Assays for studying mitochondrial health and function

Introduction

Mitochondria play a critical role in maintaining normal cellular activities. Mitochondria are pleomorphic organelles with structural variations depending on cell type, cell cycle stage, and intracellular metabolic state. The study of mitochondrial health and dysfunction is extremely important for studies in disease prevention.

In dividing cells, mitochondria can exist as ovoid-shaped organelles with a fragmented morphology (as often shown in textbooks) or as a reticulum, as a single, multibranched structure. The cell cycle– and metabolic state–dependent changes in mitochondrial morphology are controlled by a set of proteins that cause fission and fusion of the organelle mass. Studies show that mutations in these proteins are the cause of several human diseases, signifying the importance of overall morphology as an indicator of cell health.

The key function of mitochondria is energy production through oxidative phosphorylation and ATP synthase. 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; those “leaky” electrons can quickly interact with molecular oxygen to form the superoxide anion, the predominant reactive oxygen species (ROS) in mitochondria. The aberrant generation of ROS through oxidative stress has been known to cause the disruption of normal cell mechanisms and has been implicated in cardiovascular diseases, including hypertension, atherosclerosis, and diabetes-associated vascular injuries, as well as in neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, and amyotrophic lateral sclerosis (ALS) [2,3].

Although the role of autophagy in oxidative stress–induced cell death is not fully understood, the segregation and delivery of cytoplasmic cargo for degradation plays a vital role in the cell survival response during early-stage, ROS-induced stress. For many of these mitochondrial functions, there is only a partial understanding of the components involved, with even less information on mechanism and mitochondrial regulation. Here we show that a simple labeling and detection approach can be used for a variety of assays to study mitochondrial morphology, health, and function.

Materials

- Invitrogen™ CellLight™ Mitochondria-GFP, BacMam 2.0 (Cat. No. C10508)
- Invitrogen™ CellLight™ Mitochondria-RFP, BacMam 2.0 (Cat. No. C10601)
- Invitrogen™ Image-iT™ Fixation/Permeabilization Kit (Cat. No. R37602)
- Invitrogen™ BlockAid™ Blocking Solution (Cat. No. B10710)
- Invitrogen™ NucBlue™ Fixed Cell ReadyProbes™ Reagent (Cat. No. R37606)
- Invitrogen™ ProLong™ Gold Antifade Mountant (Cat. No. P10144)
- Gibco™ PBS, pH 7.4 (Cat. No. 10010023)
- Invitrogen™ ATP Synthase Beta Antibody, Alexa Fluor™ 488 Conjugate (Cat. No. MA1930A488)
- Invitrogen™ ATP Synthase Beta Antibody, Alexa Fluor™ 647 Conjugate (Cat. No. MA1930A647)
- Invitrogen™ Beta Tubulin Loading Control Antibody, Alexa Fluor™ 555 Conjugate (Cat. No. MA516308A555)
- Invitrogen™ ZO-1 Monoclonal Antibody, Alexa Fluor™ 488 Conjugate (Cat. No. MA339100A647)
- Invitrogen™ LC3B Antibody Kit for Autophagy (Cat. No. L10382)
- Invitrogen™ Goat Anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor™ 488 Conjugate (Cat. No. A11008)
- Thermo Scientific™ Paraformaldehyde (Cat. No. 416780250)
- Thermo Scientific™ 10X Permeabilization Buffer (Cat. No. 8408400)
- Thermo Scientific™ Blocker™ BSA (10X) in PBS (Cat. No. 37525)
- Greiner Bio One 96-Well CELLSTAR™ TC Treated Microplates (Cat. No. 50823589)
- Thermo Scientific™ Nunc™ Glass Bottom Dishes (Cat. No. 12567401)

Methods

Analysis of mitochondrial colocalization via high-content analysis (HCA)

