nature portfolio

Peer Review File

Cancer-associated fibroblasts shape the formation of budding cancer cells at the invasive front of human colorectal cancer

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This manuscript has been previously submitted at another journal. This document only contains information relating to versions considered at Communications Biology.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study authors have examined tumor budding at the proliferative front of the tumor for Stage II/III colon adenocarcinoma patients using spatial molecular imaging. They characterize interactions with cancer associated fibroblasts. I am impressed by the amount of validation they have done with various spatial assays, which when put together, represents an incredible body of work. I am sharing a few comments to help clarify the scope of this work:

1. A methods/results overview table/figure/section would be helpful. It can be challenging for the reader to place all of these assays and subsequent analyses and what level of validation they provide in context.
2. The abstract could be more descriptive. For instance, what does it mean for budding cells to have a different spatial distribution? These were colon adenocarcinoma cases, stage II/III, validation of certain findings was carried out through … and so forth. This could complement the methods/results overview.
3. The authors correctly point out limitations in studying tumor budding in 3D though limitations could further be stated, especially in reference to recent research disputing which cells are actually budding due to the 3D structure of the tissue, introducing uncertainty in this analysis.
4. At the beginning of the results, it should be stated how many patients were profiled and of what type. While in the methods we know which cell detection software was used, perhaps this could be mentioned briefly at the beginning of the results.
5. The authors discuss cell clusters throughout the manuscript, but it may be unclear to the reader whether clusters reflect spatial distributions of cells or expression differences. Also, motivation for why expression was used to cluster should be further elaborated as a marker approach was already taken to define cell types. It would make more sense to call cell-types then annotate the tissue for characterizing their spatial distribution, perhaps using neural network segmentation approaches of these architectures as needed. As it is written it seems like the authors are trying to further subtype tumor cells through clustering but unclear why this was done across all cells where it could be done by just considering tumor cells alone through marker based approach then clustering. If clustering is used to help assign cell type, then it should only briefly be mentioned.
6. L108, the claim that PIGR is a colon-specific epithelial marker gene is not substantiated. Wouldn't the gene just correlate with existing epithelial gene sets, why is it specific to Colon? Also the discussion with spatial locale within the crypts could benefit from reviewing prior literature on colonic cell differentiation based on spatial location and where it is expected to find stem-ness markers etc. Perhaps some of these sections that have findings that appear orthogonal to the main claims could be moved to supplementary? The presented analysis on adjacent normal epithelium does not seem to tie into the main story. It seems like the authors are trying to validate SMI but I am not convinced this provides appropriate validation of the assay. How does this section lead us to conclude that SMI provides "highly accurate spatial gene expression"? Please be explicit because this is unclear and may also be limited by variation attributed to individual patients.
7. L120 section: The selection of FOV across 4 patients: who selected these regions and how large were they? How were they annotated? The small sample size is a significant limitation given histologic and other sources of variation. What site from the Colon were they selected from? Tumor stage? N/M-stage? MSI status? BRAF/KRAS? I am assuming M=0 but else relationships are unclear.

8. What is a good operational definition of tumor budding and why is clustering then where one cluster falls spatially sufficient to call budding? Distance from tumor interface with certain density lacking certain number of other tumor cells within radius, etc?
9. “Cancer cells colocalize with PanCk-positive cells”– PanCk is an epithelial marker gene/protein though insufficient to delineate normal vs tumor epithelium. Why is this important or relevant or adds new information? Further annotation of the slide is needed to contextualize findings in relevant tissue regions.
10. L143: “unique spatial distribution” needs a more quantitative description
11. L152: FN1 as a CAF associated marker gene, is it possible that this gene is descriptive of colocalization with CAFs and that imprecise imaging/staining could be at play?
12. The use of cancer cell cluster 4 etc is confusing, would recommend giving these discovered subtypes names and also perhaps more important to also consider the spatial distribution when subtyping.
13. Serial section H&E images side-by-side with Fig1-2 and related supplementary figures would be helpful.
14. All FOV images should be in supplementary at minimum. How does spatial colocalization of cell-types vary between slides?
15. The 10X Visium analysis is qualitative and needs further assessment. For instance, could you define a tumor budding signature and other identified signatures that could be recapitulated and annotated spatially at minimum and how does it recapitulate the findings. Were these 55-micron spots? scRNASeq information could be incorporated for a cell-typing analysis. More detail on the patients are needed e.g., TNM stage, site, etc
16. What is the background set of genes for the GSEA analysis?
17. Discussion: What are limitations of SMI? The reliability of the assay has been disputed. Further review of the literature could be done here.
18. “Perfect overlap with PanCk IF staining” needs to be substantiated, I would guess would not be perfect
19. L307-312: Consider removing/revising most of these lines as this section is misleading/confusing to the readership and may not reflect our current understanding of epigenetics. The phrasing is awkward.
20. CAFs seem to be the central part of this story. They, however, have many roles and should be further subtyped to further characterize their interactions. A review of the CAF sub typing literature on tumor progression is needed. The nature of the CAFs in this analysis and their significance is unclear even though it is supposed to be central.
21. L385-399: Xenium and Visium HD could be compared and discussed here along with other related assays e.g., CODEX, COMET, validation with merScope, etc. Also, DSP could potentially characterize a neighborhood of budding cells.
22. Methods L606-609: T and N stage for patients are needed along with other relevant histological features: e.g., were tumors mucinous? Exclusion criteria? Simultaneous metastasis to the lymph nodes can impact the findings substantially.
23. Cell detection accuracy is unclear and a comparison between methods is warranted.
24. How are batch effects / normalization being accounted for?
25. What are the parameters of the neighborhood analysis? E.g., construction of spatial network, enrichment at specific distances, etc. Unclear if Leiden clustering is appropriate. Some of the cell types seem redundant and perhaps their spatial location is important, potentially necessitating spatially-informed labeling. See also my earlier comment for strategies on sub typing cell types determined through marker gene approaches versus clustering all cells then subtyping.
26. The nested nature of this analysis warrants further consideration. For instance, paired normal adjacent FOVs nested within patient with 10 additional ROIs at the primary tumor. Hierarchical regression modeling with covariate adjustment and inclusion of potential effect modifiers is warranted, or incorporation of analogous metaanalytic regression modeling. Statistical findings should be reported across all FOV rather than by one FOV at a time. Considering one FOV at a time makes it seem like results are being picked for how they best tell the story.
27. How is density calculated? How is spatial autocorrelation between cells accounted for in differential expression? Calculating the gini coefficient for differential expression analysis as a main finding seems highly unusual and unclear how this analysis considers the nested dependency structure of the data.

Reviewer #2

(Remarks to the Author)
The authors employed a 1,000-plex gene panel, and have defined major cell types in tumors and adjacent normal tissue with accurate spatial localization. They found that certain cancer cells were scattered around the periphery of the main cancer cell masses and were more frequently in contact with cancer-associated fibroblasts (CAFs). They imply that that CAFs induce pro-invasive gene expression changes in those cancer cells involved in epithelial-mesenchymal transition, extracellular matrix binding, and migration.

Major Remark: The study remains highly speculative and without any validation. The conclusions made would require in vivo tracing analysis to determine the faith of the selected cancer cells. While the reviewer understands that this is beyond the scope of the present manuscript, some level of functional analysis is clearly needed, even in vitro.

Reviewer #3

(Remarks to the Author)
This solely descriptive study deals with the spatial profiling of the invasive front of colon cancer. Normal colon is used as a model to test the capability of the method. The authors claim to identify a special gene expression profile of budding cancer cells induced by direct interaction with CAFs using the CosMx Spatial Molecular Imaging by employing a 960 plex gene

panel at the mRNA level. This is done in sections of FFPE tissues. The study is technically sound; however, I have some major concerns about the analysis and the interpretation of the data.

