of operator usage constantly monitoring an equivalent Seahorse XF sample output over time is taken into account.

(13). A further advantage of the O2k system over the traditional Clark-type oxygen electrode is that only small quantities of sample are required for metabolic analysis. However, a particular advantage specific to the O2k system is that oxygen concentration in the measurement chambers can be allowed to remain elevated during an assay or until the functional stability of the sample is impaired, enabling extended substrate–uncoupler–inhibitor–titration protocols to be applied (51). This is important because these protocols enable the evaluation of all the mitochondrial coupling and respiratory control measurements to be accurately performed. Because substrate injections are performed manually, each complex of the electron transport system can be studied independently using different substrate combinations. For example, sequential injections of glutamate, malate, and pyruvate can be used to determine the effect of each of these substrates on mitochondrial respiration when electrons are provided to complex I. A further injection of succinate can be used to assess the effect of electron input through complex I and complex II. Rotenone can then be added to specifically inhibit complex I to evaluate mitochondrial respiration from complex II. Thus, the ability to manually inject specific substrates to test different complexes for malfunction or to determine the effect of multiple substrates or drugs on the rate of mitochondrial respiration allows for an analysis of a multitude of injectable reagents.

Limitations of the O2k

There are three primary limitations to the O2k. First, operator input and labor intensity are high because constant monitoring and adjustments of concentrations of injectable reagents are required to obtain optimal signal intensity. Second, the O2k is not capable of high-throughput capability as only two samples can be performed at any one time. Because analysis using substrate–uncoupler–inhibitor–titration protocols take approximately 1 hour to complete, it is only possible to analyze 16 samples over an average 8-hour day. This can prove to be expensive for large-scale studies (ie, the testing of multiple drug compounds over multiple cell types) when the cost of an operator constantly monitoring a large number of mitochondrial assays is taken into account. A third limitation is that the O2k is incapable of recording extracellular pH levels. This represents an important measurement because it allows for a determination of glycolytic pathway utilization. Because cancer cells preferentially use glycolysis as their main source of energy (52), the testing of compounds that revert cells to oxidative phosphorylation would be beneficial for monitoring any potential therapeutic effect.

Seahorse XF Extracellular Flux Analyzer

The Seahorse XF was introduced during 2006/2007 (53) and was developed to address the need for a high-throughput system for the determination of mitochondrial malfunction. The basic principle of the system is to measure the rate of mitochondrial oxidative phosphorylation (through determination of the oxygen consumption rate measured in picomoles/minutes) and glycolysis (through determination of the extracellular acidification rate measured in milli-pH units/minutes; 54).

The Seahorse XF operates on a 24 or 96 microplate-based assay platform. A feature of the microplate-based assay is that a single plate containing multiple samples can be

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Table 2. Examples of Complex Human Diseases Analyzed for Mitochondrial Malfunction Using the Oroboros Oxygraph-2k (O2k) and the Seahorse XF Extracellular Flux Analyzer

Disease	Gene	Mutation/function studied	System (O2k*, S†)	Cells/mitochondria studied	Reference
Aging of colonic crypts	*MTCOI*	p.Y19H	O2k	Intact/permeabilized colonocytes	(17)
Aging	*Sod2*	*Sod2* knockout mouse	S	Embryonic fibroblasts	(18)
	Sod2	Heterozygous *Sod2* mouse	O2k	Permeabilized muscle fibers	(19)
	Bmi1	*Bmi1* knockout mouse	S	Thymocytes	(20)
Alzheimer's disease	*App*, *Tau*, *PS1*	Triple-transgenic knockout mutant mouse	S	Embryonic neuronal cells	(21)
	APO	Isoform E4 expression	S	Neuronal cells	(22)
	Tau	p.P301L (transgenic mouse)	O2k	Cerebellar mitochondria	(23)
Autosomal dominant optic atrophy	*OPA1*	p.R932C	O2k	Permeabilized fibroblasts	(24)
Breast cancer	*KLRK1*	Forced expression of *KLRK1*	S	MCF-7 cells	(25)
Cardiovascular disease	*Esrrg*	Small interfering RNA gene knockdown of *Esrrg*	S	Cardiomyocytes	(26)
Charcot–Marie–Tooth disease	*MFN2*	p.R468H	O2k	Permeabilized fibroblasts	(27)
Colorectal cancer	*BAX*	Homologous recombinant *BAX* knockout cells	S	HCT-116 cells	(28)
Diabetes	*Tbx15*	Forced expression of *Tbx15*	S	3T3-L1 preadipocytes	(29)
	Slc25a25	*Slc25a25* knockout mouse	S	Embryonic fibroblasts	(30)
Duchene muscular dystrophy	*mdx*	p.Q995X (*mdx* mouse)	O2k	Myoblasts	(31)
Encephalopathy	*TTC19*	c.517C>T (p.Gln173X)	S	Fibroblasts/myoblasts	(32)
Epilepsia partialis continua	*MTCOI*	p.L196I	O2k	Permeabilized muscle fibers	(33)
HLRCC‡	*FH*	p.Q396P	S	UOK 262 cells	(34)
Human carcinomas	*ATPIF1*	siRNA gene knockdown of *ATPIF1*	S	HeLa cells	(35)
Impaired autophagy in aging	*Atg7*	*Atg7* knockout mouse	S	Embryonic mitochondria/fibroblasts	(36)
Ischemic cardiomyopathy	*EGLN2*, *EGLN3*	*EGLN2*, *EGLN3* Knockout mouse	S	Cardiomyocytes	(37)
Leigh syndrome	*SURF1*	p.R230X	O2k	Intact/permeabilized fibroblasts	(38)
Liver ischemia/reperfusion injury	*Phd1*	*Phd1* knockout mouse	O2k	Hepatocytes	(39)
MDS§	*TK2*	*TK2* knockout mouse	S	Neuronal cells	(40)
Mohr-Tranebjaerg syndrome	*Tim23*	*Tim23* knockout mouse	O2k	Permeabilized embryonic stem cells	(41)
Obesity	*Ipla2γ*	*Ipla2γ* knockout mouse	O2k	Muscle/adipose tissue mitochondria	(42)
	TNFR1	*TNFR1* knockout mouse	O2k	Permeabilized muscle fibers	(43)
Parkinson's disease	*SNCA*, *Gss*	Dual transgenic inducible mouse	S	Olfactory bulb cells	(44)
	DJ-1	*DJ-1* knockout mouse	O2k	Embryonic fibroblasts	(45)
	PINK1	p.G309D	O2k	Whole-brain mitochondria	(46)
TRAPS‖	*TNFR1*	p.C33Y, p.T50M	S	Embryonic fibroblasts	(47)
Type 2 diabetes mellitus	*PARL*-β	p.R54G + p.K55S + p.R58G + p.K59S	S	RMS-13 human myoblasts	(48)
	Tfb1m	Heterozygous *Tfb1m* mice	S	INS-832/13 beta cells	(49)

