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Stable isotope-resolved metabolomics and applications for drug development

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Abstract

Advances in analytical methodologies, principally nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), during the last decade have made large-scale analysis of the human metabolome a reality. This is leading to the reawakening of the importance of metabolism in human diseases, particularly cancer. The metabolome is the functional readout of the genome, functional genome, and proteome; it is also an integral partner in molecular regulations for homeostasis. The interrogation of the metabolome, or metabolomics, is now being applied to numerous diseases, largely by metabolite profiling for biomarker discovery, but also in pharmacology and therapeutics. Recent advances in stable isotope tracer-based metabolomic approaches enable unambiguous tracking of individual atoms through compartmentalized metabolic networks directly in human subjects, which promises to decipher the complexity of the human metabolome at an unprecedented pace. This knowledge will revolutionize our understanding of complex human diseases, clinical diagnostics, as well as individualized therapeutics and drug response.

In this review, we focus on the use of stable isotope tracers with metabolomics technologies for understanding metabolic network dynamics in both model systems and in clinical applications. Atom-resolved isotope tracing via the two major analytical platforms, NMR and MS, has the power to determine novel metabolic reprogramming in diseases, discover new drug targets, and facilitates ADME studies. We also illustrate new metabolic tracer-based imaging technologies, which enable direct visualization of metabolic processes *in vivo*. We further outline current practices and future requirements for biochemoinformatics development, which is an integral part of translating stable isotope-resolved metabolomics into clinical reality.

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Keywords

Metabolomics; systems biochemistry; stable isotope tracing; drug discovery; metabolic compartmentation and regulation; pathway reconstruction

I. Introduction

I.1. Systems biochemistry and drug development

Drug development from the early stage of target identification and validation through clinical trials to clinical practice is a long, tortuous, and extremely costly process. The net yield of such process has also been poor and often comes from incremental advances on existing therapeutic agents (Adams and Brantner 2006; Adams and Brantner 2010; Arrowsmith 2011; Light and Warburton 2011; Subbaraman 2011).

The current paradigm for drug development calls for uncovering specific molecular targets, against which highly selective and potent inhibitors can be developed, with minimal off-target effects. Such agents can be synthetic small molecules that may require optimization by medicinal chemistry, or natural products and their synthetic derivatives. From target discovery, through target validation, to clinical testing and eventual clinical adoption, the whole process would logically require a systems biochemical understanding of the disease itself, pharmacological properties (i.e. absorption, distribution, metabolism, excretion, and toxicity or ADME) of the therapeutic agents, and their functional impact on the human body both on-target and off-target. Systems biochemistry can be viewed as “global biochemical networks and molecular regulations”. As with all systems approaches, this represents a tall order for drug discovery, development, and deployment using conventional approaches. The lack of systems biochemical approaches and thus functional understanding in the past presents a fundamental barrier to efficient and successful commercialization of potential therapeutic agents.

Beginning with the development of genomics, followed by functional genomics, proteomics, and now metabolomics, it is for the first time that a systems biochemical understanding of the human body may be envisioned. Once developed, this will accelerate the understanding of disease mechanisms and therapeutic development at an unprecedented pace. In this review, we will provide an overview of the metabolomics approach with the focus on the use of stable isotope tracer technologies for drug discovery and development processes from target identification and validation to pharmacodynamics and ADME.

I.2. Overview of metabolomics approaches

What is the metabolome?—The metabolome, the intersecting systems chemistry of life processes, is the functional outcome of the activity of the genome, functional genome, and the proteome. Metabolic processes are the ultimate expression of gene and protein activities to meet the physiological demands for growth and survival, including responses to environmental factors such as nutrient availability, xenobiotics, and therapeutic agents. Metabolic products are often indispensable players in maintaining metabolic homeostasis via regulating enzyme activities, as well as protein and gene expression events in a feedback loop (**Figure 1**). This is simply illustrated by the well-known insulin production in response to blood glucose levels to maintain glucose homeostasis (Lienhard, Slot et al. 1992). Thus, the metabolome is an integral part of the systems biology and a holistic understanding of metabolic processes is essential to advancing our knowledge on the molecular regulations or dysregulations as a result of disease development or therapeutic interventions, which may occur at all ‘omics levels. However, metabolomics, i.e. the study of the metabolome,

remains to be the least explored and is currently the most technologically challenged among the 'omics.

A common misconception in the past has been that the human metabolome involves only a few thousand endogenous metabolites, unlike the human genome and proteome, which consists of tens of thousands of genes and perhaps millions of different proteins (James 2000). Consequently, the metabolome seems relatively “simple” and much appears to be already known as summarized in the biochemistry textbooks. This misconception clearly does not reflect the unknown number of nutrients, xenobiotics, and anthropogenic pollutants, or therapeutic agents that human bodies need to interact with. Nor does it consider the complex dynamics, redundancy of metabolites in functionally different pathways, compartmentation including intra- and inter-organ interactions, and metabolic reprogramming that occur in the human body, particularly under stress or disease states. Metabolic reprogramming is a common feature of cancers and other metabolic diseases, which can be caused by genetic mutations. This is exemplified by the long-known aerobic glycolysis or “Warburg effect” (Warburg 1924) in many cancer types and the recent discovery of a defective mitochondrial Krebs cycle associated with a loss of function fumarate hydratase mutation (Gottlieb and Tomlinson 2005; King, Selak et al. 2006) or isocitrate dehydrogenase 1 (IDH1) mutation diverting isocitrate to 2-hydroxyglutarate (Dang, White et al. 2009). Note that this is more than an 80-year gap between discoveries. The recent findings merely represent the “tip of the iceberg” for the “undiscovered country” of the human metabolome.

Metabolomic analyses is technically challenging—Due to the diverse nature of the chemicals, metabolites, and molecular interactions that we need to cope with, the analytical demands pose extremely difficult challenges distinct from genomic and proteomic analyses. The last decade has seen unprecedented critical advances in nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) such that it is now practical to meet many of the technical challenges faced by metabolomics investigations. These include the requisite task of large-scale detection of a wide range of structurally diverse chemicals, their structure elucidation without *a priori* knowledge, and quantification across a wide concentration range, to name just a few of the major challenges. It is not surprising that studies involving both unbiased and targeted metabolite profiling have been growing exponentially since the early 2000's, many of which touched upon drug discovery and diagnostics (Fan, Lane et al. 2004; Fernie, Trethewey et al. 2004; Verhoeckx, Bijlsma et al. 2004; Whitfield, German et al. 2004; Fan, Bandura et al. 2005; Harrigan, Brackett et al. 2005; Lee and Go 2005; Lutz 2005; Rochfort 2005; Andrew Clayton, Lindon et al. 2006; Griffin 2006; Schlotterbeck, Ross et al. 2006; Beger, Hansen et al. 2009; Fan, Lane et al. 2009; Wikoff, Anfora et al. 2009; Fan 2010; Kim, Wilson et al. 2010; William J. Griffiths and Deigner 2010; Fan and Lane 2011; Psychogios, Hau et al. 2011).

Why are stable isotopic tracer approaches required for deciphering the metabolome?—Steady-state concentration-based metabolite profiling is only part of the story in the quest for deciphering the human metabolome. It is well known that many pathways intersect to form a dense network with the same chemical species participating in many interweaved pathways. Glutamate represents a good example, which can be involved in up to 55 known pathways and some 200 reactions (M. Arita, personal communication). From examining metabolic charts (such as the Metabolic Pathway Charts of D. Nicholson, <http://www.sigmaaldrich.com/life-science/metabolomics/learning-center/metabolic-pathways.html>), this is common to many compounds in central metabolism. Thus, it is mathematically impossible to discern the contributions of each pathway segment based on overall metabolite concentrations alone (**Figure 2**).

What complicates the matters even more is the largely unknown compartmentation and dynamic exchange between compartments, such as the distribution and exchange of citrate, OAA, Asp, and malate between the mitochondrion and the cytoplasm, which is essential for shuttling reducing equivalents (NADH) and acetyl CoA (for lipid biosynthesis) between the two compartments (cf. **Figure 4**). In fact, compartmentation of metabolites via membrane bound organelles, intracellular pools separated by diffusion limits, or between organs is one of the universal means for homeostatic regulation. This critical information, as well as the pathway/network information, simply does not exist in concentration-based datasets, thus it is futile to attempt to extract it from mining the datasets. Both types of information must be specifically acquired in order to decipher the human metabolome. This is also valuable to exploit from the standpoint of therapeutic targets and pharmacological efficacy. The stable isotope tracer-based metabolomics approach illustrated in **Figures 3** and **4** can reveal dynamic information on molecular-level compartmentation, which spatial discrimination-based imaging techniques have yet to achieve.

Figure 3 outlines a typical path for the stable isotope-resolved metabolomics (SIRM) approach that we have developed and applied to cell cultures, excised tissues, whole animals, and human subjects (Fan, Lane et al. 2003; Fan, Bandura et al. 2005; Fan, Kucia et al. 2008; Lane, Fan et al. 2008; Fan, Lane et al. 2009; Fan, Yuan et al. 2010; Fan, Lane et al. 2011; Lane, Fan et al. 2011; Moseley, Lane et al. 2011). The utility of this approach in exploring the toxic action of widely disparate anticancer agents such as cisplatin and sodium selenite is also illustrated. It is interesting to note that based on the ^{13}C -positional isotopomer (cf. **Figure 6**) analysis by NMR, cisplatin treatment of lung cancer A549 cells led to inhibition of ^{13}C label incorporation from uniformly ^{13}C -labeled glucose ($^{13}\text{C}_6\text{-Glc}$) into lactate (glycolytic end product), Glu, citrate, Asp, glutathione (GSH) (derived from the Krebs cycle), Gly, and adenine (AXP) and uracil nucleotides (UXP) (T.W-M Fan and M. Capece, unpublished data). These data suggest that cisplatin may block multiple pathways including glycolysis, Krebs cycle, GSH synthesis, one-carbon metabolism, and nucleotide synthesis, which may or may not be related to its ability to cross-link DNA (Poklar, Pilch et al. 1996). The ability of selenite to inhibit fatty acyl chain biosynthesis but not glycerol backbone incorporation into lipids in A549 cells is revealed by high-resolution Fourier transform-ion cyclotron resonance-MS (FT-ICR-MS) analysis of ^{13}C -mass isotopologues (cf. **Figure 6**) of lipids (**Figure 3**). This result points to one possible mechanism of selenite action, i.e. inhibition of *de novo* fatty acid biosynthesis, which involves two enzymes acetyl CoA carboxylase and fatty acid synthase.

In addition to mapping intersecting pathways to reveal specific site(s) of pathway perturbations, the SIRM or analogous approaches (Lu, Mulder et al. 2002; Jin, Jones et al. 2004; Ronnebaum, Ilkayeva et al. 2006) can help deduce pathways and their fluxes occurring in different cellular compartments or organs without having to maintain the physical integrity of cells or tissues during the measurement. Such large-scale molecular-level compartmentation and dynamics is still impractical to achieve by imaging-based technologies (cf. section IV).

Figure 4 illustrates how tracking the fate of $^{13}\text{C}_6\text{-Glc}$ delineates pathways occurring in the cytoplasm (glycolysis and lipid biosynthesis), mitochondria (Krebs cycle), and extracellular space (excretion of $^{13}\text{C}_3\text{-lactate}$). In particular, two separate pools of pyruvate are distinguished based on the cytoplasmic production of $^{13}\text{C}_3\text{-lactate}$ via LDH and mitochondrial synthesis of $^{13}\text{C}_3\text{-citrate}$ via the anaplerotic PC reaction. A 3rd pool of pyruvate can be rationalized based on the differential effect of selenite on the time course excretion of ^{13}C -lactate and ^{13}C -Ala by A549 cells (**Figure 5**). Both metabolites are derived from pyruvate and yet their excretion to the medium shows opposite behavior in response to

selenite, which indicates that lactate and Ala are synthesized from different pools of pyruvate. This aspect is not apparent in any biochemistry textbook today.

In addition, the production of all ^{12}C - (m+0), $^{13}\text{C}_2$ - (m+2), and $^{13}\text{C}_3$ - (m+3) isotopologues of citrate, Asp, fumarate, and malate from $^{13}\text{C}_6$ -Glc by A549 cells (**Figure 5**) is consistent with the operation of Krebs cycle without and with PCB activity as depicted in **Figure 4**. Note that selenite inhibits the production of $^{13}\text{C}_2$ -citrate, -fumarate, -malate, and -Asp, which suggests perturbations of the Krebs cycle activity. The reduced synthesis of the $^{13}\text{C}_3$ - analogues of these metabolites and citrate in particular (a unique marker of PC) indicate that selenite also interferes with pyruvate carboxylation. Moreover, separate pools of mitochondrial and cytoplasmic citrate and thus acetyl CoA can be deduced from the ^{13}C labeling patterns of the Krebs cycle intermediates and lipids, respectively. Cytoplasmic citrate is cleaved by ATP-citrate lyase to yield acetyl CoA for fatty acyl chain synthesis (**Figure 4**). As indicated above (**Figure 3**), the ^{13}C labeling of lipids in A549 cells derived from $^{13}\text{C}_6$ -Glc is consistent with the presence of two pools of citrate, which are in exchange across the mitochondrial membrane (**Figure 4**).

It should be noted in **Figure 4** that not only the number but also the position of ^{13}C labeled atoms vary according to the transformation pathways. Metabolites with different numbers of ^{13}C atoms are known as ^{13}C mass isotopologues, while those with different positions of ^{13}C atoms are called ^{13}C positional isotopomers, as illustrated in **Figure 6** for Ala. Mass isotopologues and positional isotopomers are best analyzed by MS and NMR, respectively. Both types of information complement each other to provide much more pathway coverage with less ambiguity than each alone. For example, with $^{13}\text{C}_6$ -Glc as tracer, $^{13}\text{C}_2$ -Glu isotopologue is produced via the Krebs cycle regardless of the PCB status (**Figure 4**). However, PCB activity can be discerned by the presence of the positional isotopomer ^{13}C -2,3-Glu versus ^{13}C -4,5-Glu in its absence (**Figures 2 and 4**). As such, NMR and MS analyses are complementary in enabling pathway reconstruction from labeled isotopologue and isotopomer patterns.

Although the stable isotope tracer approach has been applied to biological studies since the early 1900's (Rittenberg and Schoenheimer 1937), the scope of the analysis and thus interpretation have been limited to a few metabolites and pathways at a time, until the advent of metabolomics-based approaches in the 21st century. Two such approaches have been established and applied to studies related to therapeutic and chemopreventive agents, i.e. Stable isotope-based Dynamic Metabolic Profiling (SiDMAP) (Boros, Brackett et al. 2003) and Stable Isotope-Resolved Metabolomics (SIRM) (Fan, Lane et al. 2009). For reasons stated above and throughout this review, both nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) have been the quintessential analytical tools for stable isotope tracer studies.

There are now many different areas of life science research that utilize stable isotope tracers for understanding metabolic pathways and networks ranging from bacteria (Yang, Hua et al. 2002; Zamboni, Fendt et al. 2009), yeasts (Clasquin MF, Melamud E et al. 2011), to animal models (Peltz, He et al. 2005; Yang, Kombu et al. 2008; Fan, Lane et al. 2011; Olszewski, Mather et al. 2011) and humans, as indicated above. In this review, we focus on the applications relevant to pharmacological and therapeutic research. Although nutrition is an important component of human health, and where tracer methods have made seminal contributions, it is beyond the scope of this review. The reader can consult with some excellent recent reviews on this topic (Parks and Hellerstein 2006; Previs, Brunengraber et al. 2009; Zhang, Sadhukhan et al. 2011).

