EMBO reports

Combining organotypic tissue culture with light-sheet microscopy (OTCxLSFM) to study glioma invasion

Alicia Haydo, Andrej Wehle, Christel Herold-Mende, Donat Kögel, Francesco Pampaloni, and Benedikt Linder
DOI: 10.15252/embr.202356964

Corresponding author(s): Benedikt Linder (linder@med.uni-frankfurt.de) , Alicia Haydo (AliciaSusana.Haydo@kgu.de), Francesco Pampaloni (fpampalo@bio.uni-frankfurt.de)

Review Timeline:

Submission Date:	9th Feb 23
Editorial Decision:	14th Mar 23
Revision Received:	13th Sep 23
Editorial Decision:	28th Sep 23
Revision Received:	11th Oct 23
Accepted:	13th Oct 23

Editor: Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Linder

Thank you for the submission of your manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this message.

As you will see, the referees indicate that these findings are of interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed as indicated in the reports, I will not detail them here.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures (up to 8) and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

Please consult our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

3) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

9) Please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend.

10) Please also note our reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

12) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions and remove the author contributions from the manuscript. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

13) Please provide a comprehensive title with not more than 100 characters (including spaces).

14) Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section -

Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

This study by Haydo et al seeks to offer a new light sheet imaging technique to explore GBM migration after transplantation to slice cultures. New ways of exploring migration are sorely needed in the field, which makes this approach exciting. The descriptions of the approach are exciting, but the figures and details make it hard to understand how this is a measurement of migration. More clarification on this point is needed to fully support the study.

1 - Figure 1 of the paper is validating previous data and the system and could be moved to supplement.

2- Fig 2 makes it very hard to see the tumor cells - higher magnification, more angles, ideally videos of the 3 dimensions would be helpful. Also, we cannot see cell morphology in this or any of the pictures shown which is crucial for understanding migration.

3- Can cell type be associated with the migratory populations, even after the experiment?

4 - Letter labels for Figure 2 are confusing, so hard to know which panel exactly I am looking at, but the colors and the side view make it seem like very little invasion or imaging of invasion is happening.

5- In Figure 2, 3, and others, the scenarios shown involved depleting the tumor as well as the parameters of invasion. With less tumor, there will naturally be less migration. Including experiments that inhibit motility but not tumor burden (so can we watch the tumor actually expand but migrate less) would be ideal to validate this method for migration analysis. I know this is kind of the point of the CD9 experiments, but the results are variable between cell lines so it's hard to say which "should" be the result in each line.

6 - Tools for how to analyze this data are not explained in enough depth and go hand in hand with using this technique so should be included on code repositories.

7- Very hard to visualize Figure 5, and the comparison to confocal microscopy is not well explained.

8 - In general, if this is all based on light microscopy, the images of the cells, their process invading, and the morphology/cell type need to be much better demonstrated for it to be convincing.

Referee #2:

In this study, Haydo and collaborators combined two state-of-the-art techniques, adult organotypic brain tissue slice culture (OTC) and light sheet fluorescence microscopy (LSFM) of cleared tissues in a combined method termed OTCxLSFM that might be useful to understand the pathophysiology (the migration activity) of GBM tumors. In particular, authors used Arsenic Trioxide as well as genetic depletion of the tetraspanin, transmembrane receptor CD9 as examples to reveal the usefulness of the novel combined method.

This is an interesting study that reveals a novel approach to further characterize the biology of GBM. The manuscript is well written and the figures are of quality.

As a limitation, the authors used models (ATO and CD9 depletion) previously described by them to be involved in GBM migration and invasion. It would be important to use an additional approach in order to validate their model. Experiments with TMZ in GS-5 GFP/Luc-tumors and silencing of a stem cell regulator such as Sox2, Bmi1 ... would give further insight and validation of the proposed novel method.

Referee #3:

Major comments:
- LSM is able to offer information regarding depth of GBM cell invasion into the tissue slice. However, this is not being provided. Instead, metrics such as dispersion are being quantitated which only measures invasion in 2 dimensions. Its entirely possible that simply measuring invasion within the slice as opposed to across the slice is more informative.

