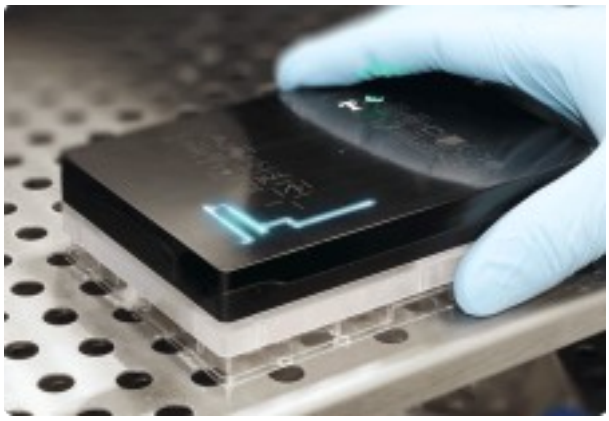
Lucid Scientific

Normalization Methods



Metabolism Matters

All cells require energy in the form of ATP to function normally. Healthy cellular mitochondria efficiently produce high levels of ATP via oxidative phosphorylation, utilizing carbon substrates as electron sources to fuel the electron transport chain, reducing oxygen to water in the process. As cells grow, develop, function and are challenged, their ATP needs change. Consequently, measures of cellular oxygen consumption rates (OCR) have provided key insights into the role of mitochondria in health and disease.

Until recently, there have been limited options for studying cellular respiration. Lucid Scientific's Resipher is a long-term, non-invasive, oxygen concentration monitoring system that enables measures of mitochondrial respiration over weeks of live cell culture in standard 96-well tissue culture plates. The Resipher system is the ONLY high throughput platform that measures OCR in living cells as they grow, divide, differentiate and are challenged, providing unique data related to metabolic behavior under ever changing conditions. This helps scientists pinpoint the factors that influence mitochondria and impact health and disease.

Researchers who study mitochondrial activity via OCR measurements realize that normalization is crucial, as there are many different factors that can affect OCR (Table 1). Normalization serves as a critical step in the analytical process, allowing for the elucidation of meaningful differences in cellular respiration arising from experimental conditions.

Normalization Methods

Lucid Scientific

Factors Affecting OCR	Assay to Determine Effect	Example Assays
Cell Proliferation	Live Cell Count Protein Quantification DNA Quantification	Hoechst, SYTO Dye Labeling BCA, Lowry, Bradford CyQuant, Qubit, QuantiFluor, Quant-iT
Cell Death	Dead Cell Count	Propidium Iodide
Cell Differentiation	Phenotype Assay	Immunohistochemistry FLOW Cytometry with multiple markers
Cell Activation	Phenotype Assay	FLOW Cytometry with multiple markers
Mitochondrial Mass	mitochondrial protein mitochondrial DNA	mitochondrial protein isolation and quantification (ex. BCA) mitotracker dye, or qtPCR
Mitochondrial Proteins	Phenotype Assay	Immunohistochemistry, Electron Microscopy
Mitochondrial Membrane Permeability	Mitochondrial Membrane Potential	TMRM, JC1
Substrate Utilization	C13 Carbon labeling of substrate	Mass Spectrometry Carbon Tracing

Table 1. Understanding factors that can influence OCR in Resipher experiments.

Overview OCR and Normalization

Normalization is a process of transforming data to remove extraneous variables and enable the detection of biologically meaningful differences across experimental conditions. In live cell culture systems, variables such as cell number, mitochondrial mass, media components, and differences in cellular activity can influence OCR (Table 1), creating challenges in data interpretation. Normalization is a key process that addresses these variables, allowing researchers to derive meaningful conclusions from data across different experimental conditions and sample types.

Normalization Methods

Lucid Scientific

There are two basic methods to normalize OCR data, internal and external. Internal normalization refers to utilizing the data itself to normalize, for example the fold-change in respiration between the first and last time points of an experiment. Internal normalization is often expressed as a percentage and results in unitless data. It is best used when no other method can be employed, or the cellular material is destroyed by the end of the experiment.

Internal normalization is fast, convenient and does not require additional assays. However, this type of normalization does not address the cause of differences. It is important to ensure that you have a baseline measurement of OCR for each experimental group. While directly imaging and counting cells is perhaps the best method of normalization, this method is very time consuming without expensive equipment to automate the process. An alternative method is to normalize respiration compared to the baseline rate (i.e. prior to any drug additions). Although this method does not allow one to compare basal respiration rates, it still enables comparisons of coupling and spare respiratory capacities.