II. Major Analytical Platforms for Stable Isotope Tracer-Based Metabolic Studies

In some studies on stable isotope tracing of metabolism, particularly those involving human subjects, isotope ratio MS (IRMS) has been a popular tool for its ability to detect and quantify a minute level of isotopic enrichment in the parent tracers such as ^{13}C -glucose, ^{13}C -cholesterol, ^{13}C , ^{15}N -amino acids and/or their labeled metabolites (Kalhan, Bier et al. 1980; Storch, Wagner et al. 1990; Yu, Wagner et al. 1990; Ostlund and Matthews 1993; Chen, Collins et al. 2005). However, this technique requires extensive isolation and sometimes even chemical degradation of the labeled metabolites before analysis can be performed (Zhang, Trierweiler et al. 1999), which precludes its application for simultaneous tracking of a large number of specifically labeled metabolites in metabolomics-based tracer studies.

Meanwhile, the isotopic ratio precision of structure-specific NMR has been shown to rival or exceed that of the structure-blind IRMS (Zhang, Trierweiler et al. 1999), and the molecular formula-specific, high-resolution accurate mass MS (e.g. Fourier transform ion cyclotron resonance-MS or FT-ICR-MS) also appears to have outstanding isotopic ratio capabilities (Lane, Fan et al. 2009). Thus, NMR, chromatography-based MS, and more recently direct infusion FT-ICR-MS, can be excellent tools for isotope ratio determination.

NMR is an extremely powerful tool for elucidating organic structures at the atom position-specific level. The versatility of multi-nuclear and multi-dimensional NMR techniques developed over the past few decades have enabled reliable and rigorous metabolite profiling of crude biological extracts without the need for sample fractionation (Fan 1996; Fan 1996; Fan and Lane 2008). Since many stable isotopes of biological importance (e.g. ^2H , ^{11}B , ^{13}C , ^{15}N , ^{77}Se) are NMR-observable and have low background of natural abundance, metabolites enriched in these isotopes can be readily tracked by NMR both in terms of their molecular structures and the positional isotopomers (**Figure 6**). Moreover, NMR measurement is non-invasive, which makes it a unique modality for *in vivo* or *in situ* metabolic analysis, particularly for pathway dynamics and real-time imaging (cf. section IV).

In addition to NMR, spectral MS (which excludes IRMS because it is a non-spectral technique) analysis is frequently used to achieve comprehensive metabolite profiling (Fan, Lane et al. 2004; Lane, Fan et al. 2008; Lane, Fan et al. 2008). NMR and MS are both complementary and confirmatory in terms of metabolomics investigations. While NMR is by far the most powerful tool in providing structural identity and positional labeling information, it is limited by sensitivity (e.g. $>1\text{-}2\text{ nmol}$ metabolite for ^1H -NMR detection), structural resolution in case of certain structures (such as highly redundant methylene groups in a long fatty acyl chain), insensitivity to NMR-inactive nuclei (e.g., ^{16}O and ^{32}S) or paramagnetically influenced nuclei (Lane, Fan et al. 2008). These limitations can be overcome by the complementary use of MS in confirming the metabolite identity and quantification by NMR, while extending the metabolite coverage beyond NMR analysis alone. Equally important is the ability of MS to supply mass isotopologue distribution, with high sensitivity and resolving power. Together with the positional isotopomer information from NMR, such capability enables a global reconstruction of metabolic pathways (Fan, Yuan et al. 2010) and quantification of pathway fluxes using flux modeling approaches (Moseley, Lane et al. 2011).

However, SIRM studies ideally require global metabolite coverage of all possible isotopomers and isotopologues. This, means that, for example, the ^{13}C mass spectral peaks must be free of all other elemental isotopologues (e.g. ^2H , ^{15}N , ^{18}O) in order to accurately

remove ^{13}C natural abundance contributions for net enrichment determination (Moseley 2010). Until recently, this basic premise has not been emphasized because of the lack of MS instrument resolution and mass accuracy, which cannot be circumvented by other means such as chromatography. The advent of “user friendly”, modern Fourier-transform (FT) MS instruments has delivered the necessary performance to globally measure such isotopically pure peaks (Pingitore, Tang et al. 2007; Lane, Fan et al. 2009; Moseley 2010). These FT-MS instruments, principally FT-ICR-MS, are capable of achieving ultra-high resolving power of 400,000 or more (at 400 m/z, 10% valley) and mass accuracies better than 0.2 ppm (i.e. 0.00008 Da @ 400 m/z). This level of performance enables unambiguous detection and quantification of thousands of metabolites and all their isotopologue species simultaneously in a high throughput fashion (e.g. < 5 min per sample) (Pingitore, Tang et al. 2007; Lane, Fan et al. 2009) (cf. **Figure 7**). As of this writing, another major type of FT-MS, Kingdon-Makarov trap MS (OrbitrapTM), are not yet available with resolutions sufficient to meet this need.

Recently, the biochemoinformatics potential of such high level performance by FT-ICR-MS was revealed for quantitatively tracking multiple convergent pathways by analyzing the ^{13}C isotopologue distribution of just a single metabolite, UDP-GlcNAc (Moseley, Lane et al. 2011). It is reasonable to expect that since hundreds of other metabolites and their isotopologues are also measured simultaneously, the information density from FT-MS analysis of stable isotope tracer-based experiments is immense, and appears sufficient to interpret multiple intersecting events at the network level.

II.1. Separation-based MS

Insufficient mass resolution of conventional MS instruments is a major impetus for coupling MS to separations technology to improve compound resolution (Watson 1985). Today, separation-based MS techniques are commonly used in stable-isotope tracer experiments. Coupling chromatographic separation with MS allows for highly sensitive (pM) detection (Shimizu and Matsuoka 2010; Psychogios, Hau et al. 2011) with added resolving dimensions that significantly exceed the performance of standalone MS instruments (Lane, Fan et al. 2008).

The oldest of the separation-based instruments, GC-MS, is a well-established technique for metabolite structure confirmation and quantification (Fan, Higashi et al. 1986; Lane, Fan et al. 2008), and it has been utilized in SIRM extensively (Boros, Brackett et al. 2003; Des Rosiers, Lloyd et al. 2004; Fan, Lane et al. 2009). This is in large part due to the highly mature derivatization technology that enhances the volatility of many polar metabolites, a prerequisite for any GC-based analysis. In addition, GC-MS such as 2-D GC-TOF (time of flight)-MS posts the highest chromatographic resolution among the different platforms of separation-based MS (Lidstrom and Guo 2008; Sumner and Bedair 2008), but remains unproven for SIRM studies. Among the many GC derivatization probes available, silylating reagents such as MTBSTFA (N-methyl-N-(tertbutyldimethylsilyl) trifluoroacetamide) are the most useful ones due to the ability for simultaneous derivatization across multiple functional groups (Fan, Colmer et al. 1993).

However, it is becoming clear that GC-based techniques are of limited utility to fill the extremely wide metabolic coverage needs of metabolomics. Without prior degradation – which may destroy critical isotopologue information – major classes of metabolites cannot be made sufficiently volatile for GC-MS analysis; these include many higher molecular mass metabolites such as polar lipids, nucleotides, oligosaccharides, peptides, etc. and metabolites that are permanently charged such as cholines (Fan, Lane et al. 2004). Thus the future utility of GC-MS in metabolomics and especially for stable isotope tracer studies will mainly reside in targeted metabolic studies.

In contrast, high-performance liquid chromatography (HPLC) coupled to various MS platforms has no such limitations, and recently has become a powerful and commonly employed method in both “unbiased” global and targeted metabolite profiling studies (Want, O'Maille et al. 2006; Frezza, Zheng et al. 2011). HPLC-MS applications in metabolomics studies have been reviewed extensively elsewhere (Chen, Gonzalez et al. 2007; Metz, Zhang et al. 2007; Ceglarek, Kortz et al. 2011; Theodoridis, Gika et al. 2011).

Despite the proven values of GC-MS and HPLC-MS in metabolite profiling, there is one major drawback to consider for accurate stable isotope ratio analysis in SIRM studies. The MS signals of analytes acquired from chromatographic separation are transient in nature (in milliseconds to seconds range), which is incompatible with the need for long signal averaging to obtain more precise isotopic profiles. This issue, together with the desire for high sample throughput, predicate the development of direct sample introduction in conjunction with high-resolution MS (Aharoni, Ric de Vos et al. 2002; Lane, Fan et al. 2009; Lane, Fan et al. 2011), particularly for stable isotope tracer studies (Pingitore, Tang et al. 2007; Lane, Fan et al. 2009; Moseley, Lane et al. 2011).

II.2. Direct MS: Continuous Ion Source

In lieu of the separation techniques described above, direct sample introduction MS analyses have been successfully and preferably employed for SIRM investigation of crude extracts (Pingitore, Tang et al. 2007; Lane, Fan et al. 2009; Moseley, Lane et al. 2011). It is clear that FT-MS is the only category of MS instruments that can achieve the need for unambiguous distinction of molecular formulae and all possible elemental isotopologues, a problem not addressable by chromatographic separation (Pingitore, Tang et al. 2007; Lane, Fan et al. 2009; Moseley, Lane et al. 2011). By coupling with continuous infusion, FT-MS can achieve high sensitivity and greater dynamic range through signal averaging, which also enables far more precise isotope ratio determination (Lane, Fan et al. 2009) than is possible from separation-based MS. Among continuous infusion ion sources, an excellent choice is the nanoelectrospray source, which greatly reduces sample consumption while enhancing the sensitivity of detection. Another added advantage of long continuous delivery of analyte ions (for up to 1 h) is the enhanced performance on tandem MS experiments such as MS² or even MSⁿ (n>2) for detailed structural confirmation or positional isotopomer determination (Pingitore, Tang et al. 2007).

Since direct continuous infusion demands high MS resolution for SIRM studies, the question is how high is sufficient resolution? We have tested this with a 7 Tesla FT-ICR-MS instrument housed in our Center for Regulatory and Environmental Analytical Metabolomics (CREAM). **Figure 7** demonstrates that glutamate simultaneously labeled with ¹³C, ¹⁵N, and ²H can be clearly distinguished from all other metabolite isotopologues present in a crude cancer cell extract. This spectrum was acquired with a mass resolving power of 1:400,000, which is routinely achieved by this instrument. Such mass resolution also allows practically all mammalian lipid species to be resolved in a single run (Lane, Fan et al. 2009). In addition, the high precision of FT-ICR-MS for isotope ratio analysis enables reliable natural abundance isotopic stripping (Moseley 2010). As described above, this level of resolving power and precision for isotope ratio is “sufficient” for deconvoluting multiple converging biosynthetic pathways based on the isotopologue distribution of a single metabolite (Moseley, Lane et al. 2011). As FT-ICR-MS spectra typically contain hundreds of other metabolites with their thousands of isotopologue peaks, the reconstruction of multiple metabolic pathways and modeling fluxes through the metabolic network appears to be within reach. However there are still many technical challenges in analyzing the extremely rich FT-ICR-MS spectral data, which may be overcome by future chemoinformatic developments (cf. section V)

II.3. Direct MS: Discontinuous Ion Source

In addition to electrospray-based ion introduction techniques, Matrix Assisted Laser Desorption Ionization (MALDI) is another direct introduction technique that allows for simultaneous detection of multiple metabolites from crude extracts. Here the metabolite ions are generated by irradiating the MALDI matrix and sample pre-concentrated on a small area of a conductive plate placed in high vacuum or in atmospheric pressure conditions. This approach is generally more tolerant to higher salt content samples than the electrospray methods, which makes it intrinsically compatible with complex biological mixtures. However, high chemical background from the matrix can interfere with the analysis of a significant fraction of the metabolites (Strupat, Karas et al. 1991; Beavis and Bridson 1993; Shroff, Rulisek et al. 2009). More recently, new MALDI matrices have been developed to minimize this issue (Shroff, Rulisek et al. 2009; Miura, Fujimura et al. 2010).

There have been a few applications of MALDI-MS to stable-isotope tracer based studies. For example ^{13}C flux analysis was performed with MALDI-TOF-MS for bacterial species *Corynebacterium Glutamicum* (Wittmann and Heinzle 2001) and other microorganisms (*S. cerevisiae*) (Hollemeyer, Velagapudi et al. 2007). There have also been some applications of MALDI-MS for stable isotope studies in mammalian cells. Estrada et al. analyzed simultaneously the incorporation of D_9 -choline into human lens epithelial cell lipids (Estrada, Borchman et al. 2006). The changes in the rates of label incorporation into phosphatidylcholine (PCh) and sphingomyelin (SM) headgroups were consistent with PCh synthesis preceding SM synthesis (Estrada, Borchman et al. 2006). However there are no comparable applications in human or model animal tissues, yet there are no intrinsic barriers for the application.

An interesting potential of the MALDI ion source has been metabolite imaging across the surface of tissues or in cells (Hsieh, Casale et al. 2006; Cornett, Reyzer et al. 2007; Reyzer and Caprioli 2007; Signor, Varesio et al. 2007; Stoeckli, Staab et al. 2007; Caprioli, Cornett et al. 2008). Again, there are no applications shown for stable isotope labeled tissue imaging in human or model animals, but there is no known barrier to such use. Currently, the spatial resolution of imaging-MALDI lies between a few μm and ca. 100 μm depending on the instrumental setup and the MALDI matrix (Heeren and Chughtai 2010). It is not trivial to achieve further improvements in spatial resolution because of detection limit problems of small sample size ionized by very thin laser beams (Dorrestein and Watrous 2011) and wavelength limits on spot size. However, it is expected that this will improve to nm resolution in the future, so that, spatial discrimination of subcellular compartments or organelle by MALDI-MS imaging coupled with stable-isotope tracer experiments may become an important tool in tracking localized changes in metabolic pathways. As discussed previously and shown in **Figures 4 and 5**, stable isotope tracer experiments can already reveal compartmentation information from tissue or cell extracts without any explicit spatial resolution. In conjunction with stable isotope tracer approach, MALDI imaging stands poised to provide metabolic network information as a function of tissue heterogeneities and even intracellular compartmentation *in-situ* without the need for metabolite extraction.

II.4. Tandem MS

In order to have the widest coverage of metabolite isotopologues, the main focus of MS in SIRM studies has been to obtain molecular ion data using one-dimensional MS mode of operation. However, there are cases where more detailed, sub-molecular structural information may be desired such as for structural confirmation or determination of positional isotopomers. Analysis by tandem MS including two-dimensional ($\text{MS}/\text{MS} = \text{MS}^2$) or higher dimensional (MS^n , $n > 2$) MS meets such demands, especially in conjunction with high-resolution FT-ICR-MS (Pingitore, Tang et al. 2007). Tandem MS accomplishes

this by generating daughter mass fragments from the isolated molecular or parent ion, which is a routine operation on all of the commercial FT-MS instruments. For example, Pingitore et al. (Pingitore, Tang et al. 2007) utilized multiple MS² mode of FT-ICR-MS under direct continuous infusion to obtain the ¹³C isotopomer labeling pattern of amino acids from soil bacterium *Desulfovibrio vulgaris* growing on [1-¹³C] lactate. However presently there is no equivalent work on human or model animal systems. Using low resolution multiple tandem MS measurements for each observable labeled metabolite, a workflow was developed for metabolomics flux analysis in heart tissue (Antoniewicz and Choi 2011), but the approach is not amenable for SIRM studies under non-steady state conditions (cf. Section V).

