nature portfolio

Peer Review File

An Organ-wide Spatiotemporal Transcriptomic and Cellular Atlas of the Regenerating Zebrafish Heart

Corresponding Author: Professor Ying Su

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 authors provide in-depth single-cell sequencing data for zebrafish heart regeneration across several time points, combined with spatial transcriptomics. This undoubtedly represents a rich and useful resource for further studies into the mechanisms of heart regeneration. However, many single cell data-sets from regenerating zebrafish hearts have been published before, eg. for cardiomyocytes (Honkoop H et al., 2019), fibroblasts (Hu B et al., 2022 and), macrophages (Wei KH et al., 2023) and epicardium (Cao J, 2019). Recently, a comparative time resolved single cell sequencing data was published between zebrafish and medaka as well (Carey CM et al., 2024). Thus, the bar is already set quite high in terms of what a new study needs to add. The integration with spatial transcriptomics is new, but it seems that the biological insight that this has made possible is rel. limited. This is exemplified by the 4D atlas that the authors provide. This is very nice for the non-injured heart, but where it gets really interesting, namely heart regeneration, it seems to lack the resolution to be of much use. Overall it seems to us that albeit the paper represents a useful resource, it is rel. weak at deriving significant novel biological insights. Definitely, the authors should further support those conclusions that they do draw from their data with additional experiments as detailed in the “major issues” below. Whether they should in addition use their data as starting point for more experimentally validated insights to make it interesting enough for Nat. Comms is for the editors to decide.

Major issues:

1. Page 3, line 79-80. The authors claim that spatial transcriptomics platforms have not been used for zebrafish heart regeneration. While it is true that certain platforms have not been utilized, spatial transcriptomics in itself (admittedly with lower resolution as the currently used technology) has been already published on the cryoinjury model at one timepoint (Wu CC et al.,2014). This has to be clarified as they do not refer to this paper.

2. The existence of several cardiomyocyte subclusters (pre-dediff, dediff and dediff-prolif) is an interesting observation. Yet, the authors should tone down their conclusions: “these results demonstrate that CMs adopt a pre-dedifferentiation state as early as 6 hpa, becoming fully dedifferentiated by 12 hpa”. How do they know that CMs are “fully dedifferentiated” by 12 hpa? What is their definition of dedifferentiation? Do the CMs show disassembly of sarcomeres at 12 hpa? In page 7, lines 205-207, the authors refer to Honkoop H et al.,2019 which described the upregulation of nppa after 3 dpa. Early upregulation of nppa in the current paper might be due to differences in injury models. What is the earliest time point when tnfrsf11b and nppa genes can be detected in cryoinjury?

3. In Figure 2G, comparing uninjured vs 7 dpa, there is a decrease in tpm4a. How is that explained?

4. The conclusion that tpm4a might be required for CM re-differentiation is potentially interesting, but should be supported by additional data.
a) finding such a rather striking phenotype in heterozygous mutant fish is unexpected. Thus, authors should have much stronger statistics to back these data. samples size for wild-types with n=4 is clearly too low. Also, in how many independent experiments (batches of fish raised) have they seen these phenotypes? Did they house and raise wild-types and mutants together and genotype and separate them only immediately prior to resection or better even; did they perform the resections on non-genotyped fish to exclude bias?
b) Further, the embMHC data should be quantified to support the claim that dedifferentiation is not effected.
c) Can they provide data showing that there are fewer differentiated CMs in the mutants at late stages? E.g more CMs

expressing embMHC or disassembled sarcomeres at 30 dpa? Smaller CMs as shown in Bertozzi et al. 2021 doi: 10.1016/j.ydbio.2020.12.004? Or do the authors observe a defect in calcium handling in mutants based on Nguyen PD et al., who describe an increase in calcium handling maturation during the course of redifferentiation.

5. Fibroblasts: Conservation of lumican expression only in regenerating tissue is interesting, yet the authors unfortunately do not venture beyond confirming lumican expression.

Reviewer #2

(Remarks to the Author)
Li et al. present a resource of spatial transcriptomics (stereo-seq) and single-cell RNA-seq data of the regenerating zebrafish heart. This is a powerful dataset in a very interesting biological system, and the data analysis is overall well executed, even if some questions remain (see below). The manuscript has the character of a resource article, since the focus is on presenting the dataset in a relatively broad manner, without focusing very much on one specific process. Most of the novelty is related to cardiomyocytes, as the authors describe a more detailed spectrum of de- and redifferentiated cardiomyocytes than in previous publications, and they also included a functional experiment related to cardiomyocyte states. By contrast, there is less novelty in the authors' findings on fibroblasts, macrophages, epi- and endocardium. I would consider this manuscript to be in principle suitable for Nature Communications, but the authors would have to address some issues regarding data analysis and presentation (as described in more detail below), with the goal of maximizing the usefulness of this resource for the scientific community.

Major comments:

1) The amount of data presented by the authors is impressive. However, given that some populations do not match between both data modalities, and given that analysis of the main populations is done from different perspectives (stereo-seq for CMs and scRNA-seq for non-myocyte populations), it would be good to perform a systematic analysis for matching the clusters of the two modalities. Please also compare abundances between the two modalities.
Also, please separate the cell types identified by the two modalities in color codes, clearly highlight which cell types are of which modality, and specify this clearly in all figure legends. As an example, Fig. S2b & d, color code and legend of the populations is difficult to follow.

2) Of the 21 unique spot identities found by stereo-seq, neither the endocardial subtypes nor the pre-dedifferentiation or dedifferentiated CMs are identified as pure clusters of a single cell type. It hence appears that these subtypes cannot be disentangled (Fig S2A-B). The labels used to reference these populations are presented in a confusing manner in the manuscript: in the text in lines 188-192 this is not written explicitly, and in Fig. 2 and Fig. S2 the labels differ and are not explained in the footnote, only Fig. S2 clarifies the mixture between CM subtypes and the endocardial activated subtype.

3) The authors perform an integrative analysis of the data by linking scRNA-seq populations to spatial transcriptomic spots with RCTD, and they conduct this analysis for two endocardial subpopulations. As already mentioned above in comment 1, a more systematic integrative analysis will help to better understand the matching populations (e.g., do pre-dedifferentiation or dedifferentiated CM found by stereo-seq match with dedifferentiated CMs found by scRNA-seq?). Would it be possible to perform a deconvolution analysis as the authors do with the proliferative endocardial subpopulation, also for cardiomyocytes?

4) CMs (dediff.-prolif.) and CMs (prolif.) (identified by stereo-seq and annotated manually as cell types) express markers of non-myocyte populations (Fig. 2E). Do these markers such as collagens or postnb colocalize with tpm4a, or could this indicate a mixture of two or more populations?

5) It would be good to integrate the authors' single-cell RNA-seq dataset with the existing scRNA-seq atlas by Hu et al., Nat Genet, 2022, which has similar resolution and is so far the biggest scRNA-seq dataset for the regenerating zebrafish heart. It would be useful for the field to match the cell types/states identified here to the annotations of the previous publication. For instance, the authors identified two predominant subtypes of fibroblasts, general and pro-regenerative, while Hu et al. performed a more fine-grained resolution. It is not very helpful if each new publication introduces a different clustering resolution without relating to previous annotations.

6) Some of the analysis and plots are difficult to follow or not fully explained. For the comparison of FB populations in regenerative and non-regenerative hearts, the authors identify a list of candidate genes upregulated in pro-regenerative FBs and then investigate the pattern of expression of those genes in regenerative and non-regenerative scenarios. The populations of FBs plotted in Fig. 4E panels 2 and 3 should be introduced in the text or footnotes, including the labels for the different conditions of myocardial infarction (BZ, RZ, LZ, FZ). The pattern of expression of lum is validated by fluorescence in situ hybridization or immunostaining on zebrafish hearts and by checking the spatial expression patterns of lum on 3 dpa, 7 dpa, 14 dpa and 21 dpa following injury of regenerative P2 neonatal mouse hearts. However, no validation is conducted in non-regenerative scenarios. To state lum upregulation as a unique feature of highly regenerative hearts, showing little to no induction in non-regenerative hearts ,we suggest to clarify the analysis in Fig. 4e and check experimentally the expression of lum in non-regeneration scenarios.

7) It's great that the raw data is available and that the analyzed data can be browsed. However, it would be important that intermediate stages of the analysis are also accessible, e.g. Seurat objects to download; also, scripts are currently not made available, please change this.

8) For a Resource paper, it is particularly important that the Methods contain sufficient detail. I have some specific questions as listed below, and in general I would ask the authors to expand the Methods:
- How long is the immersion in citric acid buffer (line 749)?
- Reference of injury area quantification missing (line 774)?
- Add pepsin concentration in U/ml (line 805).
- Superscript II reverse transcription mix (line 811): Please add manufacturer and specify if there were any custom modifications to the mix.
- Does tissue removal buffer really work without proteinase K? (line 813)?
- What is the cDNA release enzyme (line 814)?
- No second strand synthesis is mentioned. Is it true that there was none?
- Sequences of primers in line 818 & 828 (or are those in a table I have overlooked?)
- Heart dissociation is done for 2 hours at 37 C (line 834) - this sounds like a lot, and may induce considerable stress response
- I don't think red blood cell lysis buffer (line 837) is working in zebrafish, since zebrafish erythrocytes are very different from mammalian ones, e.g. they remain nucleated. Can the authors please comment on this.

9) The importance of tpm4a as a novel regulator of CM redifferentiation might be overstated since tpm4a is a structural component of the sarcomere, and it is not surprising that reduced dosage of a myosin leads to defects.

Minor comments

10) Line 320/321: "Unexpectedly, analysis of the proliferating endocardial cells at 3 dpa and 14 dpa revealed that they were somewhat scattered throughout the entire heart (Figure S10B)". How trustworthy is the finding that these cells are scattered throughout the heart? Is this based on marker genes that are unique to the cell state? It would be good to show such genes and their spatial expression patterns in the stereo-seq data, to convince the reader that these cells can be mapped accurately.

11) The authors found 21 unique spot identities with stereo-seq data. Each spot has a 3-8 cells resolution and thanks to manual annotation the authors identify a maximum of 2 cell types per spot (Line 104-107). The authors use RCTD function with doublet model (assuming at most 1-2 cell types per pixel) to assign scRNA-seq populations to spatial transcriptomic spots (Line 934). The authors should show that those spots for activated and proliferating endocardial populations correspond to unique populations or predominantly consist of fewer than two cell types.

12) Does it make sense to include the proportion of red blood cells in Table S3? RBCs vary between 13% and 35% depending on the conditions and may not be strictly related to cardiac injury.

13) Table S5 shows that GO term analysis identified several terms enriched within the gene sets defining each subtype from scRNA-seq populations. Since this paper is presented as a resource, it will be interesting to add all the identified populations, such as general macrophages, smooth muscle cells, etc. The same rationale for the stereo-seq populations, where only CM states are found.

14) Based on the information from Figure 3F, the authors mention that proliferating macrophages are first observed at 12 hours post-amputation (hpa) and then in higher abundance at 1-day post-amputation (dpa), predominantly localized to the wound area. However, we noticed some dark purple dots accumulating at the BA. Is this effect due to the visualization not accurately reflecting the lower density of proliferating macrophages in areas away from the wound, leading to a misinterpretation of their abundance in those regions?

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors describe a spatial transcriptomics dataset obtained by stereo-seq on injured zebrafish hearts at various time points after injury (resection of the ventricle). In addition, they obtained single-cell RNA-seq datasets for comparable stages of heart regeneration and deconvoluted the single-cell data on the spatial datasets. Furthermore, the authors performed stereo-seq on a single uninjured heart by sectioning through the entire heart and combining this with additional scRNA-seq data. Finally, they constructed an interactive database in which the stereo-seq data from various regeneration time points can be searched without the need to download the raw data.

Overall, the authors present a significant amount of work by generating a high-resolution spatial transcriptomics map of the regenerating zebrafish heart. This resource including the interactive searchable database will be very valuable to the research community. However, their interpretation of the data often falls short, as it is based on the incorrect assumption that local spots are similar to individual cells. I would advise the authors to focus their manuscript more on the correct description of the data and less on the interpretation of these data and over interpretations about cell type subpopulations.

- Throughout the manuscript, the authors define stereo-seq spots as being equal to single cells. Stereo-seq does not have

the resolution to detect single cells, yet the authors define the spots as cell types, which is not correct. This interpretation makes it very confusing. For example, the authors claim that they have identified eight different CM subpopulations, which seems very unlikely. This is confirmed by their own scRNA-seq data and the deconvolution of the scRNA-seq data on the stereo-seq data shown in Fig. S9. Their claim that the CM subpopulations of the stereo-seq are missed in the scRNA-seq is not valid. In Table S2 and Fig. S9, these spots are named more correctly based on the mixed cell identity markers (e.g., Cardiomyocyte & Endocardium). Instead of different cell populations, these spots represent different combinations of cell types, and this should be better acknowledged throughout the entire manuscript. The authors should therefore annotate the clusters from the stereo-seq not as cell types but more unbiased (e.g., by numbering). They also need to rewrite the entire results section describing these data properly by describing the deconvolution at the start before going into the various interpretations of the data. Interpretation often fails because spots do not represent cells. For example, nppb is described as a marker for activated endocardial cells, which is unlikely as nppb is exclusively expressed in cardiomyocytes. For that same reason, the cell trajectories that they describe and show in Fig. 1d and Fig. 2 don't make sense as these are based on spots with different cell types and not on single cells.

- Related to the above section, the description of the different CM populations in Fig. 1e is based on such incorrect interpretation of cell types based on spots containing multiple cell types. For example, the CM (proliferating) population indicated here is high in markers for fibroblasts and endothelial cells (e.g., postn1, collagens, crip2, tagln). That the border zone contains different cell types that are activated or accumulate at the border zone was described previously using TOMO-seq (PMID: 26748692) and explain these results.

- The authors introduce the term Pre-dedifferentiated CMs. In my opinion, this is incorrect as the authors do not show any evidence that these cells undergo dedifferentiation at later stages. Activated CMs may be a better name for the CMs that express TNF receptor as early as 6 hpi.

- Authors claim a novel CM population based on the co-expression of nppa, nppb, and pcna. It is not clear why they claim here a novel CM population as the co-expression of these markers has been well established.

- In the introduction, authors write: "Recently, several spatial transcriptomics platforms have emerged for characterizing gene expression patterns in multiple dimensions. However, this technology has yet to be applied to zebrafish heart regeneration." This seems incorrect as spatial transcriptomics was applied to the regenerating zebrafish heart previously using TOMO-seq (PMID: 26748692).

- In their validation of the stereo-seq data shown in S3, they use PCNA. PCNA+ CMs are claimed but not visible in S3. What about the well-characterized BZ markers like nppa, nppb, mustn1, and glycolysis genes or embryonic TF like tbx20, nkx2.5? Are these BZ genes expressed in a region around the amputation site as would be expected?

- The high-res 3D uninjured heart is interesting but not well described. It is also not included in the online search tool that the authors developed. Including this in the search tool would be very helpful to the research community.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all the issues we have raised in a satisfactory manner. In particular, the data concerning the tpm4a mutant are now much more solid. Overall, the paper certainly represents a very useful and rich resource, the data presented are now of very high quality and conclusions drawn from the transcriptomics data are well confirmed by other methods, namely in situ hybridizations. In terms of new biological and experimentally validated insight into heart regeneration, it provides evidence that tpm4a is required for heart regeneration, and identifies two genes that are expressed after injury in the regenerating zebrafish, but not in the non-regenerating mouse heart. As already stated in the review of the original submission: whether this represents enough novelty for Nat Comms. is for the editors to decide.

Reviewer #2

(Remarks to the Author)
The authors have addressed my concerns in an adequate manner. Major changes include the rewriting of an entire section, where it turns out the conclusions were incorrect because of a lacking control. Furthermore, the authors have more systematically matched and compared the two modalities (scRNA-seq and stereo-seq), they improved the graphical representation or the results and introduced more precise terms in the text, and they integrated their data with a previously published reference dataset. Finally, the computational code is now accessible. In summary, this manuscript presents a powerful resource generated with innovative technology. My only remaining minor criticism is that I find the section on their mutant of tpm4a is still somewhat overstated. I would ask the authors to tone down their interpretation. Other than that, I would now consider the manuscript as suitable for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
In this revised manuscript the authors have addressed several of my initial concerns, which were mostly related to the confusion of sports versus single cells in the Stereo-seq data description. While these concerns have been addressed by the authors, there are a few remaining concerns about the interpretation of the data that need to be addressed:

1.In this revised version the authors claim that CM dedifferentiation starts as early as 12 dpa. Page 8, line 231-232 "Together, these results demonstrate that CMs adopt an activated state as early as 6 hpa, becoming dedifferentiated by 12 hpa". Page 9, line 254-255 "Taken together, these data suggested that the dedifferentiation of CMs involves two CM cell-state transitions that occur much earlier than previously described".
This conclusion is mostly based on nppa/nppb expression, which is first detected at 6 hpa. Nppa/Nppb are expressd in embryonic CMs but their expression is also regulated by stress (PMID: 24104878) and thus cannot be used as a marker for dedifferentiated CMs (even though it is expressed during embryo development). In addition, the GO-term analysis indentifies heart development in group 3 (BZm (dediff-prol) suggesting that dedifferentiation occurs later. This is also consistent with the result on the embryonic myosin expression which starts only at 2dpa and peaks at 7 dpa and with previously published data that cardiac transcription factors (e.g. hand2, nkx2.5, tbx20) important for cardiac development start to be expressed at 3dpa (see PMID: 17081981). Can the authors make a module for the 'heart development genes' and and show their combined expression in the Stereo-seq data at various time points after amputation. This may give a better view on when the dedifferentiation process starts.

2.The authors use expression of OXPHOS genes as a proxy for cardiomyocyte maturation. What about genes regulating calcium, since rapid influx of calcium negativeley regulate cardiomyocyte proliferation and stimulate cardiomyocyte maturation during zebrafish heart regeneration (PMID: 37200435)? The authors should include this in their analysis for CM maturation.

3.The authors conclude: "Taken together, our results suggest that tpm4a functions to promote the redifferentiation of CMs following the peak of proliferation at 7 dpa".
The authors have not investigated the redifferentiation/maturation state directly (as also indicated by reviewer#1), hence they have little evidence to support this conclusion. Their observation of more embryonic myosin expression and more PCNA+ cells suggest that dedifferentiation is enhanced, not that redifferentation is impaired. Other explanation is that tpm4 is required for CM migration into the scar area or for resolving the fibrotic scar. These aspects have not been adressed nor discussed. In addition, The expression data for tpm4 shown in Fig.S8 suggests that tpm4 is expressed in non-cardiomyocytes as the staining does not overlap with MF20, also suggesting possible other (non-CM) roles for Tpm4. This should either be investigated more extensively to support these conclusions or the authors need to tune down the conclusion about the role of Tpm4.

4.It seems that the online vizualisation tool the authors generated only contains the data for the uinjured heart. The more interesting injured hearts at the various time points and the main focus of this manuscript was unaccesible to me. To make these data a valuable resource to the community, the authors should make all the data available without need to download the whole dataset.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors addressed all my questions and revised the manuscript accordingly. I have no further questions to the authors except for a last comment. When trying to access their data on the StomicsDB, I was still unable to do so. The link to the 3D models did not bring me to the correct page using different web browsers. The authors should check to make sure that the data is accessible to others.

RESPONSE TO REVIEWER COMMENTS

We sincerely appreciate the valuable suggestions and comments provided by the reviewers, which have significantly improved the quality of our manuscript. In the following text, we have responded of their comments point to point.

