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Towards High Resolution Analysis of Metabolic Flux in Cells and Tissues

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Abstract

Metabolism extracts chemical energy from nutrients, uses this energy to form building blocks for biosynthesis, and interconverts between various small molecules that coordinate the activities of cellular pathways. The metabolic state of a cell is increasingly recognized to determine the phenotype of not only metabolically active cell types such as liver, muscle, and adipose, but also other specialized cell types such as neurons and immune cells. This review focuses on methods to quantify intracellular reaction flux as a measure of cellular metabolic activity, with emphasis on studies involving cells of mammalian tissue. Two key areas are highlighted for future development, single cell metabolomics and noninvasive imaging, which could enable spatiotemporally resolved analysis and thereby overcome issues of heterogeneity, a distinctive feature of tissue metabolism.

Introduction

Metabolism refers to biochemical processes that extract chemical energy from nutrients (or solar energy in the case of phototrophic organisms), and use this energy to convert nutrients into building blocks for biosynthesis. The intermediates of these processes, i.e. metabolites, also act as regulatory molecules that coordinate the activities of metabolic pathways and other cellular pathways such as signaling. Even for cells and tissues whose primary physiological role is not metabolic *per se*, e.g. immune cells [1], there is increasing evidence that their metabolic state is a key factor in determining function.

For metabolically active cells in liver, muscle, and adipose, metabolic indicators are integral to tissue function assessment. More broadly, analysis of cellular metabolism can reveal opportunities to tailor the culture environment to fit the tissue engineering objectives, for example by identifying metabolic pathways whose activities correlate with improved function [2]. Rational redesign of cellular metabolism, termed metabolic engineering, has been successfully used in the microbial cell engineering community to upgrade the catalytic properties [3,4] or biosynthetic capabilities [5-7] of various unicellular organisms. In comparison, cells relevant to tissue engineering and regenerative medicine applications have been examined to only a limited extent from a metabolic engineering perspective.

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Fortunately, many of the methods developed to analyze microbial metabolism can be readily adopted to study tissue metabolism, as the main pathways for extracting energy and generating building block biomolecules are highly conserved across organisms [8]. One qualification is that cells of differentiated tissue require rich media with more than one carbon source. This adds complexity [9], in terms of both the number of metabolites that need to be monitored and the mathematical models that need to be formulated/solved to interpret the measurements. Another important qualification is that metabolic studies on microbes have typically assumed that the system of interest is homogeneous, or that characterization of bulk behavior is satisfactory. In the case of tissue systems, which often comprise a heterogeneous population of cells, it can be desirable to obtain spatially and/or temporally resolved information. Spatial heterogeneity in metabolic state could reflect the presence of nutrient or signaling gradients, which are nearly always present in an avascular system due to mass transfer limitations. Spatial heterogeneity is sometimes an inherent feature representing physiological or pathological variations such as tissue zonation [10,11] or tumor microenvironments [12,13]. Temporal heterogeneity can arise as cells respond to hormones and other stimuli to engage and disengage different metabolic pathways. Terminal changes in metabolic phenotypes can occur over longer time periods, as illustrated by stem cells exhibiting dramatic changes in their redox levels as they transition from a proliferative state to terminally differentiated state [14]. Ideally, these types of heterogeneities are studied using spatiotemporally resolved, noninvasive methods that enable repeated observations over time.

In this review, we first provide an overview of the most commonly used methods for quantitative characterization of cellular metabolism, highlighting representative applications in systems that are relevant to tissue engineering and regenerative medicine. We then discuss emerging methods that could enable highly resolved analysis of cellular metabolism, and thus address the issues of heterogeneity outlined above.