Human bone osteosarcoma (U2OS) cells were seeded in 96-Well CELLSTAR TC Treated Microplates and transduced with CellLight Mitochondria-RFP reagent. After incubation at 37°C and 5% CO_2 for 48 hours, cells were fixed and permeabilized with the Image-iT Fixation/Permeabilization Kit and incubated in BlockAid Blocking Solution for 1 hour. The cells were incubated another hour in a mixture of BlockAid Blocking Solution containing 5 µg/mL ATP synthase beta antibody conjugated to Alexa Fluor 488 dye along with 1 drop/mL NucBlue Fixed Cell ReadyProbes Reagent. Using the "General Colocalization Measurement Tool" assay, plates were analyzed on the Thermo Scientific™ CellInsight™ CX7 HCA Platform.

Detecting changes in mitochondrial morphology during the cell cycle

Human colorectal adenocarcinoma (Caco-2) cells were seeded onto No. 1.5 round coverslips and incubated overnight at 37°C and 5% CO_2. Cells were then fixed in 4% paraformaldehyde for 15 minutes, permeabilized for 15 minutes, and blocked in 3% BSA in PBS for 30 minutes. A mixture of labeling reagents (10 µg/mL ATP synthase beta antibody conjugated to Alexa Fluor 647 dye; 10 µg/mL beta tubulin antibody conjugated to Alexa Fluor 555 dye; 10 µg/mL ZO-1 antibody conjugated to Alexa Fluor 488 dye; 1:10,000 Hoechst™ 33342 stain) was diluted in blocking buffer and applied to cells for a minimum of 1 hour. The labeled Caco-2 cells were mounted in ProLong Gold Antifade Mountant and image stacks were acquired at 63x magnification on a ZEISS 710 Confocal Laser Scanning Microscope and displayed as maximum intensity projections.

Analysis of mitochondrial morphology disruption via HCA

U2OS cells were seeded in 96-Well CELLSTAR TC Treated Microplates and transduced with CellLight Mitochondria-RFP reagent. After incubation at 37°C and 5% CO_2 for 48 hours, cells were treated with 100 µM menadione for 1 hour, fixed and permeabilized with the Image-iT Fixation/Permeabilization Kit, and incubated in BlockAid Blocking Solution for 1 hour. Cells were then incubated in a mixture of BlockAid Blocking Solution containing 5 µg/mL ATP synthase beta antibody conjugated to Alexa Fluor 488 dye along with 1 drop/mL NucBlue Fixed Cell ReadyProbes Reagent. Using the "Compartment Analysis" assay, plates were analyzed on the CellInsight CX7 HCA Platform.

Detecting mitophagy

U2OS cells were seeded in Nunc Glass Bottom Dishes and transduced with CellLight Mitochondria-RFP reagent. After incubating at 37°C and 5% CO_2 for 24 hours, cells were treated with 30 µM chloroquine for 16 hours followed by 100 µM menadione for 1 hour. The U2OS cells were then fixed and permeabilized with the Image-iT Fixation/Permeabilization Kit and incubated in BlockAid Blocking Solution for 1 hour. The cells were then incubated in a mixture of BlockAid Blocking Solution containing 5 µg/mL ATP synthase beta antibody conjugated to Alexa Fluor 488 dye along with 1 drop/mL NucBlue Fixed Cell ReadyProbes Reagent. The labeled U2OS cells were mounted in ProLong Gold Antifade Mountant and image stacks were acquired at 63x magnification on a ZEISS 710 Confocoal Laser Scanning Microscope and displayed as maximum intensity projections.

Results

Analysis of mitochondrial colocalization via HCA

In any assay that may alter organelle function or area, it is important to confirm localization and abundance of the target of interest. As both the CellLight Mitochondria-RFP reagent and ATP synthase beta antibody were utilized to demarcate the mitochondria in all subsequent experiments, a colocalization experiment was performed and a Pearson's correlation coefficient (PCC) of 0.79 was calculated to confirm strong positive localization of both mitochondrial probes (Figure 1). A PCC is a measure of dependence between two variables, where a value of +1 indicates perfect alignment, 0 indicates no alignment, and –1 indicates an inverse relationship.

