### Title

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### Journal

Essays in Biochemistry, 68(3)

### ISSN

0071-1365



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### Publication Date

2024-11-18

### DOI

10.1042/ebc20240002

Peer reviewed

*Essays in Biochemistry* (2024) **68** 273–281
https://doi.org/10.1042/EBC20240002

PORTLAND PRESS

Review Article

# Understanding metabolic plasticity at single cell resolution

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It is increasingly clear that cellular metabolic function varies not just between cells of different tissues, but also within tissues and cell types. In this essay, we envision how differences in central carbon metabolism arise from multiple sources, including the cell cycle, circadian rhythms, intrinsic metabolic cycles, and others. We also discuss and compare methods that enable such variation to be detected, including single-cell metabolomics and RNA-sequencing. We pay particular attention to biosensors for AMPK and central carbon metabolites, which when used in combination with metabolic perturbations, provide clear evidence of cellular variance in metabolic function.

## Introduction

It has long been known that cells of the human body can vary dramatically in their metabolic function. Skeletal muscle cells and adipocytes, for example, face very different demands in their function and in their requirements for nutrients. While some of these differences arise developmentally, cells also navigate extreme metabolic changes as part of their physiological function, such as the acceleration of glycolysis in T cells upon their activation [1]. Modern data have not only underscored this concept but have also revealed that cellular variation in metabolism is even more pervasive. Heterogeneity in metabolic functions can readily be found among genetically identical cells of the same type, or even within the same cell at different points in its day-to-day life [2]. Such variation in metabolism impacts the functions of individual cells and their tissue as a whole. Consequently, an adequate description of cellular metabolism for any organ or physiological system needs to account for cellular metabolic plasticity.

Multiple approaches to quantify cell-to-cell variation in metabolism are now emerging, with some of them requiring fixation of cells or tissue [3] and others operating in live cells [4]. These approaches have led to a new appreciation of the dynamic variability of metabolic processes, both in cell culture and *in vivo*. Yet, a major challenge shared across these methods is the difficulty of inferring information about metabolic function from the measurement of a single metabolite or process. Given the many branches in metabolism, a change in a single indicator is often ambiguous in its interpretation. One solution to this challenge is to pay particular attention to the cellular energy sensors that evolution has produced, with the rationale that over millions of years they have evolved to provide metabolic information that is functionally relevant to the cell [5]. One of the best-known examples is AMPK, a key regulatory kinase that monitors multiple internal factors including energy charge (i.e., the ratio of ATP relative to ADP and AMP), glucose concentration, and calcium [6]. By integrating these inputs, it provides a useful single-point status indicator for metabolic information. Several fluorescent protein biosensors for intracellular AMPK activity are now available, making it possible to monitor this indicator in real time, for hundreds or thousands of cells, both *in vitro* and *in vivo* [2,7,8]. This technology has revealed a striking amount of variation across cells, subcellular locations, and time. The key challenge now is to place this observed variability into context and to understand its cellular and physiological significance.

As a sensor for adenylate energy charge, AMPK is closely connected to central carbon metabolism (CCM), the process by which cells use nutrients to phosphorylate ADP to generate ATP. The two major

Received: 26 July 2024
Revised: 03 October 2024
Accepted: 04 October 2024

Version of Record published:
18 November 2024

*Essays in Biochemistry* (2024) **68** 273–281
https://doi.org/10.1042/EBC20240002

