nature portfolio

Peer Review File

Spatial isotope deep tracing deciphers inter-tissue metabolic crosstalk

Corresponding Author: Professor Zeper Abliz

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This work successfully combined the spatial metabolomics technology (AFADESI-MSI) and the stable-isotope tracing metabolomics to quantitatively profile metabolic activity in a spatially-resolved manner. To do so, the authors developed an interesting computational tool, namely MSIFluxer, to achieve the identification of labeled metabolites and quantify the labeled fraction of MSI data. Finally, the authors applied the method to several applications, such as fatty acid metabolic crosstalk between tissues and metabolic alterations induced by cancer. This work provided an innovative framework to track labeled metabolites with the spatial metabolomics technology. Overall, the work is well-designed and performed, and suitable for consideration of publication in Nature Communications. Here are some comments need be addressed:

1. In Fig. 2b, the data showed the infusion of U-13C glucose led to the predominant distribution of labeled glutamate within the brain, whereas following U-13C glutamine infusion, labeled glutamate-M5 was primarily observed in the pancreas and high labeled fraction of M1 and M2 glutamate in brain were also observed. Since glutamate is an important neurotransmitter in brain and it seems like the brain does not uptake M5-glutamine directly to synthesis glutamate, could the authors further clarify the highly labeled fraction of M1 and M2 glutamate in brain after U-13C glutamine infusion?
2. The MSIFluxer functions by first extracting the intensity of targeted isotopologues. However, MSI lacks the capability to separate ions with the same m/z. In cases where two metabolites share identical m/z values, how can these metabolites be identified and assigned to their respective isotopologues? In addition, there are many isobaric interferences among different lipid species, for example, M2 of TG(56:6) showed in Fig 4h and M0 of TG(56:5). The above mentioned isomeric and isobaric overlappings would cause inaccurate quantification. The authors should discuss these limitations. In such a case, ion mobility-MS may provide additional separation and resolving power.
3. In Fig 4f, it is abnormal that FFAs were not to detected in the serum. The previous work reported that dietary glucose could contribute to newly synthesized palmitate acid in plasma (https://doi.org/10.1016/j.cmet.2023.03.007). It is necessary to provide more evidence to obtain the conclusion that PA is concurrently oxidized in the BAT and brain.
4. The hypothesis of “We set a threshold, assuming that metabolites with labeling beyond M8 733 might be challenging to detect using MSI with 13C-glucose and 13C-glutamine infusion” may not be valid. The authors are recommended to check a panel of crucial metabolites in datasets including AMP, fatty acids etc.
5. The authors utilize Supplementary Figure 1a to demonstrate isotopic steady state attainment based on M6 glucose and M5 glutamine labeled fractions. While this approach is suitable, continuous monitoring of labeled fractions at multiple time points is essential to conclusively establish isotopic steady state.
6. In Supplementary Fig. 3, the authors illustrate the capacity of MSIFluxer to choose the specific adduct and improve the quantitative accuracy. Uridine and TCA were used as examples. Could the authors provide more examples or a statistics result to further prove the advantages of MSIFluxer?
7. In Supplementary Figure 4d, glutathione presented concentrated distribution in medulla in un-infusion samples while M0 of glutathione in U-13C infusion samples were distributed in both medulla and outer cortex of kidney, suggesting potential metabolic perturbations due to glucose infusion or surgical intervention?? Please explain the potential impact of these factors on organ metabolism.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
In this study, an MSI analysis tool called MSIFluxer was developed. The authors claim that this analysis tool integrates LCMS and MSI data, overcoming the traditional weakness of mass spectrometry imaging, where isotopic metabolites are less abundant (compared to LC-MS), and allowing new insights into metabolism. However, there are a number of critical issues and, in particular, from a biochemical point of view, the validity and novelty of the results obtained are questionable.

First, the organ sampling protocol is not described in detail. Presumably, the metabolic biochemical results presented here are influenced by post-mortem degradation. Indeed, previous work has shown that after brain and heart extraction, ischaemia causes rapid degradation of ATP and phosphate glycolytic intermediates due to increased phosphatase activity, accumulation of NADH due to inhibition of the electron chain transfer system, and increased lactic acid production due to increased anaerobic glycolysis. Therefore, without appropriate organ extraction methods (Proteomics 2013 DOI: 10.1002/pmic.201300047, Proteomics 2016 DOI: 10.1002/pmic.201500402), the results obtained here would not allow for a "metabolic profile of the hypoxic response". This trend would be even more pronounced in a tracer analysis using stable isotopes. In this respect, the authors should use sample preparation methods that avoid such post-mortem degradation if possible, and even if this is difficult, it should be discussed as a limitation.

Secondly, the authors claim to have found metabolic interactions between tissues other than the Cori cycle, but it is known that fatty acid is released from the liver and used by the heart. It is also well known that implanted cancer cells can affect the metabolism of the host.

Thirdly, MSI results for 13C-labelled compounds of a given m/z should be shown simultaneously with images of the same m/z in negative controls not dosed with stable isotopes, as there is no guarantee that interference from other molecular ions can be completely avoided even when results are obtained by HRMS.

Fourthly, why is an odd number of 13C-labelled palmitic acids detected when 13C6 glucose is administered for only a few hours, since 13C6 glucose is used to synthesise 13C2 malonyl CoA, which should result in the synthesis of fatty acids containing an even number of 13Cs, and there are many such reports. How can this discrepancy be explained?

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The paper from Li et al. introduces novel methodology for whole-body metabolic flux analysis using stable isotope labelling, LC-MS, mass spectrometric imaging and a new software tool termed ‘MSI Flux’. The paper features highly interesting and valuable data, however their interpretation is speculative and ignores a number of inherent problems of the chosen methodology.
Specific problems:
Based on the description of the MSIFlux, this computational tool does not perform metabolic flux analysis. State-of-the-art software packages for metabolic flux analysis (for a recent review of the field see de Falco B, Giannino F, Carteni F, Mazzoleni S, Kim DH. Metabolic flux analysis: a comprehensive review on sample preparation, analytical techniques, data analysis, computational modelling, and main application areas. RSC Adv. 2022 Sep 7;12(39):25528-25548. doi: 10.1039/d2ra03326g.) employ complex mathematical models (mostly master equation-type models, see for instance Thommen Q, Hurbain J, Pfeuty B. Stochastic simulation algorithm for isotope-based dynamic flux analysis. Metab Eng. 2023 Jan; 75:100-109. doi: 10.1016/j.ymben.2022.11.001.) to simulate and decipher complex flux networks based on quantitative data. In contrast MSI Flux calculates accurate masses for metabolites including their adducts and labelled isotopologues, extracts these signals from raw MSI datasets and corrects the isotope distribution patterns using the native distribution. These are fairly routine arithmetic tasks not dealing with the metabolic flux analysis. The tool has its practical value, however, it is not properly validated and certainly does not form sufficient novelty for publication in Nature Communications.
The role of mass spectrometric imaging is unclear. As much as one can tell from the manuscript, the areas corresponding to the individual organs are averaged and then processed using the computational tool mentioned above. This raises a number of problems including the sampling efficiency of the individual organs and also the lack of quantitative nature of MS imaging data. The LC-MS data collected by the authors could serve as a much more appropriate basis for performing flux analysis. Furthermore, for LC-MS analysis, whole organs can be harvested, extracted and analysed. One whole body section does not and cannot represent entire organs, due to the heterogeneity of metabolite distribution and the very low efficiency of sampling. Some organs may not be represented at all in the section, for others the actual cryosection represents <0.1% of their overall weight. Also, with appropriate calibration LC-MS/MS can be fully quantitative, which is an absolute requirement for metabolic flux analysis.
Along the same lines, it is not entirely clear what the relationship is between the LC-MS and MSI data. Do the authors only use the superior quality LC-MS data to create a metabolite inventory as an input for the computational tool? It would be highly interesting to see how the two types of data correlate. Otherwise is does not seem to be sensible to use high specificity, high sensitivity, quantitative data just to facilitate the analysis of inherently lower sensitivity, lower specificity and

non-quantitative data.
The details of MSI data preprocessing are not given. The reviewer understands that the m/z values predicted by the computational tool are searched for in a +/- 5 ppm window. What happens if there are multiple peaks present in this window? Is the most intensive or the closest chosen? What happens to predictable other isotopologues, like 34S ? What happens when peaks are asymmetrical or not fully resolved? What is the general consideration for isomers ? As MSI cannot resolve them, they may elicit significant interferences, making the case for MS imaging even weaker.
The authors treat MSI data as quantitative, while the response factors for different tissue matrices and also for different adducts are significantly different. This needs to be taken into account, especially when different organs are compared. The low intensity of a metabolite signal can easily be the reason for the non-detection of isotopologues.
Error bars are missing at several places, for example in Figure 4, but error bars are an absolute pre-requisite for all 'labelled fraction data'. Also, where error bars are shown, the number of points is very low (6). Do these points correspond to biological replicates, so individual animals? For the imaging data it would be useful to calculate error using all pixels. Would also be useful to demonstrate the labelling efficiency distribution in space, not only in time. Images are helpful, but do not substitute for proper statistical analysis.
Some of the claims of the authors regarding the new finding regarding systemic effect of lactate (see for instance a recent paper Liu X, Li S, Cui Q, Guo B, Ding W, Liu J, Quan L, Li X, Xie P, Jin L, Sheng Y, Chen W, Wang K, Zeng F, Qiu Y, Liu C, Zhang Y, Lv F, Hu X, Xiao RP. Activation of GPR81 by lactate drives tumour-induced cachexia. Nat Metab. 2024 Apr;6(4):708-723. doi: 10.1038/s42255-024-01011-0.) or the liver-heart fatty acid co-metabolism (recent review: Joerg Heeren, Ludger Scheja, Metabolic-associated fatty liver disease and lipoprotein metabolism, Molecular Metabolism, 50, 2021, 101238, https://doi.org/10.1016/j.molmet.2021.101238.) are not new. Literature provides compelling evidence for these processes, while the results shown in the paper do not prove (though support) the claims of the authors.
Details for the imaging experiments are missing. For instance the pixel size and biological feature resolution as well as the time required for the analysis are not given. One is also wondering why the authors use the 'AFADESI' acronym when they are basically using the DESI technology with a modified mass spectrometer inlet.
In conclusion, the authors should consider dropping the imaging component from the paper and re-evaluate the data from the LC-MS experiments. The proper validation of the LC-MS-based method may lead to an eventually publishable paper, however the manuscript in its current form cannot be accepted for publication.

(Remarks on code availability)
I do not have the expertise to judge the quality of the code.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All comments have been successfully addressed by the authors. I feel the manuscirpt is ready for acceptance by Nature Communications. Congratulations !

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors and others have generally addressed the reviewers' comments reasonably well.

One minor comment is that the colouring of the M1-M10 graph in Figure 4g is very difficult to see. It is a very interesting finding that fatty acid synthesis from glucose occurs more prominently in the BAT, so the colouring should be such that the even and odd C13 labels can be clearly distinguished here.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors spent significant amount of time with improving the paper including answering all the reviewers' comments and even performing new experiments. I do appreciate that the authors even changed the name of their software tool to reflect more to its real function. Nevertheless, in-spite of all the changes and improvements a number of fundamental issues remain, which preclude the publication of the manuscript in Nature Communications:
1. Novelty. There has been enormous amount of literature published recently in this area. I'd particularly like to highlight papers from the Patti and Rabinowitz groups, which successfully combined LC-MS and MSI for similar purposes with similar workflows to trace metabolic pathways in tissues. The authors – very correctly – cite some of these papers, for instance refs 4 and 10 in the rebuttal letter. The exact workflows described here are not exactly the same as in those publications, however the fundamental ideas are, hence the publication of the current manuscript in Nature Communications cannot be justified. Nevertheless, it can and should be published in journals focusing more on tissue metabolism or analytical

approaches.
2. Innovation. The software tool the authors devised for extracting isotopologue data from MSI datasets is highly practical, but lacks any innovation with regard to its methodology. As the authors discuss in the Discussion part of their paper, it suffers from a number of problems, which they haven't attempted to solve. One could imagine software tools which identify isotope clusters in the data which do not follow natural distribution and annotate them in a novel way using (among others) LC-MS data, but this is not the case here. The authors argue that MSI data should be used because it features more metabolites – which is true – but their algorithm simply excludes anything which happen to show better sensitivity (e.g. because of locally high concentrations) in MSI than in LC-MS. They also exclude any unexpected adducts or fragments. Furthermore, they don't discuss the case when the LC-MS has multiple comparable intensity peaks for the same accurate mass. They mention the potential use of ion mobility, however the current dataset is full of with metabolites which have multiple different isomers with completely different metabolic functions. Additional problems include the 5 ppm m/z window (a Q-Exactive can do much better than that) and their arbitrary selection of the highest intensity peak. These problems are largely mentioned, but mentioning a problem is not equivalent with solving it. The data is burdened by this enormous "noise", which is probably behind some of the "anomalies" and unexpected results.
3. Lack of quantification. The authors could have easily solved this problem by calibrating their LC-MS assays. Instead they mixed high concentrations of labelled glucose into tissue homogenates and not too surprisingly received a nice, linear calibration. MSI is infamous about its matrix effect – Figure R11 just completely denies the existence of this well documented (cf. Anal. Chem. 2018, 90, 9, 5637-5645) phenomenon. I believe that the reported data is correct, but does not achieve its intended purpose. Spiking homogenates with high concentrations of analyte does not prove the point, especially not that the authors should have done the same for all compounds concerned in the study, across the physiological concentration range to prove anything. I am not suggesting this as the spiked homogenates are not representative of the fresh frozen tissues containing the analyte molecules, due to completely different molecular interactions (or the lack of them). Practically any comparison made between the intensity of the metabolites between any two tissue segments is highly questionable this way.
4. Lack of focus and biological validation. The authors chose to show highlights of interesting pieces of data instead of focusing on a single biological pathway and properly validate their findings. Again, the manuscript is correct, the authors are aware of the weakness of their statements, but instead of providing independent evidence for their findings, just jump for the next interesting piece of data. For instance, the authors point out the interesting localisation of fatty acid biosynthesis in the brown adipose tissue. The data actually highlights some other histological features as well, which are associated with de-novo fatty acyl synthesis, but the text does not even mention these additional areas. Nevertheless, fatty acid biosynthesis is a well understood pathway, catalysed by fatty acid synthase (FASN) a well-known enzyme complex with commercially available antibodies. The authors should have used immunohistochemistry (potentially combined with proteomics) to validate their findings regarding the tentative expression of certain genes.
5. Lack of details on data treatment. In spite of the request, the authors still failed to disclose the details of data preprocessing. Just for clarity, preprocessing includes recalibration, denoising, normalisation, peak picking and peak matching among other potential steps. The added single sentence ("These cdf files were then imported into MassImager software to reconstruct MS images, browse mass spectra, select regions of interest (ROIs), and extract the average mass spectra.") does not explain anything about preprocessing, hence makes it hard to judge the graveness of the associated errors.
In addition there are plenty of minor problems associated with inconsistencies in data labelling or the lack of information in the figure captions, but these problems were not taken into account for the reviewer's decision.

(Remarks on code availability)
Not having sufficient expertise.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The reviewer appreciates the effort the authors invested into developing the manuscript and reply to the points raised. Unfortunately the replies only partially address the problems or, in other cases, do not address them at all.
Specific points: 1) Novelty. The reviewer does not say that the current manuscript has no novel components compared to the literature. However, the combination of methodologies (isotope labelled metabolite administration, MSI, LC-MS) is not new. The actual implementation of data analysis have novel components and there is new biological information. Nevertheless, these novel components can not and do not justify the publication of the manuscript in Nature Communications.
2) The points raised criticise the principles the authors based their study on. Furthermore, offered solutions for these problems. Unfortunately the authors chose to reject these based on their observations. Point 1: This situation is not unlikely. There are histological features (e.g. pituitary gland in the brain or lymph nodes in any tissue) which contribute to <1% of the mass of the organ, but have completely different metabolic composition. This can be captured by MSI, while LC-MS tend not to detect most of these components. Point 2: Again, it is a question of principle - what is correct and what is not. It is not particularly hard to check for the shift in isotopologue distribution of arbitrary ions in MSI, if the authors already created the tools. Point3: The information about the differential labelling of isomers must be obvious from LC_MS. Point 4: The answer provided by the authors says that they wrongly annotated the metabolites in 9.3% of the cases ! (Fig R1) I don't think if it is acceptable by any means. Nevettheless, the suggestion was to calibrate the spectra (not only the instrument) to achieve better than 1 ppm resolution.

