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Isotope tracing in health and disease

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Biochemical characterization of metabolism provides molecular insights for understanding biology in health and disease. Over the past decades, metabolic perturbations have been implicated in cancer, neurodegeneration, and diabetes, among others. Isotope tracing is a technique that allows tracking of labeled atoms within metabolites through biochemical reactions. This technique has become an integral component of the contemporary metabolic research. Isotope tracing measures substrate contribution to downstream metabolites and indicates its utilization in cellular metabolic networks. In addition, isotopic labeling data are necessary for quantitative metabolic flux analysis. Here, we review recent work utilizing metabolic tracing to study health and disease, and highlight its application to interrogate subcellular, intercellular, and *in vivo* metabolism. We further discuss the current challenges and opportunities to expand the utility of isotope tracing to new research areas.

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Introduction

Cellular metabolism is the sum of biochemical reactions that support essential biological functions. Metabolic homeostasis maintains cellular health and normal organismal behavior, whereas metabolic dysfunctions lead to a multitude of diseases [1–5]. The abundance of metabolites, the functional units of metabolism, is often measured in relative or absolute quantities to gauge metabolic activities. However, deep metabolic insights, including substrate utilization, pathway branching, and metabolic flux rewiring are often missing without tracking the elemental constituents of metabolites [6]. To provide such information, isotope tracing using ^{13}C, ^{2}H, ^{15}N, and other elements has been applied to complement simple quantitation of metabolite abundance [7]. Isotope tracing reveals metabolic activities specific to a substrate as it undergoes internalization and subsequent biochemical transformations in the intracellular space. Governed by atomic transitions based on reaction mechanisms, unique labeling patterns are generated when tracers are metabolized through different pathways [6,8,9]. Experimentally, isotope tracers can be administered in a dynamic or steady-state fashion. While dynamic tracing is able to reveal a snapshot of a local metabolic state, steady-state tracing allows quantitative investigation of a metabolic network at the systems level [10,11]. In this regard, isotopic labeling data can be utilized in conjunction with extracellular flux measurements to estimate normalized biochemical reaction rates, namely metabolic fluxes [12••,13]. The main applications of isotope tracers are summarized in Figure 1.

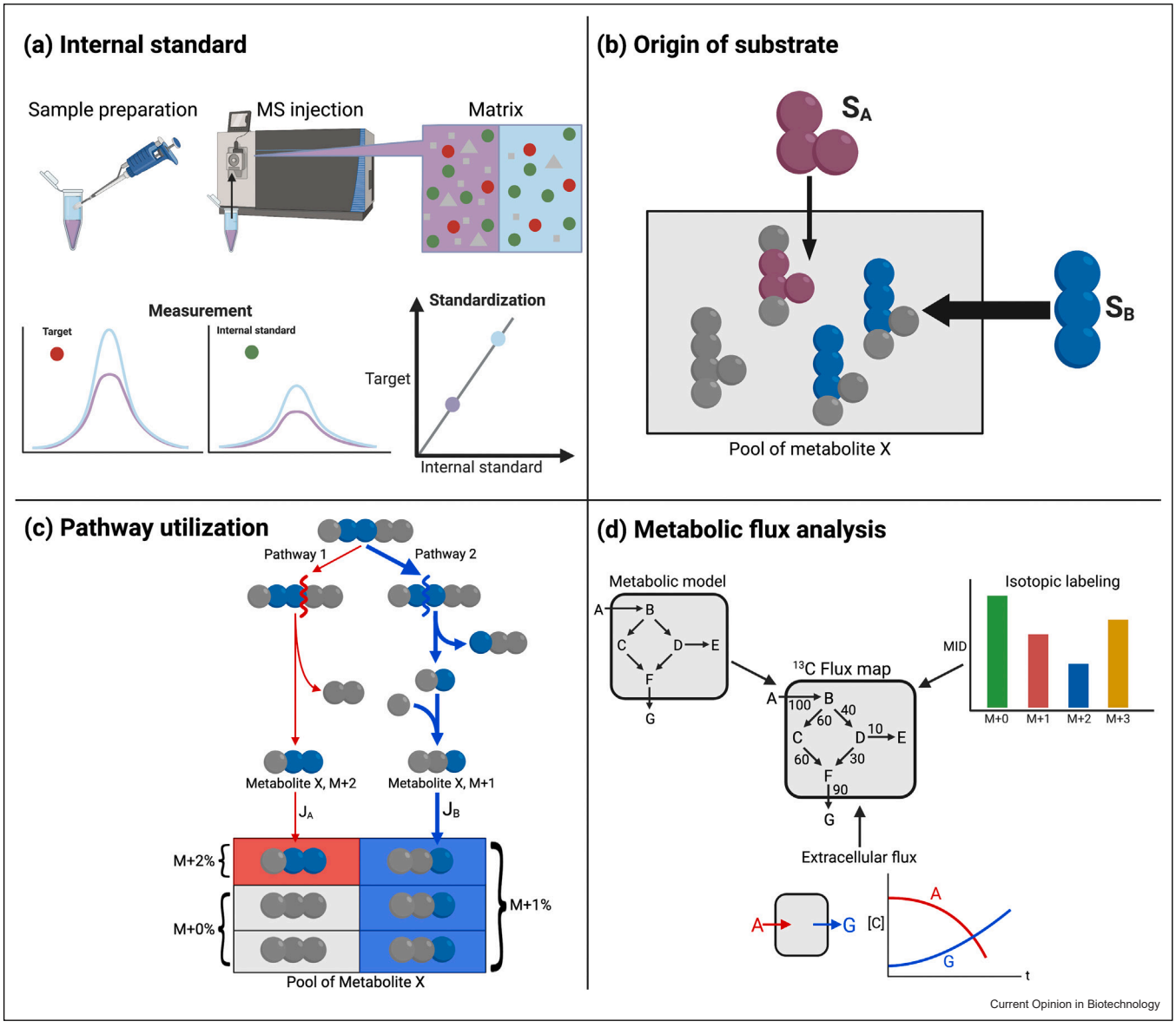
In this review, we explain isotope tracing and labeling analysis in a variety of biological and clinical contexts, including immunometabolism and metabolic reprogramming in cancer, neurodegeneration, and diabetes. Moreover, we highlight the emerging applications of isotope tracers to study subcellular, intercellular, and in *vivo* metabolism. Our goal is to illustrate the versatility of isotope tracing in these research frontiers and discuss future opportunities and challenges.