Reviewer #1 (Remarks to the Author):

The authors provide in-depth single-cell sequencing data for zebrafish heart regeneration across several time points, combined with spatial transcriptomics. This undoubtedly represents a rich and useful resource for further studies into the mechanisms of heart regeneration. However, many single cell data-sets from regenerating zebrafish hearts have been published before, eg. for cardiomyocytes (Honkoop H et al., 2019), fibroblasts (Hu B et al., 2022 and), macrophages (Wei KH et al., 2023) and epicardium (Cao J, 2019). Recently, a comparative time resolved single cell sequencing data was published between zebrafish and medaka as well (Carey CM et al., 2024). Thus, the bar is already set quite high in terms of what a new study needs to add. The integration with spatial transcriptomics is new, but it seems that the biological insight that this has made possible is rel. limited. This is exemplified by the 4D atlas that the authors provide. This is very nice for the non-injured heart, but where it gets really interesting, namely heart regeneration, it seems to lack the resolution to be of much use. Overall it seems to us that albeit the paper represents a useful resource, it is rel. weak at deriving significant novel biological insights. Definitely, the authors should further support those conclusions that they do draw from their data with additional experiments as detailed in the "major issues" below. Whether they should in addition use their data as starting point for more experimentally validated insights to make it interesting enough for Nat. Comms is for the editors to decide.

Response:

We appreciate your positive feedback regarding the richness and usefulness of our data. We have carefully considered your comments and made significant revisions to address them.

Major issues:

1. Page 3, line 79-80. The authors claim that spatial transcriptomics platforms have not been used for zebrafish heart regeneration. While it is true that certain platforms have not been utilized, spatial transcriptomics in itself (admittedly with lower resolution as the currently used technology) has been already published on the cryoinjury model at one timepoint (Wu CC et al.,2014). This has to be clarified as they do not refer to this paper.

Response：

Thank you for pointing out this inaccurate description. Our original intention was to say that high-resolution spatial transcriptomic platforms have not yet been used in zebrafish heart regeneration. We have deleted the sentence "However, this technology has yet to be applied to zebrafish heart regeneration." and added one sentence “Particularly, spatial technology tomo-seq was applied to zebrafish heart regeneration based on the cryoinjury model” to refer to the paper of Wu et al. in lines 81-82.

2. The existence of several cardiomyocyte subclusters (pre-dediff, dediff and dediff-prolif) is an interesting observation. Yet, the authors should tone down their conclusions: “these results demonstrate that CMs adopt a pre-dedifferentiation state as early as 6 hpa, becoming fully dedifferentiated by 12 hpa”. How do they know that CMs are “fully dedifferentiated” by 12 hpa? What is their definition of dedifferentiation? Do the CMs show disassembly of sarcomeres at 12 hpa? In page 7, lines 205-207, the authors refer to Honkoop H et al.,2019 which described the upregulation of nppa after 3 dpa. Early upregulation of nppa in the current paper might be due to differences in injury models. What is the earliest time point when tnfrsf11b and nppa genes can be detected in cryoinjury?

Response：

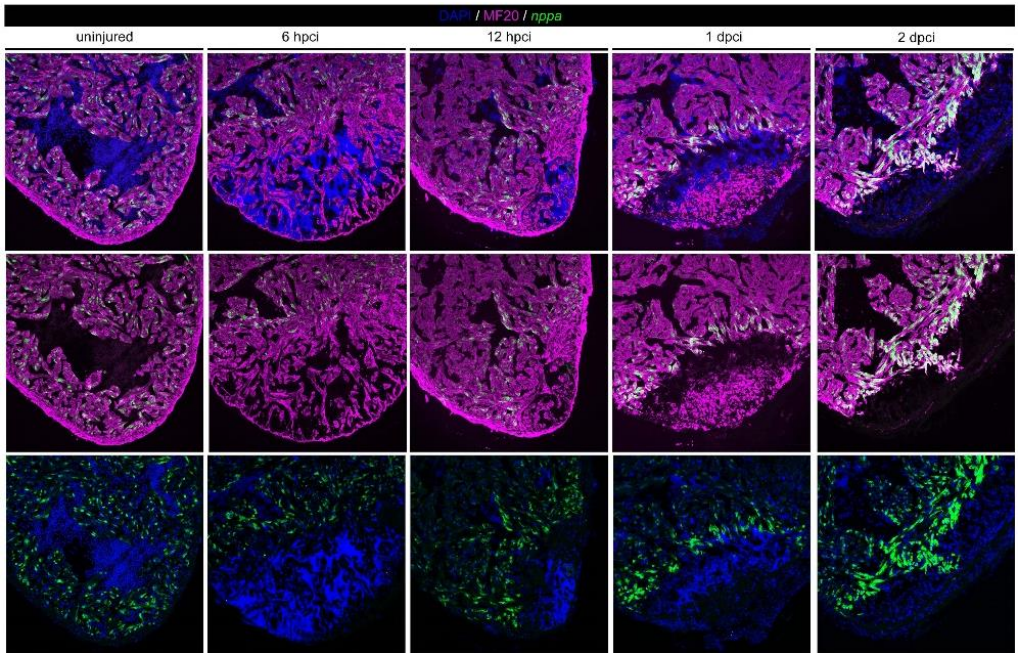
Thank you for raising this significant concern. We have revised our manuscript and toned down our conclusions. We have redefined the “pre-dediff” cluster as “activated” cardiomyocyte domain. In addition, we have completed the following experiments to address above concerns:

(1) How do they know that CMs are “fully dedifferentiated” by 12 hpa? What is their definition of dedifferentiation? Do the CMs show disassembly of sarcomeres at 12 hpa?

To clarify the dedifferentiated state of cardiomyocytes (CMs) at 12 hpa, we examined the sarcomeres of CMs at 12 hpa and 1 dpa by immunofluorescence of TPM1 and ACTN1. These immunofluorescence results showed that the disintegration of Z-disk of sarcomeres near wound appeared at 12 hpa and became severe on 1 dpa (Figure 2c and lines 243-247), indicating that the dedifferentiation state of CMs truly started as early as 12 hpa.

(2) In page 7, lines 205-207, the authors refer to Honkoop H et al.,2019 which described the upregulation of nppa after 3 dpa. Early upregulation of nppa in the current paper might be due to differences in injury models. What is the earliest time point when tnfrsf11b and nppa genes can be detected in cryoinjury?

Thank you for this suggestion. We performed fluorescence *in situ* hybridization (FISH) to detect the expression of *tnfrsf11b* and *nppa* in zebrafish cryoinjury hearts. At 6 hours post cryoinjury (hpci), we observed that *tnfrsf11b* could be detected in CMs near the injury area (Figure S7b and line250-253). And the expression of *nppa* highly increased in border zone CMs at 12 hpci (see below).



MF20 (magenta), nppa (green), DAPI (blue)

3. In Figure 2G, comparing uninjured vs 7 dpa, there is a decrease in tpm4a. How is that explained?

Response：

We apologize for the misunderstanding caused by our gene visualization methods. We re-checked the expression of *tpm4a* in our original data: Statistically speaking, the expression of *tpm4a* across all slides at 7 dpa and 14 dpa in the remote zone (RZ) was nearly unchanged after injury, but significantly increased in the border zone (BZ) of CMs (Figure 2g). To overcome the sparse nature of spatial expression data, gene expressions were imputed by scRNA-seq data using well-known Seurat *TransferData* function, but it seemed not to work well in this particular case. Thus, we updated spatial visualization of imputed expression of *tpm4a* by MAGIC package (Figure 2f and line 296-298). Additionally, we added a FISH result of the *tmp4a* expression (Figure S8d) which was also consistent with our Stereo-seq results.

We also carefully checked all gene expressions used in our paper and no more disagreement was found between raw and imputated expression. Data imputation was used to visualize genes in the Stereo-seq only. Statistic calculations were not performed based on imputed data.

4. The conclusion that tpm4a might be required for CM re-differentiation is potentially interesting, but should be supported by additional data.

a) finding such a rather striking phenotype in heterozygous mutant fish is unexpected. Thus, authors should have much stronger statistics to back these data. samples size for wild-types with n=4 is clearly too low. Also, in how many independent experiments (batches of fish raised) have they seen these phenotypes? Did they house and raise wild-types and mutants together and genotype and separate them only immediately prior to resection or better even; did they perform the resections on non-genotyped fish to exclude bias?

b) Further, the embMHC data should be quantified to support the claim that dedifferentiation is not effected.

c) Can they provide data showing that there are fewer differentiated CMs in the mutants at late stages? E.g more CMs expressing embMHC or disassembled sarcomeres at 30 dpa? Smaller CMs as shown in Bertozzi et al. 2021 doi:10.1016/j.ydbio.2020.12.004? Or do the authors observe a defect in calcium handling in mutants based on Nguyen PD et al., who describe an increase in calcium handling maturation during the course of redifferentiation.

Response：

Thank you for your advice on strengthening our conclusion about *tpm4a*.

a) We have increased the sample size for both mutant and wild-type fishes by adding eight more fishes to each group (Figure 2k-m). These eight were raised in a different batch from the original fishes, but they revealed the same regenerative defects after injury, indicating that our conclusion is reliable.

And the statistical data at 120 dpci was added (Figure 2j). WT animals successfully regenerated cryo-injured muscle, whereas mutant hearts failed to regenerate myocardium, depositing significantly higher abundant scar tissue instead (Figure S8e).

b) We quantified the fluorescence intensity of embCMHC. The statistical results indicated that the dedifferentiation of the mutant heart was greater than that of the wild types at 7 dpa (Figure 2m).

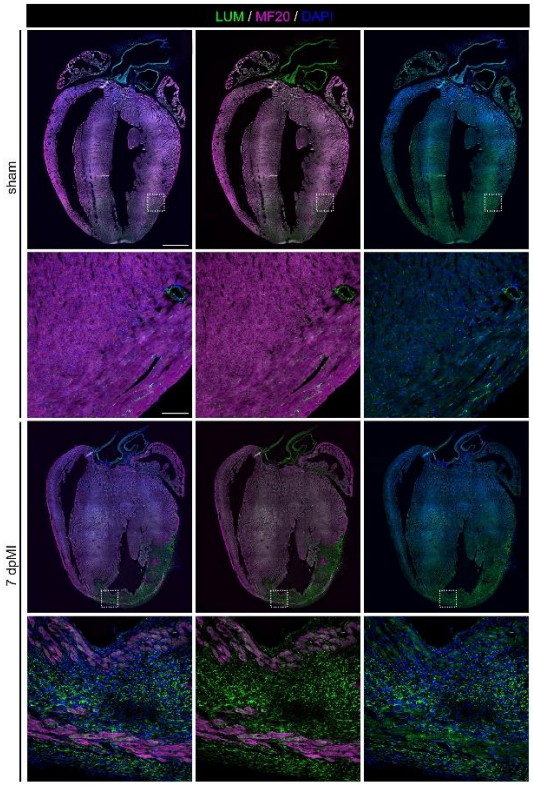
c) At 30 dpa, the expression of embCMHC in the mutant was not statistically significantly higher than that in the wild-type (Figure S8f-g). But we can clearly observe that, in the control group, the injury site has been largely regenerated, whereas in mutant group, the wound was still large and not repaired with CMs. We did not observe the smaller CMs in the mutants. The Nguyen PD et al. paper used a transgenic zebrafish to detect calcium handling. However, we are currently unable to obtain this strain since the Customs import restriction for international animal transportation, so we have not yet examined calcium handling in mutants. But, we do keep studying underlying mechanisms of Tpm4, including to generatate a similar calcium signaling transgenic line by ourself, however, which needs significant longer time to be done. We hope to report that results in future.

5. Fibroblasts: Conservation of lumican expression only in regenerating tissue is interesting, yet the authors unfortunately do not venture beyond confirming lumican expression.

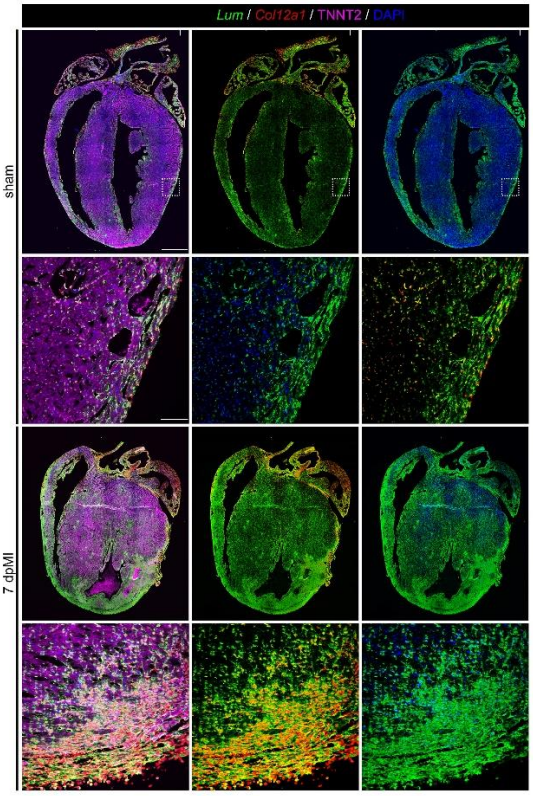
Response：

Before going further to functional study, we examined *lumican* expression in non-regenerative scenarios. However, out of our expectation, both immunofluorescence and fluorescence *in situ* hybridization revealed that the expression of *Lum* was also increased in non-myocardial

tissue near the wound in adult mice following myocardial infarction (please see pictures below). This suggests that our conclusions regarding *lumican* upregulation as a feature for heart regeneration may be not accurate. Therefore, we deleted the descriptions of *lumican*, and performed a deeper analysis to search for others genes as potential markers to distinguish regenerative and non-regenerative hearts. Eventually, we identified two genes (*atp6ap2* and *ifrd1*) that are conserved upregulated in regenerating species and have been experimentally verified to show little to no induction in non-regenerating scenarios (Figure 4c-d).



MF20 (magenta), LUM (green), DAPI (blue)



TNNT2 (magenta), LUM (green), Col12a1 (red), DAPI (blue)

Reviewer #2 (Remarks to the Author):

Li et al. present a resource of spatial transcriptomics (stereo-seq) and single-cell RNA-seq data of the regenerating zebrafish heart. This is a powerful dataset in a very interesting biological system, and the data analysis is overall well executed, even if some questions remain (see

below). The manuscript has the character of a resource article, since the focus is on presenting the dataset in a relatively broad manner, without focusing very much on one specific process. Most of the novelty is related to cardiomyocytes, as the authors describe a more detailed spectrum of de- and redifferentiated cardiomyocytes than in previous publications, and they also included a functional experiment related to cardiomyocyte states. By contrast, there is less novelty in the authors' findings on fibroblasts, macrophages, epi- and endocardium. I would consider this manuscript to be in principle suitable for Nature Communications, but the authors would have to address some issues regarding data analysis and presentation (as described in more detail below), with the goal of maximizing the usefulness of this resource for the scientific community.

Response:

We thank you for your affirmation of our resources and efforts to process them. We appreciate your feedback and would like to address your concerns below.

Major comments:

1) The amount of data presented by the authors is impressive. However, given that some populations do not match between both data modalities, and given that analysis of the main populations is done from different perspectives (stereo-seq for CMs and scRNA-seq for non-myocyte populations), it would be good to perform a systematic analysis for matching the clusters of the two modalities. Please also compare abundances between the two modalities. Also, please separate the cell types identified by the two modalities in color codes, clearly highlight which cell types are of which modality, and specify this clearly in all figure legends. As an example, Fig. S2b & d, color code and legend of the populations is difficult to follow.

Response：

Thank you for your valuable suggestions. To systematically match the two modalities, we have conducted cell-type deconvolutions of each stereo-seq slide on the basis of the annotated scRNA-seq cell types. Notably, cell types identified by scRNA-seq match well with the major cell type of domains detected by Stereo-seq (Figure 1d and lines 140-145). This was further supported by a high correlation of cellular composition between scRNA-seq and Stereo-seq

(Figure S3a and lines 141-145).

In addition, we described the deconvolution results of such as ventricular specific cardiomyocytes, atrial specific cardiomyocytes, bulbus arteriosus specific smooth muscle cells, valves and epicardium in Figure S3b and lines 141-145. We also described the cell-type deconvolution results in Figure S6b for cardiomyocytes (dediff.), Figure 3a for endocardium (activ.), Figure 3d for macrophages (prolif.) and Figure S12c for fibroblasts (reg.).

We have compared abundances between the two modalities in Table S3. There were more cardiomyocyte-related domains identified in Stereo-seq, while more non-myocyte populations were detected in scRNA-seq.

We have distinguished scRNA-seq and Stereo-seq data with different point symbols (circles for scRNA-seq and squares for Stereo-seq) in the legend of figures.

2) Of the 21 unique spot identities found by stereo-seq, neither the endocardial subtypes nor the pre-dedifferentiation or dedifferentiated CMs are identified as pure clusters of a single cell type. It hence appears that these subtypes cannot be disentangled (Fig S2A-B). The labels used to reference these populations are presented in a confusing manner in the manuscript: in the text in lines 188-192 this is not written explicitly, and in Fig. 2 and Fig. S2 the labels differ and are not explained in the footnote, only Fig. S2 clarifies the mixture between CM subtypes and the endocardial activated subtype.

Response:

We apologize for any misunderstanding caused by the loose descriptions of the unique spot identities found by Stereo-seq. We have completed the following improvements to address these concerns:

(a) Of the 21 unique spot identities found by stereo-seq, neither the endocardial subtypes nor the pre-dedifferentiation or dedifferentiated CMs are identified as pure clusters of a single cell type. It hence appears that these subtypes cannot be disentangled

Stereo-seq spots or bins are indeed composed of a mixture of cells. We have re-defined the unsupervised clusters of stereo-seq spots as major cell-type domains rather than the

previously described cell type. For certain subtypes like dedifferentiated CMs, except marker genes and FISH or antibody validation, we combined scRNA-seq and Stereo-seq by cell-type deconvolution to prove its existence and spatial distribution (Figure S6b and lines 213-217).

(b) The labels used to reference these populations are presented in a confusing manner in the manuscript: in the text in lines 188-192 this is not written explicitly, and in Fig. 2 and Fig. S2 the labels differ and are not explained in the footnote, only Fig. S2 clarifies the mixture between CM subtypes and the endocardial activated subtype.

We distinguished scRNA-seq and Stereo-seq data with different labels in Figures (for example, Figure 1 and S2). Additionally, we have rewritten the entire result part of Stereo-seq domains by performing deconvolution (Figure 1b-d). And we clarified the major cell-type compositions of each domain at the start in the main text (lines 108-124):

"In many cases, annotations contained information about anatomic location or represented cell states and types. Included in the 20 spot annotated domains were 5 border zone (BZ) domains with ventricular myocardium enriched (domain 1-5), which each contained a higher proportion of different states of cardiomyocytes along with specific endocardial cells (domain 1-5), immune cells (domain 1-2) and fibroblasts (domain 3-4) upon injury; 2 remote zone (RZ) domains with ventricular cardiomyocytes and endocardial cells enriched (domain 6-7); a ventricular (V) compact myocardium enriched domain containing cardiomyocytes, endocardial cells, fibroblasts or epicardial cells (domain 8); an atrial (A) myocardium enriched domain containing a higher proportion of atrial cardiomyocytes and endocardial cells (domain 9); a myocardium enriched domain found in both atrium and ventricle (domain 10); immune cells enriched domain, which showed a larger proportion of macrophages, neutrophils and T cells (domain 11); specific injury-related fibroblasts enriched domain (domain 12); specific immune cells, fibroblasts and endocardial cells enriched domain upon injury (domain 13); the epicardial cells enriched domain 14; the smooth muscle cells enriched domain 15; valve domain 16; three domains enriched with red blood cells (domain 17-19) and the domain 20 containing unknown cells (Figure 1b, S2a-b)."

