Peer-Review

Kim, Chaeun. 2026. “Descriptive Imaging Analysis of Glioblastoma-Associated Vascular Morphology in a Single Murine Brain Slice.” *Journal of High School Science* 10 (2): 123–39. https://doi.org/10.64336/001c.160891.

1.state biological replicates (# of mice), technical replicates (# slices per mouse) and whether tiles were averaged per mouse before statistical testing.
2.for reproducibility, state Mouse strain, sex, age, Glioma cell line used, Cell number injected, Injection coordinates and depth, Timepoint(s) post-injection, Number of animals analyzed (n).
3.Did you treat each tile as an independent observation? If yes you are artificially increasing sample size and false positives (and risking pseudorepication). Clarify the statistical unit (animal-level versus tile-level). If tile-level data were used, apply mixed-effects models or average tiles per biological replicate.
4.peritumoral region boundary is ambiguous (approx. 600 microns from apparent tumor mass..). Whether tumor boundaries should be defined more clearly i.e. by DAPI density, CD31 disruption, or anatomical landmarks. Same criterion should have been applied consistently across samples.
5.Normality was assessed (Shapiro–Wilk), but parametric tests were still used in some cases without justification. Dunnett T3 is typically used when variances are unequal, yet this is not reported. No correction for multiple comparisons across multiple figures/parameters. No post hoc test for Kruskal–Wallis results (Figure 7). Report variance homogeneity tests such as Levine’s. Use appropriate post hoc tests for nonparametric data (e.g., Dunn’s test. Clearly report exact p-values, CIs, outlier handling and effect sizes
6.DAPI labels all nuclei, not just proliferating; hence the conclusion of increased proliferation is not directly supported.
7.For manual image manipulation, you have not blinded or validated for inter-rater bias. You have also not quantitated how much data was manually altered.
8.CD31 staining reports angiogenesis; not vascular mimicry.
9.CD31 % area cannot be interpreted as “surface area”
10.Animal ethics approval (IACUC/ARRIVE compliance), housing, and welfare details are not reported and should be explicitly included.
11.Percent-area metrics partially normalize for tile area, but intensity normalization across sections, batch effects, and z-thickness/voxel size calibration are not described.

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state biological replicates (# of mice), technical replicates (# slices per mouse) and whether tiles were averaged per mouse before statistical testing.

- response: Biological replicates - 4; technical replicates - 4; tiles were not averaged per mouse before statistical testing because only one slice per mice, so four slices were selected for confocal imaging while out of the four chosen, only one was selected for image analysis.
- changes in the manuscript

✓ In section 2, subsection 2.1, first sentence description addition: We utilized the brain samples previously... □ We utilized four brain samples previously...
✓ In section 2, subsection 2.2, last sentence addition: Four technical replicates were ... for confocal imaging.

2. for reproducibility, state Mouse strain, sex, age, Glioma cell line used, Cell number injected, Injection coordinates and depth, Timepoint(s) post-injection, Number of animals analyzed (n).

- changes in the manuscript

✓ In section 2, subsection 2.1, addition of sentences following the first sentence: More specifically, glioblastoma was induced in the right hemisphere ... 3 times at 10-minute intervals.

3. Did you treat each tile as an independent observation? If yes you are artificially increasing sample size and false positives (and risking pseudorepication). Clarify the statistical unit (animal-level versus tile-level). If tile-level data were used, apply mixed-effects models or average tiles per biological replicate.

- response: Though the statistical unit was tile-level, each tile was not treated as an independent observation. Rather, the ROI (region of interest) setting allowed each *group* of tiles to be an independent observation for comparison of the mean value, with standard deviation taken into account. The contralateral region had 29 tiles, the peritumoral had 14, and the intratumoral region had 15 tiles for one brain slice, all of which were averaged, then compared between the regional groups through ANOVA or Kruskal-Wallis, not amongst the tiles themselves. Therefore, sample size was not artificially amplified.
- changes in manuscript

✓ In section 2, subsection 2.4, 6th sentence description addition: We segmented each confocal image... For tile-level analysis, we segmented each confocal image...

4. peritumoral region boundary is ambiguous (approx. 600 microns from apparent tumor mass..). Whether tumor boundaries should be defined more clearly i.e. by DAPI density, CD31 disruption, or anatomical landmarks. Same criterion should have been applied consistently across samples.

- response: The peritumoral zone was defined based on the distinct changes in nuclear density observed in DAPI staining, rather than judging by the visible appendant tumor mass. More specifically, while high nuclear density and irregular nuclei distribution were observed in the tumor core, the tumor boundary was established as the abrupt changing point of DAPI nuclear density observed between tumor region and the adjacent normal brain parenchyma. Analysis was conducted by dividing the peritumoral region based on a exact distance (600μm) from the tumor boundary defined in this manner, and this criteria were applied equally to all samples.
- changes in the manuscript

✓ In section 2, subsection 2.4, second to last sentence addition: In particular, the peritumoral zone was ... established tumor boundary.

✓ In section 2, subsection 2.4, last sentence modification: Hence, the three areas of comparison are the contralateral hemisphere, which is unaffected by metastasis, peritumoral brain zone, which is defined here as ~600 microns from the boundaries of apparent tumor mass, and the inner tumoral region. Hence, the three areas of comparison are the contraleteral hemisphere, which is unaffected by metastasis, peritumoral brain zone, and the inner tumoral region.

