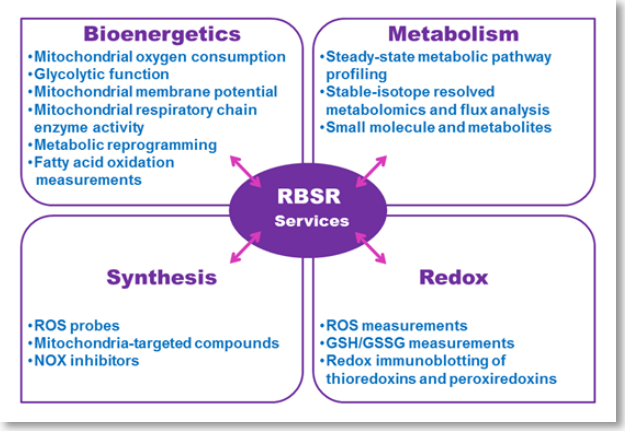
Redox and Bioenergetics Shared Resource Helps MCW Investigators Understand Cancer Cell Behavior

In 2012, the MCW Cancer Center (MCWCC), under the leadership of **Ming You, MD, PhD**, sought to develop the institution's Cancer Biology Research Program to enhance scientific discovery in the area of cancer treatment and therapeutics. The Redox and Bioenergetics Shared Resource (RBSR) was established to provide state-of-the-art instrumentation, cutting-edge techniques, and sophisticated expertise dedicated to investigating cancer cell metabolism and redox signaling.

The mission of the RBSR is to enable researchers to assess cellular bioenergetics, metabolism, ROS generation, and intracellular redox status. The RBSR provides an environment for education and training in research on oxy-radicals and redox bioenergetics. The resource supports and guides investigators in the development of anticancer treatments based on the redox profiling of cancer cell and bioenergetic status. The RBSR is one of several Core facilities supported by the MCWCC under the leadership of **Hallgeir Rui, MD, PhD**, Interim Center Co-Director. **Balaraman Kalyanaraman, PhD**, directs the RBSR with oversight by an advisory committee that reviews all services provided by the resource.

The RBSR offers services and instrumentation to assess many aspects of redox signaling and metabolic function in cancer cells, including:

- Detection of superoxide radical anion, hydrogen peroxide, and peroxynitrite
- Redox status of key cytosolic and mitochondrial antioxidant proteins including peroxiredoxins and thioredoxins
- Mitochondrial respiration and glycolytic function
- Analysis of metabolic intermediates
- Identification of altered metabolism using stable isotope-based metabolite flux analysis



The main goals of the MCWCC RBSR are to:

1. Investigate cancer cell metabolism and redox signaling and understand how cancer cells exploit metabolic pathways for survival, proliferation, differentiation, and drug resistance.
2. Provide a better understanding of the bioenergetic pathways and oxidant production in cancer cells cultured under normoxic and hypoxic microenvironments.
3. Develop new, rigorous and cost-effective assays to measure the production of ROS, redox, and bioenergetic status in cancer cells *in vitro* and in tumors *in vivo*.
4. Develop new redox- and metabolism-based strategies for inhibiting cancer cell progression and metastasis and promoting cancer prevention and therapy.
5. Promote increased collaboration in cancer metabolism research between basic scientists and clinical researchers at MCW.

Redox and Bioenergetics: Relevance in Cancer, Cardiovascular and Neuroscience Research

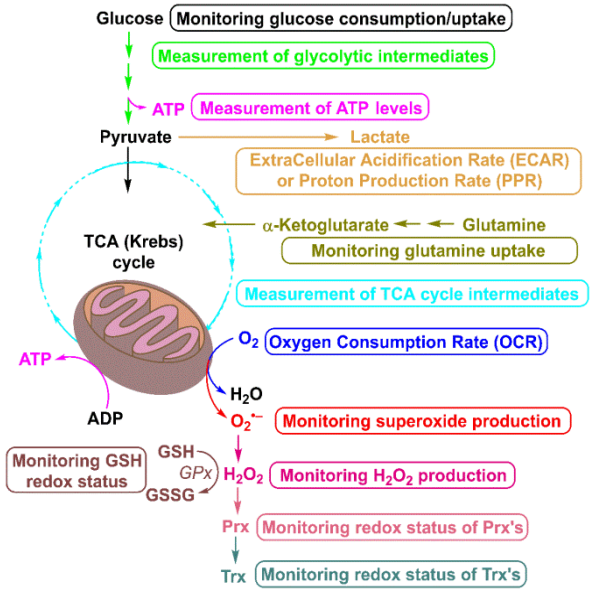
The RBSR can help analyze cancer cells of different phenotypes, including patient-derived cell lines, to determine their bioenergetic and redox profiles, with the goal of developing effective therapies. Also, understanding the differences in cell bioenergetics and redox defense mechanisms in chemo-resistant cancer cells will help in the development of redox-based therapy, mitochondrial drugs altering oxidative phosphorylation (OXPHOS) metabolism and the potential for minimizing adverse effects of chemotherapeutic drugs. The RBSR is an ideal resource for determining metabolic phenotypes and metabolic switching of cancer cells, and exploiting the difference in their metabolic needs, to develop new therapeutics.

