

High definition spatial transcriptomic profiling of immune cell populations in colorectal cancer

Corresponding Author: Dr Sarah Taylor

Version 0:

Decision Letter:

9th Jul 2024

Dear Sarah,

Your Article, "Characterization of immune cell populations in the tumor microenvironment of colorectal cancer using high definition spatial profiling" has now been seen by 3 referees. You will see from their comments copied below that while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be very interested in considering a revised version that addresses these serious concerns.

In brief, the reviews express a spectrum of enthusiasm for publication of your work, but also identify major issues, with some notable overlap between referees.

Referee #1 is the most positive, but comments on a broad range of issues, ranging from technical questions through to the biological findings.

Reviewer #2 provides no explicit opinion on publication, but asks a number of thoughtful questions for the various technical/methodological aspects of your work.

Referee #3 is the most straightforwardly negative, highlighting a lack of strong biological novelty in your CRC analysis. That said, they do some useful suggestions to improve such, e.g. C-C analysis using the higher resolution of the HD data.

In our reading of these reviews there is a path to publication, but it will clearly require both addressing all the technical comments as well as significantly improving the biological discovery to satisfy Referee #3. We note a number of overlapping technical requests across the reviews, e.g. on the spatial accuracy check, choice of bin size, unclear justification for claiming that Visium HD data does not need a matched sc reference, etc., and agree these are important and useful suggestions that would better convey the technical advance of the HD platform. For the biological novelty, we note there is not much specific guidance, but both Referees #2 and #3 suggest that the higher-resolution is not sufficiently leveraged and that using such for a deeper characterisation of the TME would be a good demonstration of the strengths of the HD technology.

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

To guide the scope of the revisions, the editors discuss the referee reports in detail within the team, including with the chief editor, with a view to identifying key priorities that should be addressed in revision and sometimes overruling referee requests that are deemed beyond the scope of the current study. We hope that you will find the prioritised set of referee points to be useful when revising your study. Please do not hesitate to get in touch if you would like to discuss these issues further.

If you choose to revise your manuscript taking into account all reviewer and editor comments, please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If revising your manuscript:

*1) Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available here. Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary: <https://www.nature.com/documents/nr-reporting-summary.pdf>
It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.
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Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

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Thank you for the opportunity to review your work.

Sincerely,

Michael Fletcher, PhD
Senior Editor, Nature Genetics
ORCID: 0000-0003-1589-7087

Referee expertise:

Referee #1: spatial and single-cell genomics; cancer, including colorectal

Referee #2: spatial methods development

Referee #3: spatial transcriptomics; immunology

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

Michelli F. Oliveira and colleagues introduce a novel spatial transcriptomics platform Visium HD and demonstrate its application in the study of colorectal cancer (CRC). Using the Visium HD platform, the authors conducted high-resolution spatial gene expression analysis of CRC and adjacent normal mucosal tissues. The results indicate that Visium HD can identify fine gene expression clusters that correspond to the morphological features of colon tissue, providing a more accurate reflection of cellular heterogeneity within the tissue compared to the Visium v2 platform. The study found two pro-tumor macrophage subpopulations with distinct gene expression profiles at the tumor periphery and precisely mapped these immune cells and their interactions with tumor cells through high-resolution spatial analysis. Additionally, combining single-

cell RNA sequencing and Xenium in situ gene expression techniques, the study further revealed the spatial distribution of clonally expanded T cells within the tumor microenvironment (TME).

The significance of this study lies in the Visium HD platform's high resolution and sensitivity, which offer a new tool for studying cell types and their interactions within the tumor microenvironment. By creating more detailed maps of cell distribution within tissues, researchers can gain a deeper understanding of tumor heterogeneity and the complexity of the immune microenvironment. This is crucial for developing new therapeutic strategies and prognostic markers.

This research is highly significant, marking the first application of the Visium HD technology and providing a basis for subsequent studies. However, the following comments were suggested to be addressed before publication.

1. In this study, the authors used 8µm bins for analysis. What were the criteria or considerations for selecting 2µm, 8µm, and 16µm bins?
2. In lines 147-148, how do the authors estimate the fraction of molecules arising from gDNA in Visium v2 and Visium HD? How to remove the probes that bind to gDNA?
3. Are the quantifications in Figure 2E goblet cell genes? Because of the regular morphology of colon glands, the authors need to consider that other marker genes also determine the spatial accuracy of mRNA detection, such as genes specifically expressed in tertiary lymph nodes.
4. Visium HD barcoded 2 x 2 µm squares. How were these cells in the single-cell scale obtained? How do the authors assign squares to cells?
5. In the method section "spot deconvolution", the authors briefly explain the use of Spacex, but it is unclear whether they deconvolute one cell type per 8 µm bin or how they do it. In addition, how do the 25 clusters in Figure 3A correspond to the cell annotations in Figure 3B?
6. The authors claimed that based on the Visium HD data alone and deconvolution using single-cell data is not required. How did the author come to this conclusion? Since the single-cell profiles have great ability to obtain the subtypes of CD8 T cells, B cells, or others, How is the performance of the Visium HD in the identification of these subtypes?
7. To reduce computational complexity and time while retaining important statistical and biological features, the authors employed a sketch-based analysis method, sampling 15% of the entire dataset. How was the 15% sampling rate determined? What considerations and selection criteria were involved in this process?
8. In the methods section "Characterizing the tumor periphery," it is mentioned that the Visium HD technology is superior to Visium v2 in tumor boundary analysis. Could the authors provide more detailed information on how the tumor periphery regions were identified and analyzed using the Visium HD platform?
9. The previous studies in spatial transcriptomics of CRC (Qi et al. Nature Communications 13 (1), 1742; Xun et al Nature Communications 14 (1), 933) have reported that SPP1+ macrophages enriched around the tumor periphery (boundary) while FOLR2+ macrophages in the stromal region or non-malignant region. Do the authors identify the FOLR2+ macrophages in Visium HD? In addition, can the high resolution of the Visium HD reveal new macrophage subsets in the slides? What are the characteristics of macrophages in the tumor area?
10. The Figure 5D show feature plot for TGFBI, REG1A, and REG1B. The authors need to quantify the expression of these genes between these two macrophage subsets.
11. It is difficult to quantify immune cells, especially T cells, in Visium, but Visium HD makes up for this deficiency. In Figure 6B, the macrophages and T cells appear to be in close proximity. Is there a crosstalk between the macrophages and T cells?
12. This study selected colorectal cancer (CRC) as the research subject for the Visium HD technology, yielding many interesting results consistent with previous literature. What impacts might arise when using the Visium HD technology on other types of cancers or tissues?
13. While the advantages of the Visium HD technology are already evident, how might this technology drive new research directions? What significant implications and value might it have for disease research and clinical applications? The authors should further discuss this in the discussion section.

Reviewer #2:

Remarks to the Author:

This manuscript utilizes the Visium HD platform to conduct spatial transcriptomics on colorectal cancer (CRC) tissues and adjacent normal mucosal tissues from formalin-fixed paraffin-embedded (FFPE) samples. The major innovation lies in the enhanced resolution of the Visium HD platform, allowing for the identification of distinct immune cell populations and their spatial distribution within the tumor microenvironment (TME). This study highlights the ability to identify pro-tumor macrophage subpopulations and clonally expanded T cells, validated through independent spatial technologies. However, there are several critical issues that need to be addressed to strengthen the manuscript.

1. Correlation Between Visium HD and Visium V2: The manuscript reports a correlation of approximately 0.8 between serial sections analyzed by Visium HD and Visium V2, whereas typical correlations for serial sections are usually above 0.9 (e.g., 0.96 for Decoder-seq). The author attributed this discrepancy to the off-target effects of probes used in Visium HD. Indeed, only considering probes spanning exon-exon junctions increased correlation from 0.82 to 0.92, indicating the substantial impact of probe specificity. This issue is particularly evident in genes where cross-exon probe design is not feasible, thus potentially skewing cell type analysis and gene quantification. Moreover, despite using adjacent sections, significant differences (4% VS 0.5% UMI from gDNA) in off-target probe effects between Visium HD and Visium V2 further suggest probe-related problems under specific conditions (line 148). A more thorough discussion and potential solutions to mitigate these off-target effects should be provided.

2. Data on Visium HD performance: The manuscript would benefit from presenting more detailed performance metrics of Visium HD, such as the number of genes or unique molecular identifiers (UMIs) detected per square or bin. Additionally, comparing these metrics with other high-resolution methods like Stereo-seq or Seq-scope could enhance the manuscript's

appeal and provide a clearer benchmark for readers.

3. More evidence on spatial accuracy: The evidence of spatial accuracy for Visium HD, based on the number of transcripts of marker genes in and around colon gland regions, is not entirely convincing. Since the colon gland size far exceeds the size of individual spots (2 μm) and bins (8 μm), more robust evidence is needed. This could include total UMI distribution or detailed comparisons of specific gene expressions with staining images. The claim that Visium HD controls analyte transfer through a controlled environment (line 160) is not substantiated by the provided reference. Clarifying this mechanism is crucial for understanding the advantages of Visium HD in high-resolution RNA capture.

4. Cell type identification without single-cell data: While the authors claim that cell types can be identified using Visium HD data alone (line 215), the resolution, even at the bin level, still contains multiple cell types (Figure 3C). Although clustering information reflects cell distribution to some extent, the current evidence does not fully resolve the tissue's cellular composition without single-cell data. If, as the authors claim, it is possible to identify cell types solely with Visium HD data, then by jointly clustering different samples and removing batch effects, uniform labels can be assigned and different samples could be compared without the need for single-cell data, as well (line 217).

5. Definition of tumor peripheral region: The authors selected 50 μm as the tumor peripheral region but did not justify why this distance was chosen over others, such as 100 μm . Different boundary selections could affect the analysis of cell composition and interactions, as seen with B cells, which are abundant in single-cell data (Supplemental Figure 2A) but scarcely mentioned at the tumor boundary. A discussion on the rationale for this choice and its implications is necessary.

6. Expression of REG1A in P2 CRC: In Figure 5D, REG1A is identified as a key gene in tumor cells near SELENOP+ macrophages but is highly expressed in some goblet cells (not in all goblet cells like PIGR). Is there any explanation for this, such as whether these cells exhibit cancerous features or other distinctive characteristics from other goblet cells not expressing REG1A?

7. Limitations of bin-based analysis: The authors mentioned that macrophages, being larger cells, can occupy a single bin, whereas T cells require assignment based on nuclei segmentations. This suggests limitations in using bins for cell type delineation within the TME. Integrating single-cell data from the same tissue batch and leveraging the 2 μm resolution of Visium HD for more precise cell type assignment could provide a more accurate representation of the TME.

8. The labeling of subregions (C/D/E/F) in Figure 7A and 7B should be checked for accuracy. Additionally, line 363 mentions SELENOP+/STAB1+ macrophages near REG1A+ tumor cells (Figures 7C, 7D), but REG1A+ tumor cells are not visible in Figure 7D. These inconsistencies need to be addressed.

Reviewer #3:

Remarks to the Author:

In this manuscript, Oliveira et al. generated spatial transcriptomics datasets in human colorectal tissue using Visium HD, which reduces the capture area size to 2x2 μm . The capture areas are continuous without any gaps. Using visium HD, the authors showed there is no need for deconvolution (as compared to visium v2) and the presence of different macrophage subgroups and T cells around/within the tumors. Compared to previous methods, this spatial transcriptomics approach represents a significant improvement and holds great potential for application.

My main concern is the lack of novelty, this manuscript provided, in CRC biology. I feel that most of the paper focused on the new technology, compared to the previous Visium v2 platform, and validated using Xenium. Thus, this manuscript would fit better in a method or resource-specialized journal rather than Nature Genetics. Moreover, the computational analysis is underdeveloped and to be considered for publication in Nature Genetics, I believe this article should include further analysis to better characterize the tumor microenvironment.

Major comments

1. The cell type annotation for Visium HD, similar to other spatial transcriptomics methods, is based on deconvolution. The 8x8 μm resolution used in the analysis is close to the size of a cell. However, the clustering results of the ST data and the deconvolution results do not correspond well, with only the main cell types matching. Deconvolution can identify more detailed cell types (Figure 3c). What is the reason for this discrepancy? The ability to deconvolute specific cell types indicates that their markers are expressed within the spots. Why can't the ST data alone detect them? Additionally, if single-cell data from specific samples are used as a reference, will the deconvolution results correspond one-to-one with the ST data? Will there be significant differences between deconvolution results based on Visium v2 data and those based on Visium HD data?

2. Like the previous comment, why CD3e and TRAC do not label the same "cells"? have the authors show other CD3 genes? The localization of CD4 and CD8 T cells restricted to the tumors might indicate low resolution in detecting T cells as they are supposed to be in the lamina propria and epithelial compartment. How does the detection of CD4 and CD8 T cells perform compared to confocal staining?

3. The authors mention that deconvolution is used due to heterogeneity between samples. Why can single-cell data be integrated, but ST gene expression data cannot? How significant are the differences in ST data between samples? Can we assess the differences arising from individual variation versus those from sequencing technology?

4. The manuscript lacks data for P5 NAT. Why is that? Supplementary Figure 1 shows that, compared to P2 CRC, the Visium HD data for P5 NAT has more detected transcripts than the Visium v2 data. Why does this phenomenon occur?

5. Although each sample has a specific tumor type identified using deconvolution results, a certain tumor type within one

sample corresponds to multiple clusters identified at the ST level. What are the differences between these clusters? Is there a spatial relationship, such as some clusters being inside the tumor and others outside?

6. The phenomenon in Figure 5 is only evident in the P1 sample. In P2, the two macrophage types have relatively consistent localization, and TGFB is widely expressed. In P3, there is only one subtype. Therefore, this conclusion may not be universally applicable and should be validated with more single-cell and spatial transcriptomics data.

7. To highlight the advantages of this new method, it would be beneficial to perform cell communication analyses. Compared to previous methods, the spatial positioning of cells with this new method is more accurate.

8. The emphasis should be on what can only be achieved with this new method, and what conclusions can only be discovered through its application, while similar analyses with other data fail to reveal these insights.

Minor comments

1. In Figure 2c, it is mentioned that Visium HD can cluster more classes compared to Visium v2. Is this due to Visium HD detecting more accurately, or because it contains significantly more spots than Visium v2, affecting the algorithm?

2. In Supplementary Figure 2, only 9 cell types are listed under level 1, whereas the article mentions 10. The group of cells labeled "qc filtered" should not be referred to as cell types since they have been filtered out.

3. The labels for P1 and P2 in Figure 4 are reversed.

Version 1:

Decision Letter:

28th Jan 2025

Dear Sarah,

Your Article, "Characterization of immune cell populations in the tumor microenvironment of colorectal cancer using high definition spatial profiling" has now been seen by the original 3 referees. You will see from their comments below that while they think your work has improved, some important points still remain unresolved. We continue to be interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

Briefly, there are still a few major requests that - to our reading - may require additional analysis.

Reviewer #3 is satisfied and supports publication.

Referee #1 appreciates the revision but still has a few minor points.

Finally, Referee #2 is similarly supportive but has some more substantive requests; we note that they overlap with some of Reviewer #1's comments (on, for example, deeper characterisation of the macrophage subtypes).

In our reading we think these new requests are not unduly onerous and we encourage you and your team to respond fully. We also acknowledge that the ST field is a very fast-moving one and that behooves all involved to get this work out sooner rather than later; hence, while we would not require new experiments or further data generation (and we do not think the referees are asking for that either), we would suggest that more and deeper computational analyses will go long way to satisfying those last two referees.

To guide the scope of the revisions, the editors discuss the referee reports in detail within the team, including with the chief editor, with a view to identifying key priorities that should be addressed in revision and sometimes overruling referee requests that are deemed beyond the scope of the current study. We hope that you will find the prioritized set of referee points to be useful when revising your study. Please do not hesitate to get in touch if you would like to discuss these issues further.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

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We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Michael Fletcher, PhD
Senior Editor, Nature Genetics
ORCID: 0000-0003-1589-7087

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed the reviewer's comments effectively, but a few minor points still require clarification.

1) The authors have defined CD8 cytotoxic T cells (Fig. 3B) in three samples, without other CD8 T cells. Should there not be a broader examination of the potential presence of other CD8 T cell subsets, especially exhausted T cells which tend to enrich tumor tissue? Knowing the specific localization of CD8 cytotoxic T cells would be helpful.

2) There is no comparison between the spatial location and function of SPP1+ macrophages and SELENOP+ macrophages. Additionally, is there any relationship between their spatial distribution and patient clinical characteristics that could be explored?

3) The authors describe interactions between two types of macrophages and tumor or T cells in the revised manuscript. However, an important question remains: Can the interaction between macrophages and CD8 cytotoxic T cells potentially inhibit T cell cytotoxicity?

Reviewer #2 (Remarks to the Author):

The authors have made substantial improvements to the manuscript, successfully addressing most of the concerns raised in my initial review. The inclusion of detailed analyses has significantly enhanced the depth and rigor of the work, particularly in elucidating immune cell interactions within the tumor microenvironment. However, a few aspects warrant further attention to solidify the conclusions and maximize the manuscript's impact.