3) The authors perform an integrative analysis of the data by linking scRNA-seq populations to

spatial transcriptomic spots with RCTD, and they conduct this analysis for two endocardial subpopulations. As already mentioned above in comment 1, a more systematic integrative analysis will help to better understand the matching populations (e.g., do pre-dedifferentiation or dedifferentiated CM found by stereo-seq match with dedifferentiated CMs found by scRNA-seq?). Would it be possible to perform a deconvolution analysis as the authors do with the proliferative endocardial subpopulation, also for cardiomyocytes?

Response：

Thanks for your suggestions. We conducted cell-type deconvolutions and correlation analysis in order to match the cellular composition between scRNA-seq and Stereo-seq. Based on systematic correlation and deconvolution analysis, cell types identified by scRNA-seq match well with major cell-type domains detected by Stereo-seq, such as ventricular specific cardiomyocytes, atrial specific cardiomyocytes, bulbus arteriosus specific smooth muscle cells appeared in the expected locations (Figure 1d and Figure S3a). Specific to dedifferentiated cardiomyocytes found by scRNA-seq, it matched well with the BZm (dediff.) domain identified by Stereo-seq based on the cell-type deconvolution results (Figure S6b).

4) CMs (dediff.-prolif.) and CMs (prolif.) (identified by stereo-seq and annotated manually as cell types) express markers of non-myocyte populations (Fig. 2E). Do these markers such as collagens or postnb colocalize with tpm4a, or could this indicate a mixture of two or more populations?

Response：

It is true that some non-myocyte markers have been detected in stereo-seq CM clusters, which indicates a mixture of two or more populations. Considering the reviewer’s suggestion and as the discussion of above issues, we have refined the cluster of stereo-seq and annotated the clusters as major cell-type “domain” more unbiasedly based on the cell-type deconvolution results and cell-type specific markers. Specifically, we provide the antibody images (Figure 2c, 2d, 2e) to prove the existence of prolif. and dediff. CMs in our domain positions.

5) It would be good to integrate the authors' single-cell RNA-seq dataset with the existing scRNA-seq atlas by Hu et al., Nat Genet, 2022, which has similar resolution and is so far the

biggest scRNA-seq dataset for the regenerating zebrafish heart. It would be useful for the field to match the cell types/states identified here to the annotations of the previous publication. For instance, the authors identified two predominant subtypes of fibroblasts, general and pro-regenerative, while Hu et al. performed a more fine-grained resolution. It is not very helpful if each new publication introduces a different clustering resolution without relating to previous annotations.

Response：

Thank you for this nice suggestion. We conducted a comprehensive correlation analysis (Figure S3c). We found the scRNA-seq clusters identified in our data were generally well correlated with the previous study of Hu et al.

Since Hu et al. classified multiple fibroblast subtypes, we further conducted unsupervised clustering (resolution=0.6) and characterized 18 different subtypes of fibroblast (Figure 3j, k and S14). After comparison, the fibroblast clusters identified in our study were well correlated with the study of Hu et al. (Figure S3c and S14b). Importantly, we identified more subtypes, such as fibroblasts (*txn*) at early stage of regeneration, fibroblasts (*steap4*) at 7dpa, fibroblasts (*col18a1b*) at 14 dpa, epicardium (*podxl*), fibroblasts (*alas2*) and fibroblasts (*scn4ab*) (Figure 3k).

6) Some of the analysis and plots are difficult to follow or not fully explained. For the comparison of FB populations in regenerative and non-regenerative hearts, the authors identify a list of candidate genes upregulated in pro-regenerative FBs and then investigate the pattern of expression of those genes in regenerative and non-regenerative scenarios. The populations of FBs plotted in Fig. 4E panels 2 and 3 should be introduced in the text or footnotes, including the labels for the different conditions of myocardial infarction (BZ, RZ, LZ, FZ). The pattern of expression of lum is validated by fluorescence in situ hybridization or immunostaining on zebrafish hearts and by checking the spatial expression patterns of lum on 3 dpa, 7 dpa, 14 dpa and 21 dpa following injury of regenerative P2 neonatal mouse hearts. However, no validation is conducted in non-regenerative scenarios. To state lum upregulation as a unique feature of highly regenerative hearts, showing little to no induction in non-

regenerative hearts, we suggest to clarify the analysis in Fig. 4e and check experimentally the expression of lum in non-regeneration scenarios.

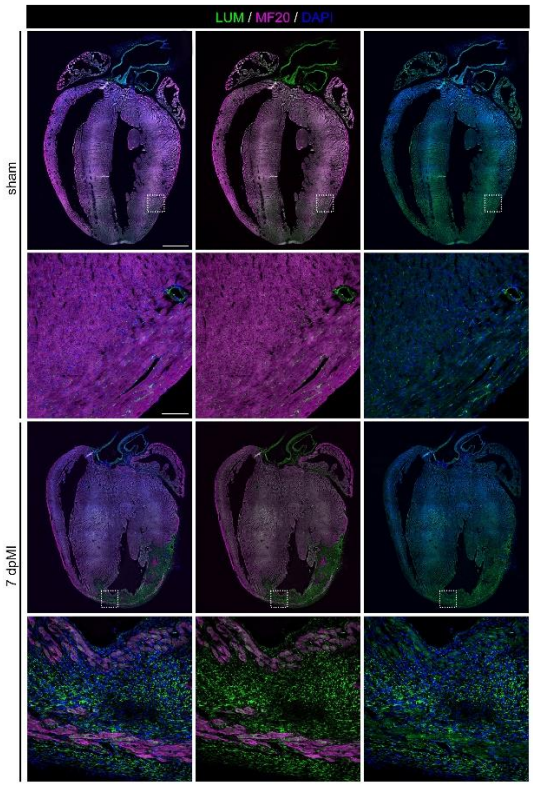
Response：

We apologize for any misunderstanding caused by lost footnotes and so on. We introduced the labels for the different conditions in the footnotes of Figure 4: Sham, sham surgeries; MI, myocardial infarction surgeries; P1, the day of birth; P8, postnatal day 8. P1-1/3 (P8-1/3), collected at 1/3 days post-surgery; BZ, border zone; RZ, remote zone; IZ, ischaemic zone; FZ, fibrotic zone; CTRL, control.

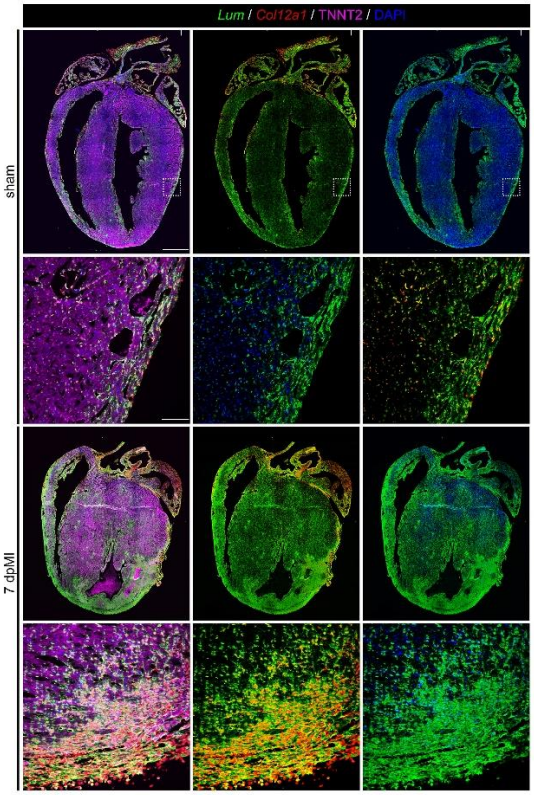
As the reviewer suggested, we detected the expression of Lum in the adult hearts of mice. Out of our expectation, the immunofluorescence and fluorescence in situ hybridization (FISH) results showed an increased expression of Lum in non-cardiac tissues near the wound 7 days after myocardial infarction (see follow). Therefore, we retracted our statement about lum upregulation as a unique feature of regenerative hearts. Alternatively, we performed a deeper analysis to search for other candidate genes. Eventually, we found two genes (*atp6ap2* and *ifrd1*) that have distinct responses in regenerative vs. non-regenerative hearts. Importantly, we have verified by experiments that they are indeed upregulated in regenerating species but show little to no induction in non-regenerating scenarios (Figure 4c, d).

Although our interpretation regarding lum is not proved eventually, we have realized, after more gene expression validation experiments, that our stereo-seq data is well reliable because the experimental results were mostly consistent with our stereo-seq data, including the expression of *lum*, *ifrd1*, *atp6ap2* and many other marker genes (Figure 3i, 4c-d).

We really appreciate the reviewer's suggestion that let us avoid making the wrong conclusion about lum. We should be careful to illustrate the data, and validation is definitely required before making any conclusion.



MF20 (magenta), LUM (green), DAPI (blue)



TNNT2 (magenta), LUM (green), Col12a1 (red), DAPI (blue)

7) It's great that the raw data is available and that the analyzed data can be browsed. However, it would be important that intermediate stages of the analysis are also accessible, e.g. Seurat objects to download; also, scripts are currently not made available, please change this.

Response：

We agree that accessibility is important. In the previous submission, all downloading urls were blocked but they are valid now. Please check:

1) All Seurat objects are available for download via

https://db.cngb.org/stomics/zebrafish_VRH/download/.

2) All related scripts and related documents are accessible via

https://github.com/BGI-Qingdao/ZebrafishHeartRegeneration_project

8) For a Resource paper, it is particularly important that the Methods contain sufficient detail. I have some specific questions as listed below, and in general I would ask the authors to expand the Methods:

- How long is the immersion in citric acid buffer (line 749)?

- Reference of injury area quantification missing (line 774)?

- Add pepsin concentration in U/ml (line 805).

- Superscript II reverse transcription mix (line 811): Please add manufacturer and specify if there were any custom modifications to the mix.

- Does tissue removal buffer really work without proteinase K? (line 813)?

- What is the cDNA release enzyme (line 814)?

- No second strand synthesis is mentioned. Is it true that there was none?

- Sequences of primers in line 818 & 828 (or are those in a table I have overlooked?)

- Heart dissociation is done for 2 hours at 37 C (line 834) - this sounds like a lot, and may induce considerable stress response

- I don't think red blood cell lysis buffer (line 837) is working in zebrafish, since zebrafish erythrocytes are very different from mammalian ones, e.g. they remain nucleated. Can the authors please comment on this.

Response：

We are so sorry for missing such details about our experiments. In the revised manuscript, we have added the related information in the Methods. For your convenience, we also pasted the information as below:

(a) For antigen retrieval, cryosections were first immersed in boiling citric acid buffer (10 mmol/L) for 15 minutes and then continued to immerse until the buffer cooled to room temperature (lines 817-819).

(b) Scar area in regenerated hearts was quantified at 30 and 120 dpi, as previously described

(lines 843-844).

(c) pepsin (Sigma, P7000, powder, ≥250 units/mg solid) (lines 874-875).

(d) Superscript II (Invitrogen, 18064-014, 10 U/µL reverse transcriptase, 1 mM dNTPs, 1 M betaine solution PCR reagent, 7.5 mM MgCl2, 5 mM DTT, 2 U/µL RNase inhibitor, 2.5 µM Stereo-seq-TSO (CTGCTGACGTACTGAGAGGC/rG//rG//iXNA_G/) and 1 x First-Strand buffer) reverse transcription (RT) mix (lines 880-883).

(e) Furthermore, tissue sections were washed twice with 0.1 x SSC buffer and digested with tissue removal buffer (lines 884-885).

(f) cDNA-containing chips were then subjected to Exonuclease I (NEB, M0293L) treatment for 1 hour at 37 °C and were finally washed once with 0.1x SSC buffer (lines 886-888).

(g) Double-stranded cDNA have been generated during the reverse transcription process.

(h) cDNA-PCR primer (CTGCTGACGTACTGAGAGGC).

Stereo-seq-Library primer (/5phos/CTGCTGACGTACTGAGAGG*C*A and GAGACGTTCTCGACTCAGCAGA) (lines 890 and 895).

(i) Enzymatically digest the cell at room temperature about 2 hours in a shaker and gently flicked every 15 minutes to help with tissue disaggregation (lines 908-908).

(j) Supernatant was removed, and the pelleted cells were then resuspended in PBS containing 0.04% BSA (lines 910-911).

9) The importance of tpm4a as a novel regulator of CM redifferentiation might be overstated since tpm4a is a structural component of the sarcomere, and it is not surprising that reduced dosage of a myosin leads to defects.

Response:

Thank you for your concern about the possible overstating of the importance of *tpm4a*. As Tpm4 has been known to be involved in cardiac muscle contraction and cardiac myofibril assembly in zebrafish embryonic heart, it is reasonable and understandable that Tpm4 deficiency can lead to regeneration defects. But striking to us, the heterogeneous mutant of

Tpm4a is able to show a defective phenotype, meaning that Tpm4 is necessary for the heart regeneration process, and also, there are no other redundant factors to compensate for Tpm4 deficiency. About Tpm4 and its cardiac specific transcriptome isoform(s), the underlying molecular mechanisms are need to be clarified. For example, Tpm1, generally, is thought to be a dominant tropmyosin protein in heart. However, the molecular relationship between Tpm1 and Tpm4 is largely unkonw. The Tpm4's functional consistency for cardiac development and regeneration still needs to be explored. Nevertheless, we thought that Tpm4 is important for heart regeneration, although it might not be surprising. And our group is indeed conducting an in-depth study on the Tpm4.

Minor comments

10) Line 320/321: "Unexpectedly, analysis of the proliferating endocardial cells at 3 dpa and 14 dpa revealed that they were somewhat scattered throughout the entire heart (Figure S10B)". How trustworthy is the finding that these cells are scattered throughout the heart? Is this based on marker genes that are unique to the cell state? It would be good to show such genes and their spatial expression patterns in the stereo-seq data, to convince the reader that these cells can be mapped accurately.

Response：

Thanks to the reviewer's suggestion. We re-analyzed this part and found that the proliferative endocardial top100 marker and specific makers (*pcna*, *spock3* and *mb*) were scattered throughout the organ but slightly concentrated in the wound site. Considering the marker distribution, we deleted this conclusion which was drawn only from the mapping results.

11) The authors found 21 unique spot identities with stereo-seq data. Each spot has a 3-8 cells resolution and thanks to manual annotation the authors identify a maximum of 2 cell types per spot (Line 104-107). The authors use RCTD function with doublet model (assuming at most 1-2 cell types per pixel) to assign scRNA-seq populations to spatial transcriptomic spots (Line 934). The authors should show that those spots for activated and proliferating endocardial populations correspond to unique populations or predominantly consist of fewer than two cell types.

Response：

We thank you for this parameter correction. We have modified the RCTD analysis and used the full mode results of RCTD for the cell type deconvolution. Full mode can discover any number of cell types per spot.

12) Does it make sense to include the proportion of red blood cells in Table S3? RBCs vary between 13% and 35% depending on the conditions and may not be strictly related to cardiac injury.

Response：

Yes, red blood cells variation between 13% and 35% doesn't make sense in this research. And we have removed Red blood cells and Others, and recalculated the relevant cell proportions in Table S3. We also checked the results of removing these two clusters from proportion analysis and no main conclusion was affected.

13) Table S5 shows that GO term analysis identified several terms enriched within the gene sets defining each subtype from scRNA-seq populations. Since this paper is presented as a resource, it will be interesting to add all the identified populations, such as general macrophages, smooth muscle cells, etc. The same rationale for the stereo-seq populations, where only CM states are found.

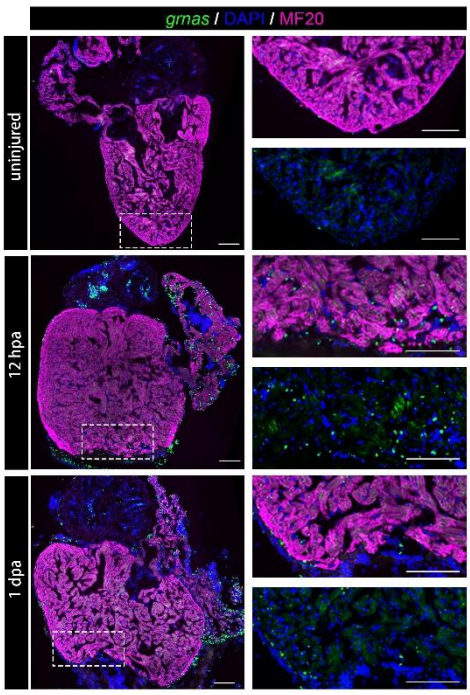
Response：

Thanks to the reviewer's suggestion. We have added all the identified populations in the enrichment term analysis in Table S5.

14) Based on the information from Figure 3F, the authors mention that proliferating macrophages are first observed at 12 hours post-amputation (hpa) and then in higher abundance at 1-day post-amputation (dpa), predominantly localized to the wound area. However, we noticed some dark purple dots accumulating at the BA. Is this effect due to the visualization not accurately reflecting the lower density of proliferating macrophages in areas away from the wound, leading to a misinterpretation of their abundance in those regions?

Response：

The experimental results also showed the same phenomenon as the spatial visualization (see below), which might be infiltrating macrophages from the circulatory system.



MF20 (magenta), *grnas* (green), DAPI (blue)

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors describe a spatial transcriptomics dataset obtained by stereo-seq on injured zebrafish hearts at various time points after injury (resection of the ventricle). In addition, they obtained single-cell RNA-seq datasets for comparable stages of heart regeneration and deconvoluted the single-cell data on the spatial datasets. Furthermore, the authors performed stereo-seq on a single uninjured heart by sectioning through the entire heart and combining this with additional scRNA-seq data. Finally, they constructed an interactive database in which the stereo-seq data from various regeneration time points can be searched without the need to download the raw data.

Overall, the authors present a significant amount of work by generating a high-resolution spatial transcriptomics map of the regenerating zebrafish heart. This resource including the interactive searchable database will be very valuable to the research community. However,

their interpretation of the data often falls short, as it is based on the incorrect assumption that local spots are similar to individual cells. I would advise the authors to focus their manuscript more on the correct description of the data and less on the interpretation of these data and over interpretations about cell type subpopulations.

Response：

We appreciate your input and have made several improvements in the revised version of the manuscript to address these concerns.

- Throughout the manuscript, the authors define stereo-seq spots as being equal to single cells. Stereo-seq does not have the resolution to detect single cells, yet the authors define the spots as cell types, which is not correct. This interpretation makes it very confusing. For example, the authors claim that they have identified eight different CM subpopulations, which seems very unlikely. This is confirmed by their own sc-RNAseq data and the deconvolution of the scRNA-seq data on the stereo-seq data shown in Fig. S9. Their claim that the CM subpopulations of the stereo-seq are missed in the scRNA-seq is not valid. In Table S2 and Fig. S9, these spots are named more correctly based on the mixed cell identity markers (e.g., Cardiomyocyte & Endocardium). Instead of different cell populations, these spots represent different combinations of cell types, and this should be better acknowledged throughout the entire manuscript. The authors should therefore annotate the clusters from the stereo-seq not as cell types but more unbiased (e.g., by numbering). They also need to rewrite the entire results section describing these data properly by describing the deconvolution at the start before going into the various interpretations of the data. Interpretation often fails because spots do not represent cells. For example, nppb is described as a marker for activated endocardial cells, which is unlikely as nppb is exclusively expressed in cardiomyocytes. For that same reason, the cell trajectories that they describe and show in Fig. 1d and Fig. 2 don't make sense as these are based on spots with different cell types and not on single cells.

Response：

Thank you for highlighting this important concern and we surely agree that spot was not equal to single cell. To more precisely describe and interpret our data, following your suggestions,

we make the below changes:

(a) Throughout the manuscript, the authors define stereo-seq spots as being equal to single cells. Stereo-seq does not have the resolution to detect single cells, yet the authors define the spots as cell types, which is not correct. This interpretation makes it very confusing.

