

Author's Reply to the Review Report (Reviewer 2)

We are grateful to the reviewer for their positive and constructive comments that have led to an improved manuscript. Here, we address only their concerns/suggestions and have tracked changes throughout the revised manuscript.

Broad comments

Point 1: I generally like the methodological approach and the general idea of the paper on the importance of local strains within scaffolds as a mechanical stimulus for tissue growth and differentiation. However, in its current form, this paper represents more a proof-of-concept study rather than an evaluation of the local mechanical state that cells may experience.

Response 1: Thank you for your comments. We agree that the sample size, simple loading scenarios and non-graded scaffold design tested in the paper merely lays the foundations for future studies to further explore the local cell environment.

In the study it was observed that even for the simple non-spatially graded implant design, there was an inhomogeneous mechanical response throughout the scaffolds which would neither be predicted or detected with standard materials testing equipment. This has informed scaffold manufacture and design. The method which has been demonstrated in this study could now be taken forwards for more complex device designs with 3D spatially-graded properties. Future work enabled by this study could include more complex loading scenarios, examining different scaffold architecture designs and evaluating cell-seeded scaffolds to further evaluate the local cell environment. Further development of the technique could provide a comprehensive toolkit of testing protocols to evaluate performance and suitability of scaffolds for regenerative medicine.

We have added further text in the abstract to clarify the simple device design evaluated in the study (lines 17 and 24-25), the study intention in the introduction on lines 83-84 in addition to text in the discussion to highlight future work (lines 710-711) and the long term potential for the technique on lines 515-517.

Point 2: Additionally, the link between 3D strains to inform scaffold design that authors claim in their aims is barely discussed.

Response 2: We agree that this was an oversight.

In the study it was observed that even for the simple non-spatially graded implant design, there was an inhomogeneous mechanical response throughout the scaffolds which would neither be predicted or detected with standard materials testing equipment. We believe that this may in part be caused by imperfect layer bonding and strut morphology manufacturing during 3D printing, which has informed further scaffold manufacture and design.

To this end text has been added to the discussion regarding how the technique assists in improving the design of scaffolds (lines 458-460 and lines 467-470), with suggestions for future studies on how to further inform scaffold design for regenerative medicine (lines 710-711) and discussion of the specific scaffold design tested in this study (lines 461-467). Additionally the link between 3D strains and scaffold design has been clarified on lines 24-25 of the abstract and line 83-84 of the introduction, and the potential for the technique in the discussion section on lines 515-517.

Point 3: An important issue I find in the manuscript relate the spatial resolution of the DVC measurement. Authors used for DVC computation a subvolume size of 48 voxels and 50% overlap. I would like to point out that overlapping the subvolumes does not increase the spatial resolution of the measurement, which remains as 48 voxels. The spacing between vectors do change to 24 voxels instead. Such spatial resolution is then in the same range of the scaffold's struts; thus, it allows the study of the struts' deformation, but not "intrastrut" strain measurements. This need to be clarify in the manuscript.

Response 3: We agree with the explanation and have amended the text throughout on lines 18, 260-261 and 437.

Point 4: Lines 12-17. It would be good to specify that the scaffolds of this study did not present a spatially-graded structure but a homogeneous one.

Response 4: Thank you for the suggestion. We have clarified this in the text on line 17.

Point 5: Lines 18-19. Displacement random and strain random. Authors forgot "errors".
Introduction

Response 5: We thank the reviewer for pointing out this oversight. Text has been added on lines 19 and 20.

Point 6: Lines 57-60. Authors should be clearer on what they mean about "DVC apply for the design of scaffolds". How was that method implemented in the manuscript? As for my understanding, local strains within the scaffolds were measured, but the implications on the scaffold design is not included in the methodology, results and/or discussion

Response 6: The described method revealed to us that even homogenously structured scaffolds provided a non-uniform strain response, even when accounting for boundary conditions and edge effects. This insight has informed our subsequent device design. Furthermore our method and included error analysis, comparison with DIC and manually-calculated strains should a good starting point for future research applied to more complex structures.