Notes: *Oroboros O2k.
† Seahorse XF Extracellular Flux Analyzer.
‡ Hereditary leiomyomatosis renal cell carcinoma.
§ Mitochondrial depletion syndrome.
‖ Tumor necrosis factor receptor-associated periodic syndrome.

analyzed with high resolution, thus providing sensitive high-throughput capability. The system utilizes a novel fluorescent sensor-containing biocartridge, which fits over a cell culture microplate. The biocartridge is analogous to a piston and is used for reagent mixing and measuring extracellular acidification and oxygen consumption. Pharmaceutical reagents or mitochondrial respiratory electron transport system inhibitors distributed in ports surrounding the sensor are then sequentially injected to each well. Background correction wells (ie, wells that have not been seeded with cells or mitochondria) are included in the assay to normalize the data to background plate noise.

Limitations of the Seahorse XF Extracellular Flux Analyzer

There are three main limitations to consider when analyzing mitochondrial respiration using the Seahorse XF. First, the cost of optimizing injectable concentrations and the subsequent cost per respiratory assay are high in comparison to the Clark-type electrode and the O2k. The Seahorse XF fluorescent plates are costly and need replacing on completion of each assay. This can become expensive, as it is essential to optimize the concentration of each injectable reagent prior to any respiratory assay. Furthermore, on completion of mitochondrial and cell optimization steps, further optimization will often be required when testing for the effect of cultured cells grown in the presence of pharmaceutical compounds. This optimization is required to ensure that pharmaceutical reagents do not interfere with the electron transport system inhibitors and produce misleading data. A second limitation is that only four injectable compounds can be used to detect mitochondrial respiratory malfunction in any single assay. Thus, it will be necessary to use multiple plates when the testing of more than four injectable reagents is required

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(ie, identifying the effect of multiple compounds on mitochondrial respiration for novel therapeutic identification), which again increases cost. A third limitation is that injectable compounds may potentially interfere with sensor fluorescence or the plastic plate and produce misleading data (55).

Analysis of Isolated Mitochondria

The definitions of the mitochondrial respiratory measured states first proposed by Chance and Williams (10) have evolved, and new conventions regarding the active measurement of mitochondrial function can be derived by the sequential injection of different substrates to isolated mitochondria (56; Supplementary Table 1). The addition of different substrates is important because it stimulates the flow of electrons from different complexes of the electron transport system, thus allowing the identification of a specific malfunctioning complex. Mitochondrial parameters are measured over five principle respiratory states, which in combination with different substrates are used to determine the functional activity of the electron transport system (Supplementary Table 1). Malfunctioning mitochondria isolated from disease tissue can therefore be identified in comparison to the normal respiratory activity of isolated control mitochondria. More detailed information on the measurement of respiratory states can be found elsewhere (10,51,57).

