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X^{13}CMS: Global Tracking of Isotopic Labels in Untargeted Metabolomics

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Supporting Information

ABSTRACT: Studies of isotopically labeled compounds have been fundamental to understanding metabolic pathways and fluxes. They have traditionally, however, been used in conjunction with targeted analyses that identify and quantify a limited number of labeled downstream metabolites. Here we describe an alternative workflow that leverages recent advances in untargeted metabolomic technologies to track the fates of isotopically labeled metabolites in a global, unbiased manner. This untargeted approach can be applied to discover novel biochemical pathways and characterize changes in the fates of labeled metabolites as a function of altered biological conditions such as disease. To facilitate the data analysis, we introduce X^{13}CMS, an extension of the widely used mass spectrometry-based metabolomic software package XCMS. X^{13}CMS uses the XCMS platform to detect metabolite peaks and perform retention-time alignment in liquid chromatography/mass spectrometry (LC/MS) data. With the use of the XCMS output, the program then identifies isotopologue groups that correspond to isotopically labeled compounds. The retrieval of these groups is done without any a priori knowledge besides the following input parameters: (i) the mass difference between the unlabeled and labeled isotopes, (ii) the mass accuracy of the instrument used in the analysis, and (iii) the estimated retention-time reproducibility of the chromatographic method. Despite its name, X^{13}CMS can be used to track any isotopic label. Additionally, it detects differential labeling patterns in biological samples collected from parallel control and experimental conditions. We validated the ability of X^{13}CMS to accurately retrieve labeled metabolites from complex biological matrices both with targeted LC/MS/MS analysis of a subset of the hits identified by the program and with labeled standards spiked into cell extracts. We demonstrate the full functionality of X^{13}CMS with an analysis of cultured rat astrocytes treated with uniformly labeled (U-)^{13}C-glucose during lipopolysaccharide (LPS) challenge. Our results show that out of 223 isotopologue groups enriched from U-^{13}C-glucose, 95 have statistically significant differential labeling patterns in astrocytes challenged with LPS compared to unchallenged control cells. Only two of these groups overlap with the 32 differentially regulated peaks identified by XCMS, indicating that X^{13}CMS uncovers different and complementary information from untargeted metabolomic studies. Like XCMS, X^{13}CMS is implemented in R. It is available from our laboratory website at http://pattilab.wustl.edu/x13cms.php.

The use of isotopically labeled compounds has yielded numerous important insights into the workings of cellular and organismal metabolism.[1] These have generally been gained through analysis of the patterns and kinetics of label incorporation into specific metabolites after introducing isotopically enriched precursors to the biological system of interest.[2] For instance, a common experimental design for metabolic pathway discovery is to monitor product compounds for the incorporation of isotopes derived from labeled potential precursors. A seminal example of this approach was the discovery of cholesterol biosynthesis from acetate.[3,4] Analogous experiments examining the labeling patterns of downstream metabolites have revealed key details of the Entner−Doudoroff pathway,[5] the tricarboxylic acid cycle,[6,7] the citramalate pathway,[8] the Calvin cycle,[9] and the pentose phosphate pathway.[10,11] It is worth emphasizing, however, that in all of these examples only one or two metabolites were monitored at a time for isotope incorporation.

The historic focus on analyzing label enrichment in only a limited number of metabolites is in part due to the availability of well-established and robust methods to measure labeling in specific compounds.[2] Although these experiments have proven invaluable to advancing our understanding of cellular metabolism, recent developments in untargeted metabolomic technologies have enabled comprehensive metabolite profiling at the systems level.[12] Through the use of LC/MS, it is now possible to detect thousands of metabolite signals from the extracts of biological specimens in a single analytical run. Critical to the development of this platform have been advances in both MS instrumentation and informatic tools for processing the large amounts of data that untargeted LC/MS experiments generate. Software solutions for performing peak detection, retention-time alignment, data annotation, and statistics have greatly increased the feasibility of applying the untargeted metabolomic approach to biological problems.[13−17]