Besides utility in endogenous metabolic studies, MSⁿ techniques have long been used in stable-isotope tracer experiments for tracking drug metabolism (Watson 1985). This is exemplified by Mutlib et al. who used LC/MS² to identify the metabolites of deuterium labeled benzylamine in bile and urine samples (Mutlib 2008). Since tandem MS is much more time-consuming than 1D MS, the MSⁿ approach is very practical for drug metabolism studies because of the very limited analyte list. This is diametrically opposed to the analytical needs of endogenous metabolomic studies, which have an open-ended analyte list.

II.5. Applications of MS to Stable Isotope Tracer Studies

II.5.1. General Applications—Since the early 1980's, MS has been applied to studying drug metabolism (Brazier, Ribon et al. 1980) and endogenous metabolic pathways and fluxes through the use of various isotopically enriched tracers (Wolfe 1984). As outlined in **Figure 3**, such studies require the administration of a labeled precursor (e.g. ¹³C labeled glucose) to cells, tissues or whole organisms. The resulting labeled xenobiotic and/or endogenous cellular metabolites are then identified by different platforms of MS (and often in combination with NMR), which can also provide unique patterns of mass isotopologues of the drug adducts and degradation products for subsequent reconstruction of relevant “metabolic fate” pathways (Fischer and Sauer 2003; Lane, Fan et al. 2008).

II.5.2. MS and SIRM applications to cancer cell metabolism—Stable isotope tracer studies that utilize MS (frequently in conjunction with NMR) have been conducted to identify key pathways altered by disease development, from which potential drug targets can be uncovered for clinical intervention. The same approach is also valuable for validating on-target effect and revealing off-target toxicity. As stated in the Overview, understanding the human metabolome including compartmentation and dynamics are vital to gaining insight into the pathogenesis of diseases, particularly for cancer, diabetes and other metabolic disorders. Described below are a few examples of studies that employed stable-isotope labeled tracers and MS to determine the distributions of labeled metabolite isotopologues and therefore the transformation pathways of the tracers in various types of cancer cells.

Boros et al used [¹³C₂-1,2]-glucose as a tracer and GC-MS extensively to probe fluxes through the pentose phosphate pathway (PPP), fatty acid synthesis, and glycolysis in tumor cells and fibroblasts, which led to the discovery on the role of thiamine deprivation in stimulating cell apoptosis (Boros, Brackett et al. 2003). A similar SiDMAP study using GC-MS assessed the effect of a glycogen phosphorylase inhibitor on pancreatic tumor cells and found that interference with glycogen mobilization led to inhibition of anabolic metabolism into macromolecules and apoptosis (Lee, Guo et al. 2004). Perturbed fluxes through PPP as well as synthesis of nucleic acids and fatty acids were also observed in myeloid leukemia cells treated with Gleevec and Isofenphos, which could constitute the mechanism of growth inhibition with these drugs (Boren, Cascante et al. 2001; Boros, Cascante et al. 2002; Boros, Lee et al. 2002). Harris et al used ¹³C glucose tracer and GC-MS to investigate the effect of fatty acid synthase inhibitor C75 and a panel of flavonoids on metabolic pathways of

pancreatic tumor cells MIA PaCa2. The pools of ^{13}C labeled palmitate and cholesterol were measured and the authors concluded that the naturally occurring luteolin was a superior inhibitor of fatty acid synthase than the targeted C75 drug. This study therefore demonstrated the utility of the targeted SIRM approach to assess differential drug response (Harris, Li et al. 2011).

Sabatini's group (Possemato, Marks et al. 2011) used a RNAi knockdown screening protocol on an orthotopic xenograft mouse model with human breast cancer cells to identify the importance of overexpression of phosphoglycerate dehydrogenase (PHGDH) for proliferation. PHGDH is the first committed step in serine biosynthesis. Using $^{15}\text{N}_2\text{-Gln}$ and $^{13}\text{C}_5\text{-Gln}$ as tracers and LC-MS analysis, it was shown that shunting 8-9% of the glycolytic flux through serine pathway contributed about 50% of the net production of 2-oxoglutarate in these tumors.

Some selenium-containing compounds have shown efficacy against cancers (Ip 1998; Hu, Jiang et al. 2005) (Clark, Combs et al. 1996; Fan, Bandura et al. 2005; Suzuki 2005). Fan et al. combined GC-MS with NMR to study the response of lung cancer cells to anti-cancer selenium compounds (Fan, Bandura et al. 2005; Fan, Higashi et al. 2006; Fan, Tan et al. in press, 2011). NMR was used to determine the positional isotopomers, while the absolute concentrations of metabolites and their isotopologue distributions were determined by GC-MS. This combined approach allowed multiple pathways in lung cancer cells impacted by Se compounds to be reconstructed in an unbiased manner. They found that the toxicity of selenite and methylseleninic acid to lung cancer cells involved multiple targets spanning across glycolysis, PPP, the Krebs cycle, and the biosynthesis of glutathione, fatty acids (**Figure 3**), and nucleotides. The efficacy of different Se compounds also depends highly on the chemical form, pointing to variations in the uptake and/or metabolism to the active form by cancer cells. The metabolic perturbations induced by Se forms were further linked to gene expression changes via Metabolomics-Edited Transcriptomic Analysis (META) (Fan, Bandura et al. 2005; Fan 2010) so that metabolic dysregulations at the transcriptional level were revealed.

II.5.3. MS and SIRM applications to central nervous systems—Apart from applications for cancer drug targets, the effect of drug treatments or disorder on the central nervous system have been a subject of many investigations involving stable isotope labeled tracers and MS (Schousboe, Waagepetersen et al. 2000; Dusick, Glenn et al. 2007; Fan, Yuan et al. 2010). For example the SIRM approach was used for probing metabolic pathway changes induced by lithium salt in rat astrocytes and neuronal cells (Fan, Yuan et al. 2010); Lithium is commonly used in the treatment of bipolar disorders (Yuan, Huang et al. 2001; Quiroz, Gould et al. 2004; Lan, McLoughlin et al. 2009). Both GC-MS and NMR were employed to follow the fate of three different ^{13}C -labeled tracers ($^{13}\text{C}_6\text{-glucose}$, $^{13}\text{C}\text{-3-lactate}$ or $^{13}\text{C}\text{-2,3-alanine}$). The study found that lithium enhanced carbon flow through glycolysis and part of the Krebs cycle in both cell types. In particular, lithium stimulated the anaplerotic PC pathway in both cell types, which may facilitate neurotransmitter Gln synthesis while maintaining energy production. Moreover, the release of fuel substrates by astrocytes and their subsequent metabolism by neurons for energy and neurotransmitter production were enhanced by lithium. Similar studies used $^{13}\text{C}_6\text{-glucose}$ tracer with MS and NMR to track the changes in the Krebs cycle in cortical neurons induced by depolarization (Schousboe, Waagepetersen et al. 2000). The differential labeling patterns of fumarate and Asp led to the hypothesis on the existence of distinct Krebs cycles with different turnover rates (compartmentation of the Krebs cycle) in the mitochondria.

II.5.4. MS and SIRM applications to whole model animals and humans—

Since ^{13}C or ^{15}N stable isotopes are naturally occurring, ^{13}C or ^{15}N labeled nutrients should

pose no toxicity to whole organisms and can be administered to human subjects for tracking metabolic pathways *in vivo*. Recently, Beger and colleagues (Beger, Hansen et al. 2009) introduced $^{13}\text{C}_6$ -glucose intraperitoneally into mice to monitor the influence of valproate (an anticonvulsant) on cholesterol, glycogen and ribose metabolism in mice. Mass isotopologue analysis by GC-MS was carried out on blood plasma, urine, and different tissues to reveal a decreased reabsorption of glucose through the kidney, reduced synthesis of glycogen and ribose RNA by the liver, and reduced carbon flux into the plasma and kidney cholesterol pools. In another recent study, Fan and co-workers intravenously infused $^{13}\text{C}_6$ -glucose tracer into non-small cell lung cancer (NSCLC) patients and followed the incorporation of ^{13}C into various metabolites of cancerous versus non-cancerous (NC) lung tissues using the SIRM approach (**Figure 3**). Based on the specific ^{13}C labeling patterns of the Krebs cycle metabolites, they found that the Krebs cycle was more active in cancerous lung than NC. They also uncovered a novel activation of pyruvate carboxylation (PC) (**Figure 4**) in patient lung cancer tissues and that such activation was regulated at the gene expression level (Fan, Lane et al. 2009; Lane, Fan et al. 2011). This SIRM study demonstrated the feasibility of directly working with human subjects for pathway resolution and metabolic target discovery, thus the promise for target validation and off-target assessment in human subjects is self-evident.

A study by Dusick et al. infused $^{13}\text{C}_2$ -1,2-glucose into patients with traumatic brain injury and control subjects. By examining the ^{13}C labeling patterns in plasma lactate, they uncovered an enhanced PPP flux associated with increased glucose uptake exhibited by brain injury patients (Dusick, Glenn et al. 2007). Such investigations promise to provide new functional insights into brain disorders, thereby revealing new targets for therapeutic interventions, particularly with studies performed on human subjects since it is difficult to emulate human brain disorders with animal models.

II.5.5. MS applications to ADMET—MS-based stable isotope tracer studies have also been conducted to evaluate the metabolic fate of therapeutic agents and their pharmacokinetics (Pons and Rey 1999; Mutlib 2008). A common approach is to introduce a mixture of labeled and unlabeled drug into the system, followed by probing their metabolites in biological fluids using appropriate MS platforms, e.g. HPLC-MS. MS detects pairs of companion peaks separated by a mass difference of the labeled isotope, which enables easy assignment of the parent drugs and their metabolites (Brazier, Ribon et al. 1980; Mimura and Baba 1980; Brazier, Salle et al. 1981; Mimura and Baba 1981; Goromaru, Matsuura et al. 1982; Goromaru, Matsuura et al. 1984; Mutlib and Nelson 1990; Mutlib and Nelson 1990; Borel and Abbott 1993; Tonn, Mutlib et al. 1993; Mutlib 2008). A similar approach involves the use of labeled precursors to assess if a particular interaction between the precursor and the drug occurs. An example study employed ^{13}C labeled GSH to identify glutathionylated drug conjugates (Mutlib, Shockcor et al. 2000; Yan and Caldwell 2004; Mutlib, Lam et al. 2005; Mutlib 2008). Another interesting approach of studying drug metabolism involves administering the ^{13}C labeled drug, followed by analyzing the ^{13}C enrichment of the expired CO_2 gas. If the ^{13}C label at specific atomic position(s) of the drug is metabolized via methylation or decarboxylation into formaldehyde, formic acid, formyltetrahydrofolate, and CO_2 , then the CO_2 released will be enriched in ^{13}C . Such measurement in conjunction with analysis of the drug clearance from biological fluids can serve to determine the enzyme activities responsible for the demethylation/decarboxylation reactions. A more in-depth description of such applications has been reviewed elsewhere (Pons and Rey 1999).

In a recent review by Chen et al., LC-MS based metabolomics approaches (without tracer use) were outlined for ADMET studies (Chen, Gonzalez et al. 2007). The principle approach is comparative and differential metabolite profiling, and statistically linking that to drug-

related events and xenobiotic metabolism, i.e. ADMET. However there is no mention of the stable isotope tracer-based metabolomic approaches to elucidating pathways and networks, although there is no technical barrier to extending the profiling workflow to SIRM studies.

From the above examples of applications, we hope to provide readers with a glimpse of the value and versatility of MS in stable isotope tracers-based metabolomic studies in the field of pharmacology and therapeutics. With continuing advances in MS technologies, particularly the high resolution, accurate mass instrumentation, coupled with biochemoinformatic developments and complementary use of NMR (section III), there can be quantum leaps in our understanding of human diseases and ability to develop highly efficacious and individualized therapeutics.

III. Application of NMR to Stable Isotope Tracer Studies

III.1. In vitro NMR approach

NMR has a long history in analytical and natural products research, and along with MS and single crystal x-ray diffraction it has become one of the main analytical tools for structure analysis (Pretsch, Bühlmann et al. 2009). The general principles and approaches have been described in great detail in many excellent texts (Bax, Freeman et al. 1981; Sanders and Hunter 1987; Claridge 1999; Pochapsky and Pochapsky 2007). Although most such texts discuss analysis of pure compounds, NMR in fact can easily be applied to mixtures, which has a number of advantages for biological studies, particularly for the stable isotope tracer approach, as described in the Overview (Fan and Lane 2008; Lane, Fan et al. 2008).

The majority of stable-isotope tracers applications in metabolism have used 1D methods, especially direct observation of ^{13}C and making use of the ^{13}C - ^{13}C coupling for isotopomer analysis. Such approaches have been successfully applied to many different disease states (Carvalho, Babcock et al. 1999; Zwingmann, Richter-Landsberg et al. 2001; Lu, Mulder et al. 2002; Mason, Petersen et al. 2002; Burgess, Weis et al. 2003; Mason, Petersen et al. 2003; Wang, Lloyd et al. 2003; Zwingmann and Leibfritz 2003; Anousis, Carvalho et al. 2004; Carvalho, Rodrigues et al. 2004; Cline, LePine et al. 2004; Des Rosiers, Lloyd et al. 2004; Goddard, Mason et al. 2004; Jin, Jones et al. 2004; Mason and Rothman 2004; Sherry, Jeffrey et al. 2004; Des Rosiers and Chatham 2005; Jin, Jones et al. 2005; Patel, de Graaf et al. 2005; Wang, Lloyd et al. 2005; Hausler, Browning et al. 2006; Hyder, Patel et al. 2006; DeBerardinis, Mancuso et al. 2007; Mason, Petersen et al. 2007; Fan and Lane 2008; Morrish, Neretti et al. 2008; Morrish, Noonan et al. 2010). However, the sensitivity and information content is significantly less than using 2D methods with proton detection (Fan 1996; Fan, Higashi et al. 1997; Carvalho, Jeffrey et al. 1998; Carvalho, Zhao et al. 2001; Lloyd, Zeng et al. 2004; Lane and Fan 2007).

The identification of various components in a mixture is greatly facilitated when using 2D NMR experiments that detect interactions between atoms within a molecule through the covalent network. These include homonuclear experiments such as ^1H COSY and TOCSY, and heteronuclear correlation experiments such as ^1H - ^{13}C HSQC/HMQC, HMBC and variants that combine two or more experimental types such as HSQC-TOCSY (Fan, Higashi et al. 1986; Fan 1996; Willker, Engelmann et al. 1996; Fan and Lane 2008; Fan, Kucia M. et al. 2008; Lane, Fan et al. 2008; Fan and Lane 2011; Fan and Lane 2012; Lane 2012). 2D NMR experiments are therefore more rigorous in metabolite identification and offer superior resolution than 1D NMR methods. The HSQC-based experiments are especially valuable for analyzing ^{13}C positional isotopomers of metabolites (**Figures 3 and 6**), because of the nuclear editing capabilities of NMR. Namely, a molecule that contains atoms enriched with ^{13}C or ^{15}N can be selectively observed in a mixture, and identified on the basis of their chemical shifts and covalent linkages expressed as the ^{13}C - ^1H (e.g. HSQC) and ^{13}C - ^1H - ^1H

(e.g. HSQC-TOCSY) covalent correlations in the 2D data (Fan, Higashi et al. 1997; Fan and Lane 2011).