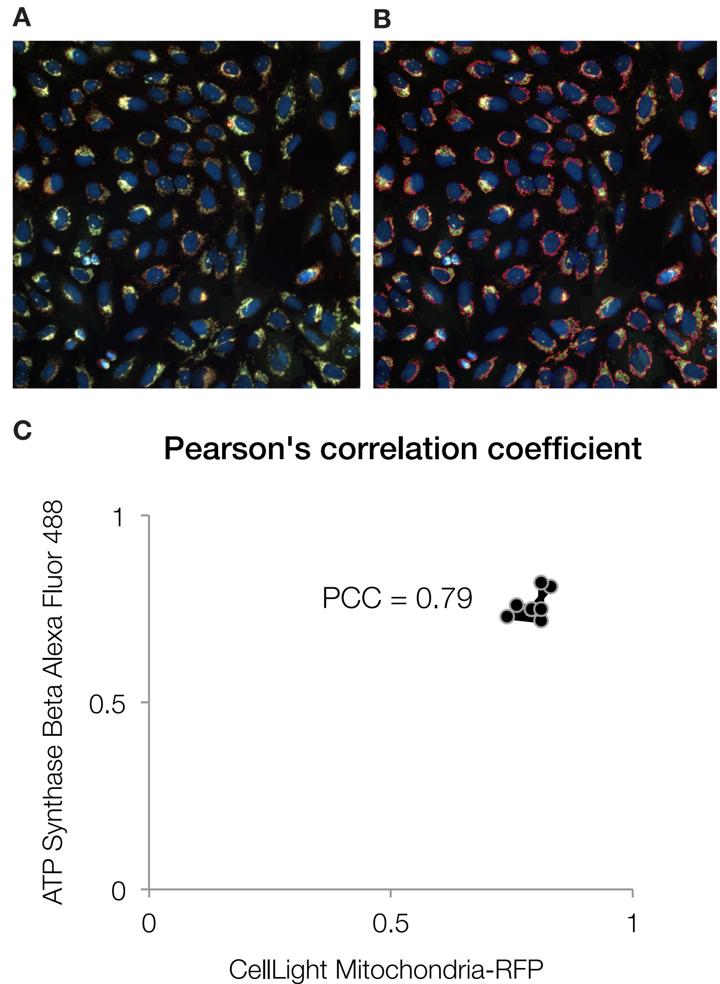

Figure 1. Colocalization of mitochondrial probes. U2OS cells were labeled with **(A)** ATP synthase beta antibody conjugated to Alexa Fluor 488 dye and **(B)** CellLight Mitochondria-RFP reagent. Cells were counterstained with NucBlue Fixed Cell ReadyProbes Reagent and analyzed on the CellInsight CX7 HCA Platform. **(C)** Graph demonstrating strong positive correlation of the probes.

Detecting changes in mitochondrial morphology during the cell cycle

Conformational changes in mitochondrial morphology are observed as a cell undergoes mitosis (Figure 2). As the cell transitions from a resting state during interphase and begins to divide, disassembly of cytoplasmic components occurs, causing the mitochondria to display both the multibranched and ovoid-shaped structures during metaphase. Fully fragmented, ovoid-shaped mitochondria are evident during telophase, as a cleavage furrow separates daughter cells prior to cytokinesis and mitosis becomes close to completion.

Analysis of mitochondrial morphology disruption via HCA

During the generation of energy through oxidative phosphorylation and ATP synthase, mitochondrial superoxide is generated when mitochondrial oxygen is incompletely reduced. Those electrons interact with molecular oxygen to generate ROS, causing the disruption of normal cell mechanisms (Figure 3). U2OS cells were treated with dilutions of the drug menadione, a redox-cycling ROS generator. As cells become oxidatively stressed, the mitochondria undergo a conformational change and condense around the perinuclear region of the cell. A statistically significant reduction in mean area is observed.