1. Supplementary Table 1 highlights that Visium HD exhibits approximately half the sensitivity (measured by captured UMI counts) of Visium V2 after normalizing for sequencing depth, spot area, and tissue size. While this reduced sensitivity may not critically affect cell population clustering, it could influence the ability to detect rare cell types and their spatial distribution in the TME (line 111).
2. The revised discussion of probe off-target binding is appreciated. However, two questions remain: (1) Given that genomic DNA (gDNA) is double-stranded, what mechanisms enable single-stranded probes in Visium V2 to bind to gDNA at the reported rate (~5%)? (2) How do the authors experimentally or computationally distinguish probe binding to gDNA from binding to unspliced, immature mRNA? Clarifying these points would strengthen confidence in the spatial transcriptomic data.
3. The new analysis of macrophage interactions with other cells (Fig. 5E) is compelling. In P2CRC, there are interactions between the two macrophage subtypes (SPP1+ and SELENOP+), with some SELENOP+ macrophages also expressing SPP1 (Fig. 4C) and not observed in other samples, raises questions about context-specific macrophage interactions or transitions. How do the authors interpret this observation? Additionally, exploring how macrophage interactions shape distinct TME types and influence tumor progression would add further value to the manuscript.
4. Given the high resolution of Visium HD, the authors could leverage spatial data to identify key factors (e.g., ligand-receptor pairs, metabolic gradients) driving macrophage transitions between pro- and anti-tumor states. Integrating these spatial insights with single-cell RNA-seq datasets might uncover mechanisms underlying macrophage reprogramming during interactions with T cells or stromal cells.
5. While the findings, such as tumorigenic goblet cells and clonally expanded T cells, are novel, the small cohort size and inter-sample heterogeneity (e.g., in macrophage subpopulations) limit broad conclusions. The authors may discuss these limitations and interpret the results with cautions.

Reviewer #3 (Remarks to the Author):

I want to thank the authors for addressing my concerns. I believe this manuscript is suitable for publication in its present form.

Version 2:

Decision Letter:

Our ref: NG-A65724R1

26th Feb 2025

Dear Sarah,

Thank you for submitting your revised manuscript "Characterization of immune cell populations in the tumor microenvironment of colorectal cancer using high definition spatial profiling" (NG-A65724R1). 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 Genetics, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

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

Thank you again for your interest in Nature Genetics. Please do not hesitate to contact me if you have any questions.

Sincerely,

Michael Fletcher, PhD
Senior Editor, Nature Genetics
ORCID: 0000-0003-1589-7087

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed the reviewer's comments. This manuscript is suitable for publication. Congratulations

Reviewer #2 (Remarks to the Author):

All my concerns have been properly addressed. I'd like to recommend it to be published. Congrats!

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Reviewer #1 (spatial and single-cell genomics; cancer, including colorectal) :

Remarks to the Author:

Michelli F. Oliveira and colleagues introduce a novel spatial transcriptomics platform Visium HD and demonstrate its application in the study of colorectal cancer (CRC). Using the Visium HD platform, the authors conducted high-resolution spatial gene expression analysis of CRC and adjacent normal mucosal tissues. The results indicate that Visium HD can identify fine gene expression clusters that correspond to the morphological features of colon tissue, providing a more accurate reflection of cellular heterogeneity within the tissue compared to the Visium v2 platform. The study found two pro-tumor macrophage subpopulations with distinct gene expression profiles at the tumor periphery and precisely mapped these immune cells and their interactions with tumor cells through high-resolution spatial analysis. Additionally, combining single-cell RNA sequencing and Xenium in situ gene expression techniques, the study further revealed the spatial distribution of clonally expanded T cells within the tumor microenvironment (TME).

The significance of this study lies in the Visium HD platform's high resolution and sensitivity, which offer a new tool for studying cell types and their interactions within the tumor microenvironment. By creating more detailed maps of cell distribution within tissues, researchers can gain a deeper understanding of tumor heterogeneity and the complexity of the immune microenvironment. This is crucial for developing new therapeutic strategies and prognostic markers.

This research is highly significant, marking the first application of the Visium HD technology and providing a basis for subsequent studies. However, the following comments were suggested to be addressed before publication:

We appreciate the feedback and acknowledgement that our work is highly significant in the field. We have addressed the comments below inline and made the relevant updates to the manuscript and figures.

1. In this study, the authors used 8µm bins for analysis. What were the criteria or considerations for selecting 2µm, 8µm, and 16µm bins?

For the majority of our analyses we used the 8 x 8 µm bin size (hereafter, 8µm data). Our rationale is that at this bin size, the data are rich enough for us to make robust cell type annotations, and the data set (specifically, the number of barcodes in the gene/barcode matrix) is of a reasonable size and therefore can be easily processed through third party data analysis tools. From a biological perspective, 8 µm bins are small enough to provide information at single cell scale for most cell types. However, the 2 x 2 µm data (hereafter, 2µm data) provide additive information, particularly when investigating smaller cell types. Indeed, we found the 2 µm data to be critical for accurate localization and classification of T and B cells, which are smaller than macrophages and often colocalize with other cell types in the 8 µm data. Conversely, for other regions of the tissue (e.g. in regions of low cellularity and low transcript density), using the data

binned to 8 μm can be more informative. We did not use 16 μm bins in this study as we would expect to lose some of the resolution advantages afforded by the Visium HD technology. Based on these points, we suggest 8 μm binned data as a starting point for analysis, as it provides sufficient information about a sample with a low compute load, and then further investigations can be performed with the 2 μm data, as exemplified in our study. The benefits of using the 2 μm data have already been demonstrated in a recent preprint: <https://www.biorxiv.org/content/10.1101/2024.06.19.599766v1>. We acknowledge that this information is important and have edited the manuscript in the Results (lines 219-225) and Methods (lines 497-503; 514-516) sections to clarify our bin size selection criteria for these analyses.

2. In lines 147-148, how do the authors estimate the fraction of molecules arising from gDNA in Visium v2 and Visium HD? How to remove the probes that bind to gDNA?

Typically, the total amount of UMIs potentially derived from gDNA is below 5%, which has been shown to have minimal impact on clustering, differential expression, and biological interpretation. To estimate the fraction of molecules arising from gDNA in the Visium assays, the 10x Genomics team has provided a technical note (Visium CytAssist Spatial Gene Expression for FFPE: Robust Data Analysis with Minimal Impact of Genomic DNA, CG000605), available [here](#). For the sensitivity analysis between Visium v2 and Visium HD in this manuscript, probes that are marked as potentially binding to gDNA were excluded from the analysis computationally.

We have added additional context to the manuscript where the reviewer pointed this out (Results, lines 112-117), and we have added the calculations used in the model to the Methods section (lines 551-570):

Briefly, the gDNA estimate is obtained by fitting a piecewise linear model to genes with both spliced and unspliced probes. The model predicts the log-transformed gene counts for unspliced probes as a function of log-transformed gene counts for spliced probes and log-transformed estimated gDNA counts per gene per 1,000 spots under tissue:

$$x_{\text{unspliced}} = \text{gDNA} + B(x_{\text{spliced}} - \hat{X}_{\text{gDNA}}) \text{II}(x_{\text{spliced}} > \hat{X}_{\text{gDNA}})$$

where:

- $\hat{X}_{\text{gDNA}}$ is the estimated gDNA UMIs per gene,
- $x_{\text{unspliced}}$ and x_{spliced} are the average number of UMIs corresponding to unspliced and spliced probes from a single gene, respectively,
- $\text{II}(x_{\text{spliced}} > \hat{X}_{\text{gDNA}})$ is an indicator function that equals one if x_{spliced} is greater than $\hat{X}_{\text{gDNA}}$, and zero otherwise.

For any fixed estimate of $\hat{X}_{\text{gDNA}}$, the model parameters gDNA and B are estimated by fitting a linear regression with a two-dimensional parameter vector. The relevant $\hat{X}_{\text{gDNA}}$ values to

consider while fitting the model are only those X_{spliced} values seen in the genes under consideration. Therefore, a linear model with every X_{spliced} value seen in the dataset as $\hat{X}_{\text{gDNA}}$ is fit, and the model with the smallest residual sum of squares is used for the estimates.

3. Are the quantifications in Figure 2E goblet cell genes? Because of the regular morphology of colon glands, the authors need to consider that other marker genes also determine the spatial accuracy of mRNA detection, such as genes specifically expressed in tertiary lymph nodes.

Yes, the quantifications in Figure 2E are based on genes that are known to be localized within glands of normal colon mucosal tissue (goblet cell gene markers: *CLCA1*, *FCGBP*, *MUC2*) (Results, lines 128-137, and Figure legend for Figure 2E). To provide additional examples for evaluation of the spatial accuracy of mRNA detection, we included analysis for tertiary lymph nodes, as suggested by the reviewer, and muscularis mucosae regions, shown by the new Supplementary Figure 2. We selected immune cell marker genes (*MS4A1*, *CD52*, *CXCR4*) known to be enriched in lymph nodes, and muscularis mucosae marker genes (*ACTA2*, *DES*, *GREM1*). Within these tissue structures, we defined three regions of interest (ROIs) for each tissue analyzed: lymph nodes (source mask) and adjacent submucosa (adjacent mask), and muscularis mucosae (source mask) and adjacent mucosa (adjacent mask) and then quantified transcript localization as described in our Methods section. In summary, we show a consistently high percentage of transcripts localized within the source mask for these structures, ranging from 93.3 to 98.1% across tissue samples evaluated.

4. Visium HD barcoded $2 \times 2 \mu\text{m}$ squares. How were these cells in the single-cell scale obtained? How do the authors assign squares to cells?

There are different approaches that can be taken to assign the squares to cells. To illustrate this and clarify different approaches that can be taken, we added a new Figure 6B to the manuscript (also shown below), by replacing the previous panel 6B (now shown in Supplemental Figure 9). We used the nuclei staining from a high-resolution H&E image to partition barcodes into $2 \times 2 \mu\text{m}$ bins that overlay a specific nuclei to that cell. The data then mimics single cell like data since the gene counts will now be reported on a per-nuclei basis (Figure 6B, right panel). In addition to including this to Figure 6, we have also clarified these different approaches in the text (Methods, lines 657-670).

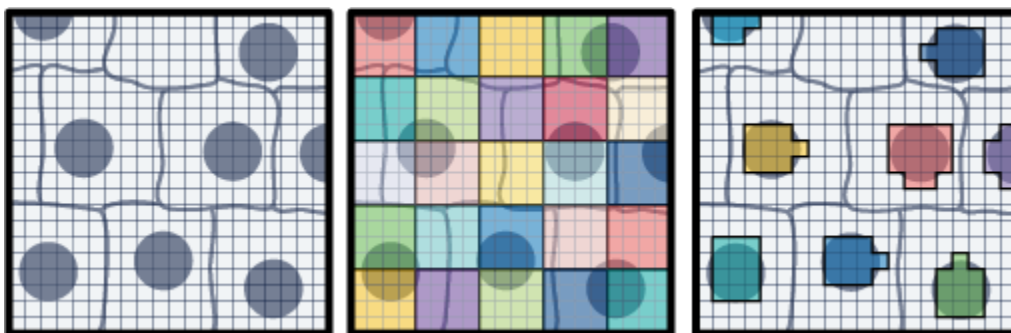


Figure 6B: Schematic representation of the two approaches for binning the 2 x 2 μm barcoded squares in Visium HD data. The capture area of a Visium HD slide is a continuous grid of barcodes (left panel). The Space Ranger pipeline by default will create square bins (shown here as 8 x 8 μm squares of a single color) that tile the entire tissue containing capture area (middle panel). This is the cell assignment method used for the 8 x 8 μm binned data. An alternative is to use the nuclei stain from a high-resolution H&E microscope image to group together barcodes that underlie the same cell nuclei based on the 2 x 2 μm data (right panel).

5. In the method section “spot deconvolution”, the authors briefly explain the use of Spacexr, but it is unclear whether they deconvolute one cell type per 8 μm bin or how they do it. In addition, how do the 25 clusters in Figure 3A correspond to the cell annotations in Figure 3B?

Deconvolution methods aim to infer the cell types and their relative proportions contributing to the signal captured in a spot, square or bin. These methods are usually paired with single cell gene expression data to be used as a reference that holds the different cell types to be assigned. For this study, we used spacexr⁴⁶ using doublet mode, which assigns 1-2 cell types per spot and is recommended for technologies with high spatial resolution such as Visium HD. Among the outputs, this mode provides the top 2 ranked cell types per spot, a matrix with the weights for all cell types in the reference in each of the spots, and a variable representing the class of every bin. The available classes are: “singlet” (1 cell type), “doublet_certain” (2 cell types), “doublet_uncertain” (2 cell types, but only confident of 1), “reject” (no prediction given). See the spacexr vignette for more details: <https://raw.githubusercontent.com/dmccable/spacexr/master/vignettes/spatial-transcriptomics.html>).

We have updated Figure 3 for clarity. We profiled three CRC samples using Visium HD and combined them into a single dataset by adopting a sketch analysis (Figure 3A). In Figure 3B, we show the deconvolution results and Figure 3C integrates these findings by displaying a heat map that shows the proportion of each unsupervised cluster (rows) within the different cell types identified through deconvolution (columns). These proportions are calculated per row, ensuring that the values in each row sum to 100%. These results are described in lines 140-161 and we have edited Methods, lines 503-513 for clarity.

6. The authors claimed that based on the Visium HD data alone and deconvolution using single-cell data is not required. How did the author come to this conclusion? Since the single-cell profiles have great ability to obtain the subtypes of CD8 T cells, B cells, or others, How is the performance of the Visium HD in the identification of these subtypes?

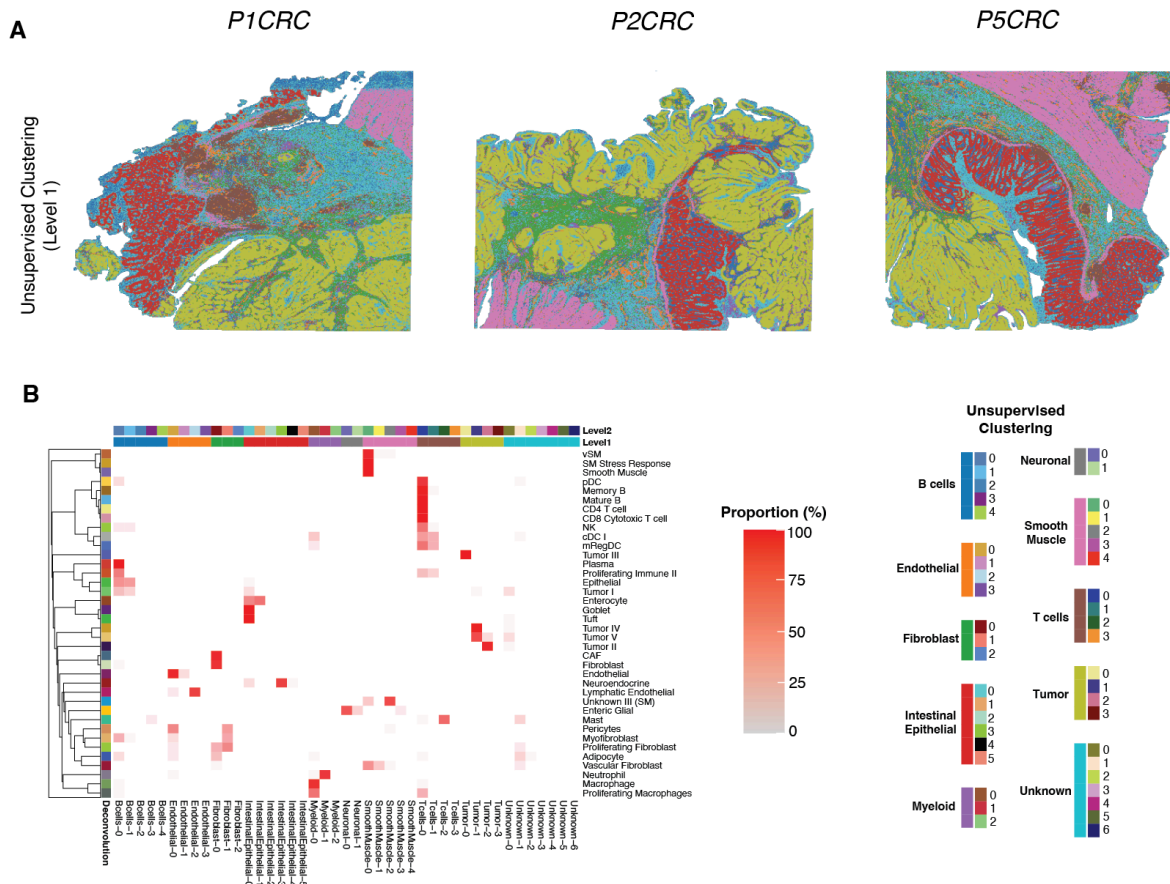
The reviewer raises a good point that we agree needs more clarification in the manuscript. To address this and really get at how the different data types compare, we applied the same Seurat sketch analysis method used for single-cell analysis to the Visium HD data.

To do this, we combined the three Visium HD sample datasets (P1CRC, P2CRC, and P5CRC) into a single dataset. Using a similar approach to the single-cell analysis, we utilized 15% of the

dataset for the sketched approach implemented in Seurat v5. Our initial clustering analysis of the combined dataset identified 23 clusters, which we then consolidated into 9 cell types (Tumor, Intestinal Epithelial, Endothelial, Smooth Muscle, T cells, Fibroblast, B cells, Myeloid, Neuronal) and 1 unknown cluster. Notably, each patient sample displayed a specific tumor cluster, consistent with findings from the single-cell data. We then mapped these clusters spatially and observed a similar cell type organization as compared to the deconvolution results. Next, we performed sub-clustering for each of the identified cell types. This process yielded 3 to 6 clusters per cell type. We found that some cell types, such as enterocytes, goblet cells, macrophages, neutrophils, tumor cells, and neuroendocrine cells, could be distinctly identified. However, one T cell cluster contained a mixture of cell types when compared to the deconvolution results and included both CD4 and CD8 T cells.

