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Peer Review File

High-Throughput Bioprinting of Spheroids for Scalable Tissue Fabrication

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The purpose of this study is to present a HITS-Bio (High-throughput Integrated Tissue Fabrication System for Bioprinting). That may be a novel multiarray spheroid bioprinting technology enabling scalable tissue fabrication by rapidly positioning a number of spheroids simultaneously using a digitally-controlled nozzle array (DCNA) platform. However, I think the present study can not be accepted.

Comments
1Title: The title is clear and descriptive. However, consider adding "High-Throughput" to the title to emphasize the key aspect of the work.
2 Abstract: Provide specific data for the speed improvement (e.g., "ten times faster").
3 Abstract: Mention the specific cell types used (e.g., "human adipose-derived stem cells").
4 Introduction: Add references to support the statement about the importance of cell density for tissue repair and regeneration.
5 Introduction: Provide more context on the limitations of current bioprinting techniques in achieving native cell densities.
6 Materials and Methods: Please add HITS-Bio System description:
7 Materials and Methods: The authors should Include detailed specifications of the DCNA, such as nozzle dimensions, material composition, and manufacturing tolerances.
8 Materials and Methods: The authors have to describe the software interface and control algorithms used for positioning the spheroids.
9 Materials and Methods: It is better to explain how the system maintains high cell viability during the bioprinting process.
10 In Spheroid Preparation: You should specify the type and source of cells used for the spheroids.
11 Please describe the methods for culturing and forming spheroids, including the duration and culture conditions.
12 Explain the rationale for choosing specific miRNAs for transfection and their role in enhancing osteogenesis.
13 In Bioprinting Protocols: The authors should provide a detailed description of the bioprinting protocols, including the steps for intraoperative bioprinting and scalable tissue fabrication.
14 Materials and Methods: The information about the composition and properties of the bioinks (BONink and CARink) used in the bioprinting process should be included.
15 Please specify the parameters for the photo-crosslinking process.
16 Results: The authors should present quantitative data on the extent of defect closure over time.
17 Histological images should be added to show the integration of bioprinted constructs with host tissue.
18 Please provide evidence of the role of miRNA in enhancing osteogenesis.
19 In Scalable Cartilage Constructs: Please show data on the mechanical properties of the bioprinted cartilage constructs.
20 Images demonstrating the uniform distribution of spheroids within the constructs should be included.
21 The authors can present data on the viability and functionality of the chondrogenic spheroids post-bioprinting.
22 Discussion section: The authors could discuss the limitations of the HITS-Bio system, such as the potential for mechanical damage to spheroids during the bioprinting process.
23 Add information about comparing the HITS-Bio system with other bioprinting technologies, highlighting the advantages and disadvantages.
24 How to address the scalability of the system for larger tissue volumes and more complex structures.
25 Add some discussion about the potential for integrating vascular networks within bioprinted tissues.
26 Conclusion: Please summarize the main findings and emphasize the significance of the HITS-Bio system in tissue

engineering. Further, it is better to outline the future directions for improving the system, such as incorporating more nozzles for higher throughput and developing methods for bioprinting on non-planar surfaces.

Reviewer #2

(Remarks to the Author)
In this study, the authors developed HITS-Bio (High-throughput Integrated Tissue 8 Fabrication System for Bioprinting) as a further development of aspiration-assisted bioprinting (AAB). The methods enables multiarray spheroid bioprinting wherein a number of spheroids are aspirated and rapidly deposited using a digitally-controlled nozzle array (DCNA) platform.

The reviewer finds that necessary important variables were addressed. However, the chosen model system (cartilage, bone) seems inadequate for the chosen printing technology and lacks experimental controls that would demonstrate superiority of this technique over more established additive manufacturing techniques. In addition, the proposed method is largely based on a previously published data and the authors miss out on demonstrating a significant contribution with regards to methodology. Lastly, the capabilities of the DCNA array, in particular for complex (multilayer, difficult-to-print) models are not further explored.

The reviewer finds that the authors have presented an overall sound set of methodologies and provided details which are both sufficient and meet the expected standards in the field.
However, the advances presented in this manuscript are only moderately innovative and present at best a natural progression of prior art. Due to several limitations outlined in the comments below, the reviewer finds the manuscript to be of moderate significance for the bioengineering/tissue engineering field.

The reviewer's comments and major revision requests are found below:
1. The HITS-Bio process is described for spheroids ranging from 300-350 µm. The authors should elaborate on the applicability, challenges and possible modifications of the system to accommodate smaller and larger spheroids?
2. What are the physical limitations of the DCNA nozzle array, and to what extent are factors such as inter-nozzle capillary reactions eventually limiting the picking of spheroids? How does the setup of the array correlate with spheroid size?
3. Page 3, line 66: The authors claim that the technique is also applicable to organoids. However, corresponding data are not shown. The reviewer is furthermore missing a quantification of shear, suction, and compressive forces that would enable to judge the usefulness of this system for very soft and fragile organoids (brain, vascular, liver etc.).
3.1. What minimum and maximum viscosity is required in this process? How are those parameters derived?
4. Fig. S3: shows the spheroid transfer process for soft support baths. How does this transfer process translate into more viscous media? In particular, how does the increased shear and compression that would be present in such a support bath interfere with the proper transfer?
5. Can the authors make a statement regarding the overall experimental duration for a physiologically relevant and complex print (multilayer deposition with non-repeating deposition patterns), corrected for the time it requires to get the DCNA nozzle array loaded spheroids in a non-repeating pattern. The reviewer assumes that at higher complexities, the loading itself becomes a central aspect that limits the efficiency of the method, especially as the loading patterns become non-repeating, i.e. non-uniform across repeated loading-deposition cycles. What alternatives to the proposed spheroid loading system can the authors propose that would enable a more efficient and targeted loading (aspiration)?
6. Page 4, lines 98ff: The authors describe a camera-based system to control and track the spheroid picking process. This process seems to allow for little control over the actual forces applied to the spheroid. A pressure-based control (via measuring the resistance or using an aspiration pressure threshold) would enable more precise and controlled pickups. What was the reason behind choosing a system based on visual interrogation and what particular benefits does this method feature vis-à-vis the aforementioned technique. The reviewer is missing a description of a dynamic pressure control setup for the controlled picking of spheroids in cases where the 3 mmHg condition fails to work.
7. Page 1, line 4f: The authors point out the relevance of high cell density in tissue engineering to replicate organ-specific architectures, yet they miss to show a corresponding application. Indeed, Figures 2G and 3F demonstrate that the process enables the deposition of spheroids in a tight pattern. However, the reviewer finds that a print with a fully cellular architecture should be included to demonstrate the capability of this method towards organ printing. In particular, this demonstration of a fully cellular print should feature tightly packed spheroids/organoids without interspaced ECM. This would help to further compare this novel setup to previously published work (DOI:10.1126/sciadv.aaw5111).
8. Herein, different stacking patterns should be explored (e.g. print patterns for volume-filling models featuring spheroids of different sizes to maximize the faction of the filled volume) to generate models with varying degrees of ECM filling.
9. Furthermore, it is not clear why this tissue type (cartilage and bone) was chosen to demonstrate this technique as other tissues (e.g. liver, muscle) would benefit more from the highly dense packing of spheroids/organoids. In that regard, it is also not clear how the showcased print (Figure 3F) would be superior to a control with randomly and homogeneously distributed spheroids (via mixing and casting). The authors should include such a control.
10. Figure 3 describes in vitro testing of the printed constructs. Eventually, both cartilage and bone quality will have to be assessed with respect to their compressive modulus, which enables them to sustain loads. The reviewer is missing a mechanical characterization of matured tissues and a comparison with previously published data featuring similar average cell densities, maturation times etc.
11. Page 13, line 400: A further claim states that 'area-to-area and batch-to-batch inconsistencies' can be mitigated using HITS-Bio, but necessary controls that would showcase spatial differences (e.g. compressive modulus, gene expression) were not included in the study. It is reasonable to assume that regions of highly dense spheroids with intercalating empty ECM would lead to a more heterogeneous tissue than single-cell deposition or stochastic deposition of spheroids at higher density. This claim can only stand if corresponding controls were included.