To improve clarity with regards to using DVC for scaffold design the text has been amended throughout the manuscript: in the abstract on lines 24-25, in the introduction on lines 43-53, 60-64, 78-81 and largely in the discussion section on lines 459-461, 467-470, 508-510, 516-518, 712-718, 723-730 to address the impact of future studies.

Point 7: Lines 70-73. Please delete.

Response 7: This template text has been deleted.

Point 8: Line 74. It would read better as implant or scaffold manufacture.

Response 8: We agree with the reviewer and have modified the title on line 97 to "Scaffold Implant Manufacture". Furthermore we have amended references throughout the document from devices to scaffold implants.

Point 9: Line 97. Could authors specify the load cell capacity?

Response 9: Thank you for the suggestion. The capacity of the load cell was 400 N.

We have added this to the text on line 147.

Point 10: Line 108-110. It is not very clear what authors mean by “manually registered at the static lowest slice”. Could you clarify how rigid registration was performed?

Response 10: We agree that this was not clear, thank you. Prior to full registration in the DVC software, scaffold features were approximately manually rigidly registered visually at the static bottom slice of the imaged scaffold to aid alignment of scans which were subsequently cropped to a volume of interest. This manual registration provided a good approximate starting point for registration and sub-voxel registration was subsequently automatically carried out in DaVis.

Clarification has been added on lines 158-161.

Point 11: Table 1. The table is a bit confusing at the moment. I would suggest to re-order it per sample rather than global strain, as imaging settings were the same independently on the applied strain.

Response 11: Table 1 has now been re-ordered with samples grouped together.

Point 12: Lines 146-150. As for my comment above, please clarify DVC spatial resolution and effect of overlap.

Response 12: The text has been amended on line 18.

Point 13: Line 149. Did the three passes used the same subvolume size?

Response 13: Yes that is correct and we have amended the text on line 260 to improve clarity.

Point 14: Line 156-157. What do authors mean by “matched to the resolution used when DVC processing the microCT data?”

Response 14: We agree that this was not well phrased. DIC pixel size was calculated and error was quantified across a range of subset sizes of equivalent physical dimensions to those of the subvolumes utilised for DVC. I.e. For a DVC subvolume size of 48 voxels, we calculated the equivalent DIC subset size so that both techniques resulted in a box of the same physical size: in this case 192 microns.

The text has been amended on lines 268-271 for clarity.

Point 15: Lines 160-165. Do the displacement random errors refer to a specific displacement component or the vector length?

Response 15: Displacement random errors were calculated using specific displacement components.

We have added some text to the manuscript to improve clarity of the equation on lines 280-282.

Point 16: Lines 176-179. Please clarify/rephrase. I do not understand how such measurement was performed.

Response 16: Local measurements were carried out using manually calculated at two loading levels at $n = 9$ separate regions in the transverse plane. This was carried out using the described global technical strain approach but rather than measuring the length change between the two platens, the length change between sample features was measured throughout the sample height. The vertical distance between scaffold features was matched to the spatial density of vectors in DVC i.e. every 24 voxels which we appreciate is not the spatial resolution but was the vector spacing. Strain measurements at each vertical location of the $n = 9$ regions were averaged to provide intra-sample strain measurements throughout the sample height.

We have added clarification to the text on lines 306-311.

Point 17: Figure 2. Could author include standard deviation of manually calculate strains in the graphs?

Response 17: Standard deviation bars have been added to both graphs and accompanying text has been added on lines 329-330.

Point 18: Lines 194-206. It is not clear to me how such measurements were obtained. Why did authors choose to test that methods on the sample imaged at 10.6 μm instead of the high-resolution ones?

Response 18: We agree that the methodology was not clear and clarification is required. We have described the method in more depth in Response 16. Sample Mech1 was chosen specifically because it was the tallest sample therefore had the most layers of struts to aid feature identification for manual tracking.