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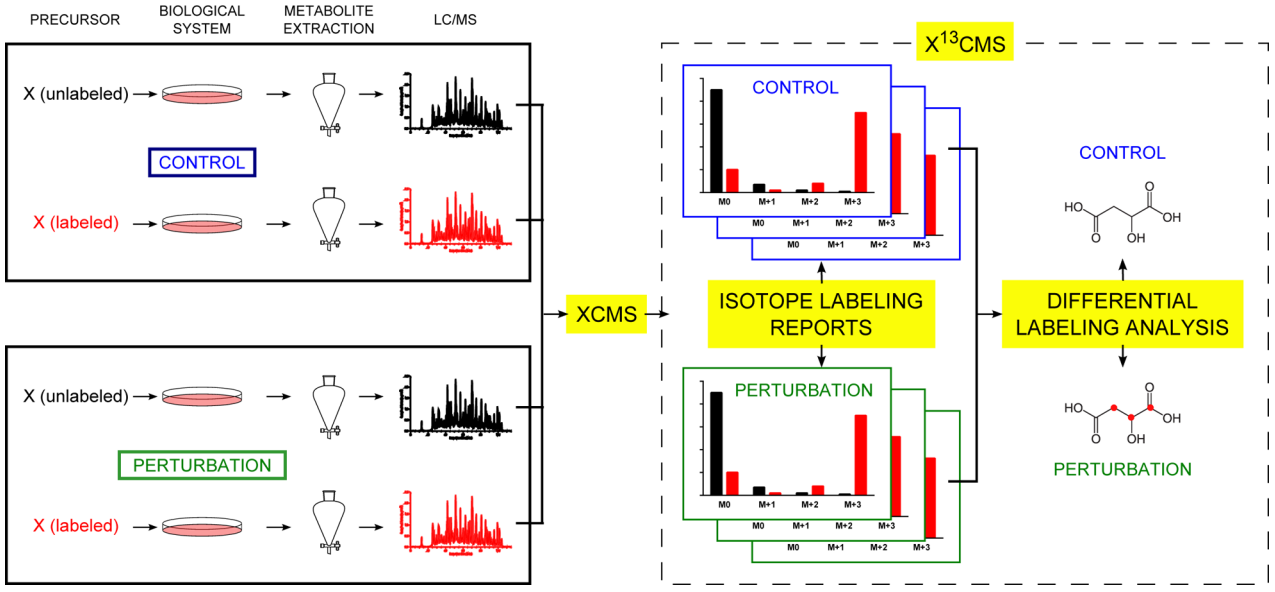

Figure 1. X^{13}CMS workflow. Samples representing control and perturbed conditions are divided into two groups, with one receiving an isotopically labeled substrate at a defined fraction of the total pool of that substrate and the other receiving the same substrate in a completely unlabeled or natural isotope abundance form. Metabolite extracts and LC/MS profiling data are collected on all samples and processed through XCMS to detect features and perform retention-time alignment. The features tables representing unlabeled and labeled samples are then paired off by biological condition and submitted to the getIsoLabelReport() routine of X^{13}CMS to detect features that have been enriched for the isotope label. To compare labeling patterns between conditions, the isotope labeling reports are processed with getIsoDiffReport().

We describe here an adaptation of the untargeted metabolomic approach to conduct isotopic labeling experiments in a more global, unbiased manner than has traditionally been available. The core of the analytical platform developed for this purpose is a software program called X^{13}CMS, an extension of the widely used untargeted metabolomic data analysis package XCMS.[13,18,19] Application of the X^{13}CMS workflow (Figure 1) allows investigators to track the fates of isotopically labeled precursors without the need of prior knowledge about pathways. To accomplish this, two biologically equivalent samples are prepared and a labeled precursor is applied to one of them. The fraction of the total precursor pool that exists in labeled form, as well as the length of time over which the label is applied, is left to the investigator to control, but generally should be set to ensure sufficient flux of the label through the cells' or tissues' metabolic pathways. After completion of the labeling protocol, replicates of both unlabeled and labeled samples are processed with the user's choice of metabolite extraction and untargeted LC/MS profiling methods. The raw LC/MS data are forwarded to XCMS for peak detection and retention-time alignment, and the resulting table representing all detected putative metabolites is forwarded to X^{13}CMS. From this table, the program retrieves groups of metabolite isotopologues; that is, it identifies metabolites that have been enriched with isotopic labels derived from the labeled precursor and groups them together by the mass-to-charge ratio (m/z) of the completely unlabeled form of each metabolite. We define an "isotopologue" as the set of all isotopomers of a compound containing a defined number of labeled atoms. We call the distribution of ion intensity among the different isotopologues of the group the "labeling pattern" of the metabolite. The algorithmic details of the isotopologue retrieval process are given in the X^{13}CMS Processing section below.

In addition to tracking the fates of labeled precursors, X^{13}CMS also performs differential labeling analysis to discover changes in these fates that arise from perturbations such as disease, genetic manipulation, or drug treatment. The analysis searches for isotopologue groups that are either uniquely represented or differently patterned in one biological condition versus another. This functionality of X^{13}CMS is analogous to the diffreport() routine of XCMS, which identifies differentially regulated metabolites from untargeted, nonlabeled profiling data.[13] To demonstrate the full capabilities of X^{13}CMS, we studied control and LPS-stimulated astrocytes with U-^{13}C-glucose. Our results reveal that integrating the untargeted metabolomic and isotopic labeling paradigms uncovers additional leads for further metabolic studies that are not available through either approach alone.