III.2. Target identification and verification by NMR profiling

There have been numerous SIRM-based studies that make use of NMR (and often MS) for drug target identification and validation in cultured cells and animal models. Based on the SIRM analysis of an experiment designed to inhibit LDH-A in breast cancer cells (which is known to be required for tumorigenesis (Fantin, St-Pierre et al. 2006; Le, Cooper et al. 2010)), Chesney's group showed that the LDHA inhibitor oxamate also impacted aspartate metabolism via the OAA/Glu transaminase, suggesting that such aminotransferases might be legitimate targets in some cancers (Thornburg, Nelson et al. 2008). These authors have also used SIRM to evaluate potential targets including choline kinase and phosphofructokinase 2, uncovered by both genetic and metabolic analyses with small molecule inhibitors specifically designed from *in silico* modeling (Telang, Yalcin et al. 2006; Clem, Telang et al. 2008; Dailey, Hait et al. 2009; Yalcin, Clem et al. 2009; Yalcin, Clem et al. 2010; Clem, Clem et al. 2011). In addition to target validation, the SIRM approach is excellently suited for assessing the off-target effects and their origin, because of the breadth in metabolic coverage with this approach (Fan and Lane 2008; Fan and Lane 2011).

III.3. NMR profiling of genetic defects in metabolism and therapeutic effects

Inborn errors of metabolism have been characterized in great detail (Wevers, Engelke et al. 1994). Less known are the germline mutations in metabolic enzymes that have been associated with cancer. These include the familial cancers with loss-of-function mutations in fumarate hydratase (FH) or succinate dehydrogenase (SDH), leading to severe defects in mitochondrial metabolism (King, Selak et al. 2006; Tennant, Duran et al. 2010), yet the resulting renal cancers grow more aggressively (Frezza, Pollard et al. 2011). Due to this defect, the tumor cells are presumably more dependent on glycolysis for energy. However, a glycolysis inhibitor, 2-deoxyglucose (2-DG), was tested on a patient with FH-deficient papillary renal carcinoma, but the treatment failed (Yamasaki, Tran et al. 2011). 2-DG appears to be more effective for hypoxic tumors, and its mode of action may not be mediated through glycolysis, but rather via the unfolded protein response (Kurtoglu, Gao et al. 2007; Kurtoglu, Maher et al. 2007; Boutrid, Jockovich et al. 2008). Clearly this demonstrates the importance of prior SIRM-based metabolic analysis before appropriate therapeutics may be designed. Indeed, a recent article reported that FH(-) mouse kidney cells utilized the Gln to heme synthesis and degradation pathway to permit a partial functioning of the mitochondrial Krebs cycle and suggested that this pathway may be a potential target for treating FH(-) cancer (Frezza, Zheng et al. 2011).

Mutations in isoforms of isocitrate dehydrogenase (IDH) have recently been found in a few cancers (Dang, White et al. 2009; Dang, Jin et al. 2010; Gross, Cairns et al. 2010). The mutations result in a change of function, i.e. the IDH variant now catalyzes the reduction of α -ketoglutarate (α -KG) to 2-hydroxyglutarate (2-HG), using NADPH as the hydride donor. 2-HG builds up to a very high concentration (Dang, White et al. 2009) and may act as a competitive inhibitor of prolyl hydroxylases for which α -KG is a co-substrate (Xu, Yang et al. 2011), thus preventing VHL (von Hippel-Lindau) binding to HIF for proteasomal degradation (Pan, Mansfield et al. 2007). Based on [U-¹³C]-Gln labeling studies, 2-HG was derived mainly from Gln, although central metabolism did not appear to be perturbed (Dang, White et al. 2009). However more recently, widespread changes in central metabolism were reported by profiling steady-state levels of a wide range of metabolites, although the authors indicated that stable isotope labeling studies are essential to define fully the metabolic consequences of the mutations (Reitman, Jin et al. 2011). As 2-HG is derived from Gln via glutaminase, inhibition of this enzyme should be a direct means to maintain a low

intracellular concentration of 2-HG. A glutaminase inhibitor (BPTES) has indeed been shown to inhibit the growth of glutamine-dependent IDH1 glioblastoma cells (Seltzer, Bennett et al. 2010).

It is also possible for isozymes of IDH to catalyze the reductive carboxylation (RC) of α -KG to isocitrate. This reaction is usually considered to be essentially irreversible. However, as the equilibrium constant of the decarboxylation reaction is near 1 M (Londesborough and Dalziel 1968), the reaction can be reversed, provided that the NADPH/NADP⁺ concentration ratio is high, the isocitrate/ α -KG ratio is low, and there is adequate dissolved CO₂ present. RC can occur in both mitochondria and the cytoplasm. In the cytoplasm, the presence of both IDH1 isoform and aconitase can convert α -KG to citrate via isocitrate without going through the mitochondrial Krebs cycle; citrate is the precursor of acetyl CoA (via ATP-dependent citrate lyase) for many acetylation reactions, as well as fatty acid biosynthesis. The RC reaction has been demonstrated in several tissues, including liver (Holleran, Briscoe et al. 1995) and brown adipose tissues (Yoo, Antoniewicz et al. 2008). When coupled with glutaminase, RC could bypass defect(s) in the Krebs cycle such as FH deficiency to supply cancer cells with citrate for acetyl CoA production and fatty acid synthesis.

It is clear that tissue specific expression of metabolic isozymes is widespread (Aledo, Gomez-Fabre et al. 2000), as is differential compartmentation of isozymes, such as mitochondrial and cytoplasmic forms of transaminase, malic enzyme, IDH, aconitase, etc. Cancers in particular are associated with altered expression of different isoforms of metabolic enzymes, such as several of the glycolytic enzymes, which makes them potential drug targets (Pelicano, Martin et al. 2006; Mathupala, Colen et al. 2007; Vander Heiden, Christofk et al. 2010; de Atauri, Benito et al. 2011). One example is the alternatively spliced pyruvate kinase. The M1 isoform of this enzyme is usually expressed in somatic tissue but in cancers the M2 splice variant is preferentially expressed (Elbers, Vanunnik et al. 1991; Mazurek, Grimm et al. 2000). PKM2 can exist as the essentially inactive dimers, or as the active tetramers (Mazurek, Grimm et al. 2002). It has been argued that the inactive form of PKM2 predominates in cancers, leading to a buildup of glycolytic intermediates prior to PEP, and the lack of lactate production from glucose. Instead, glutamine oxidation and conversion to pyruvate was proposed to account for the very high yield of lactate in tumors (Mazurek and Eigenbrodt 2003). However, SIRM experiments on different cancer cell lines show conclusively that nearly all of the lactate produced by cancers cells was derived from glucose, not from glutamine, and the same is true for tumors (DeBerardinis, Mancuso et al. 2007; Fan, Kucia M. et al. 2008; Fan, Lane et al. 2009; Fan, Tan et al. 2011; Moseley, Lane et al. 2011). Recently it was shown that a novel reaction in which PEP phosphorylates the active site histidine residue of the preceding enzyme, phosphoglycerate mutase (PGM), thereby releasing pyruvate for lactate production. This altered glycolytic pathway occurs at the expense of one less ATP produced per mol glucose consumed (Heiden, Locasale et al. 2010) and it is unclear on the overall contribution of the PGM phosphorylation to the enormous amount of lactate production by cancer cells. Nevertheless, glutamine metabolism is critical for many tumor cell types in terms of their survival and growth (Newsholme, Crabtree et al. 1985; Neermann and Wagner 1996; Cline, LePine et al. 2004; DeBerardinis, Mancuso et al. 2007; Donadio, Lobo et al. 2008; Serres, Raffard et al. 2008; Yoo, Antoniewicz et al. 2008; Yuneva 2008; Dang 2010; Seltzer, Bennett et al. 2010; Dang, Hamaker et al. 2011) (DeBerardinis, Mancuso et al. 2007; Yuneva, Zamboni et al. 2007; DeBerardinis, Lum et al. 2008; Morrish, Neretti et al. 2008; Wise, DeBerardinis et al. 2008; Dang, Le et al. 2009; DeBerardinis and Cheng 2010). Again, SIRM studies should facilitate the unraveling of the functions of glutamine metabolism in tumor development.

Furthermore, the SIRM approach is valuable in discerning the metabolic perturbations induced by a synergistic interaction of different therapeutic agents. For example, we recently conducted a SIRM study on the combined effect of anti-cancer agents paclitaxel and methylseleninic acid (MSA) on lung cancer cells. This combination has been reported to synergize apoptosis in prostate cancer cells (Hu, Jiang et al. 2005). The fate of $^{13}\text{C}_6$ -glucose was tracked in PC14PE6 cells treated with MSA or taxol alone, or MSA plus taxol. The 1D HSQC NMR analysis of the cell extracts indicates that the glucose to glutamate pathway may be synergistically inhibited by the combination treatment, as evidenced by the further reduction in the synthesis of different ^{13}C isotopomers of Glu by the combination treatment compared with either treatment alone (^{13}C -2-Glu and ^{13}C -4-Glu in **Figure 9**) (T.W-M Fan and K. Sellers, unpublished data).

III.4. ADMET of therapeutic agents monitored by NMR

An important component of the drug development concerns the absorption, distribution, metabolism, excretion, and toxicity of drugs (ADMET). Many drugs contain naturally abundant ^{19}F , which improves their ADMET properties (Bachert 1998; Park, Kitteringham et al. 2001; Mutlib 2008; Reid and Murphy 2008) and facilitates tracking of the parent drugs and their metabolites by NMR. Incorporation of stable isotopes such as ^2H and ^{13}C into the parent drugs are also frequently used to trace drug metabolism (Meese and Fischer 1990; Athersuch, Nicholson et al. 2007; Mutlib 2008). Investigating drug metabolism by NMR and MS has been reviewed extensively by Mutlib (Mutlib 2008).

Metabolism of ^{19}F -containing drugs has been extensively studied by Nicholson's group (Wade, Wilson et al. 1990; Spraul, Hofmann et al. 1993; Corcoran, Lindon et al. 2001; Blackledge, Nicholson et al. 2002; Blackledge, Nicholson et al. 2003; Keun, Athersuch et al. 2008). In these studies, the agent was introduced to an organism such as a rat, followed by the analysis of the drug and its metabolites by ^{19}F and ^1H NMR in urine and or blood plasma. As such, the extent of excretion of the untransformed drug as well as the actual metabolites transformed by the organism can be quantified (Blackledge, Nicholson et al. 2002; Blackledge, Nicholson et al. 2003). In a recent study of flucloxacillin biotransformation in humans, heteronuclear ^1H -(Fant, De Sloovere et al.) statistical correlation spectroscopy (STOCSY) on high field NMR (18.8 T) was used to evaluate the drug metabolism and excretion in urines collected at timed intervals after the drug administration (Keun, Athersuch et al. 2008). This technique can be applied to any ^1H -X nucleus pair in suitable drugs enriched with stable isotopes or ^{19}F .

^{13}C and ^{15}N enriched drug molecules can also be traced by similar approaches, as demonstrated in numerous studies (Meese and Fischer 1990; Serkova and Boros 2005; Athersuch, Nicholson et al. 2007; Mutlib 2008; Blech, Ludwig-Schwellinger et al. 2010). Identification of drug metabolites can be greatly facilitated by the use of 2D heteronuclear correlation experiments (e.g. HSQC or HSQC-TOCSY) (Fan and Lane 2008; Fan and Lane 2011) after the enriched drugs are transformed.

An important aspect of ADMET studies is the use of appropriate model system. Using SIRM technology, substantial differences were observed in metabolite utilization between rat and human hepatocytes, which was traced to hormone status and fasting in the human versus fed in the rat case (Winnike, Pediaditakis et al. 2011). As hepatocytes are the primary cells for drug metabolism, considerations for such distinction are very important in any ADMET study.

III.5. NMR-based tracer studies in whole model animals and humans

Animal models of diseases are widely available (Caron, James et al. 2002; Griffin 2006; Richmond and Su 2008; Brosius, Alpers et al. 2009) (Thelwall, Simpson et al. 2011) (Chen, Gonzalez et al. 2007). As depicted in **Figure 3**, the isotope tracing studies can be performed on whole animals, in addition to cell cultures. The analytical approach can be sampling and extracting biofluids and tissues at different time points for analysis (Beger, Hansen et al. 2009; Fan, Lane et al. 2011; Lane, Fan et al. 2011), or direct measurements in real time *in vivo* (see below). The former has considerably better resolution analytical data, but poorer time resolution and more interference from individual variations than *in vivo* spectroscopy. It should be noted that NMR is one of the few and by far the most versatile technique that can be employed for real time stable isotope tracer studies *in vivo* due to its noninvasive mode of measurement. In either case, the tracer can be administered as a bolus orally (Mason, Petersen et al. 2002) or intraperitoneally, via ad libitum food or water intake, via tail vein injection, (Fan, Lane et al. 2011; Lane, Fan et al. 2011), or by continuous infusion via a cannula (Thelwall, Yemin et al. 2005; Mason, Petersen et al. 2007; Thelwall, Simpson et al. 2011). The continuous infusion approach has the advantage of achieving a much higher and constant level of the tracer. In contrast, a bolus injection leads to a time dependent concentration of the tracer in the circulating blood due to tissue absorption and metabolism, which limits the time scale of the metabolic events that can be followed, as well as complicating the subsequent flux analysis (see below). Oral and peritoneal administration lie somewhere in between these two extremes, and give rise to a more complex convolution of the tracer input function with the subsequent metabolic transformations (Fan, Lane et al. 2011).

Another significant consideration with animal models is whether to work with alert or anesthetized animals. Both have disadvantages. For example, extensive animal handling is known to cause tachycardia and release of stress hormones into the plasma of rodents (Roizen, Moss et al. 1978; Kawashima, Miwa et al. 1985), which may affect metabolic activity of various organs, such as glycogen degradation in liver (Gruetter, Magnusson et al. 1994), synaptic release of amino acids in brain (Timmerman, Cisci et al. 1999), and energy metabolism in brain (Chance, Nakase et al. 1978). Therefore, handling stress-induced alteration of ^{13}C -glucose metabolism may contribute to the pattern of labeled products observed in Figures 2, 4-8 of (Fan, Lane et al. 2011). Particularly notable is the lack of ^{13}C -glucose and significant buildup of ^{13}C -lactate in our mouse brain extracts (cf. Fig. 6 and S3, (Fan, Lane et al. 2011)), which is distinct from that observed *in situ* in human or rat brain (Gruetter, Adriany et al. 2003). In contrast, anesthetized animals are metabolically less active than alert animals, leading to altered drug metabolism and response; the anesthetizing agent itself can also have an effect on animal's metabolism. Inserting a cannula for infusion studies can overcome some of the stress issues as the animals become acclimated, and pre-cannulated animals are often available from vendors (e.g. Charles River, Harlan Laboratories). Nevertheless, the infusion lines may still affect behavior patterns. The choice of technique has to be made according to the experimental design and information to be sought (Fan, Lane et al. 2011).