Further text has been added to the Methods section line to describe the local manual tracking approach 306-311 and rationale for choosing this sample on lines 311-312.

Point 19: Lines 200-201. Were the slices used for rigid registration at the bottom or the top of the sample? Could that explain that difference?

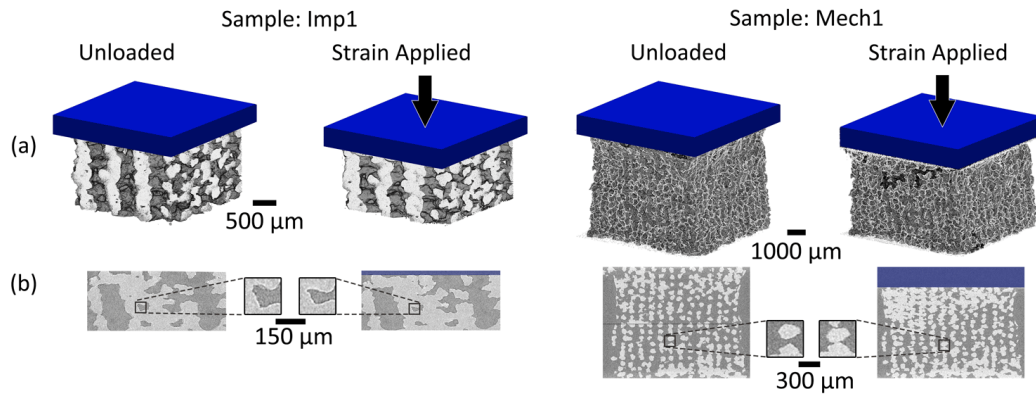
Response 19: Slices at the bottom of the sample were used for rigid registration and we agree that this could have contributed to the observed strain values.

Text has been added to the Discussion on lines 498-500 and registration location was clarified in the Methods 158-161.

Point 20: Figure 4. Could authors include microCT cross-sections of both samples in the unloaded and loaded configuration to demonstrate the strut's deformation? Which material were the platens made of?

Response 20: Thank you for the suggestion, we agree that adding micro-CT cross-sections is useful. The platens were made of polyoxymethylene (Delrin).

We have added cross-sections of both samples under both loading configurations to Figure 4 (copied below) with additional detail in the accompanying caption. Clarification of the platen material has been added to line 148.



Point 21: Line 220-221. What do authors mean by discontinuities in the scaffold?

Response 21: We agree that this phrasing was not clear. The 3-D printing process created struts with an irregular surface caused by a combination of the sol-gel ink's rheological properties and shrinkage occurring during the drying process. The resulting struts therefore included morphological imperfections whilst the scaffolds largely maintained the overall intended lattice architecture. There were rare cases during manufacturing where the layers of struts did not bond appropriately or otherwise lead to deviations from the intended architecture. The 3-D printing process is covered in depth by some of the authors in Tallia et al., 2018 which is now referenced (#24) in the manuscript at this point.

The text has been modified for clarification at lines 378-382.

Point 22: Line 222-223. Were the images affected by artefacts close to the top platen? How do authors explain the high noise in the DVC measurements, especially in the second compression step?

Response 22: Yes, we agree with the reviewer that artefacts close to the top platen are likely. A geometric mask was utilised on the top platen of all samples yet artefacts at and around the mask interface likely remain. We believe that the large variation in DVC measurements observed on the surface of the scaffold in Figure 5 are a combination of accurate (but largely variable) values due to deviation in scaffold morphology during the manufacturing process and spurious vectors from the aforementioned edge artefacts which are largely avoided in the central slices as in the supplementary figures.

We have added to the text in the methods to clarify the masking on line 264, in the discussion on lines 631-640 to address artefacts and edge effects and on lines 499-501 to address endcaps as suggested in a separate comment and in the results to address manufacturing limitations on lines 378-381.