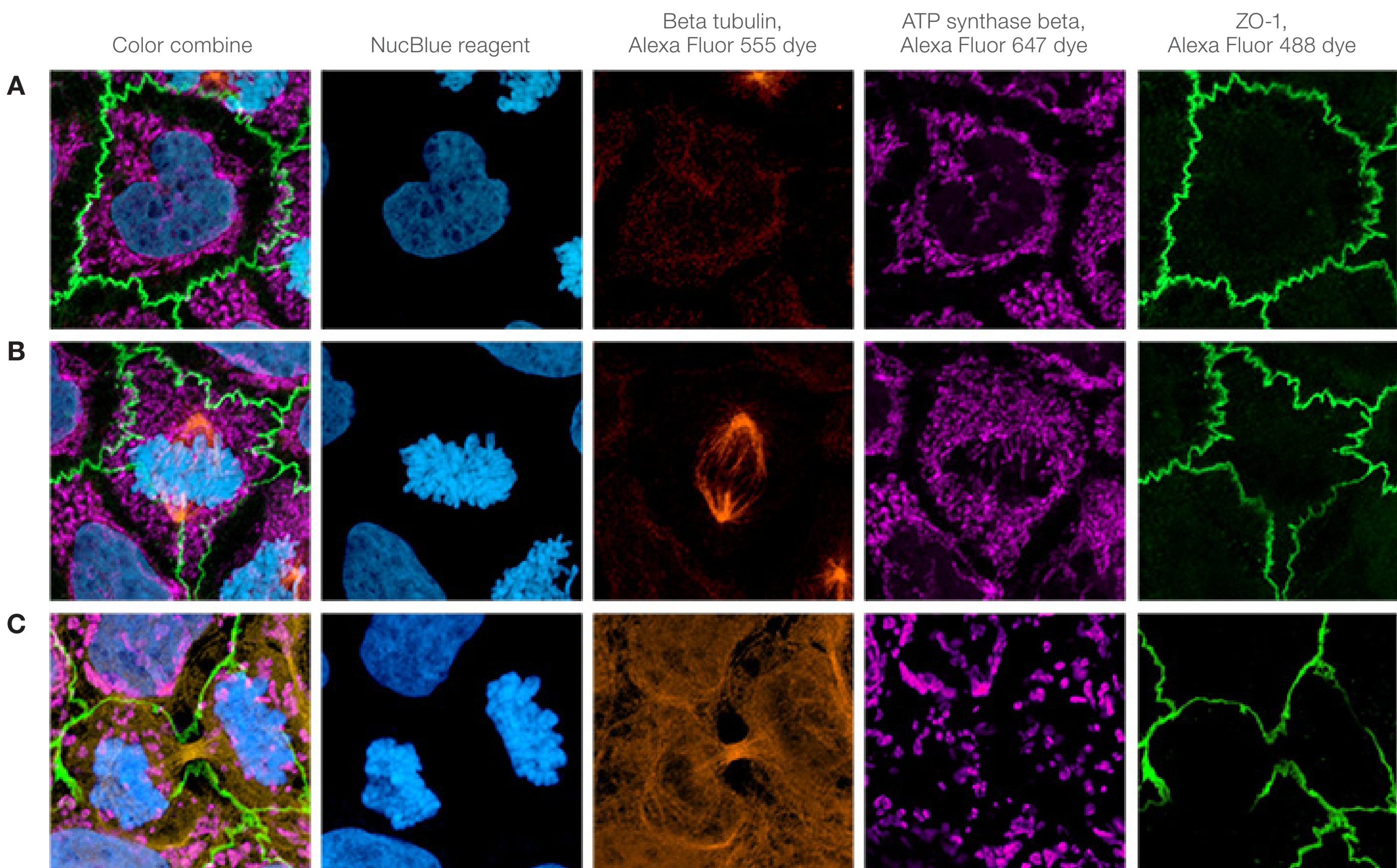

Figure 2. Mitochondrial morphology during mitosis. Caco-2 cells were labeled with ZO-1 antibody conjugated to Alexa Fluor 488 dye (green), beta tubulin antibody conjugated to Alexa Fluor 555 dye (orange), ATP synthase beta antibody conjugated to Alexa Fluor 647 dye (magenta), and NucBlue Fixed Cell ReadyProbes Reagent for nuclei (blue). The typical morphology associated with **(A)** interphase, **(B)** metaphase, and **(C)** telophase of mitosis is shown at 63x magnification. Image stacks were acquired on a ZEISS 710 Confocal Laser Scanning Microscope and displayed as a maximum intensity projection.