Major points

Figure 1
Add size bars to each image (also in Figure 2 and all Suppl. Figures with microscopic images). I wonder why in the normal colon tissue there are no endothelial cells (blood and lymphatic) and macrophages identified? These cells are known to be present in the colonic tissue in the stromal part at quite high numbers. Please comment why these cells were not identified. (they are clearly identified in the clusters from the tumor sections in Figure 2A-C). If they are not detectable this would challenge the quality of the method to identify all relevant cell types in tissues. Moreover, pericryptal fibroblasts are well established to form a thin layer of cells around the crypt, characterized by alpha SMA expression. Identification of these cells would be a perfect proof that the SMI really can detect structures at the cellular (or even subcellular …as stated by the authors) level. Maybe the unknown cluster are these cells (The marker genes like PDGFRA, CDH11, CXCL12 shown in Fig. 1F at least point to fibroblast like cells as the potential cell types for the unknown cluster. Show a closeup picture of the of the unknown cluster. How about LGR5+ CBCs? Why are these cells not identified? These cells are known to be at the crypt base in rather high numbers. For PIGR and OLFM4 show the corresponding cell types in (publicly available) datasets from single cell mRNA sequencing of normal colon. All together the presented data do not fully support the full identification of different cell types present at substantial quantity in normal colon.

Figure 2
Four patients are used for the analysis of the colon cancers. Please identify the clinical characteristics and mutational profile (at least for driver mutations) in a separate table (e.g. pathological description, age, sex, pretreated or not, mutations, type of tumor, primary or metastasis, location etc.). Why is the expression level in Figure 2 G, H always an integer? This seems not the case for Figure 1J. Please comment and explain.
An intrinsic problem of spatial profiling is always the exact topological source of expression of genes. There is always the possibility that at a certain point in the slide two or more cellular structures (meaning parts of cell bodies of different cell types) might overlap or are situated so close that the spatial profiling raster is not able to resolve this. These mixtures of cells then also lead to a mixture of expressed RNAs at the certain point and might lead to a false classification of expression signatures of the cell type, which was characterized by signature genes for the rough distinction of cell types such as epithelial cells and stromal cells or different immune cells. In the light of this study, where the major focus of interest is the tumor budding cell area at the invasive front at which there is close contact to the TME and the cells within it, one has to be extremely cautious in interpreting the obtained expression profiles. For example, one can easily see an obvious contamination of CAF and lymphoid cell signatures in the tumor cell 4 fraction in the heatmap of Figure 2f or Figure 2h. The other tumor cell types, being further away from the budding zone (as shown in Figure 3D, H do not show this contaminating signatures. This is also supported by the fact that the authors show that these cells are in closest contact with fibroblasts in Figure 5)
Thus, the analysis of the EMT state and the tumor cell type 4 association with the mesenchymal state is most likely due to the fact of the mesenchymal cell contribution at this specific budding sites. Hence, interpretation of the EMT data might be done with caution and should be clarified in the manuscript. (see below for validation of the data by single cell seq analysis).
The identification of the specific expression signature of Tumor cells 4 in Figure 2JK seems to be appropriate (i.e to exclude all other tumor cell and CAF signature genes). Some of these 11 genes are tightly connected to immune cells, thus also here it might be due to immune cell contamination.
Taken together all these points, there is need to analyze the identified subtypes from this spatial analysis presented here with single cell data and correlate the findings. In single cell sequencing, if proper single cell dissociation is done, there is no contamination issue from other cells. Therefore, a combination of these two methods has the power to clarify the raised points in this study and any other spatial analysis. Hence, for this manuscript the cell groups identified need to be found and validated in a scRNA Seq dataset (which are available). This needs to be done to validate the conclusions made in this manuscript (i.e. to find the tumor cell 4 expression profile in single cell data and to validate the findings.

Figure 3, 4
All the concerns about contaminating signatures have to be taken into account in the analysis presented in Figure 3 and 4.

Figure 5
This analysis has high potential to measure which cells really impact the budding tumor cells. And also underline the importance of cell contamination as raised before. How are the heatmaps generated? How “their neighboring cells” is defined? What is the basis of cell- cell interaction distances measurements? Does the software measure the distance between two cells? This need s to be clarified for the reader. Besides, it would be helpful to keep the nomenclature of the tumor cells from FOV1 consistent with the other FOVs. It is not intuitive that in some FOVS tumor cells 4 are the budding cells and in others Tumor cells 1 or others. Are the cells (CAFs) or other cells in closest vicinity to the bussing tumor cells also changed in expression profile? Again, still the contamination issue applies.

Figure 6
CAF contamination possibility is also here an issue. ECM remodeling is often done by fibroblasts. However, the question whether CAFs are altered in this respect (upon TC contact) needs also be addressed. Again, comparison to scSeq data is necessary here to clarify.

Some of the identified genes in the budding cells should be verified by IHC or IF.

Version 1:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors answered all my raised concerns appropriately.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The study authors have examined tumor budding at the proliferative front of the tumor for Stage II/III colon adenocarcinoma patients using spatial molecular imaging. They characterize interactions with cancer associated fibroblasts. I am impressed by the amount of validation they have done with various spatial assays, which when put together, represents an incredible body of work. I am sharing a few comments to help clarify the scope of this work:

We would like to thank the reviewer for stating that we have done an impressive amount of validation and that, together, our study represents an incredible body of work.

1. A methods/results overview table/figure/section would be helpful. It can be challenging for the reader to place all of these assays and subsequent analyses and what level of validation they provide in context.

We agree that an overview figure could be helpful for the readers. We have made such a figure, which is labelled Supplementary Fig. 1 in the new version of the manuscript.

2. The abstract could be more descriptive. For instance, what does it mean for budding cells

to have a different spatial distribution? These were colon adenocarcinoma cases, stage II/III, validation of certain findings was carried out through … and so forth. This could complement the methods/results overview.

We agree with the reviewer. The reason for our abstract being less informative was that we were under the impression that the abstract should be limited to 150 words. However, we have been informed that this is not the case, and we have therefore improved the abstract according to the reviewer's suggestions.

3. The authors correctly point out limitations in studying tumor budding in 3D though limitations could further be stated, especially in reference to recent research disputing which cells are actually budding due to the 3D structure of the tissue, introducing uncertainty in this analysis.

We appreciate this comment, and we have added a reference (PMID: 36669472) to recent research analyzing the 3D structure of the tissues, disputing which cells are actually budding, in the relevant paragraph in the discussion section.

4. At the beginning of the results, it should be stated how many patients were profiled and of what type. While in the methods we know which cell detection software was used, perhaps this could be mentioned briefly at the beginning of the results.

This information is now available in the overview figure labelled, Supplementary Fig. 1, as suggested by the reviewer. This figure is cited in the beginning of the result section.

5. The authors discuss cell clusters throughout the manuscript, but it may be unclear to the reader whether clusters reflect spatial distributions of cells or expression differences. Also, motivation for why expression was used to cluster should be further elaborated as a marker approach was already taken to define cell types. It would make more sense to call cell-types then annotate the tissue for characterizing their spatial distribution, perhaps using neural network segmentation approaches of these architectures as needed. As it is written it seems like the authors are trying to further subtype tumor cells through clustering but unclear why this was done across all cells where it could be done by just considering tumor cells alone through marker based approach then clustering. If clustering is used to help assign cell type, then it should only briefly be mentioned.

We agree that it may be useful to provide additional information on how cell clusters were defined and how cell types were called. The clustering was based on gene-expression. We performed UMAP analysis to visualize cell distribution patterns and unsupervised clustering using the Leiden algorithm to identify individual cell clusters (based on gene-expression). This has now been clarified in the new version of the manuscript. This was followed by interrogation of the top marker genes that defined each cluster as well as known canonical cell markers to label each cluster with a cell type. These annotations were further confirmed using the PanglaoDB database. Our approach showed high specificity and spatial coherence as shown in Figure 1 (Supplementary Fig. 2 in the new version of the manuscript), which displays the cosMx data from the adjacent normal colon tissue. Thereafter, we applied the same

pipeline for the adenocarcinoma tissues (no additional tweaking of the pipeline was done to tease out the different cancer cell clusters).