3)The quantification - or actually the lack of it - is the gravest problem with the study. The authors state that they always look at labelled fraction, however 90% of the manuscript is about the distribution of certain (labelled or unlabelled) isotopologues across different tissue types. This does not work, the reference provided in the previous round of review clearly explains the problem. There is not a single figure in the manuscript or the supplmentary material, which would depict the labelled fraction in any spatially resolved manner ! This also raises questions regarding the functionality of the MSITracer. The authors base their conclusions on the following axioms:
- The intensity of an ion is proportional its concentration across multiple tissue types
- The 2D sections represent the entire animal/organ
As neither is true, the conclusions of the authors are at most indicative. Mixing stable isotope labelled standards with tissue homogenates, analysing them and showing their intensity ratios does not address the problem and does not yield any new information.
4)The authors have performed a number of additional experiments in relation to this point. The reviewer appreciates both the IHC and the quantitative proteomics experiments. Unfortunately the selection of IHC targets still left a number of questions open. While ACC1 and FASN are excellent targets for capturing fatty acid biosynthesis, GPAT1 is an enzime taking part of the biosynthesis of all glycerolipids - not only triglycerides. CPT2 takes part of the transport of long-chain fatty acyls through the mitochondrial membranes - again, not an excellent marker for fatty acid oxidation. Certainly does not perform the beta-oxidation fatty acids as the authors state. The other problem is associated with the incompleteness of spatial localisation. The stains (e.g. FASN or ACC1 in BAT) shed light on the intensity of fatty acid biosynthesis in the target tissue, however if the authors state that a certain process takes place in a given organ, then they have to prove that the given pathway is only active there. Since they only tested 2-4 organs for most enzymes(except for FBPase, G6Pase and PEPCK, where they tested 9), they just don't have enough information to localise the given process, not to mention alternative pathways.
All of the pathways localised by the study are known to be expressed in the given tissue, from intensive fatty acid metabolism in BAT to highly intensive glucose metabolism in cancer - there has been no novel information revealed due to the development of the novel tools. This is not necessary a problem, however the authors should present their results in the scientific context and not pressing on the novelty of results, which are fundamentally not novel.
5)The reviewer - and likely the readership of the Journal - are interested in the steps of spectral data preprocessing in scientific terms. Unfortunately the settings of a vendor-specific data analysis software do not provide this information. It would be very interesting to see how post-acquisition recalibration of the data is performed, how the data is background-subtracted (in scientific terms) or how peak-picking was performed. There are various algorithms for these steps, and the reviewer is confident that the Thermo tool uses one or another, but citing on-screen settings in a manuscript, which focuses on the development of a data processing platform is not acceptable.
In conclusion, in the light of the remaining problems, I cannot support the acceptance of the manuscript for publication.

(Remarks on code availability)
No sufficient expertise.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The reviewer does appreciate the efforts of the authors regarding addressing the points raised in the previous round of review. However, in spite of the 11 pages long rebuttal letter, the manuscript has hardly changed as a result of the revision.
With regard to novelty, the reviewer agrees that the model the authors used gave an unprecedented opportunity to characterise the organism-level metabolism, but this opportunity was largely missed, due to inappropriate data analysis strategies. In order to perform such study, the authors (or anyone else) is expected to provide 3D, quantitative maps of all isotopologues of major metabolites at multiple time points. The reviewer agrees that it is a gargantuan challenge, but the authors should not claim of solving it when they did not.
Specific further points: In case of tissue regions, the authors gave somewhat related information with numerous citations, however did not address the point raised. With regard to the ion types, they unfortunately depend on the instrument and experimental parameters (DESI solvent, LC eluents) so if the intention is to produce a tool for the community, the pre-chosen ion types do not make sense. In case of isomers, the MSITracer could actually use the LC-MS information to address this problem. In case of false discovery rates, it is at least surprising that an order of magnitude drop in FDR does not change the outcome, which raises further serious questions about the data interpretation.
In case of tissue-specific response factors, the authors accepted the point that an whole organ or whole body intensity map is meaningless. Nevertheless, almost all figures show ion intensity maps and the conclusions are - directly or indirectly - based on this information. The use of TECs could solve the problem, however the authors tried to prove that it does not change anything (Fig R2c.). Indeed, dividing both the numerator and the denominator by the same number does not change the value of a fraction, this 3rd grade elementary school math. However, the different TECs can show that a metabolic activity of tissue A is negligible compared to the metabolic activity of tissue B. Similarly, by not analysing >99% of the body by MSI, makes any conclusions unfounded. The authors state that all MSI field uses 2D sections, however these groups are not trying to make organism-level claims.
With regard to the IHC validation, this is the only part of the manuscript where meaningful information was added. Nevertheless, the problem raised was the broad substrate specificity of some of the enzymes, which was not fully addressed.
With regard to data pre-processing, the authors are apparently unfamiliar with the complexity of peak detection, background subtraction or normalisation. These are complex tasks, with enormous literature from the last 40 years.

In conclusion, due to factual errors, the reviewer cannot support the publication of the manuscript.

(Remarks on code availability)
Lack of expertise.

Responses to Reviewers' Comments

Reviewer #1: (Remarks to the Author):

This work successfully combined the spatial metabolomics technology (AFADESI-MSI) and the stable-isotope tracing metabolomics to quantitatively profile metabolic activity in a spatially-resolved manner. To do so, the authors developed an interesting computational tool, namely MSIFluxer, to achieve the identification of labeled metabolites and quantify the labeled fraction of MSI data. Finally, the authors applied the method to several applications, such as fatty acid metabolic crosstalk between tissues and metabolic alterations induced by cancer. This work provided an innovative framework to track labeled metabolites with the spatial metabolomics technology. Overall, the work is well-designed and performed, and suitable for consideration of publication in Nature Communications. Here are some comments need be addressed:

RE: Thank you for your overall positive evaluation of our work! We deeply appreciate the time and effort you have spent in reviewing our manuscript. The manuscript has been revised comprehensively according to your and other two reviewers' comments and suggestions. We believe these revisions have significantly strengthened the manuscript and are hopeful that it now meets the criteria for acceptance and publication in *Nature Communications*.

1. In Fig. 2b, the data showed the infusion of U-^{13}C glucose led to the predominant distribution of labeled glutamate within the brain, whereas following U-^{13}C glutamine infusion, labeled glutamate-M5 was primarily observed in the pancreas and high labeled fraction of M1 and M2 glutamate in brain were also observed. Since glutamate is an important neurotransmitter in brain and it seems like the brain does not uptake M5-glutamine directly to synthesis glutamate, could the authors further clarify the highly labeled fraction of M1 and M2 glutamate in brain after U-^{13}C glutamine infusion?

RE: Thank you for your valuable comments. As you noted, it is intriguing that the labeled glutamate M5 was primarily distributed in the pancreas, whereas significant labeled fractions of glutamate M1 and M2 were found in the brain after the infusion of U-^{13}C glutamine. Given that the brain is the primary consumer of glucose, one possibility is that glutamate M1 and M2 in the brain could be produced from glucose derived from gluconeogenesis. We used glucose M3 as an indicator of gluconeogenic activity to identify the organs involved in the conversion of glutamine to glucose. AFADESI-MSI results indicate that glucose M3 was predominantly enriched in the kidney and liver (Fig. R1a). These results were further confirmed by the LC–MS (Fig. R1b). Additionally, we also observed a high labeled fraction of glucose M3 in the serum

(Fig. R1b). This suggests that glutamate M2 and M1 in the brain may originate from glucose produced via gluconeogenesis in the kidney and liver.

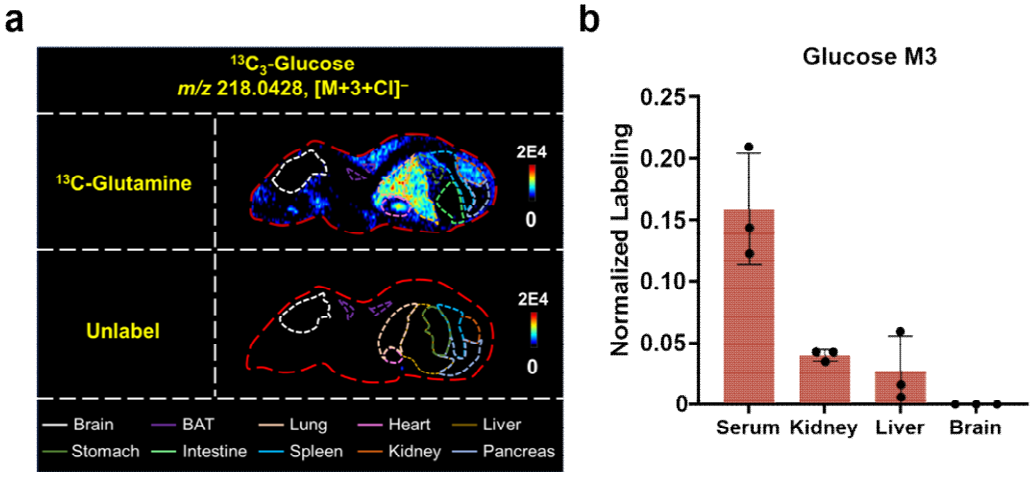
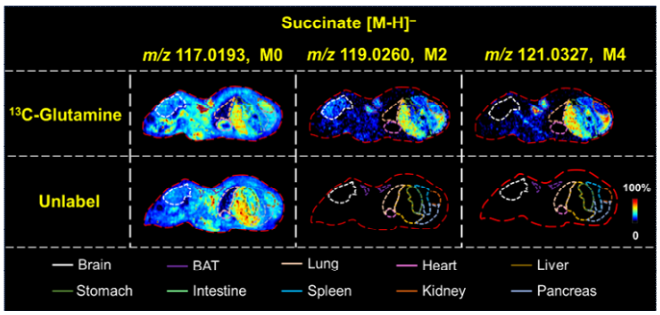


Figure R1. (a) The distribution of glucose M3 across organs following U-^{13}C glutamine infusion (upper) and the non-infusion samples (lower). (b) Normalized labeling of glucose M3 in serum, kidney, liver and brain following U-^{13}C glutamine infusion, as measured by LC–MS (n=3, mean ± SD).

Furthermore, in addition to glutamate, the spatial distribution differences of labeled isotopologues are also observed in other central carbon metabolite intermediates, such as aspartate (Fig. 3f), glutamine (Fig. 3b2, 3b3), and succinate (Fig. R2a). Specifically, higher-order labeling metabolites (M5 or M4) are predominantly found in the pancreas and spleen, while M2 isotopologues derived from gluconeogenesis-produced glucose are distributed in the brain. Collectively, these results indicate the presence of a metabolic collaboration among organs (Fig. R2b): following U-^{13}C glutamine infusion, gluconeogenesis occurs in the liver and kidney, producing ^{13}C-labeled glucose. This ^{13}C-labeled glucose is then transported through the bloodstream to the brain, where it is utilized to generate isotopologues M1 and M2. Following your suggestions, we have added more detailed explanations about this phenomenon in the revised manuscript (lines 268–283 in page 12).

a



[editorial note: panel redacted]

Figure R2. (a) AFADESI-MS images of succinate M0, M2, and M4 in whole-body mouse samples with and without U-^{13}C glutamine infusion. (b) Illustration of the glutamine-glucose metabolic coordination between kidney, liver and brain.

2. The MSIFluxer functions by first extracting the intensity of targeted isotopologues. However, MSI lacks the capability to separate ions with the same *m/z*. In cases where two metabolites share identical *m/z* values, how can these metabolites be identified and assigned to their respective isotopologues? In addition, there are many isobaric interferences among different lipid species, for example, M2 of TG (56:6) showed in Fig 4h and M0 of TG (56:5). The above mentioned isomeric and isobaric overlappings would cause inaccurate quantification. The authors should discuss these limitations. In such a case, ion mobility-MS may provide additional separation and resolving power.

RE: Thank you for your valuable comments. We fully agree with your opinion that direct mass spectrometry analysis, including MSI, cannot distinguish isomeric ions, and therefore, MSIFluxer is unable to differentiate between labeled metabolites with the same *m/z*. Following your suggestion, we have added a discussion of this limitation in the revised manuscript (line 554–558 in page 24). “Second, the current MSITracer cannot distinguish isomeric molecular species. The presence of the same molecular weights may lead to multiple tentative annotations. Integrating orthogonal techniques, such as ion mobility MS, presents a promising solution to this challenge.” (The name MSIFluxer has been revised to MSITracer according to another reviewer’s suggestion). Additionally, to obtain more accurate information, we examined all labeled metabolites with isomers, ensuring that MSITracer includes notifications for isomeric ions in its output results. For example, the metabolite name for *m/z* 191.0197 identified by MSITracer has been updated from “Citrate” to “Citrate_isomer”.

As for your concern about the isobaric interference between the M2 isotopologue of TG (56:6) (*m/z* 931.7636 $[M+Na]^+$) and the M0 of TG (56:5) (*m/z* 931.7725 $[M+Na]^+$), we are sorry for any confusion caused by our unclear explanation in the original manuscript. Fig. 4h is based on LC–MS data, which allows for clear separation of these

two metabolite ions at different retention times. To clarify the data types used, we have added more detailed descriptions in the relevant sections of the revised manuscript (line 363 in page 17, line 355 in page 16, line 325 in page 14).

3. In Fig 4f, it is abnormal that FFAs were not to detected in the serum. The previous work reported that dietary glucose could contribute to newly synthesized palmitate acid in plasma (https://doi.org/10.1016/j.cmet.2023.03.007). It is necessary to provide more evidence to obtain the conclusion that PA is concurrently oxidized in the BAT and brain.
RE: Thank you for your valuable comments. We are sorry for the inappropriate description in the original manuscript. Indeed, following U-^{13}C glucose infusion, free fatty acid can be detected in the serum, but they are not labeled. We have corrected the sentence in the revised manuscript (line 325 in page 14). Additionally, in the literature you provided, the fatty acid (FA) extraction method incorporates an additional saponification step compared to the method used in our study. Therefore, we applied the sample pretreatment method from this paper (Method 2) [1] and another one which also includes a saponification step (Method 1) [2] to re-analyze the serum FAs. We then compared these results with those obtained using our original method (Method 3). The results showed that the saponification step indeed increased the relative abundance of detected FAs. For instance, the signal intensity of palmitate was significantly higher with Methods 1 and 2 compared to Method 3 (Fig. R3). However, no labeled palmitate isotopologues were detected in the serum of U-^{13}C glucose-infused mice compared to non-infused samples even when using Method 1 (Fig. R4). This result aligns with our previous data, indicating that glucose-derived FAs did not enter the circulation.

Regarding the paper you mentioned, which detected labeled FAs in the blood of mice following ^{13}C-glucose administration, the possible reasons are as follows: As activated BAT undergoes continuous lipid remodeling, it generates molecules like triglycerides, phosphatidic acid, and phosphatidylcholine, which may then be transported into the bloodstream via lipoproteins or chylomicrons [3]. Sample pretreatment methods, such as the saponification of serum, may increase the proportion of free FAs detected in the circulation. Additionally, a longer duration of ^{13}C-glucose administration may result in more extensive ^{13}C labeling of FAs.

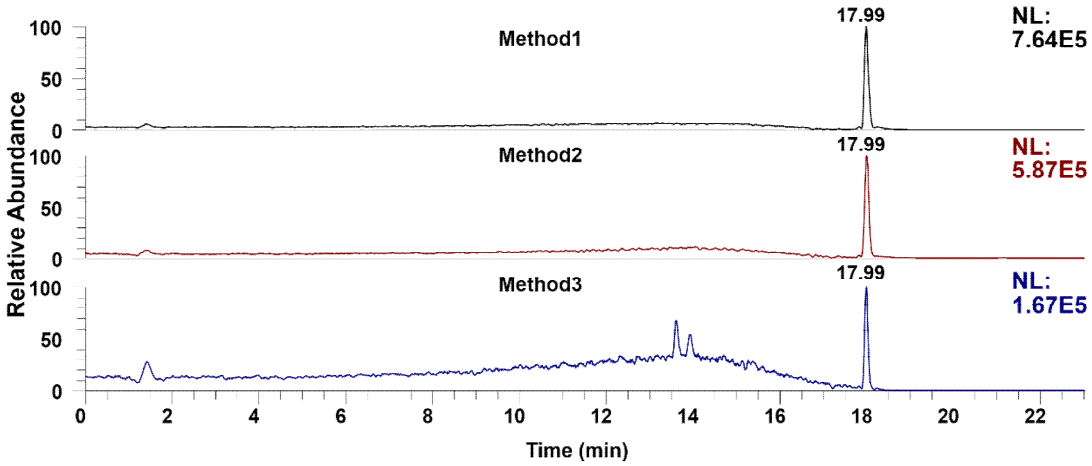


Figure R3. Extracted ion chromatograms of palmitic acid detected by LC–MS using the three different sample preprocessing methods.