EXPERIMENTAL SECTION

Cell Culture and Metabolite Extraction. All reagents and solvents were obtained from Sigma-Aldrich unless otherwise specified. Immortalized rat astrocytes (American Type Culture Collection CRL-2005) were grown to confluency at 37 °C and 5% CO_2 in T25 flasks, using high-glucose DMEM (Gibco) containing 10% FBS and 1% penicillin/streptomycin (Gibco). Isotopic labeling was achieved through 30 min treatments with media containing 4.5 g/L U-^{13}C-glucose (100% of the total glucose content; Cambridge Isotopes); parallel cultures were treated with 4.5 g/L natural-abundance glucose. Simultaneous to the introduction of the labeled substrate, subsets of both unlabeled and labeled cultures were treated with LPS (added to culture media to a final concentration of 1 μg/mL) for the duration of the labeling protocol. Cells were then washed with phosphate buffer solution and high-performance liquid chromatography (HPLC)-grade water, quenched with 1 mL cold HPLC-grade methanol, scraped from the plate, and pelleted. Pellets were dried on a SpeedVac and subsequently lyophilized. Dried samples were weighed out and extracted as previously described,[20] with the solvent volumes adjusted to maintain a ratio of 1 mL of solvent per 1 mg of dried cellular

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material. The final volume of reconstitution solvent was adjusted to 100 μL per 1 mg of dried material. All cell-culture conditions (unlabeled versus labeled, control versus LPS-stimulated) were sampled in triplicate.

LC/MS Analysis. Five microliters of each extracted sample were injected onto a Luna Aminopropyl, 3 μm, 150 × 1.0 mm i.d. column (Phenomenex) with an Agilent 1200 series HPLC system. The samples were kept at 4 °C in the autosampler and injected onto a column maintained at room temperature. The column was used in hydrophilic interaction liquid chromatography (HILIC) mode with the following buffers and linear gradients: *A* = 95% H_2O, 5% ACN, 10 mM ammonium hydroxide, 10 mM ammonium acetate, pH 9.45; *B* = 100% ACN; 85% B from 0 to 7 min (min), 85% to 60% B from 7 to 27 min, 60% to 40% B from 27 to 32 min, and 40% to 20% B from 32 to 40 min. Mass spectrometry (MS) detection was carried out on an Agilent 6520 Q-TOF in negative ESI (electrospray ionization) mode with the following settings: gas temperature 325 °C, drying gas 5 L/min, nebulizer 15 psi, fragmentor 120 V, skimmer 65 V, capillary voltage −3500 V, and scan rate 1.06 spectra/s. Tandem MS (MS/MS) analyses were carried out with identical ESI parameters, and the following fragmentation and precursor ion selection settings: collision energy 10 V, precursor isolation window 1.3 amu, and scan rate 1.00 spectra/s.

Spiking of Labeled Standards. After all cell extracts had been analyzed, the remaining unlabeled LPS-stimulated samples were pooled, and then divided into two aliquots. One aliquot was spiked with U-^{13}C-lactate to a final concentration of 50 μM and alpha-^{15}N-glutamine to a final concentration of 10 μM. The control and spiked aliquots were each analyzed twice with the LC/MS protocol described above.

LC/MS Data Preprocessing with XCMS. Agilent .d files were converted to the mzXML format with msconverter.exe.[21] All samples (unlabeled and labeled, control, and LPS-stimulated) were analyzed together via a single call to xcmsSet(), which performs peak detection and sets up the data for further processing, including most importantly retention-time alignment. The designation of sample-class identities within XCMS should indicate which samples are unlabeled and which are labeled. The values for the parameters used in each step of the XCMS analysis in the present work are listed in Table 1.

X^{13}CMS Analysis. The main functionality of X^{13}CMS is implemented in two routines, getIsoLabelReport() and getIsoDiffReport(). The values for the parameters used in each routine to analyze the astrocyte data described herein are listed in Table 1.

Table 1. XCMS and X^{13}CMS Parameters

tool	routine	parameter values
XCMS	xcmsSet()	method = "centWave"
		ppm = 20
		peakwidth = c(20,200)
	group()	bw = 5
		mzwid = 0.025
	retcor()	method = "obiwarp"
X^{13}CMS	getIsoLabelReport()	RTwin = 10
		ppm = 20
		baselineNoise = 10000
		alpha = 0.05
	getIsoDiffReport()	none
	processIsoDiff()	alpha = 0.05

getIsoLabelReport(). This routine identifies which peaks in the LC/MS data are enriched for the isotopic label. The inputs are (1) an xcmsSet object containing the grouped and retention time-aligned peaks detected in both unlabeled and labeled samples, (2) the difference in mass between the unlabeled and labeled isotopes (we define this as the mass difference of the isotopic label, e.g., 1.00335 Da for ^{13}C), (3) the mass of the unlabeled isotope (e.g., 12.00000 Da for ^{12}C), and (4) three error tolerance parameters: (i) the acceptable parts per million (ppm) error of the m/z measurements, (ii) the ion intensity of baseline noise from the MS detector, and (iii) the retention-time window (RTwin) within which all detected peaks are considered as coeluting. With the use of the following procedure, the routine outputs a table listing isotopologue groups with statistically significant enrichment of the isotopic label: (1) the peak groups of the xcmsSet object are sorted by their aligned retention times into overlapping bins of width RTwin; the overlap is half of the bin width. (2) Within each bin, all possible pairwise comparisons of peaks are conducted to determine which are potential isotopologues of each other. The criteria for calling an isotopologue pair are as follows (note: in the ensuing text, "base peak" refers to the peak with the lower m/z value of the pair and "labeled peak" to the higher m/z value): (a) The m/z difference k between the base and labeled peaks must be a whole-number multiple of the isotopic label mass difference to within the ppm error of the MS instrument,