Instead of different cell populations, these spots represent different combinations of cell types, and this should be better acknowledged throughout the entire manuscript. The authors should therefore annotate the clusters from the stereo-seq not as cell types but more unbiased (e.g., by numbering). They also need to rewrite the entire results section describing these data properly by describing the deconvolution at the start before going into the various interpretations of the data.

1) We re-defined the clusters of stereo-seq spots as major cell-type domains which was commonly used in spatial transcriptomics papers[1-3]. The domain names were also defined more precisely by a combination of number and its major cell populations. To reduce confusion, we used rectangle to label stereo-seq domains and circle to label the scRNA-seq cell types in all figures.

2) Furthermore, we have conducted cell-type deconvolutions of each stereo-seq slide leveraged the annotated scRNA-seq cell types. Notably, based on cell-type deconvolution and systematic correlation analysis (Figure 1d and S3a), cell types identified by scRNA-seq match well with the major cell-type domains detected by Stereo-seq (lines 141-143).

3) We have presented the spatial visualization of cell-type deconvolution results of such as ventricular specific cardiomyocytes, atrial specific cardiomyocytes, bulbus arteriosus specific smooth muscle cells, valves and epicardium (Figure S3b and lines 141-145). In addition, we described the key deconvolution results and rewritten the corresponding part separately at the beginning of each section: Figure S6b for cardiomyocytes (dediff.), Figure 3a for endocardium (activ.), Figure 3c for macrophages (prolif.) and Figure 12c for fibroblasts (reg.).

4) We have rewritten the entire result part of Stereo-seq domains and clarified the major cell-type compositions of each domain at the start in the main text (lines 108-124):

"In many cases, annotations contained information about anatomic location or represented cell states and types. Included in the 20 spot annotated domains were 5 border zone (BZ) domains with ventricular myocardium enriched (domain 1-5), which each contained a higher proportion of different states of cardiomyocytes along with specific endocardial cells (domain 1-5), immune cells (domain 1-2) and fibroblasts (domain 3-4) upon injury; 2 remote zone (RZ) domains with ventricular cardiomyocytes and endocardial cells enriched (domain 6-7); a ventricular (V) compact myocardium enriched domain containing cardiomyocytes, endocardial cells, fibroblasts or epicardial cells (domain 8); an atrial (A) myocardium enriched domain containing a higher proportion of atrial cardiomyocytes and endocardial cells (domain 9); a myocardium enriched domain found in both atrium and ventricle (domain 10); immune cells enriched domain, which showed a larger proportion of macrophages, neutrophils and T cells (domain 11); specific injury-related fibroblasts enriched domain (domain 12); specific immune cells, fibroblasts and endocardial cells enriched domain upon injury (domain 13); the epicardial cells enriched domain 14; the smooth muscle cells enriched domain 15; valve domain 16; three domains enriched with red blood cells (domain 17-19) and the domain 20 containing unknown cells (Figure 1b, S2a-b)."

Ref:

1. *van Dijk, D. et al. Recovering Gene Interactions from Single-Cell Data Using Data Diffusion. Cell 174, 716-729.e27 (2018).*

2. *Ma, Y. & Zhou, X. Accurate and efficient integrative reference-informed spatial domain detection for spatial transcriptomics. Nature Methods 21, 1231-1244 (2024).*

3. *Xu, C. et al. DeepST: identifying spatial domains in spatial transcriptomics by deep learning. Nucleic Acids Res 50, e131 (2022).*

(b) For example, the authors claim that they have identified eight different CM subpopulations, which seems very unlikely. This is confirmed by their own scRNAseq data and the deconvolution of the scRNA-seq data on the stereo-seq data shown in Fig. S9. Their claim that the CM subpopulations of the stereo-seq are missed in the scRNA-seq is not valid. In Table S2 and Fig. S9, these spots are named more correctly based on the mixed cell identity markers (e.g.,

Cardiomyocyte & Endocardium).

It is true that mixed cell identity markers have been detected in stereo-seq CM clusters, which indicates a mixture of two or more populations. We have refined the cluster of stereo-seq and annotated the clusters as ventricular myocardium-enriched domains. As mentioned above, we described their cell type composition at start (lines 108-116).

In addition, we describe the different myocardial states in each domain (204-212): "In total, we discovered eight ventricular myocardium-enriched domains in uninjured and regenerating hearts that were annotated based on gene-expression profiles, including previously established markers (Figure S2b, S5 and Table S2). Five ventricular myocardium-enriched domains were injury-induced, localized to the wound edge, and stage-specific (Figure 2a, S2b and S5). They were annotated as BZ myocardium-enriched domains reflecting states of activation (activ.), dedifferentiation (dediff.), dedifferentiation-proliferation (dediff.-prolif.), proliferation (prolif.), and re-differentiation (re-diff.) of cardiomyocytes at 6 hpa, 12 hpa/1 dpa, 3 dpa, 7 dpa, and 14 dpa, respectively (Figure 2a, S2b, S5 and Table S2)."

(c) Interpretation often fails because spots do not represent cells. For example, nppb is described as a marker for activated endocardial cells, which is unlikely as nppb is exclusively expressed in cardiomyocytes.

Thanks for your reminder. As shown in Figure 2b, the FISH results indicated that the expression of *nppb* was highly increased in the border zone and mostly co-expression with CM marker *MF20*. However, Figure 2b also showed that the expression of *nppb* was not exclusively in CMs. Together with our scRNA-seq data, we thought it could be true that *nppb* is upregulated also in activated endocardial cells. Anyway, for the reliability of descriptions, we deleted it from the section of activated endocardial cells, also because it does not exist in the top10 DEG list.

(d) For that same reason, the cell trajectories that they describe and show in Fig. 1d and Fig. 2 don't make sense as these are based on spots with different cell types and not on single cells.

The cell trajectories in Figure 2 based on the spatial clusters were deleted from our paper. The stereo-seq part cell lineage analysis in Figure 1 was also removed, we added a correlation analysis in Figure S6a to prove the domain similarity.

- Related to the above section, the description of the different CM populations in Fig. 1e is based on such incorrect interpretation of cell types based on spots containing multiple cell types. For example, the CM (proliferating) population indicated here is high in markers for fibroblasts and endothelial cells (e.g., postn1, collagens, crip2, tagln). That the border zone contains different cell types that are activated or accumulate at the border zone was described previously using TOMO-seq (PMID: 26748692) and explain these results.

Response：

Thanks for your reminder. We redefined the clusters of stereo-seq based on the reviewer's helpful comments and the paper of TOMO-seq (PMID: 26748692).

It is true that fibroblasts and endothelial cells markers have been detected in stereo-seq CM related clusters (Table S2). As the discussion of above issues, we have refined the cluster of stereo-seq and annotated the clusters as major cell-type "domain" more unbiasedly based on the cell-type deconvolution results and spatial location (line 987-997):

"Based on the major cell-type markers, spatial locations, regeneration time points, as well as the results of the annotated cell types of scRNA-seq, Stereo-seq clusters were also annotated. These ventricular myocardium enriched clusters near the injury area (BZ, border zone) were called BZm domains, in contrast, myocardium enriched clusters located in the remote zone (RZ) were called RZm domains…"

Specifically, we provide the antibody images (Figure 2c, 2d, 2e) to prove the existence of prolif. and dediff. CMs in our domain positions.

- The authors introduce the term Pre-dedifferentiated CMs. In my opinion, this is incorrect as the authors do not show any evidence that these cells undergo dedifferentiation at later stages. Activated CMs may be a better name for the CMs that express TNF receptor as early as 6 hpi.

Response：

Thanks for the reviewer's helpful suggestion. As suggested, we changed the description of "Pre-dedifferentiated" to "Activated" in the revised manuscript.

- Authors claim a novel CM population based on the co-expression of nppa, nppb, and pcna. It

is not clear why they claim here a novel CM population as the co-expression of these markers has been well established.

Response：

Thanks for the reviewer's helpful suggestion. As the reviewer suggested, we removed this specific claim of "novel" CM populations. We have revised our manuscript and toned down our conclusion in lines 256-259:

"We observed a subgroup of CMs in BZm (dediff. −prolif.) domain along the wound edge representing an intermediate cell state between dedifferentiated and proliferative CMs, characterized by simultaneous upregulation of *nppb* and *pcna*…"

- In the introduction, authors write: "Recently, several spatial transcriptomics platforms have emerged for characterizing gene expression patterns in multiple dimensions. However, this technology has yet to be applied to zebrafish heart regeneration." This seems incorrect as spatial transcriptomics was applied to the regenerating zebrafish heart previously using TOMO-seq (PMID: 26748692).

Response：

Thanks for the your reminder, we have deleted the sentence "However, this technology has yet to be applied to zebrafish heart regeneration." and added one sentence "Particularly, spatial technology tomo-seq was applied to zebrafish heart regeneration based on the cryoinjury model" to refer to the paper of Wu et al. in line 81-82.

- In their validation of the stereo-seq data shown in S3, they use PCNA. PCNA+ CMs are claimed but not visible in S3. What about the well-characterized BZ markers like nppa, nppb, mustn1, and glycolysis genes or embryonic TF like tbx20, nkx2.5? Are these BZ genes expressed in a region around the amputation site as would be expected?

Response：

Thank you for this valuable suggestion. The *pcna* was enriched in BZ CMs, but the plotting was inapparent due to the disturb of rare high expression outliers. we added glycolysis genes *pkma* and heart regeneration-related genes *nkx2.5, hand2, and mustn1b* in Figure S3e. We also

found them expressed in a region around the amputation site in our data.

- The high-res 3D uninjured heart is interesting but not well described. It is also not included in the online search tool that the authors developed. Including this in the search tool would be very helpful to the research community.

Response：

Thanks for the reviewer's suggestion and we have added supplementary description about the high-res 3D uninjured heart in lines 523-531:

“To integrate the scRNA-seq and stereo-seq atlas, the gene expressions of stereo-seq data were first imputed by scRNA-seq data, then the spot identities of stereo-seq data and scRNA cell type mapping results were carefully merged. This integration allowed us to construct the first high resolution organ-wide 3D transcriptional and cellular atlas of the adult zebrafish heart (Figure 5c, S19, and Video S1). In total, our integrated atlas contains spatial distributions of 18 cell types and 27661 gene expression patterns in the uninjured adult zebrafish heart. Users can query them through our online website (https://db.cngb.org/stomics/zebrafish_VRH/3d.model/).”

We have rearranged the page to make it more noticeable.

RESPONSE TO REVIEWER COMMENTS

We sincerely appreciate the valuable suggestions and comments provided by the reviewers, which have significantly improved the quality of our manuscript. In the following text, we have responded to their comments point to point.

Reviewer #1 (Remarks to the Author):

The authors have addressed all the issues we have raised in a satisfactory manner. In particular, the data concerning the tpm4a mutant are now much more solid. Overall, the paper certainly represents a very useful and rich resource, the data presented are now of very high quality and conclusions drawn from the transcriptomics data are well confirmed by other methods, namely in situ hybridizations. In terms of new biological and experimentally validated insight into heart regeneration, it provides evidence that tpm4a is required for heart regeneration, and identifies two genes that are expressed after injury in the regenerating zebrafish, but not in the non-regenerating mouse heart. As already stated in the review of the original submission: whether this represents enough novelty for Nat Comms. is for the editors to decide.

Response:

Thank you for the positive feedback on our revised manuscript. And thanks again for your affirmation regarding the richness and usefulness of our data.

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns in an adequate manner. Major changes include the rewriting of an entire section, where it turns out the conclusions were incorrect because of a lacking control. Furthermore, the authors have more systematically matched and compared the two modalities (scRNA-seq and stereo-seq), they improved the graphical representation or the results and introduced more precise terms in the text, and they integrated their data with a previously published reference dataset. Finally, the computational code is now accessible. In summary, this manuscript presents a powerful resource generated with innovative technology. My only remaining minor criticism is that I find the section on their mutant of tpm4a is still

somewhat overstated. I would ask the authors to tone down their interpretation. Other than that, I would now consider the manuscript as suitable for publication in Nature Communications.

Response:

We appreciate your positive feedback regarding our revised manuscript. As suggested by your comment, we have performed further experiments for *tpm4a* and toned down the description of *tpm4a*:

(1) We demonstrated through immunostaining of ACTN2 that the mutant heart cannot properly assemble its sarcomeres at 30 dpa (in line 327-329 and in figure 2l):

"Furthermore, immunostaining with ACTN2 revealed disorganized Z-disks of sarcomeres in BZ of *Z8*$^{+/-}$ hearts compared to WT animals at 30 dpa, indicating that the *Z8*$^{+/-}$ heart cannot properly assemble its sarcomeres at 30 dpa (Figure 2l).."

(2) "Taken together, our results suggest that *tpm4a* functions to promote the redifferentiation of CMs following the peak of proliferation at 7 dpa." was changed to "T Taken together, our results suggested that *tpm4a* is vital for cardiac regeneration process, whereas *tpm4a* mutants failed to reorganize sarcomeres at redifferentiation stage and consequently hampered heart regeneration." in line 330-332.

(3) In the Abstract, the description about *tpm4a* has been toned down as "explored a potential role for *tpm4a* in cardiomyocyte re-differentiation" in line 40-41.

(4) "Moreover, we implicated tpm4a in stimulating re-differentiation of proliferating CMs, thereby promoting heart regeneration." was changed to "Moreover, we implicated *tpm4a* may stimulate the re-differentiation of proliferating CMs, thereby promoting heart regeneration." in line 614-615.

Reviewer #3 (Remarks to the Author):

In this revised manuscript the authors have addressed several of my initial concerns, which were mostly related to the confusion of sports versus single cells in the Stereo-seq data

description. While these concerns have been addressed by the authors, there are a few remaining concerns about the interpretation of the data that need to be addressed:

Response：

Thank you for your affirmation of our revised manuscript. We appreciate your feedback and would like to address your concerns below.

1.In this revised version the authors claim that CM dedifferentiation starts as early as 12 dpa. Page 8, line 231-232 "Together, these results demonstrate that CMs adopt an activated state as early as 6 hpa, becoming dedifferentiated by 12 hpa". Page 9, line 254-255 "Taken together, these data suggested that the dedifferentiation of CMs involves two CM cell-state transitions that occur much earlier than previously described".

This conclusion is mostly based on nppa/nppb expression, which is first detected at 6 hpa. Nppa/Nppb are expressd in embryonic CMs but their expression is also regulated by stress (PMID: 24104878) and thus cannot be used as a marker for dedifferentiated CMs (even though it is expressed during embryo development). In addition, the GO-term analysis indentifies heart development in group 3 (BZm (dediff-prol) suggesting that dedifferentiation occurs later. This is also consistent with the result on the embryonic myosin expression which starts only at 2dpa and peaks at 7 dpa and with previously published data that cardiac transcription factors (e.g. hand2, nkx2.5, tbx20) important for cardiac development start to be expressed at 3dpa (see PMID: 17081981). Can the authors make a module for the 'heart development genes' and and show their combined expression in the Stereo-seq data at various time points after amputation. This may give a better view on when the dedifferentiation process starts.

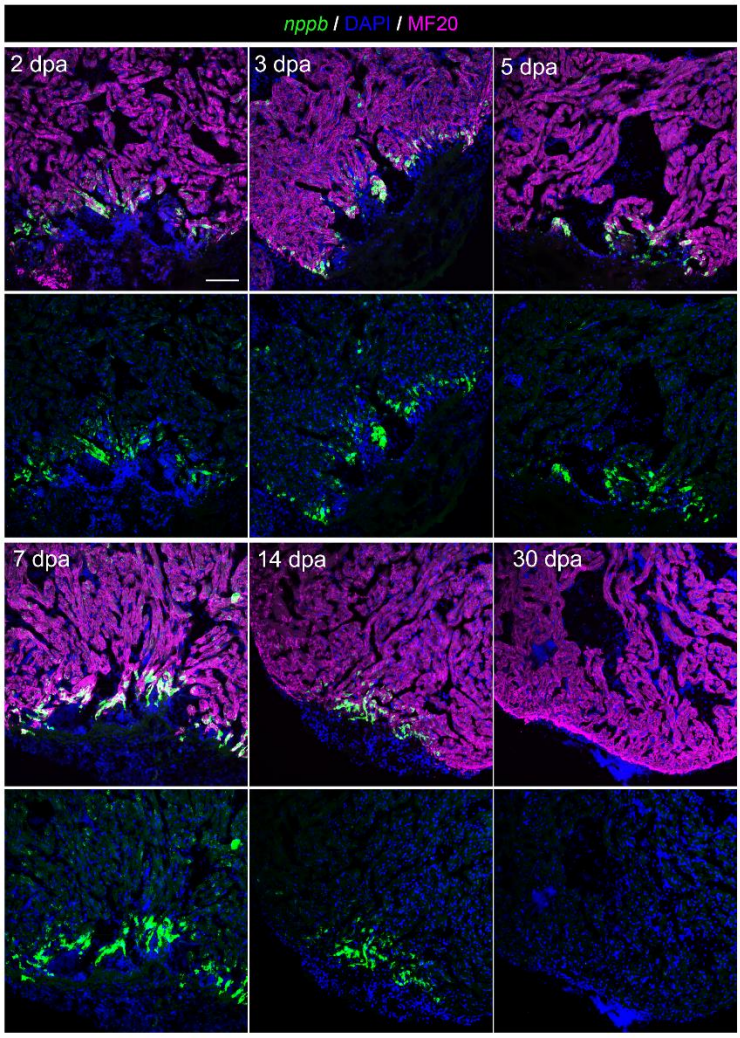
Response：

Thanks for your kind reminder. As the reviewer suggested:

(1) We made a module for the "heart development genes" and showed their combined expression in the Stereo-seq data using Seurat module score function in Figure S5d: "In addition, the module score showed that many other heart development-related genes, including hand2, tbx20, mustn1b, desma, mef2cb, and cacybp, which have been reported in

previous studies[22,31,32,43,56,57], were preferentially upregulated in the border zone starting from 12 hpa (Figure S5d), also hinting at a trend towards cellular dedifferentiation." (line 228-232).

(2) Referring to the previous studies (31,43), we observed the upregulation of embryonic myocardium-related genes *nppa* and *nppb* in BZm (dediff) based on Stereo-seq data (Figure S2a, S5c and Table S2). In another article, *nppa and nppb* were also utilized as representative genes for dedifferentiation (57). Building on this, we characterized the expression profile of the gene *nppb* throughout the cardiac regeneration process. Our findings indicate that the expression pattern of *nppb* closely resembles that of the dedifferentiation marker embCMHC at 3 dpa and at later stages (see below). Therefore, we propose that nppb may serve as a marker gene for dedifferentiation.



(3) Moreover, we performed experiments and examined the sarcomeres of CMs at 12 hpa and

1 dpa by immunofluorescence of TPM1 and ACTN1: “The results of immunofluorescence demonstrated that the Z-disk of sarcomeres near the wound disintegrated at 12 hpa, with more significant disintegration observed at 1 dpa (Figure 2c).” (line 248-250)

(4) And in order to make the results more rigorous, we changed the conclusions “Together, these results demonstrate that CMs adopt an activated state as early as 6 hpa, becoming dedifferentiated by 12 hpa” and “Taken together, these data suggested that the dedifferentiation of CMs involves two CM cell-state transitions that occur much earlier than previously described” and changed them to:

Line 235-236: “Together, these results suggest that CMs may adopt an activated state as early as 6 hpa and become dedifferentiating by 12 hpa.”

Line 258-260: “Taken together, these data, including sarcomere breakdown, activations of injury-related, development-related, or glycolysis-related genes, supported that the dedifferentiation of CMs involves two CM cell-state transitions that occur much earlier than previously described.”