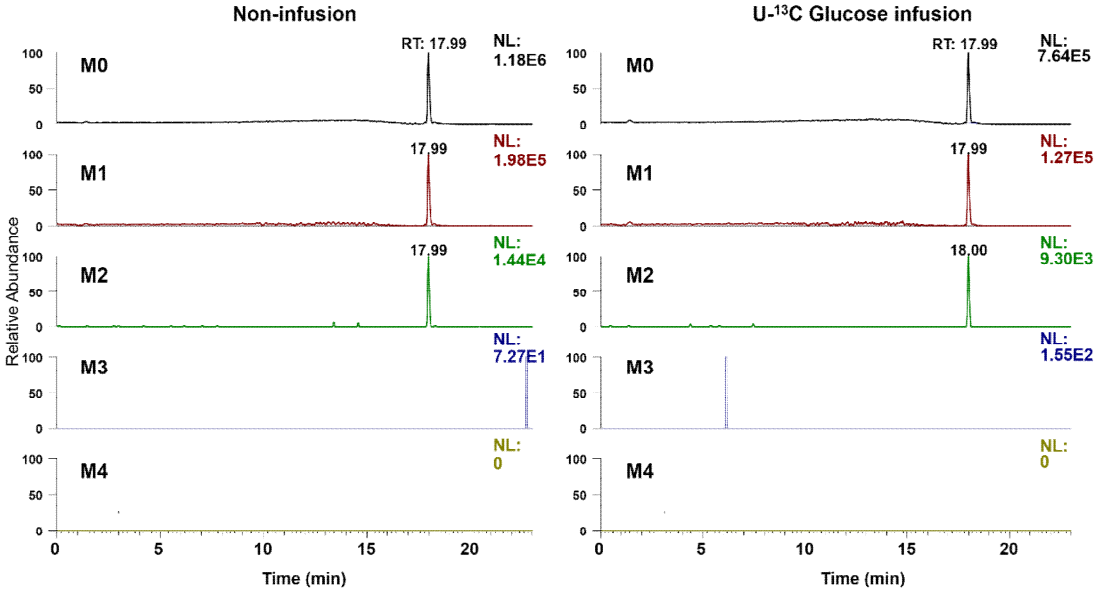


Figure R4. The extracted ion chromatograms of palmitic acid and its corresponding isotopologues detected by LC–MS using sample preprocessing Method 1.

4. The hypothesis of "We set a threshold, assuming that metabolites with labeling beyond M8 might be challenging to detect using MSI with ^{13}C-glucose and ^{13}C-glutamine infusion" may not be valid. The authors are recommended to check a panel of crucial metabolites in datasets including AMP, fatty acids etc.

RE: Thank you for your comment and suggestion. We are sorry for the initial limitation of setting the labeled metabolites in the MSI datasets to M8, which may result in some highly labeled metabolites not being identified. To enhance the annotation coverage of MSITracer, we have added all isotopologues of the labeled metabolites in the revised

manuscript (Supplementary Data 2.xlsx).

5. The authors utilize Supplementary Figure 1a to demonstrate isotopic steady state attainment based on M6 glucose and M5 glutamine labeled fractions. While this approach is suitable, continuous monitoring of labeled fractions at multiple time points is essential to conclusively establish isotopic steady state.

RE: Thank you for your valuable comments. Following your suggestion, we performed additional experiments to monitor the labeled fractions of key metabolites at six time points (10, 30, 60, 120, 180, and 240 minutes) following ^{13}C-glucose infusion. As shown in Fig. R5a, the labeling extent of glucose in the serum continuously increased. Glycolysis-related metabolites such as glucose-6-phosphate (G6P), pyruvate, and lactate reached a steady state at 180 minutes, while TCA cycle intermediates such as succinate and malate showed a slight increase in labeling at 240 minutes. Additionally, after 180 minutes, the labeling fraction of glucose M6 in the lung, heart, liver, kidney, and brain showed minimal changes, indicating that glucose M6 has reached isotopic steady state at the tissue level (Fig. R5b). For the labeled downstream metabolites in various organs, there were no significant differences in the labeling fraction of lactate M3, succinate M2, and malate M2 between 180 minutes and 240 minutes (Fig. R5c, 5d, 5e). Therefore, these results suggest that an infusion duration of 180 minutes is sufficient to establish an isotopic steady state in the body. We have added these results in the revised manuscript (Supplementary Fig. 1). Thank you again for your kind suggestions.

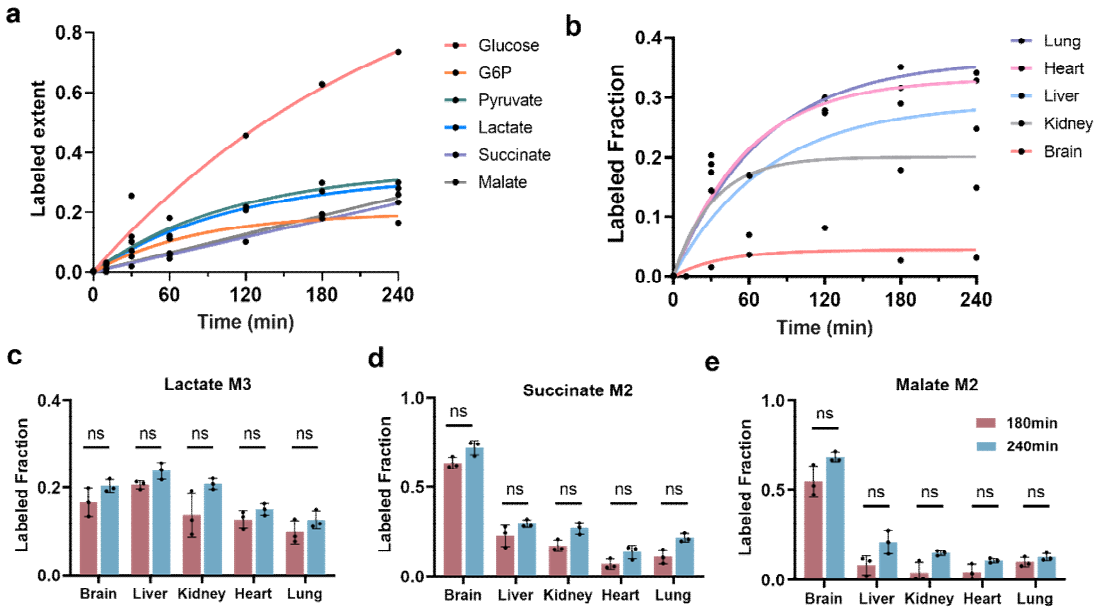


Figure R5. (a) Labeling extent of representative metabolites in serum at six time points

(10, 30, 60, 120, 180, and 240 minutes). (b) Labeling fractions of glucose M6 in various organs at six time points. (c-e) Labeling fractions of lactate M3, succinate M2, and malate M2 in various organs between 180 and 240 minutes. The data are presented as the means ± SD (n=3).

6. In Supplementary Fig. 3, the authors illustrate the capacity of MSIFluxer to choose the specific adduct and improve the quantitative accuracy. Uridine and TCA were used as examples. Could the authors provide more examples or a statistics result to further prove the advantages of MSIFluxer?

RE: Thank you for your kind comments. Following your suggestion, we provided additional representative metabolites that have multiple adduct ions, such as glycerophosphocholine and aminobutanoic acid (Fig. R6a, 6b). Specifically, glycerophosphocholine exhibited notably higher abundances in the $[M+Na]^+$ form relative to the other forms in positive ion mode, and they shared similar spatial distribution characteristics. We also counted the number of labeled metabolite ions identified by MSITracer in whole-body mouse, kidney, and brain tissues following U-^{13}C glucose and U-^{13}C glutamine infusion (Fig. R6c). We have included these results in Supplementary Fig. 5 of the revised manuscript to further demonstrate the capacity of MSITracer.

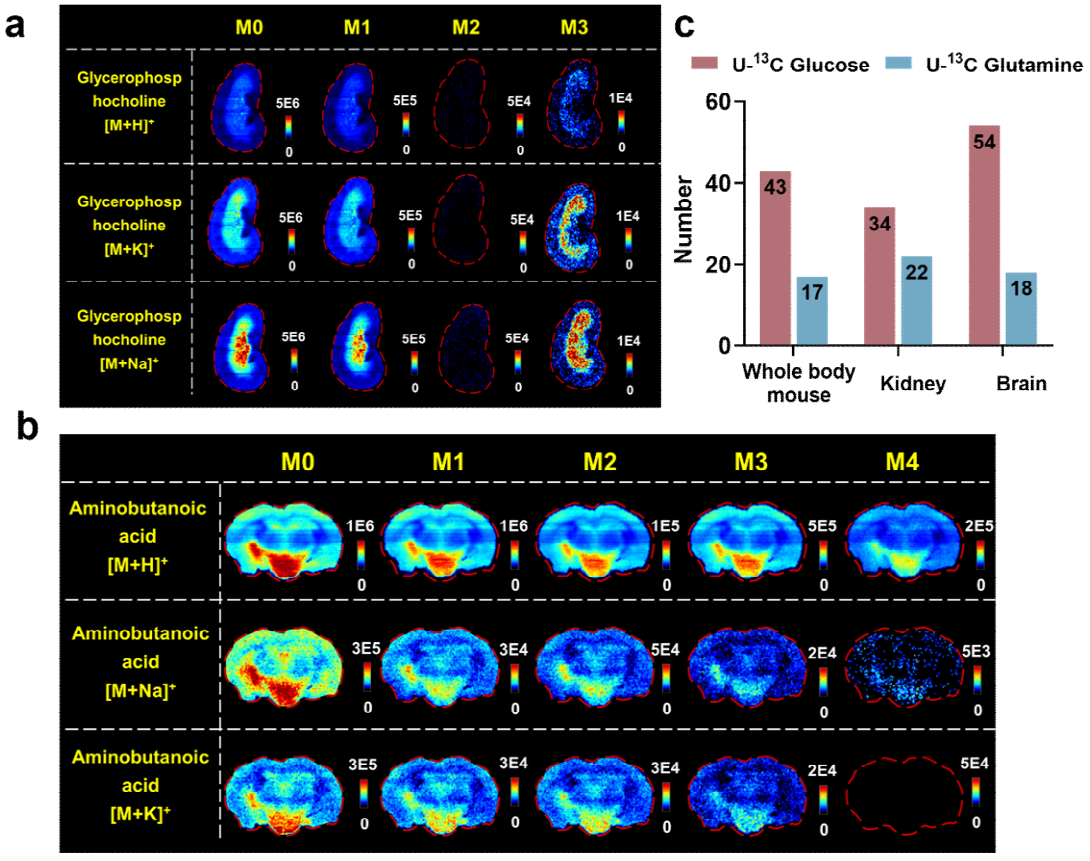


Figure R6. The annotation capacity of MSITracer for labeled metabolites with multiple adduct ions. (a) AFADESI-MS images of native glycerophosphocholine and the corresponding ^{13}C-isotopologues in the $[M+H]^+$, $[M+K]^+$ and $[M+Na]^+$ forms. (b) AFADESI-MS images of native aminobutanoic acid and the corresponding ^{13}C-isotopologues in the $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ forms. (c) The number of labeled metabolite ions identified by MSITracer in whole-body mouse, kidney, and brain tissues following U-^{13}C glucose and U-^{13}C glutamine infusion.

7. In Supplementary Figure 4d, glutathione presented concentrated distribution in medulla in un-infusion samples while M0 of glutathione in U-^{13}C infusion samples were distributed in both medulla and outer cortex of kidney, suggesting potential metabolic perturbations due to glucose infusion or surgical intervention? Please explain the potential impact of these factors on organ metabolism.

RE: Thank you for your comments. As you mentioned, U-^{13}C glucose infusion may have led to a slight alteration in the spatial distribution of glutathione in the kidney. To determine whether the infusion affected the overall metabolic characteristics of the organs, we compared the MSI data between the non-infused group (0 min) and the group infused with U-^{13}C glucose for 180 minutes (additional experiments in the response to Comment #5). The *m/z*-intensity matrices for the brain, kidney, heart, and lung were extracted and then subjected to cluster analysis to assess metabolic alteration. The principal component analysis (PCA) plots showed clear separation of the metabolic profiles among the four organs, with samples from the same organ in both groups clustering closely together (Fig. R7). These results indicate that while jugular vein infusion has some impact on *in vivo* metabolism, it does not significantly alter the overall metabolic characteristics of the organs.

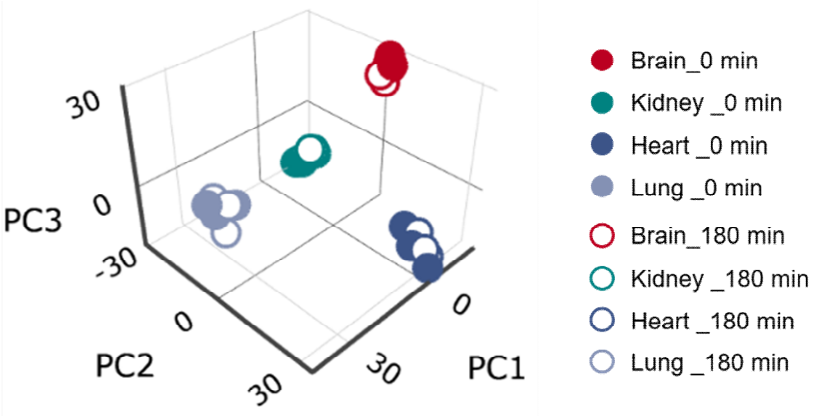


Figure R7. PCA plots of metabolic profiles in the brain, kidney, heart and lung for the non-infused group and the group infused with U-^{13}C glucose for 180 minutes.

Reviewer #2: (Remarks to the Author):

In this study, an MSI analysis tool called MSIFluxer was developed. The authors claim that this analysis tool integrates LC–MS and MSI data, overcoming the traditional weakness of mass spectrometry imaging, where isotopic metabolites are less abundant (compared to LC–MS), and allowing new insights into metabolism. However, there are a number of critical issues and, in particular, from a biochemical point of view, the validity and novelty of the results obtained are questionable.

RE: We deeply appreciate the time and effort you have spent in reviewing our manuscript. The manuscript has been revised comprehensively according to your and other reviewers' comments and suggestions. The quality of the revised manuscript has been significantly enhanced, and we are hopeful that it now meets the criteria for acceptance and publication in *Nature Communications*.

1. First, the organ sampling protocol is not described in detail. Presumably, the metabolic biochemical results presented here are influenced by post-mortem degradation. Indeed, previous work has shown that after brain and heart extraction, ischaemia causes rapid degradation of ATP and phosphate glycolytic intermediates due to increased phosphatase activity, accumulation of NADH due to inhibition of the electron chain transfer system, and increased lactic acid production due to increased anaerobic glycolysis. Therefore, without appropriate organ extraction methods (Proteomics 2013 DOI: 10.1002/pmic.201300047, Proteomics 2016 DOI: 10.1002/pmic.201500402), the results obtained here would not allow for a "metabolic profile of the hypoxic response". This trend would be even more pronounced in a tracer analysis using stable isotopes. In this respect, the authors should use sample preparation methods that avoid such post-mortem degradation if possible, and even if this is difficult, it should be discussed as a limitation.

RE: Thank you for your valuable comments. We are sorry for not providing a detailed description of the organ sampling protocol in our original manuscript. Before conducting the animal experiments, we carefully considered this issue and made great efforts to minimize post-mortem degradation of metabolites during sample collection. Following your suggestion, we have now included a detailed description of the organ sampling protocol in the revised manuscript (lines 610–621 in page 26). Additionally, we agree with your opinion that these measures may not completely avoid metabolite degradation. Therefore, we have also added a discussion on this limitation in the revised manuscript (lines 558–562 in page 24).

(i) Detailed organ sampling protocol used in our study:

For whole-body animal MSI analysis, the samples were collected as follows: After

the infusion, blood was collected from the orbital sinus, and the mouse was euthanized. The whole-body mouse was then quickly snap-frozen in liquid nitrogen within 30 seconds to halt metabolic processes. The samples were subsequently stored at −80 °C until section preparation.

For MSI and LC–MS analysis of individual organs, the samples were collected as follows: After the infusion, blood was collected from the orbital sinus, and the mouse was euthanized. Nine organs (liver, kidney, spleen, pancreas, heart, lung, brain, brown adipose tissue, and muscle) were sequentially harvested and quickly snap-frozen in liquid nitrogen to halt metabolic processes. The samples were stored at −80 °C until the preparation of tissue section for MSI analysis and tissue homogenates for LC–MS analysis.

(ii) Discussion on the limitation of the organ sampling protocol used in our study:

Moreover, we employed ex vivo rapid freezing of tissues to terminate enzymatic activity during sample collection. Since this approach cannot entirely prevent post-mortem degradation of metabolites, studies examining metabolites that rapidly change after death may consider alternative organ sampling methods, such as in-situ focused microwave irradiation, to effectively halt metabolic activities.

2. Secondly, the authors claim to have found metabolic interactions between tissues other than the Cori cycle, but it is known that fatty acid is released from the liver and used by the heart. It is also well known that implanted cancer cells can affect the metabolism of the host.

RE: Thank you for your comments. We are sorry for not clearly identifying the novel aspects of our results in the original manuscript. Based on your reminders, we have modified the relevant statements in the revised manuscript (lines 100–105 in page 5, line 320 in page 14). Regarding your concerns about the novelty of metabolic interactions in this study, we re-analyzed the experimental data using the developed method and discovered the following intriguing results:

(i) Metabolic exchange of glutamine across the kidney, liver, and brain. Following U-^{13}C glutamine infusion, MSITracer uncovered significant spatial distribution differences among various isotopologues of certain metabolites. For example, higher-order labeled isotopologues (e.g., aspartate M4, glutamine M5, glutamate M5, and succinate M4) were predominantly enriched in the spleen, pancreas, or liver, whereas their M2 isotopologues were primarily observed in the brain (Fig. 3f, 3b, 2b, Supplementary Fig. 10a). We then used glucose M3 as an indicator of gluconeogenic activity to identify the organs involved in the conversion of glutamine to glucose. A high labeled fraction of glucose M3 was observed in the kidney, liver, and serum. These

results indicate that glutamine ingested in the body is converted into glucose in the kidney and liver, which is subsequently transported to the brain to serve as an energy source (Fig. R8a). A more detailed explanation of these results has been added to the revised manuscript (lines 268–283 in page 12).