5. Normality was assessed (Shapiro-Wilk), but parametric tests were still used in some cases without justification. Dunnett T3 is typically used when variances are unequal, yet this is not reported. No correction for multiple comparisons across multiple figures/parameters. No post hoc test for Kruskal-Wallis results (Figure 7). Report variance homogeneity tests such as Levine's. Use appropriate post hoc

tests for nonparametric data (e.g., Dunn's test. Clearly report exact p-values, CIs, outlier handling and effect sizes

- response: Levine's test results were reported only for parametric tests. Since the statistical program that the author owns, IBM SPSS Statistics V22.0, does not enable Dunn's test, pairwise Mann-Whitney test with Bonferroni correction (*$p<0.0167$; **$p<0.0033$; ***$p<0.0003$) was performed. Exact p-values were

reported. Nonparametric tests do not conventionally report CIs, so 95% CIs were only reported for parametric tests. No outlier handling was implemented in the statistical analysis as despeckling and reconnection processes, both of which underwent objective standards as described under reviewer comment 7, essentially removed any outliers that can skew the results. Effect sizes were reported for all. For parametric tests, eta-squared was reported, and for nonparametric tests, both epsilon-squared value for Kruskal-Wallis H and r (correlation) value for Mann-Whitney pairwise comparison were reported.

- changes in manuscript

✓ In section 2, subsection 2.5, 2nd to last sentence addition: On that account, we performed pairwise Mann-Whitney U test with Bonferroni correction (*p<0.0167;
**p<0.0033; ***p<0.0003) for Figures 7A-F.

✓ In section 3, subsection 3.3, subsubsection 3.3.3, from 2nd sentence to the end of the paragraph: First, the number of branches per identified skeleton was evaluated and determined to be exceptionally high...

✓ In Figure descriptions, Figure 4B, addition of eta-squared values and exact p values
as well as CI intervals: $ç^2$ = 0.97, F = 853.663; p = 0.238 (contralateral vs peritumoral zone; 95% CI = -7.01, 1.29); ***p = 0 < 0.001, (contralateral vs
inside tumor; 95% CI = -79.70, -67.30); ***p = 0 < 0.001 (peritumoral zone vs
inside tumor; 95% CI = -77.40, -63.87).

✓ In Figure descriptions, Figure 5C, addition of eta-squared values and exact p values
as well as CI intervals: $ç^2$ = 0.28, F = 6.098; *p = 0.047 < 0.05 (contralateral vs peritumoral zone; 95% CI = 0.03, 5.04); *p = 0.028 < 0.05 (contralateral vs inside
tumor; 95% CI = -5.14, -0.25); ***p = 0 < 0.001 (peritumoral zone vs inside
tumor; 95% CI = -8.09, -2.37).

✓ In Figure descriptions, Figures 7A-F, addition of epsilon-squared values, r (correlation) values, exact p values from Mann-Whitney U tests with Bonferroni correction (example provided with Figure description 7A): (A) Quantification and comparison of the number of branches per identified skeleton in each of the three groups (n = 1058, n = 556, n = 432). H = 11.719; ϵ^2 = 0.01; *p = 0.091 <
0.0167 (contralateral vs peritumoral zone; r = 0.04); **p = 0.001 < 0.0033
(contralateral vs inside tumor; r = 0.08); p = 0.072 (peritumoral zone vs inside tumor; r = 0.06)...

6. DAPI labels all nuclei, not just proliferating; hence the conclusion of increased proliferation is not directly supported.

• response: As a universal nuclear staining that binds to DNA, it is well known that it is not a marker specific for cell proliferation. Therefore, it is not reasonable to directly conclude the increase in proliferation only by the increase of the number of DAPI+ cells. In this study, it is more appropriate to interpret the DAPI quantification results as a change in cell density or nuclear density, and the evaluation of the actual proliferation activity can only be clearly determined through proliferation-specific markers such as Ki-67 and EdU/BrdU incorporation. Accordingly, the author has modified the interpretation of DAPI-based results to "increase in cell density" rather than "increase in proliferation".
• changes in manuscript

✓ In section 3, subsection 3.2, last sentence modification: Nevertheless, the overwhelmingly... unregulated proliferation of tumor cells... Nevertheless, the overwhelmingly... unregulated increase of tumor cell density...
✓ In section 4, first paragraph, 4th sentence modification: The small scale of the difference... with the proliferating tumor cells' compression of their own vessels...
 The small scale of the difference... with the compression of their own vessels due to the increased cellular density...
✓ In section 4, first paragraph, second to last sentence modification: According to Stylianopoulos et al. (2013), ... exerted by the proliferating tumor mass. According to Stylianopoulos et al. (2013), ... exerted by the increasing tumor mass.

7. For manual image manipulation, you have not blinded or validated for inter-rater bias. You have also not quantitated how much data was manually altered.
• response: With alteration of CD31 images, the yardstick for the initial removal of stains was the subset of 80 to 255 on the Fiji (ImageJ) threshold grayscale, where
0 represents black and 255 represents white in binary images. Branches and skeletons were converted to show white foreground. This quantitative thresholding limit significantly reduces inter-rater bias even before manual alteration. With the manual modification itself, tumor cells exhibiting CD31 due to stem-like cells and transdifferentiation, as classified by the presence of donut-shaped distinct single cells distanced from the surrounding vascular structures, was eliminated for further reduction of bias, not for data alteration. With alteration of skeletonized images, the initial transition to the binary skeletal remnant is automatic, and therefore has no controversy of manual bias, also significantly reducing inter-rater bias even prior to manual alteration. Only those with disconnected branches due to systemic conversion through the Fiji (ImageJ) software were re-connected based on the images of vascular structures before binary reconstruction. The author has been

educated by her expert mentors, Seong-eun Ryu and Dr. Minah Suh from the Sungkyunkwan University, that manual image manipulation through these methods is sufficiently objective and valid.

