nature research

Peer Review Information

Journal: Nature Methods
Manuscript Title: Integration of Mass Cytometry and Mass Spectrometry Imaging for Spatially Resolved Single Cell Metabolic Profiling
Corresponding author name(s): Noel (F) De Miranda

Editorial Notes: None

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Noel,

Your Brief Communication entitled "Integration of Mass Cytometry and Mass Spectrometry Imaging for Spatially Resolved Single Cell Metabolic Profiling" has now been seen by 3 reviewers, whose comments are attached. While they find your work of potential interest, they have raised serious concerns which in our view are sufficiently important that they preclude publication of the work in Nature Methods, at least in its present form.

As you will see, the reviewers raise concerns about a lack of benchmarking against existing methods and that the utility of the method is unclear without metabolite identification. Should further experimental data allow you to fully address these criticisms we would be willing to look at a revised manuscript (unless, of course, something similar has by then been accepted at Nature Methods or appeared elsewhere). This includes submission or publication of a portion of this work somewhere else. We hope you understand that until we have read the revised paper in its entirety we cannot promise that it will be sent back for peer-review.

If you are interested in revising this manuscript for submission to Nature Methods in the future, please contact me to discuss your appeal before making any revisions. Otherwise, we hope that you find the reviewers' comments helpful when preparing your paper for submission elsewhere.

Sincerely,
Madhura

Madhura Mukhopadhyay, PhD
Senior Editor
Nature Methods

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors describe a methodology for analysis of fresh-frozen tissue sections using MALDI mass spectrometry imaging (MSI) to measure relative metabolite abundances, combined with imaging mass cytometry (IMC) for antibody detection. This is certainly an advanced technique, where the ability to perform both MSI and IMC on the same tissue section holds promise to identify metabolites specific to certain cell types in their native context within tissues. However, I feel that the manuscript in its current form has two major limitations. First, important information is lacking to evaluate the performance of the proposed method and how it improves upon the state-of-the art. Second, the method as described herein does not identify any metabolites from the MSI data, so nothing can really be said about metabolism from the data (beyond observing that "something" in metabolite space differs between cell populations). I think these two points should be addressed if the method is to be of broad interest to the metabolism research community.

** Major points **

The main contribution seems to be the developed protocol for performing MALDI MSI and then IMC on the same tissue section, which should improve co-registration (more precisely co-locating metabolite abundance and cell marker data). However, the article does not really analyze the pros & cons of this method compared to existing approaches that use two consecutive sections for MSI and IMC (such as ref 10). As the authors point out, a key concern is that the MALDI procedure might negatively affect antigen detection in the later IMC analysis. Unfortunately, the authors do not provide data to address this question. Suppl Fig 1 details how results improved after optimizing MALDI parameters and antibody selection, but only shows results on MALDI-treated sections. It would seem crucial to compare IMC data acquired from sections with and *without* MALDI. Are antibodies detected with comparable quality in both cases, or might the MALDI procedure be detrimental to IMC quality? Can cell phenotypes be resolved similarly in both cases? Could there be other artefacts in the IMC epitope images induced by the MALDI procedure that might skew cell segmentation or cell type recognition? This should preferably be evaluated on consecutive sections, so that images from (1) and (2) can be co-registered and compared directly. Some external validation of the cell types predicted from the MSI data would also be helpful (for example by separate IHC, or from cells with known anatomical location). Also, it was not clear to me exactly how the 19 cell types were defined (L290, "cells forming visual neighborhoods were grouped to define cellular phenotypes"), the criteria used here should be stated more clearly.

Moreover, I think the authors could strengthen their case by explicitly showing the advantages gained by performing MSI + IMC on the same section, as opposed to using existing methods on consecutive sections. I would expect that the author's procedure has better co-registration, but this should be demonstrated by comparing the two approaches side-by-side. For this, some ground truth would be required, for example demonstrating that metabolites known to be specific to certain cell types are assigned correctly by the authors' approach, and that such assignment is improved when combining MSI and IMC on a single section.

In relation to the above, and in order to evaluate the usefulness of the method for metabolism research, the authors should provide at least some metabolite identification. Could not MS/MS be used to identify some of the MS peaks with reasonable confidence? For example, can the authors identify

the top features that differed between macrophages vs. monocytes in Fig 2A? Does the method pick up on known/expected differences between cell type, for example accumulation of lactate in more glycolytic cells, or nucleotides in proliferating cells? Even a few such identified peaks could help to readers better appreciate the method and its potential applications.

** Minor points **

L104: "no clear association between the IMC and glycerophospholipid clusters, confirming the presence of metabolite variations within the same cell populations" -- this seems overstated, it may simply be that the MSIC data is noisy?

Fig 2DE: I'm not convinced that these are truly cell clusters, since it often happens that clustering methods "invent" clusters in noisy data. In Fig 2D the clusters are not that well separated, and could be an artefact of k-means method. How was the number of clusters K=10 picked? Can the clustering be confirmed by other methods, e.g. Louvain or Leiden clustering? The heatmap in Fig 2E should also show metabolite abundances per cell, not merely a cluster average, so that the reader can judge cluster separation.