These results reiterate that cell types with a clear morphological organization (e.g., Goblet cells and enterocytes in colon glands) are easily distinguishable with Visium HD data. However, identifying subtypes of cells with a more random organization or very small cells, like B and T cell subtypes, may require different methods - this could be integration with single cell data or cell segmentation approaches based on cell membrane staining. Our Analysis confirms the point we make in the manuscript that while broad cell typing is feasible with Visium HD data, additional methods are needed to identify some cell subtypes.

We have included a new Supplementary Figure 6 and modified Figure 3 to highlight these findings and have modified the text in the revised manuscript (Results, lines 140-161 and Discussion, lines 324-332) to ensure that it is clear that some small or densely packed cell types may not be completely distinguishable using Visium HD data alone at this time and we note that improvements in cell segmentation approaches for this data type may alleviate this in the future.



Supplemental Figure 6: Sketch based analysis of Visium HD data without the integration of single cell data. A. Spatial plots with bins colored by cell type identified level 1 unsupervised clustering. Scale bar = 1mm. B. Heatmap from confusion matrix showing the relationship between cell labels provided via deconvolution (rows) and unsupervised clusters (columns). Heatmap is scaled by row, summing up to 100% per row. Longer color bars represent level 1 cell type annotations and small colored squares represent the level 2 clusters identified for each level 1 cell type.

7. To reduce computational complexity and time while retaining important statistical and biological features, the authors employed a sketch-based analysis method, sampling 15% of the entire dataset. How was the 15% sampling rate determined? What considerations and selection criteria were involved in this process?

The 15% sampling rate was chosen to ensure efficient computational performance. This decision was mainly driven by the goal of keeping every dataset to less than 100,000 bins. We did not establish specific criteria for selecting bins; instead, we utilized the Seurat package with Leverage score as the sketching method. Seurat calculates a score for each barcode, representing the magnitude of its contribution to the gene-covariance matrix and its importance

to the overall dataset. This process often results in the oversampling of rare populations, thereby preserving the biological complexity of the sample. For more information on sketching approaches in single-cell analysis, refer to Hie et al., 2019 and Hao et al., 2022.

We have added additional information on our sketch analysis and cited Hie et al., 2019 (Methods, lines 506-513).

8. In the methods section “Characterizing the tumor periphery,” it is mentioned that the Visium HD technology is superior to Visium v2 in tumor boundary analysis. Could the authors provide more detailed information on how the tumor periphery regions were identified and analyzed using the Visium HD platform?

To identify and analyze tumor periphery regions using the Visium HD platform, we developed custom computational pipelines (which we have already made public as part of the published GitHub repository that was made available with this manuscript: https://github.com/10XGenomics/HumanColonCancer_VisiumHD). The algorithm primarily uses two parameters: 1) cell type/cluster to find the periphery, and 2) distance.

The process begins by selecting all bins labeled with the given cluster or cell type. For each bin, the algorithm identifies all neighboring bins within a user-defined radius. In our study, we selected all bins labeled as tumor and used a radius of 50 μm . To accurately delineate the boundary of the cluster or cell type, the algorithm excludes bins with the same label as the initial bin.

To further refine the boundary identification, we remove singlets—bins labeled with the given cluster but not in proximity to other bins with the same label. Specifically, we exclude bins that have fewer than 25 neighboring bins with the same label. This ensures the selection of well-defined regions within the tissue.

The key advantage of this method is its versatility; it is not restricted to tumors and can be applied to identify cell types in the boundaries of other tissues, such as colon glands or skeletal muscle tissue.

This method's reliance on precise distance measurements (i.e. 50 μm) highlights a significant limitation of the Visium v2 technology, where the closest spot would exceed this distance, making it less suitable for detailed boundary analysis.

We have included more detail to the main text to clarify how we did this analysis (Results, lines 167-172) and a detailed description in the Methods section (lines 603-615).

9. The previous studies in spatial transcriptomics of CRC (Qi et al. Nature Communications 13 (1), 1742; Xun et al Nature Communications 14 (1), 933) have reported that SPP1+ macrophages enriched around the tumor periphery (boundary) while FOLR2+ macrophages in

the stromal region or non-malignant region. Do the authors identify the FOLR2+ macrophages in Visium HD? In addition, can the high resolution of the Visium HD reveal new macrophage subsets in the slides?

Our original analysis was focused on understanding the cell populations around the tumor periphery. Therefore, to address this question we have now performed additional analysis. We have added a description of our findings (outlined below) to the main text (Results, lines 201-206; Methods, lines 616-627) and added Supplemental Figure 7.

To more deeply analyze the macrophage populations in our samples, we combined all the bins labeled as macrophages by the deconvolution algorithm across the entire tissue of three different patients. This would allow us to a) have greater resolving power across all patients and b) detect if any of the subpopulations we identified are found in all patients or if they are patient specific. We then used Seurat to obtain the unsupervised clusters. Once the clusters were segregated, we identified differentially expressed markers to label these subpopulations in the same way we previously performed the analysis that identified the *SPP1*⁺ and *SELENOP*⁺ macrophages in the tumor periphery.

This new analysis revealed four different macrophage subpopulations. Interestingly, *SPP1* expression was restricted to Macrophage_2, while *SELENOP* was expressed in the other three subpopulations (Macrophage_0, Macrophage_1 and Macrophage_3). We also identified other genes as markers for these subpopulations and indeed, *FOLR2* was identified as a marker for Macrophage_3, however it was expressed in a low proportion of the subpopulation. In order to better understand the spatial distribution of these subpopulations, we quantified the proportion assigned to the tumor periphery and the rest of the tissue. This analysis revealed that the *FOLR2*⁺ macrophages (Macrophage_3) were almost (>98%) exclusive to bins > 50 microns from the tumor. This finding clarifies that we did not previously identify this subpopulation as it was outside the region of the tumor periphery that our analysis was focused on. We then mapped these macrophages subpopulations to their spatial context across samples and observed that *FOLR2*⁺ macrophages were only found in the P5CRC sample. However, other subpopulations could be located in the stromal/healthy regions of the sections.

While this analysis allowed us to better define macrophages throughout the entire tissue samples, our results still indicate that in the tumor periphery (tumor area) there are two macrophage subpopulations (*SPP1*⁺ and *SELENOP*⁺) as we show in Figure 5.

What are the characteristics of macrophages in the tumor area?

The characteristics of the macrophages in the tumor area remain as described, the two *SELENOP*⁺ and *SPP1*⁺ macrophages dominate in the tumor periphery and both display some pro-tumor characteristics. We cover this in more detail in response to the question on cell-cell communication below.

10. The Figure 5D show feature plot for *TGFBI*, *REG1A*, and *REG1B*. The authors need to quantify the expression of these genes between these two macrophage subsets.

In the original Figure 5D, we showed the expression of *TGFBI*, *REG1A*, and *REG1B* in the **tumor** regions that are adjacent to the different macrophage subsets, rather than in the macrophage subsets themselves. The normalized log UMI counts for these genes in the three patient samples are now shown in Figure 5B. We revised Figure 5 legend accordingly to ensure this is clear.

11. It is difficult to quantify immune cells, especially T cells, in Visium, but Visium HD makes up for this deficiency. In Figure 6B, the macrophages and T cells appear to be in close proximity. Is there a crosstalk between the macrophages and T cells?

This is a great point that we thank the reviewer for raising and we have looked into this extensively. As we note in the manuscript, in the tumor microenvironment of the CRC samples we studied, both *SPP1*⁺ and *SELENOP*⁺ macrophages may play crucial roles in modulating immune responses and supporting tumor progression through distinct yet complementary mechanisms. To assess cell-cell communication between the two macrophages subpopulations and the neighboring tumor or T cells, we used LIANA (Dimitrov et al. 2022), which provides a framework to combine cell communication methods to infer ligand-receptor interactions. We selected all bins within the previously defined tumor periphery region (within 50 μ m from the tumor boundary) to create an independent Seurat object to be used as input for LIANA. We filtered the predicted interactions by selecting any interaction with `aggregate_rank` ≤ 0.01 . This procedure was done on a per-patient basis. The results of this new analysis were added in Figure 5E and 5F (also shown below) and in lines 207-213.

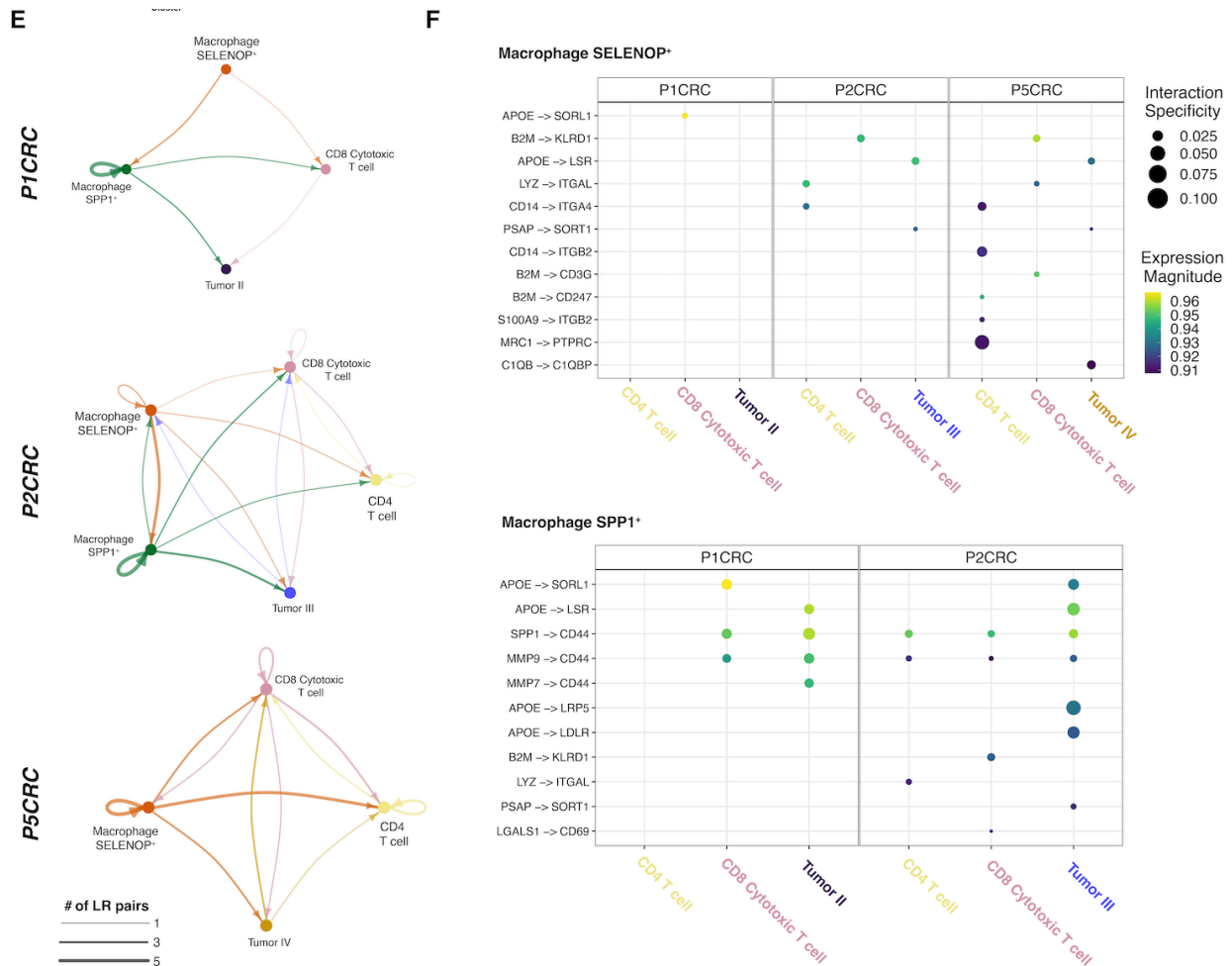


Figure 5. Cell-cell communication analysis of immune cells in the tumor microenvironment of the CRC samples. **E** Interaction networks of macrophages, T cells and tumor cells in the boundary regions (50um). Nodes represent cell types and edges represent the number of significant ligand receptor pairs. **F**. Dotplot representing the expression and specificity of ligand-receptor pairs for SPP1⁺ and SELENOP⁺ macrophages as sources.

12. This study selected colorectal cancer (CRC) as the research subject for the Visium HD technology, yielding many interesting results consistent with previous literature. What impacts might arise when using the Visium HD technology on other types of cancers or tissues?

We chose to work with CRC samples because of its high relevance for public health and because the morphological structures of colon tissue makes it a great tissue model to highlight the potential of high resolution spatial transcriptomics methods, such as Visium HD technology. However, Visium HD was developed on a wide range of sample types in both mouse and human in order to support discoveries that are relevant to diverse fields of research and

applications. We have made several of these [datasets publicly available on the 10x Genomics website](http://www.10xgenomics.com/datasets) (www.10xgenomics.com/datasets), including:

- Mouse Kidney FFPE
- Mouse Embryo FFPE
- Mouse Brain FFPE
- Mouse Small Intestine FFPE
- Human Pancreas FFPE
- Human Lung Cancer FFPE with IF staining
- Human Colorectal Cancer FFPE (the datasets that accompany this manuscript)

These tissue samples yielded high quality data and are supported in Visium HD. We have not seen consistent issues with any specific tissue type, but we have observed that in highly cellularly dense tissues, segmentation of cells and assignment of transcripts is more challenging than in more cellularly sparse tissues. In addition, we have seen (as with any spatial technology of this nature), tissues with highly abundant extracellular matrices are more prone to detachment from the slides.

13. While the advantages of the Visium HD technology are already evident, how might this technology drive new research directions? What significant implications and value might it have for disease research and clinical applications? The authors should further discuss this in the discussion section.

We thank the reviewer for raising this point. We agree that addressing it adds value, and provokes thoughts on the reality of what these technologies may enable in the future of research and clinical practice. We have significantly reworked the discussion to consider the points raised.

Reviewer #2 (spatial methods development):

Remarks to the Author:

This manuscript utilizes the Visium HD platform to conduct spatial transcriptomics on colorectal cancer (CRC) tissues and adjacent normal mucosal tissues from formalin-fixed paraffin-embedded (FFPE) samples. The major innovation lies in the enhanced resolution of the Visium HD platform, allowing for the identification of distinct immune cell populations and their spatial distribution within the tumor microenvironment (TME). This study highlights the ability to identify pro-tumor macrophage subpopulations and clonally expanded T cells, validated through independent spatial technologies. However, there are several critical issues that need to be addressed to strengthen the manuscript.

1. Correlation Between Visium HD and Visium V2: The manuscript reports a correlation of approximately 0.8 between serial sections analyzed by Visium HD and Visium V2, whereas typical correlations for serial sections are usually above 0.9 (e.g., 0.96 for Decoder-seq). The

author attributed this discrepancy to the off-target effects of probes used in Visium HD. Indeed, only considering probes spanning exon-exon junctions increased correlation from 0.82 to 0.92, indicating the substantial impact of probe specificity. This issue is particularly evident in genes where cross-exon probe design is not feasible, thus potentially skewing cell type analysis and gene quantification. Moreover, despite using adjacent sections, significant differences (4% VS 0.5% UMI from gDNA) in off-target probe effects between Visium HD and Visium V2 further suggest probe-related problems under specific conditions (line 148). A more thorough discussion and potential solutions to mitigate these off-target effects should be provided.

We appreciate the Reviewer's comments on correlations between Visium HD and Visium V2 and will address these individually below.

- The manuscript reports a correlation of approximately 0.8 between serial sections analyzed by Visium HD and Visium V2, whereas typical correlations for serial sections are usually above 0.9 (e.g., 0.96 for Decoder-seq). The author attributed this discrepancy to the off-target effects of probes used in Visium HD.

While Visium HD and Visium V2 do share a common mixture of probes, they are fundamentally different assays, based on different biochemistries, slides, workflows, and analysis pipelines. Given that the capture probes in each technology are manufactured and placed on the slides via different methods, a correlation of $R^2 \sim 0.8 - 0.9$ (exon-exon junctions spanning probes only) is good. We see similar correlations when comparing Visium assays to Chromium single-cell assays.

Importantly, when we test serial sections using the same Visium technology we see R^2 correlations well above 0.95, typically close to 0.99 (shown in Figure 13A of the Visium HD Spatial Gene Expression Performance Technical Note (CG000686, RevA): <https://www.10xgenomics.com/support/spatial-gene-expression-hd/documentation/steps/library-construction/visium-hd-spatial-gene-expression-performance>). We have added this reference to the Methods section of the manuscript, line 495.

- Moreover, despite using adjacent sections, significant differences (4% VS 0.5% UMI from gDNA) in off-target probe effects between Visium HD and Visium V2 further suggest probe-related problems under specific conditions (line 148). A more thorough discussion and potential solutions to mitigate these off-target effects should be provided.

These differences also can be attributed to the fundamental differences in the Visium assays. As we developed Visium HD, we optimized the assay's chemistry to perform on the new Visium HD arrays, which resulted in an observed decrease in the number of reads estimated to originate from off-target binding to gDNA. With regard to the difference that could be attributed to gDNA, probes that are marked as potentially binding to gDNA can be excluded from the analysis computationally. This point is similar to Reviewer 1's second comment, and to address it we have added information to the manuscript on how to account for potential gDNA and additional explanation in the Methods section (lines 540-570).