Oroboros O2k

Determination of mitochondrial functional integrity and the maximal capacity of oxidative phosphorylation has been demonstrated to be advantageous through in vitro–based assays using the O2k (58). Studies have also used isolated mitochondria for metabolic flux control analysis (59,60,61). The control of oxidative phosphorylation is shared between all the enzymes of the metabolic pathway that incorporates the phosphorylation system (ATP synthase and adenine nucleotide translocase), the electron transport system (complex I to complex IV), and the reactions that provide substrates and electrons to the system (tricarboxylic acid cycle and metabolite transporters). This represents the global flux of oxidative phosphorylation, and the manipulation of flux enables the investigation of specific metabolic pathways.

The analysis of flux requires that all enzymatic steps involved in the electron transport system be identified with the importance of each step being quantified (62). There are several approaches to achieve this goal (56,63,64), but all require the titration of the whole respiratory chain (global flux). These protocols are time consuming, quite complicated, and often limited due to functional instability after isolation. In contrast, the use of substrate–uncoupler–inhibitor–titration protocols using the O2k system greatly simplifies analysis and may allow the specific steps implicated in disease to be determined (51). Substrate–uncoupler–inhibitor–titration protocols enable the accurate determination of all the mitochondrial coupling and respiratory control parameters. For example, these protocols can control the rate of complex IV flux on oxygen dependence and can help explain the pathological phenotype of mild complex IV deficiencies observed in some mitochondrial myopathies (65). Moreover, the application of the flux control analysis using the O2k system has demonstrated that complexes I and III in mammals behave as single units, suggesting the existence of substrate channeling and supercomplex organization (66). In addition to providing a quantitative analysis of oxidative phosphorylation control (62,67), it has been further suggested that the disruption of supercomplex organization may lead to mitochondrial functional derangements and the progression of disease development (68).

Seahorse XF Extracellular Flux Analyzer

Mitochondrial analysis on the Seahorse XF can be performed using very small quantities of mitochondria (1–10 μg of mitochondrial protein per well; 69). Prior to the addition of electron transport system reagents, the basal levels of oxygen consumption by the mitochondria are first determined to establish steady state levels. The Seahorse XF incorporates a further measurement to determine oxygen tension (a similar measurement to oxygen concentration in the traditional oxygen electrode). This is a useful function that can help determine the correct concentration of mitochondria being loaded directly into each well. Once optimal concentrations are established, all the mitochondrial-measured parameters can be determined with electron transport system inhibitors. Comparisons can then be made between experimental samples. However, the results obtained from the Seahorse XF may vary in replicate wells due to clumping of mitochondria as a consequence of the isolation procedure. Thorough suspension and careful dilutions of extracted mitochondria are necessary to give robust results.

An example of the high-throughput capability of the Seahorse XF was provided by Abadir and colleagues (70) who identified respiratory malfunction in the mitochondrial rennin–angiotensin system. In addition, they present evidence of age-related changes in mitochondrial rennin–angiotensin receptor expression. Isolated rat heart or liver mitochondria from mice were plated into a Seahorse XF 96-well plate, and the ADP-activated state was determined using substrate-supported respiration. A single assay was capable of determining the effect of different substrates and mitochondrial angiotensin 2 receptor agonists and antagonists on the activation of mitochondrial respiration in multiple replicates from different mitochondrial isolations. The data from this study suggest that a mitochondrial angiotensin 2 receptor agonist was capable of reducing ADP-activated respiration in parallel with nitric oxide production. Nitric oxide production inhibits mitochondrial respiration through binding to the electron transport system complex IV (71). These data strongly suggest that inadvertent mitochondrial angiotensin 2 receptor agonist activation could lead to disease development.

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Limitations of Quantifying Mitochondrial Dysfunction in Isolated Mitochondria

Extraction of mitochondria disrupts the intricate mitochondrial network integrity leading to differences in in vitro and in vivo data (51,58,72). Mitochondria exist as large tubular assemblies in living cells, are in close contact with other organelles in the cell matrix (73,74), and undergo fusion and fission events (75). Disruption of these networks (during isolation) may have important ramifications in determining the specific mutations that cause disease. For example, previous studies have demonstrated that mutations in proteins involved in mitochondrial fusion, fission, motility, or degradation cause neurological disorders including hereditary optic neuropathies (76) and Charcot–Marie–Tooth type 2A disease (77). Thus, if the mitochondrial network integrity is disrupted during the isolation procedure, it is possible that false-negative respiratory data may be produced.

In addition to mitochondrial network disruption (78), there are other limitations of studying isolated mitochondrial preparations that may cause in vitro properties of isolated mitochondria to be different from mitochondria maintained in vivo (58). These limitations equally affect data collected from the Clark-type electrode, O2k, as well as the Seahorse XF. Mitochondrial isolation from normal tissues is performed under optimal centrifugation speeds that have been demonstrated not to damage mitochondria and thus provide the highest quality of mitochondrial integrity (intactness of outer and inner membranes). However, different pathologies may affect the membrane intactness and therefore the sensitivity of mitochondria to centrifugation (79). If this happens, centrifugation methods may result in a biased selection of a particular mitochondrial population (80). Moreover, large quantities of cells or tissues are required for the optimal yield and quality of isolated mitochondria (81). The measurement of respiratory control in cell populations has the benefit of maintaining the intricate structural network of mitochondrial connections, and the study of bioenergetic function in cultured cells may be preferable in some situations (57,82).