$$k^*(1-\varepsilon) - \frac{m_b\varepsilon}{d(1+\varepsilon)} \le k \le k^*(1+\varepsilon) + \frac{m_b\varepsilon}{d(1-\varepsilon)}$$

where k^* is the nearest whole number to k, ε is the ppm error expressed as a decimal, m_b is the m/z of the base peak, and d is the mass difference of the isotopic label. The derivation of these bounds on k is shown in Figure 1 of the Supporting Information. (b) Mean intensity of the labeled peak must be less than that of the base peak in samples treated with the unlabeled precursor. (3) The list of potential isotopologue pairs is sorted into groups that each contain a single unlabeled peak and all of that peak's observed isotopologues. This is done by grouping together all isotopologue pairs sharing a common base peak. Pairs in which both the base and labeled peaks are labeled peaks for a different base peak are discarded because they represent duplicate information. (4) Each isotopologue group is tested for statistically significant enrichment of the isotopic label in those samples generated from treatment with the labeled precursor. The intensities of each isotopologue within a group are normalized to the total ion count of the group; these relative intensities are then compared between the unlabeled samples and the labeled samples with a Welch's t-test. As long as at least one isotopologue within the group meets the user-defined p-value cutoff for significance, the whole group is called as being enriched with the label and is added to the output table. Additional quality-control measures include discarding groups in which the completely unlabeled isotopologue has a mean absolute ion intensity below baseline noise and those in which the relative intensities of the base isotopologue are higher in the labeled samples than in the unlabeled samples.

The output of getIsoLabelReport() is described in further detail in Table 2.

getIsoDiffReport(). This routine compares labeling patterns between two different biological conditions and assigns

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Table 2. Description of getIsoLabelReport() Output Table

column heading	explanation
compound	m/z of the base (unlabeled) isotopologue; value to be used for searching metabolite databases for putative identities of the group
isotopologues	list of m/z of all isotopologues in the group, including base
groupIDs	ID number of isotopologues in the xcmsSet object's "groups" slot; used for plotting EICs of individual isotopologues
RTs	retention time of each isotopologue
meanAbsIntU	mean absolute intensity of each isotopologue in the samples treated with unlabeled precursor
meanAbsIntL	mean absolute intensity of each isotopologue in the samples treated with labeled precursor
meanRelIntU	mean relative intensity (fraction of total absolute intensity of entire isotopologue group) of each isotopologue in the samples treated with unlabeled precursor
meanRelIntL	mean relative intensity of each isotopologue in the samples treated with labeled precursor
enrichmentLvsU	ratio meanRelIntL:meanRelIntU for each isotopologue
p_value	p-values from Welch's t-tests comparing relative intensities of each isotopologue in unlabeled versus labeled samples
sampleData	absolute intensities of each isotopologue in every sample

significance values to the observed differences. Its inputs are the tables of labeled isotopologue groups produced by getIsoLabelReport() from samples representing each condition. The groups are assigned into two categories: those that appear only in one sample class or the other and those that appear in both. Groups that appear only in one sample class are assigned a p-value of 0; these are potentially readouts of metabolic pathways that are uniquely activated in that biological condition. For all other groups that are enriched for the isotopic label in both conditions, getIsoDiffReport() uses an analysis analogous to that described in step (4) of getIsoLabelReport() to determine whether any isotopologues within the group are represented to a significantly different degree in samples obtained from one condition versus the other. The t-test is performed by using the relative intensities of the isotopologues in the labeled samples of each condition. The default output of getIsoDiffReport() is a table in which all labeled isotopologue groups are displayed with their categorization and associated p-values for differential labeling (see Table 3). Additional subroutines are available to extract groups

Table 3. Description of getIsoDiffReport() Output Table

column heading	explanation
sample1	list of m/z of all isotopologues in the group in samples from condition 1; empty if group is found to be enriched for label only in condition 2
sample 2	list of m/z of all isotopologues in the group in samples from condition 2; empty if group is found to be enriched for label only in condition 1
RT1	retention time of each isotopologue in condition 1
RT2	retention time of each isotopologue in condition 2
enrichment1	ratio meanRelIntL:meanRelIntU for each isotopologue in condition 1
enrichment2	Ratio meanRelIntL:meanRelIntU for each isotopologue in condition 2
p_value	p-values from Welch's t-tests comparing relative intensities of each isotopologue in labeled samples of condition 1 versus those of condition 2
sampleData	absolute intensities of each isotopologue in the labeled samples of both conditions

of interest that meet user-defined p-value cutoffs for significance and are not uniquely represented in one sample class simply due to poor chromatographic resolution producing extra shoulder peaks in those samples.