Cancer metabolism

Metabolic rewiring in cancer cells offers energetic and anabolic advantages for sustaining uncontrolled proliferation. Over the past decade, much of the knowledge about factors that alter cancer metabolism was obtained through the use of isotope tracing and flux analysis [2,14–17].

Cancer cells respond to growth signals and chemical and physical alterations in their microenvironment by

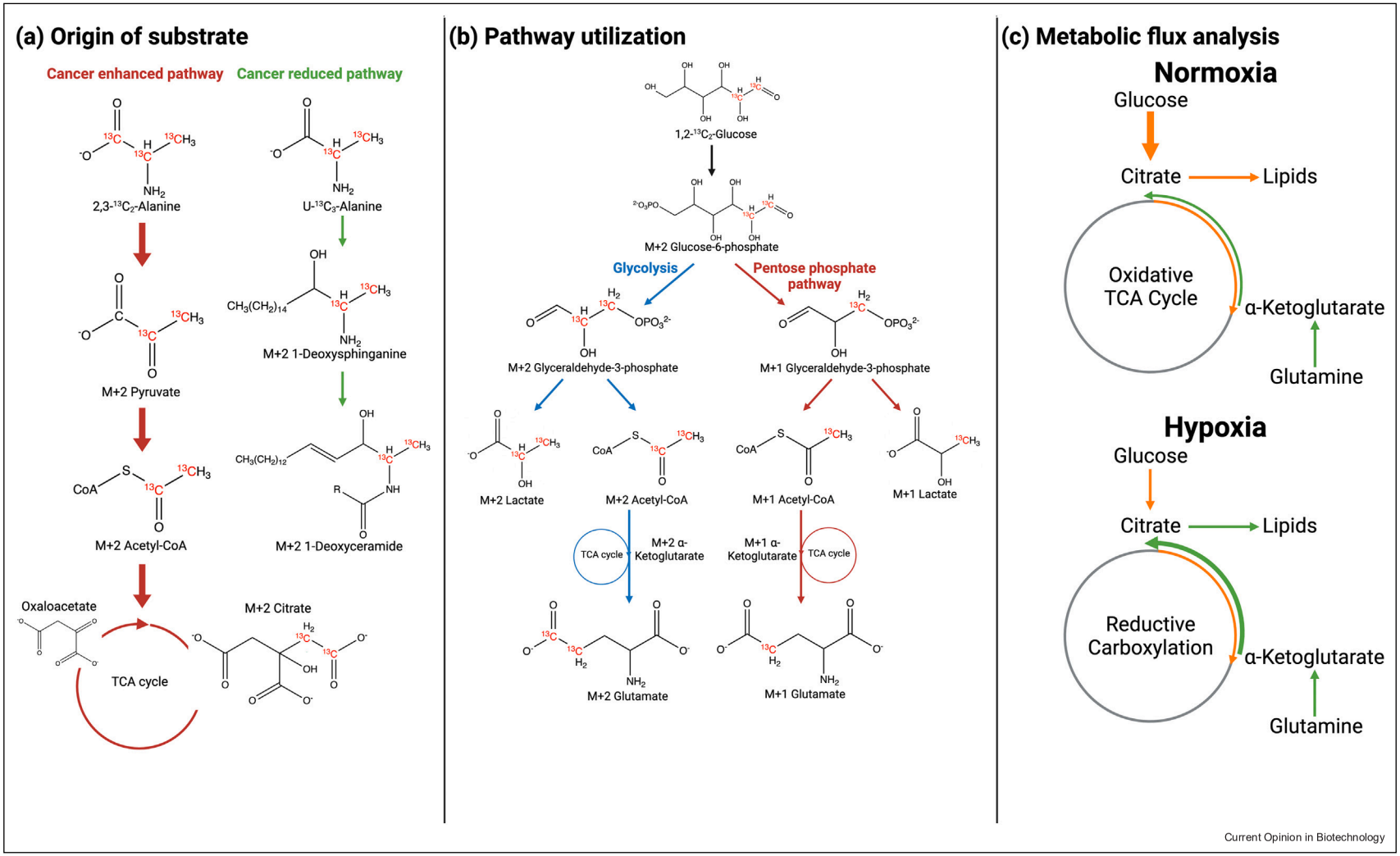
Figure 1



Applications of isotope tracers in metabolic research. **(a)** Isotope tracers serve as reliable internal standards for quantifying metabolite abundance. Signal responses for tracers and metabolites of interest scale proportionally during sample preparation and acquisition. Additionally, different sample matrices affect mass spectrometric responses for isotope tracers and analytes to the same extent. By dividing the signal of a targeted analyte to that of its isotopic internal standard, true biological responses from mass spectrometry can be obtained. **(b)** Isotope tracing reveals the relative contribution of a substrate into target metabolites. **(c)** Tracers indicate pathway activities along different metabolic routes. By quantifying the labeling percentage of isotopomers characteristic of a unique pathway, relative fluxes can be compared. **(d)** Metabolic flux analysis integrates isotope tracing data that include mass isotopomer distribution (MID) and extracellular flux data from kinetic concentration measurements [13]. This advanced analysis of isotope tracing data yields comprehensive quantitation of fluxes for all metabolic pathways of interest.

reprograming their metabolism. In rapidly dividing cells, glucose and glutamine are the main carbon substrates for mitochondrial respiration and fatty acid synthesis. A recent study showed, using uniformly labeled (U)-$^{13}C_5$-glutamine tracing, that glutamine is also actively utilized for synthesizing non-essential amino acids in growing meningiomas [18]. However, this substrate utilization pattern is changed when cellular growth stalls. For example, human mammary epithelial cells rely on branched-chain amino acids (BCAAs) for mitochondrial metabolism and lipogenesis under near-quiescent condition. This is evidenced by increased ^{13}C-enrichment of the tricarboxylic acid (TCA) cycle metabolites and free fatty acids from ^{13}C-labeled BCAAs [19]. Besides

Figure 2