More papers have been cited in Reference: (22,PMID: 17081981; 31.,PMID: 31868166; 32, PMID: 35864193; 34, PMID: 34523214; 43, PMID:26748692; 56, PMID: 22750409; 57, PMID: 37200435)

2.The authors use expression of OXPHOS genes as a proxy for cardiomyocyte maturation. What about genes regulating calcium, since rapid influx of calcium negativeley regulate cardiomyocyte proliferation and stimulate cardiomyocyte maturation during zebrafish heart regeneration (PMID: 37200435)? The authors should include this in their analysis for CM maturation.

Response：

Thanks for your reminder. Referring to the previous studies[57], we have added analysis for CM maturation about genes regulating calcium in Figure S6f and added descriptions in line 297-302：

“The previous study in zebrafish hearts has confirmed that calcium handling is essential for

CMs maturation[57]. Consistently, we observed the upregulation of Ca^{2+} handling related genes at 14 dpa (Figure S6f), such as well-established EC coupling genes *atp2a2a* (*serca2*), *slc8a1a* (*ncx1*) and *ryr2b*[57,68-74]. Overall, the upregulation of oxidative phosphorylation and calcium handling features associated with cardiac maturation were found in the late stage of regeneration."

New References have been added: (57, PMID: 37200435; 68, PMID: 17626279; 69, PMID: 22465693; 70, PMID: 2245735; 71, PMID: 28963192; 72, PMID: 28684623; 73, PMID: 32188566; 74, PMID: 34462437)

3.The authors conclude: "Taken together, our results suggest that tpm4a functions to promote the redifferentiation of CMs following the peak of proliferation at 7 dpa".

The authors have not investigated the redifferentiation/maturation state directly (as also indicated by reviewer#1), hence they have little evidence to support this conclusion. Their observation of more embryonic myosin expression and more PCNA+ cells suggest that dedifferentiation is enhanced, not that redifferentation is impaired. Other explanation is that tpm4 is required for CM migration into the scar area or for resolving the fibrotic scar. These aspects have not been adressed nor discussed. In addition, The expression data for tpm4 shown in Fig.S8 suggests that tpm4 is expressed in non-cardiomyocytes as the staining does not overlap with MF20, also suggesting possible other (non-CM) roles for Tpm4. This should either be investigated more extensively to support these conclusions or the authors need to tune down the conclusion about the role of Tpm4.

Response：

Thank you for your valuable suggestions of *tpm4a*. In the revised manuscript, we added further experiments of tpm4a and toned down the description of *tpm4a*:

(1) We demonstrated through immunostaining of ACTN2 that the mutant heart cannot properly assemble its sarcomeres at 30 dpa (in line 327-329 and in figure 2l):

"Furthermore, immunostaining with ACTN2 revealed disorganized Z-disks of sarcomeres in BZ of *Z8*$^{+/-}$ hearts compared to WT animals at 30 dpa, indicating that the *Z8*$^{+/-}$ heart cannot

properly assemble its sarcomeres at 30 dpa (Figure 2l)..”

(2) “Taken together, our results suggest that *tpm4a* functions to promote the redifferentiation of CMs following the peak of proliferation at 7 dpa.” was changed to “T Taken together, our results suggested that *tpm4a* is vital for cardiac regeneration process, whereas *tpm4a* mutants failed to reorganize sarcomeres at redifferentiation stage and consequently hampered heart regeneration.” in line 330-332.

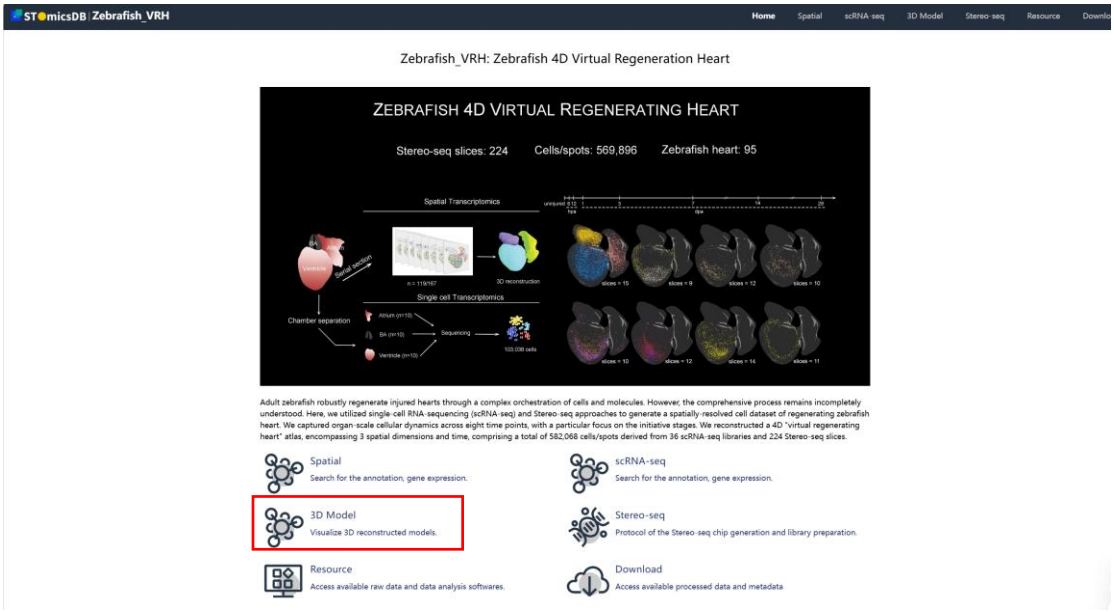
(3) In the Abstract, the description about *tpm4a* has been toned down as “explored a potential role for *tpm4a* in cardiomyocyte re-differentiation” in line 40-41.

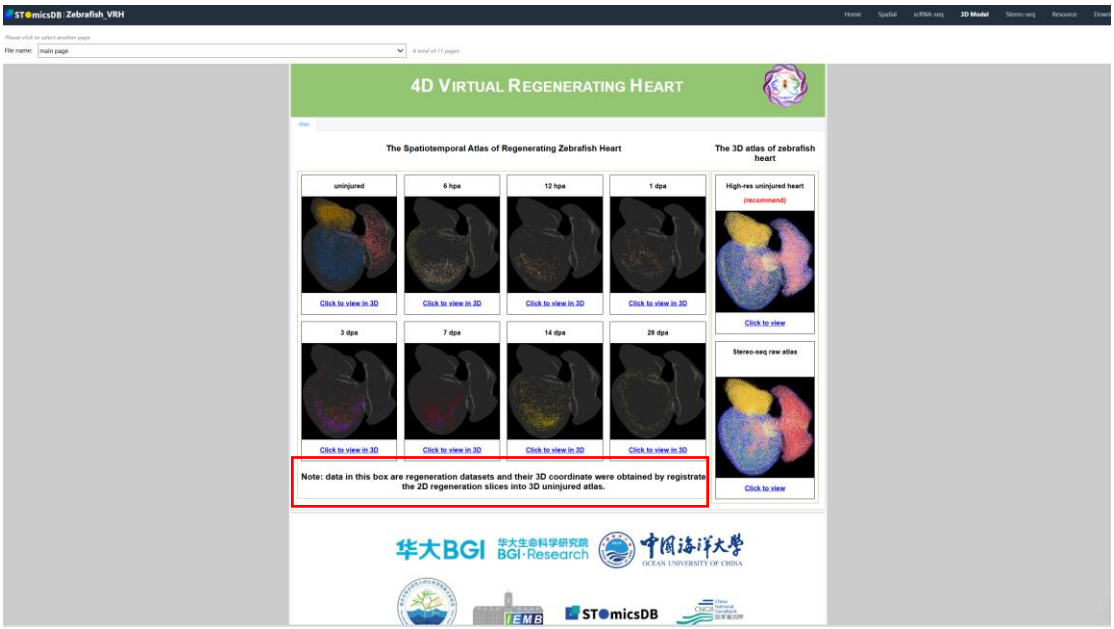
(4) “Moreover, we implicated tpm4a in stimulating re-differentiation of proliferating CMs, thereby promoting heart regeneration.” was changed to “Moreover, we implicated *tpm4a* may stimulate the re-differentiation of proliferating CMs, thereby promoting heart regeneration.” in line 614-615.

4.It seems that the online vizualisation tool the authors generated only contains the data for the uinjured heart. The more interesting injured hearts at the various time points and the main focus of this manuscript was unaccesible to me. To make these data a valuable resource to the community, the authors should make all the data available without need to download the whole dataset.

Response：

We agree the importance of making all data publicly available and accessible. All data, including the injured hearts, were accessible on the online website since our first submission. The page layout for switching data may not be very prominent, thus we have added guidance information, for example, the Stereo-seq page (see below). And in order to avoid confusing readers with 3D atlas and 2D atlas URLs, we unified the 3D URLs to https://db.cngb.org/stomics/zebrafish_VRH/ in the revised manuscript.


STomicsDB | Zebrafish_VRH
Home
Spatial
scRNA-seq
3D Model
Stereo-seq
Resource
Zebrafish_VRH: Zebrafish 4D Virtual Regeneration Heart
ZEBRAFISH 4D VIRTUAL REGENERATING HEART
Stereo-seq slices: 224
Cells/spots: 569,896
Zebrafish heart: 95
Spatial Transcriptomics
Single cell Transcriptomics
Chamber separation
Sequencing
3D reconstruction
Adult zebrafish robustly regenerate injured hearts through a complex orchestration of cells and molecules. However, the comprehensive process remains incompletely understood. Here, we utilized single-cell RNA-sequencing (scRNA-seq) and Stereo-seq approaches to generate a spatially-resolved cell dataset of regenerating zebrafish heart. We captured organ-scale cellular dynamics across eight time points, with a particular focus on the initiative stages. We reconstructed a 4D "virtual regenerating heart" atlas, encompassing 3 spatial dimensions and time, comprising a total of 582,068 cells/spots derived from 36 scRNA-seq libraries and 224 Stereo-seq slices.
Spatial
Search for the annotation, gene expression.
scRNA-seq
Search for the annotation, gene expression.
3D Model
Visualize 3D reconstructed models.
Stereo-seq
Protocol of the Stereo-seq chip generation and library preparation.
Resource
Access available raw data and data analysis softwares.
Download
Access available processed data and metadata


STomicsDB | Zebrafish_VRH
Home
Spatial
scRNA-seq
3D Model
Stereo-seq
Resource
File name: main page
4D VIRTUAL REGENERATING HEART
The Spatiotemporal Atlas of Regenerating Zebrafish Heart
The 3D atlas of zebrafish heart
uninjured
6 hpa
12 hpa
1 dpa
3 dpa
7 dpa
14 dpa
28 dpa
Click to view in 3D
High-res uninjured heart
(recommend)
Click to view
Stereo-seq raw atlas
Note: data in this box are regeneration datasets and their 3D coordinate were obtained by registrate the 2D regeneration slices into 3D uninjured atlas.
华大BGI
华大生命科学研究院
BGI·Research
中国海洋大学
OCEAN UNIVERSITY OF CHINA
IEMB
STomicsDB

Reviewer #3 (Remarks to the Author):

The authors addressed all my questions and revised the manuscript accordingly. I have no further questions to the authors except for a last comment. When trying to access their data on the StomicsDB, I was still unable to do so. The link to the 3D models did not bring me to the correct page using different web browsers. The authors should check to make sure that the data is accessible to others.

Response:

Thank you for your affirmation of our revised manuscript. We appreciate your feedback about 3D models. For the website issue, we have made efforts in three aspects:

1) Except the authors, we also invited our friends from China, Europe, and USA to visit the page of 3D models, but no failure occurred. Due to the lack of specific failure information provided by the reviewer, we are unable to carry out targeted problem fixes.
2) To prove our data accessibility, we tested it with different web browsers, including Google Chrome (Figure R1), Microsoft Edge (Figure R2), Firefox (Figure R3), and Safari (Figure R4). We also test it on the web browser of mobile phone (Figure R5).
3) We added a detailed instruction document at the Download page and also attached it at the end of this response.

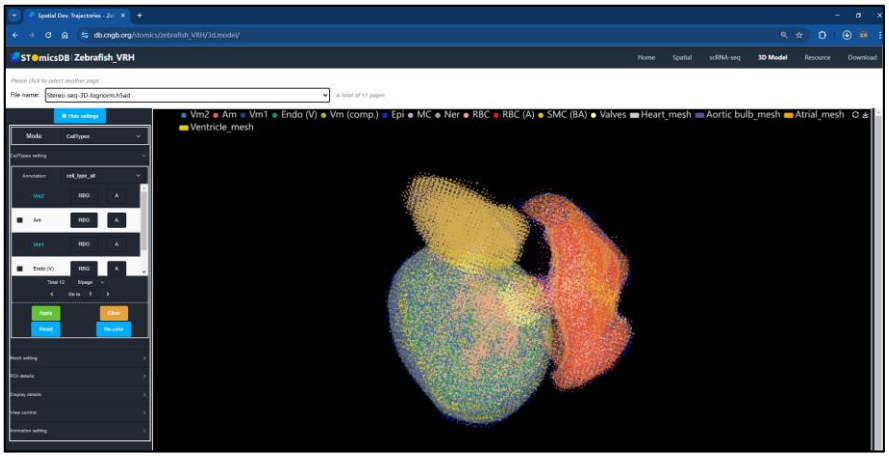


Figure R1. Screenshot of visiting the 3D model page by Google Chrome.

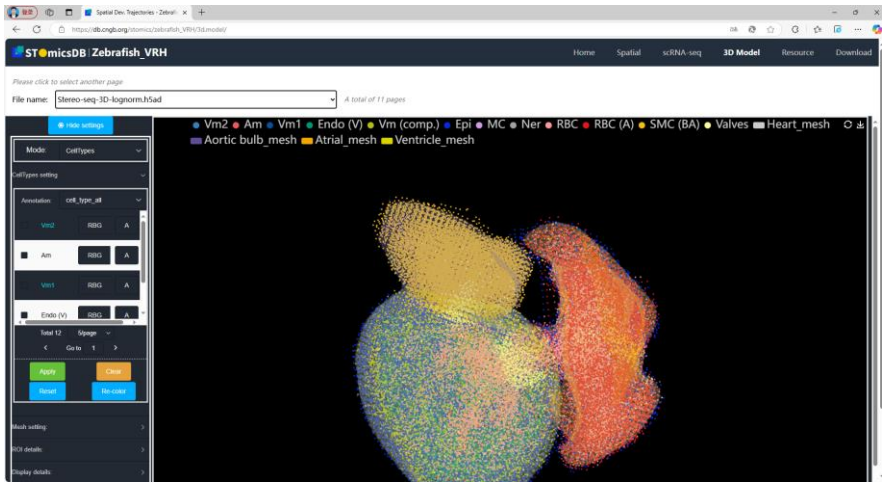


Figure R2. Screenshot of visiting the 3D model page by Microsoft Edge.

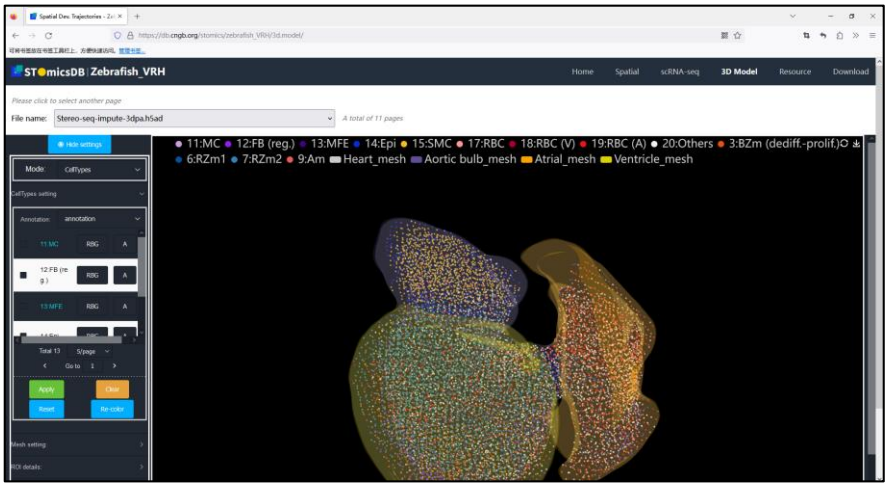


Figure R3. Screenshot of visiting the 3D model page by Firefox.

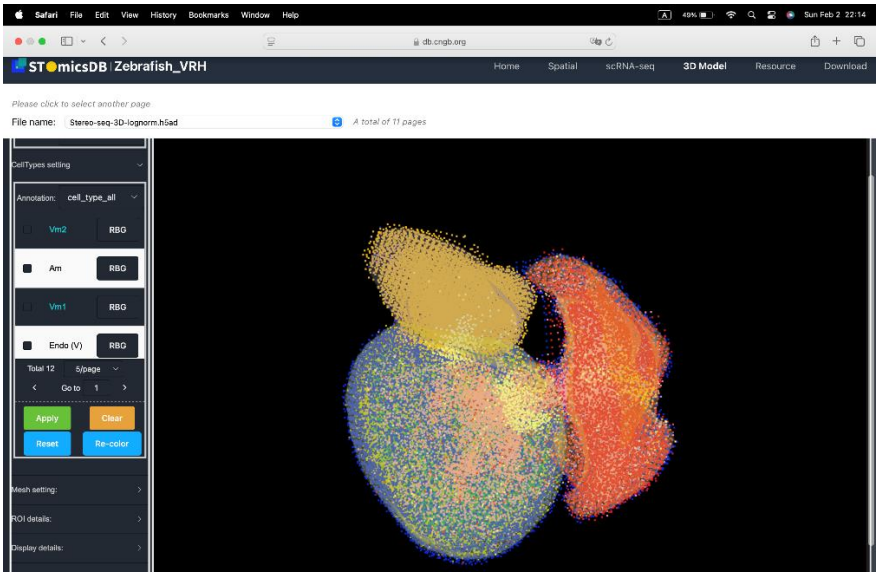


Figure R4. Screenshot of visiting the 3D model page by Safari.