L91, "separation was confirmed by a higher pairwise distance" (Fig 1E) -- are these also distances in the UMap embedding (in the 2D plane) ? If so this does not add much beyond what's already visible in Fig 1CD. It might be better to compare against the original distance matrix (the input to UMap) to asses the effect of the UMap embedding / manifold detection.

L95, "performed differential feature analysis", might be clearer to state "computed fold changes" between cancer and stromal-immune cell compartments.

L112, "based on metabolite profiles" --> based on glycerophospholipid profiles

Use of Thomson (Th) units is deprecated by IUPAC, see
https://www.degruyter.com/document/doi/10.1351/PAC-REC-06-04-06/html

** Code review **

The provided code consists of python and R scripts supplied as a zip file available on figshare. It would be preferable to use a code repository such as github / gitlab. The code does not run out-of-the-box, but I was able to get it to work after some tweaking (see below). If the codebase is intended to be used by others as a routine tool (as part of the authors' proposed method) then I think some optimization is needed. The R script is very slow, it took 40 min to process a single m/z image on my laptop. I think this step could be done much more efficiently with a better data representation; I would suggest using numpy/pandas from python, then the R component can be eliminated entirely.

Python scripts will not run as provided due to hardcoded absolute paths referring to the authors local machines, for example 'C:/TAMIM/LUMC/MSI-IMC_Integration/' and "New data/2.ROIs_normalised_only/..." Please use relative paths instead.

The R script ("1_IMC_MSI_celltype_metabolite-abundance_integration.R") is interactive due to the file.choose() call on line 87 which requires the user to pick a mass list. I believe the file to read should

be "curated-masslist.txt", and this should be a constant in the script. The script and the file curated-masslist.txt also contains hardcoded paths so does not run as provided. There are several unused dependencies, which tends to cause unnecessary trouble for users; as far as I can tell only the dplyr and stringr packages were actually needed.

Roland Nilsson
Karolinska Institutet

Reviewer #2:
Remarks to the Author:
In this manuscript, the authors describe method to integrate imaging mass cytometry and matrix-assisted laser desorption/ionization mass spectrometry imaging for single cells metabolic profiling on cancer patient biopsy slides. The authors provided a proof of principle that distinct metabolic features between different cell types in tissue can be spatially resolved on the single cell level. When it is combined with single cell spatial proteomics, it represents a powerful tool to resolve metabolic heterogeneity and cellular complexity. I have a few additional comments.
1. The authors indicated that Fig. 1D corresponded to the left UMAP of Fig. 1C. I don't see the right UMAP of Fig. 1D corresponds to the left UMAP of Fig. 1C (the shapes do not match). Please clarify the discrepancy.
2. I wonder why the authors tended to lump stromal cells and immune cell together in Fig. 1, when they have many IMC antibodies to distinguish different immune cell subsets. The rationale to focus on macrophages could be further clarified too. The descriptions in Results are a little vague. For example, "The analysis demonstrated no clear association between the IMC and glycerophospholipid clusters, confirming the presence of metabolite variations within the same cell populations. This phenomenon was particularly evident in macrophages, which formed clusters but also scattered throughout the embedding". I'm not sure how such conclusions were reached. At least visually plasma cells appeared to have distinct glycerophospholipid too.
3. I think the authors should provide a rationale why they chose to focus on glycerophospholipids but not other metabolites. There was also limited indication of specific phospholipid species. For example, Fig. 2A and Supplementary Fig. 3B did not indicate the identities of each phospholipid (or potential identities). Overall, the descriptions in Main text are a little vague. The authors simply described that they could detect different phospholipid distribution between different cell types in a tumor tissue slide. But there were few details. Any standout metabolites in any specific cells, what are the implications (even speculations could be fine). Here, Fig. 1F showed striking difference between cancer cells and stromal-immune cells. It would be great if the authors can delve a little more on what is/are the metabolites enriched in cancer cells, and what is/are those enriched in stromal-immune cells (what type of stromal-immune cells?), and what are the implications.
4. Following the above comment, the authors could discuss the limitations of their methods. Is the method limited to glycerophospholipids and small metabolites (what is defined as small metabolites)?

Reviewer #3:
Remarks to the Author:
NMETH-BC52925 Nunes et al.

Nunes et al address a very important topic in spatial biology, the precise spatial assignment of metabolites to single cells and defines cell types, followed by multivariate statistical analysis of cell populations. The authors use three CRC tissues, but focus most of their analysis on a single example.

Nunes et al use simple rapiflex MALDI-TOF MSI and NEDC matrix to acquire small molecule datasets – at a proposed pixel size of 5x5 µm2. They claim to have developed an improved workflow that enables subsequent data acquisition by imaging mass cytometry (IMC) at 1x1 µm2 pixel, for which they use a 25-plex metal-labeled antibody panel optimized for use with FFPE tissue.
Data from both imaging modalities are converted to TIFF and co-registered as TIFF. Single MSI pixels are then mapped to 25 IMC pixels. The latter are used to assign cell type labels.

Pairwise Euclidian distances suggest that cells within cancer or immune compartments are closer to each other than cancer cells are to immune cells. Bidirectional clustering analysis indicates that various cell types, in particular cancer cells and immune cells, feature distinct glycerophospholipid (GPL) and small metabolite profiles that can also be segregated by UMAP data reduction. Some m/z co-localize with IMC cell type markers (all Fig. 1).