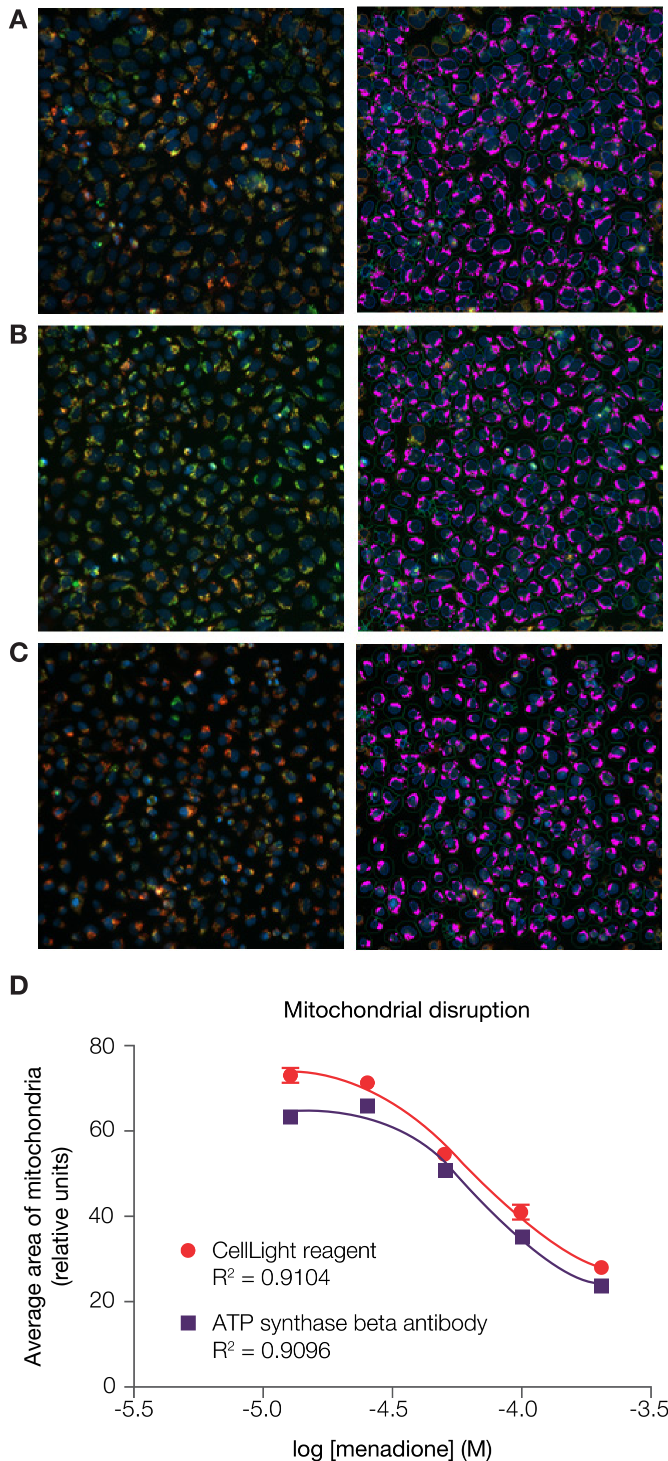

Figure 3. Disruption of mitochondrial morphology. U2OS cells were transduced with CellLight Mitochondria-RFP reagent, treated with menadione, and counterstained with ATP synthase beta antibody conjugated to Alexa Fluor 488 dye along with NucBlue Fixed Cell ReadyProbes Reagent for nuclei. The mean total area of the mitochondria was calculated on the CellInsight CX7 HCA Platform. **(A)** Untreated control sample, **(B)** low menadione concentration of 12.5 µg/mL, **(C)** high menadione concentration of 200 µg/mL, and **(D)** graph of results.