6. L108, the claim that PIGR is a colon-specific epithelial marker gene is not substantiated. Wouldn't the gene just correlate with existing epithelial gene sets, why is it specific to Colon? Also the discussion with spatial locale within the crypts could benefit from reviewing prior literature on colonic cell differentiation based on spatial location and where it is expected to find stem-ness markers etc. Perhaps some of these sections that have findings that appear orthogonal to the main claims could be moved to supplementary? The presented analysis on adjacent normal epithelium does not seem to tie into the main story. It seems like the authors are trying to validate SMI but I am not convinced this provides appropriate validation of the assay. How does this section lead us to conclude that SMI provides "highly accurate spatial gene expression"? Please be explicit because this is unclear and may also be limited by variation attributed to individual patients.

We thank the reviewer for this comment and agree that our statement on *PIGR* was not accurate. What we meant was that it is specific to epithelial cells in the colon, but we don't know if it is also a good epithelial marker in other types of tissues. This has now been clarified in the new version of the manuscript. In addition, we have now cited another paper also showing that *PIGR* is specific to epithelial cells in the colon.
We agree that the section on the adjacent normal tissue does not tie directly into the main story and, as suggested by the reviewer, we have now moved all these data to the supplementary information (Supplementary Fig. 2 in the new version of the manuscript).
We also agree that our analyses are not sufficient to thoroughly validate the SMI technology. However, this has already been done in the original *Nature Biotechnology* paper (PMID: 36203011). Rather we wanted to show its performance on colon tissues as well as use these analyses to move forward with an adequate, precise and coherent cell clustering method as described above.
Regarding stemness, we have decided not to add further analysis on this topic to keep the focus of the story on the adenocarcinoma tissues, in line with the reviewer not finding the analyses on normal tissues to be tied into the main story, but also due to the absence of key stemness markers such as *LGR5* in the 1,000-plex cosMx gene panel that we used.

7. L120 section: The selection of FOV across 4 patients: who selected these regions and how large were they? How were they annotated? The small sample size is a significant limitation given histologic and other sources of variation. What site from the Colon were they selected from? Tumor stage? N/M-stage? MSI status? BRAF/KRAS? I am assuming M=0 but else relationships are unclear.

We thank the reviewer for this comment as we agree that it is important to provide additional information about the patient samples in the manuscript. This has now been done both in the Results section and in the Methods section and we have prepared a Table with all this additional information (Supplementary Table 1 in the new version of the manuscript). The regions were selected in collaboration with an experienced pathologist (Dr. Henrik Hager).

8. What is a good operational definition of tumor budding and why is clustering then where one cluster falls spatially sufficient to call budding? Distance from tumor interface with certain density lacking certain number of other tumor cells within radius, etc?

In the clinic, a tumor bud is defined as an individual cancer cell or small clusters of up to five cancer cells that are located outside the tumor mass. With our unsupervised approach, described above, we identified at least one cluster that exhibited a markedly different spatial distribution in all FOVs. In contrast to the other cancer cell clusters, where most individual cancer cells belonging to a distinct cluster were in close physical contact, the cells were distributed throughout the tissues, encompassed most of the budding cells, and were always found at the outskirts of masses of other cancer cells belonging to other clusters. Thus, all these cells were in close contact with cells within the TME. As these differences in spatial distribution were very obvious, we decided to show all the data and all the annotations from all FOVs rather than using a strict definition.

9. “Cancer cells colocalize with PanCk-positive cells”– PanCk is an epithelial marker gene/protein though insufficient to delineate normal vs tumor epithelium. Why is this important or relevant or adds new information? Further annotation of the slide is needed to contextualize findings in relevant tissue regions.

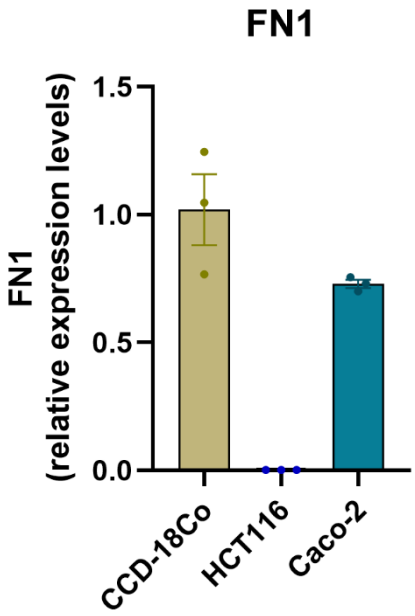
Seeing that the cells that were called as cancer cells, based on SMI data and our bioinformatics pipeline described above, were PanCk-positive by immunofluorescence was very re-assuring and serves as a validation of the SMI data (The immunofluorescence data are acquired independently and is not used for the clustering). This has been clarified in the new version of the manuscript.

10. L143: “unique spatial distribution” needs a more quantitative description.

This has been further clarified in the new version of the manuscript. It is stated that “In contrast, the cells from one of the cancer cell clusters (denoted as Cancer cells 4) appeared to be randomly distributed throughout the tissue. Interestingly, this cluster was found to encompass most of the budding cells, and upon closer inspection, it became apparent that individual cells were always found at the outskirts of masses of cancer cells belonging to other clusters. Thus, all these cells were in close contact with cells within the TME (Fig. 1E).”

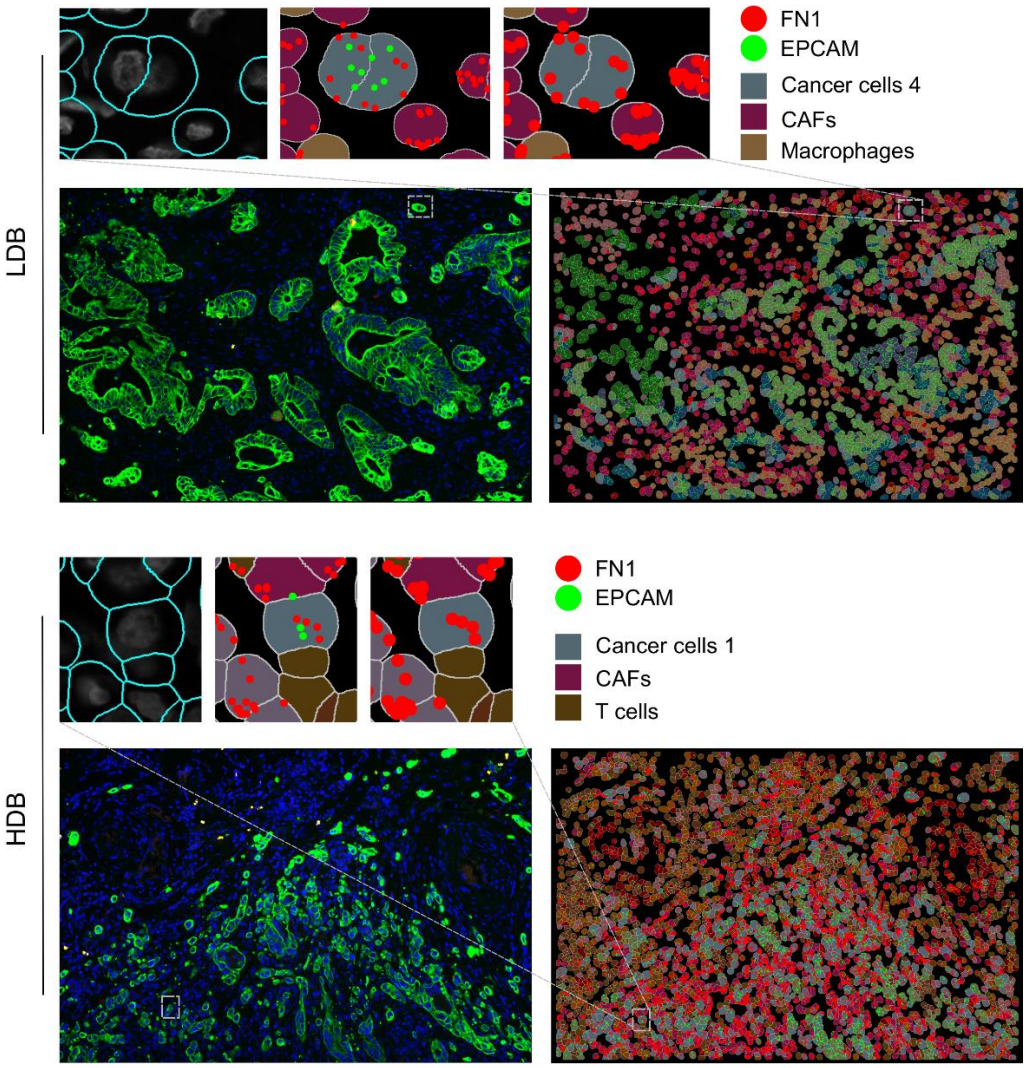
11. L152: FN1 as a CAF associated marker gene, is it possible that this gene is descriptive of colocalization with CAFs and that imprecise imaging/staining could be at play?