Some m/z clearly distinguish macrophages/monocytes from all other cell types. Interestingly, the relative abundance of certain m/z in macrophages/monocytes is much more heterogeneous than in other cell types, as assessed by several statistical methods (Fig. 2).

As important as the addressed topic may be, the study raises several major concerns that would have to be addressed:

1. MALDI-TOF MSI is not a suitable technology for spatial metabolomics, as it lacks resolving power and mass accuracy to unambiguously assign metabolite identities. The authors even acknowledge in the methods that their spectral processing included per pixel spectral alignment with maximum shifts of 25 ppm. MALDI-TOF/TOF on a rapiflex MS also does not permit any on-tissue MS/MS validation, since the precursor ion selector window in TOF/TOF is much too big. Consequently, the identity of all metabolites in this study is hypothetical at best. In line with this sentiment, the authors – while referring to GPL and small metabolites in some sub-Figures, avoid the naming of lipids/metabolites throughout the manuscript and rather refer to m/z-values with single-digit accuracy in critical Figures 1F or 2AB. They nevertheless refer to these m/z as "top differentiating metabolites" in the text (line 96). A high resolution MSI instrument or true on-tissue MS/MS capabilities would be required instead.

2. Rapiflex MALDI-TOF does not offer true 5x5 µm2 lateral step size. The instruments today are specified for 20x20 µm2 pixels and can reliably support 10x10 µm2 spatial resolution. At 5x5 µm2 very likely there will be overlaps with neighboring pixels.

3. Referencing to relevant literature could be improved considerably: Several major technologies like UMAP are not cited; work by other teams on spatial single cell metabolomics (e.g. Rappez et al. Nat Methods. 2021 Jul;18(7):799-805 or reviews like Liu et al. Recent advances in mass spectrometry imaging of single cells. Anal Bioanal Chem. 2023 Jul;415(18):4093-4110) is not cited.

4. The authors claim development of an optimized wet-lab protocol (line 40). It remains unclear though what this development really was. Just the inclusion of a formalin-fixation step? This has been used in multimodal MSI-single-label IHC work before. References 11 and 12 point to rather old papers

in this respect.

5. Judged from a biomedical perspective, for lack of metabolite IDs, this study is inconclusive: No putative cancer markers were identified in Fig. 1. More importantly, major observations in Fig. 2 remain purely descriptive and speculative. For instance, does the observed "metabolic" heterogeneity of macrophages represent different activation states (like M1 and M2), or may it be caused by technicalities? If macrophages were larger than other cells, perhaps they'd be covered by a pure macrophage MSI pixel and several other MSI pixels which to large extends represent other cell types?

6. The mapping of IMC pixels to MSI pixels is not entirely clear. How are MSI pixels assigned if half of the corresponding IMC pixels were cell type 1 and the other half was cell type 2? This refers to the formula in line 314, but should be stated more explicitly.

7. What m/z windows (corresponding to real mass accuracy) were used for the database searches against Hmdb and Lipidmaps?

Other open questions:
- How can an MSI pixel contain zero cells? (line 79)
- Why was a rather conservative S/N cutoff >15 chosen for peak picking? (line 264)
- How was recalibration against a list of known m/z features done? (line 265)
- How was peak list filtering against the expected mass defect window done, i.e. using what software? (line 266)

Author Rebuttal to Initial comments

Reviewer #1:

Comment 1: The main contribution seems to be the developed protocol for performing MALDI MSI and then IMC on the same tissue section, which should improve co-registration (more precisely co-locating metabolite abundance and cell marker data). However, the article does not really analyze the pros & cons of this method compared to existing approaches that use two consecutive sections for MSI and IMC (such as ref 10).

*As the authors point out, a key concern is that the MALDI procedure might negatively affect antigen detection in the later IMC analysis. Unfortunately, the authors do not provide data to address this question. Suppl Fig 1 details how results improved after optimizing MALDI parameters and antibody selection, but only shows results on MALDI-treated sections. It would seem crucial to compare IMC data acquired from sections with and *without* MALDI. Are antibodies detected with comparable quality in both cases, or might the MALDI procedure be detrimental to IMC quality? Can cell phenotypes be resolved similarly in both cases? Could there be other artefacts in the IMC epitope images induced by the MALDI procedure that might skew cell segmentation or cell type recognition? This should preferably be evaluated on consecutive sections, so that images from (1) and (2) can be co-registered and compared directly.*

We appreciate the Reviewer's detailed and constructive feedback on our manuscript. In agreement with the suggestion to compare our method with separate MALDI-MSI and IMC analyses on consecutive sections, we performed a new experiment on two tissue sections. One tissue section underwent MALDI-MSI/IMC processing, and the other was subjected to IMC alone. Our examination of antibody performance in both the IMC-only and MALDI-MSI/IMC datasets revealed no significant differences. We have incorporated these findings into Supplementary Figure 2 and have updated the main text accordingly, starting at line 66.

Comment 2: Some external validation of the cell types predicted from the MSI data would also be helpful (for example by separate IHC, or from cells with known anatomical location). Also, it was not clear to me exactly how the 19 cell types were defined (L290, "cells forming visual neighborhoods were grouped to define cellular phenotypes"), the criteria used here should be stated more clearly.