Analysis of Intact Cultured Cells

The respiratory rates measured in intact cultured cells vary slightly in comparison to measured rates from isolated mitochondria. For example, the coupled ADP-activated state 3 (state 3_{ADP}) rate is not measured in cultured cells because ADP is incapable of penetrating the outer membrane of cultured cells (13,58). However, the real-time analysis of in vivo basal respiration, oxygen consumption, ATP turnover, coupling efficiency, proton leak, maximum respiration rate, respiratory control ratio, spare respiratory capacity, and non-mitochondrial respiratory parameters can all be determined by the sequential addition of specific electron transport system inhibitors (eg, oligomycin, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, rotenone, and antimycin A, Supplementary Table 1). A more detailed description and analysis of these measured parameters can be found in the review by Brand and Nicholls (57).

Oroboros O2k

The assessment of mitochondrial activity in suspension cells such as skin fibroblasts (38), lymphocytes (83), HEK293 cells (84), and 32D cells (85) has been performed in real time using the O2k system to determine respiratory malfunction. Cells may be suspended in a culture medium supporting physiological in vivo oxygen consumption and growth by exogenous substrates (advantageous when aiming at near-physiological conditions for cultured cells). Alternatively, cells may be in crystalloid medium without energy substrates (eg, mitochondrial respiration medium) yielding physiological in vivo oxygen consumption with endogenous substrates (86). Inhibitors and an uncoupler are then added to evaluate the different respiratory rates.

Analysis of disease-associated mutations can readily be analyzed by the O2k for respiratory malfunction. For example, cultured COS cells (derived from kidney cells of the African green monkey) have been used to analyze the respiratory response to increased expression of the Parkinson's disease-associated α-synuclein protein (87). Furthermore, duplications and triplications of the α-synuclein gene, *SNCA*, have been associated with more severe forms of the Parkinson's disorder (88,89). However, the underlying functional consequences of these mutations remain to be determined. Nakamura and colleagues (87) found that the overexpression of α-synuclein produced an increase in mitochondrial fragmentation and reduced both the basal and maximum levels of mitochondrial respiration, thus indicating that the affected cells could no longer respond efficiently to cell stress. This observation was further confirmed in neuronal cells where an increase in α-synuclein also produced mitochondrial fragmentation and decreased cell survival (87). These data indicate that mitochondrial malfunction as a consequence of the overexpression of α-synuclein may help explain some of the underlying mechanisms responsible for the Parkinson's disease severity and progression.

Seahorse XF Extracellular Flux Analyzer

The Seahorse XF can measure cellular mitochondrial metabolism in real time and is capable of analyzing both adherent cells and, in some cases, cells in suspension (however, suspension cells first need to be centrifuged to form a monolayer on the bottom of the well prior to analysis). For cell analysis, cells are grown to 70%–80% confluency. Growth media is removed and replaced with cell assay media. The derived measured parameters in cells are then determined after the addition of electron transport system inhibitors (57).

Novel bioenergetic properties of currently available pharmaceutical compounds have been identified with the Seahorse XF, and these have the potential to treat complex

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diseases of aging. Gohil and colleagues (90) included >3,500 chemical compounds assayed across multiple cell types identified that various food and drug administration–approved compounds shifted mitochondrial respiration to glycolysis. One of these compounds was meclizine, a nausea and vertigo approved compound that is readily available over the counter. Meclizine was found to reduce oxygen consumption and increase extracellular acidification in all cell types studied. This newly identified metabolic activity for meclizine was further found to confer protection in models of cardiac ischemia–reperfusion injury and significantly reduced infarct volume in an in vivo model of cerebral ischemia when used as a prophylactic (90).

Limitations of Quantifying Mitochondrial Dysfunction in Cultured Cells

Although cultured cells maintain a cellular architecture and are physiologically relevant for mitochondrial bioenergetic analysis, there are, however, several problems associated with the construction and maintenance of such cell lines. These problems include contamination by viruses, mycoplasma, and other cell lines (91). The generation of immortalized cell lines are also limited due to loss of differentiated functions, high glycolytic rates, and reduced respiration (92). Furthermore, most often individual cell populations are analyzed, and they lack direct comparisons with whole-tissue measurements that may contain multiple cell types. Another major issue is that cultured cells do not allow certain important functional parameters of mitochondria to be studied. For example, mitochondrial responses to various substrates such as ADP, creatine, or cytochrome *c* cannot be investigated in intact preparations because they are unable to penetrate the cellular membrane (13,58). However, these issues are not specific to the O2k and the Seahorse XF but rather a general limitation to cell growth–based research.