We appreciate this comment from the reviewer as this is something we have speculated ourselves. However, *FN1* has previously been shown to be present in cancer cells, both in patient samples and in colon cancer cell lines, and to play an important role in migration and metastasis (PMID: 29274284). In addition, for the reviewer, we have tested two cancer cell lines (HCT116 and Caco-2) and a colon fibroblast cell line (CCD-18Co) running in our laboratory and found almost as high levels of *FN1* in one of the two cancer cell lines as in the fibroblasts (see data below).



Moreover, the sensitivity of the spatial molecular imager for allocating detected transcripts to individual cells is very precise, in fact the number of transcripts falsely called is estimated as low as 0.0092 per cell (PMID: 36203011). The transcript annotations are generated in a three-dimensional super-resolution of the analytes and the resulting images are Z-stacked to make the final cellular composite with the complete annotated transcript location data from the Z-stacked images imposed. In addition, the segmentation annotations are made by using the Cellpose algorithm powered by the cell membrane and nuclei antibody staining information for every cell which allows for a precise segmentation approach diminishing the error in the labelling of polygonal boundaries.

To further convince the reviewer of the specificity of the *FN1* signal, we have prepared a representative plot showing *FN1* and *EPCAM* transcripts localization in tumor budding cells surrounded by CAFs from Low-Density Budding (LDB) and a High-Density Budding (HDB) FOVs (see Figure below).



12. The use of cancer cell cluster 4 etc is confusing, would recommend giving these discovered subtypes names and also perhaps more important to also consider the spatial distribution when subtyping.

The entire idea of our cluster analyses and subtyping was to do this in an unbiased fashion as described above (using the same bioinformatic pipeline as for the normal colon tissue). We believe that giving descriptive names to all the individual cancer cell clusters in all the FOVs would be confusing. We decided to refer to the clusters as "the cancer cell clusters that encompass most of the budding cells" as it would be misleading to call them budding clusters or something similar as they also contain cells that are not budding according to the clinical definition as described above.

13. Serial section H&E images side-by-side with Fig1-2 and related supplementary figures would be helpful.

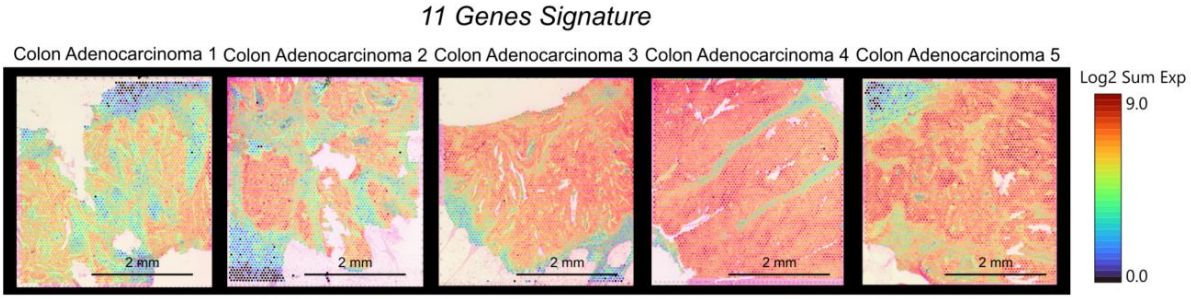
Unfortunately, we do not have the immediate adjacent tissue section slides available.

14. All FOV images should be in supplementary at minimum. How does spatial colocalization of cell-types vary between slides?

All FOV images are shown in the manuscript (See Supplementary Fig. 3, 5 and 6 in the new version of the manuscript). These images contain the spatial cell plots with cell types annotated next to the immunofluorescent composite images. We also supplied the results from the UMAP and Leiden algorithm as well as labeling of each cell type.

15. The 10X Visium analysis is qualitative and needs further assessment. For instance, could you define a tumor budding signature and other identified signatures that could be recapitulated and annotated spatially at minimum and how does it recapitulate the findings. Were these 55-micron spots? scRNASeq information could be incorporated for a cell-typing analysis. More detail on the patients are needed e.g., TNM stage, site, etc

The Visium 10X technology that we used has a total of 4,992 spots per capture area and each spot are 55 µm in diameter. This method has the advantage of performing whole transcriptomic analysis but does not have single-cell resolution. Rather each spot encompasses approximately 10 cells making it impossible to distinguish signals from tumor budding cells from the signal from surrounding stromal cells. Thus, we decided not to include data from the 11 marker genes, we found commonly upregulated in the cancer cell clusters that encompass most of the budding cells. We have included plots showing the results of these analyses for the reviewer (see figure below) but prefer not to add these to the manuscript. It can be observed that there is a higher expression of this gene signature in the invading tumor areas and in specific parts of the microenvironment suggesting the presence of budding cells. However, the low resolution prevents us from making strong claims related to these data.



Regarding the use single-cell RNAseq data for cell typing analysis, it is something that our team was discussing during the development of the project, however, the fact that the SMI technology uses a pre-designed panel consisting of only 1,000 genes, prompted us to employ a different strategy, since our agnostic (single-cell reference-based) analysis would carry a clear bias from the 1,000 genes that are present, which potentially could lead to overrepresenting cell types falsely. Rather, we decided to first apply the gene expression-based Leiden algorithm for clustering of the cells, second, to use known canonical markers to identify the main cell types, and third, to use the PangaLao database to confirm our cell type annotations.

16. What is the background set of genes for the GSEA analysis?

We did not specify a background set of genes when using WikiPathways, Reactome or Biological processes. This is a valid approach since what we analyzed were only the differentially expressed genes.

17. Discussion: What are limitations of SMI? The reliability of the assay has been disputed. Further review of the literature could be done here.

In the discussion section, we have now discussed the limitations of the analyses being done in 2D and the limitation of using the 1,000-plex CosMx panel.

18. “Perfect overlap with PanCk IF staining” needs to be substantiated, I would guess would not be perfect.

We agree with the reviewer and have now changed the wording from “perfect overlap with PanCK IF staining” to “strong overlap with PanCK IF staining”. In adjacent normal tissue (Supplementary Fig. 2A, D, E in the new version of the manuscript), PanCK immunofluorescence (green) robustly labels colon epithelial cells at the crypts. *PIGR* staining (Supplementary Fig. 2G–J) aligns strongly with this pattern. Heatmap data further confirmed the high expression of additional epithelial markers, supporting our revised claim of a "strong overlap" between spatial annotations and PanCK positive staining.

19. L307-312: Consider removing/revising most of these lines as this section is misleading/confusing to the readership and may not reflect our current understanding of epigenetics. The phrasing is awkward.

Epigenetics is generally defined as gene-expression changes that are stably inherited through somatic cell divisions without changes in the DNA sequence and may thus be traceable during clonal evolution. For instance, cancer cells often acquire DNA methylation in the promoter regions of tumor suppressor genes, which in concert with histone modifications, lead to their inactivation, which is passed on from dividing cancer cells to daughter cells. Accordingly, we do not believe that our phrasing is awkward and prefer to keep it.

20. CAFs seem to be the central part of this story. They, however, have many roles and should be further subtyped to further characterize their interactions. A review of the CAF sub typing literature on tumor progression is needed. The nature of the CAFs in this analysis and their significance is unclear even though it is supposed to be central.