In this study, cell types were identified using protein data from imaging mass cytometry (IMC), not from the MALDI-MSI data. The antibody panel we used has been previously validated and employed in various studies (PMID: 31736961, 36631610, 36442992). To further clarify how we identified different cell phenotypes, we have included Supplementary Table 2, which details the markers used for this identification. We have now also clarified this procedure (line 344)

Comment 3: Moreover, I think the authors could strengthen their case by explicitly showing the advantages gained by performing MSI + IMC on the same section, as opposed to using existing methods on consecutive sections. I would expect that the author's procedure has better co-registration, but this should be demonstrated by comparing the two approaches side-by-side. For this, some ground truth would be required, for example demonstrating that metabolites known to be specific to certain cell types are assigned correctly by the authors' approach, and that such assignment is improved when combining MSI and IMC on a single section.

We thank the Reviewer for this important suggestion. By making use of the same experiment to address the Reviewer's first comment, we compared the cell segmentation masks obtained from the MALDI-MSI/IMC section with the one from the IMC-only section. Despite the sections being consecutive and only 5 µm apart, we observed significant differences in tissue structure and cell localization. These findings are detailed in Supplementary Figures 2B & C and discussed in line 70 of the main text. Importantly, while tissue structure is relatively easy to maintain in FFPE tissue sections, the use of fresh frozen samples complicates co-registration of data derived from consecutive sections.

Comment 4: In relation to the above, and in order to evaluate the usefulness of the method for metabolism research, the authors should provide at least some metabolite identification. Could not MS/MS be used to identify some of the MS peaks with reasonable confidence? For example, can the authors identify the top features that differed between macrophages vs. monocytes in Fig 2A? Does the method pick up on known/expected differences between cell type, for example accumulation of lactate in more glycolytic cells, or nucleotides in proliferating cells? Even a few such identified peaks could help to readers better appreciate the method and its potential applications.

We appreciate the Reviewer's suggestion and recognize its importance for our methodology. Unfortunately, conducting MS/MS at a 5 µm spatial resolution is challenging in MALDI-MSI, primarily due to the high sensitivity needed for quality MS/MS spectra. However, we have conducted a new experiment on the advanced Bruker timsTOF fleX MALDI-2 platform, featuring improved mass resolution and trapped ion mobility separation (TIMS). This allowed us to identify/name 112 metabolites. For example, we discovered that CD204+ macrophages are characterized by PG(40:7) and LPI(18:1) (row 122), and a specific subset of macrophages, irrespective of their IMC phenotype, shows high abundances of various PGs (row 131). The manuscript has been updated to include these new findings, with details on the detected metabolites provided in rows 107, 122, and 131.

*** Minor points ***

Minor point 1: L104: "no clear association between the IMC and glycerophospholipid clusters, confirming the presence of metabolite variations within the same cell populations" -- this seems overstated, it may simply be that the MSIC data is noisy?

We agree with the Reviewer that this sentence was overstated. Thus, we have removed the sentence and clarified the section (starting at row 115).

Minor point 2: Fig 2DE: I'm not convinced that these are truly cell clusters, since it often happens that clustering methods "invent" clusters in noisy data. In Fig 2D the clusters are not that well separated, and could be an artefact of k-means method. How was the number of clusters K=10 picked? Can the clustering be confirmed by other methods, e.g. Louvain or Leiden clustering?

The Reviewer's concern about the potential risks of k-means clustering is well-founded. However, our new experiment yielded improved signal-to-noise ratios, enabling us to identify and name the detected metabolites more accurately. This advancement allowed for a more thorough evaluation of the clusters. For example, we observed that cluster 5 is distinguished by high levels of all phosphatidylglycerols (PGs) identified in this study. Given the biological relevance of co-abundance of these lipids, due to their role in the glycerophospholipid pathway, we believe that these clusters represent biologically significant groupings. This is further detailed in row 131 of the manuscript.

Minor point 3: L91, "separation was confirmed by a higher pairwise distance" (Fig 1E) -- are these also distances in the UMap embedding (in the 2D plane) ? If so this does not add much beyond what's already visible in Fig 1CD. It might be better to compare against the original distance matrix (the input to UMap) to assess the effect of the UMap embedding / manifold detection.

We thank the Reviewer for pointing this out and agree that it is more informative to visualise the pairwise distances in the original matrix than in the UMAP embedding. As it is a small validation, we have also moved it to the supplementary information (row 103, supplementary figure 4b).

Minor point 4: L95, "performed differential feature analysis", might be clearer to state "computed fold changes" between cancer and stromal-immune cell compartments.

We thank the Reviewer for the suggestion and have adapted this throughout the text.

Minor point 5: L112, "based on metabolite profiles" --> based on glycerophospholipid profiles
Use of Thomson (Th) units is deprecated by IUPAC, see:
https://www.degruyter.com/document/doi/10.1351/PAC-REC-06-04-06/html

We thank the Reviewer for this information and have adapted the manuscript accordingly.