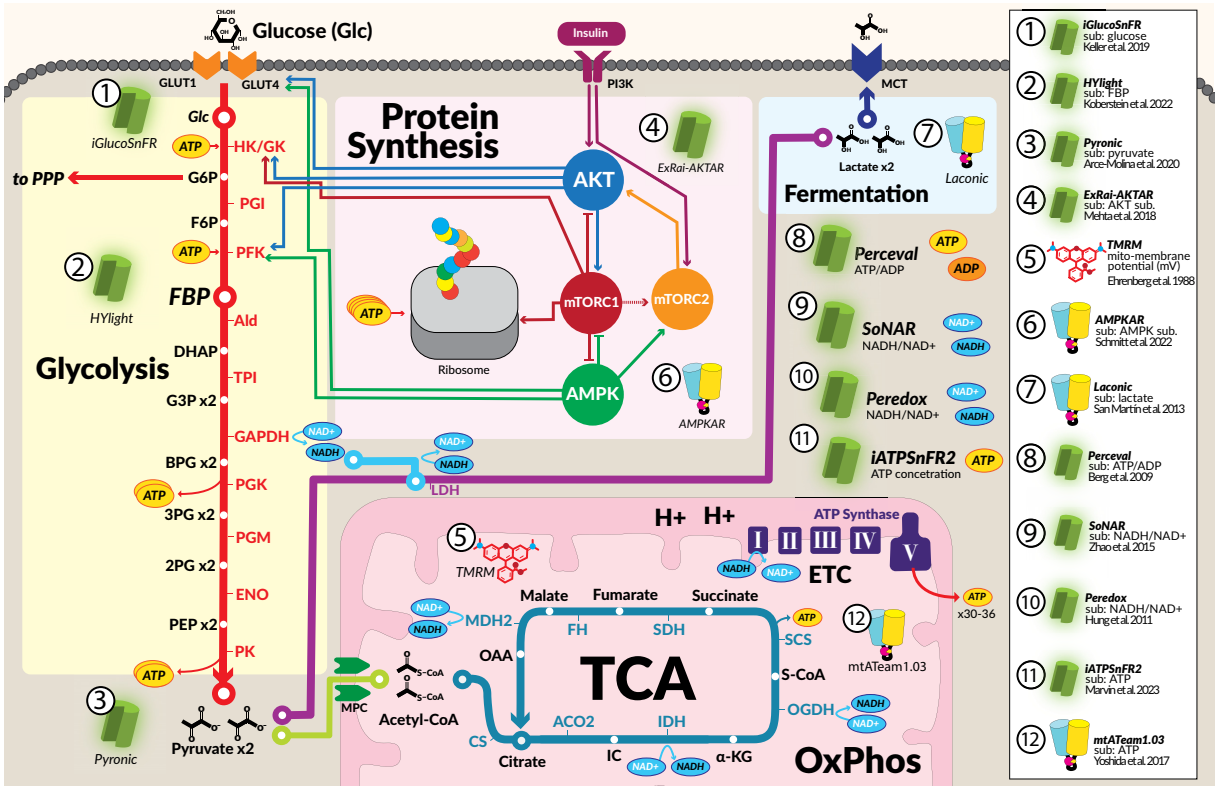


**Figure 1. Schematic diagram of CCM, associated regulators and live-cell reporters.**

Several of the major catabolic routes involved in CCM are depicted using a simplified formalism. Protein synthesis and its network of regulatory kinases are shown as an example of an ATP-consuming anabolic process that is closely interconnected with CCM. Shown throughout the diagram are examples of biosensors or other reagents that have been used to track CCM function within living cells; green cylinders represent biosensors based on cpGFP, and blue/yellow cylinders represent biosensors based on FRET interactions between cyan and yellow fluorescent proteins. Relevant references for each sensor are listed in the legend at right. The set of biosensors shown is not exhaustive, and there are many additional tools we were unable to include due to space constraints.

branches of CCM, glycolysis and the tricarboxylic acid cycle/oxidative phosphorylation (TCA/OxPhos) pathway, generate ATP as well as other essential intermediates (Figure 1). While OxPhos is more efficient at producing ATP, glycolysis can predominate in situations where cells are growing rapidly or have limited oxygen supply. Prominently, cancer cells often have a higher glycolytic rate and increased lactate secretion (also known as fermentation, Figure 1) than non-tumor cells, a shift known as the Warburg effect [9,10]. Importantly however, the balance of glycolysis and OxPhos in a cell is not a simple "either-or"; OxPhos remains essential in tumor cells and activated T cells alike [11]. A complete picture of CCM also requires accounting for ATP turnover - that is, how rapidly ATP is hydrolyzed to ADP by cellular processes. The most energy-intensive cellular processes include ion transport to maintain cellular potentials, protein synthesis by ribosomes, and nucleotide synthesis to support transcription and replication. Of these, protein and nucleotide synthesis are the most sensitive to changes in bioenergetic status [12]. As all of these processes impact the energy charge of the cell, AMPK activity is best viewed as a balance of all the incoming and outgoing rates affecting ATP. Therefore, the integrative nature of AMPK comes with a challenge: identifying which of these interlinked processes are responsible for a given change observed in AMPK activity.