We agree that this is a good suggestion. Thus, we attempted to perform CAF subtyping annotations based on Khaliq AM et al. classification (PMID: 35538548), however, as shown in the table below, most of the reference markers for CAFs subclasses were not present in the CosMx 1000-plex panel and thus we decided not to include further characterizations of CAFs in the new version of the manuscript. However, we have added a comment about this limitation in the discussion section.

CAF activation	Adhesion-Wound healing-CAF-S1	CAF-S1-iCAF	CAF-S1-MY	IL-iCAF	detox-iCAF	Perivascular CAF-S4	ecm-myCAF	TGFBeta-myCAF	Wound-myCAF
FAP	FAP	CXCL12	FAP	SCARA5	ADH1B	RGS5	GJB	CST1	SEMA3C
ACTA2	PDPN		PDPN	DLK1	GPC3	CSPG4	ANTRX1	TGFB1	ANTRX1
S100A4	PDGFRA		PDGFRA	CXCL12	CXCL12	PDGFRB	SDC1	ANTRX1	CD9
IL6			COL1A1			CD248		LAMP5	
IL1B			COL1A2			EPAS1			
NNMT									

21. L385-399: Xenium and Visium HD could be compared and discussed here along with other related assays e.g., CODEX, COMET, validation with merScope, etc. Also, DSP could potentially characterize a neighborhood of budding cells.

We do not think an extensive discussion on the different spatial transcriptomics platforms available is warranted, as it was not the scope of this study to compare the performance of the different platforms.

22. Methods L606-609: T and N stage for patients are needed along with other relevant histological features: e.g., were tumors mucinous? Exclusion criteria? Simultaneous metastasis to the lymph nodes can impact the findings substantially.

This comment has been addressed above.

23. Cell detection accuracy is unclear and a comparison between methods is warranted.

This comment has been addressed above.

24. How are batch effects / normalization being accounted for?

We do not expect any batch effects as all the data comes from the same slide. We performed a normalization by total library size, custom scale factor, log transformation and z-scoring and excluded the FOVs having a less than 250 transcripts on average per cell (as indicated in the new Supplementary Fig. 1).

25. What are the parameters of the neighborhood analysis? E.g., construction of spatial network, enrichment at specific distances, etc. Unclear if Leiden clustering is appropriate. Some of the cell types seem redundant and perhaps their spatial location is important, potentially necessitating spatially-informed labeling. See also my earlier comment for strategies on sub typing cell types determined through marker gene approaches versus clustering all cells then subtyping.

The construction of the network was performed using the Delaunay triangulation method (https://pmc.ncbi.nlm.nih.gov/articles/PMC6767241/). The spatial proximity enrichment between pairs of cell types is calculated based on the Delaunay data and calculating observed over expected frequency of cell-cell proximity interactions. This frequency is the average from the number of spatial network simulations. These simulations are performed by reshuffling the cell type labels from each cell in the

spatial network. Thereafter, the cell proximity scores show the enrichment results. The reason for performing Leiden clustering and then cell typing was explained above.

26. The nested nature of this analysis warrants further consideration. For instance, paired normal adjacent FOVs nested within patient with 10 additional ROIs at the primary tumor. Hierarchical regression modeling with covariate adjustment and inclusion of potential effect modifiers is warranted, or incorporation of analogous metaanalytic regression modeling. Statistical findings should be reported across all FOV rather than by one FOV at a time. Considering one FOV at a time makes it seem like results are being picked for how they best tell the story.

We strongly disagree with the statement that considering one FOV at a time makes it seem like results are being picked for how they best tell the story. Rather our analyses were performed completely unbiased using the same pipeline for all FOVs as described above and no additional tweaking of the pipeline was done to tease out the different cancer cell clusters observed for each FOV. The fact that we consistently rediscovered the same findings across different FOVs and samples from different patients, along with validation through multiple approaches, demonstrates the robustness of our conclusions.

27. How is density calculated? How is spatial autocorrelation between cells accounted for in differential expression? Calculating the gini coefficient for differential expression analysis as a main finding seems highly unusual and unclear how this analysis considers the nested dependency structure of the data.

The Gini coefficient is an established method for detecting rare cell populations distinct from dominant groups, aligning with our focus on tumor budding cells. As demonstrated in Jiang et al. (Genome Biol 2016, [PMID: 27368803]), the GiniClust algorithm leverages gene expression heterogeneity via the Gini index outperform conventional methods in identifying rare subtypes.

Reviewer #2 (Remarks to the Author):

The authors employed a 1,000-plex gene panel, and have defined major cell types in tumors and adjacent normal tissue with accurate spatial localization. They found that certain cancer cells were scattered around the periphery of the main cancer cell masses and were more frequently in contact with cancer-associated fibroblasts (CAFs). They imply that that CAFs induce pro-invasive gene expression changes in those cancer cells involved in epithelial-mesenchymal transition, extracellular matrix binding, and migration.

We appreciate that the reviewer has clearly understood the message of our study.

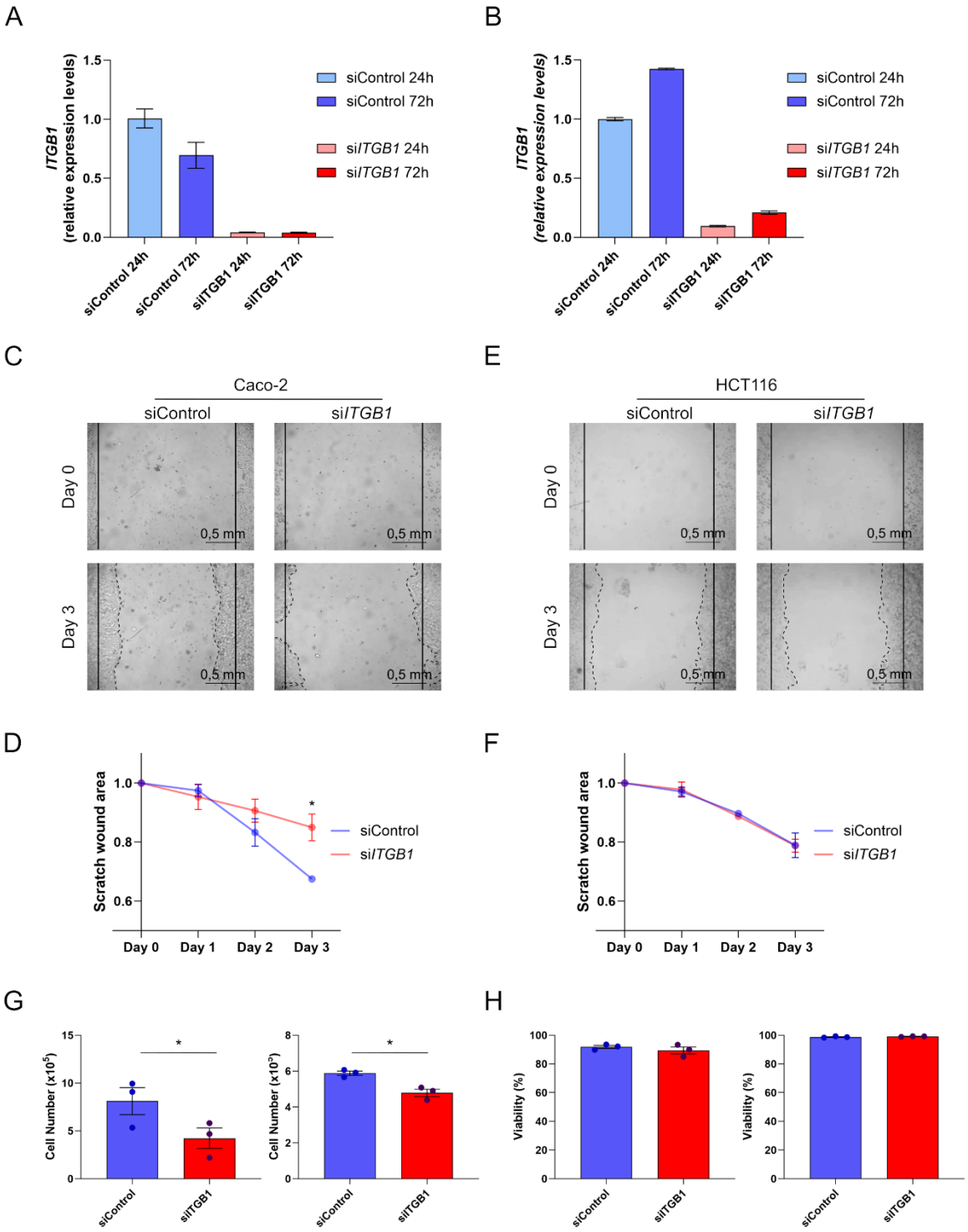
Major Remark: The study remains highly speculative and without any validation. The conclusions made would require in vivo tracing analysis to determine the faith of the selected cancer cells. While the reviewer understands that this is beyond the scope of the present

manuscript, some level of functional analysis is clearly needed, even in vitro.