** Code review **
The provided code consists of python and R scripts supplied as a zip file available on figshare. It would be preferable to use a code repository such as github / gitlab. The code does not run out-of-the-box, but I was able to get it to work after some tweaking (see below). If the codebase is intended to be used by others as a routine tool (as part of the authors' proposed method) then I think some optimization is

needed. The R script is very slow, it took 40 min to process a single m/z image on my laptop. I think this step could be done much more efficiently with a better data representation; I would suggest using numpy/pandas from python, then the R component can be eliminated entirely.

We are grateful to the Reviewer for their thorough review of our code. While the primary focus of our manuscript is to introduce a wet lab approach for combined MALDI-MSI and IMC to the scientific community, we acknowledge the importance of the computational aspect. In line with the Reviewer's suggestion, we have converted our R-script, which was used for obtaining metabolite abundances per cell, into a Python script. This change has significantly reduced the runtime, enhancing the utility of our approach. Moreover, the code will be made available on github.

Python scripts will not run as provided due to hardcoded absolute paths referring to the authors local machines, for example 'C:/TAMIM/LUMC/MSI-IMC_Integration/' and "New data/2.ROIs_normalised_only/..." Please use relative paths instead.

We have adapted the code to rely on relative paths.

The R script ("1_IMC_MSI_celltype_metabolite-abundance_integration.R") is interactive due to the file.choose() call on line 87 which requires the user to pick a mass list. I believe the file to read should be "curated-masslist.txt", and this should be a constant in the script. The script and the file curated-masslist.txt also contains hardcoded paths so does not run as provided. There are several unused dependencies, which tends to cause unnecessary trouble for users; as far as I can tell only the dplyr and stringr packages were actually needed.

Following the Reviewer's suggestion, we have cleaned up the code for improved clarity and efficiency.

Reviewer #2:

Comment 1: The authors indicated that Fig. 1D corresponded to the left UMAP of Fig. 1C. I don't see the right UMAP of Fig. 1D corresponds to the left UMAP of Fig. 1C (the shapes do not match). Please clarify the discrepancy.

We are thankful to the Reviewer for their thorough review of our manuscript and for highlighting the discrepancy in Figure 1D. We have repeated the MALDI-MSI/IMC experiments,

which also necessitated redoing the UMAP analysis. Consequently, the updated density plots that correspond to Figure 1D have been revised and are now included in Supplementary Figure 4A (row 103).

Comment 2: *I wonder why the authors tended to lump stromal cells and immune cell together in Fig. 1, when they have many IMC antibodies to distinguish different immune cell subsets. The rationale to focus on macrophages could be further clarified too. The descriptions in Results are a little vague. For example, "The analysis demonstrated no clear association between the IMC and glycerophospholipid clusters, confirming the presence of metabolite variations within the same cell populations. This phenomenon was particularly evident in macrophages, which formed clusters but also scattered throughout the embedding". I'm not sure how such conclusions were reached. At least visually plasma cells appeared to have distinct glycerophospholipid too.*

We appreciate the Reviewer's feedback and have tried to enhance the overall readability and clarity of our manuscript. To initially validate our methodology, we simplified our approach by comparing combined stromal/immune cells with cancer cells. While this simplified analysis sets the stage, our method indeed distinguishes over 20 cell types, including various immune cells, which we delve into in greater detail starting from row 115.

Regarding the previously vague and overstated sentence about the lack of clear association between IMC and glycerophospholipid clusters, we acknowledge the Reviewer's concern. We have therefore removed this sentence and have focused on improving the clarity and coherence of the relevant section, particularly from row 115 onwards. We observed that only few immune cell subsets, most clearly plasma B cells and CD204+ macrophages grouped by cell type in a UMAP generated with glycerophospholipids. With this in mind, we investigated the lipid profile in the CD204+ macrophages and found that these were characterized by PG(40:7) and LPI(18:1) (row 122). Next, as apart from these cells, little phenotype specific clustering occurred, we explored whether distinct metabolic profiles could be identified within the macrophage compartment. Indeed, different lipid clusters were found of which cluster 5 was characterised by high abundance of PG lipids (Figure 2E, Row 131).

Comment 3: *I think the authors should provide a rationale why they chose to focus on glycerophospholipids but not other metabolites. There was also limited indication of specific phospholipid species. For example, Fig. 2A and Supplementary Fig. 3B did not indicate the identities of each phospholipid (or potential identities). Overall, the descriptions in Main text are a little vague. The authors simply described that they could detect different phospholipid distribution between different cell types in a tumor tissue slide. But there were few details. Any standout metabolites in any specific cells, what are the implications (even speculations could be fine). Here, Fig. 1F showed striking difference*

between cancer cells and stromal-immune cells. It would be great if the authors can delve a little more on what is/are the metabolites enriched in cancer cells, and what is/are those enriched in stromal-immune cells (what type of stromal-immune cells?), and what are the implications.

We thank the Reviewer for this important comment and agree that this was a major limitation. As this was also pointed out by Reviewer 1 we would like to refer to our answer to comment 4 of Reviewer 1.