Analysis of Cell Fibers and Tissues

One strategy to overcome the limitations found in both mitochondrial isolations and in cultured cells is to study mitochondrial function using in situ–based approaches. Indeed, novel methods have been developed with the O2k to remove cell membrane inhibition and allow mitochondrial substrates to penetrate the cellular membrane (ie, permeabilization; 58). Methods have also been developed for the Seahorse XF that allow for the respiratory analysis of whole-tissue samples (ie, hippocampal slices; 93). A continued development of both systems to study cell fibers and tissues is expected in the next few years and will likely provide additional insight into complex diseases of aging.

Oroboros O2k

The O2k can quantify mitochondrial dysfunction in situ in permeabilized muscle fibers and in tissues. The permeabilized in situ approach is also suitable for the application of substrate–uncoupler–inhibitor–titration protocols for the analysis of ADP affinity (78,94) and for all functional studies of the electron transport system complexes (51,95). Although the in situ approach can be used with the Clark-type oxygen electrode (96), the O2k system has provided an important advance because of the stability of the signal, low instrumental background noise, and increased resolution (13,97,98,99,100).

The in situ approach weakens or permeabilizes the plasma membrane with a chemical compound so that mitochondrial function can be analyzed (96,97,98,101). The plasma membrane contains almost five times more cholesterol than the endoplasmic reticulum and 7–10 times more than the outer and inner mitochondrial membrane (102,103). Permeabilizing agents like saponin or digitonin interact specifically with cholesterol molecules abundant in the plasma membrane resulting in pore formation without affecting other cellular components like the endoplasmic reticulum, mitochondria, myofilaments, and cytoskeleton (58,78). This approach has been demonstrated to be physiologically relevant because it enables analysis of mitochondria in their normal intracellular assembly (58). Furthermore, preparations of permeabilized fibers have been shown to display mitochondrial functional stability for up to 24 hours when stored under cold conditions (104). The in situ approach is also appropriate for the assessment of mitochondrial function in model species of aging (105). For example, a constraint of many aging studies in *Drosophila* is that mitochondria increase in size as the fly ages. As a consequence, it has not been possible to study intact mitochondrial function in flies over 25 days of age (80).

The O2k has been used to quantify specific changes in mitochondrial function associated with aging disorders using permeabilized muscle fibers that was not detected using mitochondrial isolations. For example, using isolated mitochondria from skeletal muscle, it has been shown that sarcopenia, the decline of muscle mass and function with aging, is correlated with age-related changes in mitochondrial function (106,107). Sarcopenia is an important contributor to frailty and age-related disability. Using the O2k system, Picard and colleagues (72) performed a comparison of mitochondrial functions in mitochondrial isolations and in permeabilized myofibers from skeletal muscle of young adults and senescent rats. This study demonstrated that mitochondrial isolations employed to study aged muscles expose functional impairments not seen in situ in the permeabilized myofibers. Therefore, it was concluded that aging is associated with more modest changes in mitochondrial function in sarcopenic muscle using a physiologically relevant approach than previously thought using isolated mitochondria.

Seahorse XF Extracellular Flux Analyzer

An adaptation of the Seahorse XF plate assay has been the development of microplates that are capable of analyzing

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pancreatic islets for the study of insulin secretion (108). Pancreatic islets are scarce, and isolating sufficient mitochondria from them is difficult. Analyzing whole islets has the advantage of maintaining the cellular architecture. For respiratory analysis, pancreatic islets are isolated and plated into a recess in the bottom of the Seahorse XF islet capture microplate and immobilized with a securing screen. The pancreatic islets are analyzed in the same way as cultured cells to determine metabolic function. Indeed, it has been established that the reduction of cytochrome *c* in isolated pancreatic islets is necessary for insulin secretion and is unaffected by a decrease in oxygen consumption (108). A further development of the Seahorse XF islet capture microplate has enabled the analysis of hippocampal tissue slice preparations (93) and the bioenergetic profiling of whole zebra fish embryos (109). The zebra fish study demonstrated that total respiration increased with age of the embryo and was associated with an increase in mitochondrial content. Because whole embryos can be studied, there is a potential to determine respiratory malfunction in aging disorders and to test the effects of therapeutic agents (109).

A second adaptation of the Seahorse XF analyzer is the measurement of mitochondrial bioenergetics in intact skeletal muscle fibers (110). This protocol has implications for studying genotype-specific decline of function with increasing age as well as neuromuscular disorders including muscular dystrophy and cachexia. For intact muscle analysis, single muscle fibers are enzymatically isolated and seeded on to a culture microplate to cover approximately 50%–60% of the well bottom. Cellular measurements are then performed following a similar analysis as for cultured cells (110).