2. Data on Visium HD performance: The manuscript would benefit from presenting more detailed performance metrics of Visium HD, such as the number of genes or unique molecular identifiers (UMIs) detected per square or bin. Additionally, comparing these metrics with other high-resolution methods like Stereo-seq or Seq-scope could enhance the manuscript's appeal and provide a clearer benchmark for readers.

We agree with the reviewer that presenting the suggested metrics may benefit the readers and have now added Supplemental Table 1, which includes these metrics for the samples we profiled.

Supplemental Table 1. Visium metric comparison

	P1CRC	P2CRC	P2CRC	P5CRC	P3NAT	P3NAT	P5NAT	P5NAT
Assay	Visium HD	Visium HD	Visium V2	Visium HD	Visium HD	Visium V2	Visium HD	Visium V2
Number of Reads	862,469 ,618	721,219 ,716	1,584,7 28,978	926,026 ,426	831,879 ,560	326,070 ,358	1,103,4 73,733	359,834 ,785
Mean reads per 8 μ m bin	1698.8	1321.1	-	1708.6	1,706	-	2,532	-
Mean read per spot	-	-	371,218	-	-	83,887		136,404
Reads per μ m ²	26.54	20.64	42.86	26.69	26.65	9.68	39.56	15.75
Number of 8 μ m binned squares under tissue	507,684	545,943	-	541,968	487,552	-	435,773	-
Number of spots under tissue	-	-	4,269	-	-	3,887	-	2,638
Fraction of 8 μ m bins under tissue	72.30%	77.70%	-	77.20%	69.40%	-	62.10%	-
Fraction of spots under tissue	-	-	85.51%	-	-	77.86%		52.84%
Mean UMIs per 8 μ m bin	207.4	488.4	-	441.4	177	-	298	-
Mean UMIs per spot	-	-	94,824	-	-	9,695	-	14,176
Mean Genes per 8 μ m bin	161.5	393.2	-	310.7	125	-	218	-
Mean Genes per spot	-	-	10,149	-	-	2,518	-	4,693
Total genes detected	18,055	18,072	18,073	18,058	18,063	18,073	18,059	18,049

<i>Estimated UMIs from gDNA</i>	0.60%	0.50%	2.90%	0.60%	0.50%	4.10%	1.30%	5.40%
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However, comparison with other available technologies is beyond the scope of this manuscript. A true comparison would only be possible with serial sections of the same samples, which are no longer available to us. Furthermore we are unable to access technologies from BGI or modify Illumina consumables as would be required to set up Seq-Scope. However, we do note that several other recent studies have published relevant comparisons (using older Visium technologies). These include:

- [A comprehensive benchmarking with practical guidelines for cellular deconvolution of spatial transcriptomics. Li et al. 2023](#)
- [Systematic comparison of sequencing-based spatial transcriptomic methods. You et al. 2024](#)
- [Comparison of spatial transcriptomics technologies across six cancer types. Cervilla et al. 2024](#)
- [Comparison of spatial transcriptomics technologies using tumor cryosections. Rademacher et al. 2024](#)

3. More evidence on spatial accuracy: The evidence of spatial accuracy for Visium HD, based on the number of transcripts of marker genes in and around colon gland regions, is not entirely convincing. Since the colon gland size far exceeds the size of individual spots (2 μm) and bins (8 μm), more robust evidence is needed. This could include total UMI distribution or detailed comparisons of specific gene expressions with staining images.

In Figure 2E, we have evaluated transcript localization accuracy of genes that are known to be specific to cells of colon glands. This analysis considers transcripts that are outside delineated morphological regions as “mislocalized”. The delineations of Source Masks are performed on the high resolution HE stained images using QuPath and are drawn agnostic from the gene expression data. We agree with the reviewer that the delineation of a morphological structure will not perfectly align with the boundaries of some “spots” or “bins” when aligned with the Visium HD gene expression data. Therefore, both Source masks or Adjacent masks may have UMI contributions from spots or bins around the boundaries. Despite this limitation, we have observed a high proportion of transcripts were localized within their specific location in colon glands. In response to reviewer 1’s comment #3, we also provided additional examples using different sets of morphological structures and their specific genes (tertiary lymph nodes and muscularis mucosae) that showed a consistent high proportion of transcript accuracy results, which are now included as Supplementary Figure 2. Below, we also provide total UMI distribution of specific gene markers for muscle (*ACTA2*, *DES*, *GREM1*), colon glands (*CLCA1*, *FCGBP*, *MUC2*) and tumor (*CEACAM5*, *CEACAM6*, *EPCAM*) in three selected regions of a CRC sample, highlighting expression specificity of the given gene markers to their morphologic regions.

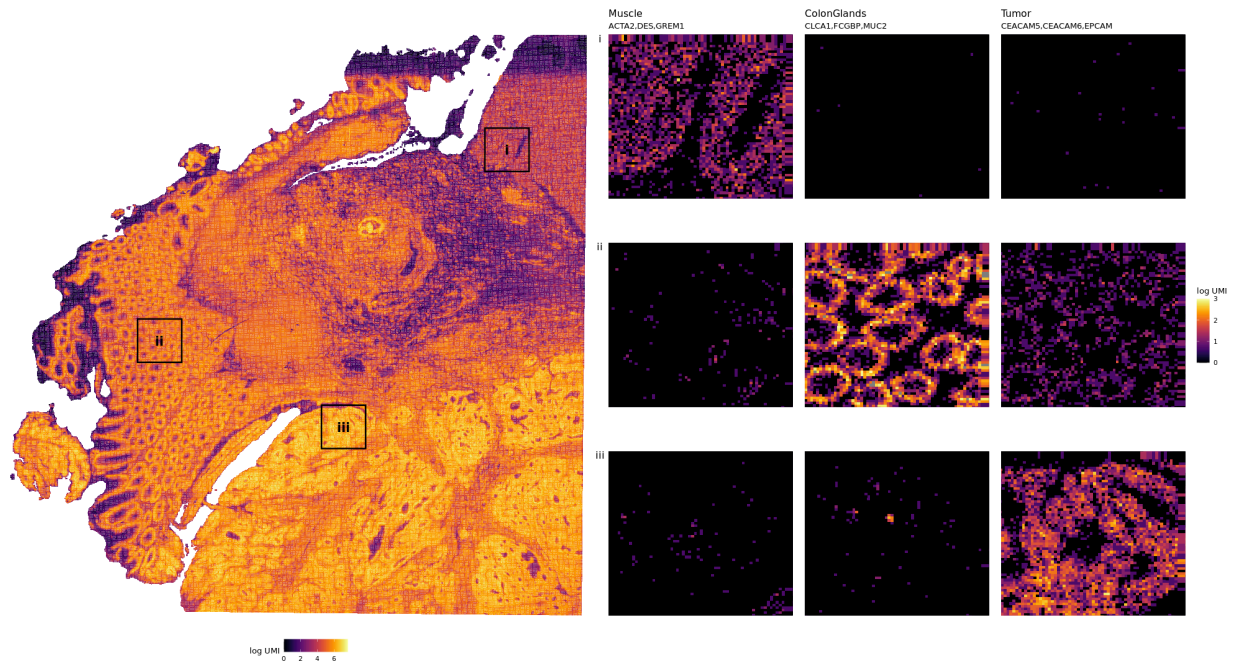


Figure. UMI distribution in Visium HD across FFPE section. (left) P1CRC section with bins colored by total log UMI counts per 8um bins. (right) Zoom in on specific regions in the section with bins colored by log UMI counts of specific cell type markers (column).

- The claim that Visium HD controls analyte transfer through a controlled environment (line 160) is not substantiated by the provided reference. Clarifying this mechanism is crucial for understanding the advantages of Visium HD in high-resolution RNA capture.

The Visium CytAssist instrument is a proprietary instrument developed by 10x Genomics that enables NGS based spatial transcriptomics assays from tissue sections placed on regular glass slides. Due to its proprietary nature, not all aspects of how the CytAssist operates are disclosed publicly. However, from first principles we can derive why the CytAssist in combination with Visium HD provides advantages regarding high-resolution RNA capture.

1. In the CytAssist configuration, target molecules released from the surface of the tissue are captured by the Visium HD capture oligos as they are released from the tissue. In contrast, when using a placing tissue directly onto slides without using this controlled transfer, there is an open solution reservoir on one side (i.e., above) of the tissue, target molecules released from the tissue are free to diffuse in all directions with some target molecules being released into this reservoir and away from the capture probes of the capture area.
2. When the tissue slide and Visium HD slide are paired together in the CytAssist instrument, this creates a configuration that substantially reduces flow during target molecule release and capture by the capture probes. In direct placement workflows, flow is not tightly controlled due to the open solution reservoir.

3. The Visium HD slide is designed to maximize resolution having no gaps or spacing between barcoded features. In barcoded arrays with gaps between features, target molecules may interact with an area of the barcoded array that contains no capture oligonucleotides and the target molecules remain free to migrate until they are captured at a distal region having capture oligos.

We do also have a Technical Note that demonstrates the higher spatial fidelity of Visium v2 using CytAssist vs. Visium v1 using direct tissue placement: <https://www.10xgenomics.com/support/cytassist-spatial-gene-expression/documentation/steps/library-construction/visium-cytassist-spatial-gene-expression-for-ffpe-reagents-workflow-data-comparison>).

We have added a more detailed explanation of the CytAssist to the main text (Results, lines 124-128) and referenced the technical note in the manuscript.

4. Cell type identification without single-cell data: While the authors claim that cell types can be identified using Visium HD data alone (line 215), the resolution, even at the bin level, still contains multiple cell types (Figure 3C). Although clustering information reflects cell distribution to some extent, the current evidence does not fully resolve the tissue's cellular composition without single-cell data. If, as the authors claim, it is possible to identify cell types solely with Visium HD data, then by jointly clustering different samples and removing batch effects, uniform labels can be assigned and different samples could be compared without the need for single-cell data, as well (line 217).

The reviewer raises a similar point to Reviewer 1 in comment #6, and we provide a detailed response in line above and have addressed this point in the manuscript with the addition of Supplemental Figure 6, highlighting the results if we run the sketch analysis alone on the Visium HD data.

Batch effects are challenging to correct for since there are no well established methods that specifically address these effects in spatial transcriptomics data. While there are good tools for single cell data sets these are not sufficient for spatial data since spatial transcriptomics datasets intrinsically contain additional variables to be accounted for such as the number of cells in each bin/spot and the varying cell type mixtures within these bins/spots. In our study we accounted for as much technical variation as possible by sequencing samples to the same depth and running all samples in parallel.

We have explored existing single cell batch correction methods like Harmony for Visium v1 (<https://www.10xgenomics.com/analysis-guides/correcting-batch-effects-in-visium-data>) and found the results to be useful. However, due to the increased data complexity in Visium HD a thorough investigation into batch effects would be required and would likely require the development of new computational tools that are beyond the scope of this manuscript.

5. Definition of tumor peripheral region: The authors selected 50 μm as the tumor peripheral region but did not justify why this distance was chosen over others, such as 100 μm . Different boundary selections could affect the analysis of cell composition and interactions, as seen with B cells, which are abundant in single-cell data (Supplemental Figure 2A) but scarcely mentioned at the tumor boundary. A discussion on the rationale for this choice and its implications is necessary.

In this analysis we wanted to characterize the cell types immediately adjacent to the tumor boundary. We reasoned that by setting a threshold of 50 μm , we would be capturing a layer no more than a few cells thick around the tumor so we would be able to draw clear conclusions about the cells that are directly in the interface of the tumor and thus allow us to gain better insights into the tumor dynamics. A boundary analysis with larger distance from the tumor would start to include cells that were multiple layers of cells away from the tumor and thus having a less direct impact of tumor progression or control.

We also wanted to fully exploit the high resolution of Visium HD. A 50 μm boundary analysis is impossible with Visium v2, which has a spot size of 55 μm .

We have added an explanation of the biological rationale to the main text (Results, lines 169 - 171), and commented on the technological reasons for this in the discussion (lines 608 - 610).

6. Expression of REG1A in P2 CRC: In Figure 5D, REG1A is identified as a key gene in tumor cells near SELENOP⁺ macrophages but is highly expressed in some goblet cells (not in all goblet cells like PIGR). Is there any explanation for this, such as whether these cells exhibit cancerous features or other distinctive characteristics from other goblet cells not expressing REG1A?

This is a great question that we dug into and found some very interesting results. We performed granular sub-clustering analysis of all goblet cells which revealed a distinct subpopulation expressing *REG1A* (Figure 5 C-D). This subpopulation exhibited features of both goblet cells and *REG1A*⁺ tumor cells. While these *REG1A*⁺ cells expressed lower levels of goblet cell markers like *CLCA1*, *FCGBP*, and *MUC2*, they also exhibited *REG1A* tumor-associated features such as *REG1A*, *REG1B*, *DMBT1*, and *OLFM4*. This goblet subpopulation localized at the normal-tumor tissue border of P1CRC and P2CRC and may represent a transitional state of tumorigenesis. We have edited the manuscript to include these findings (lines 194-200 and 294-301).

7. Limitations of bin-based analysis: The authors mentioned that macrophages, being larger cells, can occupy a single bin, whereas T cells require assignment based on nuclei segmentations. This suggests limitations in using bins for cell type delineation within the TME. Integrating single-cell data from the same tissue batch and leveraging the 2 μm resolution of

Visium HD for more precise cell type assignment could provide a more accurate representation of the TME.

This comment is similar to Reviewer 1, comment #6. We agree that there are limits to bin-based analysis and that for truly accurate cell type assignment a robust cell segmentation approach is needed, which is not currently available for Visium HD. While we demonstrate that segmentation based on nuclei and transcript assignment is possible, this is not trivial and requires significant compute power. We have added to the discussion around this point (Discussion, lines 324-332).

We have also expanded on the section where we discuss the requirement for single cell data, to articulate where single cell data does add value. While we do not find single cell data necessary for high level cell typing, it is useful in the case described here where the cells are smaller than the bins (lines 156-161).

8. The labeling of subregions (C/D/E/F) in Figure 7A and 7B should be checked for accuracy. Additionally, line 363 mentions *SELENOP*+/*STAB1*+ macrophages near *REG1A*+ tumor cells (Figures 7C, 7D), but *REG1A*+ tumor cells are not visible in Figure 7D. These inconsistencies need to be addressed.

Thank you for highlighting these errors, we have fixed the subregion labeling and corrected Figure 7D. As discussed in comment #6, we identified a subset of goblet cells that exhibited elevated levels of *REG1A*, *REG1B*, *DMBT1*, and *OLFM4*, features of *REG1A*+ tumors, suggesting a transitional state of tumorigenesis. These cells were localized in microenvironments with a higher density of *SELENOP*+/*STAB1*+ macrophages (Figure 4D). Figure 7D has been updated to show *SELENOP*+/*STAB1*+ macrophages near *REG1A*+ cells.

Reviewer #3 (spatial transcriptomics; immunology):

Remarks to the Author:

In this manuscript, Oliveira et al. generated spatial transcriptomics datasets in human colorectal tissue using Visium HD, which reduces the capture area size to 2x2 μm . The capture areas are continuous without any gaps. Using visium HD, the authors showed there is no need for deconvolution (as compared to visium v2) and the presence of different macrophage subgroups and T cells around/within the tumors. Compared to previous methods, this spatial transcriptomics approach represents a significant improvement and holds great potential for application.

My main concern is the lack of novelty, this manuscript provided, in CRC biology. I feel that most of the paper focused on the new technology, compared to the previous Visium v2 platform, and validated using Xenium. Thus, this manuscript would fit better in a method or resource-specialized journal rather than Nature Genetics. Moreover, the computational analysis is underdeveloped and to be considered for publication in Nature Genetics, I believe this article should include further analysis to better characterize the tumor microenvironment.

We appreciate the reviewers' comments and have significantly revised the manuscript by including new sets of analysis. We further identified new macrophage subpopulations across whole tissue, while we confirmed that the tumor boundaries were enriched by *SPP1*⁺ and *SELENOP*⁺ macrophage populations. Additionally, we have included a cell-to-cell communication analysis to gain insights on how these *SPP1*⁺ and *SELENOP*⁺ macrophage populations in the TME interact with adjacent tumor and T cells.

Major comments

1. The cell type annotation for Visium HD, similar to other spatial transcriptomics methods, is based on deconvolution. The 8x8 µm resolution used in the analysis is close to the size of a cell. However, the clustering results of the ST data and the deconvolution results do not correspond well, with only the main cell types matching. Deconvolution can identify more detailed cell types (Figure 3c). What is the reason for this discrepancy? The ability to deconvolute specific cell types indicates that their markers are expressed within the spots. Why can't the ST data alone detect them?

The reviewer raises a similar point to Reviewer 1 in comment #6. We provide a detailed response in line above and have added a new figure (Supplemental Figure 6) highlighting the results when run the analysis on the Visium HD data without using deconvolution to address this point in the manuscript (Results, lines 156-161). In summary, this new analysis revealed that broader cell types can be easily identified in Visium HD data, but more granular observations require more in depth and custom analyses. With deconvolution methods, we aim to assess the contribution of a given transcriptomic signature (i.e. a cell type) to the signal obtained in a single bin. As this process is done individually for every bin in the assay, it is possible to find less prominent cell types in the sample. On the other hand, standard analysis pipelines aim to find sets of observations (i.e. bins) that have a similar signature and cluster them together. If due to the spatial organization of the tissue, a set of cell types are mixed together (i.e. B and T cells), clustering will tend to group the cell types in a single cluster thus making them not individually identifiable; while deconvolution will be able to identify specific bins with larger contributions of either cell type. As new computational analysis pipelines to analyze array-based spatial data are developed and cell-segmentation approaches become available, we expect that more granular clustering will be improved.