Detecting mitophagy

A distinctive feature of the early stages of apoptosis is the disruption of the mitochondria, including changes in membrane and redox potential [1]. Although the role of autophagy in oxidative stress–induced cell death is not fully understood, the segregation and delivery of cytoplasmic cargo for degradation plays a vital role in the cell survival response. The LC3B protein generally resides in the cytosol, but following cleavage and lipidation with phosphatidylethanolamine, LC3B associates with the autophagosome to sequester oxidized or dysfunctional intracellular components for downstream degradation. This autophagosome ultimately fuses with the lysosome to form a structure known as the autolysosome for final degradation of damaged cytosolic materials.

The sequestering and degradation of oxidized materials is evident in U2OS cells treated with chloroquine to artificially generate autophagosomes and menadione to induce oxidative stress (Figure 4). Autophagy is occurring where LC3B protein (green) localizes to form a membrane surrounding the colocalized mitochondrial constituents stained with CellLight Mitochondria-RFP reagent (orange) and ATP synthase beta antibody conjugate (magenta). In the control sample not treated with menadione, autophagic vesicles accumulate within the cell, but mitophagy is not occurring.

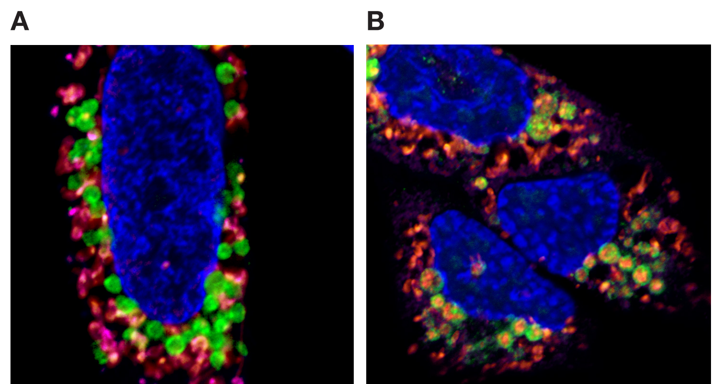

Figure 4. Detection of mitophagy. U2OS cells were transduced with CellLight Mitochondria-RFP reagent (orange) and incubated for 24 hours prior to treatment with 30 µM chloroquine for 16 hours to artificially accumulate autophagosomes. Cells were placed into **(A)** fresh medium or **(B)** fresh medium with menadione and incubated for 1 hour to induce oxidative stress. After fixation, permeabilization, and blocking, cells were labeled with ATP synthase beta antibody conjugated to Alexa Fluor 647 dye (magenta), LC3B primary antibody and goat anti-rabbit (H+L) secondary antibody conjugated to Alexa Fluor 488 dye (green), and NucBlue Fixed Cell ReadyProbes Reagent (blue).

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Conclusion

Mitochondria generate the bulk of a cell's energy and play a role in a variety of critical cell functions. The continued study of mitochondrial health and dysfunction is necessary for disease prevention. This study provided evidence that preconjugated antibodies like ATP synthase beta can be used in the detection and analysis of mitochondrial morphology, health, and function. The results correlate well with other mitochondrial detection tools like the CellLight Mitochondria-RFP reagent. The ability to generate statistically significant data regarding changes in mitochondrial conformation and function with a specific primary antibody should be extremely useful for future studies.

References

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