Fan and colleagues (Fan, Lane et al. 2011) examined glucose metabolism in SCID mice bearing an orthotopic xenograft lung tumor, using $[\text{U-}^{13}\text{C}]$ -glucose as the tracer administered via tail vein injection. This study demonstrated that the ^{13}C -edited metabolic profile obtained from 1D HSQC analysis can reveal tissue-specific metabolic networks, and many significant differences in glucose metabolism between normal and cancer-bearing lungs.

As for human applications, there have been widespread metabolomic profiling studies on human subjects for biomarker discovery, mainly involving biofluid analysis in response to

treatments or disease states, but without using tracers (Lindon 2003; Lindon, Holmes et al. 2004; Lindon, Holmes et al. 2004; Kristal BS, Shurubor YI et al. 2006; Kaddurah-Daouk, Kristal et al. 2008). As stated earlier (**Figure 2**), the chemometric approach and systemic biofluid analysis alone do not readily provide unambiguous mechanism of disease etiology or drug response. To achieve such mechanistic understanding, biofluid studies should be coupled with target tissue analysis via biopsy or surgical resection, when practical, and adopt the use of stable isotope tracers.

As described in II.3.3., Fan et al. applied $^{13}\text{C}_6$ -glucose tracer preoperatively, followed by resection of cancerous and adjacent non-cancerous (NC) lung tissue and collection of blood plasma and urine pre and perioperatively as well as at follow up (Fan, Lane et al. 2009; Lane, Fan et al. 2011). In addition to the MS analysis of tissue and plasma extracts described in II.3.3., parallel NMR analysis of the same extracts was performed. The ^{13}C labeling profiles of the cancerous and NC tissues were distinct, as revealed by their 1D HSQC spectra (**Figure 10**). Except for glucose, the ^{13}C abundance of lactate, Krebs cycle metabolites, non-essential amino acids, glutathiones, and nucleotides in the cancerous tissue was in excess of its paired NC tissues. This suggests an activation of multiple central metabolic pathways leading to the synthesis of these metabolites. In particular, the enhanced abundance of ^{13}C -2 and ^{13}C -3-Glu is consistent with the up-regulation of pyruvate carboxylase (PCB) in the cancerous tissue, as is also evidenced from the GC-MS and gene expression data described in II.3.3 and Western blot data (Fan 2010; Fan, Tan et al. 2011). The dependence of cancer cell growth on PCB has been further examined by DeBerardinis' group using ^{13}C NMR. They found that PCB activity was induced in glutaminase-suppressed glioblastoma cells, PCB overexpression sustained cell growth under Gln deprivation, and silencing PCB inhibited cell growth (Cheng, Sudderth et al. 2011). These model data support a requirement of PCB activation for cancer development, which was first uncovered by SIRM profiling of human subjects (Fan, Lane et al. 2009).

In a related study, using ^1H NMR and GC-MS, Fan et al. examined the *in vivo* metabolite profiles of two different lung lesions in the same patient with distinct responses to erlotinib, an inhibitor of the EGFR tyrosine kinase, (Fan, Lane et al. 2009). The profiles differed between the two lesions, which corresponded to different PET SUVs (standardized uptake values). The lesion with overexpression of EGFR and sensitivity to the drug was confirmed as bronchioloalveolar carcinoma (BAC), while the other was either a very early stage of lung cancer or a benign lesion. The metabolic profile of BAC showed an enhanced accumulation of many central metabolites than the erlotinib-insensitive lesion, whose metabolite profile was in turn elevated over the paired NC tissue.

III.6. *In vivo* NMR profiling of metabolism

Although detailed information can be obtained from extract analysis, many aspects of the metabolic interactions may be lost, such as the interactions between organs via the blood and lymphatic systems, pH and ion compartmentation, influence of microenvironmental conditions, and interactions between different cell types within a tissue. Such interactions are clearly important from the standpoint of metabolic homeostasis (Perriello, Jorde et al. 1995; Tayek and Katz 1997) and cooperation between neurons and astrocytes for example (Bouzier-Sore, Serres et al. 2003; Patel, de Graaf et al. 2005; Fan, Yuan et al. 2010). Furthermore, *in vivo* unidirectional rate of metabolic reactions cannot be achieved with the extract approach.

Localized ^{31}P NMR spectroscopy has long been used to obtain tissue level bioenergetics and intracellular pH information in small animals and humans, including their responses to altered physiological states or drugs (Gadian and Radda 1981; Gadian 1986; Gadian 1995; Brand, RichterLandsberg et al. 1996; Gillies, Raghunand et al. 2002; Wolfe, Hsu et al. 2002;

McClelland, MacDonald et al. 2003; Gillies and Morse 2005; Thelwall, Yemin et al. 2005; Seagle, Christie et al. 2008). The localization is readily achieved using a small surface coil to detect NMR signals from the underlying tissue (Fan, Clifford et al. 1994; Gadian 1995; Macdonald, Schmidlin et al. 2002). Typical ^{31}P signals of mammalian tissues arise from phosphocreatine, ATP, inorganic phosphate (P_i), phosphomonoesters, and phosphodiesteres. The intracellular and extracellular pH can be determined indirectly from the chemical shifts of P_i . Furthermore, the ^{31}P saturation transfer technique makes it possible to measure the unidirectional rates of ATP synthesis and utilization *in vivo* (Gadian and Radda 1981; Brindle and Radda 1987). Together, these measurements provide a detailed picture of energy metabolism, to complement stable isotope tracer studies (Mancuso, Zhu et al. 2005). *In vivo* ^{31}P and tracer-based ^{13}C NMR approaches have been applied to whole animals such as mice or other rodents, and to excised organs maintained in an appropriate physiological state (Gadian and Radda 1981; Sherry, Nunnally et al. 1985; Gadian 1986; Gadian 1995; Burgess, Babcock et al. 2001; Carvalho, Zhao et al. 2001; Macdonald, Schmidlin et al. 2002; Macdonald, Xu et al. 2002; Anousis, Carvalho et al. 2004; Carvalho, Rodrigues et al. 2004; Thelwall, Yemin et al. 2005; Hausler, Browning et al. 2006; Seagle, Christie et al. 2008).

By coupling with ^{13}C tracers, *in vivo* or *ex vivo* ^{13}C NMR has been employed to obtain the kinetics or compartmentation of metabolic reactions. Sherry and Malloy's group used multiple ^{13}C tracers to study the metabolism of isolated mouse hearts (Burgess, Babcock et al. 2001). ^{13}C -2-acetate, ^{13}C -3-lactate, $^{13}\text{C}_4$ -2,4,6,8-octanoate were infused into the hearts. Using proton detection of attached ^{13}C by HMQC, a time resolution of approximately 20 s was achieved for tracking the C3 and C4 isotopomers of glutamate. The indirect detection method provided a sensitivity enhancement of nearly 10 fold over the direct ^{13}C detection method. Based on the kinetics and a model of the TCA cycle including Glu/ α -ketoglutarate exchange reaction, the relative contributions of the substrate to TCA entry were calculated to be in the order acetate>octanoate>lactate, which is consistent with the known substrate preference of cardiac muscle for energy production. In another study, $^{13}\text{C}_6$ -glucose was used to label glycogen in the perfused rat heart (Anousis, Carvalho et al. 2004). By chasing with ^{13}C -1-glucose, ^{13}C NMR analysis of the lactate and glutamate isotopomers were used to estimate the relative contributions of glucose-dependent glycolysis from glycogenolysis. The results were consistent with functional compartmentation of glycolytic and glycogenolytic enzymes that allows pyruvate generated by these two processes to be distinguished at the level of lactate and acetyl-CoA. A third example utilized ^{13}C -2-glycine tracer and ^{13}C MRS to track glutathione and glycine metabolism in fibrosarcoma tumors implanted subcutaneously in rats (Thelwall, Yemin et al. 2005). After infusion of the Gly tracer, its uptake and metabolism was monitored *in vivo* in the tumor by surface-coil localized MRS including ^{13}C chemical shift imaging (CSI). Gly was mainly incorporated into the glycyl residue of glutathione and CSI imaging revealed spatial heterogeneity in glycine and glutathione distribution within the tumor volume.

One major disadvantage of *in vivo* NMR analysis is signal broadening due to tissue heterogeneity (thus variable magnetic susceptibility and chemical shifts), which compromises both sensitivity and resolution of NMR detection. To improve the *in vivo* NMR signal quality, high-resolution magic angle spinning (HR-MAS) spectroscopy of tissue samples has been developed. Part of the signal broadening can be overcome by spinning the tissue at moderate speeds at the so-called magic angle, which averages out spatial inhomogeneities, giving rise to much sharper (and thus more intense) and resolved NMR spectra (Millis, Maas et al. 1997; Bathen, Sitter et al. 2010; Malet-Martino and Holzgrabe 2011). This is applicable only to tissue samples, such as from biopsy and enables more metabolites to be determined than in whole organisms while largely retaining the local architecture. If combined with ^{13}C and/or ^{15}N tracer applications, the approach can give

detailed information on metabolic networks in tissues, albeit under non-physiological conditions such as low temperature and generally for relatively short periods (Martinez-Bisbal, Marti-Bonmati et al. 2004; Martinez-Granados, Monleon et al. 2006; Swanson, Zektzer et al. 2006; Levin, Albers et al. 2009; Rocha, Barros et al. 2010). HR-MAS without the use of tracers has been extensively applied to several disease states, especially in various cancers, and for assessing effects of drugs on tissue metabolism (Bathen, Sitter et al. 2010; Beckonert, Coen et al. 2010; Glunde, Artemov et al. 2010; Rocha, Barros et al. 2010; Garcia-Alvarez, Fernandez-Mayoralas et al. 2011; Malet-Martino and Holzgrabe 2011).

IV. Non-invasive imaging techniques to monitor metabolism *in vivo*

Like *in vivo* NMR, non-invasive imaging techniques have the unique ability to track real-time changes of metabolism in living organisms. In addition, some of these imaging methods, particularly magnetic resonance spectroscopy (MRS) imaging are capable of providing spatially resolved pictures of metabolic processes, which adds new dimensions to our understanding of *in vivo* metabolic perturbations caused by disease or exposure to drugs. Because many of these techniques are applicable from the cellular level to the organismal level, they translate well from the laboratory to the clinic. The clinical applications of imaging techniques have been extensively reviewed (Cai, Rao et al. 2006; Cai, Shin et al. 2006; Wessels, Busse et al. 2007; Brindle 2008; Frangioni 2008; Weissleder and Pittet 2008; Willmann, van Bruggen et al. 2008), and this section will focus on those that provide metabolic information and with the ability to sense stable isotope tracers, i.e. MRS imaging. Radioactive isotope-based positron emission tomography (PET) and optical imaging will also be briefly described. PET is a well-established method for clinical diagnostics and together with optical imaging can complement and verify information obtained from MRS. All three imaging techniques have demonstrated great utility in drug discovery process.

IV.1. PET imaging

Positron Emission Tomography (PET) images radioactive tracers that emit positrons. The positrons collide with nearby electrons, causing the production of two gamma rays. The gamma rays are emitted at a 180° angle from each other, which are detected by a pair of collinear gamma detectors. A three dimensional image can then be reconstructed using signals from multiple detectors. Because the spatial resolution (ca. 1 mm) of microPET imaging is limited by the mean free path of the positron and the number of gamma detectors, they are often combined with better-resolved anatomical data acquired from computed tomography (CT). Combined PET/CT scanners ensure that the two independent images are properly registered. PET tracers are typically analogues of biological substrates containing a radionuclide such as ^{11}C , ^{13}N , ^{15}O , and ^{18}F . Because PET is highly sensitive (10^{-12} M), tracers can be administered in doses low enough to eliminate concerns for toxicity.

The clinical potential of PET was first realized at the end of the 1970's with the development of glucose analogue ^{18}F -2-fluorodeoxy-D-glucose (2-FDG) (Reivich, Kuhl et al. 1979). Glucose-utilizing cells take up 2-FDG, which accumulates in the tissue because phosphorylation by hexokinase prevents its export and replacement of the 2' oxygen with fluorine prevents it from undergoing further glucose metabolism. Because cancer tissue has a greater glucose demand (typically by an order of magnitude) than the surrounding stromal tissues, 2-FDG provides a means to highlight tumor regions. Indeed, the standardized uptake value (SUV) is regarded as a marker of tumor aggressiveness and is valuable in tumor staging (Dhital, Saunders et al. 2000; Bares, Eschmann et al. 2002; Cermik, Mavi et al. 2008), as it reflects the well-known property of solid tumors to take up and metabolize glucose at an accelerated rate, even under aerobic conditions, i.e. the Warburg effect (Warburg 1923; Warburg 1956; Garber 2004). While 2-FDG continues to be the major tracer used in clinical PET (Gambhir 2002), other tracers have shown promise as

proliferation markers such as amino acid, nucleotide, acetate and choline-containing radionuclides, as reviewed elsewhere (Kumar, Dhanpathi et al. 2008).

In addition to its diagnostic value, 2-FDG PET has been shown to help assess therapeutic response to anti-cancer treatment in lymphoma (Juweid and Cheson 2006; Juweid, Stroobants et al. 2007), breast (Smith, Welch et al. 2000), melanoma (Tseng, Stuart et al. 2011), NSCLC (Weber, Petersen et al. 2003), and head and neck carcinoma (Brun, Kjellen et al. 2002) (e.g. **Figure 11A**). Because metabolic changes precede structural changes, PET imaging surpasses anatomical imaging techniques to assess early response to cancer treatments.

Moreover, PET has been used to identify biomarkers for disease. For instance, uptake of the amyloid-binding reagent ^{11}C -2-(4-methylaminophenyl)-6-hydroxy-benzothiazole was shown to be a positive predictor for patients who would develop Alzheimer disease (Forsberg, Engler et al. 2008). PET also has attractive properties for studying pharmacokinetics. Preclinical pharmaceuticals can be labeled with radionuclides and their tissue absorption, distribution, metabolism, and excretion can be monitored (ADME). PET tracers have been developed for cisplatin (Ginos, Cooper et al. 1987), fluorouracil (Kissel, Brix et al. 1997), tamoxifen (Inoue, Kim et al. 1996), DACA (Saleem, Harte et al. 2001), paclitaxel (Kurdziel, Kiesewetter et al. 2003), verapamil (Hoebers, Kartachova et al. 2008; Thelwall, Simpson et al. 2011), and temozolomide (Rosso, Brock et al. 2009).

However, PET has several intrinsic disadvantages in metabolomics applications. First, the metabolic information provided by PET scans is limited to a single pathway or even a single reaction and PET scans cannot differentiate between the tracer and its metabolites. Second, the availability of the radiotracers restricts PET's clinical and laboratory applications. The radionuclides for PET typically have very short half-lives, e.g. two minutes for ^{15}O , 20 minutes for ^{11}C , or 2 h for ^{18}F , requiring them to be synthesized at an on-site or regional cyclotron (Muehllehner and Karp 2006). This is not only costly but also limit PET applications to very fast metabolic reactions. The related imaging technique single photon emission computed tomography (SPECT) circumvents some of these limitations. SPECT tracers emit gamma rays directly with characteristic energies. These tracers have longer half-lives and can contain different radioisotopes for simultaneous imaging. Radiolabeled apoptosis marker annexin V has been used to evaluate *in vivo* response of paclitaxel in breast cancer mouse model (Thelwall, Simpson et al. 2011), cisplatin in head and neck cancer patients (Hoebers, Kartachova et al. 2008), and cyclophosphamide-etoposide in a lymphoma and breast cancer mouse models (Chen, Gonzalez et al. 2007). However, the spatial resolution of these techniques is low and metabolic application is still limited.