12. In addition, more complex architectures with spheroid gradients or difficult-to-cast geometries must be demonstrated to justify the use of HITS-Bio over conventional, more scalable methods (extrusion bioprinting, casting/molding).
13. Figure 4B:
13.1. The use of BONink as control is insufficient. The reviewer suggests to have controls with randomly distributed spheroids to control for any cell-mediated effect. Without these controls a proper judgment of the benefit of spatially-defined deposition of spheroids over a random distribution for the observed effect is not possible.
13.2. Figure 4B: The formation of new bone from week 3 to week 6 appears to be minimal in all conditions (a-c) and even condition (d) only features minimal additional formation. What is the explanation for the relatively inefficient closure of large defect areas? Furthermore, it is unclear where the deposited spheroids are located. Additional immunofluorescence stainings (e.g. P1NP, or procollagen type I N-propeptide; BAP, or bone-specific alkaline phosphatase; and osteocalcin) should be shown to assess matrix deposition, cell density and general maturation at and around the sites of spheroid deposition.
13.3. What explanation can be given to explain the relatively low degree of fusion between printed transplant and host cranial bone within the calvarial defect? The authors claim ‘near-complete bridging’ (page 16, line 498), however, the transplant does not seem to feature continuous bridging throughout the construct, or to the host institute.
14. Page 16, line 516: wrong reference to Figures 2Fii and 2Fiii

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have checked the response of this manuscript. The authors have revised the study according to the reviewers' suggestions. I think it can be published in NC.

Reviewer #2

(Remarks to the Author)
The author's rebuttal has addressed most of the concerns brought forward by the reviewer that related to technical aspects of DCNA. Furthermore, the additional data on the range of spheroid elastic moduli within which they can be effectively bioprinted serves as a useful reference for future studies. The additional explanations (e.g on the rational of choosing a camera-based system over a pressure-regulated alternative) are well-founded and conclusive. Overall, the newly added data seems robust and answers the open queries of the reviewer. The reviewer thus suggests to accept the manuscript for publication.

Title: High-Throughput Bioprinting of Spheroids for Scalable Tissue Fabrication

We thank the reviewers for their constructive feedback, which has significantly contributed to the improvement of this revised manuscript. We have carefully considered all comments and have addressed each one comprehensively in the revised manuscript. Below, we provide a detailed report outlining the specific revisions made in response to the reviewers' comments.

Please note: Reviewer comments are presented in **BLACK**, our in-line responses are in **BLUE**, and the revisions made to the manuscript are highlighted in **RED**.

Reviewer #1 (Remarks to the Author):

The purpose of this study is to present a HITS-Bio (High-throughput Integrated Tissue Fabrication System for Bioprinting). That may be a novel multiarray spheroid bioprinting technology enabling scalable tissue fabrication by rapidly positioning a number of spheroids simultaneously using a digitally-controlled nozzle array (DCNA) platform. However, I think the present study cannot be accepted. Comments:

1.Title: The title is clear and descriptive. However, consider adding "High-Throughput" to the title to emphasize the key aspect of the work.

Response: We appreciate the reviewer's suggestion to emphasize 'High-Throughput.' We would like to kindly point out that 'High-Throughput' was already included in the title.

2. Abstract: Provide specific data for the speed improvement (e.g., "ten times faster").

Response: We had initially described the speed improvement as 'a magnitude faster,' which is equivalent to "ten times faster". However, we have now explicitly stated this in the abstract to ensure clarity.

3. Abstract: Mention the specific cell types used (e.g., "human adipose-derived stem cells").

Response: We have now included 'human adipose-derived stem cells,' in the abstract.

4. Introduction: Add references to support the statement about the importance of cell density for tissue repair and regeneration.

Response: We have added the following references to support the statement (You et al., Science advances 2023; Daly et al., Nature communications 2021).

5. Introduction: Provide more context on the limitations of current bioprinting techniques in achieving native cell densities.

Response: We have expanded the Introduction to include the following on *Page 2*: "One key challenge is achieving physiologically-relevant cell densities (100-500 million cells/mL), which is essential for effective tissue repair and regeneration [2-4]. However, current bioprinting techniques struggle to achieve high cell densities seen in native tissues. For instance, extrusion-based bioprinting (EBB) supports higher densities but often compromises cell viability due to shear stress [5]. Inkjet bioprinting is constrained by low-viscosity bioinks that limit the cell concentration [6]. Laser-assisted bioprinting is slow, yields lower cell viability compared to other methods and better suited to small-scale constructs, limiting its use for larger tissues [7]. Light-based bioprinting usually requires lower cell densities for effective curing of photo-crosslinkable bioinks [8], while microvalve

bioprinting is limited to bioinks within a narrow range of viscosities and cell concentrations, which increases the risk of clogging [9]."

6. Materials and Methods: Please add HITS-Bio System description:

Response: We would like to note that a detailed description of the HITS-Bio System was already included in methods section **5.2 Development of the HITS-Bio platform**. However, we have added more details (See Comment #8) and are happy to provide any additional details if needed.

7. Materials and Methods: The authors should Include detailed specifications of the DCNA, such as nozzle dimensions, material composition, and manufacturing tolerances.

Response: We have included detailed specifications of the DCNA in the revised manuscript on *Page 26* as follows: "The hardware component of DCNA consisted of stainless-steel needles (30G, an inner diameter of 150 μm and an outer diameter of 305 μm) arranged in a 4 × 4 array with dimensions of nozzle array ranging from 2.8 to 4.0 mm in width, and the inter-nozzle distance between 527 and 927 μm (**Figure S6D**), which was enabled by precisely stacked multiple acrylic plates (10 mm × 10 mm, 2 mm thick), micro-manufactured by laser cutting (**Figure S1**). These nozzles were carefully inserted through the holes on the plates, and after calibrating the surface and arranging the nozzles on the same plane, were adhered to the acrylic plates for stability. Manufacturing tolerance data (**Figure S6E**), illustrated by the XY positional error before and after the acrylic plate removal, highlights the importance of plate integrity for maintaining precision."

8. Materials and Methods: The authors have to describe the software interface and control algorithms used for positioning the spheroids.

Response: The software is available at https://github.com/MHKim-software/HITS-Bio.git. The interface setup, descriptions for each button on the interface, and a detailed algorithm for the entire HITS-Bio operation have also been provided (**Supplementary Information S2**). The new content added has been as follows: "**S2. Software Interface and Control Algorithm:** The control algorithms for the DCNA involved several key functions to ensure precise operation and safe handling of spheroids. For better understanding, the software interface was marked into different Panels A-F (**Figure S3**).