Here, we discuss the potential sources of cell-to-cell variation in CCM. We then consider the techniques available for tracking such variation at the single-cell level, including AMPK biosensors, other metabolite biosensors, RNA-sequencing based methods, and mass spectrometry at single-cell resolution. We end by discussing the prospects for developing a single-cell CCM atlas across tissues.

# Sources of metabolic variation

To understand cell-to-cell metabolic variation, it is first necessary to consider the potential sources and mechanisms that underlie metabolic plasticity. We describe here factors that are in principle capable of causing cell-to-cell differences in CCM within the same cell type of a given individual.

## Cellular differentiation status

The body is comprised of hundreds of cell types that carry out a wide array of functions. Typically, differentiation entails the reorganization of gene expression programs through both transcription factor binding and epigenetic changes to the chromatin. These changes enhance the production of mRNA from genes needed for a cell's particular function and suppress the expression of genes that could interfere with that function. Metabolic enzymes are a key part of these programs. For example, the liver-specific transcription factor HNF4 is responsible in part for liver differentiation and binds to the promoter for PKL (liver-specific pyruvate kinase) [13]. The expression of PKL, which has different kinetic properties from other forms of pyruvate kinase, helps support gluconeogenesis, an important function of the liver.

However, it should be stressed that changes in differentiation status can occur continually even within a mature tissue and may be incomplete within certain cells - for example, tissue-resident stem cells undergoing asymmetric division - meaning that cells within a tissue are not permanently locked into a particular gene expression profile or metabolic behavior [14]. Moreover, metabolism can participate actively in the differentiation process. For example, during the formation of the neural crest, cells undergo a switch to glycolysis that enhances the rate of histone lactylation, which in turn triggers genes involved in the differentiation process [15].

## Cell cycle

CCM function has been shown to vary over the course of the cell cycle, and there are various points at which metabolic enzymes and cell cycle regulators communicate. For example, the glycolytic regulator enzyme PFKFB3 can be degraded by the APC-C/Cdh1 ubiquitin ligase complex that is active from the end of mitosis through late G1 phase [16]. Other studies have highlighted additional points of co-regulation [17], which are reviewed in detail here [18,19]. In most adult tissues, cells progress through the cycle asynchronously, and therefore at any point in time, cell cycle-dependent variation can be expected to contribute to a metabolically heterogeneous population.

## Circadian rhythm

Over a 24-hour period, many metabolic functions vary in their activity, regulated by hormonal processes or the oscillations in expression of the circadian clock genes. The regulatory proteins of the circadian clock are interlinked with transcription regulators of metabolic enzymes [20]. For example, expression of PFKFB3 is regulated by the circadian protein CLOCK, resulting in cyclic changes in glycolytic activity [21]. These changes may allow cells to anticipate the metabolic needs they will encounter over the course of the day. However, while these changes are in theory synchronous among all cells, in reality the variation inherent in gene expression kinetics implies that some cells will lag behind others, creating cell-to-cell variation at any given time [22].

## Autonomous metabolic cycles

There is accumulating evidence that metabolic functions undergo cyclic changes independent of both the circadian rhythm and the cell cycle [23]. Evidence for such cycles is strongest in budding yeast, where cycles on the scale of minutes [24,25] to several hours [26–29] have been well studied. In mammalian cells, glycolytic oscillations with periods ranging from 1 to 20 minutes have been observed in pancreatic beta cells, where they control cyclic secretion of insulin, and in skeletal muscle cells [25]. Cycles lasting several hours have rarely been observed in mammalian cells; however when OxPhos is inhibited in epithelial cell lines, AMPK activity undergoes a highly regular oscillation in activity with a period of about 3 hours, which is matched by rises and falls in glycolytic intermediates [30]. The exact mechanism of this cycle is not clear, but the 3-hour period clearly distinguishes this cycle from either the cell cycle or the circadian rhythm, both of which have periods of 20 hours or longer. Notably, all of these cycles are most strongly observed under specialized conditions, such as high glucose concentrations [25] or metabolic inhibition [31]; however, biosensor measurements are now revealing more subtle fluctuations with quasi-periodic behavior occurring on similar time scales, under more physiologically realistic concentrations of glucose [31].