IV.2. Optical imaging

Unlike PET probes, optical imaging probes are stable and relatively inexpensive. Fluorescence-based imaging relies on probes that absorb a photon at a particular wavelength and emit a photon at a longer wavelength. In bioluminescence, the emitting molecule does not absorb a photon but is excited biochemically, such as using the energy of ATP hydrolysis via the luciferase reaction (Chen, Gonzalez et al. 2007). Optical imaging is also highly sensitive and in principle quantitative.

Fluorescence imaging has been applied extensively in cell cultures but its application in tissues is limited because tissues absorb and scatter light and auto-fluoresce, limiting the depth these techniques can penetrate (Rao, Dragulescu-Andrasi et al. 2007). These limitations have been somewhat abated by the development of probes that fluoresce in the near infrared region (NIR), which is transparent to human tissues (Cai, Shin et al. 2006).

NIR probes increase fluorescence imaging depths from the low mm range to the several cm range, albeit with a concomitant loss of resolution by using longer wavelengths.

Recently, activatable fluorescent probes have been developed for imaging *in vivo* metabolite concentration or enzyme activity. These probes are more sensitive than constitutively active probes because they are silent until reaction with their target; thus they do not require clearance via circulation for contrast. Activatable probes rely on fluorescence resonance energy transfer (FRET) from donor to acceptor fluorophores or fluorescence self-quenching between two identical fluorophores. They typically have two or more fluorophores attached by a linker and both FRET and self-quenching depend on the distance between the fluorophores. The linker is designed to bind to a metabolite or react with an enzyme, which causes the distance between fluorophores to change, resulting in FRET or direct energy exchange for self-quenching. In both cases, the end result is a loss or gain of fluorescence intensity. These types of activatable molecular probes can be tuned for different metabolites or enzymes, providing a novel mechanism to image differences in metabolite concentration or enzyme activity as a result of disease states or in response to treatment in real-time (Elias, Thorek et al. 2008).

For example, one of the first FRET-based probes monitored calcium concentration by linking two fluorescent proteins by calmodulin. Upon calcium binding, the calmodulin linker undergoes a conformational change, bringing the donor and acceptor proteins close enough to enable FRET (Miyawaki, Llopis et al. 1997). In another example, the activity of matrix metalloproteinases (MMPs) was monitored to track tumor development and metastasis in model animals (Scherer, VanSaun et al. 2008). Scherer et al. developed a probe where Cys5.5 and AF750 were attached to a polyamidoamine backbone. Cys5.5 molecules were attached to the polymer by a peptide with a sequence specific for MMP 7 and in close proximity to permit self-quenching. Upon cleavage with MMP-7, the signal for Cys5.5 increased while AF750 served as a reference dye for the quantification of both cleaved and non-cleaved substrates.

More recently, quantum dots are being explored for their ability to serve as donors in FRET couples because they display high absorptivity, high quantum yield, resistance to photobleaching, omnidirectionality, and their emission spectra are tunable based on their size (Rao, Dragulescu-Andrasi et al. 2007). Such probes have been developed for a variety of enzymes such as trypsin (Shi, Rosenzweig et al. 2007), caspase 3 (Boeneman, Mei et al. 2009), lactate dehydrogenase (Ren, Yang et al. 2010), casein kinase and alkaline phosphatase (Freeman, Finder et al. 2010), type IV collagenase (Theodoridis, Gika et al. 2011), and histone acetyltransferase (Ghadiali, Lowe et al. 2011).

Fluorescence imaging is not limited to the large number of synthetic probes. Endogenous fluorophores arising from differential metabolic activity within cancer tissues have been investigated for tumor detection. For instance, many tumors such as melanoma, bladder, gastrointestinal, and glioma have increased capacity to synthesize and accumulate fluorescent porphyrins after administration of 5-aminolevulinic acid (5-ALA) (Stummer, Stocker et al. 1998). In an eight-year follow-up study, Denzinger and colleagues (Denzinger, Burger et al. 2007) showed that using 5-ALA-induced fluorescent-guided endoscopy, post-operative bladder carcinoma patients had significantly reduced residual tumor and increased recurrence-free survival.

It is also practical to genetically engineer fluorophore probes in cells and animal models. For example, mouse models are xenographed with cancer cells transfected with luciferase, which catalyzes the oxidation of luciferin, leading to photon emission. This allows tumor progression and treatment response to be monitored in the same animal over time (Sim,

Bibee et al. 2011). Because the luciferase reaction requires ATP, bioluminescence provides a mechanism to monitor ATP metabolism within the tumor microenvironment. Pellegatti and colleagues (Pellegatti, Raffaghello et al. 2008) engineered a plasma membrane-targeted luciferase. Using this probe, the authors quantified extracellular ATP within the tumor microenvironment in the high μM range, while it was undetectable in the extracellular matrix of normal tissue (**Figure 11B**).

As optical probes have to be developed for each reaction to be monitored, optical imaging is not suited for assaying metabolic networks, but rather for testing specific hypotheses.

IV.3. MRS and MR imaging

IV.3.1 Conventional NMR imaging—While PET and optical imaging have proven useful for monitoring specific aspects of *in vivo* metabolism, both methods can only observe a single target per probe. In contrast, MRS imaging has the potential to monitor spatially resolved flux through a large number of pathways *in vivo*. The principle of MRS or MRI is an extension of NMR with added spatial encoding. The spatial information arises from applying a magnetic field gradient across the sample to be imaged and the resonant frequency is dependent upon a nucleus's position within the gradient or from physical localization using surface coils (cf. III.6). MRI is inherently insensitive, and sensitivity versus spatial resolution is always a trade-off such that the method is usually restricted to proton detection of the most abundant molecules, i.e. water and fat. Clinically, when MRI is used to image signals derived from water or fat molecules (“chemical shift imaging”), the anatomical contrast is provided by tissue-specific water concentration and intermolecular interactions that change a proton's relaxation time.

However, MRS is intrinsically versatile for *in vivo* metabolic imaging due to the NMR's ability to resolve protons or other nuclei in different chemical environments and therefore different metabolites (Brown 1992; Li, Negendank et al. 1996; McMillan, Rogers et al. 2006). For instance, MRS analysis of breast cancer has implicated a peak at 3.2 ppm as a biomarker for malignancy (Katz-Brull, Lavin et al. 2002). Because proton peaks from *in vivo* MRS are broader than those in typical NMR spectra of extracts, this peak is a composite of several unresolved choline-containing compounds. The Bolan group (Meisamy, Bolan et al. 2004) was able to detect a positive correlation between changes in tumor sizes and total choline concentration in locally advanced breast cancer patients undergoing doxorubicin neoadjuvant chemotherapy. In addition, 75% of patients who responded to the treatment showed decreased total choline levels after one day of treatment, compared with no change or increased choline levels in 92% of non-responders (Haddadin, McIntosh et al. 2009). However, there are several obstacles for clinical applications such as poor sensitivity, the need to suppress water and lipid signals, and difficulty for accurate quantification. Furthermore, the ratio of phosphocholine to choline is not specific to breast cancer (de Molina, Gallego-Ortega et al. 2005; Glunde and Serkova 2006; Hernando, Sarmentero-Estrada et al. 2009) but more likely a marker of proliferation (Lane, Fan. T. W-M. et al. 2008). This points to a weakness of using single metabolic markers.

IV.3.2 Hyperpolarization (DNP) NMR imaging—As described above, *in vivo* NMR has the potential to measure a wide variety of metabolites noninvasively, especially when coupled with stable isotope tracers that avoid all complications of short half-lives and hazardous radioactivity. Also referred above is that its major drawback is low sensitivity.

Recently, MRS/MRI sensitivity has been dramatically improved with the use of hyperpolarized substrates. Such method is called dynamic nuclear polarization (DNP) MRS. NMR is intrinsically insensitive because of the very small energy difference between ground and excited nuclear states, which means that the population difference at thermal

equilibrium is very small (ca. 1 part in 10,000 for protons, and 4 fold lower for ^{13}C). However, the population difference can be radically enhanced by transferring the equilibrium magnetization from an electron spin radical at low temperature (typically 1-2 K). The low temperature itself increases the population difference by more than 100 fold, and the electron polarization accounts for another 660 fold (Maly, Debelouchina et al. 2008). The polarized sample must be rapidly warmed prior to injection into animals or human subjects, at which point the polarization is typically of the order 5000-10000 times that of the unpolarized sample (Hu, Balakrishnan et al. 2011; Kurhanewicz, Vigneron et al. 2011). This means that very rapid data acquisition with good sensitivity is possible for substrates present at low concentrations (Hu, Balakrishnan et al. 2011; Kurhanewicz, Vigneron et al. 2011). However, the polarization is short lived and it persists over the period of the spin-lattice relaxation time T_1 , which is about 1 second for ^{13}C directly attached to a proton. For this reason, it is much preferred that a substrate contains a carbonyl or quaternary carbon, which may have a T_1 value of 20-40 seconds or more depending on the in vivo conditions (Wilson, Keshari et al. 2010). This limits the time scale to less than 5 minutes for DNP-MRS measurements. It is also not surprising that the most commonly used substrate to date is ^{13}C labeled pyruvate. Monitoring hyperpolarized ^{13}C pyruvate metabolism in cancer models has elucidated novel biomarkers for prostate cancer (Levin, Albers et al. 2009). In addition, hyperpolarized ^{13}C pyruvate enabled tracking the action of potential anti-cancer drug dichloroacetate *in vivo* (Seth, Grant et al. 2011). Dichloroacetate attenuates lactate dehydrogenase activity by activating pyruvate dehydrogenase. The authors were able to observe a significant reduction in lactate to pyruvate signal ratio in mice treated with dichloroacetate.

Goga and colleagues (Hu, Balakrishnan et al. 2011) used ^{13}C pyruvate to monitor metabolic changes in MYC-induced liver tumors in mouse models. Doxycycline was used to suppress MYC expression. The mouse livers were histologically normal after the first four weeks of doxycycline withdrawal, even though expression of MYC mRNA was detectable. After eight weeks the mice developed primary tumors. The tissues still appeared tumorous by histological analysis after 72 hours of retreatment with doxycycline, even though MYC expression was completely inhibited. In parallel, the researchers administered hyperpolarized ^{13}C -1-pyruvate before doxycycline withdrawal (normal liver), after four weeks of doxycycline withdrawal (pre-tumor liver), after eight weeks of doxycycline withdrawal (tumor), and 72 hours after returning doxycycline treatment (tumor regression). They discovered that transamination of pyruvate to alanine was upregulated in pre-tumor tissue, which corresponded to increased alanine transaminase mRNA expression and activity. They also detected high levels of labeled lactate in the tumor tissues, while surrounding normal tissues displayed high levels of labeled pyruvate (**Figure 11C**). Increased lactate signals in tumor tissues correlated with enhanced lactate dehydrogenase (LDH) activity as well as mRNA and protein expression. Both LDH expression and ^{13}C lactate signal decreased significantly in the regressed tumor tissues. Thus, the authors were able to discover novel biomarkers for pre-MYC induced tumors and tumor regression. These biomarkers were detectable before tumor development and regression were discernable by histology. These results are consistent with reduced tumor growth elicited by LDH A knockdown *in vivo*.

Hyperpolarized ^{13}C -1-pyruvate was used to determine *ex vivo* rates of pyruvate uptake and turnover via LDH, PDH, ALT (Ala transaminase) reactions in isolated rat heart (Merritt, Harrison et al. 2007). With 1-second time resolution, the concentrations of ^{13}C labeled pyruvate, lactate (LDH), alanine (ALT), CO_2 and bicarbonate (PDH+carbonic anhydrase) could be measured (**Figure 11D**), from which metabolic fluxes could be estimated.

In another recent application, Brindle and colleagues (Gallagher, Kettunen et al. 2008) developed a novel mechanism to measure pH *in vivo*. The authors injected a mouse model with hyperpolarized $\text{H}^{13}\text{CO}_3^-$ and monitored its conversion to $^{13}\text{CO}_2$ by carbonic anhydrase. pH was estimated by the Henderson-Hasselbalch equation: $\text{pH} = \text{pK}_a + \log_{10}(\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2)$. Lymphoma-bearing mice had significantly more acidic tissue at the tumor site than the surrounding non-cancerous tissue.

Using different tracers, hyperpolarized MRI has the unique ability to monitor pathway fluxes *in vivo*, which may lead to the discovery of novel targets and validate known targets such as LDH. A recent review discusses several hyperpolarized substrates that have been developed and their subsequent metabolism as it relates to disease states (Xu, Lu et al. 2008). Hyperpolarization studies of metabolism *in vivo* have already shown values in animal-based preclinical studies. It is to be expected that they will be uniquely suited for targeted and short term translational studies in human subjects as more hyperpolarized substrates become available, multiplexing of several substrates is implemented (Wilson, Keshari et al. 2010), and the issues of toxicities are solved (Xu, Lu et al. 2008).

It is clear that all three *in vivo* imaging techniques can detect molecular markers that precede anatomical markers and that they are valuable for real-time monitoring of treatment efficacy. Early detection of treatment response has the potential to expand the number of patients eligible for clinical trials. Currently, clinical trials are conducted in patients with advance stages of disease, after established treatment methods have failed. This is the least effective population to test new drugs because these patients may be the worst responders. While these methods have serious drawbacks for comprehensive pathway analysis, they are excellently suited for hypothesis-driven investigations of specific metabolic questions. The prospect for real time tracking of specific metabolic reactions in human subjects *in situ* is exciting and will be a perfect complement to human subject-based SIRM studies.

V. Dynamic range and Sensitivity for Metabolite Detection by NMR and MS

The sensitivity of a technique depends on the ability to discriminate a signal above the noise level. This is not identical to the signal-to-noise ratio, which is defined as the integral of the signal normalized to the root mean square noise figure. In spectroscopy, sharp resonances are easier to detect above the noise than broad signals of the same integrated area or volume. The signal intensity depends on spectral resolution, in addition to the usual considerations of the quality of the detector, the efficiency of physical activation that is instrument and structure-dependant, and the intrinsic sensitivity of the spectral property being measured. For example, in NMR, only 1 nucleus in about 500,000 is typically detected, owing to the very small difference in populations between ground and excited states at room temperature. In contrast, mass spectrometry produces sub-percent to nearly 100% (for permanently ionized compounds) of measurable ions, depending on the ion source design and chemical structure.

In a mixture of metabolites extracted from tissue or in a biofluid, there will be a very wide distribution of metabolite concentrations, ranging from high mM for the abundant metabolites (e.g. lactate) to pM for some hormones. This dynamic range ($>10^9$) is extremely challenging for any analytical technique, and currently no single platform is capable of simultaneously detecting all analytes present. Indeed this is a reason why multiple platforms should be used in order to have a high coverage for metabolite analysis.