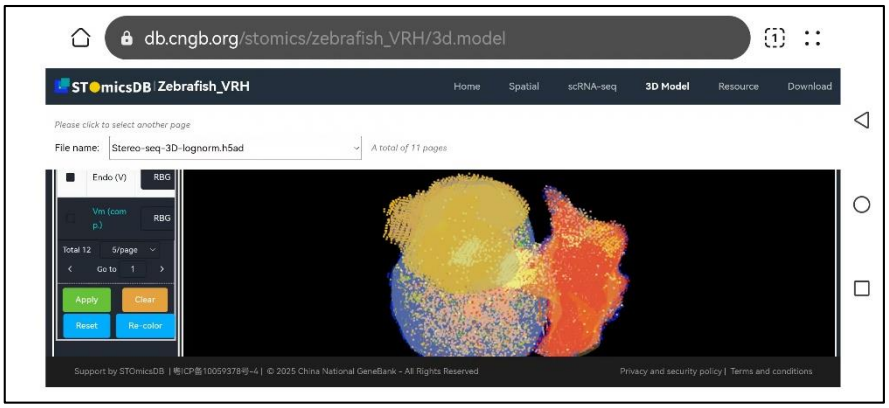
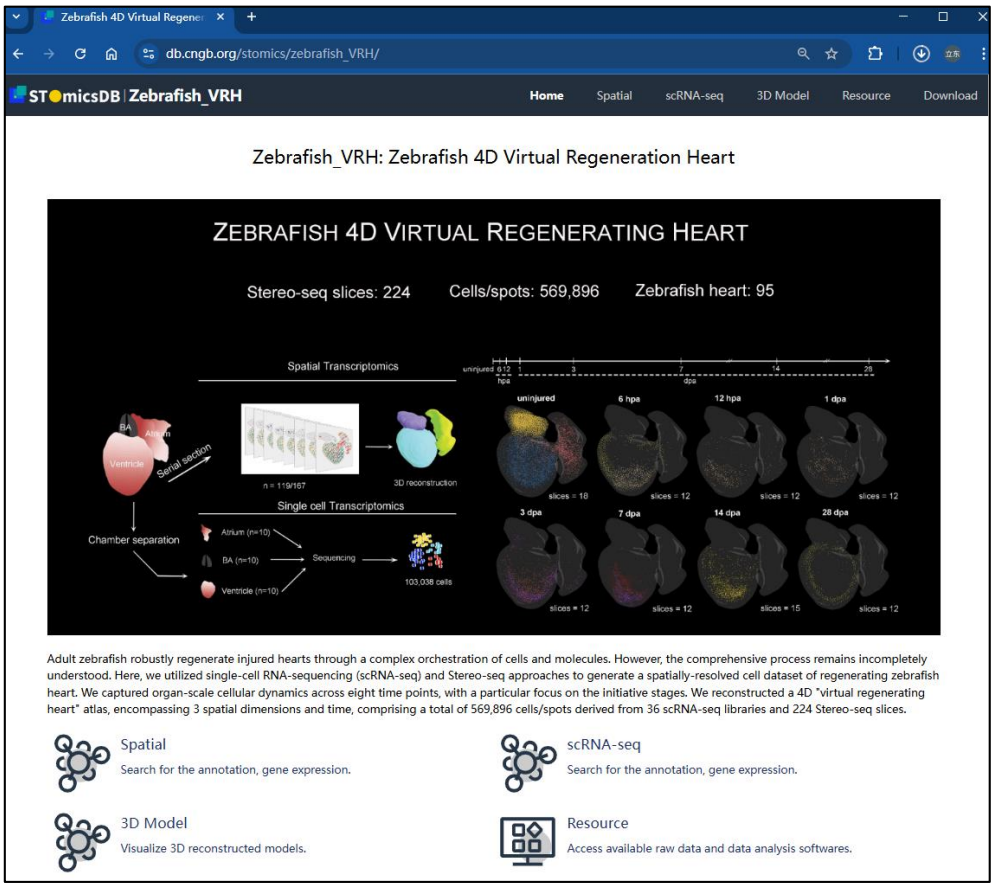


Figure R5. Screenshot of visiting the 3D model page by mobile phone.

Data Access Instructions

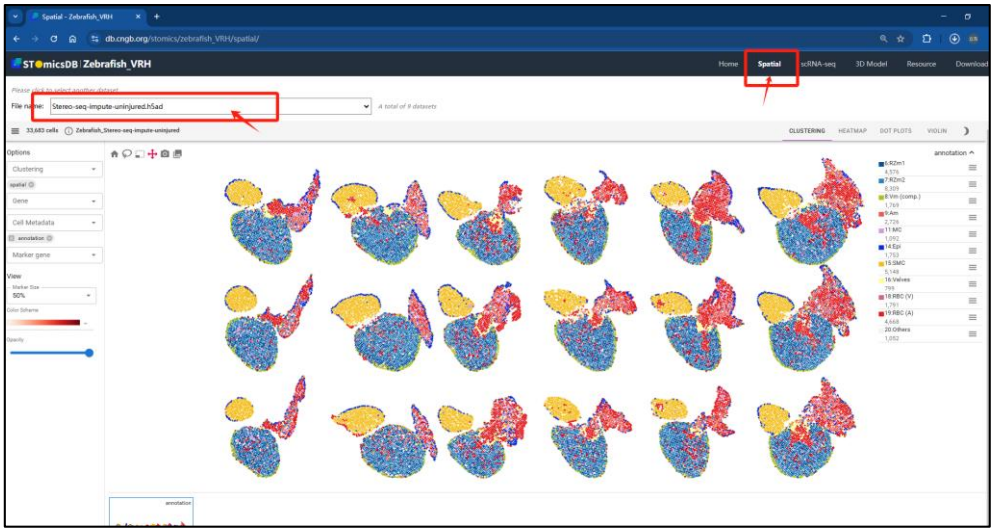
This document aims to provide step-by-step instructions for data visualization and downloading for each dataset. The main URL of our website is https://db.cngb.org/stomics/zebrafish_VRH, all below guides start this home page:



1, Stereo-seq-uninjured

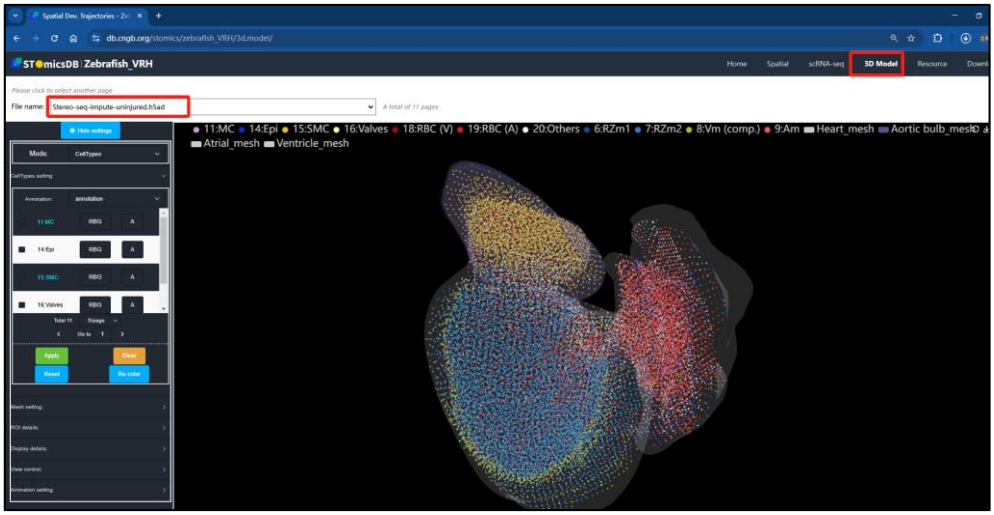
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-impute-uninjured.h5ad”



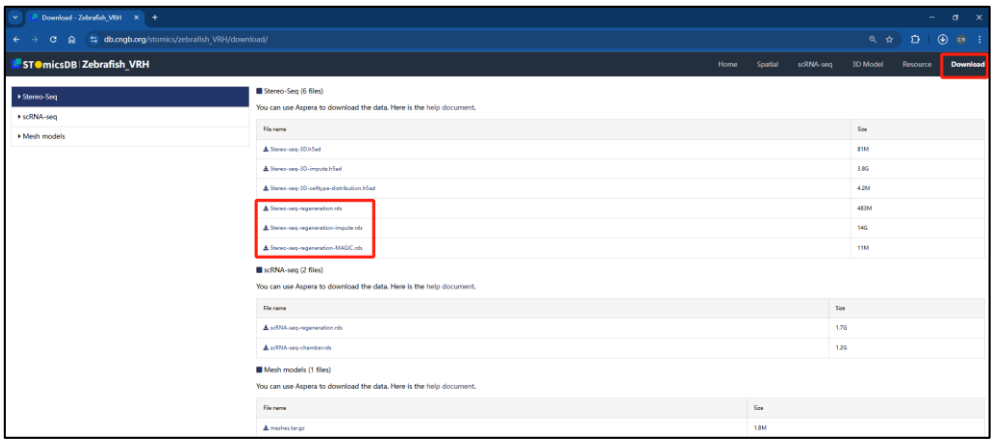
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-impute-uninjured.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-regeneration.rds/Stereo-seq-regeneration-impute.rds/Stereo-seq-regeneration-MAGIC.rds”



4) More information

a) We use the seurat-impute expression for data visualization.

b) Regeneration slices were aligned with 3D model by non-rigid registration.

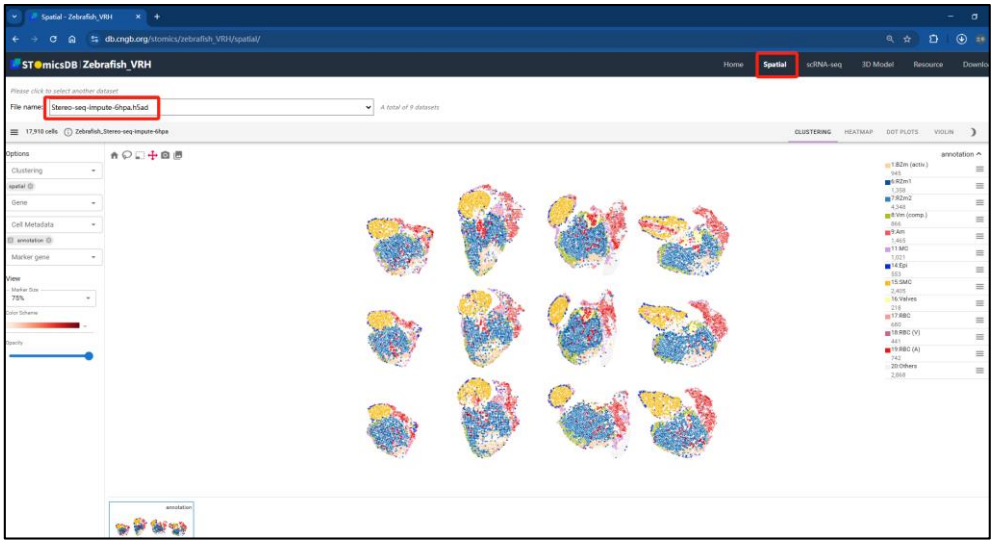
c) We provide the raw counts, Seurat impute, and MAGIC impute data for data downloading.

d) Please subset these datasets by “uninjured”.

2, Stereo-seq-6hpa

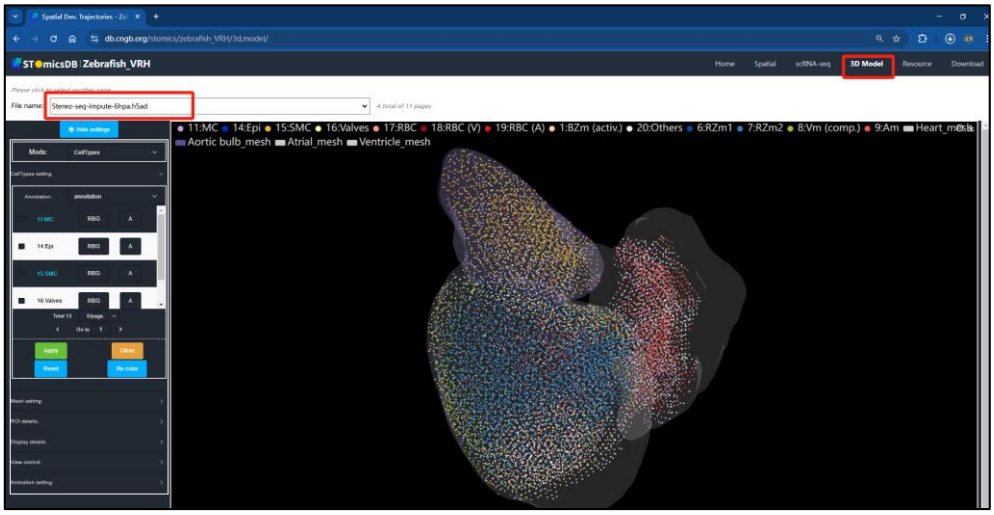
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-impute-6hpa.h5ad”



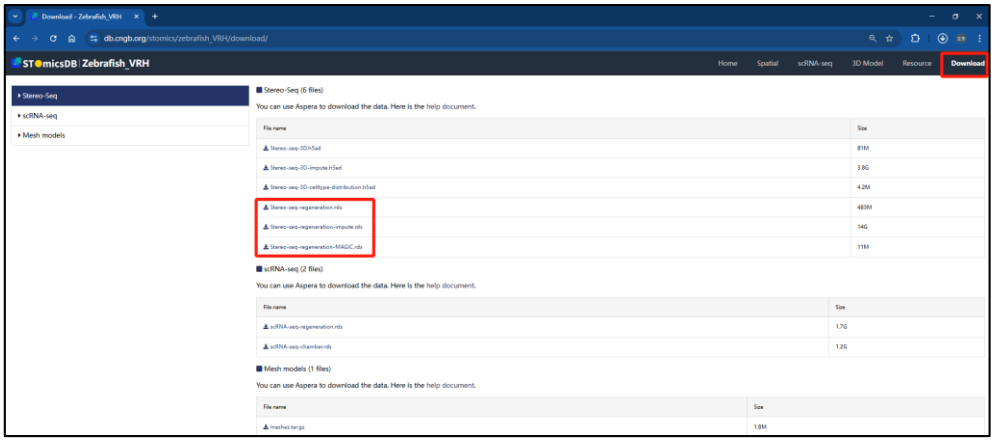
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-impute-6hpa.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-regeneration.rds/Stereo-seq-regeneration-impute.rds/Stereo-seq-regeneration-MAGIC.rds”



4) More information

a) We use the seurat-impute expression for data visualization.

b) Regeneration slices were aligned with 3D model by non-rigid registration.

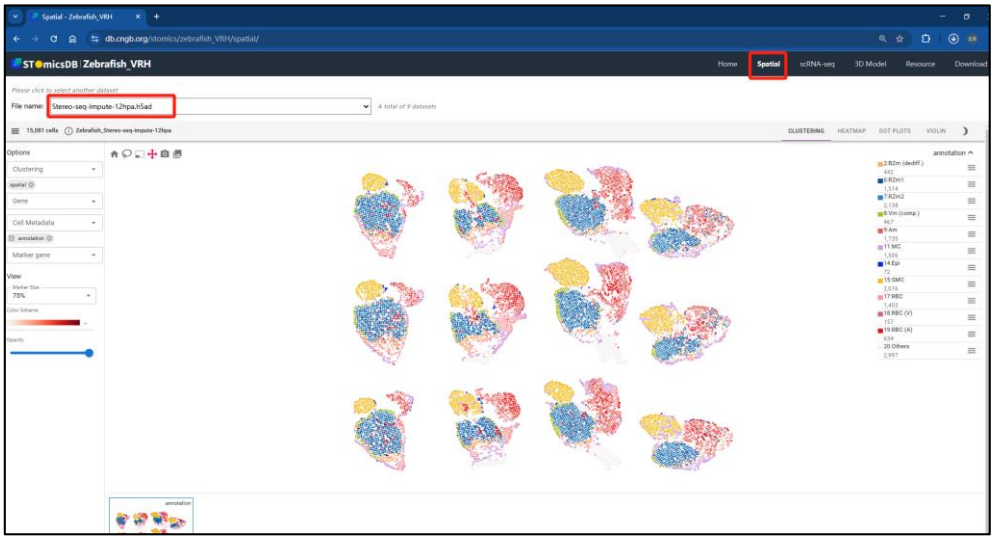
c) We provide the raw counts, Seurat impute, and MAGIC impute data for data downloading.

d) Please subset these datasets by “6 hpa”.

3, Stereo-seq-12hpa

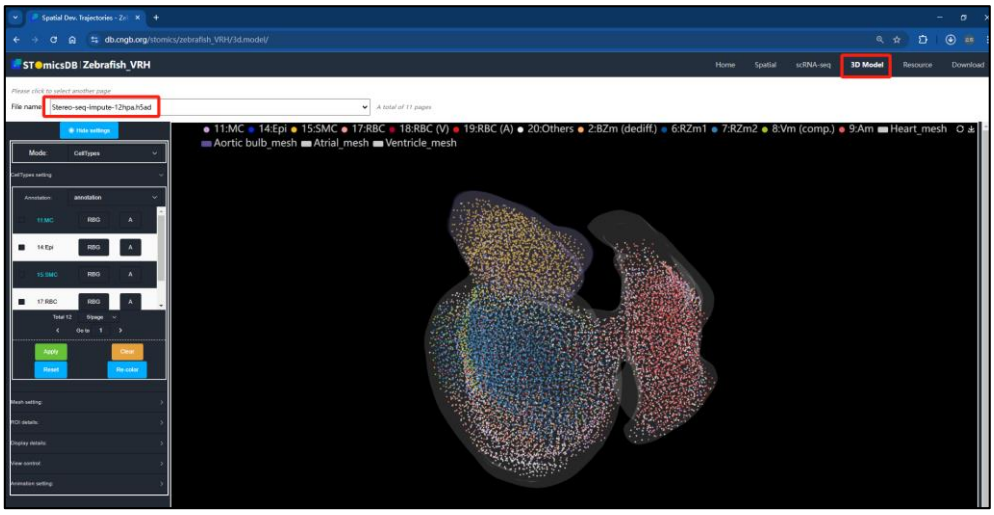
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-impute-12hpa.h5ad”



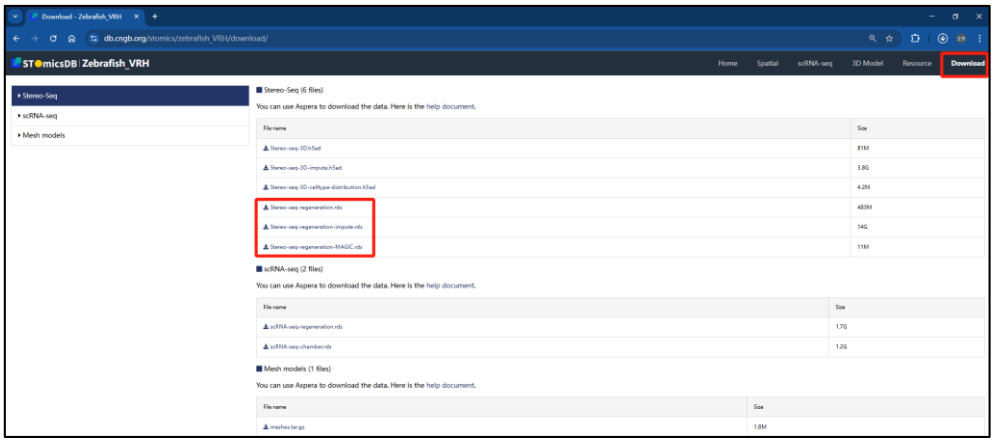
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-impute-12hpa.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-regeneration.rds/Stereo-seq-regeneration-impute.rds/Stereo-seq-regeneration-MAGIC.rds”



4) More information

a) We use the seurat-impute expression for data visualization.

b) Regeneration slices were aligned with 3D model by non-rigid registration.

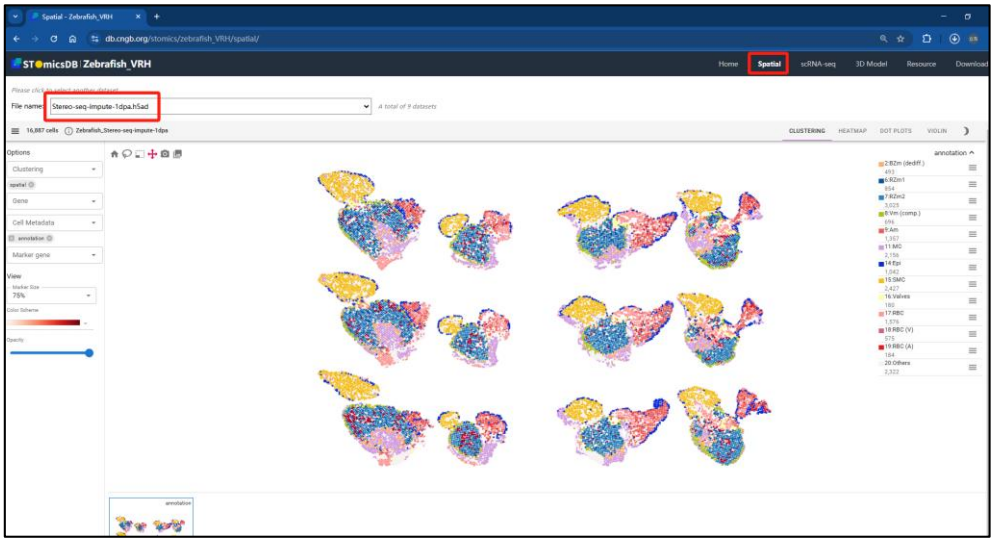
c) We provide the raw counts, Seurat impute, and MAGIC impute data for data downloading.

d) Please subset these datasets by “12 hpa”.