(ii) Metabolic coordination of glutamine between the lung and tumor. After infusing ^{13}C-glucose into tumor-bearing mice, MSITracer identified a distinct labeling profile for glutamate compared to other TCA cycle-related metabolites. Further LC–MS analysis supported extensive glutamate metabolism in tumor tissues; however, the labeling of its precursor, α-ketoglutarate, was relatively low, suggesting that glutamate in tumor tissues may originate from alternative sources. We then analyzed various potential sources of glutamate and identified glutamine as the most likely carbon supplier. Given that the labeling pattern in the lung resembled that in the serum and considering the lung's role as a major producer of glutamine, our results suggest that glutamine produced in the lung is partially released into the circulation and contributes to fueling the tumor (Fig. R8b). We have added more detailed explanations about this phenomenon in the revised manuscript (lines 387–427 in pages 18 and 19).

Overall, the main advantage of our study is that it provides a framework to intuitively decipher inter-tissue metabolic communication within the entire body of an animal. The effectiveness of our method is partially demonstrated by the fatty acid metabolism between the liver and heart. It can also extend to uncover metabolic communications between other organs as well as between tumor and organs. We believe that, with the use of additional tracers for *in vivo* metabolic tracing in the future, this method will greatly facilitate the elucidation of more complex metabolic interactions between organs.

[editorial note: figure redacted]

Figure R8. Schematic diagrams of (a) the metabolic exchange of glutamine among the kidney, liver, and brain; and (b) the metabolic coordination of glutamine between the lung and tumor.

3. Thirdly, MSI results for ^{13}C-labelled compounds of a given *m/z* should be shown simultaneously with images of the same *m/z* in negative controls not dosed with stable isotopes, as there is no guarantee that interference from other molecular ions can be completely avoided even when results are obtained by HRMS.

RE: Thank you for your comments. Following your suggestion, we have examined all the MS images from the ^{13}C-nutrients infused samples and included corresponding

negative control images in the Supplementary data (Supplementary Fig. 4e, 6e, 7c-7f, 12d, 13a of the revised manuscript) to ensure that labeled metabolite ions were not the result of interference from other ions.

4. Fourthly, why is an odd number of ^{13}C-labelled palmitic acids detected when $^{13}C_6$ glucose is administered for only a few hours, since $^{13}C_6$ glucose is used to synthesize $^{13}C_2$ malonyl CoA, which should result in the synthesis of fatty acids containing an even number of ^{13}Cs, and there are many such reports. How can this discrepancy be explained?

RE: Thank you for your professional question. As you mentioned, $^{13}C_6$-glucose generally provides two-carbon units that result in the generation of even-numbered labeled fatty acids. However, some studies have also reported the presence of low levels of odd-numbered labeled fatty acids in animal models and mammal cells,[4, 5] which was termed as "label diffusion". When glucose enters the TCA cycle via glycolysis, it can generate $^{13}C_2$-acetyl-CoA through the action of pyruvate dehydrogenase (PDH) and also incorporate three labeled carbons into oxaloacetate via pyruvate carboxylase (PC). When citrate exits the mitochondrion, two of the labeled carbons may be delivered to de novo lipogenesis as acetyl-CoA, leaving single-labeled oxaloacetate. This oxaloacetate can then potentially be converted to single-labeled pyruvate, which ultimately forms $^{13}C_1$-acetyl-CoA, resulting in the production of odd-numbered labeled fatty acids (Fig. R9).[6]

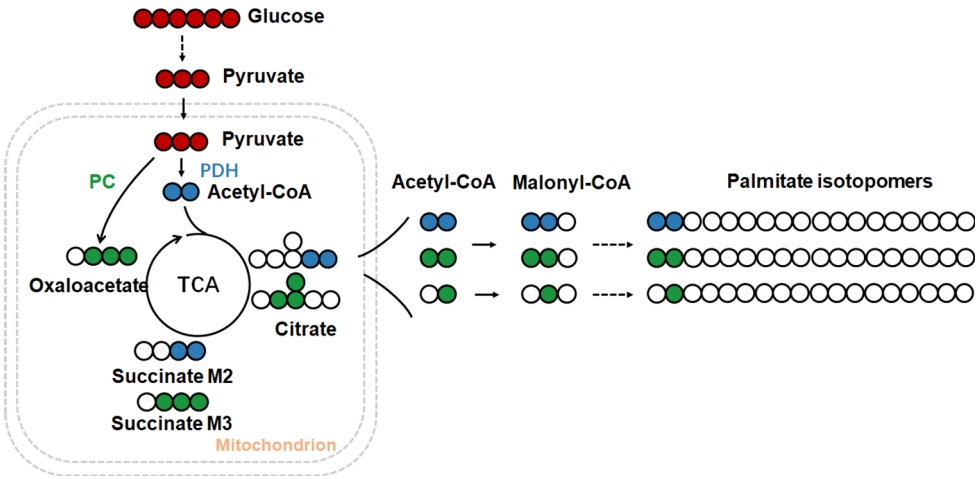


Figure R9. Diagram illustrating the formation of odd-carbon labeled fatty acids in the presence of U-^{13}C glucose.

Reviewer #3: (Remarks to the Author):

The paper from Li et al. introduces novel methodology for whole-body metabolic flux analysis using stable isotope labelling, LC–MS, mass spectrometric imaging and a new software tool termed 'MSI Flux'. The paper features highly interesting and valuable data, however their interpretation is speculative and ignores a number of inherent problems of the chosen methodology.

RE: Thank you for dedicating your time and effort to reviewing our manuscript. We greatly appreciate your overall positive evaluation and are grateful for your insightful, constructive and helpful comments. In response to your comments and major concerns, we have performed additional experiments, re-analyzed the experimental data, and added substantial supporting results into the revised manuscript to strengthen our work. We are hopeful that the revised version now meets the criteria for acceptance and publication in *Nature Communications*.

Specific problems:

1. Based on the description of the MSIFlux, this computational tool does not perform metabolic flux analysis. State-of-the-art software packages for metabolic flux analysis (for a recent review of the field see de Falco B, Giannino F, Carteni F, Mazzoleni S, Kim DH. Metabolic flux analysis: a comprehensive review on sample preparation, analytical techniques, data analysis, computational modelling, and main application areas. RSC Adv. 2022 Sep 7;12(39):25528-25548. doi: 10.1039/d2ra03326g.) employ complex mathematical models (mostly master equation-type models, see for instance Thommen Q, Hurbain J, Pfeuty B. Stochastic simulation algorithm for isotope-based dynamic flux analysis. Metab Eng. 2023 Jan; 75:100-109. doi: 10.1016/j.ymben.2022.11.001.) to simulate and decipher complex flux networks based on quantitative data. In contrast MSI Flux calculates accurate masses for metabolites including their adducts and labelled isotopologues, extracts these signals from raw MSI datasets and corrects the isotope distribution patterns using the native distribution. These are fairly routine arithmetic tasks not dealing with the metabolic flux analysis. The tool has its practical value, however, it is not properly validated and certainly does not form sufficient novelty for publication in Nature Communications.

RE: Thank you for your insightful comments. We are sorry for the unclear explanation of our tool's primary functions in the original manuscript. Generally, there are two primary strategies for studying metabolism using stable-isotope tracing. One strategy involves calculating metabolic flux to evaluate metabolic reactions by fitting the isotope incorporation patterns of known intermediates into mathematical models. Another involves comprehensively tracking the fate of isotopic labels in an unbiased manner,

which facilitates the identification of previously unreported metabolites or transformations, and enables the discovery of new biological insights. In this study, we focus on the second aspect, particularly in the field of spatially resolved isotope tracing. Therefore, complex mathematical models are not required in this scenario. Considering that the term “flux” could be confusing for these two research objectives, we have renamed the tool to “MSITracer” and updated it accordingly in both the revised manuscript and on GitHub.

In addition, this tool represents only part of our work. The other contributions of our study can be summarized as follows:

(i) The first system-level characterization of metabolic activity across multiple organs is provided. By integrating LC–MS and MSI with stable-isotope tracing, we developed a workflow for untargeted tracking of the metabolic fate of ^{13}C-nutrients and created the most comprehensive labeled metabolite database in mammals to date. By visualizing the spatial distribution of metabolite isotopologues in whole-body animal models, our study advances the understanding of nutrient metabolic turnover from the *in vitro* cellular level to that of intact tissues. Moreover, the comprehensive workflow developed in this study is applicable to other nutrient tracers.

(ii) A novel approach is proposed to elucidate inter-tissue metabolic communication by analyzing spatial distribution differences across three types of isotopically labeled metabolites: (a) original and newly synthesized metabolites (M0 and Mn), (b) various labeled isotopologues of the same metabolite (M1 and Mn), and (c) different newly synthesized metabolites within the same pathway (Mx and My).

(iii) Intriguing examples of metabolic crosstalk between organs have been revealed, such as the exchange of glutamine across the kidney, liver, and brain, as well as the metabolic coordination of glutamine between the lung and tumor (detailed examples are provided in our response to Comment #7). These findings provide valuable insights into metabolic activities underlying fundamental biological processes and disease states.

2. The role of mass spectrometric imaging is unclear. As much as one can tell from the manuscript, the areas corresponding to the individual organs are averaged and then processed using the computational tool mentioned above. This raises a number of problems including the sampling efficiency of the individual organs and also the lack of quantitative nature of MS imaging data. The LC–MS data collected by the authors could serve as a much more appropriate basis for performing flux analysis. Furthermore, for LC–MS analysis, whole organs can be harvested, extracted and analysed. One whole body section does not and cannot represent entire organs, due to the heterogeneity of metabolite distribution and the very low efficiency of sampling. Some organs may not

be represented at all in the section, for others the actual cryosection represents <0.1% of their overall weight. Also, with appropriate calibration LC–MS/MS can be fully quantitative, which is an absolute requirement for metabolic flux analysis.

RE: Thank you for your comments. We are sorry for not clearly explaining the role of mass spectrometry imaging (MSI) in the original manuscript. Based on your comments, we have added clarification on the role of MSI in the revised manuscript (line 512–526 of page 23). In this study, MSI serves two primary roles. First, it enables the *in situ* characterization of metabolic activity within specific regions of tissues (detailed examples are provided in lines 206–217 of page 9 in the revised manuscript). Second, MSI intuitively and visually uncovers clues about potential metabolic crosstalk between tissues. When combined with LC–MS, this approach allows for a systematic characterization of metabolic interactions between organs (detailed examples are provided in our response to Comment #7). To use a metaphor, MSI acts as a "flashlight," illuminating unusual areas in the dark, while LC–MS functions as a "magnifying glass," enabling the identification of metabolic exchanges and their underlying causes.

We fully agree with your point that quantification of isotope-labeled metabolites is essential for accurate metabolic flux analysis. Since calculating metabolic flux is not the primary focus of this manuscript (response to Comment #1), the labeled fraction was capable of comparing metabolic activity between different organs and to infer potential metabolic interactions. In this context, it is well-established to calculate the labeled fraction using chromatographic peak areas in LC–MS [7-9] and mass spectrometric signal intensities in MSI [4, 10].

As for the issues you mentioned regarding sampling efficiency, these are indeed common challenges faced in MSI. In some cases, sections may only represent a portion of an organ; however, the spatial information provided by MSI should not be overlooked. In traditional LC–MS based isotope tracing analysis, this type of bulk assay also involves a tradeoff between higher sensitivity and specificity and the loss of spatial information. Therefore, integrating the spatial information provided by MSI with the high sensitivity and specificity of LC–MS is the optimal strategy in this study. Based on your comments, we have emphasized the necessity of combining MSI with LC–MS in the revised manuscript (line 552–554 of page 24). Moreover, to address your concerns about the quantification in MSI, results quantified by MSITracer in this study have been validated through LC–MS data to ensure accuracy of our results (Fig. 5d and 5e of the revised manuscript).

3. Along the same lines, it is not entirely clear what the relationship is between the LC–MS and MSI data. Do the authors only use the superior quality LC–MS data to create

a metabolite inventory as an input for the computational tool? It would be highly interesting to see how the two types of data correlate. Otherwise is does not seem to be sensible to use high specificity, high sensitivity, quantitative data just to facilitate the analysis of inherently lower sensitivity, lower specificity and non-quantitative data.

RE: Thank you for your comments. As mentioned in our responses to Comment #2 regarding the relationship between LC–MS and MSI, LC–MS is not only employed to generate a list of isotope-labeled metabolites but also serves as an essential technology for identifying metabolic interactions between organs in our study. At present, the data from these two technologies have not been correlated because they do not share completely identical samples. Your question highlights an important task for this field. This will be a relatively independent and long-term fundamental study.

4. The details of MSI data preprocessing are not given. The reviewer understands that the *m/z* values predicted by the computational tool are searched for in a +/- 5 ppm window. What happens if there are multiple peaks present in this window? Is the most intensive or the closest chosen? What happens to predictable other isotopologues, like ^{34}S ? What happens when peaks are asymmetrical or not fully resolved? What is the general consideration for isomers? As MSI cannot resolve them, they may elicit significant interferences, making the case for MS imaging even weaker.

RE: Thank you for your valuable comments. We are sorry for not providing a detailed description of the MSI data preprocessing process in the original manuscript. Following your suggestions, we have provided additional details about preprocessing MSI data in the Methods section of the revised manuscript (lines 742–745 in page 31).

Regarding your other questions:

(i) If multiple peaks are present in the specified window, MSITracer will record the most intensive signal rather than the one with the closest *m/z* value. We have incorporated this explanation into the revised manuscript (lines 758–759 in page 31).

(ii) The current version of our tool does not support the prediction of sulfur isotopologues (^{34}S) because we used ^{13}C-labeled nutrients as input database. We will continuously enhance this tool to improve its ability to identify metabolites labeled with other elements, such as ^{34}S and ^{2}H, in future work.

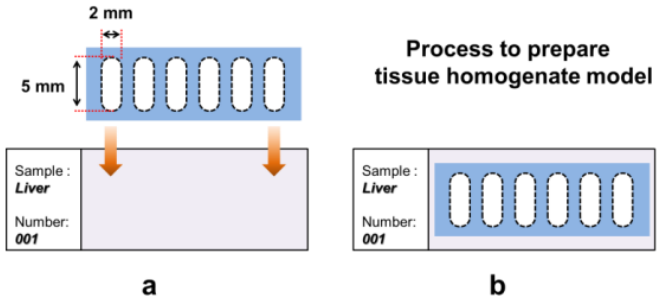
(iii) Since we did not incorporate online separation devices, it is not a common practice to evaluate peak shape (whether symmetric or not) in MSI data.

(iv) Direct mass spectrometry analysis, including MSI, cannot distinguish isomeric ions, which represents a major challenge in this field. Following the suggestions from you and another reviewer, we have added a discussion of this limitation in the revised manuscript (line 554–558 in page 24). “Second, the current MSITracer cannot

distinguish isomeric molecular species. The presence of the same or similar molecular weights may lead to multiple tentative annotations. Integrating orthogonal techniques, such as ion mobility MS, presents a promising solution to this challenge." Additionally, we have examined all labeled metabolites with isomers, ensuring that MSITracer includes notifications for isomeric ions in its output results. For example, the metabolite name for *m/z* 191.0197 identified by MSITracer has been updated from "Citrate" to "Citrate_isomer". This information will help researchers interpret the data more accurately, and determine whether to incorporate additional techniques to avoid potential interference.

5. The authors treat MSI data as quantitative, while the response factors for different tissue matrices and also for different adducts are significantly different. This needs to be taken into account, especially when different organs are compared. The low intensity of a metabolite signal can easily be the reason for the non-detection of isotopologues.

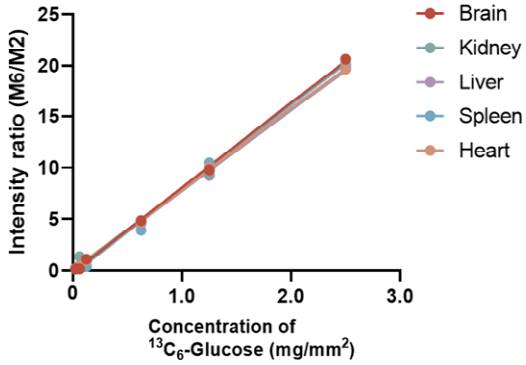
RE: Thank you for your valuable comments. Matrix effect is an important and fundamental problem in mass spectrometry analysis. As described in the Methods section of the original manuscript, we used "normalized labeling" or "labeled fraction" to quantify the relative proportions of certain metabolite isotopologues, a method analogous to standard calibration. In response to your concern, we performed additional experiments to evaluate whether the method used in this study can mitigate the matrix effect. Specifically, we prepared homogenates of five organs (brain, kidney, liver, spleen, and heart), which is a uniform and reproducible model to mimic biological tissue sections [11]. 1, 6-$^{13}C_2$ glucose and a series of U-$^{13}C_6$ glucose were added to these tissue homogenates. The final concentration of 1, 6-$^{13}C_2$ glucose on the surface of the slides was 0.125 mg/mm², while U-$^{13}C_6$ glucose was applied in a gradient of 2.5, 1.25, 0.625, 0.125, 0.0625, and 0.025 mg/mm². All samples were dried under vacuum for 30 minutes, and then subjected to AFADESI-MSI analysis. The detailed process for preparing the tissue homogenate model is shown in Fig. R10. Using the concentration of U-$^{13}C_6$ glucose as the x-axis and the ion intensity ratio of U-$^{13}C_6$ glucose to 1, 6-$^{13}C_2$ glucose (M6/M2) as the y-axis, linear regression analysis was performed for each organ. The results showed a good linear correlation between concentration and ratio across different tissues ($R>0.997$), with the fitting lines nearly overlapping (Fig. R11a, 11b). This indicates that calculating the ratios of different isotopologues of the same metabolite can effectively compensate the impact of matrix effect. In other words, matrix effect between tissues do not affect the accuracy of labeling fraction, thereby ensuring the reliability of inter-tissue comparisons in our study.