Highlighted isotope tracing studies. **(a)** Tracing by 2,3-$^{13}C_2$-alanine pinpoints the substrate origin for TCA cycle intermediates and deoxysphingolipids in colon cancer cells. Labeling by 2,3-$^{13}C_2$-alanine suggests that colon cancer cells enhance alanine incorporation into the TCA cycle. Concomitantly, reduced availability of 2,3-$^{13}C_2$-alanine results in decreased synthesis of ^{13}C-labeled deoxysphingolipids, which exhibit cytotoxic effects in cancer cells [23••]. **(b)** The use of 1,2-$^{13}C_2$-glucose provides quantitative assessment of relative pathway activities along glycolysis and the PPP. This labeling work indicates highly active PPP in primary oligodendrocytes [39••] **(c)** Metabolic flux analysis suggests activated reductive carboxylation of glutamine into *de novo* lipogenesis in hypoxic cancer cells [12••,74].

cellular growth, diet also profoundly rewires cancer metabolism. Ketogenic diet causes elevation of β-hydroxybutyrate in plasma and tumor interstitial fluid. Due to restricted carbohydrate availability, AL1376 pancreatic ductal adenocarcinoma (PDAC) cells utilize ketone bodies for fueling the TCA cycle as shown by increased labeling from U-$^{13}C_4$-β-hydroxybutyrate [20].

Additionally, cancer metabolism is responsive to oxygen and proton availability, a mechanism that has been elucidated by ^{13}C-tracing. Under hypoxia, the reductive pathway from glutamine is enhanced over the canonical oxidative route from glucose. This is evidenced by reduced M+2 labeling from U-$^{13}C_6$-glucose with a concomitant increase in M+1 labeling from 1-^{13}C-glutamine, as these isotopomers are specific to these two distinct metabolic pathways (Figure 2c) [12••]. Similar to reductive carboxylation, nonoxidative pentose phosphate pathways (PPPs) are also modulated by hypoxia. When 1-^{13}C-glucose is metabolized along glycolysis, the labeling pattern of fructose-6-phosphate/fructose-1,6-bisphosphate (F6/BP) indicates relative flux along nonoxidative PPPs. Specifically, nonoxidative PPP flux increases the M+1 isotopomer abundance of the fragment at mass-to-charge ratio (*m/z*) 307, an ion that carries the carbons at positions 4, 5, and 6 of the original molecule. Similarly, the fractional abundance of the M+1 fragment at *m/z* 364 is reduced due to isotopic dilution at carbon positions 1, 2, 3, and 4 through nonoxidative PPPs [21]. Besides oxygen, the level of protons also alters glycolytic and PPP flux. For example, depletion of monocarboxylate transporter 4 in acute myeloid leukemia cells results in reacidification of the intracellular space that further leads to attenuated ^{13}C-fractional enrichment of glycolytic metabolites [22].