External normalization is done by transforming the data by a known factor or standard to place all data on a common scale, for example dividing the OCR by the cell number in each well and reading data across wells and conditions as OCR/cell. External normalization possesses units, enables comparisons of assays completed on different days, and may address cause and effect. External normalization requires additional assays and expertise. It is a powerful process with many different options available.

A good external normalizing standard is a measurable factor within the experiment that is uninfluenced or only mildly influenced by the treatment conditions of the experiment. There are three strong external normalization methods for OCR data, 1) cell number, 2) protein quantification and 3) DNA quantification. Each has advantages and disadvantages. The best practice is to utilize the approach most familiar to the laboratory. After normalization, follow up or phenotype and mitochondria specific measures can be run to identify the potential cause for these differences. These assays include measuring mitochondrial mass, mitochondrial membrane potential, specific transcripts or protein levels, etc. (Table 1). Based on the primary mode of normalization, phenotype and mitochondria specific assays may or may not be performed on the same plates as the OCR data. Phenotype and mitochondria specific assays may need normalization as well. For example, mitochondrial mass per cell is a more powerful measurement than mitochondrial mass per plate.

Care should be taken when choosing a normalization method because many of the phenotype and mitochondria specific methods listed in Table 1 directly influence OCR. For instance, normalizing directly to mitochondrial membrane potential could erase the results. For this reason, normalization should first be done with one of the three techniques listed followed by another more specific assay.

Normalization Methods

Lucid Scientific

Resipher OCR Units

Resipher measures the rate of oxygen transported across a cross-sectional slice of the well plate where the probes operate. Units of OCR are reported per square millimeter of this cross-section. Assuming a consistent spatial distribution of oxygen across the culture area, a good assumption for a 96-well plate, the total oxygen consumption (transport) is found by multiplying the oxygen consumption rate per sq mm by the cross section of the well (31 mm^2 for a standard 96-well plate). Before normalizing with another external technique, it is important to first multiply by the cross-section to obtain total oxygen consumption. Once the conversion to total oxygen consumption is made, the result cannot be compared with another plate style (e.g. a 24 well) until another external normalization technique is employed.

Cell Number Normalization Methods

Cell numbers can be quantified numerous ways. The units of these cell counts are typically reported as total number of cells per well or number of cells per unit of area (i.e. the "density"). The units to report for normalized OCR per cell count are OCR per cell (fmol O_2/s/cell), or OCR per 1000 cells (fmol O_2/s/1000 cells). If the cell density is known, then the OCR per cell is simply the cross-sectional OCR units of the Resipher (fmol O_2/mm^2/s) divided by the number of cells per mm^2 unit area (cells/mm^2).

$$\text{OCR per cell (fmol } O_2\text{/cell/s)} = \frac{\text{Resipher OCR (fmol } O_2\text{/mm}^2\text{/s)}}{\text{Cell Density (cells/mm}^2\text{)}}$$

Note how this eliminates the unit-area component (mm^2) of the units that appears in both the numerator and denominator.

If only the total cell count per well is available, then this count must be first normalized to cells per unit area (cells/mm^2) by dividing by the surface area of the well (typically 31 mm^2 for standard 96 well plates). Dividing the OCR by this density is equivalent to multiplying the OCR by the well area and dividing by the cell count.

$$\text{OCR per cell (fmol } O_2\text{/cell/s)} = \frac{\text{Resipher OCR (fmol } O_2\text{/mm}^2\text{/s)} \times \text{Well Cross Sectional Area (mm}^2\text{/well)}}{\text{Cell Count (cells/well)}}$$

Normalization Methods

Lucid Scientific

Microscopy Techniques

Hemocytometer

Using an inverted microscope with phase contrast, cells can be trypsinized and counted by hand with a hemocytometer. This is the least expensive method of counting cells from tissue culture plates.


Easy, inexpensive, counts live cells


Error prone, time consuming, cells need to be trypsinized, cells are destroyed

Benchtop Cell Counter

Cells are trypsinized, deposited onto cell counter specific slides that are imaged and counted in a specialized light microscopic system (ex. ThermoFisher Countess 3 part# A49865).


Easy, inexpensive, counts live and dead cells separately


Error prone, time consuming for 96 well plates, cells need to be trypsinized, destructive

Note: When using trypsinization, it is important to remove all of the cells. If adherent molecules are used during culturing, this can be challenging. View the microplate prior to counting to ensure all material has been removed.

Light Microscopy Digital Imaging

Cells are imaged using a phase contrast microscope equipped with a digital camera. Image analysis software thresholds the images, outlining cells for easy hand or automated counting (ex. https://mediacy.com/app-center/cell-count-label-free/).