Methods for Quantitative Analysis of Metabolic Flux

Metabolic flux, typically expressed as a rate per unit amount of cell or tissue, measures the degree of engagement of various metabolic pathways in the intact cell. Compared to gene expression level or enzyme amount, metabolic flux provides a more immediate description of cellular activity. In the absence of detailed knowledge of enzyme kinetics, which is rarely available, steady-state fluxes provide useful quantitative snapshots of cellular metabolism. In the context of engineering microbial cells, metabolic flux analysis is already an established methodology, and has been discussed extensively in a number of excellent reviews [15,16]. Broadly, there are three groups of methods: metabolic flux analysis, flux balance analysis (FBA), and isotopomer analysis. Metabolic flux analysis (MFA) refers to the quantification of intracellular reaction rates from measurements on the rates of uptake or output of major nutrients and metabolic products in conjunction with a stoichiometric model of the major metabolic pathways. Given sufficient measurements, the intracellular reactions rates can be calculated from stoichiometric mass balances. The major limitations are that the measurement requirements can be high if broad coverage of metabolism is desired and that resolution is limited.

Flux balance analysis [17] incorporates the stoichiometric mass balances as constraints into a linear programming framework to predict intracellular reaction rates when measurements are insufficient to completely determine the system. This method is especially useful for large-scale metabolic models, which cannot be fully constrained using measurements. The main limitation is that FBA depends on identifying one or more recognizable metabolic objectives for the system of interest, which is not always possible for mammalian cells. In the context of tissue engineering, FBA can be viewed as a valuable analysis and design tool

to explore possible (e.g. genetic) modifications that could redirect metabolic resources for a desired engineering objective. An interesting variant is flux variability analysis (FVA), which estimates the possible ranges of reaction rates by iteratively maximizing and minimizing the flux through each reaction in the metabolic model, thereby exploring alternate metabolic states for a given set of uptake and output measurements [18,19]. A useful outcome of FVA is to discriminate between well and poorly constrained parts of the metabolic network, which can then be used to focus additional experiments to improve the resolution of specific pathways.

Isotopic labeling is the current gold standard for experimental resolution of metabolic fluxes. Isotopomer (isotopic isomer) analysis exploits asymmetries in atom transfers of enzymatic reactions to relate reaction fluxes in a cell to the distribution of label from an input substrate (typically a ^{13}C labeled sugar, amino acid, or fatty acid) to various metabolic pathway intermediates. The relationship between reaction fluxes and label distribution is defined by a mathematical model, which is usually solved computationally to obtain the flux estimates. A clear advantage of isotopomer analysis is that details such as exchange fluxes of reversible reactions can be quantified with good confidence. The drawbacks are that the computational, experimental, and analytical efforts can be substantial.

Metabolic Flux Analysis of Mammalian Cells and Tissues

There have been numerous metabolic studies on the liver, which performs catabolic and anabolic functions essential for whole body homeostasis, including fasting glucose production, ammonia clearance, and xenobiotic transformation. While the hepatocyte is the dominant parenchymal cell type, other cell types also contribute to the organ's metabolism. Therefore, MFA studies aimed at characterizing alterations in liver metabolism under different physiological [20] or pathological [21] conditions have often utilized *ex vivo* organ perfusion to isolate systemic influences while preserving the *in vivo* cell composition. Hepatocyte specific metabolism has been studied using cultures of established cell lines as well as primary cells from rodents, pigs and humans. One of the most widely utilized cell lines is HepG2, which has been used to study the metabolic effects of various stresses such as fatty acid toxicity [22] and drug challenge [23]. The latter study is particularly interesting, as MFA revealed alterations in central metabolism, specifically TCA cycle flux, even for sub-toxic doses of drugs, suggesting that an examination of metabolic fluxes could provide insights into more subtle impacts of a drug before onset of outright toxicity. Isotopic labeling experiments have long been used to measure metabolic flux in the liver, although historically this approach has focused on a small subset of reactions in central metabolism [24,25]. In recent years, technological advances in high-resolution tandem mass spectrometry (MS), paired with new methods for extracting positional labeling information [26] as well as developments in modeling isotopic label distribution in metabolic networks [27] have steadily improved both resolution and breadth to enable increasingly sophisticated and comprehensive flux quantification experiments. For example, utilizing transient label enrichment data can facilitate the analysis of pathways whose intermediates do not rapidly reach isotopic steady state [28]. This approach was used to examine the effect of a statin drug on both cholesterol synthesis as well as central carbon metabolism in primary rat hepatocytes, confirming that the drug exerts only minor effects outside of its target pathway [29].