[editorial note: panel redacted]

Figure R10. The process to prepare tissue homogenate model [11]. (a) A PVC adhesive sticker with 2×5 mm quasi-rectangular hole was prepared. (b) Adhesive sticker was posted on microscope slide. (c) 5 μL tissue homogenate spiked with 1, 6-$^{13}C_2$ glucose and U-$^{13}C_6$ glucose was add to the quasi-rectangular hole by micro-pipette. Three replicates from each tissue were prepared.

a



b

Organ	Slope	R
Brain	8.254	0.9994
Kidney	7.821	0.9977
Liver	7.841	0.9993
Spleen	8.228	0.9980
Heart	7.901	0.9997

Figure R11. (a) Linear regression analysis of U-$^{13}C_6$ glucose concentration and the ion intensity ratio of U-$^{13}C_6$ glucose to 1, 6-$^{13}C_2$ glucose (M6/M2) across brain, kidney, liver, spleen, and heart. (b) The slopes and R-values of the linear regression equations across five organs.

Furthermore, we fully agree with your points regarding the varying responses of metabolite adducts in MSI. As shown in Supplementary Fig. 4a of the revised manuscript, the ion intensity of uridine in its $[M+Cl]^-$ form is significantly higher than that of $[M-H]^-$, allowing the detection of the M5 isotopologue in the $[M+Cl]^-$ form. We noted that, although the ion intensity of the same metabolite varies with different adduct forms, similar distribution trends are observed across tissues. For example, regardless of whether detected as $[M+H]^+$, $[M+Na]^+$, or $[M+K]^+$, glycerophosphocholine is most enriched in the renal medulla (Supplementary Fig. 5a of the revised manuscript), while aminobutanoic acid is most abundant in the hypothalamus (Supplementary Fig. 5b of

the revised manuscript). Therefore, it is crucial to utilize the same adduct form when comparing labeling patterns between organs.

6. Error bars are missing at several places, for example in Figure 4, but error bars are an absolute pre-requisite for all 'labelled fraction data'. Also, where error bars are shown, the number of points is very low (6). Do these points correspond to biological replicates, so individual animals? For the imaging data it would be useful to calculate error using all pixels. Would also be useful to demonstrate the labelling efficiency distribution in space, not only in time. Images are helpful, but do not substitute for proper statistical analysis.

RE: Thank you for your comments. Following your suggestions, we have added error bars to Figure 4d, 4e, 4f, and 4g. These data points correspond to biological replicates as all experiments were conducted using three individual animals in this study. Despite this, to clearly illustrate the metabolic characteristics of tissue microregions, we selected two regions of interest (ROIs) in each kidney and brain sample (Fig. 2c and Fig. 2d), and three ROIs in each tumor sample (Fig. 4c and Fig. 4e). These data points do not exactly correspond to biological replicates. We have included this detailed information about these points in the revised manuscript (line 226 in page 10, line 456 in page 21).

We are sorry for the limited number of biological replicates in our study. One reason for this is the cost associated with stable isotope tracing in animal experiments. Additionally, studies utilizing these techniques published in *Nature Methods* [10], *Nature Metabolism* [12], and *Nature Communications* [4] also employed three biological replicates. We believe that the number of replicates does not compromise the conclusions of this study. Nevertheless, we will consider including more animals in future research to further strengthen the reliability of our data. Thanks again for your kind reminder.

7. Some of the claims of the authors regarding the new finding regarding systemic effect of lactate (see for instance a recent paper Liu X, Li S, Cui Q, Guo B, Ding W, Liu J, Quan L, Li X, Xie P, Jin L, Sheng Y, Chen W, Wang K, Zeng F, Qiu Y, Liu C, Zhang Y, Lv F, Hu X, Xiao RP. Activation of GPR81 by lactate drives tumour-induced cachexia. Nat Metab. 2024 Apr;6(4):708-723. doi: 10.1038/s42255-024-01011-0.) or the liver-heart fatty acid co-metabolism (recent review: Joerg Heeren, Ludger Scheja, Metabolic-associated fatty liver disease and lipoprotein metabolism, Molecular Metabolism, 50, 2021, 101238, https://doi.org/10.1016/j.molmet.2021.101238.) are not new. Literature provides compelling evidence for these processes, while the results shown in the paper

do not prove (though support) the claims of the authors.

RE: Thank you for your insightful comments. We are sorry for the inappropriate description of our results. The effectiveness of our approach is partially demonstrated by the lactate metabolism and fatty acid communication. Moreover, it has the potential to reveal metabolic interactions between other organs. In response to your and the other reviewer's concerns regarding the novelty of the metabolic crosstalk observed in this study, we re-analyzed the experimental data and discovered the following intriguing results:

(i) Metabolic exchange of glutamine across the kidney, liver, and brain. Following U-^{13}C glutamine infusion, MSITracer uncovered significant spatial distribution differences among various isotopologues of certain metabolites. For example, higher-order labeled isotopologues (e.g., aspartate M4, glutamine M5, glutamate M5, and succinate M4) were predominantly enriched in the spleen, pancreas, or liver, whereas their M2 isotopologues were primarily observed in the brain (Fig. 3f, 3b, 2b, Supplementary Fig. 10a). We then used glucose M3 as an indicator of gluconeogenic activity to identify the organs involved in the conversion of glutamine to glucose. A high labeled fraction of glucose M3 was observed in the kidney, liver, and serum. These results indicate that glutamine ingested in the body is converted into glucose in the kidney and liver, which is subsequently transported to the brain to serve as an energy source (Fig. R8a). A more detailed explanation of these results has been added to the revised manuscript (lines 268–283 in page 12).

(ii) Metabolic coordination of glutamine between the lung and tumor. After infusing ^{13}C-glucose into tumor-bearing mice, MSITracer identified a distinct labeling profile for glutamate compared to other TCA cycle-related metabolites. Further LC–MS analysis supported extensive glutamate metabolism in tumor tissues; however, the labeling of its precursor, α-ketoglutarate, was relatively low, suggesting that glutamate in tumor tissues may originate from alternative sources. We then analyzed various potential sources of glutamate and identified glutamine as the most likely carbon source. Given that the labeling pattern in the lung resembled that in the serum and considering the lung's role as a major producer of glutamine, our results suggest that glutamine produced in the lung is partially released into the circulation and contributes to fueling the tumor. (Fig. R8b). We have added more detailed explanations about this phenomenon in the revised manuscript (lines 387–427 in pages 18 and 19).

[editorial note: figure redacted]

Figure R8. Schematic diagrams of (a) the metabolic exchange of glutamine among the

kidney, liver, and brain; and (b) the metabolic coordination of glutamine between the lung and tumor.

8. Details for the imaging experiments are missing. For instance the pixel size and biological feature resolution as well as the time required for the analysis are not given. One is also wondering why the authors use the 'AFADESI' acronym when they are basically using the DESI technology with a modified mass spectrometer inlet.

RE: Thank you for your kind comments. According to your suggestions, we have provided a detailed description of the MSI experimental procedures below, which has been incorporated into the revised manuscript (line 681–702 in page 29).

The AFADESI-MSI platform mainly comprises a home-built AFADESI ion source, an ESI sprayer, a sample carrier platform, a 3D translational stage, a height-adjustable desk, and an electricity control module. The AFADESI source was constructed with a stainless-steel transport tube (i.d. 3 mm, o.d. 4 mm, length 50 cm) and a vacuum pump that produces the extracting gas for remote ion transport, with a flow rate of 45 L/min. The spray solution was ACN/H_2O (80:20, v/v), and was delivered to the sprayer at a flow rate of 10 μL/min for WBA samples and 5 μL/min for tissue samples. The spray gas was N_2 and the flow rate was set at 2 L/min. The spray voltage was set at 7.0 kV in positive ion mode and −7.0 kV in negative ion mode. The sprayer and glass slide were mounted on a 3D translational stage to allow their positioning relative to the transport tube with submillimeter precision. For WBA sample analysis, the 3D translational stage moved continuously at a horizontal speed of 0.35 mm/s with a vertical step size of 0.50 mm. For tissue sample analysis, the 3D translational stage moved continuously at a horizontal speed of 0.10 mm/s with a vertical step size of 0.15 mm. Accordingly, the time required for mass spectrometry imaging of each whole-body mouse, brain, kidney, and tumor section is approximately 140 minutes, 130 minutes, 100 minutes, and 120 minutes, respectively. The pixel sizes for AFADESI-MSI are 100 μm × 76 μm for a tissue section and 500 μm × 270 μm for a whole-body animal section.

The main difference between AFADESI and DESI is that AFADESI incorporates air flow to assist ionization. Therefore, when we first published the paper on this technique in 2011, it was abbreviated as "AFAI" [13]. After Professor R. Graham Cooks' group introduced DESI in *Science* in 2004 [14], our research group began focusing on ambient ionization and MSI studies. We noticed that under ambient conditions, the gas flow and atmospheric pressure significantly affect the ionization efficiency and focusing of ions, leading to reduced sensitivity in mass spectrometry detection. To address this, we developed the airflow-assisted desorption electrospray ionization (AFADESI) technology. This technology enables the remote transport of charged droplets and ions

at atmospheric pressure through high-speed air flow in conjunction with solvent removal, thus improving ionization efficiency and detection sensitivity [11]. Furthermore, this technology expands the sampling space and enhances the flexibility of operation, making it suitable for imaging large biological samples [15]. Due to these advantages, AFADESI-MSI has been applied in various research fields [16-24]. Initially, we used the abbreviation AFAI; however, after consulting with Professor R. Graham Cooks, we adopted the abbreviation AFADESI and have continued to use it.

9. In conclusion, the authors should consider dropping the imaging component from the paper and re-evaluate the data from the LC–MS experiments. The proper validation of the LC–MS-based method may lead to an eventually publishable paper, however the manuscript in its current form cannot be accepted for publication.

RE: Thank you for your comments. As mentioned above, we may not have clearly articulated the role of MSI and the key issues it addresses in this work in the original manuscript, which may have led to some misunderstandings. We are sorry for this oversight. We hope that through the above responses, particularly to Comments #1 and #2, we have sufficiently clarified that MSI is an indispensable part of this study. We fully understand your point that LC–MS provides more accurate quantitative information. Yet, the spatial information of isotopically labeled metabolites provided by MSI does offer valuable insights into inter-tissue metabolic communication. As MSI-driven spatial metabolomics has become a crucial tool in deciphering the fundamental biological processes, our work advances current knowledge about the potential and functions of MSI. Moreover, we have conducted a detailed analysis of the LC–MS data (response to Comment #7), as you suggested. We hope that you find this revised version to meet the criteria of *Nature Communications*. Thank you once again for your overall helpful comments.

Reviewer #3 (Remarks on code availability):

I do not have the expertise to judge the quality of the code.

RE: Thank you for your response.

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Responses to Reviewers' Comments

Reviewer #1: (Remarks to the Author):
All comments have been successfully addressed by the authors. I feel the manuscript is ready for acceptance by *Nature Communications*. Congratulations!
RE: Thank you again for your valuable comments.

Reviewer #2: (Remarks to the Author):
The authors and others have generally addressed the reviewers' comments reasonably well. One minor comment is that the colouring of the M1-M10 graph in Figure 4g is very difficult to see. It is a very interesting finding that fatty acid synthesis from glucose occurs more prominently in the BAT, so the colouring should be such that the even and odd C^{13} labels can be clearly distinguished here.
RE: Thank you for the valuable suggestion. We have adjusted the color scheme of Fig. 4g in the revised manuscript.

Reviewer #3: (Remarks to the Author):
The authors spent significant amount of time with improving the paper including answering all the reviewers' comments and even performing new experiments. I do appreciate that the authors even changed the name of their software tool to reflect more to its real function. Nevertheless, in-spite of all the changes and improvements a number of fundamental issues remain, which preclude the publication of the manuscript in *Nature Communications*:
RE: Thank you for dedicating your time and effort to review our revised manuscript. We sincerely appreciate your insightful, constructive and helpful comments. In response to your comments and major concerns, we have performed additional experiments and added substantial supporting data into the revised manuscript. We believe these revisions have further strengthened the manuscript, and we are hopeful that it now meets the criteria for acceptance and publication in *Nature Communications*.

1. Novelty. There has been enormous amount of literature published recently in this area. I'd particularly like to highlight papers from the Patti and Rabinowitz groups, which successfully combined LC-MS and MSI for similar purposes with similar workflows to trace metabolic pathways in tissues. The authors – very correctly – cite some of these papers, for instance refs 4 and 10 in the rebuttal letter. The exact workflows described here are not exactly the same as in those publications, however the fundamental ideas are, hence the publication of the current manuscript in *Nature*

Communications cannot be justified. Nevertheless, it can and should be published in journals focusing more on tissue metabolism or analytical approaches.

RE: Thank you for your comments. We thank you for tracking and highlighting the works of the Patti and Rabinowitz groups on spatial stable isotope imaging. While their contributions have indeed greatly inspired our research, the current study introduces several novel aspects and differs in key areas, including: 1) In terms of research objectives, their studies primarily combine MSI with isotope tracing to visualize metabolic fluxes within or between tissues, such as gluconeogenic and glycolytic fluxes (Rabinowitz group), as well as fatty acid synthesis and elongation fluxes (Patti group). Our approach leverages the spatial differences in isotopologue distribution to elucidate inter-organ communication, a novel concept that, to our knowledge, has not been previously applied in this context. 2) Their research focuses on specific metabolic pathways, whereas our study introduces a comprehensive workflow that shifts traditional targeted pathway analysis toward a more holistic approach. As more labeled metabolites are identified in mammals, this approach enhances our understanding of nutrient metabolic turnover within the organism. 3) Moreover, our study expands the application of spatial isotope tracing to the whole-animal scale, offering the first system-level characterization of metabolic activity and communication across multiple organs. Therefore, our work fundamentally differs from previously published studies. It also leads to intriguing biological discoveries, which we have preliminarily validated (refer to our response to Comment #4), rather than serving solely as a methodology development. We believe our study exemplifies a successful integration of analytical chemistry and biology, aligning well with the scope of *Nature Communications*.

2. Innovation. The software tool the authors devised for extracting isotopologue data from MSI datasets is highly practical, but lacks any innovation with regard to its methodology. As the authors discuss in the Discussion part of their paper, it suffers from a number of problems, which they haven't attempted to solve. One could imagine software tools which identify isotope clusters in the data which do not follow natural distribution and annotate them in a novel way using (among others) LC-MS data, but this is not the case here. The authors argue that MSI data should be used because it features more metabolites – which is true – but their algorithm simply excludes anything which happen to show better sensitivity (e.g. because of locally high concentrations) in MSI than in LC-MS. They also exclude any unexpected adducts or fragments. Furthermore, they don't discuss the case when the LC-MS has multiple comparable intensity peaks for the same accurate mass. They mention the potential use of ion mobility, however the current dataset is full of with metabolites which have

multiple different isomers with completely different metabolic functions. Additional problems include the 5 ppm *m/z* window (a Q-Exactive can do much better than that) and their arbitrary selection of the highest intensity peak. These problems are largely mentioned, but mentioning a problem is not equivalent with solving it. The data is burdened by this enormous "noise", which is probably behind some of the "anomalies" and unexpected results.

RE: Thank you for your insightful comments and for recognizing the practicality of our tool. Regarding the concerns you raised about our tool:

1) We understand your point that using an LC−MS based metabolite database as a reference may result in the loss of MSI-specific labeled metabolites. However, due to the enrichment and separation steps in LC−MS, its detection sensitivity surpasses that of MSI. Nearly all metabolites and isotopologues detected by MSI can also be identified by LC−MS. The likelihood of the situation you mentioned is low, but we will monitor it closely in future work.

2) We acknowledge that other ion adducts may be present in the MSI datasets. Nevertheless, based on our previous studies [1], the eight selected ion forms represent the most predominant species. While additional adducts, such as $[2M+H]^+$ or $[2M-H]^-$, may also occur, their ion intensities are expected to be weaker than those of the selected forms. Hence, excluding these adduct ion forms is unlikely to impact the annotation coverage of labeled metabolites.

3) As you probably mentioned, our current tool lacks the capability to distinguish isomers. While incorporating other instruments could better address this interference, it falls outside the scope of this study and is part of a separate fundamental investigation. From our end, we approached this issue by marking the labeled metabolites that have isomers. This information will assist researchers in more efficiently identifying potential interferences in the data and deciding whether to incorporate additional techniques for improved results.