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https://doi.org/10.1042/EBC20240002

### Microenvironment

The local surroundings of a cell provide a milieu of fuel sources and regulatory signals. For example, cells proximal to the bloodstream are exposed to the nutrients being carried by the blood and circulating hormones such as insulin, while also receiving an abundant supply of oxygen. Cells further distal are more buffered from feeding-induced changes in blood chemistry and have a lower availability of oxygen. Thus, even two initially identical cells placed into different regions of a tissue could have differences in their metabolic fluxes depending on their microenvironments. Presumably, over time these differences in fluxes could result in gene expression changes that enable the two cells to adapt to their different environments. Metabolic outputs, such as secretion of lactate due to increased glycolysis, can also contribute to a continual interplay between microenvironment and cell state. Tumors represent an extreme version of this model; within the disorganized setting of a tumor there are many microenvironments, which can be quite different from the native habitat of the original tumor cell. These microenvironments force tumor cells to adapt with differential metabolic behavior. A key question is which mechanisms allow tumor cells to increase their capacity for metabolic plasticity. For example, expression of the transcription factor MYC is frequently increased and regulates the expression of many metabolic enzymes [32], suggesting a potential mechanism for this adaptability.

### Mutations

Genetic changes that alter enzyme function can drastically change how metabolism works by allowing new metabolites to be produced. For example, the TCA enzyme isocitrate dehydrogenase (IDH), which normally converts isocitrate to alpha-ketoglutarate, is often mutated in cancers to create a neomorphic activity that produces D-2-hydroxygluatrate (D2HG) [33]. D2HG can interfere with chromatin-modifying enzymes, for which a-ketoglutarate normally serves as a co-factor [34]. Therefore, especially in tumor contexts, CCM function can vary from cell to cell based on the specific mutations carried by individual cells.

### Noise in gene expression

While not often considered in traditional studies of metabolic regulation, the expression of any given gene is variable among individual cells. Studies of such variation have found that the protein copy numbers of genes vary with a lognormal (heavy tailed) distribution with coefficients of variation between 15% and 30% [35]. Practically, this means that for any given gene there is more than a 2-fold difference in expression level between the $10^{th}$ and $90^{th}$ percentiles of a cell population [36]. Such variation, considered across all the genes of a given metabolic pathway, could lead to substantial differences in flux. Predicting how much actual impact this variation would have is challenging, as enzymatic reactions vary in the degree that they are controlled by the concentration of enzyme vs. substrate [37]. Nonetheless, this potent source of variation, which is known to affect processes such as cell death [38], needs to be considered.

Altogether, there is ample opportunity for cells within the same tissue to vary in metabolic function. The sources of variation considered here are not mutually exclusive, so they can act additively to create dispersion around the central tendency for any one cell type. However, in most cases, the data collected have been restricted to a small number of measurements, and there is no "atlas" of single-cell metabolic function yet. Achieving such a broad view will require accurate and accessible single-cell measurements of metabolism, which we catalog in the next section.

## Detecting and evaluating single-cell variation in metabolism

### Single-cell RNA sequencing

The ability to quantify the copy number of the vast majority of mRNAs within a single cell has opened up many new possibilities in understanding cellular states. The major utility of this method is the identification of differentiation states of individual cells within a tissue, which has revealed a number of previously unknown cell types [39]. Aspects of a cell's microenvironment and signaling activity can also be inferred, by examining gene expression patterns for enrichment of regulons downstream of signaling pathways [40]. It also stands to reason that information about the metabolic state can be inferred as well, and several studies have explored this possibility [3,41,42]. However, there are inherent limitations to this approach. Most importantly, metabolic flux is regulated heavily both by substrate concentration and by posttranslational modification of enzymes, both of which are not detectable in RNA-seq data. Furthermore, not all changes in enzyme expression affect rate limiting steps of metabolic pathways. Finally, the often limited correlation of mRNA and protein [43] creates difficulties for the assumption that the activity of an enzyme can be inferred from its mRNA copy number. Thus, while single-cell gene expression profiles will continue to provide important hints of differential metabolic regulation, such observations must be interpreted with caution and should ideally be followed up with further analysis by other methods.