- changes in manuscript

✓ In section 4, last paragraph, 3rd sentence modification and 4th sentence addition: In particular, the relinking of separated vascular structures... following skeletonization

 Nevertheless, for CD31 images, the objective target for stain removal... binary reconstruction. With these intentional measures, ... invaded statistical authority.

✓ In section 4, last paragraph last sentence description addition: Further studies can also focus... In any case, further studies can also focus...

8. CD31 staining reports angiogenesis; not vascular mimicry.

- response: CD31 is a representative marker of endothelial cells, and CD31+ structures refer to blood vessels consisting of endothelial cells, typically reflecting existing blood vessel structures. On the other hand, vascular mimicry is a phenomenon in which tumor cells form vascular-like structures without endothelial cells, generally judged based on the CD31- structures. It is not appropriate to interpret it as an indicator of vasculature by CD31 staining alone. Accordingly, the author has deleted expressions concerning vascular mimicry or modified them to clearly state that the CD31-based result is a change in blood vessel density.
- changes in manuscript

✓ In section 4, 2nd paragraph, 2nd sentence fragment deletion: The high number of... induced by both vascular mimicry, and aberrant angiogenesis following vessel co-option... The high number of... induced by aberrant angiogenesis following vessel co-option...

✓ In section 4, 2nd paragraph, last sentence fragment deletion: This stages the methods of angiogenesis, intussusceptive angiogenesis, and vascular mimicry as... This stages the methods of angiogenesis and intussusceptive angiogenesis as...

9. CD31 % area cannot be interpreted as “surface area”

- response: The CD31+ area ratio (% area) measured by the 2D tissue sections refers to the ratio of the area within the image, and it is not conceptually accurate to interpret this as the surface area of the actual blood vessels. Surface area should be calculated based on the 3D-structural information, and the analysis performed in this study is not applicable. Therefore, expressions of CD31% area

have been modified as "CD31+ area fraction" or "vascular area ratio" to suit the exact concept of the terminology.

- changes in manuscript

✓ In abstract, 2nd sentence modification: For a better understanding... such as the surface area of the endothelium... □ For a better understanding... such as the vascular area ratio of the endothelium

✓ In section 3, subsection 3.3, subsubsection 3.3.1, last sentence modification: Our results revealed the highest surface area of CD31+ expression to be... □ Our results revealed the highest CD31+ area fraction to be...

✓ In section 4, first paragraph, 2nd sentence modification: Our results demonstrate that the tumor vasculature has a larger surface area compared... □ Our results demonstrate that the tumor vasculature has a larger vascular area ratio compared...

✓ In section 4, first paragraph, 4th sentence modification: The small scale of the difference between the surface area of intratumor vessels... □ The small scale of the difference bewteen the CD31+ area fraction of intratumor vessels...

10. Animal ethics approval (IACUC/ARRIVE compliance), housing, and welfare details are not reported and should be explicitly included.

- response: The author (I) is a high school student, and institutional regulations do not allow high school students to conduct animal experiments registered in the IACUC protocol directly or to handle and sacrifice animals. Scholars who enter the IACUC protocol must go through a reasonable curriculum, but high school students cannot participate in this curriculum. As a result, no new animal experiments were conducted and no direct handling or treatment of mice occurred in this study, and therefore, IACUC information is not applicable.

11. Percent-area metrics partially normalize for tile area, but intensity normalization across sections, batch effects, and z-thickness/voxel size calibration are not described.

- changes in manuscript

✓ In section 2, subsection 2.4, first 4 sentences addition: With the ipsilateral cortex, the image metrics on the confocal microscope (Leica TCS SP8) were ... With the contralteral cortex, the image metrics were ... and magnification was 0.25.

✓ In section 2, subsection 2.4, 5th sentence modification: Images acquired through confocal microscopy (Leica TCS SP8) were analyzed... □ These images acquired through confocal microscopy were analyzed..

Thank you for trying to address my previous comments, however, the paper still has fundamental issues with statistical validity, independence of observations, and experimental design clarity.

1.Your design is severely underpowered as is. You have not presented any statistical power calculations, yet you have very strong claims in the results. Your analysis is effectively based on tile-derived aggregates from a single slice per mouse. It is still unclear whether the final statistical unit is n=4 (mice) or n=3 regions × pooled tiles. The description suggests within-slice aggregation, not across-mouse replication. Did you compute one value per region per mouse? - this would be correct. Or did you pool tiles across mice? This would be incorrect. Your explanation is ambiguous and there is consequently a high risk of pseudoreplication.

2.If only one slice per mouse was used for analysis, then there is no variance at slice level, hence calling this ‘technical replicates’ is misleading and incorrect.
3.You have redefined boundary via DAPI density transition + 600 μm rule. However, you have not presented any inter-sample validation or inter-rater agreement. Tile assignment (1–14 vs 15–29) appears fixed spatially, not biologically adaptive, this can misclassify regions if tumor geometry varies.
4.[1] You say Shapiro–Wilk was used. However, you do not report test statistics or decisions. ANOVA is still used without clear justification. [2] You mention Levene’s test but p-value etc. is not reported [3] Bonferroni for MWU is good addition but there is no correction across figures or endpoints. [4] $F = 853.663$; $p = 0.238$ is statistically impossible. Check OR error or calculation error. [5] in figure 7, you have listed n = 1058, 556, 432. These are tile or pixel derived, not biological replicates; this is pseudoreplication.
5.You state that Thresholding reduces bias and also state mentor validation for image processing. However, you still have no blinding, Inter-rater validation, Quantification of % pixels altered and the Number of edits. This appears to be a high-risk bias source.
6.You state “No IACUC because you didn’t perform experiments”. However, you must still present Original study’s IACUC approval and Protocol number (or citation confirmation). Your Mentor can provide you with that information.
7.You over-reach with your claims. For example, you claim hypoxia, solid stess and angiogeneis but you have only measured morphology. You would have needed to measure perfusion, hypoxia markers to claim the former.