Besides elucidating intrinsic and environmental cues that influence metabolism in cancer cells, isotope tracing revealed a nontraditional manifestation of cancer metabolism; in addition to increasing metabolic activities along certain pathways that provide growth advantage, cancer cells also attenuate metabolic pathways with growth-suppressing effects. Indeed, it was recently found that cytotoxic deoxysphingolipids are synthesized by serine palmitoyltransferase (SPT) due to enzyme promiscuity. Instead of using serine as the canonical substrate, SPT combines alanine with palmitoyl-CoA to produce toxic deoxysphingolipids in response to high alanine/serine ratio. This metabolic connection was elucidated by ^{13}C-alanine tracing. Reduced expression of the mitochondrial pyruvate carrier (MPC) has been reported in colon cancer. This transcriptional change accelerates alanine consumption by the TCA cycle as the labeling of citrate from 2,3-$^{13}C_2$-alanine is increased. Decreased alanine availability further results in the depletion of deoxysphingolipids and enhanced tumor growth (Figure 2a) [23••]. Therefore, evading the metabolic programs that produce cytotoxic compounds is another strategy that cancer cells use to metabolically maintain their survival and growth.

As for emerging frontiers, there have been several major breakthroughs in exploring metabolic coupling in cancer over the past few years. Metabolic coupling exists between different pathways as well as between distinct cell types. At the pathway level, the crosstalk between cystine metabolism and PPP was lately uncovered by 1,2-$^{13}C_2$-glucose tracing. Cancer cells with SLC7A11 overexpression exhibit increased M+1 and decreased M+2 abundance for lactate, indicating enhanced flux through the oxidative PPP. Mechanistically, PPP provides NADPH to reduce the insoluble cystine imported by SLC7A11 to cysteine, a function required to avoid oxidative stress in SLC7A11-overexpressing cells [24•]. Similar metabolic crosstalk in the broader context of redox homeostasis has also been reported [25,26]. As for the intercellular level, the tumor microenvironment (TME) has been of great interest since metabolic interactions between cancer and surrounding cells exist in physiological contexts and are capable of modulating tumor progression [3]. Previously, alanine has been shown to be secreted from stellate cells to replenish the TCA cycle in PDAC cells. Indeed, a substantial amount of TCA cycle intermediates are labeled by exogenous U-$^{13}C_3$-alanine [27]. A recent study further revealed that PDAC metabolism is also maintained by cancer-associated fibroblasts (CAFs) that secrete branched-chain α-ketoacids (BCKAs). Specifically, BCKAs contribute to both protein synthesis and the TCA cycle as evidenced by the presence of labeled BCAAs and TCA cycle intermediates from ^{13}C-BCKA tracing [28••]. It thus appears that a diverse class of metabolites may be exchanged between cancer and surrounding cells, which warrants further investigation by isotope tracing analysis.

To characterize cancer metabolism in physiologically relevant settings, more recent efforts have led to a substantial progress in the field of *in vivo* isotope tracing. For instance, the first tracing experiment in patients with clear cell renal cell carcinoma (ccRCC) demonstrated that the Warburg effect is indeed conserved in tumors infused with U-$^{13}C_6$-glucose at the time of nephrectomy. Specifically, increased glycolysis and suppressed TCA oxidation were observed in ccRCC tumors [29•]. Another *in vivo* tumor study in zebrafish uncovered a tumor-liver alanine cycle during which alanine is excreted from tumors and regenerated to glucose via gluconeogenesis in the liver. U-$^{13}C_6$-glucose tracing showed that the amount of circulating M+3 glucose was increased in the fish harboring BRAF mutation and p53 deficiency. Such isotopic labeling pattern suggests enhanced gluconeogenesis. In addition, compared with other tricarbon substrates, M+3 alanine was markedly increased in both the liver and serum, indicating that

alanine acts as a circulating carrier that allows the removal of excess nitrogen from tumor while supporting hepatic gluconeogenesis to fulfill the high demand for glucose in cancer cells [30]. We believe that future advancements in *in vivo* tracing will be necessary to provide novel and comprehensive understanding of metabolic dependencies in cancer.