4) We agree that the Q-Exactive can achieve a resolution higher than 5 ppm. However, 5 ppm is a commonly used threshold for metabolite annotation, as a larger *m/z* tolerance is typically applied for ions with *m/z* values below 100. Notably, this parameter can be manually adjusted according to specific experimental conditions, enabling compatibility with various mass spectrometers. Based on your question, we further evaluated the impact of using either the highest peak intensity within a 5 ppm tolerance or the intensity of the closest peak on the results. Using the built-in example dataset of this tool as an example, both methods matched peak intensities for 3,373 ions, with 90.7% of these ions exhibiting identical intensity values (Fig.R1). By taking the ratio of ion intensities obtained from the two methods, the intensity ratio (IR) was calculated.

Only 0.5% of the ions had an IR greater than 100, which may indicate a certain degree of intensity differences. Therefore, with the intensity filtering step incorporated into this tool, the choice of intensity extraction method has minimal impact on the final results, and the differences remain within an acceptable range.

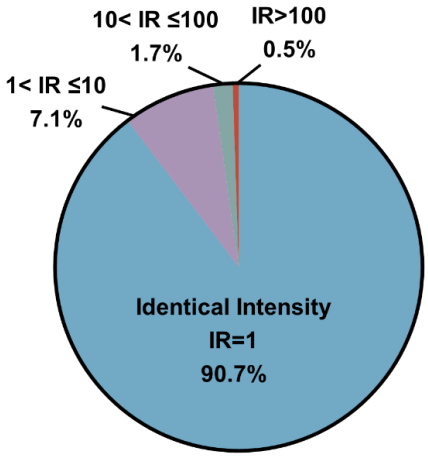

Figure R1. Comparison of annotation results using either the highest peak intensity or the intensity of the closest peak within a 5 ppm tolerance. IR: the ratio of ion intensities obtained from the two methods.

Finally, we wish you could understand that the tool was only a part of our manuscript. In our study, the MSI results were validated by LC−MS, and the major biological conclusions were validated using immunohistochemistry and quantitative proteomics (refer to our response to Comment #4), which had no doubt on accuracy. In the future, we will further improve our tool's performance based on your valuable suggestions.

3. Lack of quantification. The authors could have easily solved this problem by calibrating their LC−MS assays. Instead they mixed high concentrations of labelled glucose into tissue homogenates and not too surprisingly received a nice, linear calibration. MSI is infamous about its matrix effect – Figure R11 just completely denies the existence of this well documented (cf. *Anal. Chem*. 2018, 90, 9, 5637-5645) phenomenon. I believe that the reported data is correct, but does not achieve its intended purpose. Spiking homogenates with high concentrations of analyte does not prove the point, especially not that the authors should have done the same for all compounds concerned in the study, across the physiological concentration range to prove anything. I am not suggesting this as the spiked homogenates are not representative of the fresh frozen tissues containing the analyte molecules, due to completely different molecular interactions (or the lack of them). Practically any comparison made between the

intensity of the metabolites between any two tissue segments is highly questionable this way.

RE: Thank you for your valuable comments. Given the matrix effect is a fundamental challenge in mass spectrometry analysis, we understand your concern about the quantitative accuracy, particularly when making comparisons across different tissues. However, it is important to emphasize that the statistical analysis results of MSI and LC−MS in this study focuses on comparing the "labeling fraction" of specific metabolites rather than the "ion intensity". This approach is recognized for mitigating the impact of matrix effects. As mentioned in Patti's group's paper: "Because isotopologues of a metabolite have the same ionization efficiency, differences in the physicochemical properties across a tissue will not confound quantitative assessment of metabolite labeling, unlike metabolite pool sizes" [2]. To make it easier for you and the readers to understand, we performed additional experiments to demonstrate this phenomenon.

For LC−MS analysis, 25 mg of fresh brain, heart, kidney, liver, lung, and spleen were homogenized with 480 μL of pre-cooled extraction solution, along with 10 μL each of 50 mg/mL $^{13}C_2$-glucose and $^{13}C_6$-glucose. The remaining steps were the same as the "Metabolite extraction" section described in the methods. The concentration of exogenous ^{13}C-glucose was carefully assessed and found to be at similar levels in the liver (data not shown). Following LC−MS analysis, we observed variations in the ion intensities of glucose M2 and M6 across the six organs, with the highest intensity detected in the liver and the lowest in the lung (Fig. R2a). This indicates that tissue-specific characteristics indeed influence the detection of peak intensities. Next, we aimed to determine whether calculating the labeled fraction could reduce the matrix effect. In theory, the labeled fraction can be calculated by Eq. 1 as follows:

$$\text{Labled fraction of Mi} = \frac{I_{Mi}}{\sum_{i=0}^{N} I_{Mi}} \quad (1)$$

where I_{Mi} is the peak intensity of isotopologue Mi and N represents the carbon count of the metabolite. Considering the variation in M0 and M1 intensities across tissues, we simplified the formula, as shown in Eq. 2 and Eq. 3.

$$\text{Labled fraction}' \text{ of M2} = \frac{I_2}{I_2+I_6} \quad (2)$$

$$\text{Labled fraction}' \text{ of M6} = \frac{I_6}{I_2+I_6} \quad (3)$$

The results indicate that the modified labeled fraction of glucose M2 and M6 showed no significant differences across the various tissues (Fig. R2b, 2c), suggesting that the matrix effect was effectively minimized.

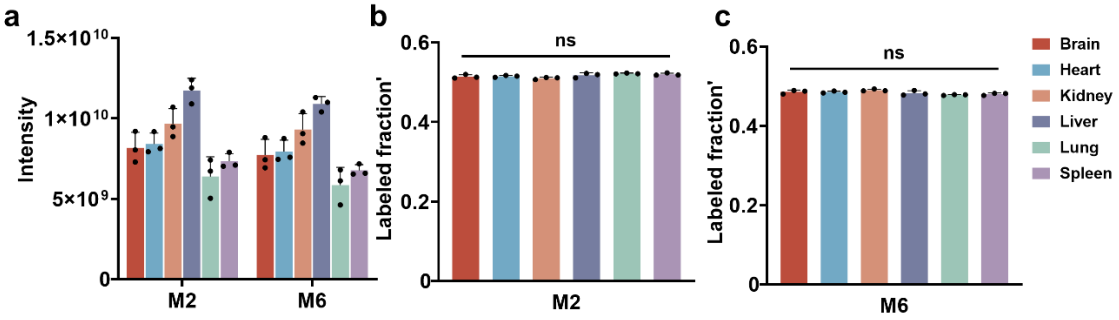

Figure R2. (a) Ion intensities of externally added $^{13}C_2$-glucose and $^{13}C_6$-glucose in the homogenates of brain, heart, kidney, liver, lung, and spleen, measured by LC−MS. (b-c) The modified labeled fraction of glucose M2 and M6 calculated in the brain, heart, kidney, liver, lung, and spleen (n=3, mean ± SD).

Similarly, for the AFADESI-MSI analysis, we prepared 15 μm-thick fresh tissue sections of brain, heart, kidney, liver, and spleen. A 1:1 mixture of $^{13}C_2$-glucose and $^{13}C_6$-glucose solutions was evenly sprayed onto the sample surface, ensuring that the concentration of exogenous ^{13}C-glucose detected by AFADESI-MSI approximated the physiological levels in liver tissue (data not shown). The results showed that despite the same concentration of glucose M2 and M6 being sprayed, their ion intensities varied significantly across different tissues, indicating the influence of matrix effects (Fig. R3a). However, after applying formulas (2) and (3) for calculation, the modified labeled fraction values remained consistent across the tissues, with no significant variation observed (Fig. R3c, 3d).

In summary, whether using MSI or LC−MS, calculating labeled fractions, rather than relying on ion intensities, proves to be a more effective approach for minimizing matrix effects. These matrix effects across tissues did not compromise the accuracy of our results.

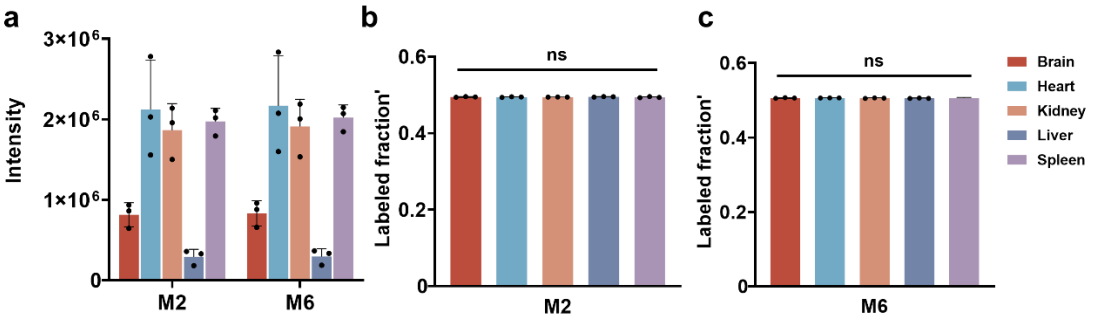

Figure R3. (a) Ion intensities of externally added $^{13}C_2$-glucose and $^{13}C_6$-glucose in the tissue sections of brain, heart, kidney, liver, and spleen, measured by AFADESI-MSI. (b-c) The modified labeled fraction of glucose M2 and M6 calculated in the brain,

kidney, liver, and spleen (n=3, mean ± SD).

4. Lack of focus and biological validation. The authors chose to show highlights of interesting pieces of data instead of focusing on a single biological pathway and properly validate their findings. Again, the manuscript is correct, the authors are aware of the weakness of their statements, but instead of providing independent evidence for their findings, just jump for the next interesting piece of data. For instance, the authors point out the interesting localisation of fatty acid biosynthesis in the brown adipose tissue. The data actually highlights some other histological features as well, which are associated with de-novo fatty acyl synthesis, but the text does not even mention these additional areas. Nevertheless, fatty acid biosynthesis is a well understood pathway, catalysed by fatty acid synthase (FASN) a well-known enzyme complex with commercially available antibodies. The authors should have used immunohistochemistry (potentially combined with proteomics) to validate their findings regarding the tentative expression of certain genes.

RE: Thank you for your valuable comments. The purpose of presenting multiple intriguing biological discoveries is to demonstrate the effectiveness of our approach in uncovering metabolic crosstalk between organs. Following your suggestion, we performed immunohistochemistry (IHC) staining and quantitative proteomics to validate the metabolic communications proposed here. Fatty acid synthase (FASN) and acetyl-CoA carboxylase 1 (ACC1) are essential enzymes in the *de-novo* fatty acid synthesis. The IHC assays demonstrated that these two proteins were most abundant in BAT, with the lowest expression observed in the heart (Fig. R4a). Quantitative proteomics further confirms that FASN and ACC1 expression levels were significantly enhanced in BAT compared to the brain and heart (Fig. R4b, 4c). These observations align with the distribution features of labeled free fatty acids observed in this study (Fig. 4g), supporting the conclusion that BAT serves as a major producer of fatty acid.

Next, we investigated the key proteins involved in triglyceride synthesis and oxidation. GPAT1, the enzyme responsible for initiating triglyceride biosynthesis by transferring an acyl group from acyl-CoA to glycerol-3-phosphate, shows significantly higher expression in BAT and the liver (Fig. R4d), consistent with the liver's observed capacity for triglyceride synthesis (Fig. 4g). Moreover, the lipoprotein lipase (LPL) and carnitine O-palmitoyltransferase (CPT2) are responsible for the hydrolysis of triglycerides into free fatty acids and the β-oxidation of long-chain fatty acids, respectively. Their high expression in the heart supports the notion presented in this study that the heart utilizes fatty acids derived from triglycerides as an energy source (Fig. R4e, 4f).

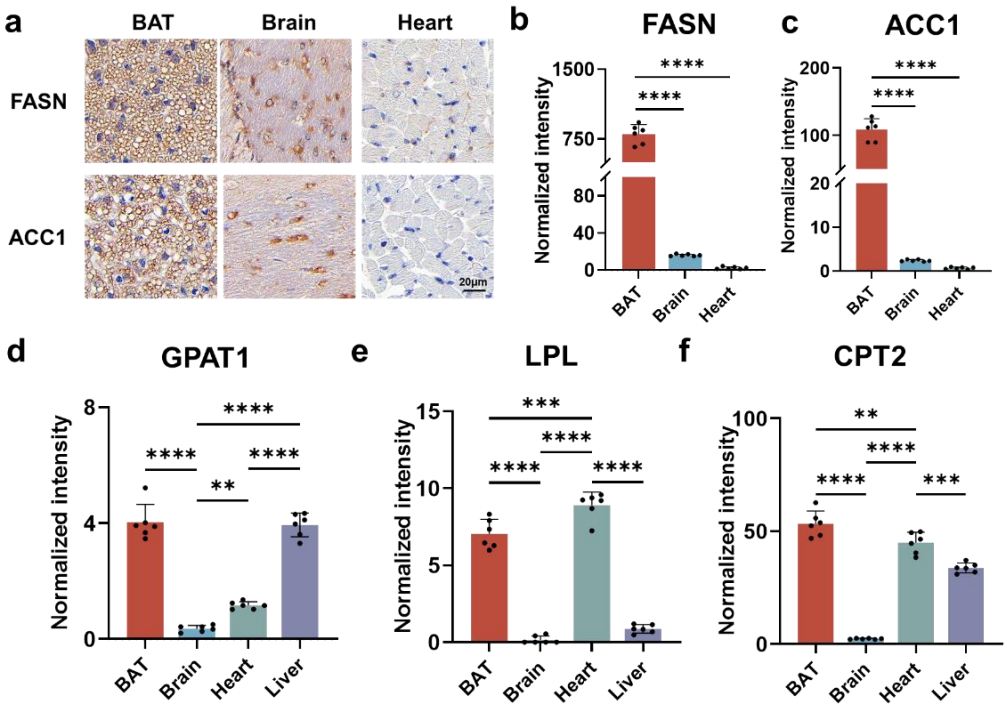

Figure R4. a) Representative IHC staining of FASN and ACC1 in the BAT, brain and heart of normal mice (n = 3). Scale bar, 20 μm. (b-c) Expression levels of FASN and ACC1 in BAT, brain, and heart, measured by quantitative proteomics. (d-f) Expression levels of GPAT1, LPL, and CPT2 in BAT, brain, heart, and liver, measured by quantitative proteomics (n = 6). Bar graphs are mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA with Tukey's post hoc test.

To validate the interaction of glutamine metabolism among the liver, kidney, and brain, we examined glycolysis-related proteins in our proteomics dataset. We observed markedly higher expression levels of fructose-1,6-bisphosphatase (FBPase), glucose-6-phosphatase (G6Pase), and phosphoenolpyruvate carboxykinase (PEPCK) in the liver and kidney compared to other organs (Fig. R5). This result is consistent with our findings, which employ glucose M3 as an indicator of gluconeogenic activity (Supplementary Fig. 10b, 10c), further supporting the hypothesis that glucose produced through gluconeogenesis in the liver and kidney can serve as an energy source for the brain.

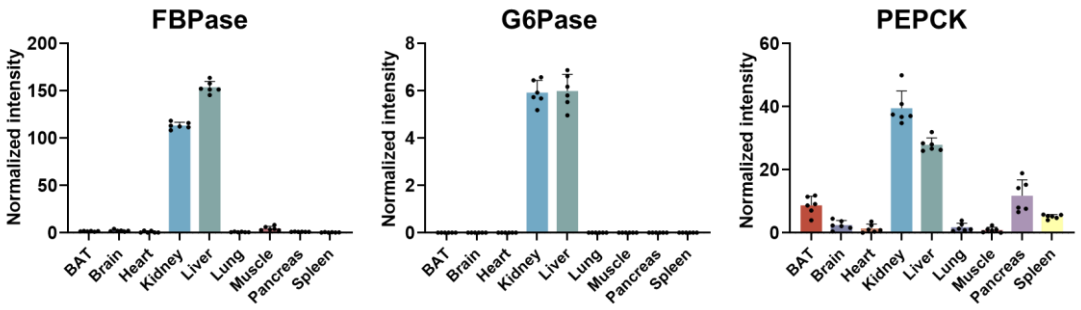

Figure R5. Expression levels of FBPase, G6Pase, and PEPCK in BAT, brain, heart, kidney, liver, lung, muscle, pancreas, and spleen, measured by quantitative proteomics (n = 6).

In HepG2 tumor-bearing mice, spatial isotope tracing data inspired the discovery that the host supplies glutamine to the tumor. To determine if this was the case, we conducted quantitative proteomic analysis on the tumors and the livers of the corresponding tumor-bearing mice. Our results indicate that the glutamine transporters responsible for shuttling glutamine into the cytosol (SLC1A5, SLC38A1) are significantly elevated in tumors (Fig. R6a, 6b). Glutamine is further converted to glutamate within the mitochondria by glutaminase (GLS). Our analysis reveals that GLS expression is increased in tumor tissues as well (Fig. R6c), consistent with the higher glutamate labeling observed in this study (Fig. 5e). In contrast, the expression of glutamine synthetase (GS) in tumors is much lower than that in livers (Fig. R6d), suggesting that HepG2 tumor cells shift away from synthesizing glutamine internally. Instead, they rely on increased expression of transporters to import glutamine from the extracellular environment.