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12. Your design is severely underpowered as is. You have not presented any statistical power calculations, yet you have very strong claims in the results. Your analysis is effectively based on tile-derived aggregates from a single slice per mouse. It is still unclear whether the final statistical unit is n=4 (mice) or n=3 regions × pooled tiles. The description suggests within-slice aggregation, not across-mouse replication. Did you compute one value per region per mouse? - this would be correct. Or did you pool tiles across mice? This would be incorrect. Your explanation is ambiguous and there is consequently a high risk of pseudoreplication.

- Response: Even though statistical power calculations have not been presented, p-values, different types of effect sizes, and 95% confidence intervals have been demonstrated. These calculations support the validity of the study and the results even without power analysis as traditional power calculations may be unreliable due to the limited number of biological replicates, as is common for these types of imaging-based biological studies. Only 3% of neuroimaging research papers published in 2017-2018 had power calculations, and they were mostly for t-tests and correlations (Szucs and Ioannidis, 2020). The author apologizes for the misunderstanding and confusion she has caused, realizing now that her explanations have been poor and incoherent at times. Though 4 distinct brain slices were collected from each of the 4 different mice, only four of the sixteen brain slices was selected for imaging, one from each mouse. Then, out of those four, only one was selected for image analysis. During the image analysis itself, a z-stack was created for both the contralateral and the ipsilateral hemisphere, generating 39 images for the former and 40 images for the latter. During image analysis, each z-stack was processed and summarized by ‘maximum intensity’ for each optimal visualization of every pixel point. Therefore, since technically speaking, only one biological replicate was used for image analysis, n should equal to 1 across all parameters.

The author acknoweldges the comparative lack of scientific rigor in terms of the number of mice used for the study, but as a high-schooler, it was realistically unfeasible to acquire a high number of brain samples. The author still believes that her research can

contribute positively to biological literature and therefore has authentic scientific value because it has carefully documented the basic steps of imaging-based neuroscience experiments for other high-school students. Moreover, and perhaps most importantly, her findings are consistently aligned with the results that other researchers have discussed in a large range of previous literature. The author's mentor, Dr. Minah Suh, also agrees with these views and implores that the journal thoughfully considers these practical limitations as a high school student.

- Changes in manuscript

✓ In section 2, subsection 2.4, fifth sentence addition: A Z-stack of 39 slices was created for… for the ipsilateral hemisphere

✓ In section 2, subsection 2.4, sixth sentence modification: These images acquired through confocal microscopy were analyzed with Fiji (ImageJ) software (Figure 2A, 2B). → Out of the four sets of brain slice images obtained from confocal microscopy, only one was selected for image analysis, and its Z-stacks were analyzed with Fiji (ImageJ) software (Figure 2A, 2B).

✓ In section 2, subsection 2.4, seventh sentence addition: Each Z-stack was processed by projecting 'Maximum Intensity' for optimal visualization of each cell.

✓ In section 2, subsection 2.4, eighth sentence modification: For tile-level analysis, we segmented each confocal image… → For tile-level analysis, we segmented the confocal image…

✓ In Figure Descriptions, Figure 4 and Figure 5 sample sizes modification: n = 29, n = 14, n = 15 → n = 1

✓ In Figure Descriptions, Figure 6 sample size modification: n = 277, n = 124, n = 94 → n = 1

✓ In Figure Descriptions, Figure 7 sample size modification: n = 1058, n = 556, n = 432 → n = 1

13. If only one slice per mouse was used for analysis, then there is no variance at slice level, hence calling this 'technical replicates' is misleading and incorrect.

- Response: The definition of technical replicates are repeated measurements of a sample. Collecting four brain slices cannot be considered repeated measurements due to differences in tumor geometry; therefore, despite the fact that they are all from the same mouse, they cannot be considered as 'technical replicates'. In accordance, any mention of technical replicates has been deleted.
- Changes in manuscript

✓ In section 2, subsection 2.2, last sentence modification: Four technical replicates were… → Four brain slices were…

14. You have redefined boundary via DAPI density transition + 600 μm rule. However, you have not presented any inter-sample validation or inter-rater agreement. Tile assignment (1–14 vs 15–29) appears fixed spatially, not biologically adaptive, this can misclassify regions if tumor geometry varies.

- Response: Even though the z-stack for the ipsilateral hemisphere was created, essentially only one brain slice was used for imaging, which means that the there is only one tumor arrangement to address. Therefore, as long as the designated boundary is appropriate for the sample presented, biological adaptation is not necessary and tumor geomtery would not vary.

15. [1] You say Shapiro–Wilk was used. However, you do not report test statistics or decisions. ANOVA is still used without clear justification. [2] You mention Levene's test but p-value etc. is not reported [3] Bonferroni for MWU is good addition but there is no correction across figures or endpoints. [4] F = 853.663; p = 0.238 is statistically impossible. Check OR error or calculation error. [5] in figure 7, you have listed n = 1058, 556, 432. These are tile or pixel derived, not biological replicates; this is pseudoreplication.