- Consistency in the brain slice is missing. It is assumed that while adjacent tissue sections are being used, it is less clear whereabouts from the brain the slice originates. This does matter if invasion is being measured.

- Histologic analysis of the brain slice used is not discussed. Viability of this microenvironment should be presented.

-More discussion on how this method correlates with non-tissue findings is recommended.

Minor comments:
- Legend for Figure 2 does not match Figure 2 in the pdf.
- Images for Figure 1D are not placed properly and overlaps with Y-axis label for graph adjacent to it.
- Figure 5D represents data from tumors grown on brain slices in Figure 5C, but there were 11 and 7 tumors that could have been selected for analysis. Instead only 3 and 2 were selected. No rationale was provided for this selection and could lead to bias. This is also observed for Figure 6.
- Figure 5E should also list the N= if based from Figure 5D.
- Some of the Supplementary Figures are also inconsistent with the results text and legends.

Revision EMBO Reports

1. Referee #1:

This study by Haydo et al seeks to offer a new light sheet imaging technique to explore GBM migration after transplantation to slice cultures. New ways of exploring migration are sorely needed in the field, which makes this approach exciting. The descriptions of the approach are exciting, but the figures and details make it hard to understand how this is a measurement of migration. More clarification on this point is needed to fully support the study.

1.1.

Figure 1 of the paper is validating previous data and the system and could be moved to supplement.

Answer: Thank you for this suggestion. However, we would like to point out that we have not directly demonstrated anti-migratory effects of ATO in our previous work. The data shown in Fig. 1 rather confirm conclusions drawn from a prior proteome analysis (Linder et al, 2019a) and we believe that the assay providing proof for ATO-dependent effects on GBM migration represents a novel development. We have therefore revised the respective text passage to make this clearer and would prefer to have this figure remaining in the main article (Page 4: Results: Arsenic trioxide inhibits migration in vitro and tissue infiltration ex vivo)

1.2.

Fig 2 makes it very hard to see the tumor cells - higher magnification, more angles, ideally videos of the 3 dimensions would be helpful. Also, we cannot see cell morphology in this or any of the pictures shown which is crucial for understanding migration.

Answer: The now provided Source Data includes videos of the 3D-Projection for all light sheet images for better visualization, as well as the segmented color-coded cells for Fig. 2.

1.3.

Can cell type be associated with the migratory populations, even after the experiment?

Answer: That is an interesting question. We assume the author is referring to several cell states (e.g. proneural vs mesenchymal). We believe that these cell states are highly plastic and this would require specific marker/reporters rather than morphology. The biggest caveat by using antibody-based detection of proteins is that generally each staining needs to be optimized and requires extensive incubation times, which likely negatively affect the tissue complicating downstream analyses. For this reason, we used GFP-expressing tumor cells. To analyze different cell types, proteins tagged with fluorescent tags could be used as genetic reporters for different cell types in follow-up studies. Accordingly, Schmitt et al (2021) developed reporter cassettes based on glioblastoma subtype signatures (proneural, mesenchymal and classical) to investigate tumor heterogeneity and cellular plasticity (e.g. proneural-to-mesenchymal shift) in vitro and in vivo. We have now expanded our discussion section to address this point properly.

1.4.

Letter labels for Figure 2 are confusing, so hard to know which panel exactly I am looking at, but the colors and the side view make it seem like very little invasion or imaging of invasion is happening.

Answer: We apologize for this error. We mistakenly had uploaded a pre-final version of this figure. The conclusions previously drawn from these experiments are not affected by this change, but the new (correct) figure now fits to the figure legends and the main text.

1.5.

In Figure 2, 3, and others, the scenarios shown involved depleting the tumor as well as the parameters of invasion. With less tumor, there will naturally be less migration. Including experiments that inhibit motility but not tumor burden (so can we watch the tumor actually expand but migrate less) would be ideal to validate this method for migration analysis. I know this is kind of the point of the CD9 experiments, but the results are variable between cell lines so it's hard to say which "should" be the result in each line.