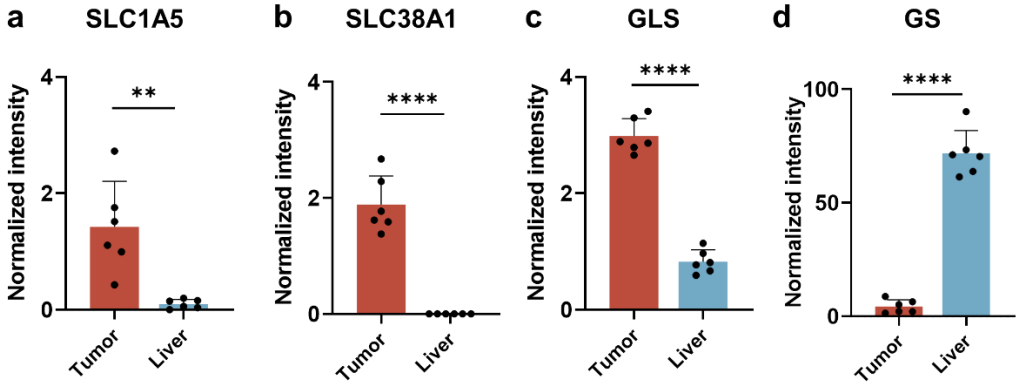

Figure R6. (a-d) Expression levels of SLC1A5, SLC38A1, GLS and GS in tumors and tumor-bearing livers, measured by quantitative proteomics (n = 6; $^{**}p < 0.01$, $^{***}p < 0.001$; $^{****}p < 0.0001$; by two-tailed Student's t-test; mean ± SD).

Taken together, the biological validation experiments provide additional compelling

support for the metabolic crosstalk proposed in this study. These results also further demonstrate the practicality and reliability of the approach we developed. Following your helpful suggestion, new data and discussion have been added into the revised manuscript (lines 277-285 in page 12, lines 353-366 in pages 15-16, lines 432-438 in pages 19, lines 841-893 in pages 34-36).

5. Lack of details on data treatment. In spite of the request, the authors still failed to disclose the details of data preprocessing. Just for clarity, preprocessing includes recalibration, denoising, normalisation, peak picking and peak matching among other potential steps. The added single sentence ("These cdf files were then imported into MassImager software to reconstruct MS images, browse mass spectra, select regions of interest (ROIs), and extract the average mass spectra.") does not explain anything about preprocessing, hence makes it hard to judge the graveness of the associated errors. In addition there are plenty of minor problems associated with inconsistencies in data labelling or the lack of information in the figure captions, but these problems were not taken into account for the reviewer's decision.

RE: Thank you for your valuable comments. We are sorry for not providing a detailed description of the MSI data preprocessing. Following your suggestions, we have added the following description to the Method section of the revised manuscript:

Before data collection, mass calibration was conducted in both positive and negative ion modes via electrospray ionization, using the respective calibration solution (Pierce, Thermo Scientific). The mass error was maintained below 3 ppm within the *m/z* range of 70 to 900 (lines 720-723 in page 30).

In the AFADESI-MSI experiment, the tissue sample is scanned line by line, with each line's data saved as a raw file. These raw files were converted to .cdf format using Xcalibur (Thermo Fisher Scientific) and then imported into MassImager software [3]. By typing the physical length and height of the tissue sample, the scan count and sampling time can be checked automatically by the MassImager upon completing line sequence creation. Four parameters were set for peak detection and integration: a slope threshold of 100, a minimum peak intensity of 0, "total intensity" selected for intensity calculation, and *m/z* calculation performed using weighted interpolation based on peak shape. To reduce background interference, a non-tissue background area was selected, and background subtraction was performed with a proportional coefficient of k = 1. The *m/z* tolerance for background subtraction is set to 0.001 Da. The images were subsequently denoised by choosing the "high" image quality button in the software, which applies pixel alignment and 2D-interpolation filter algorithms. The "global image" and "intensity threshold" methods were applied as appropriate to normalize the

intensity of each mass pixel. After selecting the region of interest (ROI), the *m/z* and peak intensity list were exported into a new text file for further analysis (lines 773-789 in page 32).

Additionally, we have thoroughly reviewed all the labels and legends in the revised manuscript and corrected the inconsistencies to ensure clarity and consistency (lines 372, 376-378, 381 in page 17, line 485,490-492 in page 22). We believe that the readability and accuracy of the manuscript have been further improved. Thank you again for your helpful suggestions!

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Reviewer #3 (Remarks on code availability):

Not having sufficient expertise.

RE: Thank you for your response.

Responses to Reviewers' Comments

Reviewer #3 (Remarks to the Author):

The reviewer appreciates the effort the authors invested into developing the manuscript and reply to the points raised. Unfortunately the replies only partially address the problems or, in other cases, do not address them at all.

RE: Thank you for taking the time and effort to review our manuscript. We have carefully considered your comments and made additional modifications to address your concerns comprehensively. We hope these revisions meet your expectations. Once again, thank you for your feedback and for helping us enhance the quality of our work.

Specific points:

1) Novelty. The reviewer does not say that the current manuscript has no novel components compared to the literature. However, the combination of methodologies (isotope labelled metabolite administration, MSI, LC-MS) is not new. The actual implementation of data analysis have novel components and there is new biological information. Nevertheless, these novel components can not and do not justify the publication of the manuscript in *Nature Communications*.

RE: Thank you for your comments and for recognizing the novelty of our data analysis approach and the new biological insights presented in our study. While the combination of methodologies has been explored previously, it is not the primary focus of this manuscript. Instead, we emphasize a novel perspective and a practical tool that not only presents data but also aids in understanding the distribution of metabolomic variables, offering insights beyond statistical features. Specifically, our study leverages spatial distribution differences among various types of isotopically labeled metabolites to elucidate inter-tissue metabolic communication. Additionally, we present the first system-level characterization of metabolic activity and communication on a whole-animal scale. Our workflow is uniquely designed for untargeted tracking of the metabolic fate of ^{13}C-nutrients in MSI datasets. We believe this tool will be highly beneficial for researchers in the MSI field, bringing a fresh viewpoint on data

processing and interpretation. We are confident it will capture substantial interest among *Nature Communications* readers.

2) The points raised criticise the principles the authors based their study on. Furthermore, offered solutions for these problems. Unfortunately the authors chose to reject these based on their observations. Point 1: This situation is not unlikely. There are histological features (e.g. pituitary gland in the brain or lymph nodes in any tissue) which contribute to <1% of the mass of the organ, but have completely different metabolic composition. This can be captured by MSI, while LC-MS tend not to detect most of these components. Point 2: Again, it is a question of principle - what is correct and what is not. It is not particularly hard to check for the shift in isotopologue distribution of arbitrary ions in MSI, if the authors already created the tools. Point3: The information about the differential labelling of isomers must be obvious from LC_MS. Point 4: The answer provided by the authors says that they wrongly annotated the metabolites in 9.3% of the cases ! (Fig R1) I don't think if it is acceptable by any means. Nevettheless, the suggestion was to calibrate the spectra (not only the instrument) to achieve better than 1 ppm resolution.

RE: Thank you for your critical comments. We have carefully considered the issues you raised in the previous rounds and made the necessary adjustments. But, for certain suggestions, we believe the proposed solutions are not fully compatible with the specific context of our research.

Point 1: We agree with the reviewer that certain histological regions may exhibit distinct metabolic compositions compared to others. However, as the reviewer noted, these regions constitute less than 1% of the organ's total mass. Given this minimal contribution, we believe that our tool's results are unlikely to affect the overall findings. For imaging the pituitary gland and lymph nodes, MSI is primarily employed to assess the biochemical profiles of these tissues for potential intraoperative applications [1, 2], which required more specialized sample preparation methods. We further examined current MSI studies on brain tissues and found that, regardless of whether sagittal [3], coronal [4], or transverse sections were used [5], the maximum number of microregions

characterized was 11. However, the pituitary gland was not included, likely due to limitations in spatial resolution and sampling challenges. Similarly, in the most comprehensive brain metabolomics analysis using LC−MS to date [6], the pituitary gland was also excluded, highlighting the difficulty of analyzing this structure despite its critical functional importance. Since one of our ultimate goals is to build a more comprehensive labeling database, we will focus on these microregions in our future work. We encourage researchers who are interested the metabolism of those regions to obtain the characteristics using LC−MS first and they can still freely use the method proposed here.

Point 2: To determine the predominant ions in MSI, we have already conducted a detailed analysis of the isotopologue distribution for multiple arbitrary ions in our previous work [7]. We concluded that a substantial number of $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, and $[M+Cl]^-$ adduct ions were detected in MSI compared to LC−MS, providing the foundational basis for this study (Fig. R1).

[editorial note: figure redacted]

Figure R1. (a) Number and percentage of adduct ion forms of identified metabolites in positive mode (left) and negative mode (right) in LC−MS. (b) Number and percentage of adduct ion forms of annotated metabolites in positive mode (left) and negative mode (right) in AFADESI-MSI.[7]

Point 3: That's true. Using LC−MS, some isomers can be effectively distinguished based on their retention times. In this study, they were identified as distinct metabolites and are clearly documented in Supplementary Data 1. We have added a discussion on this point in the revised manuscript: Secondly, unlike LC−MS, which can differentiate metabolites based on retention times, the current MSITracer cannot distinguish isomeric molecular species (lines 607-608 in page 25).

Point 4: We appreciate your valuable suggestions. We optimized our data processing workflow by incorporating data alignment steps. Specifically, after extracting the average mass spectra of the areas of interest, the data were imported into MarkerView software (Sciex) for peak alignment (mass tolerance: 5 ppm; minimum required response: 100). This step moves the exclusion of low-intensity peaks to occur before *m/z* matching rather than after, reducing the inconsistent matching rate from 9.3% to 0.12%. As demonstrated in our built-in example dataset, this additional step does not affect the final results but confirms that the principles underlying our method are acceptable. We have included the modified processing steps in the revised manuscript (lines 820-822 in page 32). Additionally, we revisited our data and confirmed that the spectral resolution was better than 1 ppm.

3) The quantification - or actually the lack of it - is the gravest problem with the study. The authors state that they always look at labelled fraction, however 90% of the manuscript is about the distribution of certain (labelled or unlabelled) isotopologues across different tissue types. This does not work, the reference provided in the previous round of review clearly explains the problem. There is not a single figure in the manuscript or the supplmentary material, which would depict the labelled fraction in any spatially resolved manner ! This also raises questions regarding the functionality of the MSITracer. The authors base their conclusions on the following axioms:

- The intensity of an ion is proportional its concentration across multiple tissue types
- The 2D sections represent the entire animal/organ

As neither is true, the conclusions of the authors are at most indicative. Mixing stable isotope labelled standards with tissue homogenates, analysing them and showing their

intensity ratios does not address the problem and does not yield any new information.

RE: Thank you for your comments and for sharing the relevant article [8]. We fully agree with the paper that incorporating the tissue extinction coefficient (TEC) is an effective approach to mitigating matrix effects. As our supplemented results from the previous two rounds were not accepted, we reanalyzed the data and applied the TEC to recalibrate the original intensity. As shown in Figure R2a, although the $^{13}C_6$-glucose was uniformly deposited across the sample, variations in the intensity of the intact $[M+Cl]^-$ molecular ion were observed across different organs. To address this, we calculated the TECs for each organ by dividing the mean intensity of the $^{13}C_6$-glucose $[M+Cl]^-$ peak within the organ by the intensity of the same peak in the off-tissue background region. The brain and liver exhibited TEC values below one, indicating ion suppression of the $^{13}C_6$-glucose peak relative to the off-tissue region. In contrast, the kidney and heart showed TEC values above one, suggesting relative ion enhancement (Fig. R2b). The extracted intensity was normalized using the corresponding TECs for the four organs, followed by ^{13}C natural isotope abundance correction. The results showed that the labeled fraction of $^{13}C_6$-glucose was identical to the previous data without TEC normalization, suggesting that the labeled fraction itself compensates for matrix effects, thereby confirming the accuracy of the quantitative results in this study (Fig. R2c).

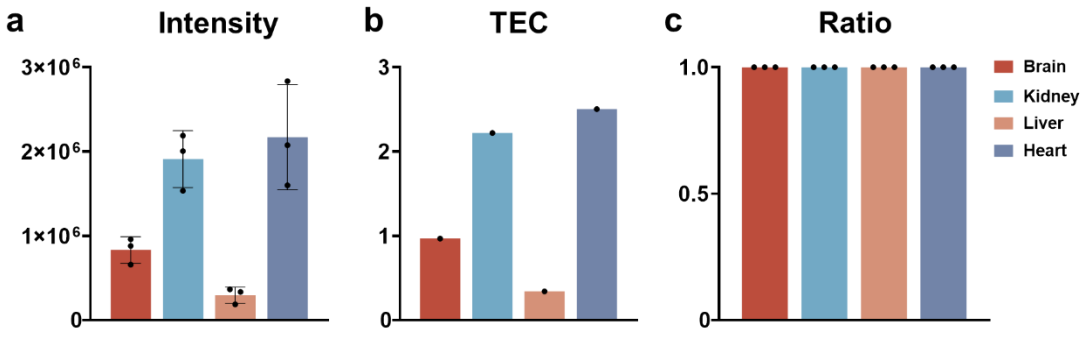

Figure R2. (a) Ion intensities of externally added $^{13}C_6$-glucose in the tissue sections of brain, kidney, liver, and heart, measured by AFADESI-MSI. (b) Calculated TECs for the four organs. (c)The ratio of the labeled fraction of glucose M6 calculated from TEC-normalized intensity to that calculated from unnormalized intensity across organs (n=3).

Additionally, papers published by Rabinowitz and Patti's groups also utilized labeled fractions to compare metabolic activity within heterogeneous tissues (e.g., kidney)[4] or between different tissues (e.g., brain and tumor)[9]. We also benchmarked our MSI results against LC−MS assays, which demonstrated similar labeling trends, further validating the effectiveness of this method.

We understand your concerns that ion intensities are not always directly proportional to their concentrations across tissues. However, as demonstrated in the calculations above, the labeled fractions are independent of the response factors from different organs, making this approach applicable in this scenario. Meanwhile, in spatial metabolomics, MSI analyses are generally conducted using 2D sections. This is not unique to spatial metabolomics, as spatial transcriptomics, proteomics, and even the pathological gold standard also typically rely on 2D sections rather than entire organs. Despite the potential limitations, these approaches have been extensively recognized and used to study tissue heterogeneity, tumor microenvironments, and other biological contexts.

4) The authors have performed a number of additional experiments in relation to this point. The reviewer appreciates both the IHC and the quantitative proteomics experiments. Unfortunately the selection of IHC targets still left a number of questions open. While ACC1 and FASN are excellent targets for capturing fatty acid biosynthesis, GPAT1 is an enzime taking part of the biosynthesis of all glycerolipids - not only triglycerides. CPT2 takes part of the transport of long-chain fatty acyls through the mitochondrial membranes - again, not an excellent marker for fatty acid oxidation. Certainly does not perform the beta-oxidation fatty acids as the authors state. The other problem is associated with the incompleteness of spatial localisation. The stains (e.g. FASN or ACC1 in BAT) shed light on the intensity of fatty acid biosynthesis in the target tissue, however if the authors state that a certain process takes place in a given organ, then they have to prove that the given pathway is only active there. Since they only tested 2-4 organs for most enzymes (except for FBPase, G6Pase and PEPCK, where they tested 9), they just don't have enough information to localise the given

process, not to mention alternative pathways.

All of the pathways localised by the study are known to be expressed in the given tissue, from intensive fatty acid metabolism in BAT to highly intensive glucose metabolism in cancer - there has been no novel information revealed due to the development of the novel tools. This is not necessary a problem, however the authors should present their results in the scientific context and not pressing on the novelty of results, which are fundamentally not novel.

RE: Thank you for the valuable comments. In the revised manuscript, DGAT2 and CPT1b have been chosen to represent triglyceride synthesis and fatty acid oxidation, respectively. UCP1, ATP5F1A, and ATP5F1B are also analyzed to further illustrate the distinct metabolic roles of CPT1b in BAT and the heart. The reason for presenting enzyme distribution in only four organs in the previous round (Supplementary Fig. 11d-h) is that the BAT, heart, liver, and brain are the primary organs involved in fatty acid metabolic interactions. Following your suggestions, we have now included enzyme levels from more organs (Supplementary Fig. 11d-k) and revised the manuscript accordingly as follows: Diacylglycerol O-acyltransferase 2 (DGAT2), recognized as the rate-limiting enzyme in triglyceride biosynthesis with greater substrate affinity than DGAT1[10], is more highly expressed in BAT and the liver (Figure R3a), consistent with the triglyceride synthesis function observed in this study. Moreover, the lipoprotein lipase (LPL) and carnitine O-palmitoyltransferase 1b (CPT1b) are responsible for the hydrolysis of triglycerides and the β-oxidation of long-chain FAs, respectively[11]. In BAT and the heart, the elevated expression of LPL and CPT1b underscores their dependence on fatty acids as a primary energy source (Figure R3b, 3c). This is further supported by the uniquely enhanced levels of uncoupling protein 1 (UCP1) in BAT, highlighting its role in non-shivering thermogenesis by converting energy into heat[12] (Figure R3d). By comparison, the heart exhibits greater levels of ATP synthase F1 subunit alpha (ATP5F1A) and ATP5F1B, reflecting its reliance on ATP production for contractile activity[13] (Figure R3e, 3f) (lines 359-372 in pages 15-16).