Additionally, if single-cell data from specific samples are used as a reference, will the deconvolution results correspond one-to-one with the ST data?

As mentioned by the reviewer, deconvolution methods heavily depend on the selected reference. For our study, we created a specific dataset that combines tumor and healthy sections in order to have a full representation of the different cell types found both in healthy and tumoral colon samples from the same set of patients. However, subsetting the reference to a specific sample would not guarantee one-to-one correspondence between the single cell and spatial data because: 1. single cell and spatial data are generated from serial sections, which

implies a different set of cells being sampled across the FFPE block and 2. there is a 1:n relationship between bins and cells. The heatmap in Supplemental Figure 6B only includes bins classified as “singlets” (the deconvolution algorithm is able to identify only one cell type) and it excludes all the multiplet bins. This also affects the 1:1 correspondence with single cell data.

Will there be significant differences between deconvolution results based on Visium v2 data and those based on Visium HD data?

To address this question, we performed deconvolution on the Visium v2 data from a serial section of the same patient’s block (P2CRC) for comparison. While we observed similar results regarding the spatial localization of cell types by both assays, with Visium HD we are now able to accurately localize cell types in the different morphological structures of the tissue, due to its increased resolution. As in the example below, goblet cells are accurately located within the colon glands by both assays; however, while Visium v2 locates the cells in the correct position, it is unable to resolve the structure of the glands.

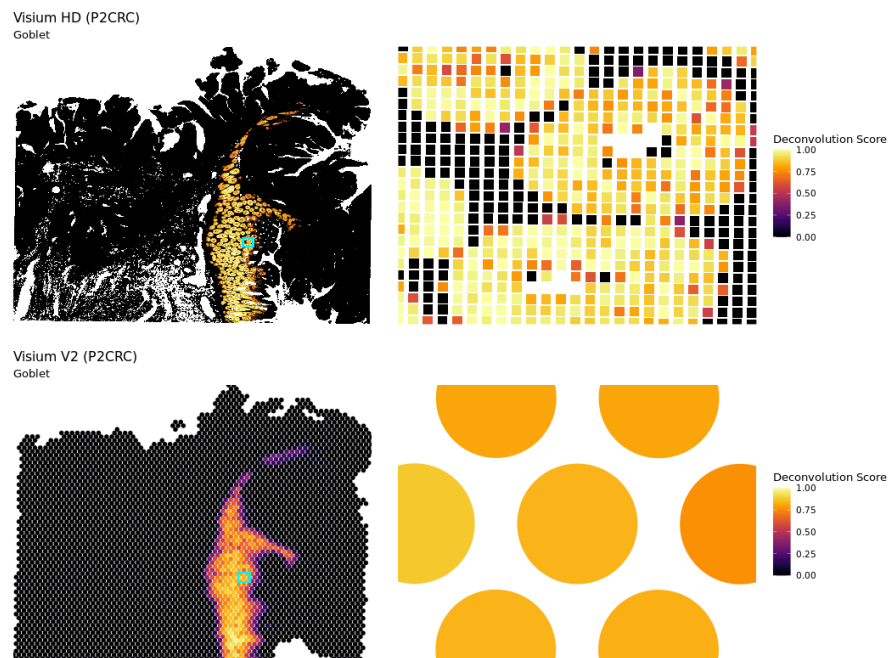


Figure. Deconvolution comparison for goblet cells between Visium HD and Visium V2. Top left: Deconvolution score for goblet cells in P2CRC section in Visium HD. Top right: 200 micron zoom in region in colon glands, with 8um bins colored by goblet cell deconvolution score. Bottom left: Deconvolution score for goblet cells in P2CRC section in Visium V2. Bottom right: 200 micron zoom in region in colon glands, with spots colored by goblet cell deconvolution score.

2. Like the previous comment, why CD3e and TRAC do not label the same “cells”? have the authors show other CD3 genes?

We appreciate the reviewer's insightful comment. In response, we expanded our analysis to include additional CD3 genes (*CD3D*, *CD3E*, and *CD3G*) as well as a set of TRA-related genes (*TRAC*, *TRBC1*, and *TRBC2*). We examined the coexpression of all possible pairs from these two gene sets. This analysis revealed an increase in the number of nuclei exhibiting coexpression (920 compared to 172 nuclei). However, the detection of genes from the TRA gene set was more frequent than those from the CD3 gene set (15,397 nuclei versus 3,257 nuclei).

Further analysis indicated that the overall detection of *CD3* transcripts is relatively low, resulting in a limited probability $P(\text{TRAC} > 0 \ \& \ \text{CD3E} > 0) = 1.75\text{e-}03$ of detecting both *CD3E* and *TRAC* molecules within the same 8 micron bin. In contrast, single-cell data showed higher detection rates for these molecules, yielding an increased coexpression probability $P(\text{TRAC} > 0 \ \& \ \text{CD3E} > 0) = 0.05412916$. We suspect that this discrepancy is related to sensitivity differences between the platforms.

To validate this observation, we subsampled the single-cell UMIs to match the capture efficiency of Visium HD and Flex platforms so that $P(\text{TRAC} > 0)$ and $P(\text{CD3E} > 0)$ were similar across platforms. Under these adjusted conditions, the co-expression probability in single-cell data also decreased $P(\text{TRAC} > 0 \ \& \ \text{CD3E} > 0) = 1.87\text{e-}03$, aligning with our observations in spatial data.

Additionally, *CD3E* is a lowly expressed gene, which further impacts co-expression detection. In Visium HD, the mean UMI count per nuclei bin for *CD3E* is low (0.004339107), and while it is also low in single-cell data (0.1107654), it is still comparatively higher than in Visium HD. This overall low expression level highlights the challenge of detecting lowly expressed genes and reinforces our observation of reduced coexpression probabilities in Visium HD compared to single-cell data. We have summarized the findings in the table below (P1CRC).

	Flex	Flex (Downsampled*)	Visium HD (8um)	Visium HD (2um)	Visium HD (Nuclei bin)
Mean CD3E UMIs per barcode	1.11e-01	6.88e-03	7.21e-03	4.51e-04	4.34e-03
$P(\text{TRAC} \ \& \ \text{CD3E} > 0)$	5.41e-02	1.87e-03	1.75e-03	1.54e-05	5.95e-04
$P(\text{CD3E} > 0)$	7.20e-02	6.40e-03	6.93e-03	4.48e-04	4.26e-03
$P(\text{TRAC} > 0)$	1.39e-01	3.55e-02	3.47e-02	2.51e-03	2.27e-02

* Flex UMI count matrix was downsampled based on Visium HD (8um) probabilities

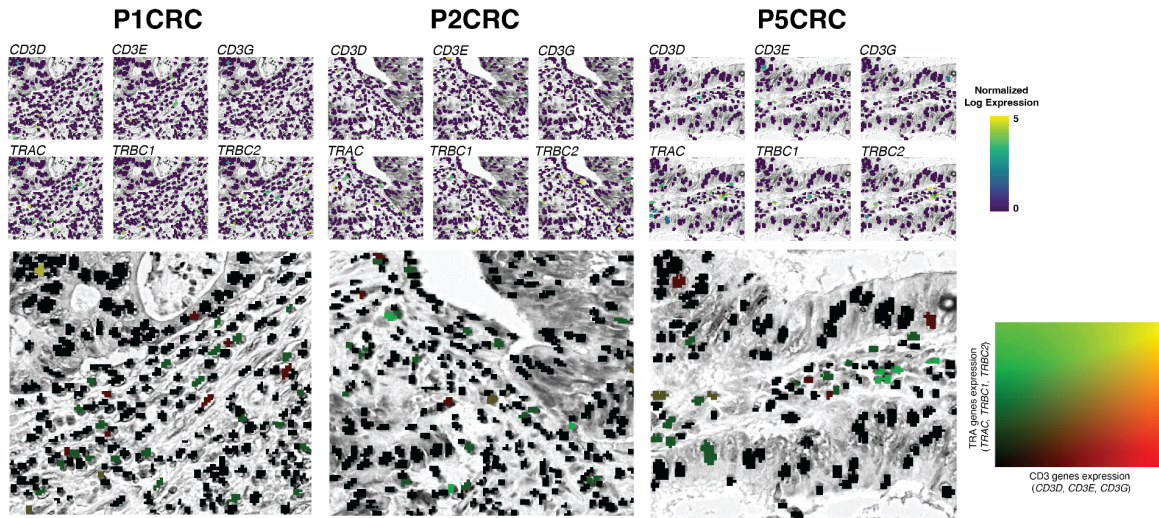


Figure. Coexpression analysis of *CD3* and *TRA* genes in Visium HD CRC sections. For each patient (P1CRC, P2CRC, P5CRC) we show 2 micron bins grouped by nuclei and colored by the expression of *CD3D*, *CD3E*, *CD3G*, *TRAC*, *TRBC1* and *TRBC2*. (Bottom) Nuclei colored by coexpression of *CD3* and *TRA* molecules.

The localization of CD4 and CD8 T cells restricted to the tumors might indicate low resolution in detecting T cells as they are supposed to be in the lamina propria and epithelial compartment. How does the detection of CD4 and CD8 T cells perform compared to confocal staining?

The density maps in original Figure 6 (included now in the new Supplemental Figure 9) focus on T cells within the tumor periphery, within a 50 μm radius from the tumor. When we look at the lamina propria and the epithelial compartment we do see T cells distributed throughout the tissue as expected. We were not able to perform confocal microscopy due to sample limitations but used our Xenium data (which is a high resolution microscopy based technology rather than a sequencing based one) from the same samples and compared the data.

As shown below, *CD3D*, *CD3E*, *CD3G*, *CD4*, and *CD8A* genes were indeed detected in the lamina propria. We observed a similar expression pattern in the Visium HD samples as we did in an adjacent tissue section using the Xenium, which is an imaging-based gene detection platform.

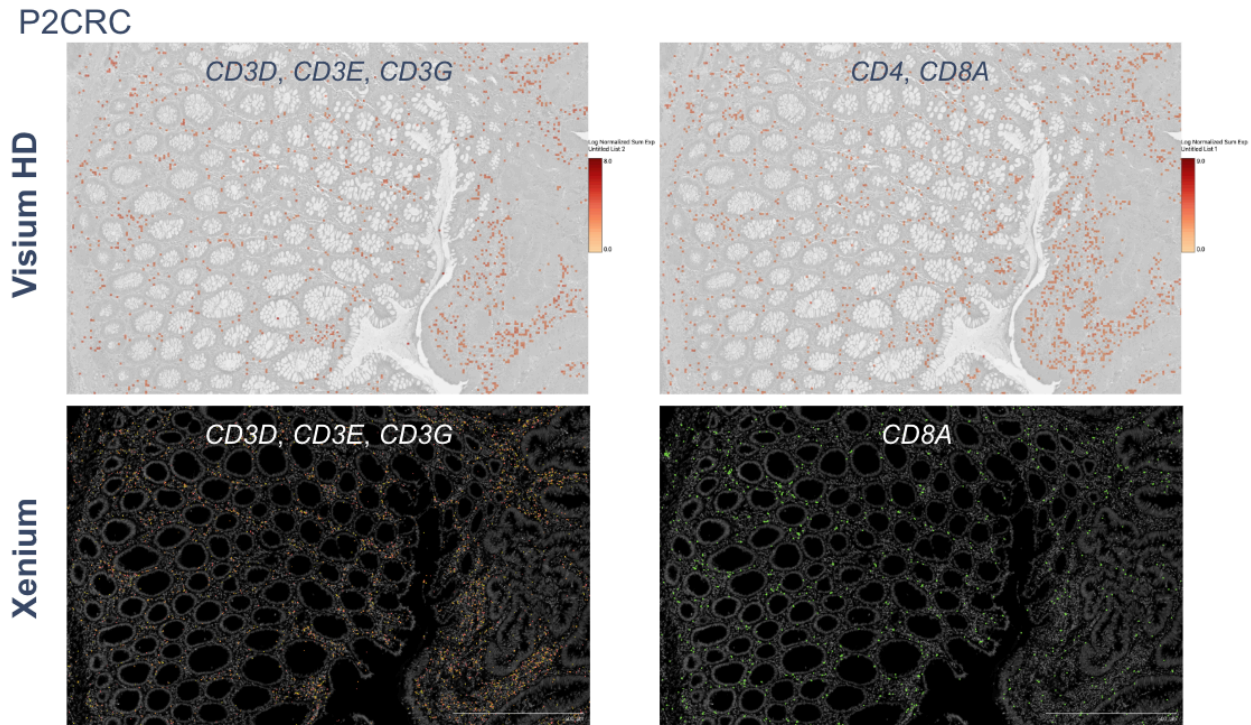


Figure. Spatial localization of *CD3D*, *CD3E*, *CD3G*, *CD4*, and *CD8A* gene expression. Top: Images generated using Loupe Browser, counts are summed for the displayed genes and log normalized. Bottom: Images generated using Xenium Explorer, counts are shown. *CD4* was not included in the Xenium gene panel and thus is not shown.

3. The authors mention that deconvolution is used due to heterogeneity between samples. Why can single-cell data be integrated, but ST gene expression data cannot?

The reviewer raises a similar point to Reviewer 2 in comment #4, we provide a detailed response in line above and have addressed this point in the manuscript Results, lines 140-161. We have also modified the original figures highlighting the results if we run the analysis alone on Visium HD data (Figure 3 and Supplemental Figure 6) without using deconvolution.

While spatial transcriptomics datasets can be integrated, they intrinsically contain additional variables to be accounted for (in comparison to single cell) such as the number of cells in each bin/spot and the varying cell type mixtures within these bins/spots when integrating datasets. Currently there are not well established methods that specifically address these effects. We have explored existing single cell batch correction methods like Harmony for Visium v1 (<https://www.10xgenomics.com/analysis-guides/correcting-batch-effects-in-visium-data>) and found the results to be useful. However, due to the increased data complexity in Visium HD a thorough investigation into batch effects would be required and would likely require the development of new tools.

How significant are the differences in ST data between samples? Can we assess the differences arising from individual variation versus those from sequencing technology?

When we control for sequencing depth and run the samples in parallel the technical variability is limited. We have previously conducted an in depth study where we compared sections from the same samples run across different slide lots, in different storage conditions and on different thermal cyclers and found the data to be highly comparable with very high R2 (0.99-1). See Figure 13 and legend from technical note below (Visium HD Spatial Gene Expression Performance Technical Note (CG000686, Rev A). In this work we processed the samples in the same fashion and controlled for sequencing depth, therefore we believe that any differences we see between samples truly arise due to biological sample variance rather than any inherent variability arising from the Visium HD technology and workflow.

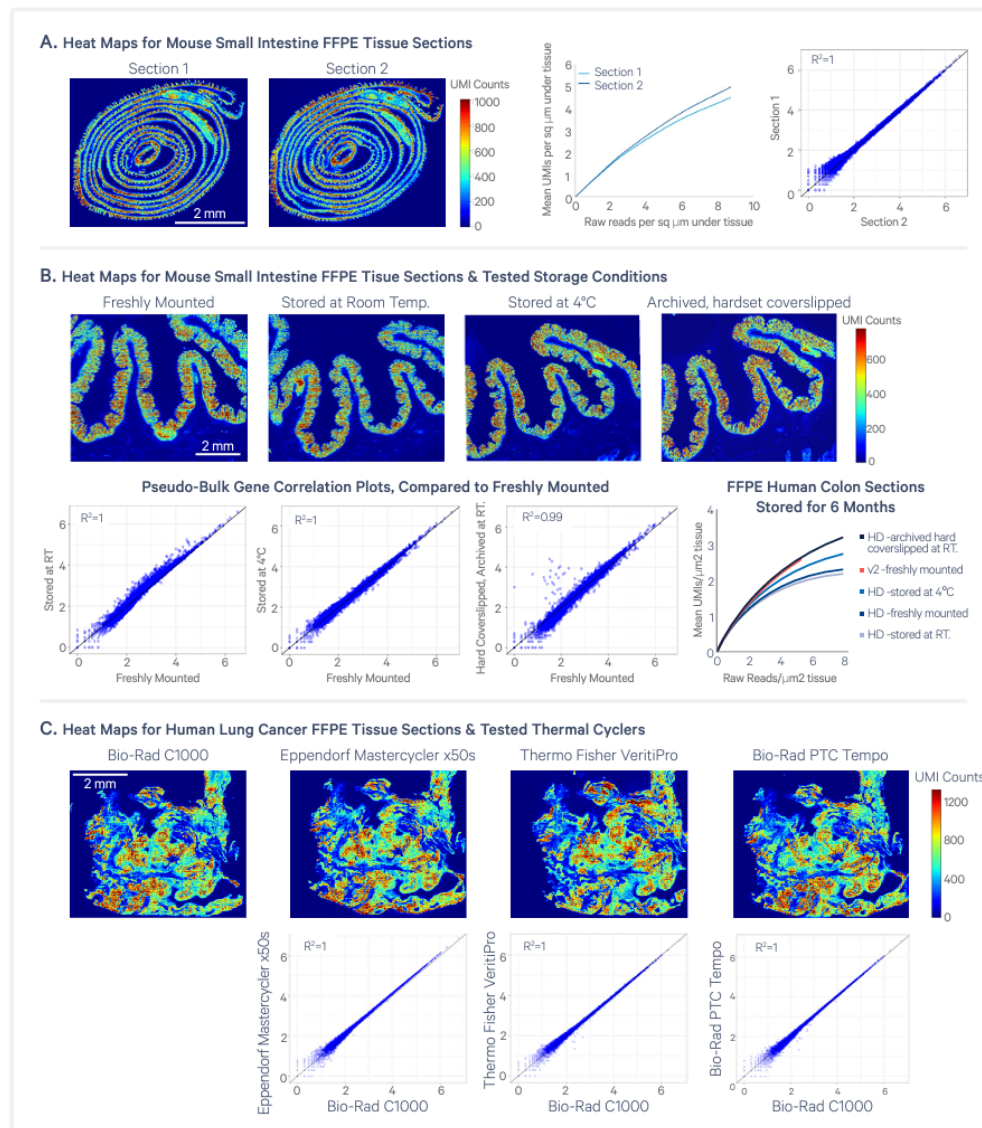


Figure 13. Assay reproducibility across slide lots, storage conditions, and thermal cyclers. (A) Two sections from the same mouse small intestine FFPE block have similar UMI counts, mean UMIs per square μm under tissue, and have strongly correlated gene expression profiles across slide lots. (B) Human colon FFPE tissue was processed under the indicated conditions. Unless otherwise noted, samples were not coverslipped. At 6 months, archived sections were processed using a freshly-sectioned/mounted tissue from the same block as control on different CytAssist instruments. UMI heat maps and pseudo bulk gene correlation plots between replicates show high correlation. (C) Consecutive sections from a human lung cancer FFPE tissue block were used to test the indicated thermal cyclers. Each condition was run on a different CytAssist instrument. Replicates have consistent UMI heat maps as well as high gene expression correlation between the tested thermal cyclers. Source: Visium HD Spatial Gene Expression Performance Technical Note (CG000686, Rev A).