The RBSR is also utilized by researchers investigating diabetes, obesity, stem cell therapy, autism, angiogenesis, and many other areas of biomedical research. Most of the studies are related to understanding mitochondrial function, glucose metabolism, and nutrient metabolism.

Researchers **Jennifer Strande, MD, PhD**, and **Meetha Medhora, PhD**, are investigating mitochondrial function disparities and increased cardiovascular toxicity resulting from radiation therapy. Neuroscience researchers **Gisela Chelimsky, MD**, and **Thomas Chelimsky, MD**, are investigating bioenergetic changes in the central autonomic network of autistic patients.

Major Services, Technologies, Equipment, and Expertise Provided by the RBSR

The RBSR facility is managed and operated by **Monika Zielonka** (Research Technologist III). **Jacek Zielonka, PhD** (Assistant Professor, Biophysics) provides the analytical expertise in redox measurements. **Gang Cheng, PhD** (Research Scientist II, Biophysics) provides the expertise in the measurement of OXPHOS and glycolytic metabolism.



Cellular bioenergetics and redox status services provided.

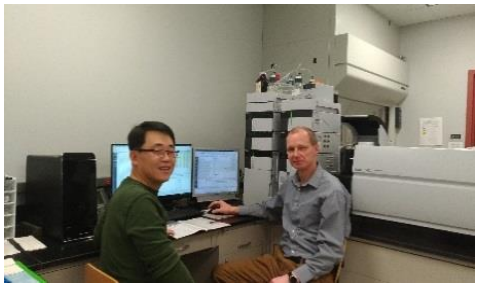
A wide range of experimental applications of extracellular flux, metabolomics, and redox signaling studies can be performed utilizing the instruments and expertise in the RBSR. The RBSR facility can be utilized to examine mitochondrial function, glycolytic function, fatty acid oxidation, glucose metabolism, glutamine metabolism, and metabolic phenotypes in cancer models. Bioenergetic flux analysis, conducted on the Seahorse XF analyzer, consists of multiple assay types (e.g., mitochondrial stress test, glycolytic stress test, permeabilized cell assay, or fuel flux analysis) to determine major pathways fueling mitochondrial respiration. LC-MS analyses include small molecule/drug measurements and snapshot metabolic profiling or metabolomic flux analyses using isotope-labeled substrates, such as ^{13}C- glucose and ^{13}C-pyruvate.

Major equipment in RBSR:

- Seahorse Bioscience XF96 and XFe96 extracellular flux analyzers
- Shimadzu Nexera-2 UHPLC system with UV-vis absorption and MS/MS detectors
- ESA HPLC system with electrochemical CoulArray® detector
- Beckman Coulter DTX 880 multimode plate reader
- Agilent 1100 HPLC systems with UV-vis absorption and fluorescence detectors
- Nikon Eclipse Ti Fluorescence Microscope
- Bruker EMX electron paramagnetic resonance (EPR) spectrometer
- Perkin Elmer LS55 luminescence spectrometer

For ROS measurements, the available assays enable detection of superoxide radical anion, hydrogen peroxide, and peroxynitrite. These include plate reader-based fluorescence measurements for real-time ROS monitoring, and HPLC-based analyses of the specific oxidation/nitration products produced using commercially unavailable probes and analytical standards. HPLC- and LC-MS-based assays provide rigorous methodology for detection and characterization of ROS and provide validation for plate reader-based assays. In the field of redox biology, the MCWCC RBSR is a unique, internationally renowned, and trusted resource for method development and validation.

Seahorse XF96 and XF^{e}96 instruments.