Similar to the liver, metabolic studies of muscle cells, notably cardiomyocytes, have centered on energy metabolism in the context of overall tissue function [30] and metabolic consequences of drug challenge [31,32]. In a recent study, Strigun et al. used ^{13}C labeling experiments using a murine atrial cell line (HL-1) to examine the effects of verapamil, a calcium channel blocker used to treat hypertension, and found that the drug reduces flux

through glycolysis while minimally affecting the TCA cycle, suggesting a possible explanation for the drug's potential anti-cancer activity [32].

For adipocytes, isotopic labeling experiments have been used to specifically interrogate lipid synthesis [33] and, in conjunction with MFA, to broadly profile the changes in central metabolism as the cells mature phenotypically through differentiation [34]. More recently, we have utilized MFA as a screening tool to identify and characterize enzyme targets for reducing lipid accumulation in adipocytes in the context of obesity [35].

For neuronal cell types, a major focus has been on glutamate metabolism, due to the metabolite's central role as a neurotransmitter. *In vivo*, glucose is first converted to glutamine in astrocytes, and then taken up and hydrolyzed to glutamate in neurons [36]. This compartmentalization presents a challenge for conventional MFA [37], which assumes that metabolite pools are homogeneous. Thus, isotopic labeling has been the method of choice. For *in vivo* studies, NMR spectroscopy has been particularly useful as a noninvasive analytical tool. In an earlier study, Shen et al. used this approach to estimate that glutamine/glutamate cycling rate is ~80% of glucose oxidation in resting human brains, confirming the quantitative importance of this metabolic cycle [38]. *In vitro* studies where destructive sampling is an option have typically used MS to take advantage of increased sensitivity. Recently, Amaral et al. analyzed the dynamics of ^{13}C label enrichment in cell extracts collected over time to determine the quantitative significance of branched chain amino acid metabolism in cultured primary rat cortical astrocytes [39].

In comparison to liver, muscle, and neuronal cells, relatively little is known regarding immune cell metabolism. The field of immunometabolism is relatively new, having emerged only a few years ago. However, the field has already proposed compelling hypotheses [40] linking cellular energy metabolism to the regulation of immune cell phenotypes. Here, we focus on macrophages, although metabolism appears to play an important role in the functions of dendritic cells [41] and T-cells [42]. For macrophages, it has been suggested that the metabolic state of the cell influences its activation state [43]. A recent isotopic labeling study in cultured primary murine macrophages showed that activated macrophages exhibit a tumor cell-like tendency favoring glycolysis over oxidative metabolism [44]. Another intriguing observation was that classical (M1) activation led to a significant shift to glucose catabolism via glycolysis, whereas alternative activation had minimal impact. Such a metabolic "switch" has been also identified in the pentose phosphate pathway (PPP), where the switch can direct the cell towards M1 polarization by rebalancing the carbon fluxes through glycolysis and PPP [45].

Genome-scale Metabolic Analysis

Since the publication of the first genome-scale model of *Escherichia coli* MG1655 more than a decade ago [46], a number of such models have been assembled for many industrially significant unicellular organisms, and used to characterize, design or optimize cellular metabolism, very often in conjunction with FBA or related constraint-based methods. In recent years, several models reflecting a global reconstruction of enzyme-catalyzed pathways have also appeared for mammalian species, including humans [47]. A major challenge in the construction of tissue-specific models has been the need to manually curate the reconstruction based on literature reports due to the complexities arising from tissue-specific distribution of enzymes [48]. A notable example of a large-scale tissue-specific model is HepatoNet1, the first comprehensive reconstruction of human hepatocyte metabolism capable of simulating a large number of the liver's canonical metabolic functions [48]. As a demonstration of potential utility, this model was used to identify metabolic enzymes that are essential to a pathogen, but nonessential to the liver, and thus