RESULTS AND DISCUSSION

Untargeted Retrieval of Isotopically Labeled Metabolites from Astrocytes. Several approaches to the problem of detecting isotopically labeled peaks in complex LC/MS data have been described in the literature,[22,23] but they conduct the isotopologue search prescriptively, which prevents them from being robust untargeted methods suitable for the discovery of new metabolites, uncharacteristic metabolic pathways, or metabolic byproducts of xenobiotics. In contrast, nontargeted tracer fate detection (NTFD) has been described for metabolomic workflows based on gas chromatography/mass spectrometry (GC/MS).[24] NTFD detects isotopically labeled features based on differences in mass spectra collected from samples that have been treated with a labeled tracer versus those that have not. In the present work, we present a similarly unbiased approach to retrieving isotopically labeled metabolites from LC/MS data, which differs from GC/MS data in that metabolites are more frequently represented as intact molecular ions than as ion fragments. Moreover, the NTFD method relies on the extraction of pure compound spectra, which remains a challenge with LC/MS data.[25−27] To accommodate these complexities, X^{13}CMS uses the feature-grouping and retention-time alignment capabilities of XCMS to reduce the search space for isotopologue groups. The search is then conducted exhaustively, using a small number of conservative assumptions about the ion-intensity patterns expected for isotopologue groups to filter out false calls based on mass difference and retention-time alignment alone.

To validate this untargeted approach to isotope-label detection, we examined the fates of U-^{13}C-glucose in rat astrocytes grown under standard culture conditions. Using getIsoLabelReport() on LC/MS profiling data collected from parallel cultures of cells treated with natural-abundance or U-^{13}C-glucose for 30 min, we identified 193 isotopologue groups that were significantly enriched for ^{13}C at a p-value cutoff of 0.05. To confirm that these groups correspond to actual labeled metabolites, we performed targeted MS/MS analyses on selected base isotopologues, their most abundant labeled isotopologues, and unlabeled standards matching the base isotopologues. A set of these analyses for the group with a base m/z of 146.045 is shown in Figure 2. Querying this m/z in METLIN returned glutamate as a possible identity,[28] which was supported with the observation of identical retention times of the standard and the group as well as the strong match between the 10 V MS/MS fragmentation patterns of the standard (Figure 2a) and the base isotopologue (Figure 2b). Furthermore, the predicted fragmentation pattern of the most abundant isotopologue, 148.050 m/z, was also observed (Figure 2c). This isotopologue corresponds to the presence of two ^{13}C atoms in the molecule, both of which are retained in the fragment that arises from a water loss (128.035 m/z). The corresponding fragment of the 148.050 isotopologue is thus predicted to have an m/z of 130.042, and this was indeed observed. The other predominant peak in the fragmentation pattern, 102.056 m/z, corresponds to a CO_2 loss containing either of the carboxylate carbons of glutamate. Depending on whether the lost carbon is labeled or not, this peak is expected to shift to 103.059 or 104.063 m/z, both of which are observed

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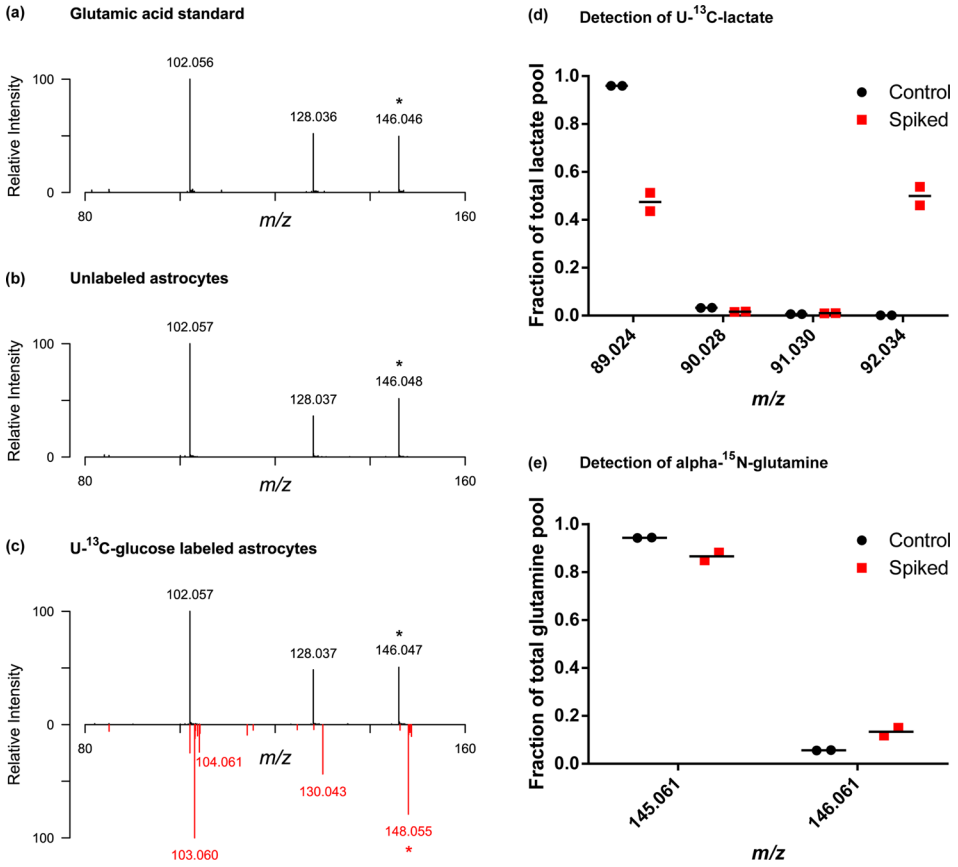