We have validated the robustness of the SMI technology in several ways and were able to consistently rediscover the same findings across different FOVs and patient samples, demonstrating the strength of our conclusions. While we agree that substantial functional analyses are beyond the scope of this study, we have performed some in vitro studies to further substantiate our findings.

In particular, we have interrogated two human colorectal cancer cell lines for the pro-budding role of Subunit β Integrin gene (*ITGB1*), which is one of the 11 genes part of the budding signature that we found to be commonly upregulated in the clusters that encompasses most of the budding cells.

***ITGB1* has been described to be key for the metastatic progression in several types of tumor cells and it has been especially well described in breast cancer showing its key role in metastasis through focal adhesion stabilization and cell survival (PMID: 30719702). However, the effects of *ITGB1* on tumor budding in CRC requires further research. Thus, we targeted *ITGB1* by transfecting specific siRNAs using siPOOLs technology on Caco-2 and HCT116 cell lines to then study cell viability, cell proliferation and changes in cell migration by performing a wound healing assay. The results are shown as Supple. Fig. 12 in the new version of the manuscript and also shown below. It can be observed that the silencing was effective and sustained the time of the experiments (72h) (See panels A and B). The wound healing assay revealed a dependency on the *ITGB1* gene for the migratory potential of Caco-2 cells, being statistically significant after 72h (See panels C and E). However, this was not observed in HCT116 cells after silencing (See panels D and F). Regarding cell numbers, both cell lines showed a significant reduction in the growth (See panel G), whereas the cell viability was unaffected (See panel H). These results suggest that different mechanisms for migration might be used by these cell lines, and that ITGB1 is important for this process in Caco-2 cells. Noteworthy, both cell lines have very different molecular backgrounds. Caco-2 cells are characterized by *APC* and Beta-Catenin (*CTNNB1*) mutations, whereas HCT116 are driven by a *KRAS* mutation and MMR defects, which may explain the different response found upon *ITGB1* silencing.**



Reviewer #3 (Remarks to the Author):

This solely descriptive study deals with the spatial profiling of the invasive front of colon cancer. Normal colon is used as a model to test the capability of the method. The authors claim to identify a special gene expression profile of budding cancer cells induced by direct interaction with CAFs using the CosMx Spatial Molecular Imaging by employing a 960 plex gene panel at the mRNA level. This is done in sections of FFPE tissues. The study is technically sound; however, I have some major concerns about the analysis and the

interpretation of the data.

We appreciate that the reviewer finds our study technically sound.

Major points

Figure 1
Add size bars to each image (also in Figure 2 and all Suppl. Figures with microscopic images).

This has now been done.

I wonder why in the normal colon tissue there are no endothelial cells (blood and lymphatic) and macrophages identified? These cells are known to be present in the colonic tissue in the stromal part at quite high numbers. Please comment why these cells were not identified. (they are clearly identified in the clusters from the tumor sections in Figure 2A-C). If they are not detectable this would challenge the quality of the method to identify all relevant cell types in tissues.

We agree with the reviewer that we may expect to see these cell types. However, we only analyzed one FOV from adjacent normal tissue and it is entirely possible that they're not present in this particular area. Likewise, there was also a quite large area where no T-cells were detected (See Supplementary Fig. 2 in the new version of the manuscript). In line with this, the heatmap shows that the top marker genes defining each cluster do not include any markers for blood endothelial cells (BECs) or lymphatic endothelial cells (LECs), namely CD31 (PECAM1) and VWF for BECs and LYVE1 and PROX1 for LECs. Indeed, LYVE1 and PROX1 were not detected at all in the whole FOV after filtering. On the other hand, we had a robust detection of endothelial cells and macrophages in other FOVs indicating that the method is capable of detecting these cell types.

Moreover, pericryptal fibroblasts are well established to form a thin layer of cells around the crypt, characterized by alpha SMA expression. Identification of these cells would be a perfect proof that the SMI really can detect structures at the cellular (or even subcellular …as stated by the authors) level. Maybe the unknown cluster are these cells (The marker genes like PDGFRA, CDH11, CXCL12 shown in Fig. 1F at least point to **fibroblast like cells** as the potential cell types for the unknown cluster. Show a closeup picture of the of the unknown cluster.

From our analyses (Supplementary Fig. 4 in the new version of the manuscript), it is clear that the "unknown cluster" contains a mixture of different cell types with different spatial localization. Thus, it will not be appropriate to label the unknown cluster as pericryptal fibroblasts. However, we have made a comment that some of the cells within this cluster could be pericryptal fibroblasts in the new version of the manuscript. At this point, we feel that the limitation of having performed the analyses using a 1,000-plex panel preclude us from making firm statements regarding the cells present in the "unknown cluster".
We are not using subcellular information in our manuscript, and we have therefore deleted this statement in the new version of the manuscript.

How about LGR5+ CBCs? Why are these cells not identified? These cells are known to be at the crypt base in rather high numbers. For PIGR and OLFM4 show the corresponding cell types in (publicly available) datasets from single cell mRNA sequencing of normal colon. All together the presented data do not fully support the full identification of different cell types present at substantial quantity in normal colon.

Regarding the LGR5+ population, unfortunately this gene is not part of the panel, thus not allowing us to further characterize these cells. Again, the limitation of having performed the analyses using a 1,000-plex panel preclude a perfect identification of all possible cell types.

Figure 2
Four patients are used for the analysis of the colon cancers. Please identify the clinical characteristics and mutational profile (at least for driver mutations) in a separate table (e.g. pathological description, age, sex, pretreated or not, mutations, type of tumor, primary or metastasis, location etc.).

We thank the reviewer for this comment and all of this information is now available in Supplementary Table 1 in the new version of the manuscript.