Limitations of Quantifying Mitochondrial Dysfunction in Cell Fibers and Tissues

Analysis of cell fibers and tissues for mitochondrial functional determination has at least three major limitations that should be considered before this approach is initiated. First, for permeabilized cells and tissues, respiratory parameters of different mitochondrial subpopulations cannot be distinguished. This may be a serious problem in cases of mitochondrial myopathy where mitochondrial DNA heteroplasmy rates can differ within and between cells. Second, the impact of cytosolic factors cannot be studied because the cytoplasm and all cellular soluble components are lost during the permeabilization procedure (58). This is particularly important because normal mitochondrial function requires crosstalk with cytosolic factors to maintain cellular homeostasis. Thus, misleading respiratory data may be produced when using permeabilized cells. Third, cell fibers and tissues cannot interact with neighboring tissues. This means that the influence of multiple tissue/organ types cannot be analyzed for mitochondrial functional analysis. These limitations are equally applicable to all biochemical and gene expression–based assays.

Respiratory Analysis in Age-Associated Complex Human Disease

Respiratory analysis of mitochondrial function has been performed on multiple tissue types across various complex human diseases (Table 2). These studies offer insights into the underlying mechanisms of disease progression and development. Here we consider the contributions that the O2k and Seahorse XF systems have made in our understanding of two aging-associated complex diseases: Alzheimer's disease and type 2 diabetes mellitus. We further discuss the role of mitochondrial malfunction as a consequence of autophagy dysfunction in aging. In each section, we briefly review the role of each disorder and conclude by considering possible future directions. More complete reviews of Alzheimer's disease, type 2 diabetes mellitus, and autophagy dysfunction in disease can be found elsewhere (111,112,113).

Alzheimer's Disease

Alzheimer's disease is the most common form of dementia in the elderly and is characterized by a decline in memory function and cognitive ability. It tends to occur in individuals older than 65 years of age. The underlying mechanisms of Alzheimer's disease are degeneration of brain tissue with extensive neuronal loss, accumulation of senile plaques (caused by aggregates of the amyloid-β protein), and the presence of filamentous inclusions known as neurofibrillary tangles due to hyperphosphorylation of Tau proteins (114). The amyloid-β protein is derived from the amyloid precursor protein (APP) through an initial β-secretase cleavage, followed by an intramembranous cleavage by the presinilin 1 (PS1) and presinilin 2 (PS2) subunits of the α-secretase complex. The amyloid-β protein is also known to be a potent inhibitor of mitochondrial enzyme activity and therefore serves to reduce the flow of electrons through the electron transport system and decreases the rate of ATP production and oxygen consumption (115,116). Moreover, mitochondrial dysfunction is one of the earliest features identified in the pathology of Alzheimer's disease (117,118). Mutations in mitochondrial DNA in the brains of Alzheimer's disease patients are also detected with higher frequency than age-matched controls, which is suggestive of a mitochondrial genetic link in Alzheimer's disease progression (119,120).

Respiratory analysis of extracted mitochondria using the O2k system has demonstrated that both double- (APP_{SW}/$PS2_{N141I}$) and triple (APP_{SW}/$PS2_{N141I}$/Tau_{P301L})-transgenic Alzheimer's disease mice exhibit a pronounced decrease in coupling efficiency and in the uncoupled state relative to non-mitochondrial respiration by the age of 8 months (121). Furthermore, the ADP-activated state and the uncoupled state are decreased at 8 months of age in the double-transgenic mice and at 12 months of age in the triple-transgenic mice (121). In combination, these data suggest that amyloid-β protein aggregation in the transgenic mice has a stronger biochemical effect in the double- (APP_{SW}/$PS2_{N141I}$) mutant

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pathway than the (Tau_{P301L}) pathway (121). This example shows that different genetic pathways altering cellular bioenergetics can be determined using the O2k system and can help improve our understanding of the underlying mechanisms leading to mitochondrial dysfunction in Alzheimer's disease.

Data generated from Seahorse XF also indicate that mitochondrial dysfunction may be a causal factor in Alzheimer's disease pathology (21). Studies have established that mitochondrial malfunction is severely compromised in a triple-transgenic (3xTg, $APP_{SW}/PS1_{M146V}/Tau_{P301L}$) mutant Alzheimer's disease mouse model (21). Yao and colleagues (21) determined that mitochondrial respiration is decreased in primary hippocampal neurons cultured from female transgenic (3xTg) Alzheimer's disease mice compared with neurons in age-matched nontransgenic (non-Tg) controls (21). The Seahorse XF data derived from the 3xTg Alzheimer's disease mice identified reduced levels of steady state basal respiration, ATP turnover (uncoupled), respiratory control ratio, uncoupled state, and spare respiratory capacity in comparison to the non-Tg controls. Furthermore, the primary neuronal cultures used in the study derived from embryonic day 14 mice indicate that the mitochondrial dysfunction can be detected early in the pathogenesis of Alzheimer's disease. These data strongly suggest that mitochondrial malfunction precedes the development of plaque formation, which is the classical histopathological marker of Alzheimer's disease.