Answer: This is a valid point and we agree that one confounding factor is that reduced tumor sizes will result in an apparent reduction of migration. We tried to approach this issue by focusing on maximally migrating cells in both pharmacological and genetic targeting. To our knowledge, there is no distinct inhibitor of GBM migration exclusively targeting only cell migration making it rather difficult to analyze migration independent of other cancer hallmarks. To better approach the question raised, we chose to target the JAK/STAT3 signaling pathway that is known to act as a key player of cell migration and infiltrative tumor growth in GBM. In line with this well known function of JAK2/STAT3 signaling, pharmacological blockade of JAK2 activity with WP-1066 (ClinicalTrials.gov ID: NCT01904123) and CRISPR-Cas-mediated ablation of STAT3 led to robust and very similar inhibitory effects on tumor dispersion in the OTCxLSFM model (EV3), further underscoring the validity of our obtained results.

1.6.

Tools for how to analyze this data are not explained in enough depth and go hand in hand with using this technique so should be included on code repositories.

Answer: Thank you for pointing this out. We have now further elaborated the materials and methods section detailing how the images were processed. The used Macros for Fiji are able to access via Zenodo: https://doi.org/10.5281/zenodo.8332648.

1.7.

Very hard to visualize Figure 5, and the comparison to confocal microscopy is not well explained.

Answer: We apologize for the sub-optimal presentation of Fig. 5. We have now revised this figure and improved the part on comparison to confocal microscopy in the discussion. We added the source data, videos of the 3D projection, as well as arrows in the montages.

1.8.

In general, if this is all based on light microscopy, the images of the cells, their process invading, and the morphology/cell type need to be much better demonstrated for it to be convincing.

Answer: Thank you for this contextualizing concluding remark and the input given. We are confident that having addressed all the previous points as outlined in the answers above, we can now provide a much improved version of the manuscript.

---

2. Referee #2:

In this study, Haydo and collaborators combined two state-of-the-art techniques, adult organotypic brain tissue slice culture (OTC) and light sheet fluorescence microscopy (LSFM) of cleared tissues in a combined method termed OTCxLSFM that might be useful to understand the pathophysiology (the migration activity) of GBM tumors. In particular, authors used Arsenic Trioxide as well as genetic depletion of the tetraspanin, transmembrane receptor CD9 as examples to reveal the usefulness of the novel combined method. This is an interesting study that reveals a novel approach to further characterize the biology of GBM. The manuscript is well written and the figures are of quality.

Answer: We would like to thank the reviewer for this appreciation.

2.1.

As a limitation, the authors used models (ATO and CD9 depletion) previously described by them to be involved in GBM migration and invasion. It would be important to use an additional approach in order to validate their model. Experiments with TMZ in GS-5 GFP/Luc-tumors and silencing of a stem cell regulator such as Sox2, Bmi1 ... would give further insight and validation of the proposed novel method.

Answer: Thank you for these excellent suggestions. Based on our previous work, we chose to target the JAK/STAT3 signaling pathway that is known to act as a key player of tumor stemness, cell migration and invasion in GBM (see response to Reviewer # 1). A dual approach to interfere with this pathway using pharmacological blockade of JAK2 activity with WP-1066 (ClinicalTrials.gov ID: NCT01904123) and CRISPR-Cas-mediated ablation of STAT3 led to robust and very similar inhibitory effects on tumor dispersion in the OTCxLSFM model (EV3), further underscoring the validity of our model and our obtained results.

3. Referee #3:

3.1.

LSM is able to offer information regarding depth of GBM cell invasion into the tissue slice. However, this is not being provided. Instead, metrics such as dispersion are being quantitated which only measures invasion in 2 dimensions. Its entirely possible that simply measuring invasion within the slice as opposed to across the slice is more informative.