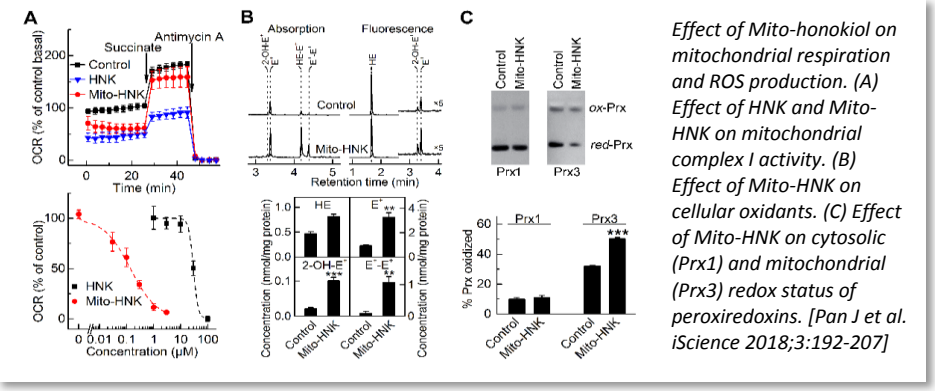


Shimadzu Nexera-2 UHPLC-LCMS 8030 instrument.

The RBSR is equipped with the instruments needed to provide sample acquisition and analysis to generate high-quality data related to cancer cell redox and bioenergetic function.

New collaborations, publications, grant funding and scientific breakthroughs by Core users

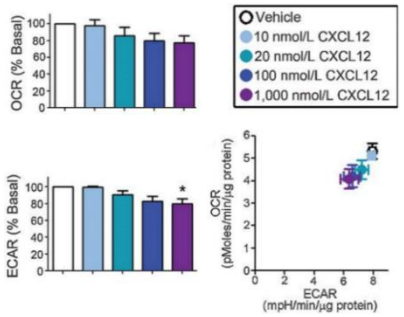
The RBSR supports MCWCC members and other MCW researchers by providing access to specialized, state-of-the-art instruments and expertise in cellular redox and bioenergetic function. The RBSR promotes collaboration between MCW investigators interested in cancer metabolism, bioenergetics, and redox mechanisms.



Effect of Mito-honokiol on mitochondrial respiration and ROS production. (A) Effect of HNK and Mito-HNK on mitochondrial complex I activity. (B) Effect of Mito-HNK on cellular oxidants. (C) Effect of Mito-HNK on cytosolic (Prx1) and mitochondrial (Prx3) redox status of peroxiredoxins. [Pan J et al. iScience 2018;3:192-207]

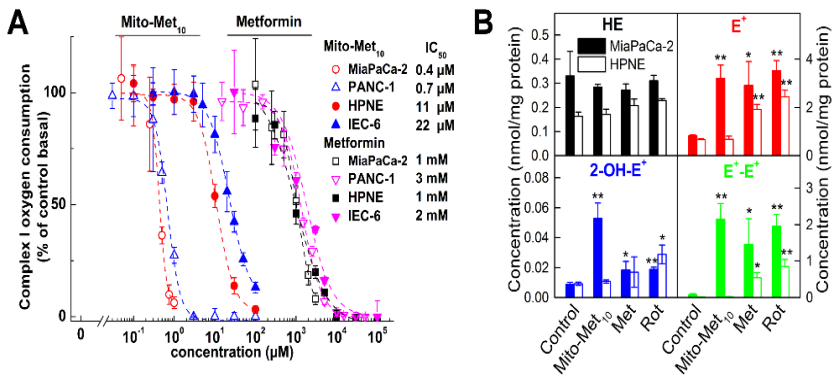
Dr. Ming You and colleagues reported that mitochondria-targeted honokiol (Mito-HNK), a synthetic derivative of the active component of magnolia bark extract, inhibits mitochondrial function (A) in lung cancer cells, stimulates ROS formation (B), and induces oxidation of mitochondrial peroxiredoxin (C), leading to inhibition of lung cancer growth and prevention of brain metastasis. **This is the first study demonstrating the simultaneous measurements of mitochondrial function and ROS in chemoprevention.**

Michael Dwinell, PhD, and colleagues found that certain chemokines decrease mitochondrial respiration and glycolytic function in pancreatic cancer cells, resulting in activation of AMPK. The services offered by the RBSR allowed the authors to implicate the causative role of decreased cell bioenergetic status in inhibiting pancreatic cancer cell migration and metastasis upon *in vitro* and *in vivo* treatment with ataxic doses of CXCL12. **This is the first paper demonstrating the effects of chemokines on the bioenergetic function of human pancreatic cancer cells.**