Neurometabolism

The correlation between metabolic alterations and neuropathies is well-established [4,31,32], however, the study of neurometabolism that characterizes the metabolic features of the central nervous system (CNS) is still in its nascency. Traditionally, metabolic studies in the CNS have focused on the biochemistry of neurotransmitters and brain energetics. Brain has a high energy demand, which is about 20% of individual's expenditure, although it represents only 2% of body weight [33]. More recent work, using isotope tracing, has provided novel insights into neuronal metabolism. The metabolic flexibility of neurons has been revealed by tracing with U-$^{13}C_6$-glucose and U-$^{13}C_5$-glutamine. As a therapeutic intervention to treat excitotoxic neurodegeneration, MPC was inhibited to promote the oxidation of glutamate, the accumulation of which can lead to neuronal toxicity. Upon MPC inhibition, the ^{13}C-enrichment of TCA cycle metabolites from U-$^{13}C_6$-glucose was reduced. In order to maintain TCA activity, neurons upregulate glutamate oxidation as evidenced by significant increase in ^{13}C-enrichment from U-$^{13}C_5$-glutamine. This metabolic rewiring decreases the availability of glutamate used for excitatory neurotransmission without impairing bioenergetics, and hence provides a therapeutic strategy that leverages this metabolic flexibility to modulate neuronal excitotoxicity. [34]. In addition to glutamate metabolism, the importance of one-carbon metabolism for neural development has been recently uncovered through the use of 1,2-$^{13}C_2$-glycine. Genetic depletion of glycine decarboxylase prevents one-carbon transfer from 1,2-$^{13}C_2$-glycine to its methyl group receptors, as the ^{13}C-incorporation into serine and purines is reduced. Inhibition of the methionine cycle also leads to dysfunctional neural tube closure. This work highlights the important role of glycine and methionine cycle in supplying one-carbon units during embryogenesis [35].

In addition to neuronal cell metabolism, it is well appreciated that glial cells also support metabolic homeostasis in the CNS. Lately, several metabolic pathways have been studied in primary oligodendrocyte precursor cells. Labeling by 1,2-$^{13}C_2$-glucose indicated highly active oxidative PPP by measuring the M+1/M+2 ratio of glutamate (Figure 2b). Pyruvate carboxylation activity was also estimated by examining the ^{13}C-enrichment level of pyruvate from 1-^{13}C-lactate. M+1 pyruvate is only generated through the pyruvate carboxylation reaction due to the retention of ^{13}C atom that will otherwise undergo decarboxylation via the pyruvate dehydrogenase activity [9,36,37]. This further suggests that pyruvate anaplerosis is also active in oligodendrocyte precursors in addition to astrocytes, where such mechanism is important [38]. Moreover, oligodendrocytes also incorporate carbons from 1,2-$^{13}C_2$-acetate into the TCA cycle [39••]. This metabolic activity has also been observed in astrocytes [40], indicating that several metabolic features may be shared by multiple types of glial cells.

An important characteristic of neurometabolism is its heterogeneous nature. Isotope tracing in freshly dissected mouse brain slices revealed similar labeling patterns by U-$^{13}C_6$-glucose between cortex and hippocampus. However, increased labeling percentage of M+2 TCA metabolites was observed in the hippocampus compared to the cortex when tracing was performed using 1,2-$^{13}C_2$-acetate [41]. At the cellular level, neuronal metabolism is different from that of astrocytes. For example, cerebellar astrocytes exhibit a higher citrate excretion rate compared to neurons [42]. Furthermore, the labeling of glutamate from U-$^{13}C_3$-lacate is higher in neurons than that in astrocytes [43]. The metabolic heterogeneity among brain regions and cell types requires metabolic crosstalk and cooperation (division of labor) within the CNS to maintain metabolic homeostasis. Consistent with this notion, a recent study reported a mechanism by which astrocytic ApoE-mediated microRNA delivery can inhibit neuronal cholesterol biosynthesis. Specifically, neurons cultured with astrocyte-conditioned medium exhibited reduced levels of ^{13}C-labeled cholesterol and its biosynthetic precursors from U-$^{13}C_6$-glucose [44]. Another study showed that ApoE mediates shuttling of lipids from neurons to astrocytes for lipid detoxification [45]. These results highlight the dynamic cooperation of glial cells and neurons for concerted maintenance of brain metabolic homeostasis.

Besides elucidating the communication between different brain cell types, isotope tracing also revealed metabolic connections between mitochondrial dysfunction and neuropathy. Through dynamic labeling by U-$^{13}C_6$-glucose in mice with *Polg* mutation, a model for mitochondria-associated neuropathy, enhanced glycolysis and gluconeogenesis were observed as determined by the increased levels of ^{13}C-labeled glycolytic intermediates and glucose isotopomers, which indicate gluconeogenic scrambling. Moreover, ^{13}C-labeled lactate and alanine were significantly increased in plasma but not in other tissues, suggesting activated Cahill cycling under mitochondrial dysfunction. In addition, labeling by ^{15}N-ammonium chloride also showed decreased isotope enrichment in urea, reflecting an impaired urea

cycle. These results suggest strong correlation between mitochondrial defects and neuropathy manifested by both carbon and nitrogen metabolic abnormalities [46].