Point 23: Figure 6. Could authors include also comparison between DIC and DVC data for the strain?

Response 23: We thank the reviewer for the suggestion. We have elected to compare strain side by side for both DIC and DVC in Figure 2, with correlation to the manually-calculated strains including equations and R^2 for the correlation for both techniques. This provides a superior quantitative comparison of strain measurements between the two techniques which we believe provides sufficient data for comparison. Figure 6 provides a complimentary visual comparison of displacements which is not plotted elsewhere in the manuscript.

On lines 642-643 in the discussion we have added a suggestion that further work comparing mechanical response between the techniques could be beneficial.

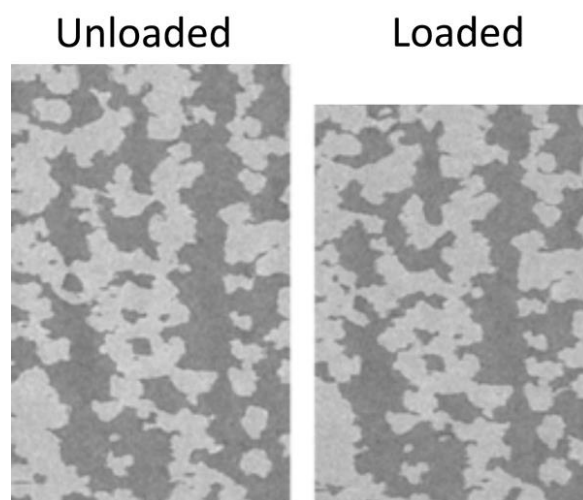
Point 24: Line 236. What does author mean by intra-strut level?

Response 24: In light of the reviewer's useful previous feedback regarding overlap/resolution (Point 3) we have now removed this text and amended line 404-405 to reflect the reduced spatial capabilities.

Point 25: Figure 10. How to authors explain the noise at the top of Mech 2 implant? What did they observe in the microCT images?

Response 25: We thank the reviewer for this is very good question. The micro-CT images themselves were satisfactory and showed the top platen compressing the sample as intended. For the reviewer's interest we have included below an example region showing the sample undergoing loading. We believe that the inconsistent deformation demonstrated at the top of Mech2 was caused by a combination of: the large single loading step causing trouble with feature tracking and unusual scaffold behaviour in certain regions with this specific sample caused during the manufacturing process.

We have elaborated on potential causes on lines 322-324 and 650-659 in the revised manuscript.



Point 26: Line 252. As for my previous comments, please clarify the spatial resolution of the DVC measurement.

Response 26: We have updated the stated spatial resolution values on lines 437 and provided a comment in the discussion section about further optimising the scanning parameters (lines 541-543) to aid spatial resolution.

Point 27: Lines 274-277. Authors should expand on how the strain quantification would aid the design of the novel implants/scaffolds.

Response 27: We agree that further clarification is necessary. In summary, strain quantification could be utilised to ensure scaffold deformation provides an appropriate biological microenvironment for regenerative medicine applications, forming part of the device development process.

Further text has been added on lines 458-460 and 467-470 to address how the described technique could aid scaffold design and insights provided by the technique on the scaffolds tested in this study (lines 461-467). Additionally, suggestions for future studies to improve capabilities of the technique to influence scaffold design are provided on lines 710-711.

Point 28: Lines 278-280. Were there image artefacts in proximity to the platens that could have caused such strain concentrations? Authors should comment on how to avoid such issue (i.e. using endcaps for the samples that provide mechanical stability and flat surface).

Response 28: The reviewer is correct that proximity to the platens likely contributed, in addition to the imperfect scaffold morphology creating regional strain concentrations. In line with what was discussed for Point 22, clarification has been provided in the text on lines 631-640, with masking discussed on line 264 and end caps on lines 499-501 including the reviewer's suggestions. Thank you.