targets for antibiotics, demonstrating an intriguing possibility to analyze tissue metabolism in the context of infectious disease studies. Another example is a model of human macrophage metabolism [1], which was recently used to show that oxidative phosphorylation is more important for alternative (M2) activation, whereas shuttling of glycolytic NADH from cytosol to mitochondria is more important for M1 activation, underscoring the quantitative role of metabolism in determining macrophage phenotype.

Beyond cell-type specific analysis, an exciting development in the use of genome-scale models has been to examine the integration of metabolism across multiple tissues in the body. For example, the HepatoNet1 model was combined with a physiologically based pharmacokinetic (PBPK) model to examine the consequences of perturbing specific enzymes in the liver on the metabolic profile of the whole body [49]. Using a related approach, Bordbar et al. tailored a genome-scale reconstruction of human metabolism into cell-type specific models for adipocytes, hepatocytes and myocytes, which was then used to simulate several known metabolic cycles that integrate the different cell types [50]. The integrated model predicted that alterations in metabolic gene expression in disease (e.g. obesity) lead to differentially active sets of reactions in these cycles, demonstrating potential uses for multi-tissue models in characterizing and understanding complex metabolic diseases. The predictions of these multi-scale FBA models remain to be experimentally validated, and would almost certainly benefit from the inclusion of other important cell types and regulatory (e.g. endocrine) mechanisms. In this regard, recent advances in micro-physiological lab-on-chip devices could offer timely opportunities to validate the multi-tissue models by isolating the interactions of selected cell types under controlled conditions.

Spatiotemporally Resolved Metabolic Analysis

The metabolic analysis methods discussed thus far are inherently limited in their ability to address cellular heterogeneity. One way to address this limitation could be to measure isotopic enrichment of metabolites at the single cell level for calculation of metabolic fluxes. Single cell metabolomics is a relatively recent development (reviewed in [51]). To date, a majority of single cell metabolomics efforts have targeted unicellular organisms such as yeast [52] and simple algae [53], which exist in a population, but not bound cohesively as in tissue. Examples of animal cell studies involve zebra fish embryos [54] and mollusk neurons. In the latter study, capillary electrophoresis coupled electrospray ionization MS was used to detect several hundred distinct metabolite signatures in individual neurons of a mollusk [55]. These studies demonstrate that metabolite detection at subcellular concentration is possible; however, challenges remain in isolating the cells and quantifying the metabolites [56].

An obvious limitation of MS based single cell metabolomics is that the analysis of intracellular metabolites is destructive, thus precluding repeated observations over time on the same cell. An alternative is to utilize noninvasive imaging methods, which additionally offer the benefit of providing spatially resolved information. There are many established fluorescent chemical probes used to quantify metabolic flux through specific pathways, with earlier studies relying on non-metabolized analogs of native substrates. An interesting variation involves fluorescence generating reporter substrates competing with the native substrate of the target enzyme. Sames and coworkers, who have reported extensively on this approach, demonstrated that the synthetic chemical coumestrol selectively competes for human hydroxysteroid dehydrogenases of the aldo-keto reductase (AKR) superfamily, and thus can be used to infer the flux of enzymes that couple with AKRs [57]. In principle, this approach could be extended to other enzymes, provided that a fluorescence generating substrate with suitable affinity similar to the native substrate is available for the enzyme of interest.