- Response: [1] Shairo-Wilk test values are now reported. Those considered significant used Kruskal-Wallis while those considered insignificant used One-way ANOVA. [2] The author apologizes for her shortcoming; she did calculate the Levene's test values, but had mistakenly not included them in the paper. The numbers are present now for the parametric tests. [3] Bonferroni for MWU was only used for the Kruskal-Wallis data as a substitue of a post-hoc test, as Dunn's test was not available on IBM SPSS Statistics V22.0. Each endpoint deserves a separate analysis without interlinkage because they measure and represent distinct biological parameters. Therefore, there is no need for correction across figures and endpoints. [4] This is statistically possible because $F = 853.663$ for the intergroup analysis for the parameter of DAPI % area, and overall p-value was 0.000. After post-hoc was performed, $p = 0.238$ only for contralateral vs peritumoral zone while $***p = 0 < 0.001$ for contralateral vs inside tumor and $***p = 0 < 0.001$ for peritumoral zone vs inside tumor. [5] That has changed to n=1 because brain slice from only 1 mouse was used.
- Changes in manuscript
✓ In section 2, subsection 2.5, second and third sentence addition: Parameters significant for the Shapiro-Wilk test… used One-way ANOVA. For the parameters that applied One-way ANOVA… used the Scheffe test.
✓ In section 2, subsection 2.5, fourth sentence Shapiro-Wilk and Levene's test p-values addition: …(contralateral, peritumoral zone, inside tumor, respectively: $p = 0.071$, 0.129, 0.130; all > 0.05), the parametric method One-way ANOVA, Levene's test for equal variances ($**p = 0.002 < 0.01$)…
✓ In section 2, subsection 2.5, fifth sentence Shapiro-Wilk and Levene's test p-values addition: …(contralateral, peritumoral zone, inside tumor, respectively: $p = 0.302$, 0.381, 0.862; all > 0.05), the parametric method One-way ANOVA, Levene's test for equal variances ($p = 0.25 > 0.05$)…
✓ In section 2, subsection 2.5, seventh sentence modification Shapiro-Wilk test p-values addition: …($***p = 0.000 < 0.001$ for all groups of all parameters) and the nonparametric method…
✓ See 8th change in manuscript for reviewer comment #1

16. You state that Thresholding reduces bias and also state mentor validation for image processing. However, you still have no blinding, Inter-rater validation, Quantification of % pixels altered and the Number of edits. This appears to be a high-risk bias source.

- Response: Quantification of % pixels altered has now been discussed. About 20% of the tiles for each of the three groups of the contralateral (6 tiles), peritumoral zone (3 tiles), and the inside tumor (3 tiles) regions were randomly selected for analysis for the two imaging frameworks of CD31 immunohistochemistry and skeletonization. For CD31 tiles, 0.0992 ± 0.049%, 0.106 ± 0.18%, and 3.18 ± 0.21% were reported for the contralateral, peritumoral, and inside tumor region, respectively. For skeletonized tiles, 11.3 ± 8.7%, 15.7 ± 5.0%, and 5.00 ± 5.0%, were reported for the contralateral, peritumoral, and inside tumor region, respectively. Considering the inherently invasive nature of the manipulation itself as a constructional correction method, these numbers indicate minimal to moderate image processes.
- Changes in manuscript
✓ In section 4, fourth paragraph, third sentence modification to third and fourth sentences: Nevertheless, for CD31 images… based on binary reconstruction. → Nevertheless, for CD31 tiles, the objective target for… indicating minimal fluctuation. Additionally, re-connected branches were only… generative manual correction to attenuate false negatives.

17. You state "No IACUC because you didn't perform experiments". However, you must still present Original study's IACUC approval and Protocol number (or citation confirmation). Your Mentor can provide you with that information.

• Response: A statement of IACUC approval along with the Protocol number of the original study, Yei et al. (2025), was added in the paper.
• Changes in manuscript
✓ In section 2, subsection 2.1, second sentence addition: In Yei et al. (2025), all animal experimental procedures were approved by the Animal Care and Use Committee (IACUC) of Sungkyunkwan University (SKKUIACUC 2023-08-37-1).
✓ In section 2, subsection 2.1, third sentence lead-in modification: More specifically, glioblastoma… → Glioblastoma…

18. You over-reach with your claims. For example, you claim hypoxia, solid stess and angiogeneis but you have only measured morphology. You would have needed to measure perfusion, hypoxia markers to claim the former.
• Response: The author recognizes that her claims may have been over-reaching without empirical data to support them, especially in terms of hypoxia and solid stress. Therefore, claims have been modified to attenuate assertions of solid stress merely as merely suggested "potential" pathways rather than as factual statements. Mention of hypoxia in the discussion has been deleted.
• Changes in manuscript
✓ In section 4, first paragraph, second sentence modification: … suggesting larger caliber due to vascular dilation in presumably hypoxic regions and the abundance… → …suggesting larger caliber due to vascular dilation and the abundance…
✓ In section 4, first paragraph, fourth sentence modification: … may be associated with the compression of their own vessels due to the increased cellular density, causing potential collapses and diminished abilities due to solid stress… → … may be associated with the compression of their own vessels due to the increased cellular density and solid stress, potentially inducing vessel collapses and diminished abilities…
✓ In section 4, first paragraph, fifth sentence modification: Similarly, the decrease of CD31 % area… is also explainable by solid stress… → Similarly, the decrease of CD31 % area in the peritumoral zone… may also be understood in the context of solid stress…
✓

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I appreciate you trying to address my comments. However, my original comments are still valid. This paper cannot be accepted
unless the manuscript is Completely reframed as:
a methodological demonstration, or
a single-sample exploratory case study
with: removal of ALL inferential statistics and the elimination of p-values and ALL hypothesis testing (Including all error bars from figures).
All inferential claims will need to be removed. The title will need to be changed to something like "Descriptive Imaging Analysis of Tumor-Associated Vascular Morphology in a Single Murine Brain Slice"
At this point, the manuscript does lose most of its contributory value. I have included a revised abstract below that you can build your revised manuscript around, should you still choose to go this route.