Positioning: The DCNA was mounted on the Z-axis and moved using keyboard arrow keys. The arrow keys moved the X and Y axes, while the Page Up and Page Down keys move the Z-axis. Users can set the step size, ranging from 0.01 to 20 mm, via designated buttons in Panel C. For a customized step size, users can input values followed by pressing the 'Input' button. Additionally, velocity and acceleration parameters were entered in the 'Vel' and 'Acce' fields within the same Panel.

Saving and Retrieving Positions: Users can save up to ten positions during the operation by pressing the corresponding 'Pos #' button in Panel D after setting the DCNA's coordinates. These saved positions can be easily accessed and moved by clicking the play buttons next to each position button in Panel D.

Safety Controls: To ensure that spheroids were not damaged during handling, a maximum Z position was set by pressing the 'Set Max Z' button once the DCNA reached the required height. This position was entered and activated by toggling the activation switch in Panel E to prevent potential damage to spheroids and DCNA.

Pressure and Aspiration Controls: Pressure levels, aspiration, and minimum vacuum were controlled via buttons in Panel F2. The 'Minimum Vacuum' button was specifically designed to hold aspirated media and to prevent leakage when spheroids were placed. Real-time monitoring of these pressure values was available in Panel A., The 'Initialization' button in Panel A calibrates the pressure sensor to zero.
Solenoid Valve Connection: The interface included a direct connection to the solenoid valve controller, displayed in Panel B. Users can connect or disconnect the controller with 'Connect' and 'Disconnect' buttons, respectively. The 'Initialize Controller' button resets the controller and closes all valves, while the 'Switch OFF Controller' opens all of them to turn off the controller.
Nozzle Configuration and Operation: Individual nozzles on DCNA can be controlled via buttons labeled '1' to '16' in Panel F1. The arrangement of these nozzles into a desired array was set in Panel F4, and their positions were confirmed through a camera view matched to a 4 × 4 light-emitting diode (LED) array in Panel F3. After selecting the desired channels, solenoid valves were activated by pressing 'Aspiration On/OFF' and 'Aspiration Set Coils' sequentially. To deactivate the selected channels, the user can press again 'Aspiration On/OFF' and 'Aspiration Set Coils' sequentially."

9. Materials and Methods: It is better to explain how the system maintains high cell viability during the bioprinting process.

Response: We have added the following on *Page 32*: "To ensure high cell viability during bioprinting, we optimized the aspiration pressure, identifying ~10 mmHg as the optimal level. This pressure did not affect the spheroid structural integrity while enabling lifting from the culture medium." Under these conditions, spheroids maintained high viability for 14 days post bioprinting, as shown in **Figures 5B** and **5E**.

10. In Spheroid Preparation: You should specify the type and source of cells used for the spheroids.

Response: We would like to kindly point out that the asked information was provided already. Specifically, on *Page 25* Section 5.1: "Human adipose-derived stem cells (hADSCs, PT-5006, Lonza, Walkersville, MD) were obtained and cultured in a basal medium…" Additionally, we have now added the preparation methods for other new spheroid types, in Section 5.1, *Pages 25-26* as follows: "Similarly, other spheroids were prepared by taking 8,000 cells in each well of a cell repellent 96-well plate. In the first group, MDA-MB-231 (MDA; gift from Dr. Danny Welch, University of Kansas) and human lung fibroblasts (HLFs; Lonza, Walkersville, MD) were mixed in ratios of 1:0, 3:1, 1:1, 1:3, and 0:1, respectively. In the second group, MDA and human dermal fibroblasts (HDFs; Lonza) were combined in ratios of 3:1, 1:1, 1:3, and 0:1. In the third group, human umbilical vein endothelial cells (HUVECs; Lonza), transduced to express tdTomato (tdTomato HUVECs) following a previously established protocol [65], were mixed with HDFs in a 1:2 ratio. The last group of spheroids consisted of HDFs only. MDAs, HLFs, and HDFs were cultured in the Dulbecco's Modified Eagle Medium (DMEM; Corning) supplemented with 1% penicillin-streptomycin (PS), 10% FBS, while HDF/HUVECs were cultured in the MCDB 131 medium (Corning) supplemented with 10% FBS, 1 mM Glutamine, 1 mM PS, 0.5 mm bovine brain extract (BBE, Lonza), 1200 U mL^{-1} heparin (Sigma-Aldrich, St. Louis, MO) and 0.25 mM endothelial cell growth supplement (ECGS, Sigma-Aldrich). The cells were then allowed to

aggregate and form spheroids over 24-48 h in an incubator set to 37°C with 5% CO_2, ensuring consistent spheroid formation across all groups. For fabrication of pyramidal architectures, spheroids were generated using ADSCs with varying cell numbers. ADSC aliquots containing 7,000, 14,000, 20,000, and 30,000 cells were prepared for spheroid formation by seeding them into low-attachment 96-well plates. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24-48 h to allow spheroid formation.

Induced pluripotent stem cell (iPSC)-derived vascular organoids were generated as per an established protocol [66]. Briefly, iPSCs (IMR90C4 iPSCs, WiCell Research Institute Inc, Madison, WI, USA) were aggregated and mesoderm differentiation was induced using CHIR99021 and BMP-4. For vascular differentiation, a combination of growth factors including VEGF-A and forskolin over a period of 3-5 days were used."

11. Please describe the methods for culturing and forming spheroids, including the duration and culture conditions.

Response: We would like to kindly point out that the asked information was already provided in Section 5.1 Cell culture and spheroid/organoid fabrication. As suggested, we have now specified the duration as: "24-48 h."

12. Explain the rationale for choosing specific miRNAs for transfection and their role in enhancing osteogenesis.

Response: We would like to kindly point out that the asked information was already provided. Specifically, on *Pages 17-18*, Discussion section – "A combination of miR-196a-5p and 21 was used for the co-transfection of hADSCs to create osteogenically-committed spheroids. miR-196a-5p plays a crucial role in bone homeostasis and is highly expressed in osteoclast precursors [24]. Kim et al. reported that miR-196a-5p regulates the proliferation and osteogenic differentiation of human ADSCs, which may be mediated through HOXC8 [25]. Concurrently, miR-21 has been proven to play a role in bone formation by mediating mesenchymal stem cell proliferation and differentiation [26,27]. It activates the ERK-MAPK (extracellular signal-regulated kinases (ERKs)-mitogen-activated protein kinases (MAPKs)) signaling pathway, promoting osteogenesis by suppressing the expression of its target gene SPRY1 [28]. In a study, when combined, miR-196a-5p and -21 exhibit synergistic effects to enhanced osteogenesis, where miR-196a-5p stimulates osteogenic ability, while miR-21 further supports osteoblastic differentiation and amplified proliferation rate, confirming the hypothesis of Abu-laban et al. [29] and Celik et al. [17]."

13. In Bioprinting Protocols: The authors should provide a detailed description of the bioprinting protocols, including the steps for intraoperative bioprinting and scalable tissue fabrication.