Point 29: Lines 293-294. Using the FFT+DC DVC method, you are in fact employing a multi-pass DVC approach with different subvolume sizes (larger for FFT compared to the final DC). Could that be the reason why errors were lower? How many passes and of which sub-volume sizes were the DVC schemes for the FFT or DC approaches. It has been previously shown that the use of multi-pass schemes reduces uncertainties (Palanca et al. 2015, Pena Fernandez et al 2018) and benefit DVC computation.

Response 29: We agree that FFT+DC effectively employs a multi-pass approach with multiple subvolume size steps which certainly would contribute to lower errors as shown in those previous studies. Errors could very likely be reduced further by utilising more passes with subsequently smaller subvolume sizes.

Text has been added, with references, at lines 613-616 to address FFT+DC employing a multi-pass approach.

For FFT or DC approaches the same number of passes and subvolume size were used as each FFT+DC run (i.e. 3 passes at 48 voxel subvolume size), though of course not benefitting from the additional larger FFT "pre-shift" pass (as demonstrated in Figure7, now

Supplementary Figure S1). Further text has been added to clarify the parameters for FFT or DC approaches at lines 202-203 in the methods section.

Point 30: Lines 301-310. To improve resolution on DIC measurement, generally speckle patterns are used on the surface of the samples. Authors should comment on that, as generally DIC provided higher spatial resolution than DVC and therefore, local strain within the struts at the surface could have been examined.

Response 30: Text and an appropriate reference has been added to lines 539-541 of the discussion to address the use of speckle patterns for DIC.

Point 31: Line 305-306. “This difference may potentially be due to change to the samples during scanning process X-ray interactions causing morphological changes”. Please rephrase, it is not clear.

Response 31: Thank you for the suggestion to rephrase this sentence. This has been rephased to “The increased systematic error in strain measurements observed with DVC in comparison to DIC may potentially have been caused by morphological changes in the samples resulting from X-ray interactions experienced during image capture using micro-CT”.

Wording has been updated on lines (621-623) to improve clarity and a reference to potential X-ray damage is included on the following line.

Point 32: Line 322. I think the sentence “Error was quantified...” is just a repetition from previous paragraph.

Response 32: We agree with the reviewer and this statement has been removed.

Point 33: Line 326. Which issues?

Response 33: Thank you for bring this oversight to our attention. As discussed in Point 25, the mechanical response of sample Mech2 under load was unexpected (Supplementary Information Figure S4) and we believe one of the causes was the single large loading step (8% compression) which contributed to issues tracking features in the sample, leading to inconsistent results.

More detail regarding the issues in tracking features has been added to the text on lines 322-324 and 650-657.

Point 34: Lines 335-339. Again, how does it allow do improve scaffold design?

Response 34: We agree that this was not well explained in the manuscript. The method provides a complimentary means of device testing allowing quantification and visualisation of localised mechanics both throughout the height and internal volume of scaffolds. Therefore the technique could be incorporated as part of the medical device design process to enable testing and mechanical demonstration of scaffolds with the intention to provide a targeted three dimensional strain gradient on cells for tissue regeneration.

To this end text has been added to the discussion regarding how the technique assists in improving the design of scaffolds (lines 458-460 and lines 467-470), with suggestions for

future studies on how to further inform scaffold design for regenerative medicine (lines 710-711) and discussion of the specific scaffold design tested in this study (lines 461-467). Additionally the link between 3D strains and scaffold design has been clarified on lines 24-25 of the abstract and line 83-84 of the introduction, and the potential for the technique in the discussion section on lines 515-517.

Point 35: Figure 5, 6, 9, 10. Figure 5. It'd be interesting to see the 3D render of the scaffold and/or microCT cross-section to relate have a qualitative view of the deformation next to the strain quantification.

Response 35: We thank the reviewer for the suggestion.

Micro-CT cross-sections of both this sample and another have now been added to Figure 4 (as shown in Response 20) to accompany the 3D render on the previous page as suggested in a separate comment.