Abstract

Glioblastoma is characterized by pronounced spatial heterogeneity in vascular structure, which is often assessed using imaging-based approaches. In this study, we present a descriptive analysis of vascular morphology within a single murine brain section containing a tumor region, using confocal microscopy and image-based quantification. A single brain slice (n = 1) was selected for detailed analysis, and spatially resolved measurements were obtained across contralateral, peritumoral, and intratumoral regions using tile-based segmentation and processing workflows implemented in Fiji (ImageJ). Across this specimen, regional variations in vascular-associated metrics, including CD31-positive area and skeletonized network features, were observed. The intratumoral region exhibited higher apparent vascular density and altered structural characteristics relative to

surrounding regions, while the peritumoral zone showed intermediate patterns. These observations are consistent with previously reported spatial heterogeneity in tumor-associated vasculature, although no statistical inference can be made due to the single-sample design.

This work is intended as an exploratory and methodological case study demonstrating a reproducible pipeline for spatial quantification of vascular features in confocal brain images. The findings are descriptive and hypothesis-generating, highlighting patterns that may inform future studies with appropriate biological replication and statistical power.

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19. Response: Considering the substantial issue of n = 1, the author has decided to accept the reviewer's offer of reframing the paper as a methodological demonstration with a single-sample case study. Accordingly, all inferential statistics and p-values have been removed. No hypothesis testing has been included and all inferential claims have been removed or completely rearranged so that it does not directly connect to the sample findings.
20. Changes in manuscript
21. Title: Morphological Changes of Cerebral Vasculature Induced by Glioblastoma Descriptive Imaging Analysis of Glioblastoma-Associated Vascular Morphology in a Single Murine Brain Slice
22. Abstract: Though glioblastoma is the most common malignant … contralateral and peritumoral zones. Glioblastoma is characterized by pronounced spatial … replication and statistical power.
23. Keyword addition: methodological case study
24. Section 1, third paragraph, first sentence revision: Due to these involvements, the GBM demonstrates aberrant blood vessel formation, with fenestrated and diluted walls, irregular patterns of endothelium, and poorly perfused overall. Due to these involvements, the GBM demonstrates aberrant blood vessel formation, with fenestrated and diluted walls as well as irregular patterns of endothelium.
25. Section 1, fourth paragraph revision: In this study, we investigated … GBM to its environment Recognizing the need for further investigations … growth of GBM to its environment.
26. Section 2 name: Materials and Methods Case Study Design
27. Section 2 first paragraph addition: This section outlines the standardized … corroboration with previous literature.
28. Section 2, subsection 2.1, first sentence deletion: In Yei et al. (2025), all … (SKKUIACUC 2023-08-37-1). Glioblastoma … Glioblastoma …
29. Section 2, subsection 2.5 addition (separation from 2.4 and addition): 2.4 … A Z-stack of 39 slices … inner tumoral region. 2.5 … A Z-stack of 39 slices … 'Paintbrush' function on Fiji (ImageJ) software.
30. Section 2, subsection 2.6 revision: 2.5 Statistical … The data … Deviation except for Figure 6. 2.6 Statistical … The data … statistical analysis as post hoc.
31. Section 3 name: Results Sample Findings
32. Section 3, subsection 3.2, first sentence modification: To statistically affirm the tumor's presence … To affirm the tumor's presence …
33. Section 3, subsection 3.2, fourth sentence modification: Subsequent Fiji (ImageJ) analysis … One-way ANOVA (Figure 4B). Fiji (ImageJ) analysis … respective average values (Figure 4B).
34. Section 3, subsection 3.2, fifth sentence modification: Conclusively, we found that the tumoral zone displayed significantly higher mean values … Conclusively, we found that the tumoral zone displayed higher mean values …
35. Section 3, subsection 3.3, subsubsection 3.3.1, third sentence modification: In addition, the % area of CD31+ regions … through One-way ANOVA (Figure 5C). In

addition, the % area of CD31+ regions … ultimately averaged for comparison (Figure 5C).

36. Section 3, subsection 3.3, subsubsection 3.3.3, second sentence modification: First, the number of branches per identified skeleton was evaluated and determined to be exceptionally high for the inner tumor region compared to the contralateral region, signifying a higher frequency of ramification (Figure 7A). □ First, the number of branches per identified skeleton was evaluated and determined to be higher for the inner tumor region compared to the contralateral region (Figure 7A).

37. Section 3, subsection 3.3, subsubsection 3.3.3, third sentence modification: The number of end-point voxels … tumoral vascular tips (Figure 7B). □ The number of end-point voxels … contralateral region (Figure 7B).

38. Section 3, subsection 3.3, subsubsection 3.3.3, fourth sentence modification: The number of pixels between … additional oxygen and nutrients (Figure 7C). □ : The number of pixels between … then the peritumoral zones (Figure 7C).