Reviewer #3:

Comment 1: MALDI-TOF MSI is not a suitable technology for spatial metabolomics, as it lacks resolving power and mass accuracy to unambiguously assign metabolite identities. The authors even acknowledge in the methods that their spectral processing included per pixel spectral alignment with maximum shifts of 25 ppm. MALDI-TOF/TOF on a rapiflex MS also does not permit any on-tissue MS/MS validation, since the precursor ion selector window in TOF/TOF is much too big. Consequently, the identity of all metabolites in this study is hypothetical at best. In line with this sentiment, the authors – while referring to GPL and small metabolites in some sub-Figures, avoid the naming of lipids/metabolites throughout the manuscript and rather refer to m/z-values with single-digit accuracy in critical Figures 1F or 2AB. They nevertheless refer to these m/z as "top differentiating metabolites" in the text (line 96). A high resolution MSI instrument or true on-tissue MS/MS capabilities would be required instead.

We thank the Reviewer for the thorough review and insightful comments on our manuscript. The Reviewer has highlighted a key issue regarding the limitations of the MALDI-MSI machine initially used, particularly its challenges with MS/MS due to inadequate mass selection capabilities and insufficient sensitivity for high-quality spectral generation for feature identification. In response, we conducted a new experiment using an advanced MALDI-MSI machine, the timsTOF fleX MALDI-2, equipped with microGRID and enhanced mass resolution and trapped ion mobility separation. This upgrade enabled us to successfully identify/name 112 detected metabolites. The manuscript has been updated to reflect these new findings, with detailed information on the identified metabolites now included in rows 107, 122, and 131.

Comment 2: Rapiflex MALDI-TOF does not offer true 5x5 µm2 lateral step size. The instruments today are specified for 20x20 µm2 pixels and can reliably support 10x10 µm2 spatial resolution. At 5x5 µm2 very likely there will be overlaps with neighboring pixels.

We appreciate the Reviewer highlighting the potential issue of oversampling with the rapifleX system. As mentioned earlier, we have repeated the measurements on the timsTOF

fleX MALDI-2 platform, now enhanced with microGRID technology. This system is specifically designed to measure true pixels at a 5 µm spatial resolution, effectively eliminating the risk of oversampling.

Comment 3: Referencing to relevant literature could be improved considerably: Several major technologies like UMAP are not cited; work by other teams on spatial single cell metabolomics (e.g. Rappez et al. Nat Methods. 2021 Jul;18(7):799-805 or reviews like Liu et al. Recent advances in mass spectrometry imaging of single cells. Anal Bioanal Chem. 2023 Jul;415(18):4093-4110) is not cited.

We appreciate the Reviewer for bringing these articles to our attention. Given the constraints on reference numbers in short report formats, we have carefully selected and included the suggested and newly published studies in our list of references to enrich our manuscript (starting at row 142).

Comment 4: The authors claim development of an optimized wet-lab protocol (line 40). It remains unclear though what this development really was. Just the inclusion of a formalin-fixation step? This has been used in multimodal MSI-single-label IHC work before. References 11 and 12 point to rather old papers in this respect.

The primary aim of our manuscript is to offer the scientific community an accessible approach for simultaneous imaging and integration of metabolites and protein markers on the same tissue section. While previous studies have successfully combined MALDI-MSI with IHC or IF, they often face limitations in the number of markers that can be analyzed. Our work introduces a methodology for comprehensive multiplex immunophenotyping using IMC, a technique that typically presents challenges when combined with others due to its non-amplified signal and the destructive nature of the process.

Comment 5: Judged from a biomedical perspective, for lack of metabolite IDs, this study is inconclusive: No putative cancer markers were identified in Fig. 1. More importantly, major observations in Fig. 2 remain purely descriptive and speculative. For instance, does the observed "metabolic" heterogeneity of macrophages represent different activation states (like M1 and M2), or may it be caused by technicalities? If macrophages were larger than other cells, perhaps they'd be covered by a pure macrophage MSI pixel and several other MSI pixels which to large extends represent other cell types?

While the main focus of our manuscript was different, as noted in our response to Reviewers 1 and 2, we concur with the Reviewer that identifying specific metabolites is of biomedical interest. With the successful identification of 112 metabolites in our revised study, we have been able to pinpoint the metabolites that are upregulated in cancer cells, immune/stromal cells, and in specific metabolite subsets, as detailed in rows 107, 122, and 131.

Comment 6: The mapping of IMC pixels to MSI pixels is not entirely clear. How are MSI pixels assigned if half of the corresponding IMC pixels were cell type 1 and the other half was cell type 2? This refers to the formula in line 314, but should be stated more explicitly.

To address this, we have incorporated the following additional sentences, including an example, into the methods section (line 359):

"To calculate metabolite abundance per cell, the peak intensity of the overlapping 5x5 µm MALDI-MSI pixel was assigned to each 1×1 µm IMC-pixel. The IMC cell segmentation mask and the assigned peak intensities of each 1×1 µm were combined, and the relative metabolite abundance per cell was calculated using the mean of all pixels within a cell. For instance, if a cell consists of 10 IMC pixels, and 5 of those fall into a MALDI-MSI pixel with peak intensity 1, and 5 IMC pixels, fall in a MALDI-MSI pixel with peak intensity 0, then the mean intensity of the cell is (5*1) + (5*0) / 10 = 0.5 (exemplified in Supplementary figure 3B)."

We believe that these revisions, along with Supplementary Figure 3B, will provide a clearer understanding of the calculation process.