Figure 2. Validating isotopologue retrieval by X^{13}CMS. MS/MS spectra at a collision energy of 10 V for (a) glutamic acid standard, (b) base isotopologue of the 146 m/z group in unlabeled astrocytes, (c) base isotopologue of the 146 m/z group in U-^{13}C-glucose-labeled astrocytes (top) and 148 isotopologue (bottom) in labeled astrocytes. Star indicates precursor ion. Intensities are expressed relative to the most intense fragment. Detection of (d) U-^{13}C-lactate and (e) alpha-^{15}N-glutamine spiked into unlabeled, LPS-stimulated astrocytes.

in the fragmentation pattern of the 148.050 m/z isotopologue. Taken together, these data indicate that the 146.045 m/z group was correctly identified by getIsoLabelReport() as a labeled metabolite.

To further validate the untargeted label retrieval process, we spiked cell extracts obtained from unlabeled, LPS-treated astrocytes with known amounts of U-^{13}C-lactate and alpha-^{15}N-glutamine and analyzed them along with unspiked controls by LC/MS. The data were processed with getIsoLabelReport() twice, once to search for ^{13}C and once for ^{15}N. In the former case, we recovered the isotopologue group with base m/z 89.024 and determined that in the spiked samples, approximately half of the total lactate pool was present in the fully labeled form (Figure 2d). The search for ^{15}N recovered the isotopologue group with base m/z 145.061, which was enriched for the +1 ^{15}N peak (146.061 m/z) in the spiked samples approximately 2.5 times more than in the unspiked samples (Figure 2e). It should be noted that the resolution of the Q-TOF used in these analyses is insufficient to differentiate between the 0.99704 m/z difference between ^{14}N and ^{15}N and the 1.00335 m/z difference between ^{12}C and ^{13}C. Consequently, the intensity of the 146.061 m/z peak in the spiked samples is a mix of signal from both the natural abundance of singly ^{13}C-labeled glutamine as well as alpha-^{15}N-glutamine. This circumstance did not prevent getIsoLabelReport() from finding the +1 ^{15}N isotopologue, due to its use of the control samples containing natural-abundance distributions of isotopologues as a basis for comparison with the labeled or spiked samples.

Differential Labeling Analysis of LPS-Challenged Astrocytes. To illustrate the complete X^{13}CMS workflow, we investigated the use of glucose in astrocytes challenged with the inflammatory stimulus LPS. Astrocytes, the most abundant cell type in the central nervous system, are metabolically linked to neurons through the delivery of energy substrates, reuptake of neurotransmitters, and defense against oxidative stress.[29] In particular, inflammation-induced astrocyte dysfunction has been associated with neurological pathologies such as stroke, Alzheimer's, and Parkinson's disease.[30] The exact nature of the astrocyte response to inflammatory stimuli remains incompletely understood, but it is known that their glucose utilization is increased.[29] Our aim in the current work was to use an untargeted isotopic labeling approach to generate leads into the downstream metabolic consequences of this increased glucose utilization.

In parallel, we cultured immortalized rat astrocytes in media containing either U-^{13}C-glucose or natural-abundance glucose. Subsets of both unlabeled and labeled cultures were treated with LPS for the duration of the labeling period (Figure 3a). Analysis of the LC/MS profiling data of these samples with getIsoLabelReport() yielded 193 and 160 ^{13}C-enriched isotopologue groups in control and LPS-challenged astrocytes, respectively. Of these, 130 were found in samples from both conditions (Figure 3b). Differential labeling analysis using getIsoDiffReport() and the additional filtering subroutines