Response: We would like to respectfully note that the requested details have already been provided. Specifically, **Figure 1** includes a schematic outlining the steps involved, and we have now added a detailed algorithm for the entire HITS-Bio operation (**Figure S3-S4**). Please see above Comment #8 for more details. Additionally, Sections 5.12 (Intraoperative Bioprinting of Bone) and 5.18 (Scalable Cartilage Tissue Fabrication) offer further detailed explanations of the processes. In addition, we have now expanded the description to give step wise protocol as mentioned in **Figure 1**. The inclusion in the manuscript on *Page 5* is as follows: "…For IOB (**Figure 1C**), a critical-sized calvarial defect (Step 3.1) was created. Firstly, the bone ink (BONink) was extruded at the defect area (Step 3.2) and the DCNA loaded with spheroids was positioned over the defect (Step 3.3). The spheroids were deposited at two different spheroid densities (low

- 16 spheroids and high - 64 spheroids) using the DCNA (Step 3.4 – 3.5). Then, another layer of the BONink was extruded over the spheroids (**Figure 1C**, Step 3.6), followed by photo-crosslinking and suturing of the skin (Step 3.7). For scalable tissue bioprinting (**Figure 1D**), scalable cartilage tissues (SCTs) were created, using a cartilage ink (CARink). Firstly, the CARink was extruded (Step 4.1), followed by the precise placement of 64 chondrogenic spheroids (Step 4.2-4.3). This iterative process (Step 4.2 – 4.4) was repeated nine times to assemble a construct comprising nine stacked layers and a total of 576 spheroids, followed by photo-crosslinking (Step 4.5)..."

14. Materials and Methods: The information about the composition and properties of the bioinks (BONink and CARink) used in the bioprinting process should be included.

Response: We would like to kindly point out that the composition of the bioinks was already provided in Section 5.3 Preparation of Bioinks (BONink and CARink), and illustrated in **Figure S14A-B**, while the bioink properties were characterized as shown in Results Section 2.2 *In vitro* development and characterization of bioinks as a substrate for spheroid bioprinting.

15. Please specify the parameters for the photo-crosslinking process.

Response: We have already provided the parameters as follows: In Section 5.3, "0.25% (w/v) lithium phenyl (2,4,6-trimethylbenzoyl) phosphinate (LAP, L0290, TCI Chemicals, OR) as a photoinitiator" and "photo-crosslinking using a 405 nm LED light source for 1 min (**Figure 1B**, Step 2.5)", while the scalable cartilage tissues were photo-crosslinked using the same light for 3 min (Section 5.18).

16. Results: The authors should present quantitative data on the extent of defect closure over time.

Response: We already included quantitative data on the extent of defect closure over time in the original submission, as detailed in Section 2.3, *Pages 12-13*, as follows: "As a quantitative metric to assess the efficacy and extent of bone tissue formation, bone volume to total volume (BV/TV) was calculated, which revealed significantly higher bone regeneration of ~38 and 33% in Week 3, and ~39 and 39% in Week 6 for low- and high-density group, respectively, compared to the BONink-only ($p \leq 0.05$) and empty group ($p \leq 0.01$) (**Figure 4Ci**). Moreover, the normalized bone mineral density (BMD), reflecting the density of regenerated bone normalized to the native bone density, exhibited ~29 and 28% at Week 3, and ~34 and 34% at Week 6 for low and high-density groups, respectively, compared to the BONink-only ($p \leq 0.05$) and empty group ($p \leq 0.01$) (**Figure 4Cii**). Additionally, the bone coverage area (%) for the low-density and high-density groups was ~90 and 91% at Week 3, and ~88 and 96% at Week 6, respectively (**Figure 4Ciii**). Moreover, the maximum intensity projections generated from µCT data (**Figure 4B**) of each group were used for the scoring (1-4) of bony bridging across the defect. The results showed significantly higher scores for spheroid-involved groups compared to empty defect and BONink-only groups at both Weeks 3 and 6 (**Figure 4Civ**)."

17. Histological images should be added to show the integration of bioprinted constructs with host tissue.

Response: We had originally provided histological imaging data to show the integration of bioprinted constructs with host tissue in Sections 2.3 and 2.4. Moreover, we have now provided new immunohistochemical staining data for Procollagen type I N-terminal propeptide (P1NP) and Osteocalcin (OCN), the key markers for bone formation (**Figure 4E**), further attesting the same.

18. Please provide evidence of the role of miRNA in enhancing osteogenesis.

Response: We would like to kindly point out that this has been addressed in your Comment #12.

19. In Scalable Cartilage Constructs: Please show data on the mechanical properties of the bioprinted cartilage constructs.

Response: We have now included new data on the mechanical properties of SCT constructs as follows (*Page 14*): "The compressive modulus of the bioprinted SCT was found to be 116.8 ± 22.1 kPa (**Figure S19C**)." Further, we have discussed and compared the mechanical properties of our samples with literature and native values on *Page 22* (as per Reviewer 2 Comment #10).

20. Images demonstrating the uniform distribution of spheroids within the constructs should be included.

Response: We have already provided data demonstrating uniform distribution of spheroids within constructs *in vitro*, as shown in **Figures 3F, 5A, S17**, and **Supplementary Videos 6-7**. However, this was not technically possible *in vivo*. We have now discussed this in the revised manuscript on *Page 21*, as follows: "…Additionally, although the spheroids were deposited uniformly, their direct visualization and distribution *in vivo* was not feasible due to technical limitations associated with embedding constructs within the tissue matrix. Moreover, the uniformity of the original spheroid distribution may be challenging to assess in histological evaluations of retrieved explants, as tissue remodeling and integration processes can alter the initial arrangement of spheroids...."

21. The authors can present data on the viability and functionality of the chondrogenic spheroids post-bioprinting.

Response: The viability and functionality of the chondrogenic spheroids post-bioprinting were already presented in Section 2.4 and **Figure 5**. Specifically, **Figures 5A** and **5E** illustrate the viability of the spheroids, while **Figures 5C** and **5G** demonstrate their functionality by showing the detection of extracellular matrix components, such as glycosaminoglycans (GAGs) and proteoglycans.

22. Discussion section: The authors could discuss the limitations of the HITS-Bio system, such as the potential for mechanical damage to spheroids during the bioprinting process.

Response: In the final paragraph of the Discussion section, we have already addressed the limitations of the HITS-Bio system. In addition, we have now included the following on *Page 23*: "Third, as with most pressure-driven systems, HITS-Bio is susceptible to potential damage to spheroids due to the aspiration forces applied during bioprinting. To mitigate this risk, we optimized several key parameters, including nozzle size and pressure, ensuring that all nozzles in the DCNA are aligned on a uniform plane to minimize stress on spheroids and preserve their structural integrity. Through this optimized approach, we identified a range of elastic moduli suitable for effective bioprinting with HITS-Bio. Specifically, spheroids with an elastic modulus greater than ~50 Pa were successfully bioprinted, while those below 40 Pa were not suitable (**Figure S9**). Also, we demonstrated the successful bioprinting of iPSC-derived vascular organoids with an elastic modulus of 133 ± 20 Pa (**Figures 2J and S12**), showing the system's applicability to bioprinting of organoids."

23. Add information about comparing the HITS-Bio system with other bioprinting technologies, highlighting the advantages and disadvantages.