Comment 7: . What m/z windows (corresponding to real mass accuracy) were used for the database searches against Hmdb and Lipidmaps?

Molecular annotations were based on a maximum mass error tolerance of 10 ppm and a CCS error tolerance of 5%. This has been added to the methods section (line 322).

Minor points:
Minor point 1: How can an MSI pixel contain zero cells? (line 79)

As all pixels of an image are included, areas exist where no tissue or, in for instance necrotic areas, cells are present.

Minor point 2: Why was a rather conservative S/N cutoff >15 chosen for peak picking? (line 264). How was recalibration against a list of known m/z features done? (line 265)

How was peak list filtering against the expected mass defect window done, i.e. using what software? (line 266)

Since the resubmitted version of the manuscript is based on newly acquired MALDI-MSI data, the data processing of the MALDI-MSI data has been changed substantially. This has now been described in the methodology section. In short, feature selection was done on the timsON data, and based on the T-ReX^3 4D feature finding algorithm (referenced here: https://www.nature.com/articles/s41467-019-14044-x), and as such has a different parameterization. Due to the improved feature finding, the peak list filtering was deemed unnecessary. The text has been updated to reflect these changes (line 315).

Decision Letter, first revision:

Dear Noel

Thank you for submitting your revised manuscript "Integration of Mass Cytometry and Mass Spectrometry Imaging for Spatially Resolved Single Cell Metabolic Profiling" (NMETH-BC52925B). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Methods, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements within two weeks or so. Please do not upload the final materials and make any revisions until you receive this additional information from us.

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Nature Methods offers a transparent peer review option for new original research manuscripts submitted from 17th February 2021. We encourage increased transparency in peer review by publishing the reviewer comments, author rebuttal letters and editorial decision letters if the authors agree. Such peer review material is made available as a supplementary peer review file. **Please state in the cover letter 'I wish to participate in transparent peer review' if you want to opt in, or 'I do not wish to participate in transparent peer review' if you don't.** Failure to state your preference will result in delays in accepting your manuscript for publication.

Please note: we allow redactions to authors' rebuttal and reviewer comments in the interest of confidentiality. If you are concerned about the release of confidential data, please let us know specifically what information you would like to have removed. Please note that we cannot incorporate redactions for any other reasons. Reviewer names will be published in the peer review files if the reviewer signed the comments to authors, or if reviewers explicitly agree to release their name. For more information, please refer to our FAQ page.

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described in the following link prior to acceptance:
https://www.springernature.com/gp/researchers/orcid/orcid-for-nature-research

Thank you again for your interest in Nature Methods. Please do not hesitate to contact me if you have any questions. We will be in touch again soon.

Sincerely,
Madhura

Madhura Mukhopadhyay, PhD
Senior Editor
Nature Methods

Reviewer #1 (Remarks to the Author):

In the revised version, the authors have addressed most criticisms, and I think the paper is now more mature. There are however a couple of remaining issues that the authors may want to address before publication.

To demonstrate the advantage of their method vs. performing MSI and IMC on consecutive slices, the authors presented IMC images from two consecutive slices (Suppl Fig 2BC) and argue that consecutive slices are too different to permit co-registration ("significant differences in tissue structure and cell localization even in sections just 5 µm apart"). Unfortunately Suppl Fig 2C does not seem to be correct: the legend states that the figure "is rotated to match the orientation" and mentions boxes and arrows, but the image shown does not match in orientation and no arrows or boxes are visible; perhaps there was some technical error? After rotating the figure, I found the images to be quite similar in structure, while some "scratches" can be seen in MALDI-MSI treated section when zoomed in. It is rather difficult to discern smaller distortions at this low magnification though, and the authors might want to show a zoomed-in image instead to better demonstrate their point.

I appreciate the additional experimental effort that went into identifying some of the metabolites reported in the study. As it turns out, the identified peaks are all phospholipids, which usually reflect membrane lipid composition rather than metabolism. (I think that most readers will take "metabolism" to mean the processing of central, soluble metabolites such as sugars, amino acids, nucleotides etc.) Since membrane lipids form the bulk of the presented data, I feel that the study does not really capture "metabolic heterogeneity", "metabolic processes" etc, as the authors state, but rather shows differences in membrane composition between cell types. For example, Figure 1 essentially shows that MSI can distinguish cancer cells from immune cells by their membrane composition, while immune cells (except maybe B cells) are more similar in this regard. I would suggest re-wording the text accordingly. It might also be helpful to explain in discussion whether the technique could be used to evaluate central metabolism, even though this is not covered in the present study.

Regarding the clustering analysis shown in Figures 2DE, I'm still not entirely convinced that these are actual clusters. While the heatmap in Fig 2E at first sight does seem to indicate clusters, it shows only a single value per cluster (a mean or centroid?) which makes it impossible to tell if any clusters are

truly separated. Given that the corresponding UMap does not indicate any clusters, it seems essential to show the individual cell values as columns in Fig 2E, as is commonly done in single-cell gene expression analysis. This would allow the reader to judge within-cluster variation and cluster separation.

Roland Nilsson
Karolinska Institutet

Reviewer #1 (Remarks on code availability):

I briefly reviewed the github repository which includes python code for MALDI and IMC image processing. However, it was not possible to reproduce the results using this code since the underlying image data was not available.