Among structure-based analytical platforms capable of isotopomer analysis, NMR is the least sensitive technique, and does not usually detect molecules present at sub μM concentrations. However, NMR detection is linear in response over nearly 5 orders of magnitude. Without any further sample manipulation, the dynamic range is determined by

the digital resolution of the receiver, which in modern NMR spectrometers is at least 16 bits (range = 65,536), and may be closer to 20 bits effectively. As some of the digitization is used to represent noise, the actual dynamic range for detection is less than the number of bits available. In practice the effective dynamic range is closer to 5×10^3 owing to limitations in spectral resolution (defining as spectral width/peak width), which is of the order 10^4 and much worse for dilute species resonating next to very abundant ones. Some of the dynamic range problems can be alleviated by suppressing unwanted signals via solvent exchange (e.g. D₂O for H₂O) and weak irradiation ("saturation") or by targeting desired signals using tailored excitation profiles. For isotopomer analysis, the isotope selection procedure itself (e.g. indirect proton detection of attached ¹³C) removes a large number of unlabeled signals (see above and (Fan and Lane 2011; Lane 2012)). Furthermore, as described above, targeted enrichment with DNP can enhance specific signals 5000-10000 fold, so that low abundance metabolites can be selectively detected.

Relative to NMR, MS has always been considered as a more sensitive technique for isotopologue structure-based analysis and its sensitivity is highly platform-dependent. With advancement in novel nanomechanical MS, detection of a single protein molecule is within reach (Naik, Hanay et al. 2009). However, it is unclear if such innovation will soon be applicable to small molecules and how the high matrix (e.g. abundant non-targeted analytes and salts) interference (Lagerwerf, van Dongen et al. 2000; Taylor 2005; Vogeser and Seger 2010) can be dealt with. As stated earlier, the latter has been a major hurdle to overcome for direct analysis by MS without coupling to separation methods such as GC, LC, or capillary electrophoresis. With excellent separation such as 2D GCxGC, routine MS detection can now reach down to sub pM (Sumner and Bedair 2008; Psychogios, Hau et al. 2011).

However, the digital dynamic range of the MS detectors is typically 2^{12} (4096) to 2^{14} (16384), which falls far short of the dynamic range (pM to mM) of metabolite concentrations in crude biological extracts. As part of the digitization is used for noise definition, this means that a 12-bit or a 14-bit digitizer has effective dynamic range of the order 256 to 1024 or 1024-4096, respectively, for a single spectrum, i.e. no signal averaging. However, with signal averaging via direct infusion from a continuous ion source, the dynamic range can approach the upper values quoted, and the isotope ratio precision is also greatly improved (see section II.2). It must be kept in mind that this benefit of direct infusion is not practical for complex metabolomic analysis unless it is coupled with an MS of resolving power >200,000. The upshot of dynamic range limitations is that trace-level metabolites will need to be enriched before MS detection. Affinity chromatography or other forms of separation methods as described above is a common but usually more time consuming strategy to pre-concentrate metabolites or to remove interfering matrix (Want, Smith et al. 2006; Pucci, Di Palma et al. 2009). Selective chemical derivatization is another good strategy, not only for metabolite enrichment but also for stabilization of labile metabolites and increasing ionization efficiency (Fu, Li et al. 2011). We have utilized this strategy to enrich metabolites with carbonyl functionality for direct detection by FT-ICR-MS (e.g. pyruvate and oxaloacetate) (Mattingly et al., accepted for Metabolomics J. with minor revision). A summary of additional derivatization strategies for improving LC-MS detection of metabolites with other functional groups can be found in the review by Santa (Santa 2011).

For the MS analysis of relative isotopologue distribution, the typical effective dynamic limit is constrained not only by the sensitivity but also by the digitization range of the MS detector, which is at best a few % of the most abundant ions. In contrast, with the very high mass resolution techniques, such as FT-ICR-MS, the effective dynamic range can be extended to 0.05% under superior mass resolution and a 14-bit digitizer detector and signal averaging of transients (Fan et al., unpublished data).

VI. Biochemoinformatics and Modeling

VI.1. Computational requirement for tracer studies

Informatics is an integral component of all 'omics approaches from raw data reduction through interoperability of different data streams, standardization, statistical analysis to biological interpretation (Sansone, Fan et al. 2007; Sumner, Amberg et al. 2007; Scalbert, Brennan et al. 2009; Redestig, Kusano et al. 2010). For metabolomics, this involves primarily analysis of NMR and MS raw data, reconstruction of metabolic networks, and modeling of metabolic flux and regulations in extended networks. There has been considerable effort expended on the various technical problems of MS and NMR data analyses, such as spectral alignment, semi-automated metabolite identification and quantification (Weljie, Newton et al. 2006) (Coulier, Bas et al. 2006; Van der Greef, Hankemeier et al. 2006; Zhao, Stoyanova et al. 2006; Schiavo, Ebbel et al. 2008; Kind, Wohlgemuth et al. 2009; Ebbel, Leymarie et al. 2010; Kumari, Stevens et al. 2011; Sands, Coen et al. 2011; Veselkov, Vingara et al. 2011). It is commonly recognized that many features in NMR and MS spectra are unassigned, and therefore difficult to quantify rigorously and uninterpretable in the biochemical context. In SIRM studies, this problem is further aggravated by the need for analyzing mass isotopologues and positional isotopomers of metabolites to track the fate of individual atoms from a labeled tracer (cf. **Figures 4 and 6**), which requires new computational resources to deconvolute. It is also non-trivial to remove natural abundance contribution when quantifying fractional label enrichment in metabolites such as lipids, particularly for species with multiple labeled isotopes. For mass isotopologue data acquired from FT-ICR-MS, natural abundance stripping for ^{13}C isotope has been solved algorithmically (Lane, Fan et al. 2009; Moseley 2010). However, the chemoinformatic development for automated determination of the isotopomers and isotopologues of different metabolites is at its infancy and is platform dependent, whether they be different types of MS (Lane, Fan et al. 2009; Moseley, Lane et al. 2011) or NMR (Fan and Lane 2008). Furthermore, to reconstruct metabolic pathways from labeled isotopomer and isotopologue profiles by computational means, *in silico* atom-resolved databases of metabolic networks are required and have been developed for *E. coli* by Arita (Arita 2003; Arita 2004) as well as for several organisms in the KEGG database (<http://www.genome.jp/kegg/pathway.html>). However, to date, there are no appropriate atom-tracking databases available for human that encompasses such essential elements as compartmentation and interorgan interactions.

VI.2. Computational analyses of tracer data

Various levels of computational analyses are applicable to MS and NMR data obtained from tracer experiments. For profiling total metabolite levels, various data mining tools (e.g. principal components analysis, orthogonal partial least square-discriminant analysis) can be applied to both assigned and unassigned MS and NMR data. These are by far the most common tools for uncovering biomarkers using non-tracer types of NMR and MS data for a variety of diseases including cancers (Griffin and Shockcor 2004; Lindon and Nicholson 2008; Fan, Lane et al. 2009). These methods may also be useful for initial biomarker discovery with isotopomer and isotopologue data from stable isotope tracer studies. However, such purely statistical data mining approaches do not provide robust understanding of disease initiation and progression for drug target discovery. The next level of data mining is required to identify stable isotope labeling patterns and to quantify their distribution for subsequent pathway reconstruction. This type of chemoinformatic tools is not yet available for NMR or MS analysis, neither are the tools available for automated reconstruction of human metabolic networks from labeled isotopomers and isotopologues of metabolites.

However, there is a number of methodologies developed for quantitative flux modeling of specific pathways based on stable isotope labeling data. This is epitomized in the SiDMAP (Boros, Brackett et al. 2003) and metabolic control analysis (MCA) approaches (Fell 1992; Cascante, Boros et al. 2002). Moreover, a quantitative flux analysis of stable isotope incorporation into metabolites of cancer cells has been pursued by a variety of related computational methodologies under the umbrella of metabolic flux analysis (MFA) (Wiechert, Mollney et al. 2001; Papin, Price et al. 2003; Blank, Kuepfer et al. 2005; Antoniewicz, Kelleher et al. 2006; Lee, Gianchandani et al. 2006; Antoniewicz, Kelleher et al. 2007). These methodologies rely on detailed human metabolic networks which are available from public metabolic databases such as KEGG (Kanehisa and Goto 2000; Goto, Okuno et al. 2002), HumanCyc (Romero, Wagg et al. 2004), and the human metabolome database (Wishart, Tzur et al. 2007; Psychogios, Hau et al. 2011). However, none of these databases are adequate in terms of compartmentalized metabolic reactions, or take into account interorgan interactions.

A more comprehensive modeling of cellular metabolism also requires very complete and accurate metabolic models, which have been developed for multiple prokaryotic species, a few eukaryotic species, but are still under heavy development for human cells (Duarte, Becker et al. 2007; Oberhardt, Palsson et al. 2009). Nevertheless, significant strides have been made in developing cancer-relevant models of cellular metabolism which have already demonstrated the utility for identifying new drug targets for cancer (Folger, Jerby et al. 2011). The majority of the model-based metabolic flux analysis (MFA) on human cells has been limited to central carbon metabolism under metabolic steady-state conditions, which requires high concentrations of labeled tracers, typically ^{13}C -labeled glucose and for long periods (Maier, Hofmann et al. 2008; Meadows, Kong et al. 2008; Yang, Richardson et al. 2008; Metallo, Walther et al. 2009). Even when isotopic steady state is established for metabolic end products such as lactate, there is no assurance that other metabolites in the modeled metabolic networks are in the required isotopic equilibrium. This is particularly the case for “hub” metabolites such as glutamate, which can participate in more than 200 metabolic reactions (M. Arita, personal communication); its isotopic steady-state was not reached in lung cancer A549 cells even after 96 h of growth in $^{13}\text{C}_6$ -glucose (Fan, unpublished data). Therefore, the early successes of ^{13}C MFA in prokaryotic systems under chemostat conditions (Tang, Martin et al. 2009; Dauner 2010) cannot be directly applied to almost all tissue culture experiments in complex media due to the metabolically non-steady-state (non-stationary or instationary) conditions resulting in part from batch-fed, unstirred nature of the experimental set up (Wahl, Nöh et al. 2008; Noack, Nöh et al. 2010; Zamboni 2010). It is self-evident that the applicability to whole organisms such as humans is even further distant.

Moreover, it may not be practical to establish isotopic steady state when cell metabolism is perturbed by drug treatments or by fluctuations of nutrient status to be expected in tissue microenvironment. Nutrient deficiency can dictate non-steady-state conditions because the transporters typically have K_m values in the range 1-10 mM (Shepherd and Kahn 1999). Finally, it is unrealistic to expect isotopic steady-state conditions for human tracer studies since not only this is cost-prohibitive but also incompatible with ethical standards.

A recent development by Moseley et al. demonstrated a novel approach to non-steady state modeling of relative flux through intersecting metabolic pathways (Moseley, Lane et al. 2011; Moseley, Higashi et al. 2011). The approach involves tracing biochemical subunits (e.g. acetyl, ribosyl, uracil) through metabolic networks from the labeled tracer (e.g. $^{13}\text{C}_6$ -glucose) to the detected metabolite (e.g. uridine diphosphate N-acetylglucosamine or UDP-GlcNAc), which allows computational deconvolution of a set of mass isotopologue data acquired from FT-ICR-MS into fractional labeling of these subunits in the detected

metabolite. This deconvolution enables modeling of fractional labeling patterns into relative flux through relevant metabolic pathways. This approach (Genetic Algorithm for Isotopologues in Metabolic Systems or GAIMS) was demonstrated in prostate cancer cells where a time series of FT-ICR-MS isotopologue data for UDP-GlcNAc were deconvoluted into time-dependent fractional labeling of its four subunits (glucose, ribose, uracil, and acetyl). The label incorporation into the subunits was also confirmed by NMR. The modeling outcome revealed a large relative flux (>80%) through the PPP and hexosamine biosynthetic pathway versus half to a third of the flux through glycolysis and the Krebs cycle. In addition, the robustness of this approach was tested by selecting the correct deconvolution out of 40 alternatives (Moseley, Lane et al. 2011). These results demonstrate the promise of the GAIMS approach for non-steady state modeling of fluxes through metabolic networks and its value as a validation tool for models built from known metabolic networks (Moseley, Lane et al. 2011; Moseley, Higashi et al. 2011).

To facilitate metabolic flux modeling in mammalian systems including human, future development should therefore focus on i) automation of isotopologue and isotopomer assignments and quantification from large MS and NMR datasets; (ii) developing robust metabolic flux analyses that can deal with metabolically non-stationary states of these systems; iii) improving network models of human metabolism, particularly regarding compartmentation for a variety of cell and tissue types; and iv) making these models available in open standards like the systems biology markup language or SBML (Hucka, Finney et al. 2003). Already there is progress in the extension and development of a variety of methods to handle the metabolic realities of cell culture and whole organism experiments (Selivanov, Marin et al. 2006; Baxter, Liu et al. 2007; Lee, Gianchandani et al. 2008; Wahl, Nöh et al. 2008). Recently, non-stationary methods were applied to metabolic flux analysis for cancer cells (Selivanov, Vizan et al. 2010). These approaches generally solve the differential equations for a set of biochemical reactions that represent a realistic model of the relevant metabolic processes (Maher, Kuchel et al. 2003; Selivanov, Puigjaner et al. 2004; Selivanov, Meshalkina et al. 2005; Possemato, Marks et al. 2011). For small networks models, it is possible to solve the differential equations analytically (Yuan, Bennett et al. 2008; Lane, Fan et al. 2011) but such approaches are necessarily limited. This is because as the model become more complex, the number of differential equations will expand rapidly to render such computational approaches intractable. For larger, more biologically realistic models, numerical solutions are essential. Given the interrelatedness of metabolic networks (Arita 2004), it remains an art determining how large the model should encompass to be realistic, while computationally tractable. In the past, metabolic models tend to be simplistic because they do not account for dynamic compartmentation, even though the problem has been long recognized (Wolfe 1992), and isotope tracing can detect different intracellular pools of metabolites (cf. **Figure 5**) (Fan and Lane 2008). Although a selected set of compartmentalized events have been modeled in humans, such as the glutamate cycling between astrocytes and neurons (Mason and Rothman 2004; Patel, de Graaf et al. 2005; Mason, Petersen et al. 2007), dynamic compartmentation issues will remain to be the “Achilles heel” of large-scale network modeling for cells, not to say of tissues or whole organisms.

VII. Future Directions

It is clear that complementary and confirmatory structure-based tools such as NMR and MS are indispensable for resolving complex metabolic networks. Past metabolic studies have tended to utilize either tool alone, which cannot benefit from the synergism of the combined approaches. Facing a multitude of difficult challenges in deciphering human disease metabolome and its response to therapeutics, it is most efficient and reliable to integrate

stable isotope tracers, MS, NMR, and any other relevant approaches (such as those outlined in this review) in future research.

As the emerging stable isotope tracer-based metabolomic approach matures, it is foreseeable that the full complexity of the mammalian metabolic networks may begin to unfold for the first time in biomedical history. For this to be of translational and clinical relevance, our understanding of the metabolic networks on models must be ultimately validated by *in vivo* studies directly on human subjects, a trend that has already started (Fan, Lane et al. 2009). This is where one area of future tracer-based metabolomics research should be emphasized. A second area is the development of appropriate atom-tracking human metabolome databases and biochemoinformatic tools for analyzing metabolite labeling patterns from raw data and for reconstructing and modeling the networks. A third area is to develop computational tools for integrating the metabolic network functions with functional genomics and proteomics information into comprehensive metabolic, signaling, and gene regulatory models that can be used to discern key nodes in network regulation. These nodes are crucial to realizing the full benefit of systems biochemical understanding of human diseases in therapeutic development and validation, such as timely, cost-effective, and efficacious drug development with optimal on-target effects and minimal off-target toxicity. It is our view that the exciting technologies presented in the review will help revolutionize the field of pharmacology and therapeutics in the 21st century.