Answer: Thank you for this comment. We agree that our previous presentation of data wasn't concise enough. We now investigated invasion depth and dispersion following measurement across all three dimensions as requested (Fig. 3 and 5, Fig. EV 3 and 4). We have now revised the text and improved graphical data presentation accordingly. Our analyses is performed as a 3D segmentation, meaning all data sets were analysed in all dimensions, resulting in x-, y- and z-coordinates after generating the tumor dispersion and migrated distance.

3.2.

Consistency in the brain slice is missing. It is assumed that while adjacent tissue sections are being used, it is less clear whereabouts from the brain the slice originates. This does matter if invasion is being measured.

Answer: By cutting the brain slice into 4 sections, it is shown that within technical replicates there is a difference in invasion and migration due to limitations of the overall size of the sample. However, samples from all regions were used for every condition. Even further, distinct studies focusing on specific regions of the brain could select the region of interest and could only use slices from desired region (EV1).

3.3.

Histologic analysis of the brain slice used is not discussed. Viability of this microenvironment should be presented.

Answer: Tissue integrity of mouse brain slices over time was validated by propidium iodide (PI) staining of tumor-free cultures, revealing small rims of PI-positive (dead) cells at the cutting edge of the slices already on d0, while inner regions of the slices remain fully intact (EV 5A and B, left panel). Only minor integrity loss at the maximum incubation time of 10 days, as indicated by single PI-positive cells in the inner region of slices, is visible (EV 5A and B, middle panel). Treatment with 5 µM stauroporine on d9 for 16 h served as a positive control for cell death (EV 5A and B, right panel).

3.4.

More discussion on how this method correlates with non-tissue findings is recommended.

Answer: Thank you for pointing this out. As outlined in the answers to Reviewers # 1 and # 2, we now added new data on interfering with the JAK2/STAT3 pathway in our OTCxLSFM model, demonstrating robust inhibitory effects of pharmacological (WP-1066) and genetic (CRISPR/Cas mediated ablation of STAT3) blockade of this pathway on tumor infiltration (EV3). Since we extensively studied the role of this pathway in GBM migration/invasion in our

previous work using GBM in vitro cell culture models, organotypic transplantation models and syngeneic/xenograft orthotopic in vivo mouse models in combination with pharmacological inhibitors and genetic interference approaches (Linder *et al*, 2019b; Priester *et al*, 2013; Weissenberger *et al*, 2010), *this allows a direct comparison of the OTCxLSFM data obtained in the present study. Overall, the congruence of obtained data appears to be very high, demonstrating broad anti-migratory, anti-invasive effects of JAK2/STAT3 inhibition in vivo, ex vivo and in vivo* (Linder *et al.*, 2019b; Priester *et al.*, 2013; Weissenberger *et al.*, 2010) *that are faithfully recapitulated in OTCxLSFM, our new model system allowing quick and reproducible analysis of GBM cell infiltration in an authentic brain environment. We have added the discussion section to better address these points. This is now discussed in detail in the manuscript (Discussion: Page 9).*

3.5.

- Legend for Figure 2 does not match Figure 2 in the pdf.

Answer: We apologize for this error. We uploaded a pre-final version of the figure and have now changed this to the final version.

3.6.

- Images for Figure 1D are not placed properly and overlaps with Y-axis label for graph adjacent to it.

Answer: Thank you for pointing this out. We have now addressed this issue and revised this figure.

3.7.

- Figure 5D represents data from tumors grown on brain slices in Figure 5C, but there were 11 and 7 tumors that could have been selected for analysis. Instead only 3 and 2 were selected. No rationale was provided for this selection and could lead to bias. This is also observed for Figure 6.

Answer: Another excellent observation. The data presented in Fig. 5B and 5C was obtained using epifluorescence microscopy (as stated in the figure legends) and Fig. 5B and C lower part is a subset of slices. The main reason for this selection is that not all slides/tumors could be analysed in their entirety, sometimes due to technical struggles (e.g. floating of the slides in CUBIC2 solution, software glitches of the microscope) as well as occasionally slices got destroyed during the transferral process.