Response: We have added a comparative analysis of the HITS-Bio system with other spheroid bioprinting technologies on *Pages 2-3*, as follows: "Several bioprinting techniques, each with specific advantages, have been explored for spheroid-based approaches; however, certain challenges limit their applications. For example, EBB offers high throughput by randomly mixing spheroids within a bioink and extruding them into various architectures, but this exposes cells to substantial shear stress and lacks control over the precise number and placement of spheroids [5]. The Kenzan method, which uses needle arrays for spheroid placement, faces limitations due to its low throughput as it assembles spheroids one at a time, often causing damage, and the fixed needle arrangement restricts its versatility [12]. Similarly, droplet-based bioprinting (DBB) is constrained by droplet formation, bioink viscosity, and limited precision, making it less suitable for applications requiring high spatial resolution or scalability [13]. Other spheroid assembly strategies, such as magnetic and acoustic methods, offer innovative approaches but come with challenges [14]. For example, while the magnetic technique enables spatial patterning of organoids, it requires the use of biocompatible magnetic particles, which may not be consistent across different cell types. Additionally, this method is low-throughput, as it typically manipulates one spheroid at a time, requires specialized molds, and may impact spheroid shape fidelity and viability due to magnetic forces [15]. On the other hand, the acoustic technique offers non-invasive, high-throughput spheroid patterning. However, it relies on acoustic nodes, which limits the ability to create fully compact cellular structures and restricts interactions between spheroids. It limits the flexibility needed for achieving desired spatial arrangements in complex geometries [16]." While AAB shows promise to address some of these limitations, it remains constrained by throughput. Thus, the HITS-Bio system was specifically developed to overcome these challenges, offering a more versatile option for spheroid-based tissue biofabrication applications.

24. How to address the scalability of the system for larger tissue volumes and more complex structures.

Response: We already demonstrated the scalability potential of HITS-Bio by fabricating a 1 cm^3 cartilage construct containing ~600 chondrogenic spheroids in under 40 min per construct. In addition, we have now added new data and showed the capability to create more complex structures, including a 4-layered pyramidal shape using four different spheroid sizes and a total of 171 spheroids (**Figure S13**), fully cellular architectures incorporating vascular cells (**Figure S10**), and void-filling constructs (**Figure S11**). However, achieving full scalability for larger tissue volumes and more intricate structures will require further enhancements to the current platform, as discussed in the last two paragraphs of the Discussion Section.

25. Add some discussion about the potential for integrating vascular networks within bioprinted tissues.

Response: We have discussed this and now expanded upon on *Page 24* as follows: "Using HITS-Bio, a fully cellular architecture composed of HDF/HUVEC spheroids (**Figure S10**), and iPSC-derived vascular organoids (**Figures 2J, S12**) were bioprinted, demonstrating its potential for integration of vascularization within bioprinted constructs. Its precision in co-bioprinting vascular spheroids alongside other tissue-specific cells can support vascularization, which is critical for sustaining the viability of large-scale tissues. Looking ahead, the current work aligns with the urgent need to advance spheroid bioprinting techniques for rapid fabrication of scalable,

vascularized tissues. Integrating vascular networks within large-scale bioprinted tissues, particularly for organs with high metabolic demands such as the heart, pancreas, and liver, is a crucial step achieving clinically-relevant tissues for transplantation."

26. Conclusion: Please summarize the main findings and emphasize the significance of the HITS-Bio system in tissue engineering. Further, it is better to outline the future directions for improving the system, such as incorporating more nozzles for higher throughput and developing methods for bioprinting on non-planar surfaces.

Response: We have now added the following to the Conclusion Section: "…To further expand its capabilities, future improvements could include incorporating additional nozzles to increase throughput, developing height-adjustable platforms for bioprinting on non-planar surfaces, and increasing automation. These technological enhancements are critical toward fully realizing the potential of HITS-Bio in scalable tissue biofabrication applications."

Reviewer #2 (Remarks to the Author):

In this study, the authors developed HITS-Bio (High-throughput Integrated Tissue 8 Fabrication System for Bioprinting) as a further development of aspiration-assisted bioprinting (AAB). The methods enables multiarray spheroid bioprinting wherein a number of spheroids are aspirated and rapidly deposited using a digitally-controlled nozzle array (DCNA) platform. The reviewer finds that necessary important variables were addressed. However, the chosen model system (cartilage, bone) seems inadequate for the chosen printing technology and lacks experimental controls that would demonstrate superiority of this technique over more established additive manufacturing techniques. In addition, the proposed method is largely based on a previously published data and the authors miss out on demonstrating a significant contribution with regards to methodology. Lastly, the capabilities of the DCNA array, in particular for complex (multilayer, difficult-to-print) models are not further explored.

The reviewer finds that the authors have presented an overall sound set of methodologies and provided details which are both sufficient and meet the expected standards in the field.

However, the advances presented in this manuscript are only moderately innovative and present at best a natural progression of prior art. Due to several limitations outlined in the comments below, the reviewer finds the manuscript to be of moderate significance for the bioengineering/tissue engineering field. The reviewer's comments and major revision requests are found below:

Response: We thank the reviewer for the comments aimed at improving the manuscript. We would like to highlight that the advances presented herein are significant within the biofabrication field. Using the HITS-Bio platform, we have, for the first time, successfully demonstrated the scalable fabrication of constructs using spheroids, as well as the intraoperative bioprinting of spheroids in a high-throughput manner, unlike the one-by-one approach of conventional methods. These findings indicate the platform's versatility, even in surgical settings (which is highly challenging compared to *in vitro* bioprinting), and represent a substantial advancement over existing techniques, opening new possibilities for scalable tissue engineering applications.

1. The HITS-Bio process is described for spheroids ranging from 300-350 µm. The authors should elaborate on the applicability, challenges and possible modifications of the system to accommodate smaller and larger spheroids?

Response: In this study, the HITS-Bio process was primarily optimized for spheroids ranging from 300-350 µm in diameter. However, we have demonstrated its applicability to larger spheroids, as shown in **Figure 2H** and detailed on *Page 7* of the manuscript, where we successfully bioprinted spheroids with diameters of 350 µm (Green), 425 µm (Blue), and 500 µm (Red). Additionally, through further experimentation, we have extended the range of spheroid sizes that can be accommodated by the HITS-Bio system to 300-735 µm in diameter (see your Comments #7, 8, and 12). Regarding the challenges and possible modifications of the system to accommodate smaller and larger spheroids, we have elaborated the following on *Page 16*: "The HITS-Bio process was optimized for spheroids within the 300-350 µm range using a 30G needle, which was proven effective for this size range. It is important to note that the spheroid size must be larger than the inner diameter of the nozzle used. For smaller spheroids, adjustments in nozzle size would be necessary to prevent them from being inadvertently drawn into the nozzles, though this is currently limited by the availability of commercial nozzles with a diameter smaller than 30G. Larger spheroids may require modifications in nozzle size and spacing to avoid spatial interference and ensure efficient transfer. This can also be managed by selectively controlling (On/Off) adjacent nozzles in DCNA, to accommodate the transfer of larger spheroids. Additionally, while the current 4-mm exposed nozzle length was sufficient for bioprinting onto gel surfaces, extending the exposed nozzle length could optimize the system for embedded bioprinting applications, enabling more complex or deeper spheroid placements. Future iterations of DCNA could benefit from the inclusion of independently height-adjustable nozzles and an increased number of nozzles, enhancing the system's flexibility and versatility across a broader range of applications."

2. What are the physical limitations of the DCNA nozzle array, and to what extent are factors such as inter-nozzle capillary reactions eventually limiting the picking of spheroids? How does the setup of the array correlate with spheroid size?