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Abbreviations

SIRM	Stable Isotope Resolved Metabolomics
META	Metabolomics Edited Transcriptomic Analysis
SiDMAP	Stable isotope-based Dynamic Metabolic Profiling
FT-ICR	Fourier Transform-Ion Cyclotron Resonance
IRMS	isotope ratio MS
PET	Positron Emission Tomography
CT	computed tomography
MALDI	matrix-assisted laser desorption ionization
TOCSY	total correlation spectroscopy
HSQC	heteronuclear single quantum coherence spectroscopy
HR-MAS	high-resolution magic angle spinning
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
CSI	chemical shift imaging
DNP	Dynamic Nuclear Polarization

NIR	near infrared region
FRET	fluorescence resonance energy transfer
α-KG	α -ketoglutarate
2-HG	2-hydroxyglutarate
GSH	reduced glutathione
GSSG	oxidized glutathione
AXP	adenine nucleotides
UXP	uracil nucleotides
2-DG	2-deoxyglucose
2-FDG	2-fluorodeoxy-D-glucose
MSA	methylseleninic acid
$^{13}\text{C}_6\text{-Glc}$	uniformly ^{13}C labeled glucose
PC	pyruvate carboxylation
PCh	phosphatidylcholines
RC	reductive carboxylation
PEP	phosphoenolpyruvate
OAA	oxaloacetate
SM	sphingomyelins
UDP-GlcNAc	uridine diphosphate N-acetylglucosamine
P_i	inorganic phosphate
5-ALA	5-aminolevulinic acid
MTBSTFA	N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide
BPTES	bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide
FH	fumarate hydratase
LDH	lactate dehydrogenase
PCB	pyruvate carboxylase
PDH	pyruvate dehydrogenase
IDH	isocitrate dehydrogenase
SDH	succinate dehydrogenase
PGM	phosphoglycerate mutase
EGFR	epithelial growth factor receptor
MMP	metalloproteinase
ALT	alanine transaminase
PPP	pentose phosphate pathway
ADMET	absorption, distribution, metabolism, excretion, and toxicity
NSCLC	non-small cell lung cancer

BAC	bronchioloadenocarcinoma
NC	non-cancerous
SUV	standardized uptake values
MCA	metabolic control analysis
MFA	metabolic flux analysis

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Figure 1. Integration of 'Omics approaches for systems biology

The arrows show the interrelationships between different 'omics levels represented by DNA (genomics), transfer RNA (transcriptomics and protein translation or functional genomics), the enzyme PCB (proteomics), metabolic pathway chart (Metabolomics) and physiology. The DNA molecule is from 1GIP, Protein Data Bank <http://www.wwpdb.org/> (Ceglarek, Kortz et al. 2011). The metabolic chart is from "<http://www.sigmaaldrich.com/life-science/metabolomics/learning-center/metabolic-pathways.html>"; transfer RNA from "http://en.wikipedia.org/wiki/Transfer_RNA"; Vitruvian from "<http://en.wikipedia.org/wiki/File:Vitruvian.jpg>"; PCB from "http://en.wikipedia.org/wiki/File:Pyruvate_Carboxylase_fromPDB_2QF7.png"; single and double headed arrows: one-way and two-way interactions, respectively

Figure 2. Intersecting metabolic pathways can only be resolved by isotopic labeling approach (adapted from (Fan and Lane 2011))

Illustrated are four intersecting pathways, glycolysis, the Krebs cycle, serinolysis, and glutaminolysis. Common metabolites are produced from these pathways, including pyruvate, lactate, glutamate, and malate. Without labeled tracers such as uniformly labeled glucose ($^{13}\text{C}_6$ -glucose), it would not be practical to resolve the metabolites derived from these pathways. Also noted is the ability of positional ^{13}C -isotopomers of Glu (i.e. ^{13}C -2,3-Glu and ^{13}C -4,5-Glu, respectively) to distinguish the presence and absence of anaplerotic pyruvate carboxylation (PC). Moreover, glutamate can be utilized for many other pathways (up to 55) such as the synthesis of glutathione (GSH), amino acids (AA), and proteins. Thus, chemical identification of metabolites does not equate to identification of their biochemical role. Colored carbons: ^{13}C ; double-headed arrows: ^{13}C labeled and unlabeled pools of the same metabolite; dashed or multiple arrows: multi-step reactions; OAA: oxaloacetate; α -KG: α -ketoglutarate.

Figure 3. Stable isotope-resolved metabolomics (SIRM) pipelines

SIRM approach can be implemented by administering stable isotope tracers such as uniformly ^{13}C -labeled glucose ($^{13}\text{C}_6\text{-Glc}$) or uniformly $^{13}\text{C},^{15}\text{N}$ -labeled glutamine ($^{13}\text{C}_5,^{15}\text{N}_2\text{-Gln}$) via addition to the culture medium for cells and excised tissues or via intravenous injection into whole organisms including human subjects. Therapeutic agents such as cisplatin or sodium selenite (structures as shown) can be included in the treatment to observe their impact on the metabolome. Alternatively, labeled therapeutic agents can be used to trace their metabolic fate in the same systems. The tracers are allowed to be metabolized for a duration, which can range from 15 min. (for mice) to 24 h or longer (for cells and tissues). After tracer incubation, cells are harvested by centrifugation or solvent quenching (Fan, Lane et al. 2011), followed by pulverization in liquid N_2 or scraping in solvents to break cells. Tissues are dissected from animals or resected from human subjects via surgery (Fan, Lane et al. 2009) and immediately flash-frozen in liquid N_2 , followed by pulverization in liquid N_2 to facilitate subsequent extraction while maintaining biochemical integrity. Aliquots of ground frozen tissues or broken cells can be extracted for polar and non-polar metabolites using a solvent partition method (Fan, Lane et al. 2011). Extracted metabolites are subject to various NMR and MS analyses for labeling patterns of various metabolites (Fan and Lane 2008; Lane, Fan et al. 2008; Lane, Fan et al. 2009). Illustrated here are the 1-D $^1\text{H}\{-^{13}\text{C}\}$ HSQC NMR spectra of polar extracts acquired from control and cisplatin-treated human lung adenocarcinoma A549 cells and the high-resolution FT-ICR-MS spectra of non-polar extracts obtained from control and selenite-treated A549 cells grown in $^{13}\text{C}_6\text{-Glc}$ (cf. **Figure 7**).

Figure 4. Stable isotope tracing can reveal metabolic pathways occurring in different cellular compartments

The fate of $^{13}\text{C}_6\text{-Glc}$ is tracked from glycolysis in the cytoplasm, lactate excretion into the medium, to the Krebs cycle in the mitochondria without and with the input of pyruvate carboxylation (PC), and then to lipid biosynthesis in the cytoplasm. The expected ^{13}C labeling patterns for various representative metabolites are deduced based on known enzyme reaction mechanisms (McMurry & Begley, 2005). The labeling patterns for the Krebs cycle metabolites are drawn for 1 cycle turn only, which will change with additional turns of the cycle (Fan et al., 2010). Dashed arrows: multi-step reactions; double-headed arrows: reversible reactions; double vertical dashed lines: membrane separation; ●: carbon-12; ●: carbon-13, ●: carbon-13 derived from PC; { }: scrambled ^{13}C labeling patterns due to the molecular symmetry of succinate; R_1 , R_2 : fatty acyl chains of lipids; R_3 : fatty acyl chains for neutral lipids or polar head groups of phospholipids; Glc: glucose; AcCoA: acetyl CoA; OAA: oxaloacetate; αKG : α -ketoglutarate; LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase; PCB: pyruvate carboxylase.

Figure 5. ^{13}C labeling patterns of various metabolites in A549 cells indicate separate pools of pyruvate and operation of Krebs cycle with and without pyruvate carboxylation
A549 cells were grown in $^{13}\text{C}_6$ -Glc with or without sodium selenite (SeO_3) for 24 hr. Medium samples were taken for ^1H NMR analysis at 0, 3, 6, 12, and 24 h in panel A. Cells were harvested by acetonitrile quenching, followed by extraction in acetonitrile: H_2O :chloroform (2:1.5:1) for polar metabolites, derivatization in MTBSTFA, and GC-MS analysis for ^{13}C mass isotopologues of metabolites in panel B. m+0, m+2, and m+3 represent monoisotopic, doubly, and triply ^{13}C -labeled isotopologues. Each value was an average of 2 duplicates.

Figure 6. Mass isotopologues and positional isotopomers

Illustrated are examples of ^{13}C mass isotopologues and positional isotopomers of Ala. ^{13}C mass isotopologues of Ala are Ala molecules with different numbers of ^{13}C atoms regardless of the atomic position (a total of 4), including the all ^{12}C (monoisotopic), singly ($^{13}\text{C}_1$ -), doubly ($^{13}\text{C}_2$ -), and triply ($^{13}\text{C}_3$ -) labeled species, as shown. ^{13}C positional isotopomers of Ala are Ala molecules with ^{13}C labeled at different atomic positions regardless of the number of ^{13}C atoms (a total of 8), such as ^{13}C at 1 (^{13}C -1), 2 (^{13}C -2), 3 (^{13}C -3), and 1 and 3 (^{13}C -1,3) positions, as shown. MS and NMR analyses are excellently suited for determination of mass isotopologues and positional isotopomers, respectively.

Figure 7. FT-ICR-MS analysis reveals reduced synthesis of fatty acyl chains but not glycerol backbone in cancer cell lipids in response to anti-cancer selenite treatment

Lung adenocarcinoma A549 cells were grown in $^{13}\text{C}_6$ -Glc in the absence (control) or presence of 6.25 μM sodium selenite. Cellular lipids were extracted with methanol and analyzed by a 7 Tesla FT-ICR-MS. A full profile of phospholipids and triacylglycerides along with their ^{13}C mass isotopologues were resolved. The spectral region for a phosphatidylinositol (PI, structure as shown) species is blown up for details. Each cycle of fatty acyl synthesis via fatty acid synthase is expected to add two ^{13}C neutron masses (from $^{13}\text{C}_2$ -acetyl CoA) to the lipid. M+3: monoisotopic (all ^{12}C) mass plus three ^{13}C neutron masses, which corresponds to $^{13}\text{C}_3$ -glycerol backbone; curved dashed arrows: cleavage of fatty acyl chains by tandem MS to confirm C18:0 (stearate) and C20:4 (arachidonate) acyl chain composition. Exact masses higher than M+3 in the insets are derived from ^{13}C -labeled fatty acyl chains with or without ^{13}C labeling in the glycerol backbone.

Figure 8. FT-ICR-MS resolves the neutron mass of ^{13}C , ^{15}N , and ^2H isotopes in glutamate
 The example given is the FT-ICR-MS spectrum of Glu simultaneously labeled with ^{13}C , ^{15}N , and ^2H in a cancer cell extract (red line). The nominal neutron mass of these three isotopes is 1 amu, which are resolved by conventional MS. The mass resolution of the FT-ICR-MS measurement was 1:200,000 or 0.5 ppm, which gave rise to three well-resolved m+1 Glu peaks, each containing one ^{13}C (1.003355 amu), one ^{15}N (0.997034), or one ^2H (1.006175 amu). Also shown is the simulated Glu spectrum with natural abundance distribution of ^{13}C , ^{15}N , and ^2H (black line).

Figure 9. 1D ^1H - $\{^{13}\text{C}\}$ HSQC NMR analysis reveals interactive effects of anti-cancer agents on cancer cell metabolism

Human lung cancer PC14PE6 cells were grown in $^{13}\text{C}_6$ -glucose for 24 h under four different treatments, control, MSA or taxol alone, and MSA+taxol. 1D HSQC analysis of the four polar cell extracts shows reduced ^{13}C abundance of the positional isotopomers of various metabolites induced by MSA or taxol alone and by the combined treatment. These changes suggest perturbations to glycolysis ($\downarrow^{13}\text{C}$ -3 and -2-lactate, ^{13}C -3-Ala), Krebs cycle ($\downarrow^{13}\text{C}$ -2-acetate, ^{13}C -2, -3, and -4-Glu, and ^{13}C -3-Asp), glutathione synthesis ($\downarrow^{13}\text{C}$ -4-GSH+GSSG), and PPP/nucleotide synthesis ($\downarrow^{13}\text{C}$ -1'-AXP, ^{13}C -1'-UXP) (cf. (Fan and Lane 2008) for assignment). The combined treatment appears to be synergistic in attenuating the synthesis of labeled Glu from the glucose tracer.

Figure 10. $^1\text{H}\{-^{13}\text{C}\}$ -HSQC NMR analysis reveals activation of multiple metabolic pathways in human lung cancer tissues

A lung cancer patient was infused with $^{13}\text{C}_6$ -glucose for 3 h before surgical resection of the cancerous and surrounding non-cancerous (NC) tissues. 1D HSQC analysis of the tissue extracts revealed increased ^{13}C abundance of all but ^{13}C -glucose isotopomers (e.g. Glc $^{13}\text{C}1$) in the cancerous tissue relative to the NC tissue. This suggests an activation of glycolysis, Krebs cycle, glutathione synthesis, PPP, and nucleotide biosynthesis in the cancerous tissue. In particular, the enhanced ^{13}C abundance of Glu-C2 and Glu-C3 isotopomers is consistent with an up-regulation of the anaplerotic pyruvate carboxylation pathway.

The data is adapted from Fan et al. (2009a, 2009b, 2009c).

Figure 11. *In vivo* metabolic imaging with PET, optical, and DNP-MRS methods

PET image (panel A) is reproduced from (Weber, Petersen et al. 2003), reprinted with permission. © 2008 American Society of Clinical Oncology. All rights reserved. The image was obtained using 2-¹⁸FDG as the PET tracer on an advanced NSCLC patient. The reduction of the PET SUV at 3 weeks after treatment with Pt-based chemotherapy is shown, and the SUV reduction precedes anatomical evidence of tumor regression.

Optical image (panel B) is reproduced from (Pellegatti, Raffaghello et al. 2008). The ATP in the extracellular space of tumors was imaged using a membrane-targeted luciferase plasmid. The extracellular fluid in the tumor contains ATP in the hundreds of micromolar, whereas healthy tissue showed much lower levels of ATP.

DNP-MRS image (panel C) is reproduced from (Hu, Balakrishnan et al. 2011) with permission from Elsevier. Hyperpolarized ¹³C pyruvate was injected into mice bearing a switchable MYC-driven liver cancer. High levels of lactate were observed in the tumor, due to the enhanced expression of LDH. Also evident was the accumulation of pyruvate in non-tumor tissues.

DNP-¹³C NMR spectrum (panel D) is reproduced from Figure 1 (Merritt, Harrison et al. 2007). The ¹³C NMR spectrum of isolated rat heart was acquired with only 100 scans after injection with hyperpolarized ¹³C-1-pyruvate. Resonances of pyruvate and its metabolites lactate, alanine, bicarbonate, and CO₂ are shown. The production of CO₂ and bicarbonate reflects pyruvate dehydrogenase activity, which also produces acetyl CoA as the entry substrate to the Krebs cycle.