Immunometabolism

As the effectiveness of immunotherapy has been significantly improving over the last decade, the effort to understand the metabolism of immune cells has gained increasing popularity. Through U-$^{13}C_6$-glucose and U-$^{13}C_5$-glutamine tracing, divergent metabolic fates have been uncovered in tumor and effector T cells under glutamine blockade. As expected, the contribution from U-$^{13}C_5$-glutamine to central carbon metabolites was reduced in both cell types treated by the glutamine antagonist JHU-083. The ^{13}C-enrichment from U-$^{13}C_6$-glucose to TCA intermediates was also attenuated by glutamine restriction in tumor cells. However, effector T cells activated anaplerosis from U-$^{13}C_6$-glucose to replenish the TCA cycle. Furthermore, increased ^{13}C-labeling from 1,2-$^{13}C_2$-acetate was also observed in T cells, suggesting greater metabolic flexibility of T cells to maintain TCA cycle activity using other carbon sources. This metabolic divergence provides an opportunity to improve immunotherapy as glutamine blockade not only leads to metabolic stress for tumor cells, but also promotes antitumor functions by T cells [47•].

Another isotope labeling work revealed distinct metabolic phenotypes between *in vivo* CD8+ T cells compared with those activated *in vitro*. Although T cells *in vitro* show high glucose uptake and lactate production rates, effector T cells differentiated *in vivo* exhibit higher flux from U-$^{13}C_6$-glucose into multiple anabolic pathways, including *de novo* serine and nucleotide biosynthesis. In addition, inhibition of serine biosynthesis as reflected by reduced levels of ^{13}C-serine isotopomers resulted in suppressed T cell proliferation *in vivo* [48]. Besides glucose and glutamine tracing, labeling using $^{13}C_3$- and ^{15}N-alanine reveals that extracellular alanine is required for T cell activation. Quantitation of ^{13}C-labeled and ^{15}N-labeled isotopomers shows that alanine is mostly used for protein synthesis rather than being catabolized into pyruvate and TCA cycle intermediates [49]. These findings collectively corroborate the important role of cellular metabolism in supporting proper immuno-oncological functions.

Metabolism in diabetes mellitus

Traditionally, insulin signaling has been the major focus of diabetes research. Lately, direct characterization of metabolic pathways in pancreatic β cells and adipocytes, one of the major types of cells responsive to insulin actions, has also begun to draw attention. Specifically, insulin resistance rewires adipocytic TCA cycle for enhanced incorporation of U-$^{13}C_6$-glucose to M+2 and M +5 citrate. This suggests increased TCA metabolism at both the first and third rounds of the cycle [50]. Moreover, kinetic studies using U-$^{13}C_6$-glucose revealed that insulin signaling acts as a driving force to prime anabolic shift toward pyruvate carboxylation and oxidative PPP in adipocytes [51•].

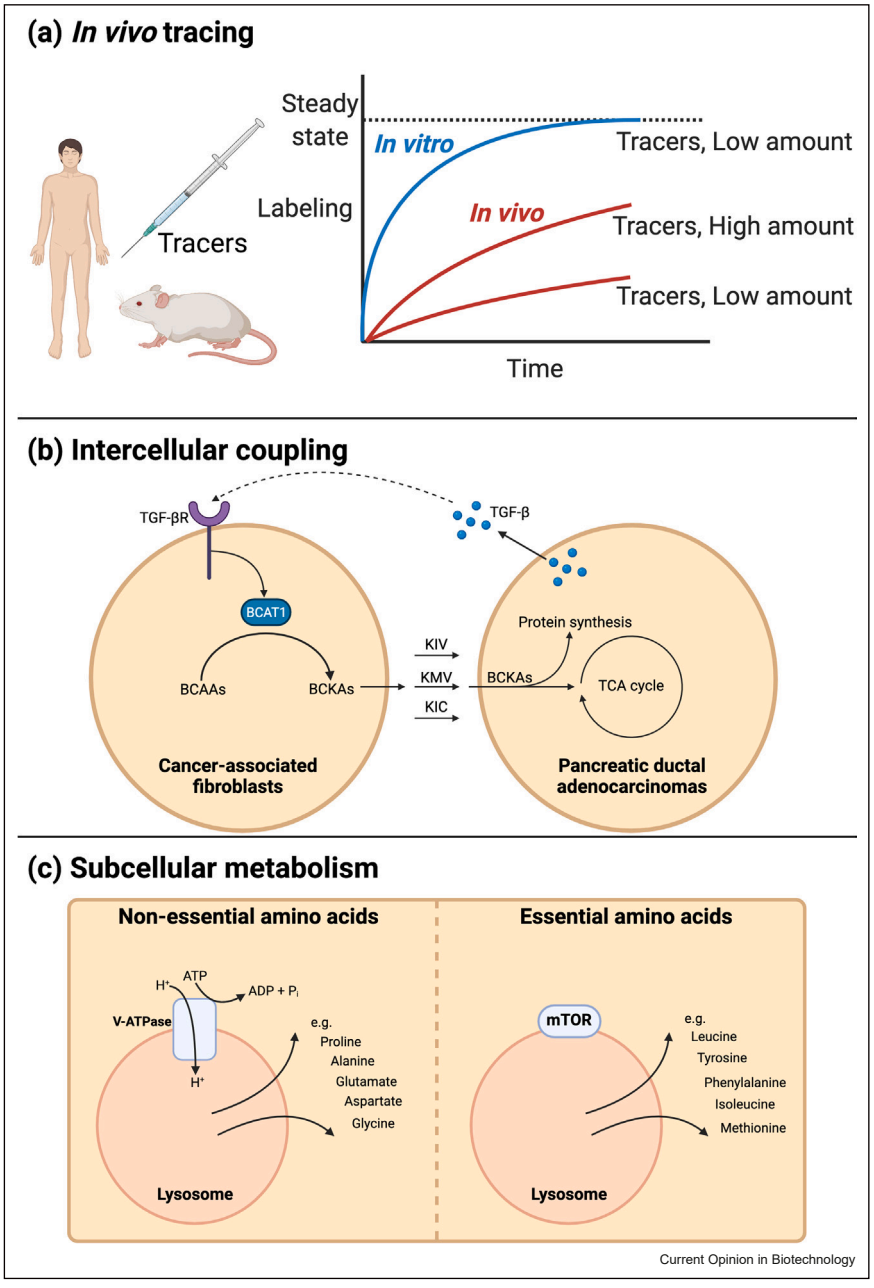
In addition to insulin, temperature-induced metabolic rewiring is also reported in adipocytes. *In vivo* metabolic tracing by U-$^{13}C_6$-glucose in brown adipose tissue indicates that glucose flux into glycolysis, PPP, and the TCA cycle is increased under acute cold treatment, since the abundance of total ^{13}C-labeled metabolites was increased [52]. *In vivo* tracing using U-$^{13}C_6$-glucose in mouse pancreatic islets revealed that pancreatic β cells from 1-year-old mice exhibit increased molar percentage enrichment of ^{13}C for central carbon metabolites and coupling factors (cofactors and metabolites required for pancreatic insulin secretion) [53] compared with their juvenile counterparts. This result suggests that aging also triggers metabolic reprogramming in pancreatic β cells [54]. We believe that as the interest in studying diabetic molecular metabolism continues to grow, isotope tracing using substrates beyond glucose will shed light on important pathogenic pathways to better understand this metabolic disease.