3.8.

Figure 5E should also list the N= if based from Figure 5D.

Answer: We have now added the required information and rearranged this plot to Figure 5B and C lower part.

3.9.

Some of the Supplementary Figures are also inconsistent with the results text and legends.

Answer: We apologize for any difficulties arising from our figure legends and we have carefully triple-checked all figures and figure legends in the revised version.

References

Linder B, Wehle A, Hehlgans S, Bonn F, Dikic I, Rodel F, Seifert V, Kogel D (2019a) Arsenic Trioxide and (-)-Gossypol Synergistically Target Glioma Stem-Like Cells via Inhibition of Hedgehog and Notch Signaling. *Cancers (Basel)* 11

Linder B, Weirauch U, Ewe A, Uhmann A, Seifert V, Mittelbronn M, Harter PN, Aigner A, Kogel D (2019b) Therapeutic Targeting of Stat3 Using Lipopolyplex Nanoparticle-Formulated siRNA in a Syngeneic Orthotopic Mouse Glioma Model. *Cancers (Basel)* 11

Priester M, Copanaki E, Vafaizadeh V, Hensel S, Bernreuther C, Glatzel M, Seifert V, Groner B, Kogel D, Weissenberger J (2013) STAT3 silencing inhibits glioma single cell infiltration and tumor growth. *Neuro Oncol* 15: 840-852

Schmitt MJ, Company C, Dramaretska Y, Barozzi I, Göhrig A, Kertalli S, Großmann M, Naumann H, Sanchez-Bailon MP, Hulsman D *et al* (2021) Phenotypic Mapping of Pathologic Cross-Talk between Glioblastoma and Innate Immune Cells by Synthetic Genetic Tracing. *Cancer Discovery* 11: 754-777

Weissenberger J, Priester M, Bernreuther C, Rakel S, Glatzel M, Seifert V, Kogel D (2010) Dietary curcumin attenuates glioma growth in a syngeneic mouse model by inhibition of the JAK1,2/STAT3 signaling pathway. *Clin Cancer Res* 16: 5781-5795

Dear Dr. Linder,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports.

Before I can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Do we need the abbreviation in the title? How about:
Combining organotypic tissue culture with light-sheet microscopy to study glioma invasion

- Please reduce the number of keywords to five.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box in the submission system to provide more detailed descriptions and do NOT provide your final manuscript text file with an author contributions section. See also our guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Please include all the funding information in the acknowledgements section and make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. Presently, Project-ID 259130777-SFB 1177 is missing in the submission system. Finally, please remove the section 'Funding' from the manuscript text file.

- Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section (DAS) - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (for main, EV and Appendix figures) of the final revised manuscript. Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

Presently, the annotated p value * is not defined in the legend of figure 5c. Moreover, that information related to n is missing in the legend of figures 2g-h (n=7?) and the data points shown in the violin plots in 5b-c, EV3c and EV4a-b is not mentioned. Please check or clarify.

- Please format the figure legends according to our journal style. See the respective section in our guide to authors (please find the link below). Please separate each panel description by a line brake and make sure that the panels are listed in alphabetic order. Moreover, please add to each legend a 'Data Information' section explaining the statistics used or providing information regarding replicates and scale.

https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please make sure that all figure panels are called out separately and sequentially (main, EV and Appendix figures), using the appropriate name (Figure X, Figure EVx and Appendix Figure X). Presently, EV and Appendix figures are not called our properly (see also below). Moreover, panels A-C of Figure 2 are called out before Figure 1 A, B and C, Figures 3, 4 and 5 are called out before Figure 2E and F, and there seems to be no callout for Fig. 1D. Please check.

- Please provide the Appendix data only as a single pdf file labelled Appendix. Please do not upload Appendix figures as separate files.