Our biological collaborators appreciate that our method effectively correlates and validates previously scattered physiological observations of organ interactions through

whole-body animal MSI analysis. Given the deep matching of metabolome and proteome data, we believe that this work will make a substantial contribution to advancing MSI-based spatial metabolomics and its broader applications.

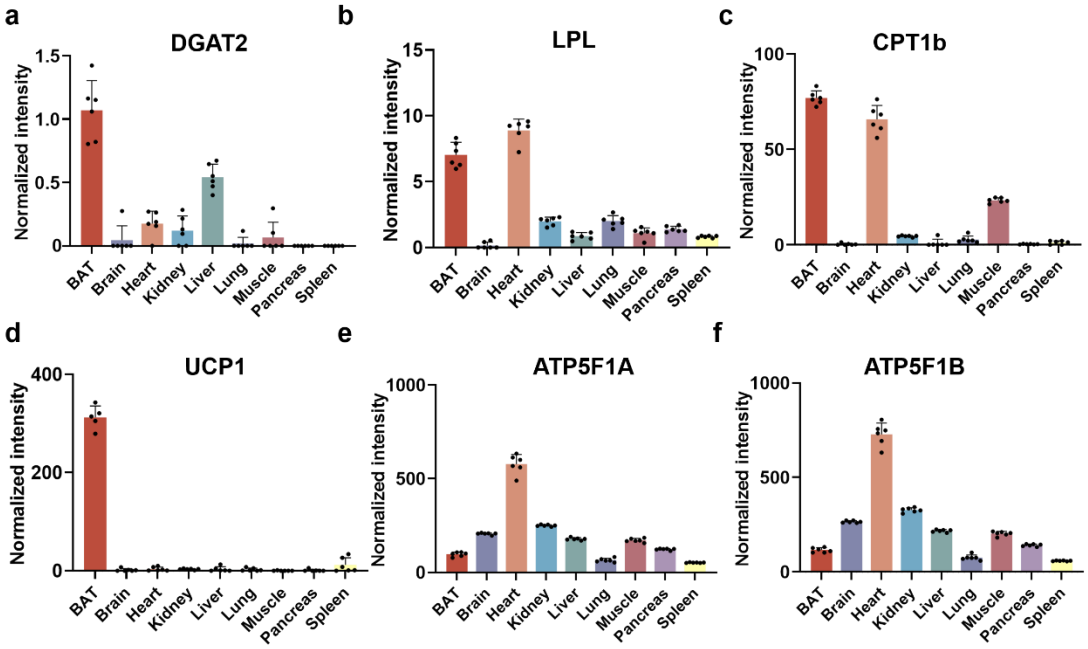

Figure R3. (a) – (f) Expression levels of DGAT2, LPL, CPT1b, UCP1, ATP5F1A, and ATP5F1B in BAT, brain, heart, kidney, liver, lung, muscle, pancreas, and spleen, measured by quantitative proteomics (n = 6).

5) The reviewer - and likely the readership of the Journal - are interested in the steps of spectral data preprocessing in scientific terms. Unfortunately the settings of a vendor-specific data analysis software do not provide this information. It would be very interesting to see how post-acquisition recalibration of the data is performed, how the data is background-subtracted (in scientific terms) or how peak-picking was performed. There are various algorithms for these steps, and the reviewer is confident that the Thermo tool uses one or another, but citing on-screen settings in a manuscript, which focuses on the development of a data processing platform is not acceptable.

RE: Thank you for your insightful comments. According to your suggestions, we revisited our data and confirmed that the resolution of our spectra was better than 1 ppm, achieved through instrument calibration and lock mass calibration during data acquisition (positive mode: *m/z* 149.0233, 279.1591, 301.1413; negative mode: *m/z*

283.2637). Consequently, no post-acquisition recalibration was performed in this study. Background-subtraction was performed by selecting a non-tissue background area and subtracting the background using a proportional coefficient (k = 1). The deduction formula is: Adjusted intensity = Original intensity- k × Background average intensity (k: 1–100). Peak picking was conducted by identifying local maxima with a slope threshold of 100, determined based on the peak shape. Moreover, data normalization was performed on the entire data matrix to further reduce the mass intensity variant in each mass pixel. We have modified the description in the revised manuscript (lines 812-820 in page 32).

On a final note, we hope the reviewer can understand that while our data processing workflow differs from commercial tools (e.g., Bruker SCiLs Lab) in certain aspects, these distinction does not compromise the accuracy of our results. The technical details described in the manuscript are fully sufficient to ensure reproducible results. MassImager is a lab-developed software specifically designed for data analysis in AFADESI, forming the foundation for this study. Its utility has been validated through multiple publications that have successfully employed this tool.

References

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Responses to Reviewers' Comments

Reviewer #3 (Remarks to the Author):

The reviewer does appreciate the efforts of the authors regarding addressing the points raised in the previous round of review. However, in spite of the 11 pages long rebuttal letter, the manuscript has hardly changed as a result of the revision.

RE: Thank you for taking the time and effort to review our manuscript again. Throughout three rounds of revision, we have made substantial and comprehensive changes to the manuscript. These include reanalyzing the data to enhance the biological contributions of our study, improving the data processing methods, and refining figure clarity. Additionally, we performed new experiments to further demonstrate the effectiveness and reliability of our analytical approach. We believe that these revisions have significantly strengthened the manuscript.

1. With regard to novelty, the reviewer agrees that the model the authors used gave an unprecedented opportunity to characterise the organism-level metabolism, but this opportunity was largely missed, due to inappropriate data analysis strategies. In order to perform such study, the authors (or anyone else) is expected to provide 3D, quantitative maps of all isotopologues of major metabolites at multiple time points. The reviewer agrees that it is a gargantuan challenge, but the authors should not claim of solving it when they did not.

RE: We thank the reviewer for recognizing the unique value of our model for organism-level metabolic analysis, but we respectfully disagree with the view that generating 3D, quantitative maps of all isotopologues across multiple time points is the only valid or necessary path to gain meaningful insights into organism-level metabolism. Instead, several high-impact studies have demonstrated the feasibility of organism-level metabolic inference without 3D quantitative reconstruction. For example, Wang et al. revealed age-dependent metabolic reprogramming in *Drosophila* through multi-organ isotopic profiling[1]. Similarly, Faubert et al. showed that lactate utilization by tumors could be robustly inferred from tissue-level isotope enrichment following [^{13}C]-lactate

infusion[2]. Moreover, a recent landmark *Nature* study systematically mapped metabolic rewiring across organs using transcriptomics, revealing comprehensive shifts in metabolic networks at both global and local levels[3]. Together, these studies support the scientific value and feasibility of our approach.

In this study, we aimed to extract meaningful metabolic information at the tissue level by analyzing isotope labeling fractions with spatial resolution, supported by LC-MS validation. We believe this approach offers a practical and biologically informative framework that complements existing efforts. It can also avoid the technical and interpretive challenges associated with full 3D isotopologue mapping.

2. Specific further points: In case of tissue regions, the authors gave somewhat related information with numerous citations, however did not address the point raised. With regard to the ion types, they unfortunately depend on the instrument and experimental parameters (DESI solvent, LC eluents) so if the intention is to produce a tool for the community, the pre-chosen ion types do not make sense.

RE: Thank you for your comment. We would like to point out a key misunderstanding in the reviewer's comment regarding MSI. While we fully agree that ion types in MSI can be influenced by parameters such as the AFADESI spray solvent, they are unlikely to be affected by LC eluents in our case, as no LC setup was involved in the MSI experiments.

With this in mind, the ion types used in our analysis were selected because they are commonly reported in the MSI literature[4], consistently observed across our dataset[5], and offer high signal-to-noise ratios, which are essential for reliable isotopologue quantification. To make our approach broadly adaptable for different laboratories and instrumentation platforms (e.g., varying MALDI matrices), we have updated MSITracer to allow users to flexibly define and customize ion types according to their own experimental setup. This functionality is now described in the Methods section in the revised manuscript (lines 809-810 in page 33).

We want to highlight that the preset ion selection strategy substantially reduces the computational burden of processing large MSI datasets, while maintaining high

metabolite coverage and minimal bias in isotopologue quantification. As a practical and widely applicable tool for the MSI community, its advantages should not be overlooked.

3. In case of isomers, the MSITracer could actually use the LC-MS information to address this problem.

RE: Thank you for your comment. As we explained in the previous round, the identification of isomeric compounds is not the focus of this study. It is technically unfeasible to resolve this challenge solely through LC-MS retention time matching without additional orthogonal techniques such as ion mobility separation or on-tissue MS/MS. Combining the advice from you and Reviewer 1, we have clearly acknowledged this limitation and responded by explicitly marking metabolites with known isomeric forms, and providing LC-MS-based annotations in Supplementary Data 1, where isomers are documented as distinct entries. These efforts aim to inform users about potential signal overlaps and promote more careful interpretation of MSI results.

While isomer interference remains an inherent limitation in direct mass spectrometry analyses (including MSI), we maintain that this limitation does not compromise the main conclusions of our study. Systematic isomer resolution constitutes a distinct research direction requiring specialized technical development, and further integration of isomer resolution strategies falls outside the scope of this study.

4. In case of false discovery rates, it is at least surprising that an order of magnitude drop in FDR does not change the outcome, which raises further serious questions about the data interpretation.

RE: Thank you for your comment. FDR control is indeed critical for reliable data interpretation. In our revised workflow, the additional step of peak alignment and low-intensity peak exclusion prior to importing data into MSITracer was designed to improve the consistency of peak matching and reduce noise. (lines 797-800 in page 32). This optimization does not alter the core matching algorithm or the fundamental logic used to identify isotopic features. Therefore, it is expected that the final outcome

remains largely unchanged, since the filtered peaks were primarily low-intensity or non-informative signals that did not contribute meaningfully to the matched isotope clusters. The observed consistency in results before and after the optimization reflects the stability of our approach rather than indicating a flaw in data interpretation.

5. In case of tissue-specific response factors, the authors accepted the point that an whole organ or whole body intensity map is meaningless. Nevertheless, almost all figures show ion intensity maps and the conclusions are - directly or indirectly - based on this information. The use of TECs could solve the problem, however the authors tried to prove that it does not change anything (Fig R2c.). Indeed, dividing both the numerator and the denominator by the same number does not change the value of a fraction, this 3rd grade elementary school math. However, the different TECs can show that a metabolic activity of tissue A is negligible compared to the metabolic activity of tissue B. Similarly, by not analysing >99% of the body by MSI, makes any conclusions unfounded. The authors state that all MSI field uses 2D sections, however these groups are not trying to make organism-level claims.

RE: Thanks for your comments. We find it somewhat unexpected that, after two previous rounds of detailed clarification, matrix effects are still considered a major concern regarding the accuracy of our results. Again, as clearly stated in the study from Gary's group[6], "We wish to emphasize that relative pool-size distributions have no effect on our ability to interpret labeling data. Analysis of labeling data only requires quantitation of isotope enrichment, **which are not influenced by matrix effects.**" This view is also supported by other MSI-based isotope tracing studies[7], which routinely apply fractional labeling ratios (e.g., labeled / total) to assess metabolic activity across diverse tissues—including liver, muscle, and pancreas—despite differences in matrix composition and biochemical properties. Thus, labeled fraction values provide a robust and commonly accepted measure for comparative metabolic analysis.

Additionally, we would like to clarify that we did not state or accept that whole-organ or whole-body ion intensity maps are meaningless. On the contrary, we emphasized that intensity-based visualization provides important insights into tissue-specific metabolic

patterns and potential inter-organ metabolic interactions.

To further address the concern, we reanalyzed our data following the methodology described in "Spatially resolved isotope tracing reveals tissue metabolic activity" (Nature Methods)[8]. Specifically, we converted our original datasets into .mat files and performed the analysis using the Isoscope software as described in that study. As illustrated by Fig. 2b and Fig. 3c in the manuscript, the results obtained using Isoscope were highly consistent with those generated from our original intensity maps and MSITracer-based calculations (Figure R1). Specifically, the infusion of U-^{13}C glucose resulted in a predominant distribution of labeled glutamate within the brain, whereas the infusion of U-^{13}C glutamine led to a primary localization of labeled glutamate in the pancreas. In addition, highly enriched M4 isotopologues of malate, succinate, aspartate, glutamate, and N-acetylaspartate (NAA) were predominantly detected in the brain following U-^{13}C glucose infusion. Altogether, our tissue sampling strategy is consistent with those employed in other high-impact studies involving organism-level metabolic analysis using stable isotope tracing. Our core biological findings are robust and do not depend on the specific normalization strategy employed.

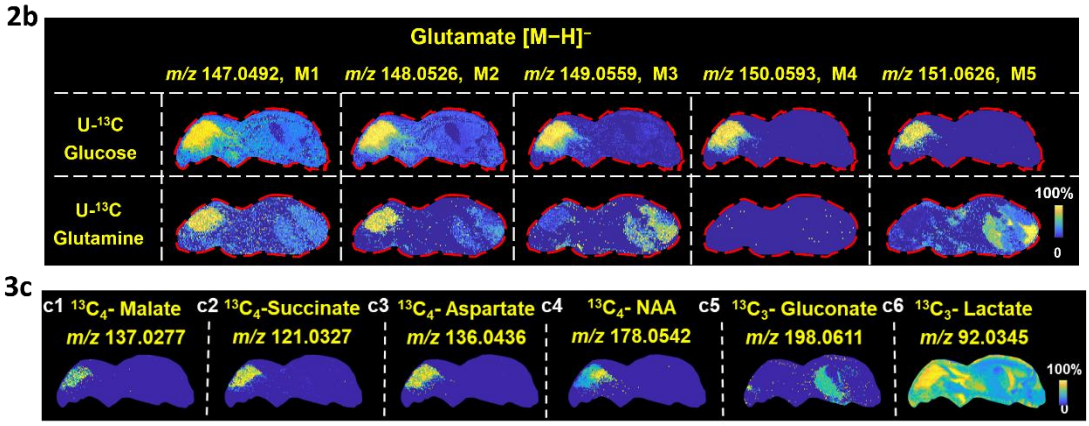

Figure R1. Validation of metabolic labeling trends using isoScope.

6. With regard to the IHC validation, this is the only part of the manuscript where meaningful information was added. Nevertheless, the problem raised was the broad substrate specificity of some of the enzymes, which was not fully addressed.

RE: Thank you for acknowledging the added IHC validation data. Regarding the

enzymes we selected, the majority (FASN, ACC1, UCP1, ATP5F1A, and ATP5F1B) exhibit strict specificity toward their physiological substrates. Only DGAT2, LPL, and CPT1b demonstrate moderate substrate flexibility; however, this flexibility remains biologically constrained: DGAT2 accepts various fatty acyl-CoAs but strictly requires diacylglycerol as the acyl acceptor[9]; LPL exclusively hydrolyzes triglycerides albeit across different species[10]; and CPT1b transfers long-chain fatty acyl-CoAs while retaining specificity for carnitine conjugation[11].

Importantly, despite these substrate characteristics, the selected enzymes effectively represent the biological activities of the metabolic processes we aim to highlight. For example, high DGAT2 expression supports active triglyceride biosynthesis; and increased LPL and CPT1b levels in BAT and heart indicate a reliance on fatty acid turnover and oxidation as major energy sources. These observations are consistent with our intended focus on tissue-specific lipid metabolism dynamics.

The selection of these enzymes was primarily aimed at reinforcing the key metabolic differences between tissues, rather than resolving the fine biochemical properties of each enzyme. As such, we believe that the substrate characteristics of these enzymes do not compromise the validity of our conclusions. On the contrary, their expression patterns further support the metabolic trends observed in our study.

7. With regard to data pre-processing, the authors are apparently unfamiliar with the complexity of peak detection, background subtraction or normalisation. These are complex tasks, with enormous literature from the last 40 years.

RE: We disagree with your conclusion that we are unfamiliar with the complexity of the data processing. We hope the reviewer can understand that there is more than one reasonable and reproducible approach to pre-processing mass spectrometry data. The AFADESI-MSI system used in this study is a custom-developed imaging platform from our laboratory, and its data characteristics differ from those of commonly used commercial instruments, such as those from Waters or Bruker. The corresponding software and data-processing workflow have been specifically optimized for this platform and have been successfully applied in diverse biomedical research areas,

including tumor metabolism and studies of drug mechanisms. As a result, the AFADESI-MSI technique and its derived spatial metabolomics approach are now widely recognized in the MSI field, with applications reported in approximately 100 peer-reviewed publications.

We also wish to clarify that the development and application of MSI in biomedical research began in 1997 with Caprioli et al.'s MALDI-MSI work[12], and thus the field's history spans less than 40 years. In the previous round of revision, we provided detailed descriptions of our data preprocessing procedures. We believe that our methods are consistent with established practices in the MSI studies, and have been transparently documented to ensure reproducibility. We respectfully note that continued concerns about the validity of our preprocessing approach, in the absence of specific evidence or examples, are not well-founded.

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