Easy, fast with automation, non-destructive (cells can be reused)


Error prone, costly for automation, best when cells are sub-confluent, does not distinguish between live and dead cells, may only count a portion of the well, may not work well with some cell types (ex. fibroblasts, suspension cells)

Normalization Methods

Lucid Scientific

Fluorescent Microscopy

Cells are labeled with a dye and imaged on a microscope equipped with an appropriate camera, filters for dye imaging, and image analysis software. This is a very flexible and accurate technique. The best dyes for cell counting are nuclear dyes because they are high contrast and easily distinguishable (Table 2).

Easy, fast with automation, accurate, some dyes are usable with live cells allowing continuation of growth or use of the culture in other applications, can distinguish between live and dead cells, some dyes are retained for long periods allowing for quantification of other variables such as proliferation, migration and long-term viability, can be combined with immunohistochemistry

Costly, may not count entire well, despite labeling live cells some dyes can be photo-toxic, less accurate when cells are confluent or super-confluent, difficult with suspension cells

Dye	Type	Specifications
Hoechst Dyes (e.g., Hoechst 33342)	Cell Permeable, Live Cell, Nuclear	UV (excitation 350/emission 461 nm)
4',6-diamidino-2-phenylindole (DAPI)	Cell Permeable, Fixed Cells, Nuclear	UV (excitation 358/emission 461 nm)
SYTO Dyes (e.g., SYTO 9 and SYTOX Green)	SYTO 9: Cell Permeable, Live Cell, Nuclear SYTOX Green: Cell Permeable, Dead Cell, Nuclear	SYTO 9 Red (excitation 485nm/emission 498nm), SYTOX Green (excitation 504/emission 523nm)
Calcein AM	Cell Permeable, Live Cell*, Cytoplasmic	Green (excitation 494/emission 517 nm)
CFSE (Carboxyfluorescein Diacetate Succinimidyl Ester)	Cell Permeable, Live Cell*, Cytoplasmic	Green (excitation 492nm/emission 517nm)
CellTracker Dyes (e.g., CellTracker Green, CellTracker Orange)	Cell Permeable, Live Cell*, Cytoplasmic**	CellTracker Green (excitation 492nm/emission 515nm)

*These dyes only label living cells. They are converted to fluorescent forms by esterases.
**Cell tracker dyes remain in the cytoplasm for long periods.

Table 2. Commonly used fluorescent dyes for cell counting.

Normalization Methods

Lucid Scientific

Microscopy offers convenient, flexible and accurate counts of cells growing in culture with the benefits of retaining the cells for future assays and study (Table 1). These techniques can be costly and may require specialized equipment and software. The imaging technique selected needs to be carefully considered to match the cells and aims of the study.

Flow Cytometry

Flow cytometry is a powerful analytical technique widely employed in biological and medical research to investigate cell populations by measuring various cell characteristics including number, size, morphology, cell cycle kinetics, and identities. It is a rapid, high throughput technique capitalizing on principles of fluid dynamics, optics, and image analysis. Cells suspended in fluid are pumped one cell at a time through a laser beam. Cells can be prelabeled with dyes or immunohistochemically. The technique can therefore simultaneously measure cell number, size, shape and label intensity among a large population of cells thereby creating population distributions, providing quantitative and qualitative information about individual cells within a heterogeneous population.



Fast and accurate, can evaluate other cell parameters in addition to counting for richer more informative data, cells can be sorted and reused



Costly equipment, requires expertise for accurate quantification without harming cells, cells may need to be trypsinized in order to quantify

Protein-Based Normalization

Normalization of OCR data can also be done using protein quantification. Although specific proteins may be up or down regulated, total protein per cell is relatively constant, making this a very good choice for normalization. The most common protein quantification assays are the Bradford assay, the BCA (Bicinchoninic Acid) assay, and the Lowry assay (Table 3). These assays measure protein content per well in µg resulting in OCR (fmol O_2/µg protein/s).



Inexpensive, quick, insensitive to cell size



May be inconsistent, prone to user error, may need to pool wells to obtain enough protein for limits of detection, destructive

Normalization Methods

The choice of protein quantification assay depends on factors such as the nature of the sample, the presence of interfering substances, and the limits of detection. Many cell lysis buffers and experimental conditions involve detergents that may interfere with protein quantification assays. Further, BSA and other sera used during culturing may interfere as well. For 96 well culture plates, wells may need to be pooled to obtain enough protein.

Good lab practices are essential for these assays. Cells should be rinsed well with sera free media prior to protein quantification, and protocols should be standardized for reproducibility. Variations in incubation times, temperatures, and pipetting can affect the accuracy of normalization.