4, Stereo-seq-1dpa

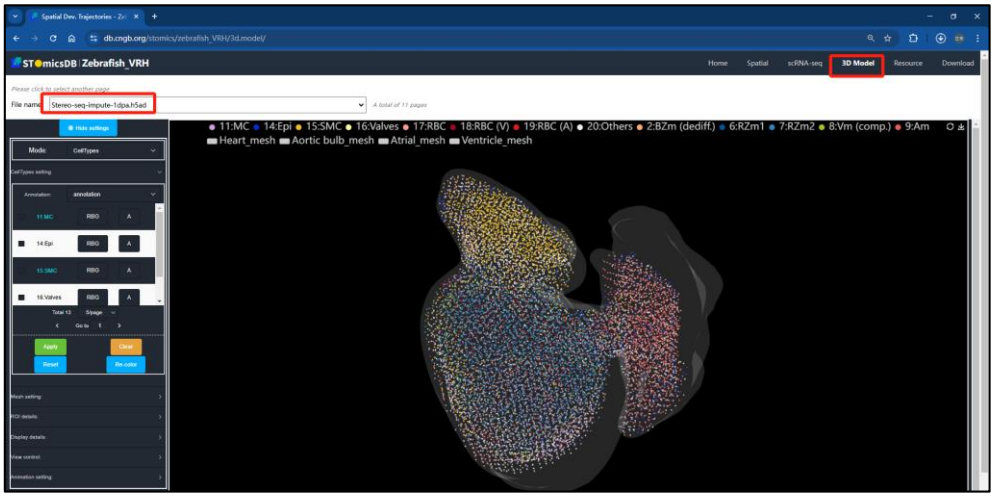
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-impute-1dpa.h5ad”



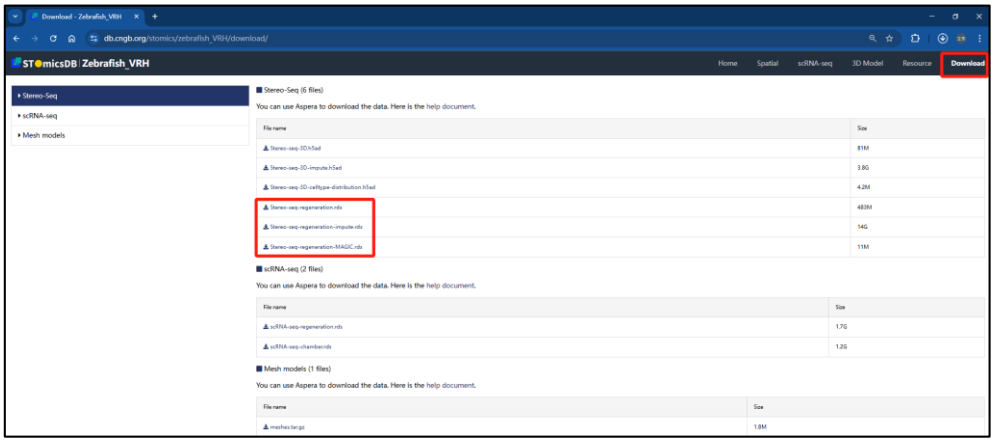
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-impute-1dpa.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-regeneration.rds/Stereo-seq-regeneration-impute.rds/Stereo-seq-regeneration-MAGIC.rds”



4) More information

a) We use the seurat-impute expression for data visualization.

b) Regeneration slices were aligned with 3D model by non-rigid registration.

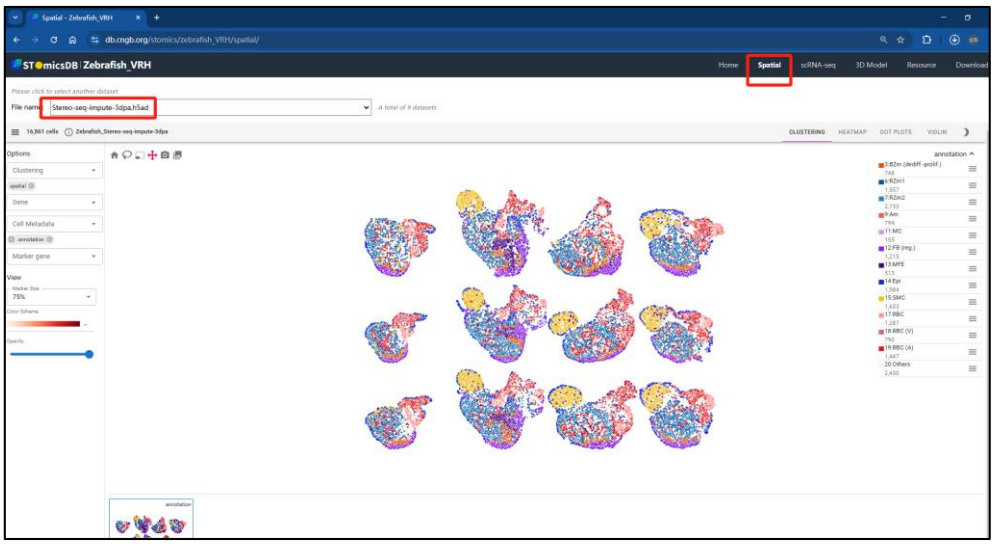
c) We provide the raw counts, Seurat impute, and MAGIC impute data for data downloading.

d) Please subset these datasets by “1 dpa”.

5, Stereo-seq-3dpa

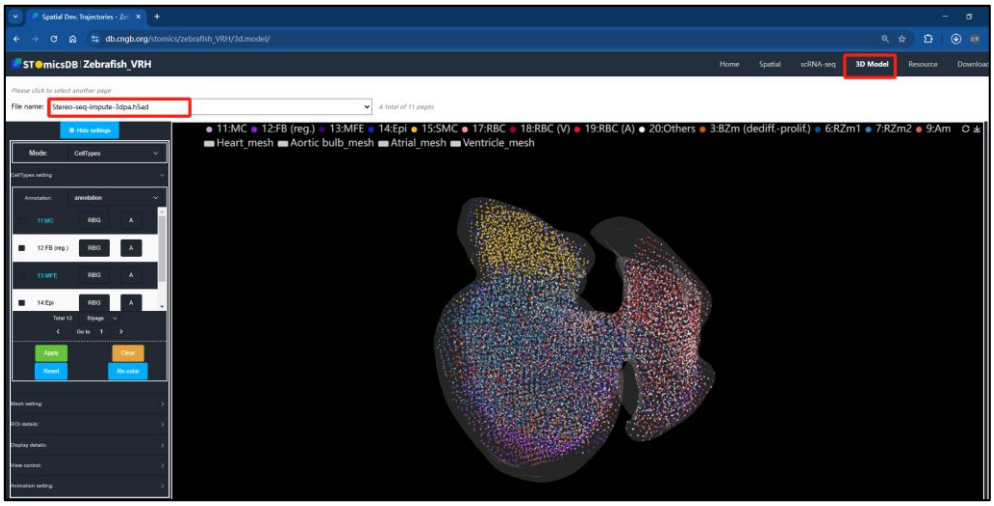
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-impute-3dpa.h5ad”



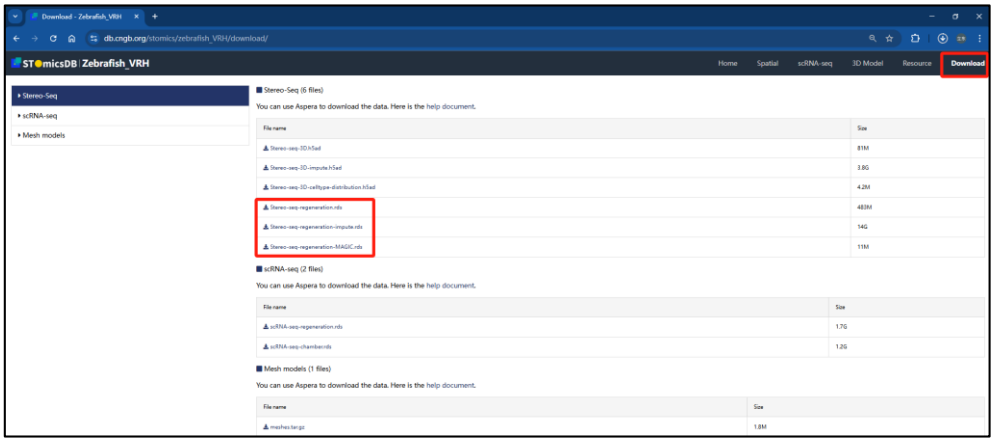
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-impute-3dpa.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-regeneration.rds/Stereo-seq-regeneration-impute.rds/Stereo-seq-regeneration-MAGIC.rds”



4) More information

a) We use the seurat-impute expression for data visualization.

b) Regeneration slices were aligned with 3D model by non-rigid registration.

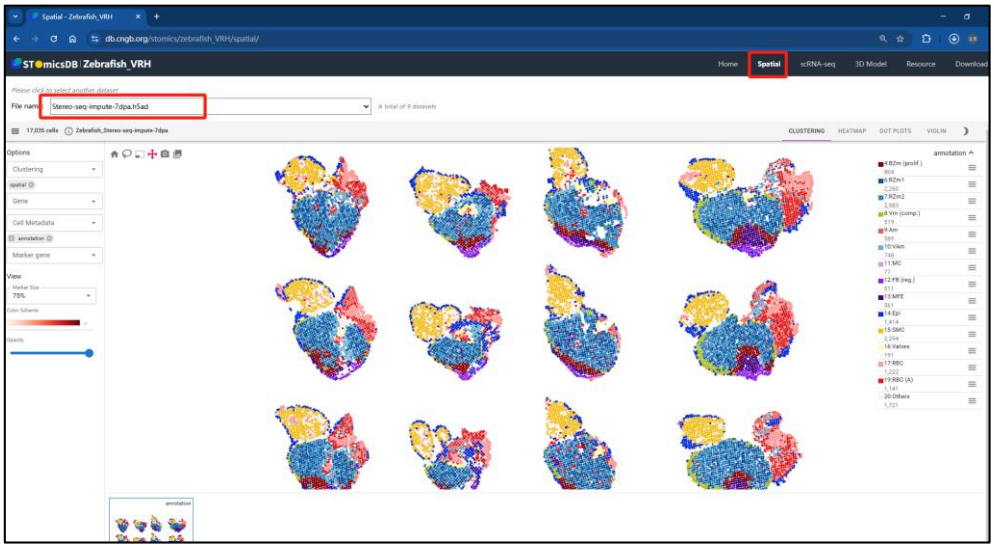
c) We provide the raw counts, Seurat impute, and MAGIC impute data for data downloading.

d) Please subset these datasets by “3 dpa”.

6, Stereo-seq-7dpa

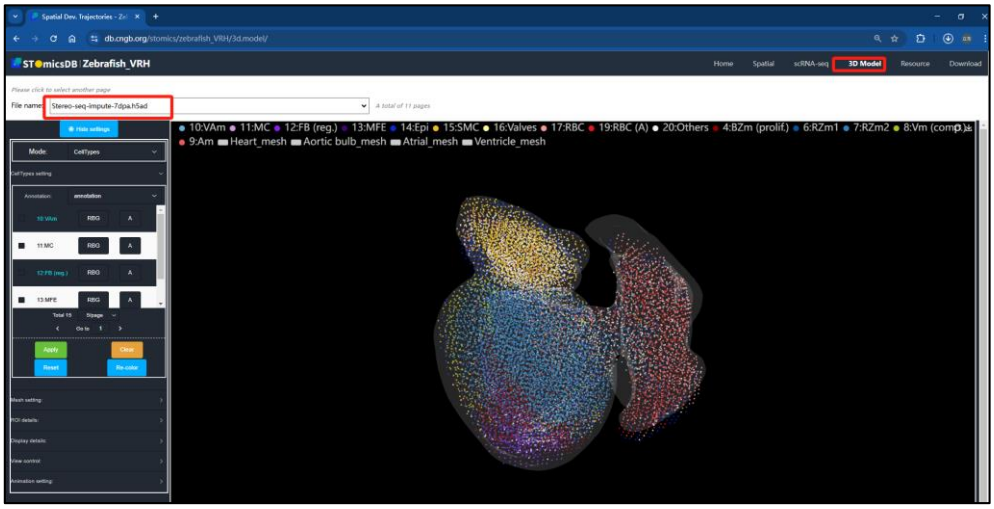
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-impute-7dpa.h5ad”



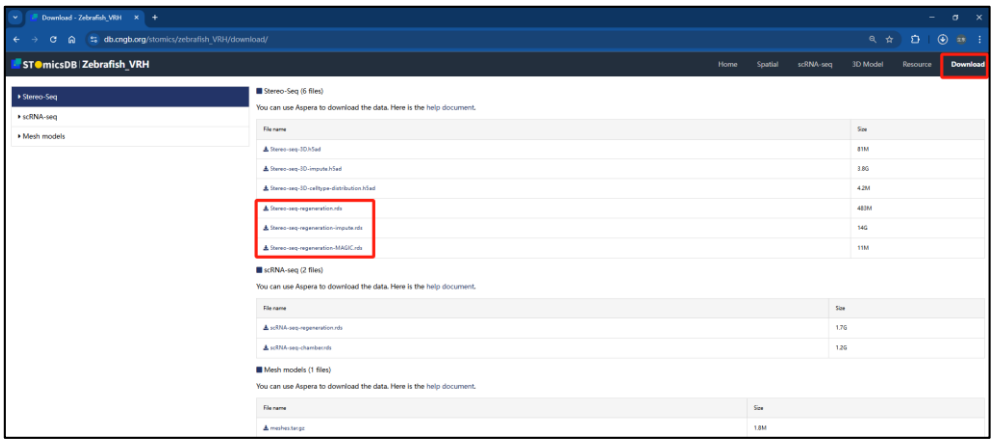
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-impute-7dpa.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-regeneration.rds/Stereo-seq-regeneration-impute.rds/Stereo-seq-regeneration-MAGIC.rds”



4) More information

a) We use the seurat-impute expression for data visualization.

b) Regeneration slices were aligned with 3D model by non-rigid registration.

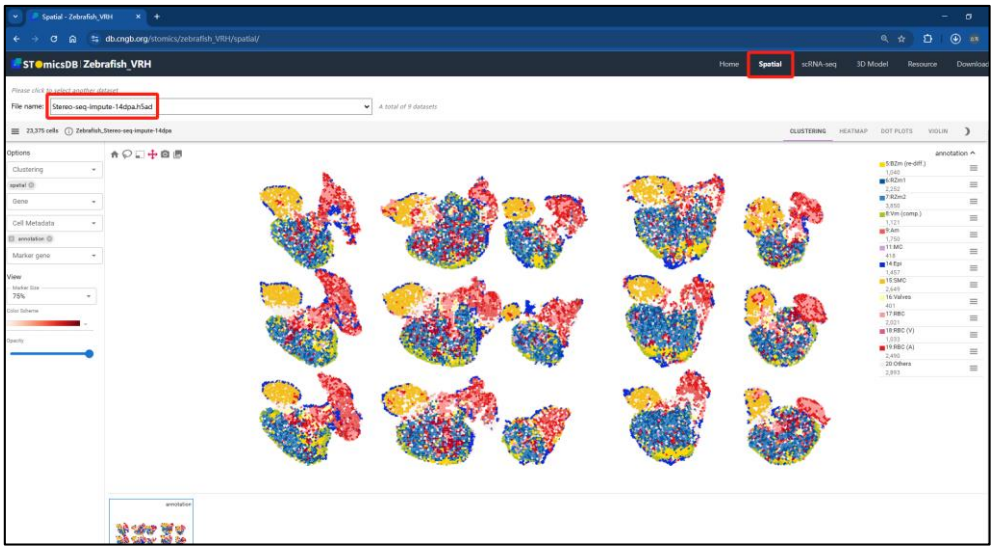
c) We provide the raw counts, Seurat impute, and MAGIC impute data for data downloading.

d) Please subset these datasets by “7 dpa”.

7, Stereo-seq-14dpa

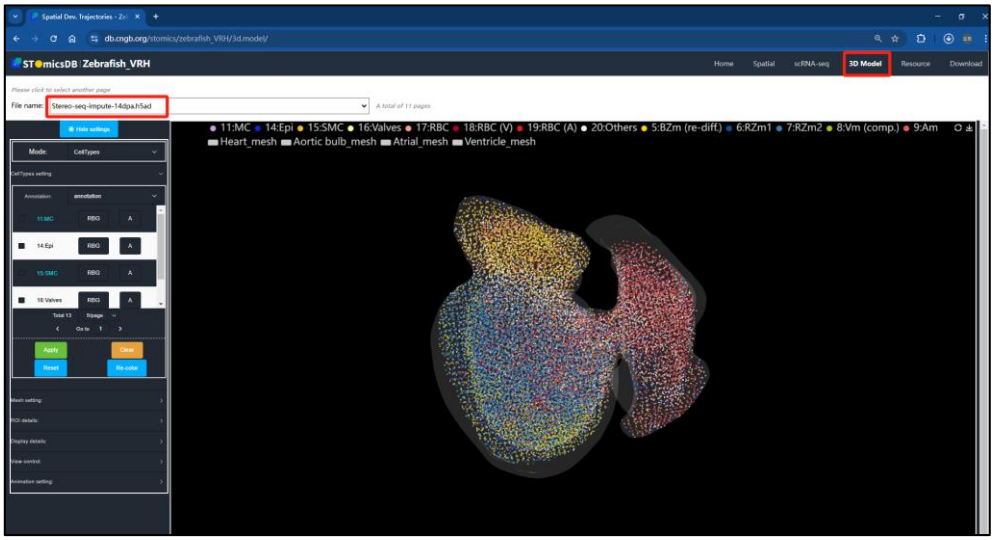
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-impute-14dpa.h5ad”



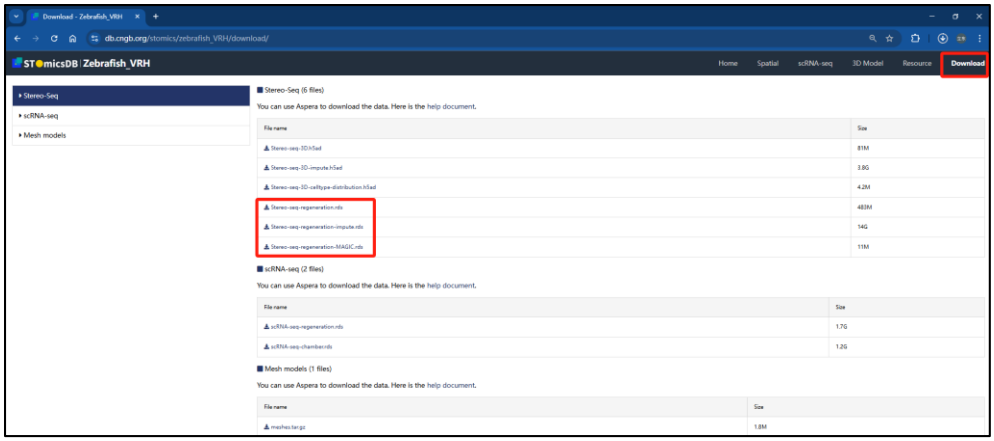
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-impute-14dpa.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-regeneration.rds/Stereo-seq-regeneration-impute.rds/Stereo-seq-regeneration-MAGIC.rds”



4) More information

a) We use the seurat-impute expression for data visualization.

b) Regeneration slices were aligned with 3D model by non-rigid registration.