39. Section 3, subsection 3.3, subsubsection 3.3.3, fifth sentence modification: Average and maximum branch lengths … manipulated vascular environment (Figure 7D, 7E). □ Average and maximum branch lengths … then inner tumoral region (Figure 7D, 7E).

40. Section 4, first paragraph, first to second to last sentences: In this study, to understand how the aggressive … exerted by the increasing tumor mass (16). □ In this study, to understand how the aggressive … compression of peritumoral blood vessels (18).

41. Section 4, second paragraph: The high frequency of ramification, reflected … preventing GBM growth and infiltration. □ The findings from skeletonized vessels and the high … angiogenesis and intussusceptive angiogenesis (15).

42. Section 4, fourth paragraph, second sentence modification: Though initially purposed to reduce statistical error by … □ Though initially purposed to reduce error by …

43. Section 4, fourth paragraph, fifth sentence modification: With these intentional measures, manual image manipulation would not have invaded statistical authority. □ With these intentional measures, manual image manipulation would not have invaded the validity of the descriptive analysis.

44. Section 4, fifth paragraph addition: Another limitation is that because this … statistically confirm the sample findings provided.

45. Section 5, first paragraph, second to fourth sentences revision: Therein, a better understanding of the vascular changes it induces is imperative … particularly the presence of neovascularization. □ Therein, a better understanding of its vascular changes through a more … methodology is effective and accurate.

46. References, #10 and #11 addition: 10. Fedchenko, N., & Reifenrath … 11. Mebratie, D. Y., & Dagnaw, G. G. …

47. Figure Descriptions, Figures 4 deletion of statistical references

48. Figure Descriptions, Figures 5 deletion of statistical references

49. Figure Descriptions, Figures 7 deletion of statistical references

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Thank you for addressing my comments. This paper is now defensible. Please address my remaining concerns:

Either remove Section 2.6 entirely, or change the subtitle to “Statistical procedures that may be used in future scaled studies”

Add "Tile-level measurements are not independent replicates and are used only for spatial description within this specimen.” at an appropriate place in the manuscript.

Replace “confirm” with “consistent with”, “illustrative of”, “qualitatively resembles”……

Since you have measured junction distribution, here's an idea that maybe you would like to include in your paper ? "Future studies may evaluate whether junction-order distributions of tumor vasculature correlate with clinical outcomes such as overall survival". Please see attached chatgpt file.

_____

Either remove Section 2.6 entirely, or change the subtitle to "Statistical procedures that may be used in future scaled studies"

- Response: The author has changed Section 2.6 to "Statistical procedures for future scaled studies" because "Statistical procedures that may be used in future scaled studies" seemed a little bit long for a subtitle.
- Changes in manuscript:
- ✓ Section 2, subsection 2.6, subtitle: Statistical Analysis Statistical procedures for future scaled studies

Add "Tile-level measurements are not independent replicates and are used only for spatial description within this specimen." at an appropriate place in the manuscript.

- Response: The author has accepted this comment and has adopted this accordingly in her manuscript.
- Changes in manuscript:
- ✓ Section 2, first paragraph, last sentence addition: It is important to note here though, that tile-level measurements are not independent replicates and were used only for spatial description within this specimen for the case study.

Replace "confirm" with "consistent with", "illustrative of", "qualitatively resembles"……

- Response: The author recognizes that the phrase 'confirm' may be misleading and have changed accordingly in her manuscript.
- Changes in manuscript:
- ✓ Section 3, subsection 3.1, subtitle: Visual Confirmation of Glioblastoma Visualization of the Glioblastoma Mass
- ✓ Section 3, subsection 3.1, second sentence: This visually confirmed, even without the aid of further magnification, GBM's … Even without the aid of further magnification, this is illustrative of GBM's …
- ✓ Section 5, first paragraph, second to last sentence: Overall, our data confirmed previous observations … Overall, our data is in line with previous observations …

Since you have measured junction distribution, here's an idea that maybe you would like to include in your paper ? "Future studies may evaluate whether junction-order distributions of tumor vasculature correlate with clinical outcomes such as overall survival". Please see attached chatgpt file.

- Response: The author views positively of this addition and has adopted this accordingly to her manuscript, with reference to the given chatgpt file.
- Changes in manuscript:
- ✓ Section 4, third paragraph, second and third sentences addition: One direction of research we propose is the … selection based on mechanistic predictions.

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Thank you for addressing my comments. I appreciate the incorporation of the branching index/network topology discussion. However, your verbiage tries to interpret biology. Would you please consider replacing that pargraph with the one below where it focuses on defining and contextualizing a measurable, falsifiable and implementable feature (as a biomarker).

"One direction for future research is the evaluation of vascular network topology using quantitative descriptors such as junction-order distributions and aggregate measures of branching complexity. In this study, we operationalized branching structure using skeletonized vascular graphs, where junctions correspond to nodes and vessel segments to edges. The degree of each node (k) was defined as the number of connected segments, and the junction-order distribution was characterized as the empirical distribution P(k) for k≥3. In addition, we define a Branching Index (BI) as a scalar summary of network complexity, given by
“BI = (sum over k≥3 of kN_k) divided by (sum over k≥1 of N_k)” (for math representation, see pdf)
denotes the number of nodes with degree k. Within this framework, the intratumoral region in this specimen exhibited a higher relative frequency of higher-degree nodes compared to surrounding regions, suggesting increased local branching complexity. While this qualitative pattern is consistent with prior descriptions of disorganized tumor vasculature, the present single-sample design and the use of semi-manual image processing steps preclude assessment of reproducibility, sensitivity to preprocessing parameters, or association with biological or clinical outcomes. Accordingly, these topological features should be interpreted strictly as descriptive characteristics of this specimen. Future studies incorporating multiple biological replicates, fully standardized segmentation pipelines, and outcome-linked datasets would be required to determine whether such network-based metrics, such as BI, are robust and whether they hold potential as candidate biomarkers of tumor behavior. "