The data generated from both the O2k and the Seahorse XF have identified altered bioenergetics in mouse models of Alzheimer's disease. Both sets of data suggest that genetic risk factors for mitochondrial bioenergetics could be critical in the development of Alzheimer's disease. This is supported by studies that found a relationship between a brain hypometabolic profile and an increased risk of Alzheimer's disease development in offspring from females with a family history of Alzheimer's disease (122,123). Combined, these studies suggest that a potential therapeutic strategy in the prevention of Alzheimer's disease would be to enhance mitochondrial metabolic function in individuals with Alzheimer's disease. Estrogen is one such metabolite that has been found to enhance mitochondrial function by upregulating key enzymes in brain metabolism (124,125,126).

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is the most common metabolic disease found in an aging population (127). Although the primary cause of type 2 diabetes remains unknown, the development of insulin resistance in both skeletal muscle and liver is pivotal in the disease progression prior to the onset of pancreatic β cell dysfunction (128). Several studies have further implicated type 2 diabetes with mitochondrial malfunction including reduced mitochondrial density (129), reduced mitochondrial oxidative phosphorylation (130,131), and increased levels of reactive oxygen species (132,133). However, the underlying molecular mechanisms responsible for mitochondrial malfunction remain to be identified.

Decreased mitochondrial function has been suggested to play an important role in the pathogenesis of insulin resistance (134). However, it is not clear whether the observed reduction in oxidative capacity is due to impairment in the functional properties of mitochondria or to a reduction in mitochondrial DNA copy number. The O2k system has demonstrated that mitochondrial respiration is altered in type 2 diabetes patients (135,136). Using the in situ approach on permeabilized muscle fibers, a decrease in the ADP-activated and in the uncoupled state was observed in type 2 diabetes patients compared with age- and body mass index–matched control subjects (136). However, oxygen consumption was the same for both type 2 diabetes patients and healthy control subjects after normalization for citrate synthase activity (a marker of mitochondrial content; 135, 136). The O2k data therefore suggest that mitochondrial malfunction is not an underlying mechanism involved with type 2 diabetes.

In contrast, data from the Seahorse XF suggest that mitochondrial bioenergetics may be altered in type 2 diabetes as a consequence of the decreased expression of the human presenilin-associated rhomboid-like (*PARL*) gene (48). *PARL* malfunction is associated with type 2 diabetes (72,137), and analysis of skeletal muscle biopsies taken from type 2 diabetes patients has revealed that *PARL* messenger RNA and mitochondrial DNA content is reduced in comparison to healthy controls (48). The enzymatic cleaved product of PARL (PARL-β) is thought to mediate mitochondria-to-nucleus crosstalk and regulate the expression of mitochondrial-specific genes (138). The Seahorse XF study by Civitarese and colleagues (48), using human RMS-13 myoblast cells transfected with either normal *PARL*-β or mutant *PARL*-β constructs, reported that oxygen consumption is reduced in the mutant-transfected cells after normalization to citrate synthase (48). Furthermore, the RMS-13 myoblast cells expressing the synthetic normal PARL-β peptide exhibited increased levels of sirtuin-1 (48). This is an interesting finding because sirtuin-1 is an activator of peroxisome proliferator–activated receptor gamma, coactivator 1 alpha, a transcriptional coactivator responsible for mitochondrial biogenesis (139,140). These data therefore suggest that PARL activates the sirtuin pathway and that a decrease in the expression of PARL may reduce mitochondrial biogenesis.

The data generated from the O2k and Seahorse XF appear to conflict, and three explanations would appear possible. One explanation for the differences is that the O2K study assayed the parameters of ADP-activated state and uncoupled state using excess concentration of substrates where the Seahorse XF study assayed for oxygen consumption using the endogenously expressed substrates within the cells. A second explanation is that the O2k used permeabilized fibers that lack nuclear factors and the cytosol, but the Seahorse XF study used intact cultured cells. A third possible

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explanation is that O2k is more physiologically relevant because human biopsies were directly assayed (136). In contrast, the Seahorse XF study represents an artificial system in that synthetic constructs were forcibly expressed within cultured cells (48). What is clear is that further analyses using the same cells and measured parameters are required to determine system sensitivity and complementarity.

Aging-Associated Autophagy Impairment and Mitochondrial Malfunction

Autophagy is a process whereby components of a cell that are no longer required for cell maintenance are lysed and digested by lysosomes and recycled for essential cellular activities (141). A further autophagic mechanism leading to the specific elimination of mitochondria is mitophagy (142). These form part of the normal homeostatic mechanism within cells, but evidence is growing that impairment of the autophagy system may be associated with disease progression (137,143,144). Indeed, impaired autophagy is associated with the pathologies of Alzheimer's, Huntington's, and Parkinson's disease (145). Of particular relevance was the observation that a decrease in autophagy is associated with an increase in age and age-related disorders (146). Moreover, the aging process is linked with accumulated morphological and enzymatic mitochondrial defects (147,148,149). Thus, impairment of mitochondrial function may be a major contributing factor in the aging process and age-associated diseases.