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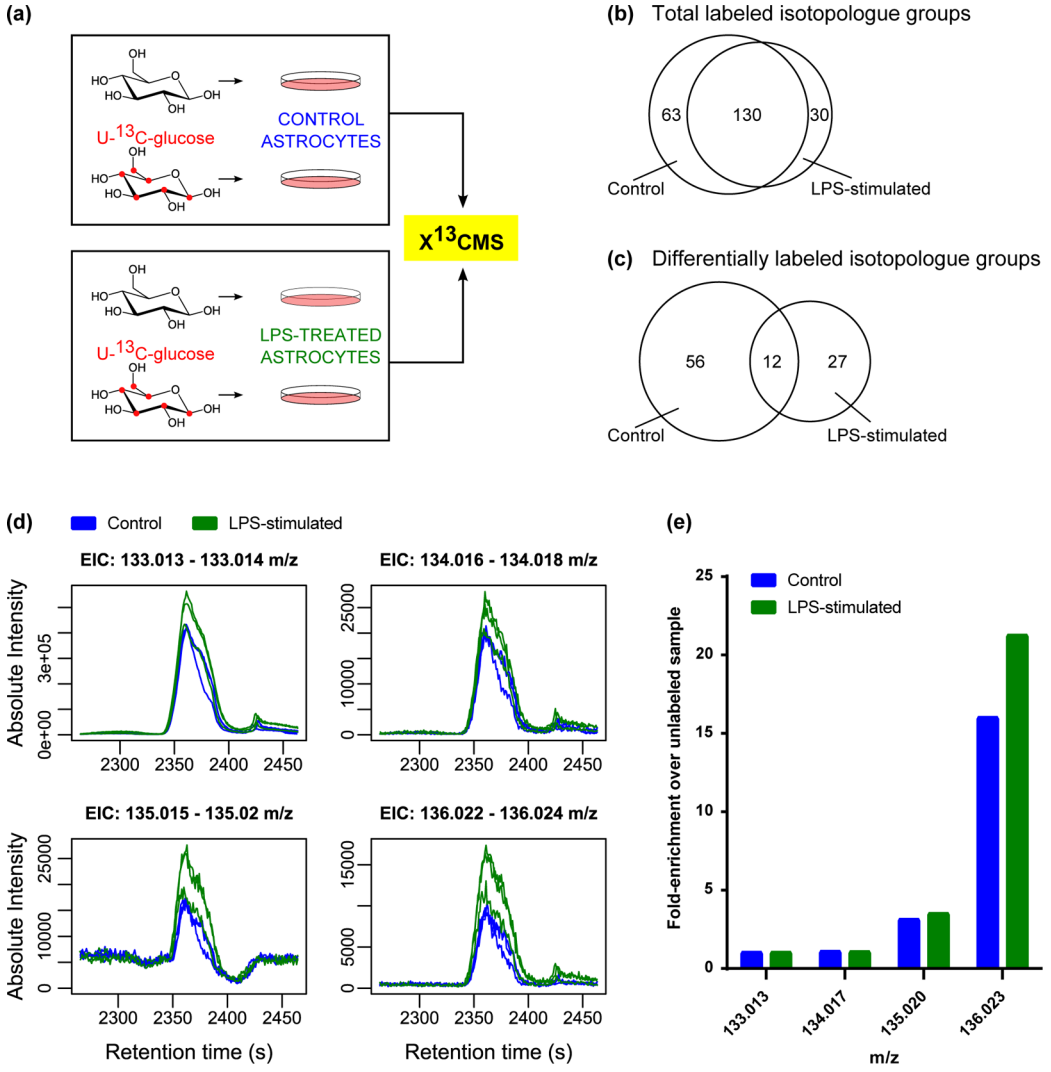

Figure 3. Differential labeling analysis of control and LPS-challenged rat astrocytes. (a) Experimental design. (b) All label-enriched isotopologue groups identified in control and LPS-stimulated cells. (c) Isotopologue groups identified as being differentially labeled in control versus LPS-stimulated cells. (d) Extracted ion chromatograms for the isotopologue group with base m/z of 133.013. (e) Enrichment of each isotopologue of the 133.013 m/z group in labeled cells compared to unlabeled cells of each culture condition (control vs LPS-stimulated).

described in the Experimental Section revealed that 95 groups had statistically significant different labeling patterns ($p < 0.05$) in control versus LPS-challenged cells. These were divided into 56 groups uniquely found in control cells and not in LPS-treated cells, 27 groups uniquely found in LPS-treated cells and not in control cells, and 12 found in both cell cultures (Figure 3c). The structural characterization of each of these leads is beyond the scope of the current work, but for illustrative purposes we show the extracted ion chromatograms for the group with a base isotopologue of 133.013 m/z, which is one of the 12 shared but differentially labeled groups (Figure 3d). The enrichment of the highest mass isotopologue of the group, 136.023 m/z, occurs to a greater extent in the LPS-treated astrocytes than in the controls (Figure 3e). Although MS/MS analysis suggested that this isotopologue group corresponds to the tricarboxylic acid (TCA) cycle intermediate malate, we note also that the majority of the differentially labeled isotopologue groups (62 out of 95) contain a base peak of over 250 m/z. These groups are unlikely to correspond to the intermediates of central carbon metabolism (glycolysis and the TCA cycle), and their predominance suggests that the increased glucose utilization of LPS-challenged astrocytes is not being directed exclusively toward energy-producing pathways. As is typical for untargeted metabolomic workflows, these outputs of X^{13}CMS are not definitive phenotypic readouts but rather leads that require further investigation to determine their chemical identities and validate their biological significance.