Assay	Principle	Advantages	Disadvantages
Bradford	Coomassie Blue Dye shift in absorbance due to protein binding	Quick and simple, suitable for rapid analysis of protein concentration	Sensitivity may vary for different protein types. Interference from certain substances (e.g., detergents) can affect accuracy. Limited linear range for quantification
BCA	Reduction of Cu2+ ions by proteins to form a colored complex quantified spectrophotometrically	Highest sensitivity, broad dynamic range, compatible with detergents	Interference from reducing agents may impact accuracy. The assay requires heating. The color development time may vary.
Lowry	Reduction of Folin-Ciocalteu by proteins, leading to a colored complex measured spectrophotometrically	Broad dynamic range, ideal for cells with high protein composition variations, stable reagents for better consistency	Complex set up. Longer assay time compared to other protein assays. Sensitivity may vary for different protein types. Generally considered less sensitive than the Bradford assay. Interference from certain substances can affect accuracy.

Table 3. Comparison of protein quantification techniques.

Normalization Methods

DNA Content Normalization

DNA content is remarkably constant within a cell and is an excellent, convenient choice for normalization. The amount of DNA can be used directly (fmol O_2/µg DNA/s) or converted to cell number (fmol O_2/cell/s). DNA quantification kits offer a quick, simple and convenient way to quantify cells and normalize OCR data (Table 4).

DNA quantification kits are based on the principle of detecting and measuring the fluorescence intensity of DNA-binding dyes. These dyes selectively bind to double-stranded DNA (dsDNA), and the intensity of the fluorescence signal is measured in a plate reader or spectrofluorometer and converted to DNA content (pg to µg) or converted to cell number (cells in sample).

Simple, quick, consistent

May not be a direct measure, costly equipment, destructive

Kit	Manufacturer	Advantages	Disadvantages
CyQUANT Cell Proliferation Assay	ThermoFisher #C7026	Simple set up, excellent repeatability, widely used	Not as sensitive as other techniques
Qubit dsDNA Quantification Kit	ThermoFisher #Q32854	Can be multiplexed with RNA and protein, Sub mg sensitivity , excellent dynamic range	Costly, limited multiplexing, designed for use with Qubit Fluorometer
QuantiFluor® dsDNA System	Promega #E2670	Sub mg sensitivity and dynamic range	Time consuming setup, requires calibration, costly
Quant-iT PicoGreen dsDNA Assay Kit	ThermoFisher #P7589	pg sensitivity, broad dynamic range	Time consuming setup, requires calibration, costly

Table 4. Common kits used to measure DNA content and estimate cell number from microplate assays.

These DNA quantification kits are excellent choices for tissue culture microplates. However, some labs transfer the material to tubes for measurement in fluorometers or to black 96 well plates for measurement in plate readers to obtain better signal to noise than clear plates.

Normalization Methods

Drawing Conclusions

Note that cell loss and super-confluence due to long term growth can confound results. It is important to decide when to run the normalization assay.

Once the data are normalized to one of these standards (cell number, protein content or DNA content), significant differences observed in OCR across conditions are interpreted as meaningful biological changes influenced by the independent variable of the experiment. Further experimentation can help identify potential causes for the change in OCR among conditions and control (Table 1). The choice of next assay largely depends on observed normalized results and the nature of the independent variable. For example, if the independent variable is a knockout of a specific gene, there is no change in cell number between conditions, and OCR is observed to drop significantly after normalization to cell number, then a recommended follow would be to examine changes in mitochondrial mass between the control and knockout normalized to cell number (Table 1). Normalization of the follow up experiment should be consistent with the original normalization procedure, and if a non-destructive technique is used to normalize the OCR data, the same plate could be used in follow up assays.

Normalizing Resipher Data

Lucid Scientific's Resipher measures OCR over hours to weeks in standard 96-well tissue culture plates. The plates can be removed for observation at any time point and then replaced in the Resipher system. Some recommendations to normalize this data are:

- Light or fluorescent microscopic cell counting can be performed at predetermined time points.
- Prespecified wells can be set aside for normalization using protein or DNA quantification at predetermined intervals.
- Experiment is concluded during mid to late log-phase of cell growth and any of the methods described can be applied for normalization.

Resipher is a powerful instrument for measuring mitochondrial activity over weeks as cells undergo various changes associated with growth, differentiation, activation and death. It is essential to normalize this data for unambiguous interpretation.

Feel free to contact us if you would like assistance purchasing a system or guidance with designing an experiment at info@lucidsci.com or visit us at https://www.lucidsci.com/resipher.