Reviewer #2 (Remarks to the Author):

I think the authors have addressed most of my concerns. My only remaining comment is that the authors should provide a short perspective on the current limitations and future development (the next step) on the technology, because the technology does not appear to be ready for wide use yet (but maybe on the cusp?). I would recommend its publication at Nature Methods.

Author Rebuttal, first revision:

Point-to-point response

Reviewer #1:

In the revised version, the authors have addressed most criticisms, and I think the paper is now more mature. There are however a couple of remaining issues that the authors may want to address before publication.

To demonstrate the advantage of their method vs. performing MSI and IMC on consecutive slices, the authors presented IMC images from two consecutive slices (Suppl Fig 2BC) and argue that consecutive slices are too different to permit co-registration ("significant differences in tissue structure and cell localization even in sections just 5 µm apart"). Unfortunately Suppl Fig 2C does not seem to be correct: the legend states that the figure "is rotated to match the orientation" and mentions boxes and arrows, but the image shown does not match in orientation and no arrows or boxes are visible; perhaps there was some technical error? After rotating the figure, I found the images to be quite similar in structure, while some "scratches" can be seen in MALDI-MSI treated section when zoomed in. It is rather difficult to discern smaller distortions at this low magnification though, and the authors might want to show a zoomed-in image instead to better demonstrate their point.

We would like to thank the reviewer for pointing out the discrepancy between supplementary figure 2 and the corresponding legend. We have adapted this and now included the figure containing examples of how the consecutive sections differ which may influence coregistration and interpretation.

I appreciate the additional experimental effort that went into identifying some of the metabolites reported in the study. As it turns out, the identified peaks are all phospholipids, which usually reflect membrane lipid composition rather than metabolism. (I think that most readers will take "metabolism" to mean the processing of central, soluble metabolites such as sugars, amino acids, nucleotides etc.) Since membrane lipids form the bulk of the presented data, I feel that the study does not really capture "metabolic heterogeneity", "metabolic processes" etc, as the authors state, but rather shows differences in membrane composition between cell types. For example, Figure 1 essentially shows that MSI can distinguish cancer cells from immune cells by their membrane composition, while immune cells (except maybe B cells) are more similar in this regard. I would suggest re-wording the text accordingly. It might also be helpful to explain in discussion whether the technique could be used to evaluate central metabolism, even though this is not covered in the present study.

We agree with the reviewer, as the manuscript focusses on glycerophospholipids, this should be reflected in the text. We therefore have reworded this in the main text. Furthermore, we have added a sentence to the discussion to highlight that the methodology is not restricted to glycerophospholipids but can be utilised to identify the full metabolic spectrum.

Commented [MNd(1]: You still need to harmonize whether you are going to make it about phospholipids or glycerophospholypids,

Regarding the clustering analysis shown in Figures 2DE, I'm still not entirely convinced that these are actual clusters. While the heatmap in Fig 2E at first sight does seem to indicate clusters, it shows only a single value per cluster (a mean or centroid?) which makes it impossible to tell if any clusters are truly separated. Given that the corresponding UMap does not indicate any clusters, it seems essential to show the individual cell values as columns in Fig 2E, as is commonly done in single-cell gene expression analysis. This would allow the reader to judge within-cluster variation and cluster separation.

We apologise to the reviewer for misunderstanding their comment in the first round of revisions. We agree that visualising the heatmap using individual cells is of great importance for the interpretation of the data and have thus adapted this in figure 2, extended data figure 3 and in the main text.

Reviewer #2:

I think the authors have addressed most of my concerns. My only remaining comment is that the authors should provide a short perspective on the current limitations and future development (the next step) on

the technology, because the technology does not appear to be ready for wide use yet (but maybe on the cusp?). I would recommend its publication at Nature Methods.

We thank reviewer 2 for their positive feedback. We agree with their remaining comment and have added a few sentences to the discussion to address this: “The here proposed methodology is directly applicable for the characterization of the metabolite composition of single cells in situ. This work focused on lipids, but the full metabolic spectrum can be analysed using this approach. Furthermore, to further entangle metabolic heterogeneity in specific immune cells, the provided 26 target IMC panel can be extended to over 40 cellular targets.”

Final Decision Letter:

Date:	26th Jul 24 11:18:53
Last Sent:	26th Jul 24 11:18:53
Triggered By:	Madhura Mukhopadhyay
From:	madhura.mukhopadhyay@us.nature.com
To:	n.f.de_miranda@lumc.nl
CC:	methods@us.nature.com
BCC:	rjsproduction@springernature.com
Subject:	Decision on Nature Methods submission NMETH-BC52925C
Message:	26th Jul 2024

Dear Noel,

I am pleased to inform you that your Brief Communication, "Integration of Mass Cytometry and Mass Spectrometry Imaging for Spatially Resolved Single Cell Metabolic Profiling", has now been accepted for publication in Nature Methods. The received and accepted dates will be June 23, 2023 and Jul 26, 2024. This note is intended to let you know what to expect from us over the next month or so, and to let you know where to address any further questions.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Methods style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Please feel free to contact me if you have questions about any of these points.

Best regards,
Madhura

Madhura Mukhopadhyay, PhD
Senior Editor
Nature Methods

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