Integrating Untargeted Isotopic Labeling Analysis with Label-Free Metabolomics. To interrogate the degree of complementarity between the isotopic labeling platform described here to the standard untargeted metabolomic approach applied to nonlabeled samples, we compared a set of results from each type of analysis performed on the astrocyte profiling data. Comparing unlabeled control to unlabeled LPS-challenged cultures with the XCMS routine diffreport(), we identified 32 differentially regulated features (defined by p-value < 0.05 for difference in absolute intensity values between sample classes; fold change > 1.5 between sample classes; and mean absolute intensity > 10000 in at least one sample class). Comparing these 32 features with the 95 identified as being differentially labeled by X^{13}CMS, we discovered that only two were recovered by both analyses (Figure 4). Taken together,

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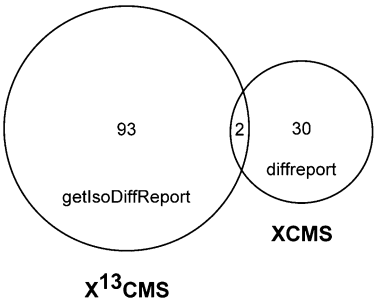

Figure 4. Comparison of outputs from untargeted isotope labeling analysis and label-free untargeted metabolomics.

these results suggest that two broad classes of metabolic shifts occur in LPS-challenged astrocytes. In the first type, identified by the traditional label-free approach, the LPS challenge alters the regulation of pathways that do not use carbon from glucose, which leads to different-sized pools of unlabeled metabolites. In the second type of shift, identified by tracing the fates of labeled glucose, treatment with LPS alters the regulation of a number of pathways that are ultimately linked to glucose, but the total pools of the metabolites involved in these pathways do not change presumably because of compensatory flux through other pathways that do not involve glucose.

The lack of a large overlap in the outputs of the standard label-free workflow and our untargeted isotopic labeling analysis highlights the complementarity of the two approaches. The former platform generates leads by discovering metabolites whose levels change significantly as a function of biological perturbation.[31,32] While a powerful approach, the consideration of only altered metabolite levels overlooks the potentially important roles played by metabolites whose levels do not change. A cell or organism may activate numerous compensatory pathways to maintain steady, homeostatic levels of critical metabolites when challenged with disruptive conditions. For example, acetyl-coenzyme A (CoA) is a central metabolite found at the convergence of several catabolic and anabolic pathways.[33] Disruption of any one of these pathways (e.g., glycolysis) may be insufficient to alter acetyl-CoA levels due to compensatory changes in the other pathways that connect to this metabolite (e.g., upregulation of fatty acid oxidation[34]). In theory, a comprehensive measurement of all metabolite levels would reveal changes in the levels of metabolites that act in compensatory pathways, thereby implicating them as part of the biochemical response to the perturbation. However, the current state of global-profiling technology remains inadequate to realize this type of analysis. Not all metabolites can be detected by LC/MS, and of those that can, only a small subset is typically assigned definitive chemical identities. Furthermore, the dynamics of cellular metabolism can be highly nonlinear,[35] which precludes drawing mechanistic conclusions from simple correlative models between metabolite levels. To overcome these limitations, isotopic labeling analysis can be applied to track the altered metabolic fluxes that arise from activation of compensatory pathways. In the example of acetyl-CoA, use of a labeled upstream substrate may reveal movement of the label to acetyl-CoA in one condition but not another, despite total acetyl-CoA levels remaining the same in both conditions.

CONCLUSIONS

Standard untargeted metabolomic approaches typically report the static levels of a broad range of metabolites from unlabeled samples. In contrast, isotopic labeling analysis provides information about pathway fluxes but most commonly only for a limited number of metabolites that are measured with targeted methodologies. Here, we present an approach that conceptually integrates characteristics from each of these experimental designs to provide insights into isotopic labeling at the systems level. Our approach leverages recent advances in untargeted metabolomics to track the metabolism of isotopically labeled substrates in an unbiased manner and to determine if this metabolism changes as a function of biological perturbation. These analyses are facilitated by the software program presented here, X^{13}CMS. We have shown that X^{13}CMS can identify features that are enriched for the precursor-derived isotopic label, as well as analyze differences in their labeling patterns between samples arising from different biological contexts. This latter application of X^{13}CMS can reveal metabolic insights that are not gained by standard untargeted metabolomic approaches that only use unlabeled samples. Thus, X^{13}CMS provides valuable insights into metabolic mechanisms that are highly complementary to those available through existing metabolomic technologies.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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