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- Response: The author has, for now, accepted the reviewer’s suggestion of replacing her current discussion of branching index into her paper. However, the author has some concerns. Though the equation may be logical in other branching studies, the author’s particular case study employs a unique type of junction order, assuming that this Branching Index is an extension of the categorization created in Figure 6.
- First, the categorization is based on the number of junctions on each branch, where branches with one junction were defined as Junction 1, branches with two junctions were defined as Junction 2, and branches with three junctions or more were all defined simply as Junction 3. The number of branches in each category were displayed as junction type distribution in Figure 6. In this light, the author does not understand why each node (k) was defined as the number of connected segments, which does not appear anywhere in the rest of the paper.
- Second, because all branches with three junctions or more were all defined as Junction 3, the author believes it is invalid to take all k values above three into account, as seen as the numerator in BI.
- Third, the author is uncertain whether the incorporation of this equation is justifiable considering that the author cannot demonstrated any calculations regarding this formula. The sentence, “Within this framework, the intratumoral region in this specimen exhibited a higher relative frequency of higher-degree nodes compared to surrounding regions, suggesting increased local branching complexity.” will most likely be misleading.
- Fourth, the author apologizes for her frankness, but one major concern is that she cannot help but wonder if this equation should even be used in her paper in the first place since she did not devise it herself. Would it be truly necessary in order for this manuscript to be accepted?
-
- Changes in manuscript:
- Section 4, third paragraph, second to second to last sentences addition: One direction for future research is … tumor behavior and determiners of treatment selection.
- _____

Thank your for your response. NO, this equation or any recommended wording is NOT necessary to be included in order for your manuscript to be accepted.
If you would prefer to revert back to your previous iteration, it is perfectly fine, just let me know.
It would help though, if you could operationalize to a mathematical form. How about a simple: Branching ratio = # of branches/# of junctions ?
You already have this in the description. Please see below for the relevant paragraph. Again, this is not necessary, just recommended.
"One direction for future research is the evaluation of vascular network topology using quantitative descriptors such as junction-order distributions and aggregate measures of branching complexity. In this study, we operationalized branching structure using skeletonized vascular graphs, where junctions correspond to branching points and vessel segments to branches. The junction-order distribution was characterized by categorizing the number of junctions per branch. In addition, we define a Branching Ratio (BR) as a simple scalar summary of network complexity, given by BR = (number of branches) / (number of junctions). Within this framework, the intratumoral region in this specimen exhibited a higher relative frequency of higher-order junctions compared to surrounding regions, suggesting increased local branching complexity. While this qualitative pattern is consistent with prior descriptions of disorganized tumor vasculature, the present single-sample design and the use of semi-manual image processing steps preclude assessment of reproducibility, sensitivity to preprocessing parameters, or association with biological or clinical outcomes. Accordingly, these topological features should be interpreted strictly as descriptive characteristics of this specimen. Future studies incorporating multiple biological replicates, fully standardized segmentation pipelines, and outcome-linked datasets would be required to determine whether such network-based metrics, such as BR, are robust and whether they hold potential as candidate biomarkers of tumor behavior."

Response: The author is grateful to the reviewer for their suggestion of junction-order operationalization. However, the new index also does not seem to be completely compatible with our model because higher number of branches and junctions both demonstrate increased local branching complexity, and setting the number of junctions in the denominator seems contradictory in this sense. The author though is positive of the notion that future studies may be able to employ quantification of junction-order as a new metrics in their own studies. Therefore, the author hopes that this adjustment is acceptable.

Changes in manuscript:
Section 4, third paragraph, second to second to last sentences revision: One specific direction for future research is the evaluation of vascular network topology using quantitative descriptors such as junction-order distributions and aggregate measures of branching complexity. In our current study, we operationalized branching structure using skeletonized vascular graphs, where branching points correspond to junctions, vessel segments to branches, and junction-order distribution was categorized by the number of junctions per branch. Within this framework, the intratumoral region in this specimen exhibited a higher relative frequency of higher-degree nodes compared to surrounding regions, suggesting increased local branching complexity. While this pattern is consistent with prior descriptions of disorganized tumor vasculature, the present single-sample design and the use of semi-manual image processing steps preclude quantitative statistical analysis, assessment of reproducibility, sensitivity to preprocessing parameters, and association with biological or clinical outcomes. Accordingly, these topological features should be interpreted strictly as descriptive characteristics of this specimen. Future

studies incorporating multiple biological replicates, fully standardized segmentation pipelines, and outcome-linked datasets would be required to perhaps produce a quantitative network-based metric based on this categorization and determine whether they hold potential as candidate biomarkers of tumor behavior and determiners of treatment selection.

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accepted.
I made changes to section 2.6 statistics and wrote that section (for the most part) in future tense.
I made changes to the verbiage in the Introduction section.
Also changed interpretation of the numbers in section 4. Those numbers were changed as well to accurately represent significant figures for uncertainty and the agreement betweeen decimal places between the mean and uncertainty.
I recommend leaving out Figure 8 since it is confusing and does not add value to the paper.