## Single-cell and imaging mass spectrometry

Mass spectrometry has long been the gold standard for analysis of metabolites, as it can detect thousands of metabolite species. Advances in sensitivity have made it possible to perform analysis on single cells. However, given the very small amount of material available from a single cell, there are still significant limitations on which metabolites can be detected accurately. While there are more than 200,000 metabolites in the current human metabolome database [44], single-cell mass spectrometry studies have detected up to several hundred metabolites [45], which are typically predominated by lipid species [46]. Mass spectrometry imaging has also advanced, making it possible to analyze metabolite composition of cells within tissue sections [47]. However, resolution for such imaging is still limited, such that individual cells are not fully resolvable. Another challenge for single-cell mass spectrometry is the difficulty of implementing flux tracing of metabolites, a key method for assessing CCM function [48].

## Biosensors for AMPK and other metabolic regulators

Fluorescent protein biosensors have the distinct advantage of providing continuous monitoring of biochemical events in single cells over time. Numerous sensors have now been developed that allow detection of the various kinase activities, based on several different design formats [49]. These sensors detect the kinase activity of AMPK and other key metabolic regulators including AKT, mTORC1, or PKA. Of these, AMPK biosensors have thus far revealed the most information about metabolic plasticity in individual cells. Notably, AMPK activity has been observed to fluctuate under various forms of metabolic stresses, including low glucose or low growth factors, and in the presence of inhibitors of glycolysis or OxPhos [31]. Cell-to-cell variation in cellular metabolism was also observed *in vivo* using intravital imaging of an AMPK biosensor [2]. Multiplexed analysis of AMPK, mTOR, and AKT biosensors within the same cells demonstrated concerted fluctuations in all of these pathways, revealing the dynamics by which these regulators help to maintain cellular homeostasis [50]. Further extending these analyses, a new AMPK biosensor design has enabled high dynamic range measurements of AMPK activity in subcellular locations [7]. This biosensor showed more rapid activation when targeted to the lysosome, where both AMPK and mTOR are frequently localized, relative to the cytoplasm or mitochondria. Thus, signaling biosensor analyses reveal both spatial and temporal detail in CCM regulation, along with substantial differences between cells.

## Metabolite biosensors

The engineering of fluorescent proteins that bind to key metabolites has achieved a number of elegant successes [51]. Many of these efforts have centered on linking a metabolite-binding protein domain to a circularly-permuted green fluorescent protein (cpGFP), such that the conformational change occurring upon metabolite binding is coupled to the physical environment (and thus the fluorescence properties) of the fluorophore (see Figure 1 for a partial list of such sensors). This approach has yielded biosensors for ATP, ATP/ADP, NADH/NAD+, glucose, fructose 1,6-bisphosphate (FBP), lactate, and others [52–59]. These sensors have proved useful in many systems, for example, demonstrating variation in glycolytic activity between cells within a tumor model *in vivo* [59]. Certain chemical dyes also provide useful information, such as tetra-methyl rhodamine methyl-ester (TMRM), whose fluorescence varies with mitochondrial membrane potential. TMRM has been widely used to track mitochondrial bioenergetics, enabling heterogeneity in CCM flux to be linked to cell death execution, for example [60]. However, a drawback to all of these tools is that many biosensors have overlapping excitation and emission spectra, complicating their multiplexed usage. Furthermore, imaging the abundance of a single metabolite is often of limited utility. Interpretation of a single readout is difficult without additional context: does an observed increase in ATP reflect an increased rate of ATP production, a decrease in ATP consumption, or both? Finally, some metabolites are highly buffered; for example, intracellular ATP concentration is very stable and will often not reflect even substantial perturbations to metabolism [61,62]. Thus, gaining insight from biosensors demands clever experimental design, rather than simple monitoring of biosensor or fluorescent dye signals alone; we highlight several such strategies below.