Response: As highlighted in your Comment #1, the physical limitations of the DCNA nozzle array were primarily influenced by the nozzle size, and spacing of the nozzles, which were crucial for efficient and precise spheroid manipulation. The DCNA was designed to operate effectively within a specific range of spheroid sizes optimal for our applications, and while there were certain factors that influence its performance, these were not inherent limitations but rather aspects that could be optimized for broader applications. Apart from the inclusion of Comment #1, we have added the following text regarding the inter-nozzle capillary reactions on *Page 23*: "…the inter-nozzle capillary interactions can affect spheroid picking precision, especially for closely spaced nozzles. However, by coating the nozzles with silicon and adjusting their spacing, as demonstrated in our study, and potentially incorporating advanced fluid dynamics management, these interactions can be effectively controlled. These approaches will reduce liquid elevation between nozzles, ensuring accurate and efficient spheroid manipulation. Building on these optimizations, the DCNA setup was further calibrated to correlate with spheroid size, ensuring optimal bioprinting performance. For larger spheroids, increased spacing between nozzles is necessary to prevent physical interference, while smaller spheroids benefit from tighter nozzle spacing and smaller nozzle sizes

to maintain precision and avoid unintended suction. In cases where larger spheroids were difficult to lift due to the surface tension, selective controlling of adjacent nozzles was performed, such that only alternate nozzles were active. Using this method, we bioprinting red spheroids (735 µm in diameter) in a pyramidal structure (**Figure S13**), validating its effectiveness in handling larger spheroids.."

3. Page 3, line 66: The authors claim that the technique is also applicable to organoids. However, corresponding data are not shown. The reviewer is furthermore missing a quantification of shear, suction, and compressive forces that would enable to judge the usefulness of this system for very soft and fragile organoids (brain, vascular, liver etc.).

Response: While this study primarily focused on spheroids, we acknowledge the importance of demonstrating the technique's broader applicability to soft and fragile organoids. To address this, we have now provided the range of spheroid elastic moduli within which they can be effectively bioprinted on *Pages 7-8* as follows: "To test the broader applicability of HITS-Bio to soft and fragile spheroids and organoids, a wide range of samples made of different cell types were screened. Our findings indicate that spheroids with an elastic modulus greater than ~50 Pa were printable while those below 40 Pa were not (**Figure S9**). For example, spheroids of MDA-MB-231 breast cancer cells (MDA) and human lung fibroblasts (HLF) mixed in an 8:0 ratio were not bioprintable while spheroids of the 2:6 ratio were bioprintable. The transfected osteogenic and chondrogenic spheroids had an elastic modulus of 978 ± 96 Pa and 641 ± 110 Pa, respectively. Along with various spheroids, elastic modulus of iPSC-derived vascular organoids was also tested, which was measured to be 133 ± 20 Pa and fell within the range of printable elastic moduli…"

3. What minimum and maximum viscosity is required in this process? How are those parameters derived?

Response: Since we can place the spheroid on any solid substrate including tissue culture plates, viscosity of the bioink may not be a directly relevant parameter for our process. However, assuming the query to be the minimum and maximum stiffness, we have now provided new data and discussed this in Comment #3.

4. Fig. S3: shows the spheroid transfer process for soft support baths. How does this transfer process translate into more viscous media? In particular, how does the increased shear and compression that would be present in such a support bath interfere with the proper transfer?

Response: Figure S3 (now **Figure S5**) illustrates spheroids suspended in a standard cell culture medium. We have specified this in the figure caption and manuscript. No support bath was utilized; instead, spheroids were picked from the culture medium and placed directly onto a gel substrate. We have now added this discussion on *Page 4*, as follows: "The spheroid transfer process was specifically designed to function within the culture medium, eliminating the need for a viscous fluid support bath. This approach simplifies handling and avoids the challenges associated with increased shear and compression forces, ensuring the integrity of spheroids during transfer."

5. Can the authors make a statement regarding the overall experimental duration for a physiologically relevant and complex print (multilayer deposition with non-repeating deposition patterns), corrected for the time it requires to get the DCNA nozzle array loaded spheroids in a non-repeating pattern. The reviewer assumes that at higher complexities, the loading itself becomes a central aspect that limits the efficiency of the method, especially as the loading

patterns become non-repeating, i.e. non-uniform across repeated loading-deposition cycles. What alternatives to the proposed spheroid loading system can the authors propose that would enable a more efficient and targeted loading (aspiration)?

Response: We have now discussed this on *Page 17*, as follows: "In terms of experimental duration, it is pertinent to note that as the complexity increases, particularly with multilayer deposition and non-repeating patterns, the loading process becomes a critical factor influencing the overall efficiency. For physiologically relevant and complex constructs, the experimental duration can vary significantly based on the intricacy of deposition patterns. While the DCNA streamlines simultaneous loading and deposition of multiple spheroids, non-repeating patterns introduce additional challenges. In this context, a total of 50 spheroids were successfully bioprinted in 8 min using two different types and sizes of spheroids (**Figure S10**). This process involved non-repeating patterns to fill gaps (without a predesigned travel path) for a fully cellular architecture and took ~8 min compared to ~5 min for a repetitive pattern of 64 spheroids using DCNA and ~25 min using a single nozzle AAB. Although the efficiency of non-repeating patterns was not as high as with repeated patterns, it was still significantly faster than the existing benchmark. The use of pre-designed travel path for bioprinting and enhancing the system with more nozzles and incorporating height-adjustable nozzles could significantly improve versatility, particularly for non-repeating patterns."

6. Page 4, lines 98ff: The authors describe a camera-based system to control and track the spheroid picking process. This process seems to allow for little control over the actual forces applied to the spheroid. A pressure-based control (via measuring the resistance or using an aspiration pressure threshold) would enable more precise and controlled pickups. What was the reason behind choosing a system based on visual interrogation and what particular benefits does this method feature vis-à-vis the aforementioned technique. The reviewer is missing a description of a dynamic pressure control setup for the controlled picking of spheroids in cases where the 3 mmHg condition fails to work.

Response: We utilized a pressure sensor to monitor the internal pressure in the platform, but we did not rely on its feedback for spheroid picking, rather we have a visual confirmation using a camera. Our system used a pressure control mechanism through solenoid valves to manage pressure, vacuum, and individual nozzle control in the DCNA. We chose a camera-based system for spheroid picking and placement due to its real-time visual feedback, which ensures precise positioning and adaptability across various spheroid types. However, we are considering integrating pressure control as a complementary feature in future iterations to enhance automation. Additionally, we would like to clarify that the 3 mmHg pressure mentioned in the above comment was optimized to hold media and prevent media leakage after cutting off the aspiration pressure (10-15 mmHg) without interfering spheroid placement. We have now added the following on *Pages 15-16*: "…A pressure sensor was used to monitor internal pressure in the platform, but the primary method for spheroid picking relied on a visual confirmation via a camera-based system. This camera-based system provided real-time visual feedback, ensuring precise positioning and adaptability across various spheroid types, regardless of differences in shape, size, or surface texture. While dynamic pressure control could be advantageous in certain situations, such as monitoring pressure changes during spheroid loading, we found the camera-based approach to be more versatile. Additionally, spheroid debris can interfere with pressure-based sensors, leading to false positives during spheroid placement."

7. Page 1, line 4f: The authors point out the relevance of high cell density in tissue engineering to replicate organ-specific architectures, yet they miss to show a corresponding application. Indeed, Figures 2G and 3F demonstrate that the process enables the deposition of spheroids in a tight pattern. However, the reviewer finds that a print with a fully cellular architecture should be included to demonstrate the capability of this method towards organ printing. In particular, this demonstration of a fully cellular print should feature tightly packed spheroids/organoids without interspaced ECM. This would help to further compare this novel setup to previously published work (DOI:10.1126/sciadv.aaw5111).