Subcellular metabolism

In addition to investigating metabolic rewiring in disease, the use of isotope tracing has also made substantial contribution to our understanding of compartmentalized metabolism. There are multiple approaches to achieve subcellular resolution for metabolic studies. Traditionally, metabolites enriched in certain compartments are used as a proxy to characterize subcellular metabolism. Well-known examples are TCA cycle and glycolytic intermediates for mitochondria and cytoplasm, respectively. However, studies have shown that certain metabolites and metabolic pathways may not exist exclusively in one subcellular compartment [12••,55•]. Nevertheless, this approach is still widely used in conjunction with isotope tracing to gain subcellular understanding. For instance, examination of the labeling patterns of uridine diphosphate N-acetylglucosamine from U-$^{13}C_6$-glucose suggests differential contribution from cytosolic and mitochondrial pathways to hexosamine synthesis in macrophages polarized by classic and alternative means [56].

Improved subcellular resolution for metabolism has been made by coupling compartment-specific reporters with specially designed isotope tracers. Tracing of deuterium in cells with a compartment-specific isocitrate dehydrogenase (IDH)-mutant reporter system revealed that the primary subcellular location for serine-to-glycine conversion is within mitochondria in A549 cells [57].

Figure 3



Emerging frontiers in isotope tracing. **(a)** *In vivo* isotope labeling provides physiological insights for cellular and organismal metabolism. However, it faces a major challenge to reach isotopic steady states, given the amount of tracers available due to cost considerations. **(b)** Intercellular metabolic coupling has been resolved by tracing studies. PDAC tumor cells receive BCKAs from CAFs to fuel TCA cycling and protein synthesis [28••]. **(c)** Isotope tracing plays an important role in deciphering subcellular metabolism. Pulse-chase experiment by labeled amino acids reveals that V-ATPase and mTOR modulate the lysosomal efflux of non-essential and essential amino acids, respectively [56].

Lately, different technologies, including Raman spectroscopy, secondary ion mass spectrometry, as well as fluorescent labeling of metabolites and computational modeling, have led to significant progress in dissecting subcellular metabolism. For example, the use of Raman microspectroscopy identified an endoplasmic reticulum (ER)-specific phospholipid dysregulation in infiltrating gliomas harboring IDH mutations [58]. In addition, subcellular localization of glycerophospholipids and their sulfatides was pinpointed in mouse hippocampus by employing secondary ion and Orbitrap mass spectrometry [59]. Furthermore, a recent study that integrates

fluorescence sensing and ^{13}C-labeling results via mathematical analysis suggested that enhanced oxidative PPP in the cytoplasm and glucose anaplerosis may support redox balance in response to mitochondria-specific oxidative stress [60].