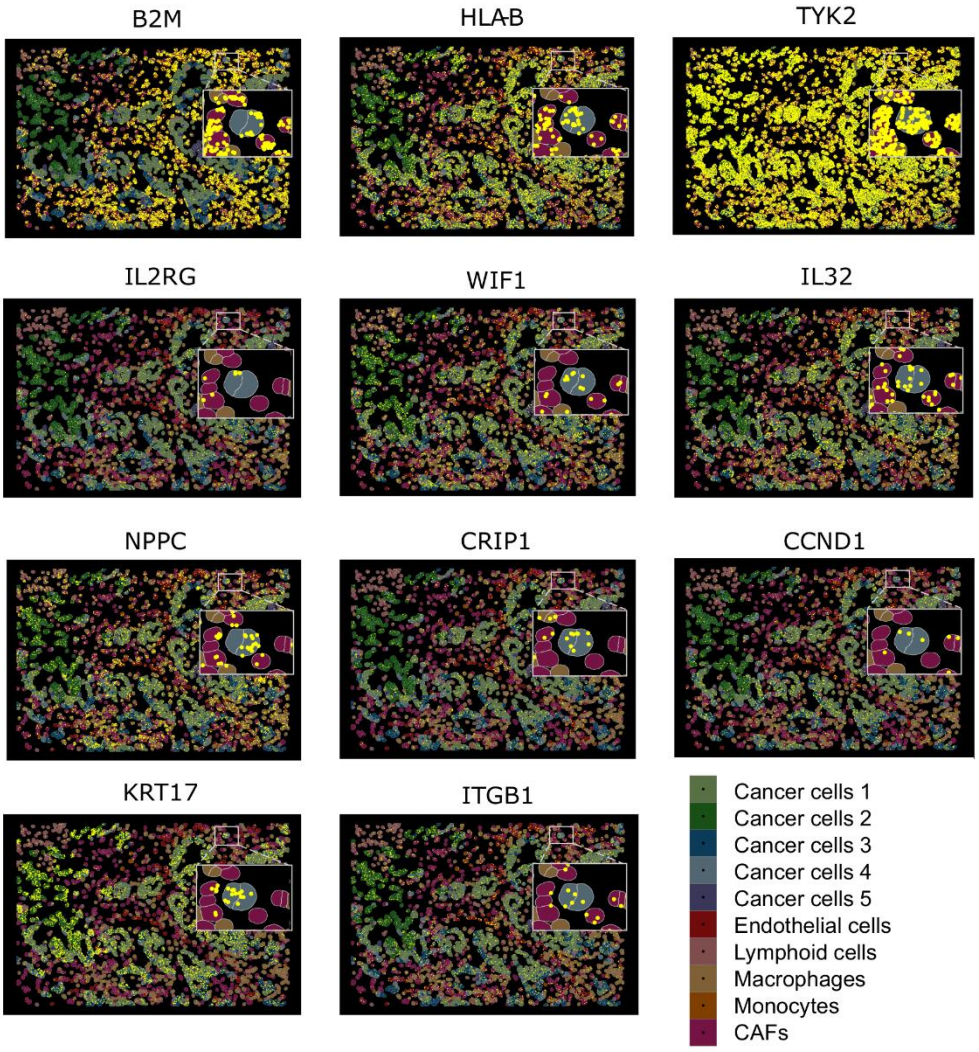
Why is the expression level in Figure 2 G, H always an integer? This seems not the case for Figure 1J. Please comment and explain.

This is because the data matrix is sparse, meaning that this technology produces count-based data, which after normalization can result in integer-like distributions. This is due to the CosMx technology being image-based, making the gene detection ranges lower than other sequencing-based methods (Visium 10X, for example). On the other hand, for highly abundant genes, such as *PIGR* in the adjacent normal tissue, it appears as more continuous range of values after normalization.

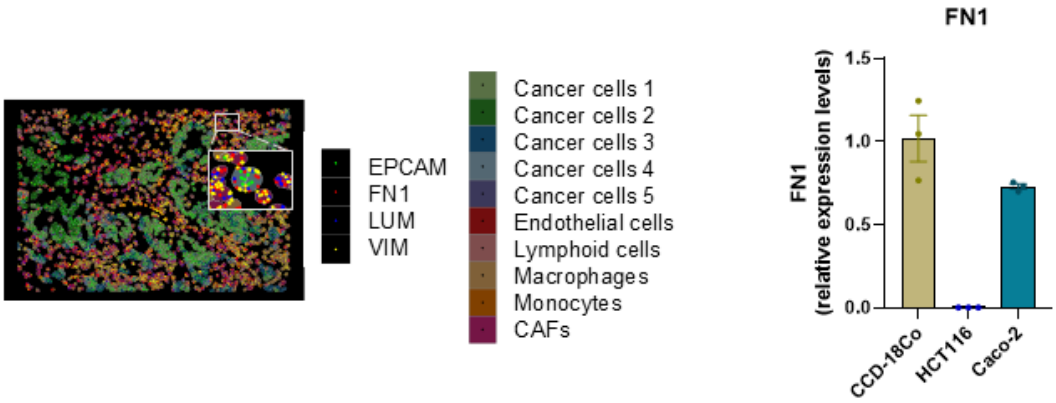
An intrinsic problem of spatial profiling is always the exact topological source of expression of genes. There is always the possibility that at a certain point in the slide two or more cellular structures (meaning parts of cell bodies of different cell types) might overlap or are situated so close that the spatial profiling raster is not able to resolve this. These mixtures of cells then also lead to a mixture of expressed RNAs at the certain point and might lead to a false classification of expression signatures of the cell type, which was characterized by signature genes for the rough distinction of cell types such as epithelial cells and stromal cells or different immune cells. In the light of this study, where the major focus of interest is the tumor budding cell area at the invasive front at which there is close contact to the TME and the cells within it, one has to be extremely cautious in interpreting the obtained expression profiles. For example, one can easily see an obvious contamination of CAF and lymphoid cell signatures in the tumor cell 4 fraction in the heatmap of Figure 2f or Figure 2h. The other tumor cell types, being further away from the budding zone (as shown in Figure 3D, H do not show this contaminating signatures. This is also supported by the fact that the authors show that these cells are in closest contact with fibroblasts in Figure 5)
Thus, the analysis of the EMT state and the tumor cell type 4 association with the mesenchymal state is most likely due to the fact of the mesenchymal cell contribution at this

specific budding sites. Hence, interpretation of the EMT data might be done with caution and should be clarified in the manuscript. (see below for validation of the data by single cell seq analysis).
The identification of the specific expression signature of Tumor cells 4 in Figure 2JK seems to be appropriate (i.e to exclude all other tumor cell and CAF signature genes). Some of these 11 genes are tightly connected to immune cells, thus also here it might be due to immune cell contamination.
Taken together all these points, there is need to analyze the identified subtypes from this spatial analysis presented here with single cell data and correlate the findings. In single cell sequencing, if proper single cell dissociation is done, there is no contamination issue from other cells. Therefore, a combination of these two methods has the power to clarify the raised points in this study and any other spatial analysis. Hence, for this manuscript the cell groups identified need to be found and validated in a scRNA Seq dataset (which are available). This needs to be done to validate the conclusions made in this manuscript (i.e. to find the tumor cell 4 expression profile in single cell data and to validate the findings.

We appreciate this comment from the reviewer and understand the concerns about potential cell contamination and to be cautious with our findings using the Spatial Molecular Imager (SMI). However, it is important to bear in mind two factors here. First, the SMI sensitivity for allocating detected transcripts is very precise, in fact the number of transcripts falsely called is estimated as low as 0.0092 per cell (PMID: 36203011). These transcripts annotations are generated in three-dimensional super-resolution of the analytes and the resulting images are Z-stacked to make the final cellular composite with the complete annotated transcript location data from the Z-stacked superimposed images. Secondly, the cell segmentations are made by using the Cellpose algorithm powered by cell membrane markers (CD298/B2M) and nuclei marker antibody (DAPI) staining for every cell allowing for a precise segmentation approach, diminishing the error in the labelling of polygonal boundaries even in high density cellular areas. Furthermore, it should be noted that the EMT transition of cancer cells is an event that has been captured many times using different methods during many years of research on metastasis and that it has also been validated using various *in vivo* and *in vitro* model systems.
Nevertheless, we do acknowledge the possibility of having some level of cross-contamination, especially in high density areas in the TME where the tumor buds are in contact with other cell types such as CAFs or immune cells opening the fair discussion on whether these transcripts are barely cross-contamination. For this reason, we have prepared representative spatial plots zooming in on two budding cells with no immune cells nearby showing each of the 11 genes that were commonly upregulated in the Cancer cells 4 cluster from FOV1 (see figure below, which is also now included in the new version of the manuscript). It can be observed that the transcripts are clearly located within the boundaries of the cells and that the three genes, which we expect the reviewer to consider part of a lymphoid cell signature (HLA-B, IL2RG, and IL32) are clearly expressed in these (panCK positive) cancer cells.