Genetically encoded probes have only recently been to be utilized to directly measure metabolite fluxes. In pioneering work, Frommer and coworkers developed a glucose sensor based on Förster Resonance Energy Transfer (FRET). The sensor comprised a glucose recognition element from a bacterial periplasmic glucose/galactose binding protein fused with a cyan version GFP (CFP) and a yellow version of GFP (YFP). Upon binding of a glucose molecule, the fusion protein undergoes a conformational change, leading to increased FRET when CFP is excited [58]. More recently, a similarly constructed FRET sensor for lactate has also been described, and used to quantitatively compare lactate flux in primary and cancerous glial cells at a single cell resolution [59,60].

While achieving impressive sensitivity without the use of exogenously introduced dyes, the genetically encoded FRET sensors present the drawback that they require at least one transfection step, which may introduce artifacts from uneven transfection efficacy. In this regard, an attractive option is to utilize methods that exploit the auto-fluorescence of endogenous metabolites. This advantage is especially important for applications involving three-dimensional (3D) systems, where uniform labeling or transfection is challenging. Well-known examples of auto-fluorescent metabolites are nicotinamide and flavin adenine dinucleotides, which emit fluorescence upon near infrared excitation. These cofactor metabolites participate ubiquitously in cellular metabolism, coordinating the regulation of virtually every major pathway. Ratios of these and related cofactors have been used as a measure of metabolic activity in numerous studies over the last several decades. With the advent of sophisticated imaging techniques such as two-photon excited fluorescence (TPEF) microscopy, it has now become possible to image ratios of auto-fluorescent cofactors at substantial tissue depths. Very recently, Quinn et al. utilized TPEF microscopy to correlate a decrease in cellular redox with stem cell differentiation into adipocytes in a 3D vascularized human adipose construct, reporting time- and tissue depth-dependent differences in the redox ratio [61].

Future Outlook

Quantitative metabolic analysis of heterogeneous tissue remains challenging, although substantial progress has been made in analytical technologies and mathematical modeling, with exciting developments in multi-tissue/multi-scale analysis and subcellular resolution measurements. Clearly, each of the methods discussed in this review has drawbacks that limit its applicability. For example, imaging methods offer the greatest potential for spatiotemporally resolved characterization of “thick” 3D tissue constructs, but are currently limited to either general assessment via auto-fluorescent redox cofactors or select pathways via available fluorescent probes. In this regard, the next advances in analyzing tissue metabolism will likely have to involve the integration of new technologies along at least two dimensions. First, cell separation technologies will be needed to selectively sample different cell populations, which would enable the application of high-resolution MS driven isotopomer analysis. Second, efficient molecular delivery technologies will be needed to expand the number of pathways that can be “visualized” by labeling with chemical probes or even genetic circuits. Integrating these technologies should fully exploit the power of current metabolic flux quantification methods, ultimately providing new insights to analyze, design, and optimize engineered or native tissue function.

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•: Papers of special interest

••: Papers of outstanding interest

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Highlights

- The metabolic state of a cell is an important determinant of cellular phenotype.
- Current methods for global metabolic flux quantification characterize bulk behavior.
- Genome-scale models offer promise for analyses involving multiple cell types.
- Advances in metabolic imaging could afford spatiotemporally resolved analysis.

Figure 1.

Cellular metabolic state influences the function of metabolically active and other specialized cell types. (a) Macrophages alter the carbon flux distribution in glycolysis and the pentose phosphate pathway (PPP) in support of classical or alternative activation. (b) A core metabolic function of adipocytes is to sequester excess nutrients as neutral lipids in the form of lipid inclusion bodies, which are fed by both de novo fatty acid synthesis and esterification of fatty acids trafficked from other tissues. (c) Oxidative phosphorylation of nucleotide cofactors is critical for ATP-dependent contractile function of cardiomyocytes. (d) Glucose, which can cross the blood-brain barrier, is first converted to glutamine in astrocytes, and then taken up and hydrolyzed to glutamate in neurons, where glutamate is released as an excitatory neurotransmitter at chemical synapses. Ensuring proper metabolic functions will be critical in engineering tissues capable of performing their physiological roles.