4. The manuscript lacks data for P5 NAT. Why is that?

The omission of P5NAT was unintentional, it has now been added to the published dataset.

Supplementary Figure 1 shows that, compared to P2 CRC, the Visium HD data for P5 NAT has more detected transcripts than the Visium v2 data. Why does this phenomenon occur?

As noted by the reviewer, we do see a slight increase in detected transcripts in the P5NAT Visium HD sample when compared to the Visium v2 sample but the increase is not significant. When we look across the range of samples tested the $\log_{10}(\text{UMIs per probe} + 1)$ detected in Supplemental Figure 1 is approximately equivalent and thus we consider the sensitivity of the assays is comparable.

To address a related point raised by Reviewer 2 (comment #2) on sharing explicit metrics, we have included a table that highlights the key assay metrics as a new Supplemental Table (Supplemental Table 1).

5. Although each sample has a specific tumor type identified using deconvolution results, a certain tumor type within one sample corresponds to multiple clusters identified at the ST level. What are the differences between these clusters? Is there a spatial relationship, such as some clusters being inside the tumor and others outside?

We observed this phenomenon consistently across all samples. This primarily occurs at the boundary between the tumor and adjacent tissues where multiple cell types map to a single bin. There is a spatial relationship that we highlighted in Figure 6A, where we show that the tumor periphery (within $50\mu\text{m}$ from tumor cells, blue in the plot) is less represented in singlet bins (containing only one cell type per bin) than the tumor (red) or the surrounding tissue (gray). The different clusters in the spatial data contain bins of tumor cells mixed with different cell types. Whether a tumor cell is adjacent to a vascular cell or a T cell (for example) would influence how it clusters. As a result a small fraction of bins at the tumor periphery that are more likely to contain multiple cell types, tend to be clustered separately from the tumor.

6. The phenomenon in Figure 5 is only evident in the P1 sample. In P2, the two macrophage types have relatively consistent localization, and *TGFB* is widely expressed. In P3, there is only one subtype. Therefore, this conclusion may not be universally applicable and should be validated with more single-cell and spatial transcriptomics data.

We agree with this reviewer's observation. The point we wanted to convey with our original Figure 5C is that the two different macrophage subtypes are differently spatially located. In P1CRC there is overlap in their location in the lower right region of the tumor, but elsewhere their localization is distinct. In P2CRC there is overlap in their localization in the middle left region of the tumor, but in the upper right of the tumor *SELENOP*⁺ macrophages are detected but *SPP1*⁺ macrophages are not. In P5CRC only *SELENOP*⁺ macrophages are detected. Analysis of the tumor cells revealed that the different macrophage subpopulations were localized in tumor regions that also had distinct gene expression profiles. Tumor cells in areas enriched for *SPP1*⁺ macrophages showed differential expression of *TGFB* and *PERP* genes (updated Figure 5B), while tumor regions closer to *SELENOP*⁺ macrophages were enriched for *REG1A* and *REG1B* genes (patient P1CRC) or *LCN2* gene (P2CRC and P5CRC; updated Figure 5B).

TGFB expression is more widely expressed but is expressed more highly in the *SPP1*⁺ macrophage regions. We have updated Figure 5B to highlight these findings.

We agree that the relatively low n in our study means that we cannot make a strong statement that this finding is universally applicable but the point is to highlight the biological resolving power that can be achieved and what we can see in these samples. We have updated the text to ensure this is clear (lines 319-323). In addition, the new analysis we performed on *REG1* and its expression in goblet cells (raised by Reviewer 2, comment #6) identified variable *REG1* expression profiles in the goblet cell cluster which (in line with previous reports) is suggestive of tumorigenesis in a subset of these cells. We have added this to Results (lines 194-200 and 294-301) and Figure 5C.

7. To highlight the advantages of this new method, it would be beneficial to perform cell communication analyses. Compared to previous methods, the spatial positioning of cells with this new method is more accurate.

We thank the reviewer for the comment, this is a great point that was also raised by Reviewer 1. We have performed extensive analysis focusing on the communication of cells in the tumor microenvironment, a description of what we have done is noted above in the response to Reviewer 1, comment #11. We have added the results of this analysis to Figure 5E - F and edited the manuscript (lines 207-213).

8. The emphasis should be on what can only be achieved with this new method, and what conclusions can only be discovered through its application, while similar analyses with other data fail to reveal these insights.

The reviewer raises a similar point to Reviewer 1, comment #13. We have significantly reworked the discussion to emphasize what can be learned using this new method and where other methods and analyses are lacking.

Minor comments

1. In Figure 2c, it is mentioned that Visium HD can cluster more classes compared to Visium v2. Is this due to Visium HD detecting more accurately, or because it contains significantly more spots than Visium v2, affecting the algorithm?

This is due to both the data density and the increased resolution which result in a greatly reduced amount of cell-type specific mixtures per barcode in HD as compared to v2. The continuous nature of the barcoded array on the Visium HD slides means that we collect more data and as such more subtle changes are more easily identifiable. The increased resolution allows us to more accurately pinpoint where a transcript is localized and assign it more cleanly to a specific cell type.

2. In Supplementary Figure 2, only 9 cell types are listed under level 1, whereas the article mentions 10. The group of cells labeled "qc filtered" should not be referred to as cell types since they have been filtered out.

This has been corrected.

3. The labels for P1 and P2 in Figure 4 are reversed.

This has been corrected.

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed the reviewer's comments effectively, but a few minor points still require clarification.

1) The authors have defined CD8 cytotoxic T cells (Fig. 3B) in three samples, without other CD8 T cells. Should there not be a broader examination of the potential presence of other CD8 T cell subsets, especially exhausted T cells which tend to enrich tumor tissue? Knowing the specific localization of CD8 cytotoxic T cells would be helpful.

We thank the reviewer for the comment. In our initial labeling of the single cell (Flex) data, we labeled CD8 T cells as “CD8 Cytotoxic T cells” but we realize this labeling can cause confusion. We have relabeled this cluster as “CD8 T cells” throughout this revised version of the manuscript. In response to this reviewer, we performed an unsupervised clustering approach to analyze the Visium HD data and examine the presence of other CD8 T cell subtypes/states. However, the similarity in the expression profile across the selected bins, and the reduced number of singlet CD8 T cell bins (1828 across the three samples), provided unmeaningful results which we did not think warranted being included in the manuscript. For any downstream analysis included in the manuscript, we remain to use the CD8 T cell cluster. Here, we show the expression of known gene markers, that could be used as an alternative to infer the location of different CD8 T cell states: *GZMB* and *PRF1* (CD8 Cytotoxic T cells), *IFNG* and *TNF* (Activated CD8 T cells), *CCR7* and *LEF1* (Naive CD8 T cells), *PDCD1* and *LAG3* (Exhausted CD8 T cells), as exemplified in selected tumor regions shown below (Figures 1-2 of this document).

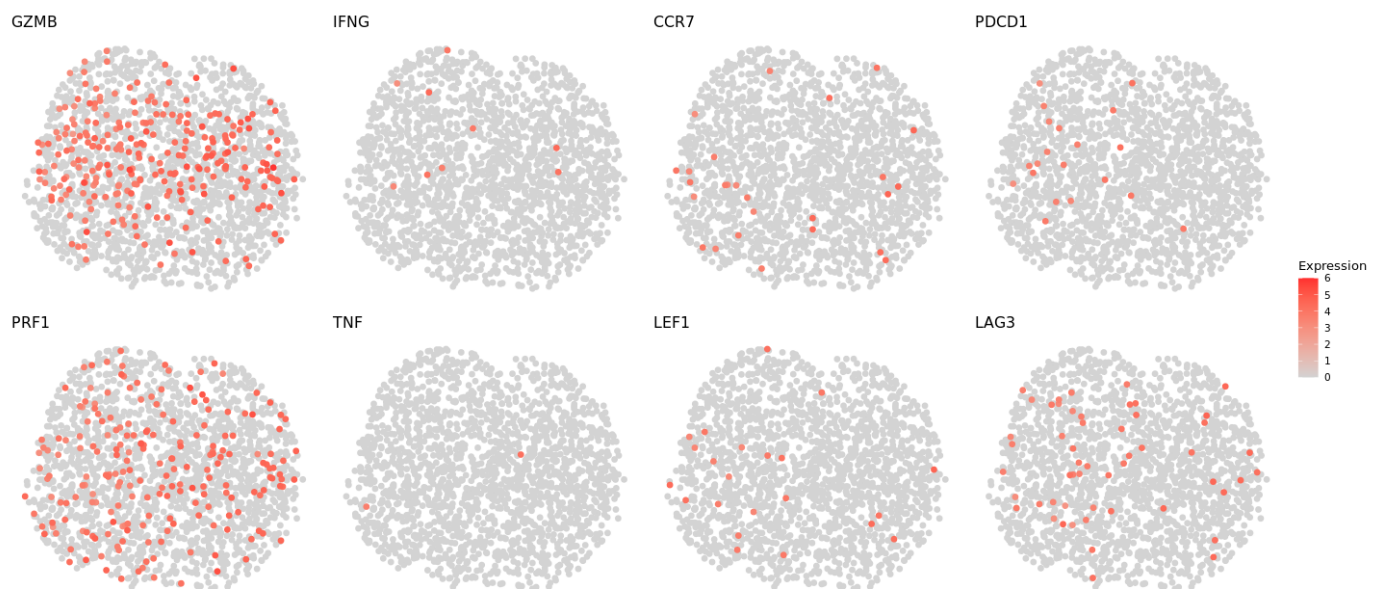


Figure 1: UMAP plot of combined CD8 T cell bins across 3 samples (1828 bins; P1CRC, P2CRC and P5CRC samples). Data points are colored by the normalized log expression of different genes used to identify T cell states. The dataset is composed of singlet CD8 T cell bins across 3 samples (1828 bins; P1CRC, P2CRC and P5CRC samples).

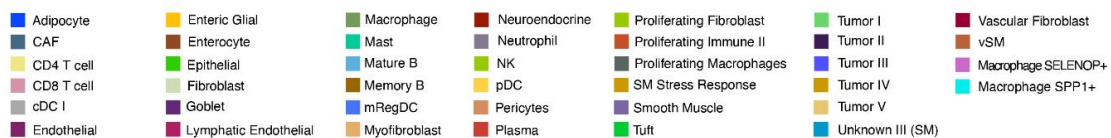
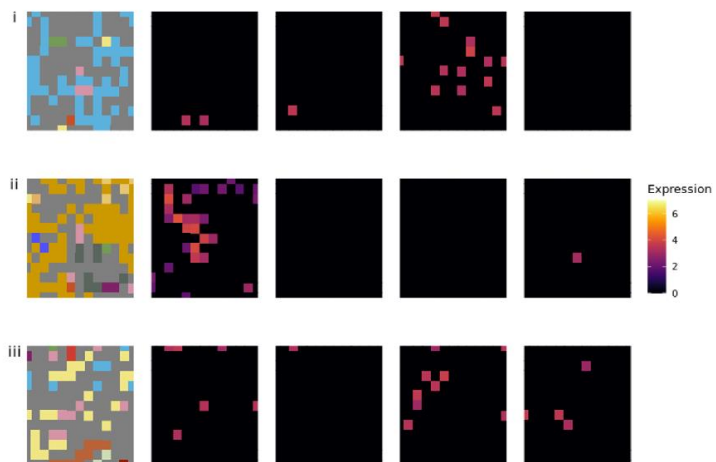
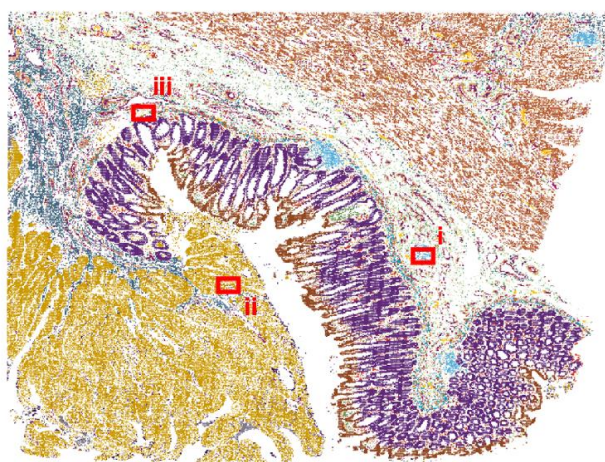
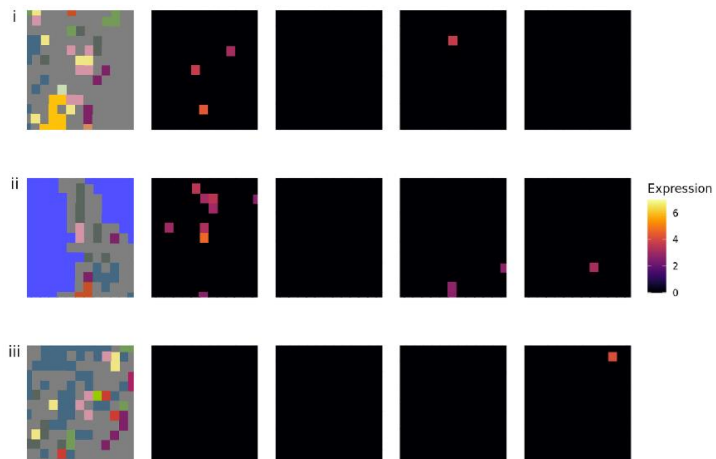
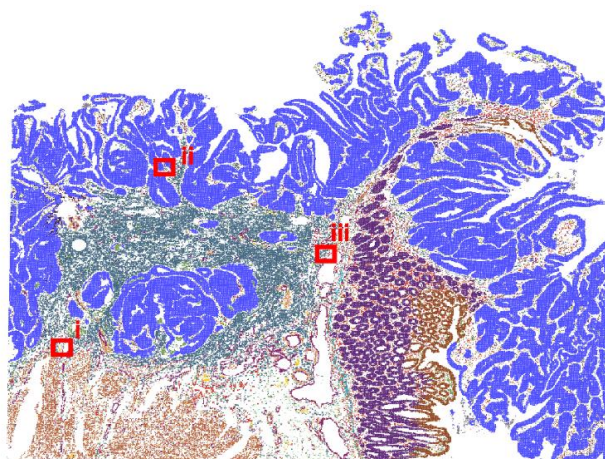
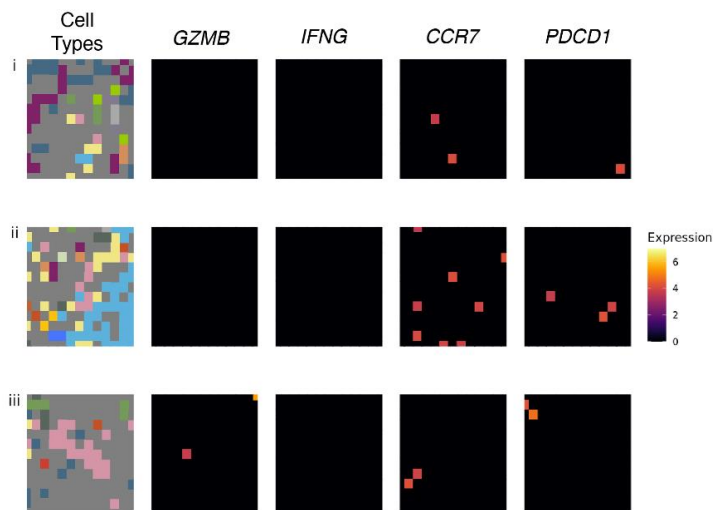
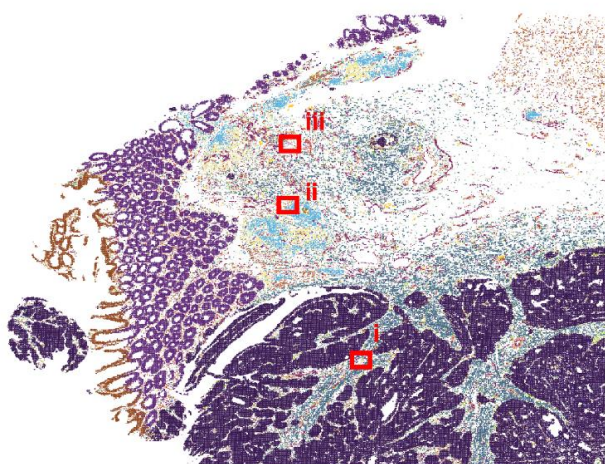


Figure 2: Visium HD spatial plots with bins colored by deconvolution label for the full section (left); zoom-in (100 micron squares) of regions where CD8 T cells were identified. The same regions are also colored by the normalized log expression of *GZMB*, *IFNG*, *CCR7* and *PDCD1*.