In addition, we have included other canonical markers of CAFs such as *VIM*, *LUM* and *FN1* and also show the epithelial marker *EPCAM* transcript location in representative tumor budding cells surrounded by CAFs. Moreover, it should also be noted that *FN1* has previously been shown to be present in cancer cells, both in patient samples and in colon cancer cell lines, and to play an important role in migration and metastasis (PMID: 29274284). In addition, for the reviewer, we have tested two cancer cell lines (HCT116 and Caco-2) and a colon fibroblast cell line (CCD-18Co) running in our laboratory and found almost as high levels of *FN1* in one of the two cancer cell lines as in the fibroblasts (see data below).



We believe that this data further supports the strength of our findings regarding the transcriptomic changes occurring in the budding cells.

We also analyzed these 11 genes using another spatial platform, called GeoMx, where we also captured an increased signal from cancer cells at the invasive front areas relative to the central parts of the tumors for seven of nine genes detectable by GeoMx (see Supplementary Figure 9 in the new version of the manuscript).
As kindly suggested by the reviewer, to further substantiate the robustness of our results, we have also validated our findings using single-cell RNA seq data from colon cancer patients, namely from Pelka et al (PMID: 34450029). This dataset consists of 371,223 cells from colorectal tumors and adjacent normal tissues. Again, we found a higher expression of the gene-signature in cancer cells undergoing EMT and higher expression in the more advanced T3 and T4 tumors compared to T1 and T2 tumors and compared to epithelial cells from adjacent normal tissues (see Figure 2E-G in the new version of the manuscript).

Figure 3, 4
All the concerns about contaminating signatures have to be taken into account in the analysis presented in Figure 3 and 4.

These concerns are addressed above.

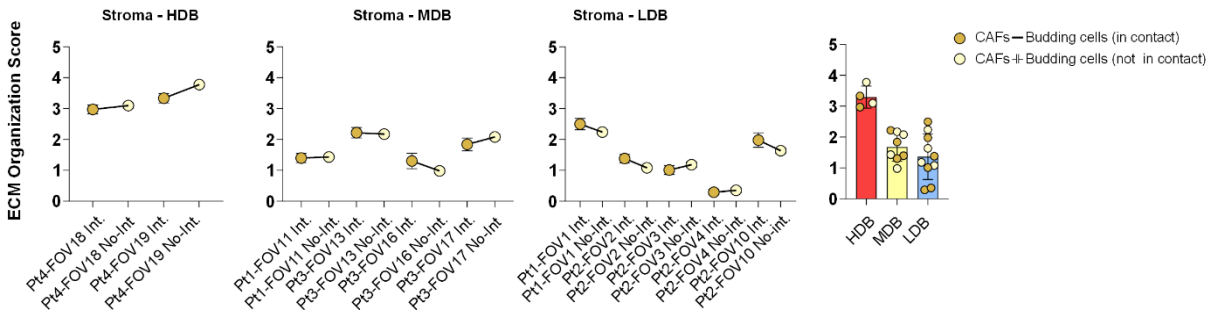
Figure 5
This analysis has high potential to measure which cells really impact the budding tumor cells. And also underline the importance of cell contamination as raised before. How are the heatmaps generated? How “their neighboring cells” is defined? What is the basis of cell- cell interaction distances measurements? Does the software measure the distance between two cells? This need s to be clarified for the reader. Besides, it would be helpful to keep the nomenclature of the tumor cells from FOV1 consistent with the other FOVs. It is not intuitive that in some FOVS tumor cells 4 are the budding cells and in others Tumor cells 1 or others. Are the cells (CAFs) or other cells in closest vicinity to the bussing tumor cells also changed in expression profile? Again, still the contamination issue applies.

We have now added a more detailed explanation on how the cell to cell interaction approach were annotated. We agree with the reviewer that this will help streamline the reading and understanding of the manuscript.

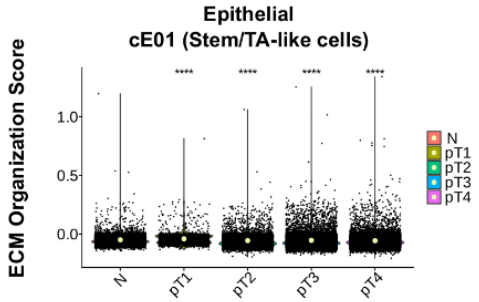
The idea of our cluster analyses and subtyping was to do this in an unbiased fashion using the same bioinformatic pipeline as for the normal colon tissue. We believe that giving descriptive names to all the individual cancer cell clusters in all the FOVs would be confusing. Thus, we decided to refer to the clusters as “the cancer cell clusters that encompass most of the budding cells” as it would be miss leading to call them budding clusters or something similar as they also contain cells that are not budding according to the clinical definition of budding cells.

Figure 6
CAF contamination possibility is also here an issue. ECM remodeling is often done by fibroblasts. However, the question whether CAFs are altered in this respect (upon TC contact) needs also be addressed. Again, comparison to scSeq data is necessary here to clarify.

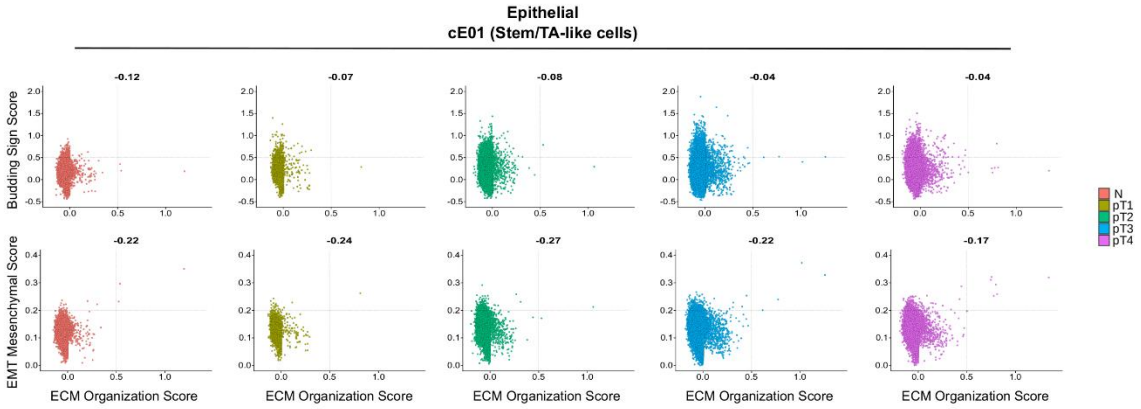
We have created a figure to clarify the reviewer’s point regarding ECM organization and potential CAF contamination (see below). First, we present data derived from stromal cells either in contact with tumor budding cells or not, scored for "ECM organization" (panel A). Overall, stromal cells exhibited higher levels of ECM organization compared to budding cells, which aligns with the reviewer’s observation that CAFs are main players in ECM remodeling. However, the ECM organization scores for budding cells (Figure 6A) appear different from with those of stromal cells in contact with budding cells (see figure below and compare with Figure 6A). Thus, since their distributions differ, this highlights the specificity of this signal rather than indicating that it results from a confounding signal from the stromal cells.



Additionally, as suggested by the reviewer we also analyzed the scSeq dataset described above with respect to these findings. Here, we again focused on the epithelial cell subset cE01 (Stem/TA-like), which shares similar characteristics with budding cells as based on our CosMx data (new Figure 2). The violin plot shown below reveals that some epithelial cE01 (Stem/TA-like) cells, in advanced stages (pT3 and pT4), exhibit higher scores for "ECM organization," thus supporting our findings.



Besides, scatter plots revealed that those cE01 cells scoring high for “ECM organization” also score high for "Budding sign" and "ECM Mesenchymal state" in tumor stages 3 and 4 (pT3 and pT4), suggesting that these epithelial cells, likely budding cells, may contribute to ECM organization.



Some of the identified genes in the budding cells should be verified by IHC or IF.

We agree with this comment and have analyzed the expression of the genes at the protein level using the human protein atlas (proteinatlas.org). This was done for 8 genes (of the 11-gene signature) as data were not present for WIF1, and for CCND1 and IL2RG no identifiable budding areas were found in any of the colorectal cancer tissues available in the database. These protein in situ data further support our findings, as we generally found higher staining intensities at invasive front areas and in budding cells (see Figure 2H in the new version of the manuscript).