Response: While our initial results effectively demonstrate the system's ability to deposit spheroids in precise patterns, we acknowledge the importance of demonstrating fully cellular architectures with tightly packed spheroids. We have now conducted additional experiments utilizing spheroids of various sizes. The inclusion in manuscript on *Page 8* are as follows: "…Moreover, the system's ability to precisely deposit spheroids in tightly packed, fully cellular architectures in order to better replicate tissue-specific structures was demonstrated. Human dermal fibroblast (HDF) spheroids (530 µm) were arranged in rows via bioprinting them onto a pre-crosslinked GelMA (10%) substrate, with gaps filled by smaller spheroids of HDF with human umbilical vein endothelial cells (HUVEC), HDF/HUVEC (300 µm) (**Figure S10A**). These spheroids fused to form a continuous, densely packed structure, highlighting the potential for creating vascularized tissues, as tdTomato^{+} HUVECs successfully migrated and formed pre-vascularized-like structures within fused spheroids. This was further supported by Von Willebrand Factor (VWF) and fibronectin (FN) staining (**Figure S10B**), where VWF staining was used to identify and confirm the presence of endothelial cells and the formation of pre-vascularized-like structures. The integration of HDF and HDF/HUVEC spheroids into a fully cellular architecture was clearly visible at the interface, supporting HITS-Bio's capability to generate complex tissue constructs…"

8. Herein, different stacking patterns should be explored (e.g. print patterns for volume-filling models featuring spheroids of different sizes to maximize the faction of the filled volume) to generate models with varying degrees of ECM filling.

Response: We have incorporated additional data showing a range of print patterns, each designed to maximize cellular density. The inclusion in manuscript on *Page 8* is as follows: "Furthermore, examples were demonstrated for optimization of area-filling models with spheroids of varying sizes to maximize the filled area fraction on the GelMA substrate. As shown in **Figure S11**, area filling of 37.03% was achieved using large spheroids (~735 µm in diameter), which increased to 63.71% when large and small spheroids (~300 µm) were mixed. This further improved to 86.91% using small spheroids alone and ultimately reached 98.11% by combining two different sizes (~530 µm and ~300 µm). These tailored patterns were designed to optimize cellular density while strategically integrating filler hydrogel substrate, utilizing various nozzle configurations to precisely balance cellular content and filler hydrogel distribution, which can be further expanded to 3D volume-filling models."

9. Furthermore, it is not clear why this tissue type (cartilage and bone) was chosen to demonstrate this technique as other tissues (e.g. liver, muscle) would benefit more from the highly dense packing of spheroids/organoids. In that regard, it is also not clear how the showcased print (Figure 3F) would be superior to a control with randomly and homogeneously distributed spheroids (via mixing and casting). The authors should include such a control.

Response: Evidence from both our lab and others has demonstrated the effectiveness of spheroid-based approaches for applications in bone, cartilage, and the osteochondral interface. For tissues like liver and muscle, there may be additional challenges due to their highly vascularized and anisotropic nature, making them less amenable to spheroid-based models compared to the relatively uncomplicated applications in cartilage and bone. We have included the following details on *Page 17*: "The spheroid-based approach offers significant advantages for bone and cartilage tissue engineering by effectively mimicking the hierarchical native structures essential for these tissues [20,34]. Spheroids enable physiologically relevant cell-cell and cell-matrix interactions, which are crucial for the differentiation and maturation of chondrocytes and osteoblasts [10]. Moreover, the relative avascularity of cartilage and the minimal vascularization requirements in early-stage bone tissue reduce complexity. Additionally, bone and cartilage are among the most extensively studied tissues, offering a great amount of reference data, making them ideal models for developing and validating new bioprinting technologies."

We recognize the importance of comparing the HITS-Bio samples with a control involving randomly distributed spheroids. To address this, we initially included a manual control group, where spheroids were mixed and cast, yielding a random distribution. However, we observed spheroid loss during transfer at pipette tips, particularly in more viscous bioinks, and spheroid aggregation was observed as well. Additionally, air bubble formation during the mixing process further compromised the construct integrity. The H&E-stained images from the manual control group, demonstrating these limitations, have been included in the revised manuscript. The inclusion in the manuscript on *Page 20* is as follows: "...In this study, a control group with manually loaded spheroids was initially included with randomly distributed spheroids, but this method encountered significant technical challenges, such as spheroid loss during their transfer, spheroid aggregation, and air bubble formation, leading to uneven distribution and reduced reproducibility as also highlighted in *in vitro* studies (**Figures S7-S8**). These issues compromised the reproducibility and data accuracy. Consequently, we did not pursue this method further in animal studies, in accordance with the principles of the 3Rs, to minimize unnecessary animal experimentation…"

10. Figure 3 describes in vitro testing of the printed constructs. Eventually, both cartilage and bone quality will have to be assessed with respect to their compressive modulus, which enables them to sustain loads. The reviewer is missing a mechanical characterization of matured tissues and a comparison with previously published data featuring similar average cell densities, maturation times etc.

Response: We have now provided a comparison of the mechanical properties with published data on *Page 22*: "Regarding mechanical properties, the BONink (GM20HA30) formulation without spheroids exhibited a compressive modulus of 360.7 ± 66.6 kPa. When compared to engineered bone reported in the literature, which ranges from 0.1 to 10 MPa [55], our *in vitro* results were within the expected range for early-stage bone constructs. Further, the *in vivo* retrieved explants demonstrated a significantly higher shear modulus of ~20 MPa, which corresponds to a compressive modulus of ~52 MPa [56]. For reference, the compressive modulus of cranial or flat bone in rats ranges from 5 to 9 MPa (depending on the age) [57-59], while in humans, cortical bone ranges from 10 to 20 GPa and trabecular bone from 0.1 to 2 GPa [56,60]. It is important to note that mechanical properties of the implanted constructs do not necessarily need to mimic those of native bone, as they can be supplemented with plate osteosynthesis during implantation [61].

However, the constructs must possess sufficient mechanical strength to provide initial support ensuring stability and facilitating proper integration with the surrounding tissue during the early stages of bone regeneration. For human articular cartilage, the aggregate equilibrium compressive modulus was reported to be in the range of 0.1 to 2 MPa depending on the specific location and health of the tissue [62]. Reported values for bioprinted cartilage are lower with a compressive modulus typically ranging from 50 to 500 kPa [63]. In this study, the compressive modulus of CARink (GM20) without spheroids was measured at 88.3 ± 34.7 kPa, which increased in SCTs containing spheroids with a compressive modulus of 116.8 ± 22.1 kPa after 2 weeks of *in vitro* maturation. This limited increase in compressive modulus was likely due to insufficient localized ECM formation, indicating that longer maturation might be needed for further mechanical improvements. It is also crucial to recognize that standardized protocols for mechanical testing of bioprinted constructs are still being developed, with American Society for Testing and Materials (ASTM) standards yet to be fully established (latest as of April 2024, ASTM F3659-24). Therefore, comparisons with other studies may not be entirely accurate without standardized methodologies."

11. Page 13, line 400: A further claim states that 'area-to-area and batch-to-batch inconsistencies' can be mitigated using HITS-Bio, but necessary controls that would showcase spatial differences (e.g. compressive modulus, gene expression) were not included in the study. It is reasonable to assume that regions of highly dense spheroids with intercalating empty ECM would lead to a more heterogeneous tissue than single-cell deposition or stochastic deposition of spheroids at higher density. This claim can only stand if corresponding controls were included.

Response: As suggested, we have reconsidered the statement and since the necessary controls to substantiate this claim were not included in the study, we have decided to remove this claim from the manuscript.