Effects of chemokine on PDAC bioenergetics and metastasis. Ataxic doses of CXCL12 alter pancreatic cancer cell bioenergetics and metastasis of pancreatic tumor in xenograft mice model. [Roy I et al. Cancer Res 2015;75:3529-42]

Dr. Kalyanaraman and colleagues have developed mitochondria-targeted metformin analogs as potential drugs for pancreatic cancer (A). Both metformin and Mito-metformin selectively inhibit complex I, but Mito-metformin is ~1,000-fold more potent than metformin. Inhibition of complex I leads to increased generation of superoxide (A and B), resulting in the formation of hydrogen peroxide and oxidation of mitochondrial peroxiredoxin (Prx3). **This is one of the first studies reporting IC^{50} determination for inhibition of mitochondrial function (including complex I activity) using the Seahorse instrument.**



Effects of metformin and Mito-metformin on PDAC proliferation. (A) Mito-metformin is 1000-fold more potent than metformin in selective inhibition mitochondrial complex I in pancreatic cancer cells. (B) Mito-metformin induces superoxide generation as measured by HPLC-based assay of hydroethidine oxidation. [Cheng G et al. Cancer Res 2016;76:3904-15]

Numerous high-impact publications authored by MCWCC members were made possible due to the services provided by the RBSR; the complete list of publications, 58 in total, is available on the RBSR website. The RBSR services were used to generate the data used in several grant NIH-funded applications. Several cancer bioenergetics-related R01 grant proposals and a P01 grant proposal are currently under review.

Funded NCI and NHLBI Grants Using the RBSR:

- Dwinell MB & Kalyanaraman B; "Targeting Pancreatic Cancer Energy Metabolism, Tumor Growth, and Metastasis" (NCI)
- See W; "BCG-Induced Oxidative Stress as a Target for Improved Clinical Efficacy" (VA Merit Award)
- Park J; "Mechanisms of MEK/ERK Growth Arrest Signaling" (NCI)
- You M & Kalyanaraman B; "Chemoprevention of Lung Cancer with Mitochondria-Targeted Honokiol" (NCI)
- You M, Kalyanaraman B & Kresty L; "Chemoprevention of Lung Cancer by Targeting Lonidamine to Mitochondria" (NCI)
- Chitambar C & Stolley M; "Every Day Counts: A Lifestyle Program for Women with Metastatic Breast Cancer" (NCI)
- Silverstein R; "ERK5 AND CD36 Link Oxidative Stress to Platelet Dysfunction and Ischemic Injury" (NHLBI)

Going forward…

Future plans include measurements of bioenergetic function and ROS production under hypoxia and in 3D spheroids, quantitative analyzes of mitochondrial membrane potential, *in vivo* monitoring of cancer cell ATP levels in tumor xenograft models, and ROS measurements *in vivo* in cancer cells and the tumor microenvironment.

Low-Temperature EPR

This advanced technique provides the ability to measure mitochondrial biomarkers of redox and bioenergetic status in tissue samples from *in vivo* models. Mitochondrial complex inhibition can be monitored using this technique, which no other Cancer Center currently has the instrumentation or expertise to handle. Once the tissue is collected, the unique instrumentation from the National Biomedical EPR Center will be used to measure mitochondrial biomarkers to gain a better understanding of cancer bioenergetics and ROS production and their response to treatment in tumor *in vivo*.

Bioenergetics Under Hypoxia

We are in the process of upgrading the hypoxia chamber to house analytical instrumentation to monitor bioenergetic status (e.g., OXPHOS, ECAR, ATP) and mitochondrial complex activity and autophagy under hypoxic conditions to better mimic the *in vivo* tumor microenvironment under *in vitro* settings.

Metabolic Flux Using 3-D Spheroids

The 3-D tumor spheroid model more accurately mimics the tumor microenvironment than 2-D monolayer cell cultures. Additionally, the 3-D tumor spheroid model can more accurately predict the *in vivo* response to drug treatments.

***In vivo* Bioenergetics and ROS Measurements**

We are currently optimizing the experimental conditions for using luciferin and its ROS-activable precursor, PCL-1 probe, to monitor changes in bioenergetic status and ROS production during tumor growth and in response to treatment, by *in vivo* bioluminescence imaging.

The RBSR has been and will continue to be an important resource for advancing studies in cancer biology. MCW investigators are fortunate to have access to this cutting-edge resource right here on campus. For RBSR comments or inquires, please contact a team member.