2) There is no comparison between the spatial location and function of *SPP1*⁺ macrophages and *SELENOP*⁺ macrophages.

This comparison was included in our revisions, but may not have been clear, so we have reworded this section to emphasize the comparisons that we performed (lines 291-298). As described in the Results (lines 167-172) and Methods sections (lines 606-618), we performed our analysis on macrophage populations found within 50 μ m of the tumor cells (which we define as the tumor boundary region). We focused on the tumor boundary region to better understand the interactions of immune cells in the immediate vicinity of the tumor. We found two macrophage populations characterized by high expression of *SPP1* or *SELENOP* genes, respectively, that were mostly located in spatially distinct tumor niches (Figure 4D of the manuscript), as revealed by density estimation analysis. We also found that the two distinct macrophage subpopulations were located near tumor cells with distinct gene signatures (Figure 5B of the manuscript). *SPP1*⁺ macrophages co-localized with *TGFBI* and *PERP*-expressing tumor cells, and both genes have been associated with poorer prognosis. *SELENOP*⁺ macrophages co-localized with tumor cells enriched with *REG* family genes or *LCN2* gene, both of which can be highly expressed in CRC and have been associated with metastasis, advanced tumor stage, and poor prognosis. To investigate the function of the different macrophage populations, we used their gene expression profiles (Figure 4C of the manuscript) to characterize which predicted signalling pathways were upregulated. While *SELENOP*⁺ macrophages were enriched for pathways of inflammatory response, TNF- α signaling via NFK- β , apoptosis, and response to ultraviolet (UV) radiation, *SPP1*⁺ macrophages were enriched for coagulation, cholesterol homeostasis, and upregulation of KRAS signaling pathways (Figure 5A of the manuscript). These results suggest that while both macrophage subpopulations display pro-tumor gene expression features, they are doing so via different mechanisms in different TME niches. We updated the Discussion (lines 291-298) to clarify these points.

Additionally, is there any relationship between their spatial distribution and patient clinical characteristics that could be explored?

Given the small sample size (n=3) and the limited clinical information available to us, we do not think we can properly evaluate associations between spatial localization of macrophages and clinical characteristics to draw concrete conclusions. In brief, all three samples consist of colorectal adenocarcinoma that were obtained pre-treatment from resections. Each sample was classified by different disease stages: P1CRC at stage II, P2CRC stage IIIB and P5CRC at stage IVA. We calculated the percentage of *SPP1*⁺ and *SELENOP*⁺ in the tumor boundaries across patients and did not observe any trend that could be associated with disease stage

(Figure 3 of this document). However, we do not feel confident in adding this to the manuscript due to the single sample per stage and potential limitations with sampling. We do believe that in the future, larger cohort studies including multiple samples will leverage these technologies for exactly this approach. In response to this comment, we revised the Discussion to emphasize this limitation (lines 324-328).

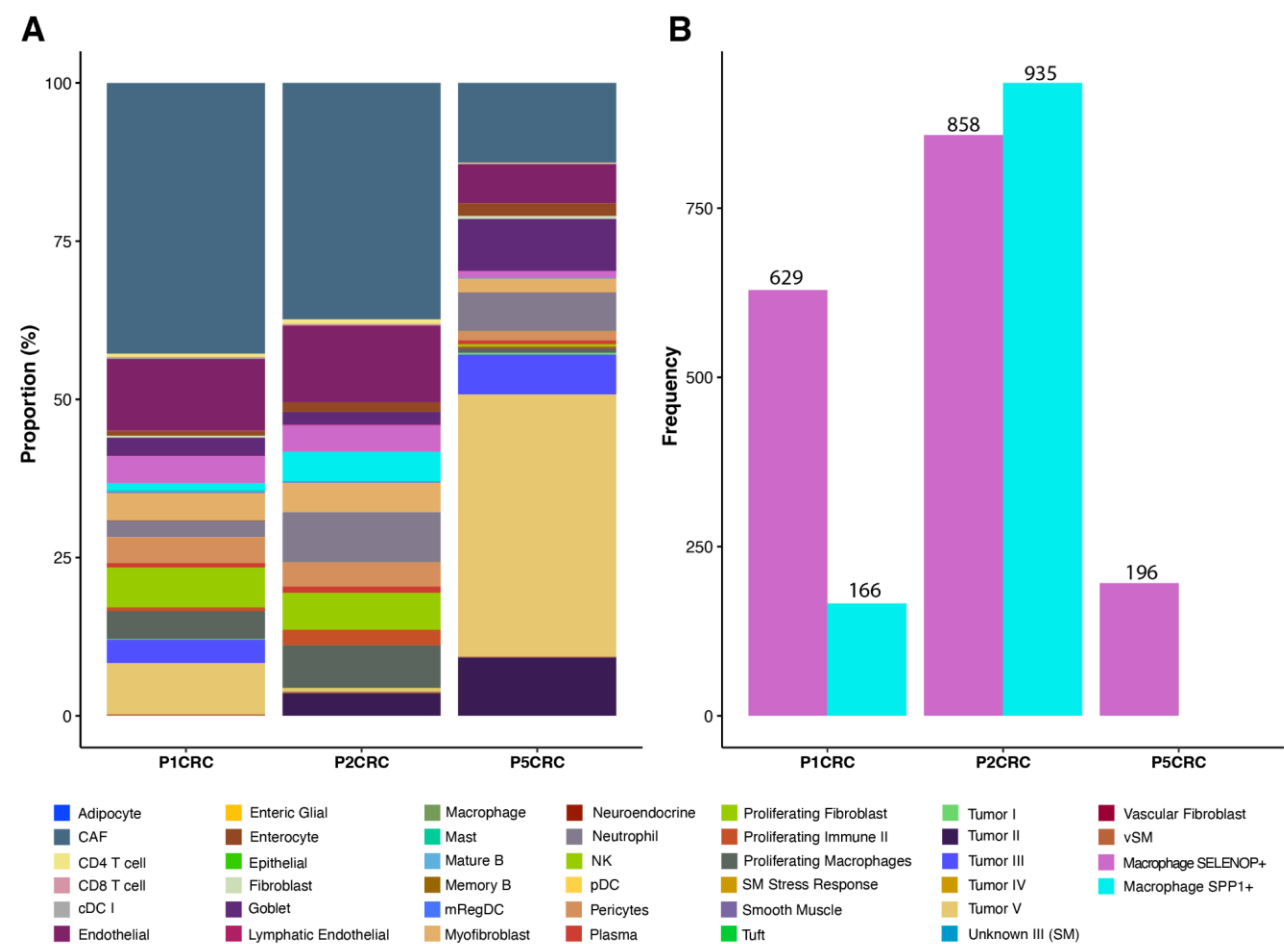


Figure 3: A. Proportions of all cell types across the tumor periphery (50 microns) of P1CRC, P2CRC and P5CRC samples. B. Frequency of *SPP1*⁺ and *SELENOP*⁺ macrophages in the tumor periphery per sample.

3) The authors describe interactions between two types of macrophages and tumor or T cells in the revised manuscript. However, an important question remains: Can the interaction between macrophages and CD8 cytotoxic T cells potentially inhibit T cell cytotoxicity?

Collectively, our data suggest that different pro-tumor mechanisms of immune response suppression and tumor progression are at play in the TME niches of these two macrophage populations. Particularly relevant to the function of CD8 T cells, communication between *SPP1*⁺ macrophages and T cells via predicted SPP1-CD44 interaction could inhibit the activity of cytotoxic CD8+T lymphocytes as suggested by previous studies (Zhao *et al.* 2024). Interactions

between B2M and KLRD1, primarily enriched in *SELENOP*⁺ macrophages in our study, can inhibit the cytotoxic activity of T cells. These predicted cell-to-cell interactions between macrophages and CD8 T cells in the TME can ultimately lead to reduced immune surveillance and evasion of the immune system. We have added some detail on this to the discussion lines 294-298.

Reviewer #2 (Remarks to the Author):

The authors have made substantial improvements to the manuscript, successfully addressing most of the concerns raised in my initial review. The inclusion of detailed analyses has significantly enhanced the depth and rigor of the work, particularly in elucidating immune cell interactions within the tumor microenvironment. However, a few aspects warrant further attention to solidify the conclusions and maximize the manuscript's impact.

1. Supplementary Table 1 highlights that Visium HD exhibits approximately half the sensitivity (measured by captured UMI counts) of Visium V2 after normalizing for sequencing depth, spot area, and tissue size. While this reduced sensitivity may not critically affect cell population clustering, it could influence the ability to detect rare cell types and their spatial distribution in the TME (line 111).

We appreciate the reviewer's comment and we realized that the title for Supplemental Table 1 is misleading. Supplemental Table 1 simply displays the output metrics from the Space Ranger web summary that we obtained from each sample, without any normalization. The table is not a sample comparison as the title may suggest (we have updated the caption title to "Visium Space Ranger summary metrics per sample" to remove this ambiguity). Sensitivity comparisons between Visium v2 and Visium HD are instead presented in Figure 2D and Supplemental Figure 1 of the manuscript. These comparisons were made on a gene-by-gene basis and were normalized for sequencing depth. Matched areas were manually selected in Loupe Browser v8.0, and probe-barcode matrices from each dataset, generated by Space Ranger, were imported into Seurat v5 using the `Read10X_h5()` function. We utilized the ggplot2 R package to graph per-probe UMI counts (outlined in Methods, lines 543-552). The data we present show a strong correlation between the two technologies (Figure 2D, $R^2 = 0.82$; Supplemental Figure 1, $R^2 = 0.81$ and 0.90), indicating that the Visium HD assay maintains the high assay sensitivity of Visium v2.

2. The revised discussion of probe off-target binding is appreciated. However, two questions remain: (1) Given that genomic DNA (gDNA) is double-stranded, what mechanisms enable single-stranded probes in Visium V2 to bind to gDNA at the reported rate (~5%)?

The most likely mechanism for probes binding to gDNA is through the denaturation of gDNA to ssDNA and then interaction with the probes, although off-target mRNA binding is a possibility. We believe it is likely due to denaturation of gDNA because, as part of the development of Visium HD, we optimized the sample preparation steps to minimize gDNA denaturation and

observe a drop in the estimated percentage UMI from gDNA (lines 112-114). Specifically, we optimized the decrosslinking step by formulating a new proprietary reagent and updating the protocol to decrease the decrosslinking temperature and duration. We believe these improvements to the Visium HD protocol over the Visium v2 protocol, lead to a reduction of gDNA denaturation and therefore a reduction in the accessibility of target sequences in the gDNA to Visium probes in Visium HD.

(2) How do the authors experimentally or computationally distinguish probe binding to gDNA from binding to unspliced, immature mRNA? Clarifying these points would strengthen confidence in the spatial transcriptomic data.

Technically is it not possible to differentiate between gDNA binding and other off-target binding events; however, as outlined above, the most likely candidate for off-target binding is gDNA and the estimated number of UMIs arising from gDNA binding is very low. We describe how our gDNA metrics are calculated in the Methods section (lines 553-572).

3. The new analysis of macrophage interactions with other cells (Fig. 5E) is compelling. In P2CRC, there are interactions between the two macrophage subtypes (*SPP1*⁺ and *SELENOP*⁺), with some *SELENOP*⁺ macrophages also expressing *SPP1* (Fig. 4C) and not observed in other samples, raises questions about context-specific macrophage interactions or transitions. How do the authors interpret this observation? Additionally, exploring how macrophage interactions shape distinct TME types and influence tumor progression would add further value to the manuscript.

The reviewer raises a good point, similar to reviewer #1 comments #2 and #3 and we provide a detailed response in-line above. Both *SPP1*⁺ and *SELENOP*⁺ macrophages have previously been shown to be enriched in tumor tissues, and are more likely to adopt an M2-like phenotype exerting pro-tumor and pro-metastatic roles. In our study, the gene expression profiles and distribution of these macrophage sub-populations further support their pro-tumor role in the TME, however through different predicted mechanisms. Communication between *SPP1*⁺ macrophages with tumor cells and T cells via the predicted *SPP1*-*CD44* interaction supports this potential pro-tumor mechanism in CRC. *SPP1* and *CD44* ligand-receptor interaction can enhance expression and activation of coagulation and complement components as well as the production of matrix metalloproteinases (MMPs) and other enzymes that can regulate EMT and extracellular matrix degradation, promoting tumor progression, metastasis, and hinder T cell activation (Klement et al. 2018, Zhao et al. 2018). In contrast, *SELENOP*⁺ macrophages interact with tumor cells primarily via *APOE*. This interaction may modulate various pro-tumor processes related to cell survival, proliferation, migration, immune modulation, lipid metabolism and ECM remodeling (Zheng et al. 2018, Kemp et al. 2021, He et al. 2023). Enrichment of genes involved in EMT may contribute to overexpression of genes involved in cell growth and regeneration, such as the *REG* gene family and *LCN2* genes in tumor cells, and could ultimately contribute to tumor progression. Additionally, interactions between B2M and *KLRD1* can inhibit the cytotoxic activity of T cells, as discussed above and now included in the discussion.

To address the point about the *SELENOP*⁺ macrophages expressing the *SPP1* gene (double positive macrophages), we performed a new analysis to further classify these cells. These double positive macrophages represented a low proportion (74 double+ , 814 *SELENOP*⁺ and 905 *SPP1*⁺) of the macrophages in the tumor periphery for P2CRC. We first identified these double positive macrophages bins and plotted their expression profile together with the *SPP1*⁺ and *SELENOP*⁺ groups. This highlighted that the double positive bins in fact expressed both signatures almost identically (Figure 4 of this document).

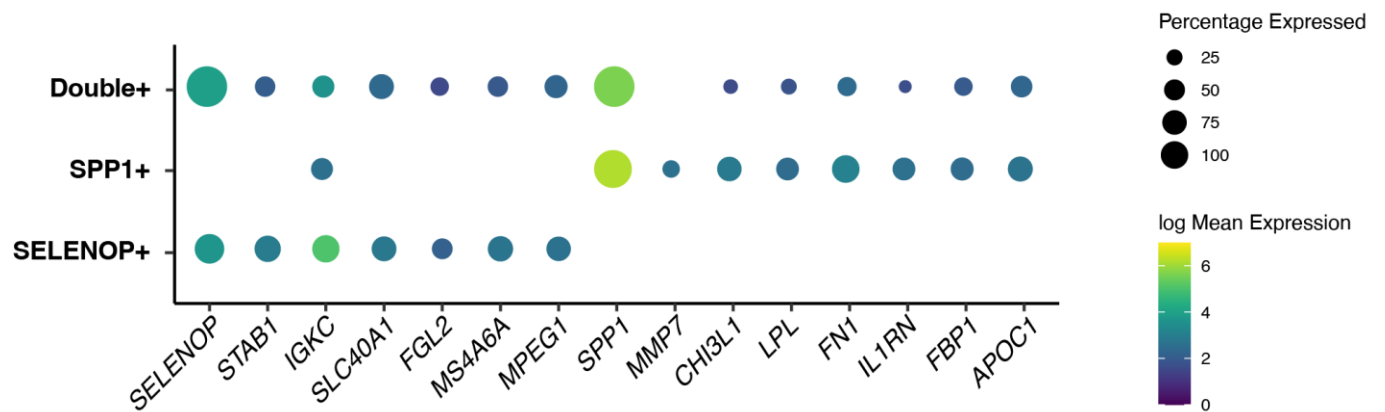


Figure 4: Dotplot with the expression profile of *SELENOP*⁺ macrophages (n=814), *SPP1*⁺ macrophages (n=905) and macrophages expressing both *SELENOP* and *SPP1* (n=74). Size represents the proportions of bins expressing a gene for every group and color denotes the normalized log expression.

We then identified the neighboring bins (within 15 microns from double positive macrophage bins) and observed that they were mostly surrounded by *SPP1*⁺ macrophages, *SELENOP*⁺ macrophages and Tumor labeled bins (Figure 5 of this document). This observation suggests that these double positive macrophages bins may be capturing the signal from cells that span more than 1 bin.