- The 'Data Availability' section (DAS) should only mention new datasets created during the study and submitted to public repositories. I think it is fine to provide the link to the Fiji Macros here. However, all the other information (regarding Laboratory Animals, Kits, Safety Regulations and Crispr/Cas9-knockouts) should be moved to the Methods section or already present respective parts of the Methods section. Please also remove the mention of the published dataset (Linder et al., 2019a) from the

DAS (see below).

- The dataset (Linder et al., 2019a) needs to be mentioned as part of the main reference list. This should be included below the citation of the paper, with the tag 'DATASET' at the start of the data citation in the reference section and mentioned accordingly in the text. Please see:

https://www.embopress.org/page/journal/14693178/authorguide#datacitation

Finally, could you please clarify how Figure 5A is represented. Is there the same sample shown from different angles over different days, which might explain very similar parts in these images?

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (!) bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have sufficiently addressed my concerns and I now endorse publication of this study.

Referee #2:

Authors addressed the main concerns and improved the manuscript.

Referee #3:

Afer reading all three reviewers comments (#2 did not offer any useful feedback though), my assessment is that the revised manuscript is substantially improved and will be very useful to others in this space. Organoids is truly becoming a more relied upon technique and the imaging techniques have not fully caught up with it. I especially commend the inclusion of the Fiji macros and I think these will be very useful for students/fellows. It will encourage others to fully use their technique. While there are still minor limitations to the technique (inability to readily track clones of interest), its benefits will still have significance to future users. This manuscript describes state-of-the-art techniques and shows that they desire knowledge translation of their work across the organoid field.

The authors have addressed all minor editorial requests.

Dr. Benedikt Linder
Goethe University Frankfurt, Goethe University Hospital
Experimental Neurosurgery, Neuroscience Center
Heinrich-Hoffmann-Straße 7
Frankfurt am Main, Hessen 60528
Germany

Dear Dr. Linder,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

****

THINGS TO DO NOW:

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/er_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2023-56964V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO Press Author Checklist

Corresponding Author Name: Benedikt Linder
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2023-56964V2

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods (Page: 10-14)

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods (SDS-PAGE and Western Plot; Page: 10-11)

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods (Page 11)

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods (Page 10)
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	17/02 are the only primary cell line and descirbed in Linder et al. 2019.
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Experiements started in 2018 and where therfore checked via STR profiling. Mycoplasma contamination is routinely checked in laboratiers.

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods (Page 13)
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods (Page 13), Data Availability Section (Page 15)

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	No plants were used in this study
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	No plants were used in this study

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	No Human participants were generated for this study

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements (Page 15)

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Yes	BioXriv: https://doi.org/10.1101/2023.02.09.527810
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	No clinical trial was performed for this study

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods via citation

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure Legend 1-5, Extend View Figure Legend 1-4, Appendix Figure Legend 1-2
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Methods (Page 12-13)
Include a statement about **blinding** even if no blinding was done.	Not Applicable	We do not have a study where blinding shloud be applied.
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods (Page 10)
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure Legend 1-5, Extended View Figure Legend 1-4, Appendix Figure Legend 1-2, Materials and Methods (Page 14)

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legend 1-5, Extended View Figure Legend 1-4, Appendix Figure Legend 1-2
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legend 1-5, Extended View Figure Legend 1-4, Appendix Figure Legend 1-2

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	No human participants were needed for this study.
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	No human participants were needed for this study.
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	No human participants were needed for this study.
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	The OTC experiments performed in our study require animal killing for scientific purposes which do not require, according to the German animal protection law, prior approval of an application for animal experiments at the local administration (Regierungspräsidium Darmstadt)
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Yes	Materials and Methods (Page 10-14)
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Yes	Data Availability Section (Page 15)
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	CRediT author statement (Page 15), Acknowledgements (Page 15)
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section (Page 15). References DATASET Proteomic Data of Linder et al. 2019 https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD009249 (Page 19)
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	Data Availability Section, Fiji Macros accessible via Zenodo: https://doi.org/10.5281/zenodo.8332648 (Page 15)
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	