The application of the O2k system has demonstrated that loss of the Parkinson's associated *DJ-1* (Parkinson protein 7, *PARK7*) gene (in a *DJ-1* knockout mouse model) is associated with both reduced autophagy levels and mitochondrial respiratory rates (45). Initial bioenergetic analysis on embryonal fibroblasts derived from the *DJ-1* knockout mice demonstrated that endogenous respiration was reduced by 33% in comparison to normal control cells. Furthermore, rates of uncoupled respiration were also reduced by 34%. O2k substrate–uncoupler–inhibitor–titration protocols using multiple substrates were further applied to determine complex I and complex II electron transport system malfunction. The ADP-activated state for complex I was found to be reduced by 50% in the *DJ-1* knockout embryonal fibroblasts compared with the normal controls. Analysis of complex II was, however, found to be normal in both cell types. These data indicate that mitochondrial respiration is altered in the absence of DJ-1 expression and that mitochondrial metabolic malfunction is restricted to complex I (45). Together, these data suggest that impaired mitochondrial respiration is associated with reduced levels of autophagy in a Parkinson's disease model and indicates that DJ-1 functions in maintaining mitochondrial homeostasis.

A Seahorse XF study on mouse models expressing a mutant form of *Atg7*, an essential gene required for autophagosome formation (150), has also revealed impaired autophagy with associated mitochondrial malfunction in aging mice (36). The bioenergetic profile of isolated mitochondria from $Atg7^{-/-}$ skeletal cells and from embryonic fibroblasts derived from $Atg7^{-/-}$ mice was determined against normal control mice. The isolated mitochondria from $Atg7^{-/-}$ skeletal cells revealed that basal mitochondrial respiration levels were significantly reduced in comparison to normal controls, and further, the ADP-activated state failed to induce respiration rates above the basal level. The derived data thus indicate that the respiratory control ratio and the spare respiratory capacity are also decreased. Similarly, the embryonic fibroblasts derived from the $Atg7^{-/-}$ mice also demonstrated reduced basal levels of oxygen consumption with associated increased levels of extracellular acidification on addition of oligomycin indicating a preference for the glycolytic pathway for ATP generation (36). The mitochondria were also found to be damaged and produced increased levels of reactive oxygen species. Increases in reactive oxygen species are associated with further damage to mitochondria, which in turn produce yet more reactive oxygen species in a damaging cycle (120). In addition, Wu and colleagues (36) found that treatment of $Atg7^{-/-}$ cells with the antioxidant *N*-acetylcysteine, a reactive oxygen species inhibitor, helped stimulate mitochondrial respiration in $Atg7^{-/-}$ cells. This suggests that antioxidant-targeted therapy might be beneficial in pathologies with impaired autophagy. Indeed, an autophagic defect associated with mitochondrial malfunction has also recently been identified in Huntington's disease patients (151).

The data generated from both the O2k and the Seahorse XF studies indicate that mutations in nuclear genes lead to malfunctioning mitochondria and that this is further associated with impaired autophagy. The $Atg7^{-/-}$ murine studies also suggest that increasing the autophagic flux cycle may offer a protective strategy in ameliorating the onset of disease (36). However, She and colleagues (152) found elevated levels of Atg7 in the skeletal muscle of a mouse model of Huntington's disease and that this is associated with severe muscle wasting. This further suggests that therapies aimed at increasing the ATG7-associated autophagy system in the skeletal muscle of patients with Huntington's disease could be severely damaging. Further studies are therefore warranted to determine the functional role of autophagy in the Huntington's disease phenotype.

Conclusion

The study of cellular metabolism and specifically the functional properties of mitochondria is likely to provide a greater understanding of the underlying metabolic malfunctions associated with the progression of many complex diseases of aging. Central in this increased understanding of basic bioenergetics is the development of two parallel systems for studying mitochondrial dysfunction. The O2k and Seahorse XF represent technological advances in comparison to the traditional oxygen electrode. Determination

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of the etiology of complex disease requires detailed analyses of evidence generated from multiple strategies designed to disentangle the complex nature of underlying disease-causing mechanisms including data from model organisms. Animal models that we include in this review can provide a starting point for investigating human disease. However, carefully controlled studies of well-characterized human populations are needed to test the hypotheses generated from animal models. One such study is the Concord Health and Ageing in Men Project. Concord Health and Ageing in Men Project is a population-based longitudinal study designed to provide a wide range of new information about the health of older men (153).

Both methodologies have advantages and limitations (Table 1). Each of the systems can be used to analyze mitochondrial malfunction in aging and complex human genetic disorders (Table 2) and have been shown to produce comparable results, at least under certain circumstances. However, both systems have notable limitations, and future strategies may incorporate the complementary use of both systems in multiple biological systems to provide a powerful strategy for identifying respiratory dysfunction. Likewise, the biological systems employed by these methodologies have strengths and weaknesses. In this article, we consider the mitochondria, cultured cells, and cell fibers and tissues and conclude that researchers may need to examine multiple biological systems to unravel complex diseases of aging.

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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