In contrast to the methods above, the direct assessment of subcellular metabolism can be achieved by rapid immunoprecipitation of organelles followed by downstream metabolite profiling. This technique has been developed and optimized for metabolite analysis in mitochondria (MitoIP), lysosomes (LysoIP), and peroxisomes (PeroxoIP) [61–64]. MitoIP enriches for metabolites that are consistent with the metabolic function of the organelle [62]. Although it is still feasible to probe some aspects of the mitochondrial metabolism at the whole-cell level by profiling TCA cycle intermediates, metabolic investigation of lysosomes requires subcellular resolution, mainly because of their small size compared to that of the cell. Consistent with this, acute disruption of the lysosomal proton gradient using selective V-ATPase inhibitors, including Concanamycin A or Bafilomycin A1, has minimal effects on the whole-cell metabolome, while it dramatically increases the levels of several metabolites in the lysosome as determined using LysoIP followed by metabolite profiling. Using a combination of compartment-specific immunoprecipitation and isotope tracing, this accumulation was shown to be a result of slower efflux of affected metabolites across the lysosomal membrane. Dynamic tracing using LysoIP also identified differential mechanisms governing lysosomal amino acid egress. While proton gradient disruption led to reduced efflux of non-essential amino acids, the export of several essential amino acids was modulated by mTOR activity [63]. We believe that the efforts to characterize subcellular metabolism using isotope tracing, immunoprecipitation, and hybrid mass spectrometry techniques will improve our understanding of compartmentalized biochemistry with precision and mechanistic insights.

Concluding remarks

With the advancement of metabolic research in different areas of biology, isotope tracing has become increasingly popular. In addition to its basic application for internal standardization of metabolite quantitation, the use of isotopically labeled metabolites offers opportunities to distinguish molecules derived from a specific substrate and processed along a distinct metabolic pathway. Additionally, labeling data contain rich information that can be fully leveraged to estimate metabolic flux, a technique that requires computational work such as metabolic flux analysis. Although additional modeling effort is needed to fully extract what isotope tracers have to offer, simple metrics such as percentage of isotope enrichment and isotopomer ratios already offer deep metabolic insights needed for mechanistic studies. It is worth noting that due to error propagation, isotopomer ratios should be computed and interpreted with caution as they are sensitive to experimental errors [65].

Experimental guidelines for carrying out tracing studies have been discussed in previous publications [6,8,10]. For applications in mammalian cell systems, tracers are usually introduced via cell culture media that contains defined chemical components. Tracer-containing media should be prepared fresh and administered after complete removal of spent media. In addition, metabolite harvesting needs swift and precise laboratory techniques to ensure rapid quenching and metabolite extraction. For steady-state tracing, environmental perturbations such as temperature and humidity changes should be avoided. For metabolic flux analysis, isotopic steady states should be experimentally validated before computational analysis. Last, for *in vivo* tracing, the choice of diet as well as the timing of feeding, fasting, and harvesting are also important experimental considerations.

By summarizing recent literature that has employed isotope tracing in cancer, neuro-, immuno-, and diabetic metabolism, several themes have emerged. First, using isotope tracing *in vivo* to obtain physiologically relevant insights is the overarching objective across all these fields. Despite several major breakthroughs in the field of *in vivo* tracing, the cost of tracers at large quantity and the difficulty to reach isotopic steady state for pathways whose flux is low or characterized by slow turnover rates, such as lipid and nucleotide biosynthesis, still remain challenging (Figure 3a). Additionally, recent efforts have also aimed to address discrepancies between *in vivo* and *in vitro* tracing studies [66]. One example is high glutamine contribution to the TCA cycle *in vitro* in contrast to its minimal utilization in *vivo*. This was uncovered by both ^{13}C-isotope infusion and metabolite quantitation in the TME in mice [67,68]. In addition, isotope tracing in physiologically relevant media has provided more accurate metabolic insights [69]. Furthermore, *in vivo* tracing studies have also unveiled tissue-dependent metabolic phenotypes. Although glucose tracing in ccRCC patients showed that the Warburg effect is conserved in this specific tumor type [29•], reports in patients with other cancer types suggest that the TCA cycle activity is higher in tumor cells compared with surrounding benign lesions [70–72]. Future tracing work will shed light on the heterogeneous nature of cancer metabolism with respect to tumor type and tissue of origin.

In addition to *in vivo* metabolic studies, intercellular metabolic communication is another emerging theme. Arguably, the best example of this endeavor is the characterization of the TME. Isotope tracing is essential for studying metabolite-based cellular communication

between different cell types as each cell type can be considered a distinct metabolic compartment (Figure 3b). The challenge for such studies is that cell-type-specific contributions to metabolite pools remain difficult to resolve in bulk tissue analysis. Recently, compartmentalized metabolism has also been characterized in the placenta and embryo, thus paving the road for investigating inter-tissue metabolic coupling [73]. Another emerging theme that we observed is the study of subcellular metabolism and interorganellar metabolic crosstalk. While mitochondria had been historically the major focus of subcellular metabolic research, recently, specially designed tracers and rapid immunoprecipitation of organelles have allowed metabolic characterization of other compartments such as the ER, Golgi, peroxisomes, and lysosomes (Figure 3c). We believe that these technical developments will pave the way to profiling other specialized organelles and subcellular compartments whose metabolic roles remain elusive, yet fascinating to uncover.

Conflict of interest statement

Nothing declared.

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