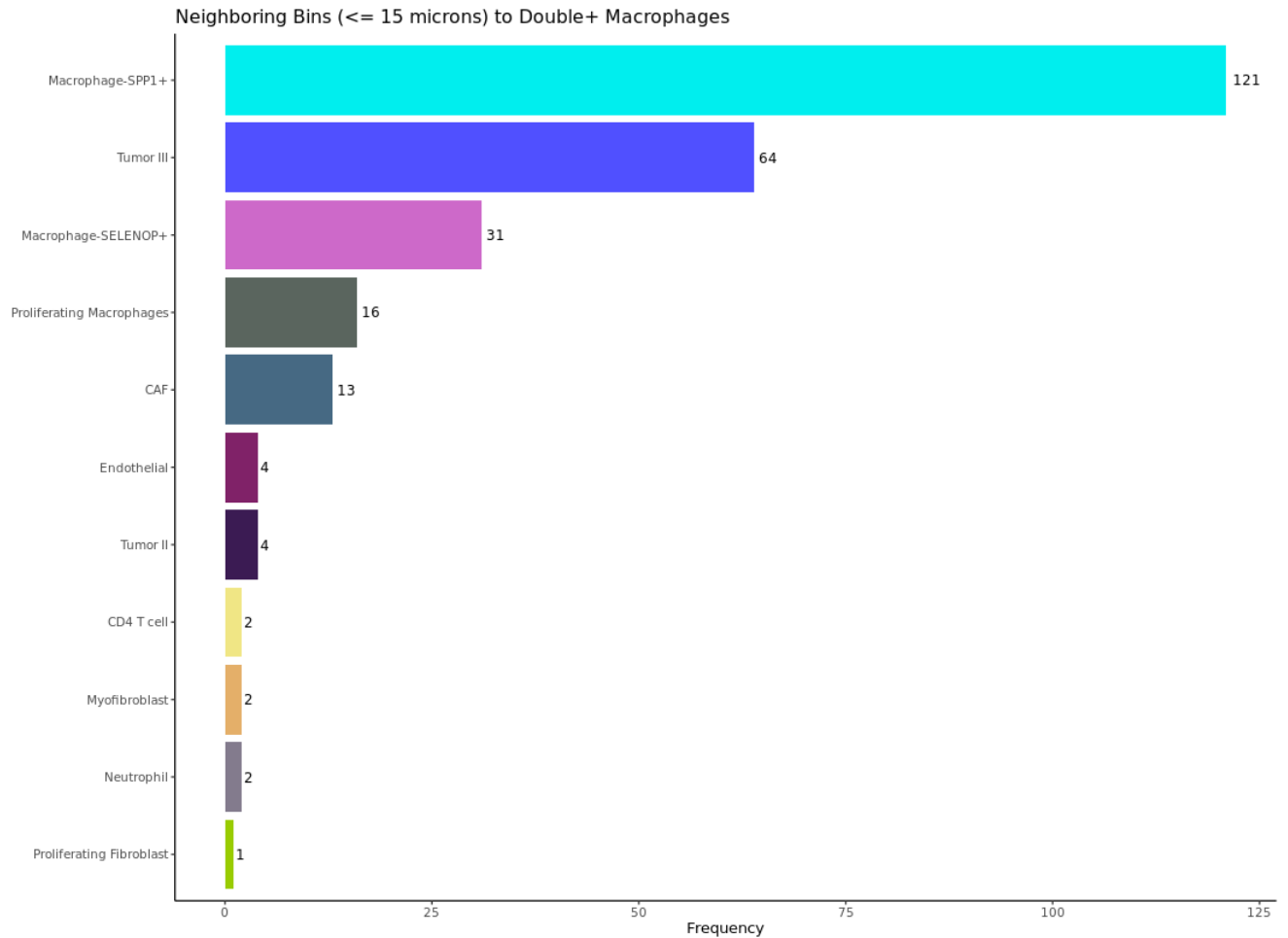


Figure 5: Barplots representing the frequency of cell type of bins located within 15 microns of double positive macrophage bins (expressing both *SPP1* and *SELENOP*) within the tumor boundary region.

Finally, we zoomed in to regions where these double positive macrophage bins were located and observed that they were mostly surrounded by either any of the macrophage subtypes or doublet/rejected bins (bins that were excluded from our analysis). We also included the corresponding H&E images for such zoom-ins for reference (Figure 6 of this document). It's important to clarify that, as described in our Methods section, we only included deconvolved singlet bins in our analysis. The subclustering of macrophages into more specific subtypes (*SELENOP*⁺ and *SPP1*⁺) may result in the emergence of new doublet bins that were not previously identified, as they were formerly categorized under a general macrophage label. The figure below illustrates examples of these newly identified doublet bins.

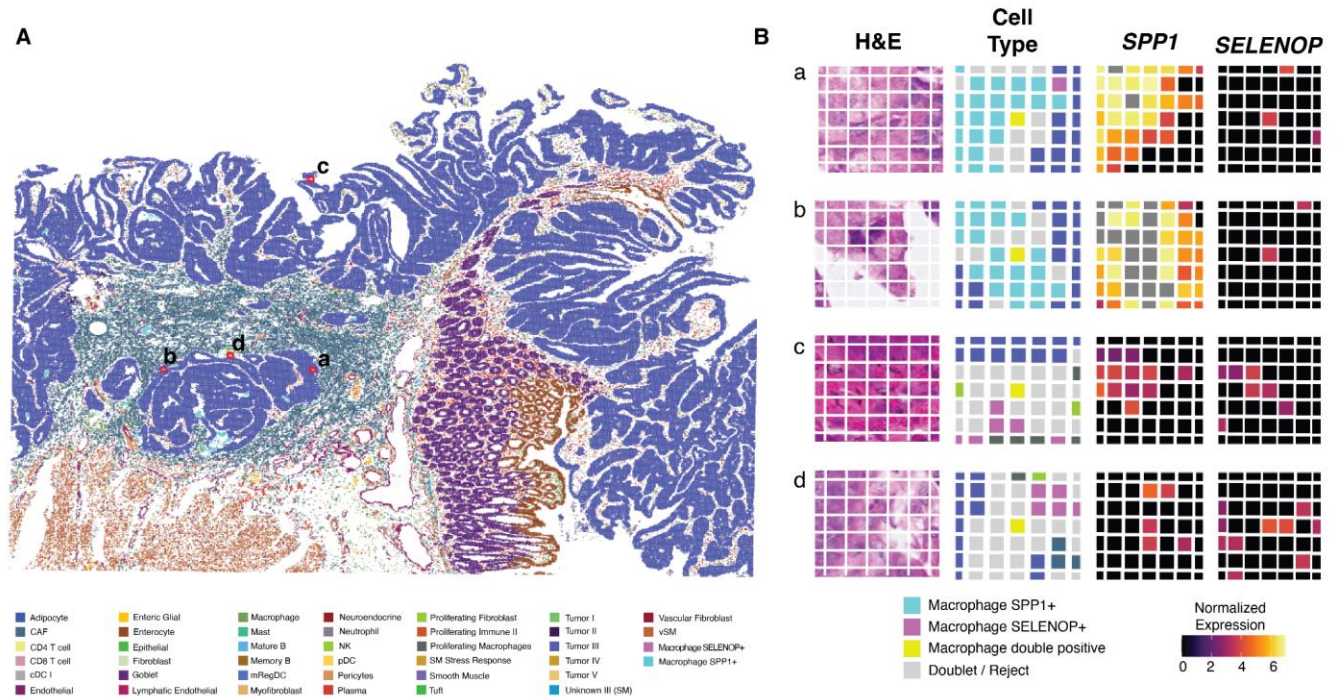
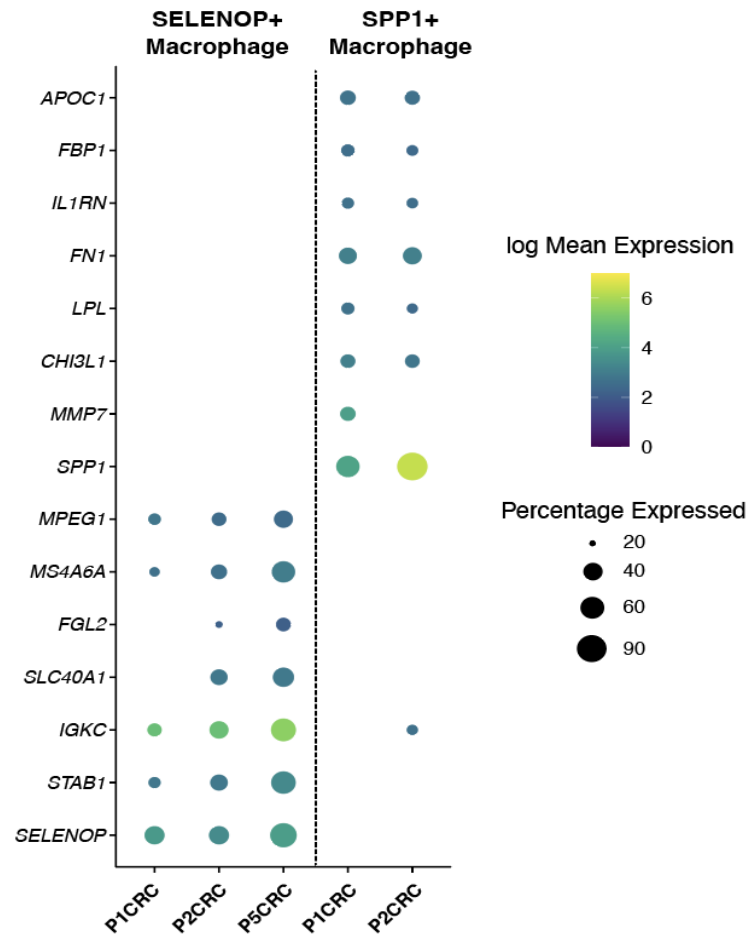


Figure 6: Analysis and localization of double positive macrophage bins in the P2CRC sample. A. Spatial plot with bins colored by the cell type identified via deconvolution. Macrophage bins in the tumor periphery (50 microns) were then subclustered into two distinct subpopulations ($SPP1^+$ and $SELENOP^+$). Red squares identify regions where some double positive bins are located. B. Zoom in regions (squares of 100 microns) displaying the H&E, cell type and expression of $SPP1$ and $SELENOP$ in the selected regions.

In order to remove any potential misinterpretation, we have updated Figure 4C of the manuscript to only show dots where a given gene is expressed in at least 20% of the cells of a given group. We have added additional clarification to the figure legend to explain this.



Updated Figure 4C of the manuscript. Dot plot showing expression profiles of two distinct macrophage subpopulations identified at the boundary of the tumor samples studied. Dots are shown if a gene is expressed in at least 20% of the cells of a given group.

References:

- Klement, J. *et al.* An osteopontin/CD44 immune checkpoint controls CD8⁺ T cell activation and tumor immune evasion. *J. Clin. Invest.* **128**, 5549-5560 (2018).
- Zhao, H. *et al.* The role of osteopontin in the progression of solid organ tumour. *Cell Death Dis.* **9**, 356 (2018).
- Zheng, P. *et al.* Tumor-associated macrophages-derived exosomes promote the migration of gastric cancer cells by transfer of functional Apolipoprotein E. *Cell Death Dis.* **9**, 434 (2018).
- Kemp, S. *et al.* Apolipoprotein E Promotes Immune Suppression in Pancreatic Cancer through NF- κ B-Mediated Production of CXCL1. *Cancer Res.* **81**, 4305-4318 (2021).
- He, L. *et al.* Jun-APOE-LRP1 axis promotes tumor metastasis in colorectal cancer. *Biomol. Biomed.* **23**, 1026-1037 (2023)

4. Given the high resolution of Visium HD, the authors could leverage spatial data to identify key factors (e.g., ligand-receptor pairs, metabolic gradients) driving macrophage transitions between

pro- and anti-tumor states. Integrating these spatial insights with single-cell RNA-seq datasets might uncover mechanisms underlying macrophage reprogramming during interactions with T cells or stromal cells.

We thank the reviewer for the comment. In order to address these suggestions, we have added the spatial information to the ligand-receptor analysis to take advantage of the high resolution capabilities of Visium HD. Using this new approach we first identified all the bins that are within 15 microns of any *SPP1*⁺ or *SELENOP*⁺ macrophage in the tumor periphery (within 50 microns from the tumor border). Once all these neighbors are selected, we are able to create different networks or communities that contain multiple bins which are physically close to each other. As we are interested in exploring the interplay between macrophages and T cells, we then filtered these communities by selecting those that contained the most T cells (either CD4 or CD8). Finally we used LIANA to identify ligand-receptor pairs that could be driving the different functions of the cells in the networks. This analysis approach allows us to investigate direct cell-to-cell interactions within the tumor boundaries. We observed that the interplay between macrophages subtypes and T cells can vary across niche-specific communities in the tumor microenvironment (Figure 8 of this document).

Figure 8 of this document displays the results obtained with this new methodology:

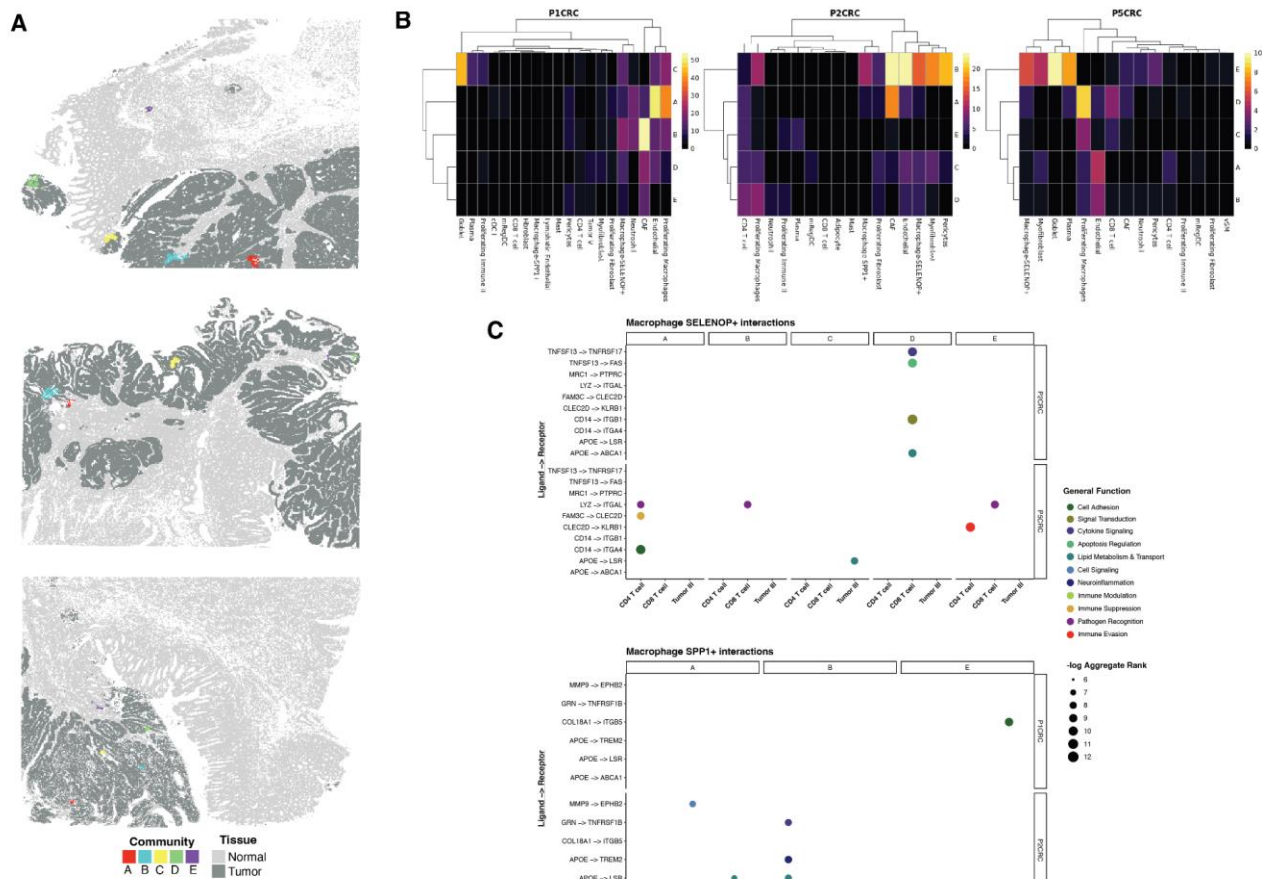


Figure 8: Community based cell-cell interactions via ligand-receptor interactions. A. Spatial localization of the cellular networks identified in each section. To create each community, we identified all bins within 15 microns of any macrophage bin in the tumor periphery. B. Heatmaps with the cell type distribution in each patient and community. Rows denote the different communities and columns the cell types. C. Dot plot with the inferred ligand-receptor interactions for each community taking the *SELENOP*⁺ macrophage (top) or *SPP1*⁺ macrophage (bottom) as source. Color represents the general function of the interaction and size the -log of the LIANA aggregate rank. Only interactions with aggregate rank $\leq 1e-3$ are shown.

While this new approach improves existing methods, as we selectively evaluate cells that are physically close to each other, we also believe it is biased due to multiple reasons: The current approach considers communities that contain the most T cells, however, the number of cells within these communities is limited. Further, the approach excludes communities with lower proportions of T cells. For these reasons, we believe that the approach of selecting all bins in the tumor periphery, as we performed in the manuscript, is a better approach.

Regarding the integration with single-cell datasets, we believe that generating conclusions relating to cellular interactions where we have the spatial context and then extrapolating the results to single cells (where there is no spatial information) would not yield reliable results. The spatiality is what adds to this analysis.

5. While the findings, such as tumorigenic goblet cells and clonally expanded T cells, are novel, the small cohort size and inter-sample heterogeneity (e.g., in macrophage subpopulations) limit broad conclusions. The authors may discuss these limitations and interpret the results with cautions.

The reviewer raises a good point that we acknowledge. We have extended our discussion on the limitations of our study to emphasize this (Discussion lines 324-328).

Reviewer #3 (Remarks to the Author):

I want to thank the authors for addressing my concerns. I believe this manuscript is suitable for publication in its present form.

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