12. In addition, more complex architectures with spheroid gradients or difficult-to-cast geometries must be demonstrated to justify the use of HITS-Bio over conventional, more scalable methods (extrusion bioprinting, casting/molding).

Response: We have now provided additional data that illustrates the formation of complex architectures. However, further improvements to the system will require additional optimization, as outlined in the final paragraph of the Discussion section. The relevant inclusion in the manuscript on *Page 9* is as follows: "As shown in **Figure S13**, the capability of HITS-Bio to create complex architectures was also demonstrated by assembling a pyramid construct composed of 171 spheroids, each varying in size and color-tagged for distinction. Specifically, the bottom layer comprised 121 300-µm-spheroids, followed by a 2nd layer of 36 540-µm-spheroids, a 3rd layer containing 9 735-µm-spheroids and 4 540-µm-spheroids, and the top layer featuring a single 445-µm-spheroid. This intricate structure was meticulously assembled by precisely controlling different nozzles of DCNA. The ability to accurately position and layer this number of spheroids, with such varied sizes and specific arrangements, is not feasible using conventional EBB or casting/molding techniques. This demonstrates HITS-Bio's potential not only for constructing intricate, multi-layered structures but also for creating complex tissue architectures that require highly controlled spatial organization and varied cellular compositions."

13. Figure 4B:

13.1. The use of BONink as control is insufficient. The reviewer suggests to have controls with randomly distributed spheroids to control for any cell-mediated effect. Without these controls a proper judgment of the benefit of spatially-defined deposition of spheroids over a random distribution for the observed effect is not possible.

Response: We respectfully maintain that using BONink (ink only) as a control was selected for this study due to reasons discussed below. We have previously discussed this in Comment #9, as follows: "…In this study, a control group with manually loaded spheroids was initially included with randomly distributed spheroids, but this method encountered significant technical challenges, such as spheroid loss during their transfer, spheroid aggregation, and air bubble formation, leading to uneven distribution and reduced reproducibility as also highlighted in *in vitro* studies (**Figures S7-S8**). These issues compromised the reproducibility and data accuracy. Consequently, we did not pursue this method further in animal studies, in accordance with the principles of the 3Rs, to minimize unnecessary animal experimentation.…" In continuation to Comment #9, we have now added the following about BONink as control: "…Instead, the BONink (ink only) was selected as the control, which provided a consistent, cell-free matrix that isolated the effects directly attributable to material properties without interference from the addition of exogenous cells (hADSCs). This approach allowed for a more reliable and clear assessment of the impact of spheroid deposition using HITS-Bio…"

13.2. Figure 4B: The formation of new bone from week 3 to week 6 appears to be minimal in all conditions (a-c) and even condition (d) only features minimal additional formation. What is the explanation for the relatively inefficient closure of large defect areas? Furthermore, it is unclear where the deposited spheroids are located. Additional immunofluorescence stainings (e.g. P1NP, or procollagen type I N-propeptide; BAP, or bone-specific alkaline phosphatase; and osteocalcin) should be shown to assess matrix deposition, cell density and general maturation at and around the sites of spheroid deposition.

Response: As added on *Page 20* of revised manuscript, "…Bioprinting using transfected spheroids contributed to earlier defect closure, particularly by Week 3, which is a significant improvement over existing approaches. This early bone formation reduced the difference observed between Weeks 3 and 6, which is consistent with our approach's ability to expedite bone healing compared to other approaches in the literature..." Thus, we respectfully disagree with the observation regarding inefficient closure of large defect areas. The µCT data clearly demonstrates substantial bone coverage, with 91% at 3 weeks and 96% at 6 weeks, along with near-complete bridging, evidenced by a score of 3.85 ± 0.37 out of 4. Additionally, the IHC analyses for bone markers such as RUNX2, OSTERIX, P1NP, and OCN provide further evidence of efficient defect closure, highlighting active osteogenesis and bone remodeling processes within the defect area. Additionally, spheroid distribution in the retrieved explants can be challenging due to potential tissue remodeling and integration. However, as suggested, we have now performed additional IHC staining for P1NP and OCN. The inclusion in the manuscript is as follows on *Pages 13-14*: "…Procollagen type I N-terminal propeptide (P1NP) and Osteocalcin (OCN), synthesized and secreted by osteoblasts, are well-established markers for bone formation. IHC images revealed that OCN staining intensity was lowest in the empty and BONink-only groups, increased in the low-density group, and was highest in the high-density group

(**Figure 4E**). Notably, both native and newly formed bone tissues were stained with OCN, suggesting that OCN plays a crucial role not only in the formation of new bone but also in the ongoing maintenance of the existing bone matrix [24]. This could be attributed to OCN binding to HA, which is more prominent in areas of active bone formation. Similarly, P1NP staining followed a comparable pattern, with increased intensity in spheroid-containing groups, particularly in the high-density group, where the intensity was highest. Importantly, P1NP staining was predominantly observed in the newly formed tissue, suggesting its specific involvement in early collagen synthesis during the initial stages of bone formation [25]. Furthermore, higher expression levels of RUNX2 were observed in the bioprinted groups compared to the empty defect and BONink-only groups. In contrast, OSTERIX was predominantly localized in the host bone rather than the regenerated bone (**Figure S18**), suggesting that the newly formed bone was in an early developmental stage, consistent with the MT staining results. Overall, the IHC analysis for P1NP, OCN, RUNX2 and OSTERIX, offer a robust assessment of different stages of osteogenesis, from early osteoblast differentiation to collagen synthesis and mineral deposition, ensuring a thorough evaluation of new bone formation and its integration with the native bone."

13.3. What explanation can be given to explain the relatively low degree of fusion between printed transplant and host cranial bone within the calvarial defect? The authors claim 'near-complete bridging' (page 16, line 498), however, the transplant does not seem to feature continuous bridging throughout the construct, or to the host institute.

Response: In addition to the µCT analysis, the extensive IHC data clearly demonstrates the fusion of the implant with the host bone. However, continuous bone visualization in the transverse plane presents challenges due to the complex 3D architecture, variations in bone density, and the presence of potential gaps. Minor misalignments during scanning can further complicate the assessment by obscuring bone continuity, making it difficult to evaluate uniform bridging. To mitigate these issues, we used both sagittal and transverse planes for a more comprehensive evaluation. Nevertheless, further optimization of scanning protocols may be necessary to enhance the accuracy and reliability of these assessments. While significant regeneration was achieved, variations in fusion between the transplant and host bone may be attributed to localized differences in mechanical loading and ongoing remodeling. The formation of dense, immature bone tissue, as indicated by histology, suggests that complete maturation and integration may require a longer timeframe. We have revised the manuscript to clarify these findings and acknowledge that while substantial bridging occurred, continuous and uniform fusion across the construct remains a challenge for further investigation. For greater clarity, we have now provided improved images (**Figure 4B**) from other scanned planes of the µCT sections, clearly illustrating the relatively high degree of fusion between the bioprinted transplant and the host cranial bone within the calvarial defect. The inclusion in the manuscript on *Page 20* is as follows: "…Despite significant bone regeneration, we observed variations in fusion between the implant and host bone, which may be attributed to localized mechanical loading differences and ongoing remodeling. The histological evidence of dense, immature bone tissue suggests that complete maturation and integration could require long-term studies..."

14. Page 16, line 516: wrong reference to Figures 2Fii and 2Fiii

Response: We have now corrected this to **Figures 3Fii** and **3Fiii**.