## Biosensors with perturbations

Because they can be sampled repeatedly in the same living cell over time, biosensors make possible a class of experiment that pointedly reveals functional information, which cannot be done with destructive methods. In such experiments, biosensor readouts are used to record each cell's reaction to a directed perturbation, such as pharmacological inhibition of a particular pathway or addition/withdrawal of a carbon source. In one example of this, an AMPK reporter was monitored as cells were treated with oligomycin, an OxPhos inhibitor [30]. The rationale for this approach is that cells that rely heavily on OxPhos for ATP production will suffer an immediate decrease in energy charge, resulting in AMPK activation. In contrast, cells capable of meeting their ATP needs through glycolysis alone

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will not experience a large change in energy charge or activation of AMPK. This concept was extensively validated in multiple cell types, suggesting that it may serve as a broadly useful tool for interrogating CCM [30]. In another example, a lactate biosensor was used to detect the rate of increase in intracellular lactate following pharmacological inhibition of lactate transporters [63,64]. The rate of this rise varied between cells, indicating different rates of glycolysis. Still another study used an ATP biosensor targeted to the mitochondrial matrix [65]. Upon glucose withdrawal, the mitochondrial ATP signal rose briefly and then fell precipitously. The kinetics of this change varied between cells and were linked to different capacities for OxPhos. Therefore, as a general strategy, observing acute biosensor signal responses following a perturbation is one of the most powerful ways to reveal cellular metabolic variation.

### Protein synthesis changes in response to metabolic perturbation

An orthogonal approach to interrogating metabolism is to make use of the connection of metabolism to protein synthesis. Because protein synthesis is an energy-intensive process, its overall rate is thought to be closely connected to ATP production processes. Total cellular protein synthesis can be assayed at the single cell level, using puromycin (or related analogs), which is incorporated into actively translating peptides, and which can be fluorescently stained and detected by imaging or flow cytometry following cell fixation. Argüello et al demonstrated an approach termed SCENITH (Single Cell ENergetIc metabolism by profiling Translation inHibition), in which labeling with puromycin in the presence of either 2-deoxyglucose or oligomycin, or both, can be used to distinguish cells' reliance on glycolysis, respiration, or other pathways to support protein synthesis [66]. Because this labeling can be performed in living tissues prior to their fixation, these states can be quantified under physiological conditions. However, such interpretations must be approached with caution as they assume a tight correlation of CCM processes with protein synthesis. Such a correlation has been shown at the bulk level [12], but other studies have indicated that protein synthesis and respiration are unlinked under some conditions [67]. Moreover, there is still little evidence available to evaluate the strength of this correlation at the single-cell level.

## The future of single-cell metabolic analysis

A key major need is now a reference dataset that compares the available single-cell technologies for metabolism. Ideally, large samples of multiple cell types, representing each of the variation sources described above, would be interrogated with multiple modalities of single-cell metabolic measurements. The resulting dataset would reveal not only how states observed in one measurement modality correspond to states in the other, but also whether the assays vary in their applicability to different contexts. In particular, because biosensors often provide the most functionally interpretable data, we would argue that there is a key need to compare biosensor readouts to scRNA-seq and puromycin-based indications. Such a dataset is quite feasible, as scRNA-seq analysis following live-cell imaging has been demonstrated [68]; puromycin labeling in tandem with live imaging is also practicable. Performing similar experiments in physiological settings will be more challenging, but is likely achievable based on elegant *in vivo* work thus far [2,59]. It should be possible to combine such imaging with post-fixation analysis by spatial transcriptomics or mass spectrometry imaging. Together, we expect these methods to provide a high level of single-cell resolution in metabolic function, revealing subsets of cells that contribute in functionally different ways to the overall function of tissues [7,52–55,57,58,69–73].

## Summary

- Multiple technologies for single-cell metabolism measurement are emerging.
- Biosensor experiments reveal cell-to-cell variation in central carbon metabolism.
- Cell cycle, circadian rhythm, and gene expression fluctuations contribute to cellular variation in metabolism.
- Autonomous metabolic cycles drive cellular metabolic variation but are not well understood.
- There is a key need for cross-platform comparisons in single-cell metabolic methods.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

## Funding

Funding for this work was provided by the National Institute of General Medical Sciences (R35GM139621 and T32GM007377) and the National Heart, Lung, and Blood Institute (R01HL151983).

## Open Access

Open access for this article was enabled through a transformative open access agreement between Portland Press and the University of California.

## Abbreviations

AMPK, 5' adenosine monophosphate-activated protein kinase; CCM, central carbon metabolism; cpGFP, circularly-permuted green fluorescent protein; IDH, isocitrate dehydrogenase; OxPhos, oxidative phosphorylation; SCENITH, single cell energetic metabolism by profiling translation inhibition; TCA, tricarboxylic acid cycle; TMRM, tetra-methyl rhodamine methyl-ester.

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