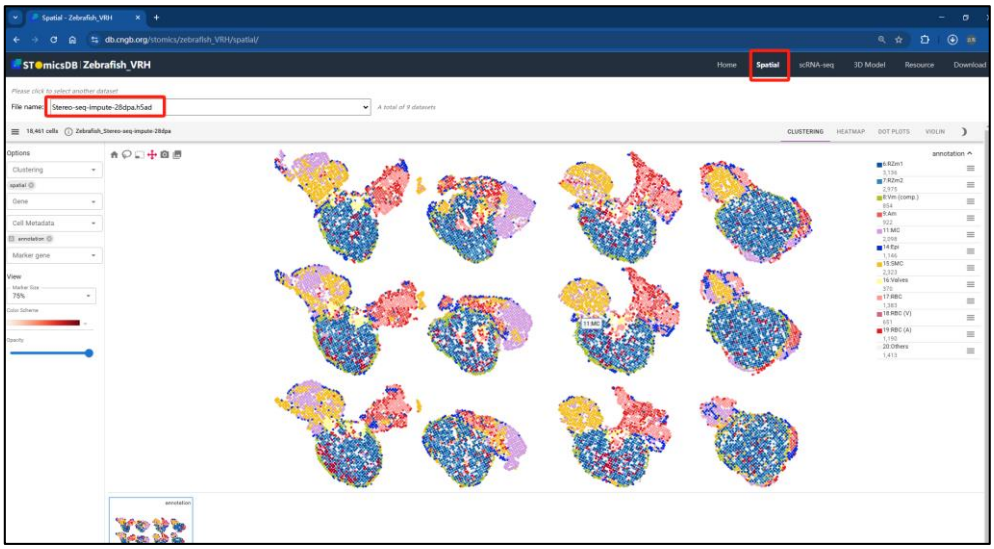
c) We provide the raw counts, Seurat impute, and MAGIC impute data for data downloading.

d) Please subset these datasets by "14 dpa".

8, Stereo-seq-28dpa

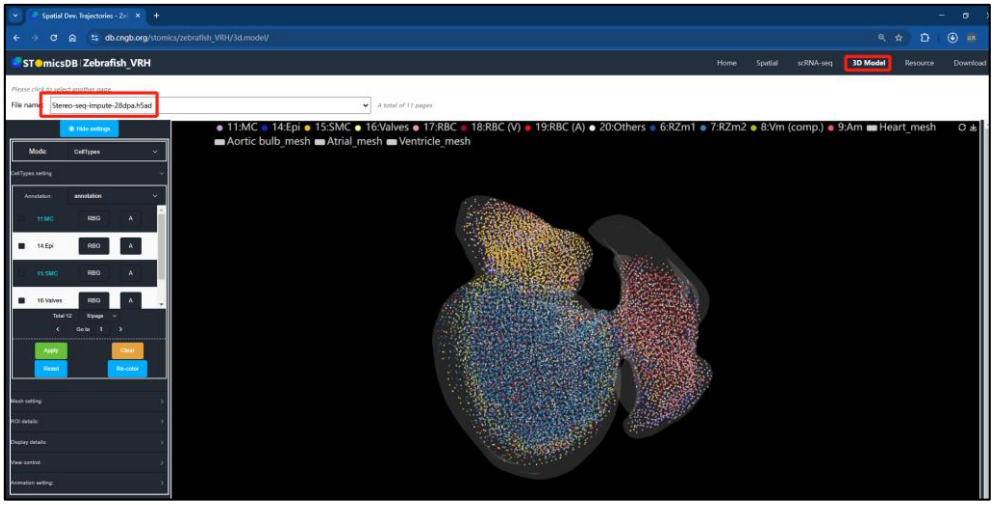
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-impute-28dpa.h5ad”



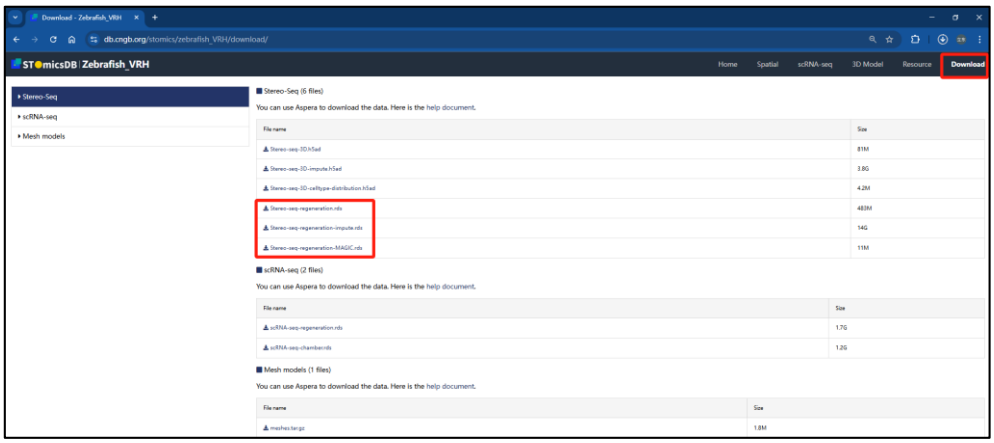
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-impute-28dpa.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-regeneration.rds/Stereo-seq-regeneration-impute.rds/Stereo-seq-regeneration-MAGIC.rds”



4) More information

a) We use the seurat-impute expression for data visualization.

b) Regeneration slices were aligned with 3D model by non-rigid registration.

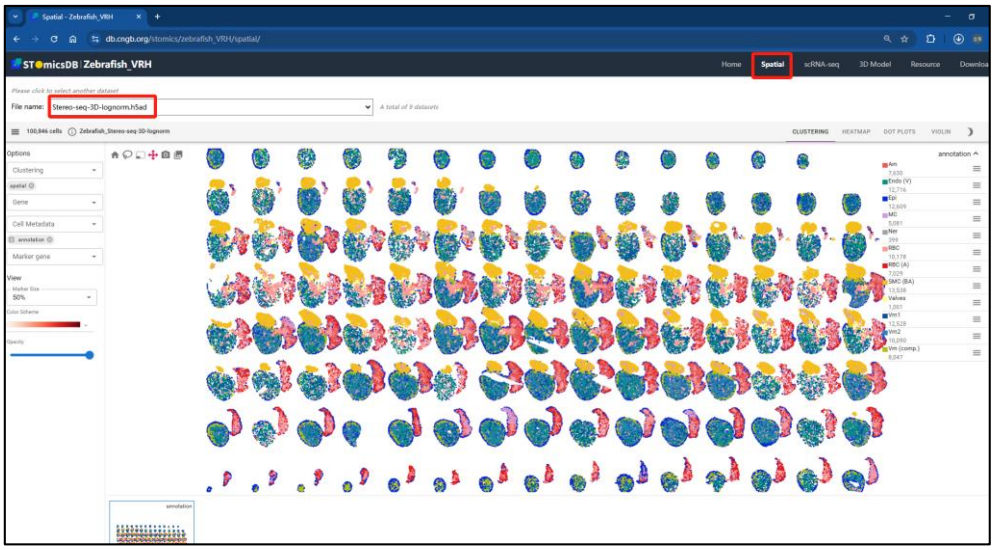
c) We provide the raw counts, Seurat impute, and MAGIC impute data for data downloading.

d) Please subset these datasets by “28 dpa”.

9, Stereo-seq-3D

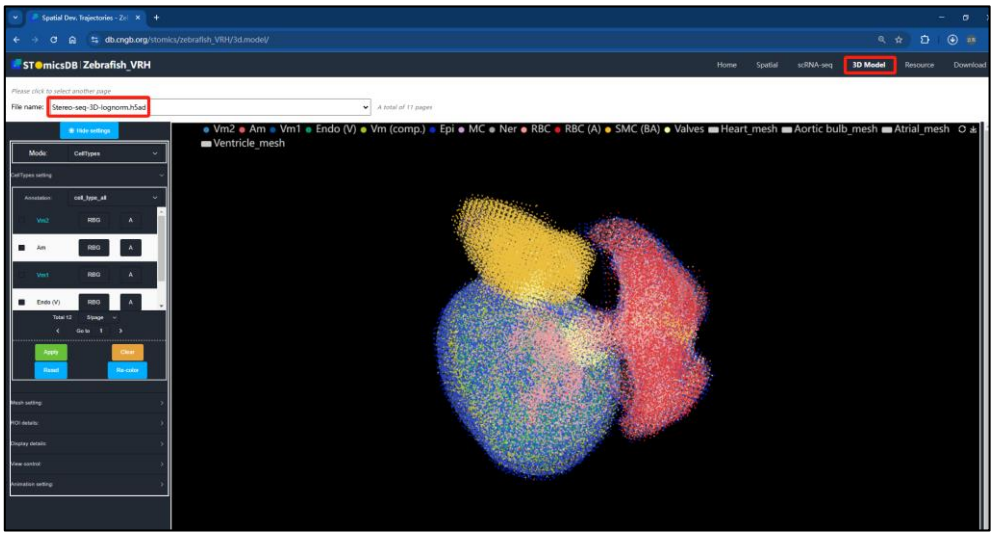
1) Visualization in 2D view

Click “Spatial” tab → Choose “Stereo-seq-3D-lognorm.h5ad”



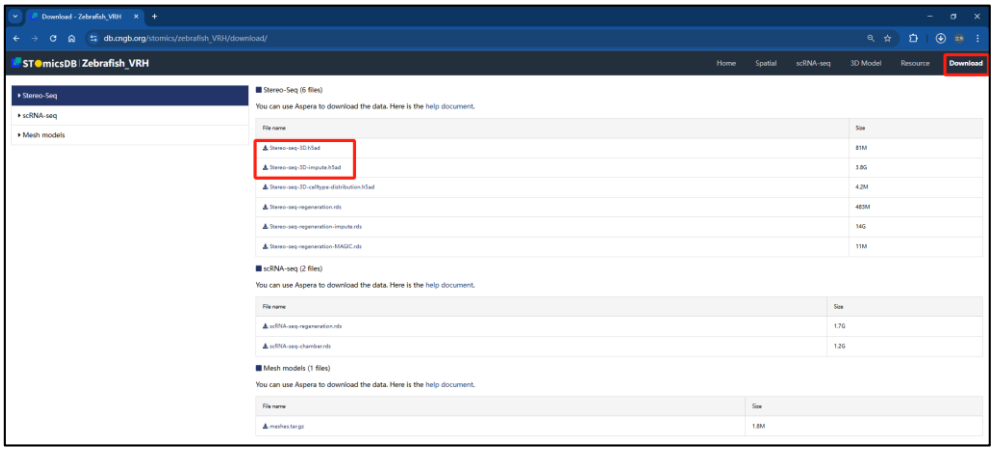
2) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-3D-lognorm.h5ad”



3) Downloading

Click “Download” tab → Choose “Stereo-seq-3D.h5ad or Stereo-seq-3D-impute.h5ad”



4) More information

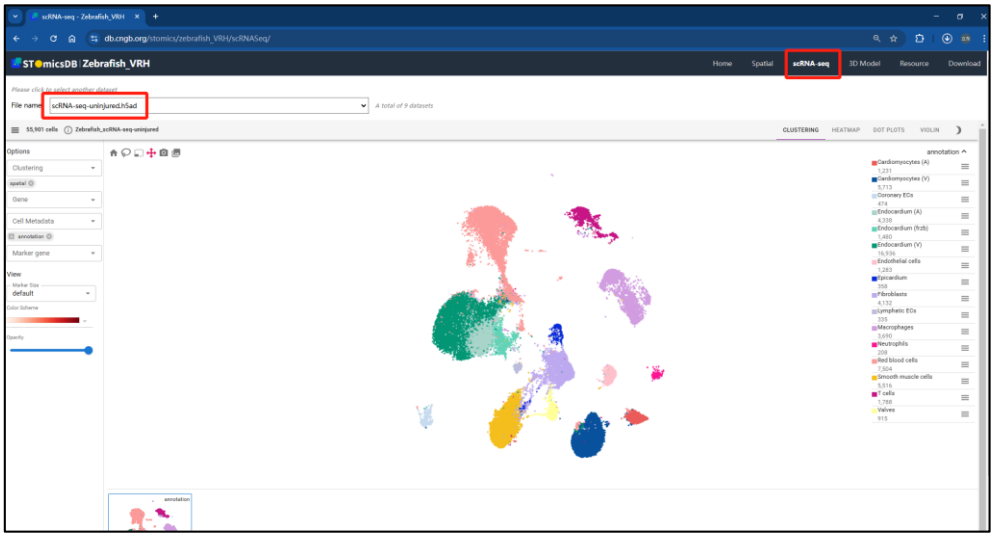
a) We use the seurat-impute expression for data visualization.

b) We provide the raw counts, and Seurat impute data for data downloading.

10, scRNA-uninjured

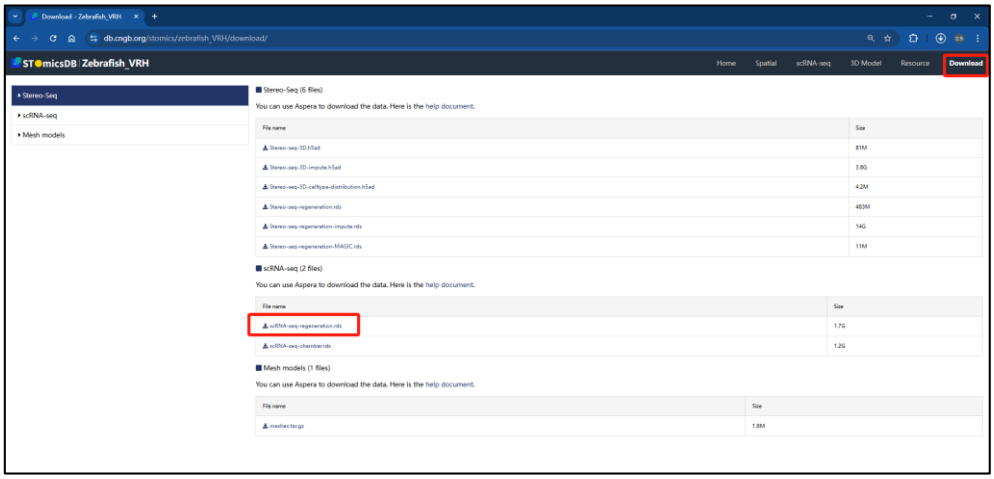
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-uninjured.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-regeneration.rds”



3) More information

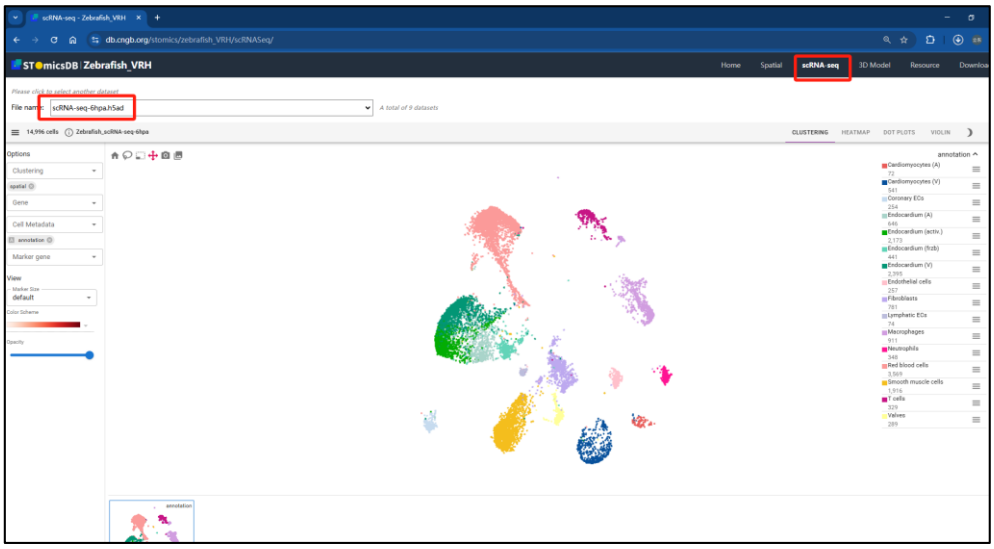
a) We use the log-normed expression for data visualization.

b) Please subset this dataset by “uninjured”.

11, scRNA-6hpa

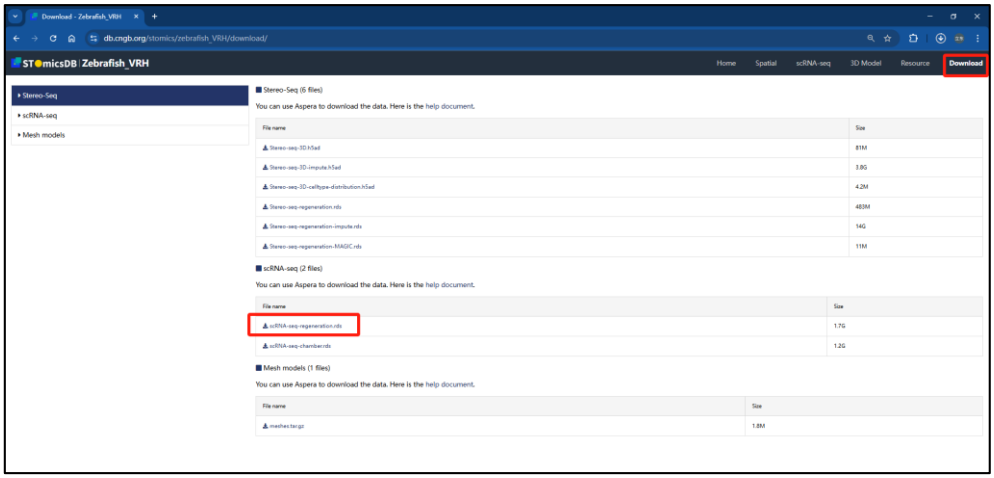
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-6hpa.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-regeneration.rds”



3) More information

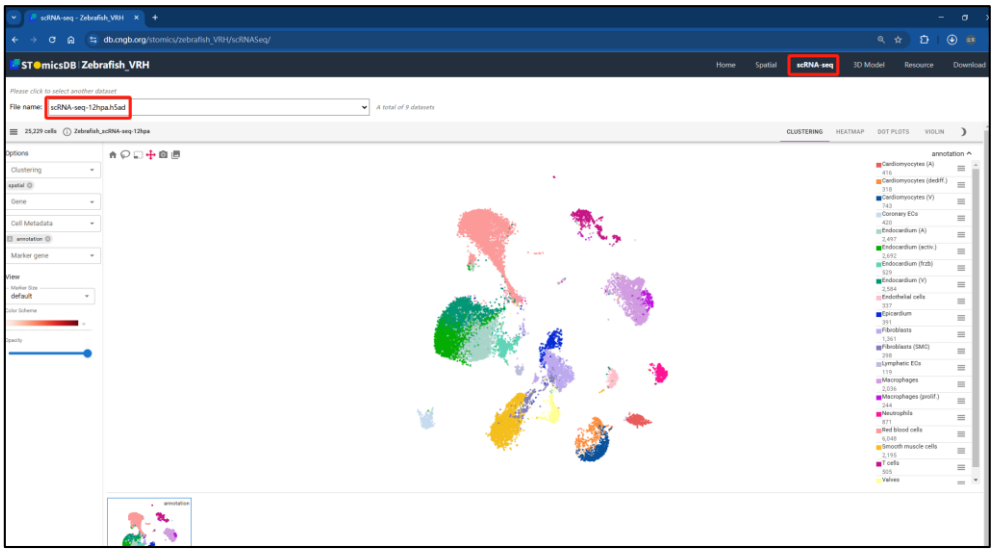
a) We use the log-normed expression for data visualization.

b) Please subset this dataset by “6 hpa”.

12, scRNA-12hpa

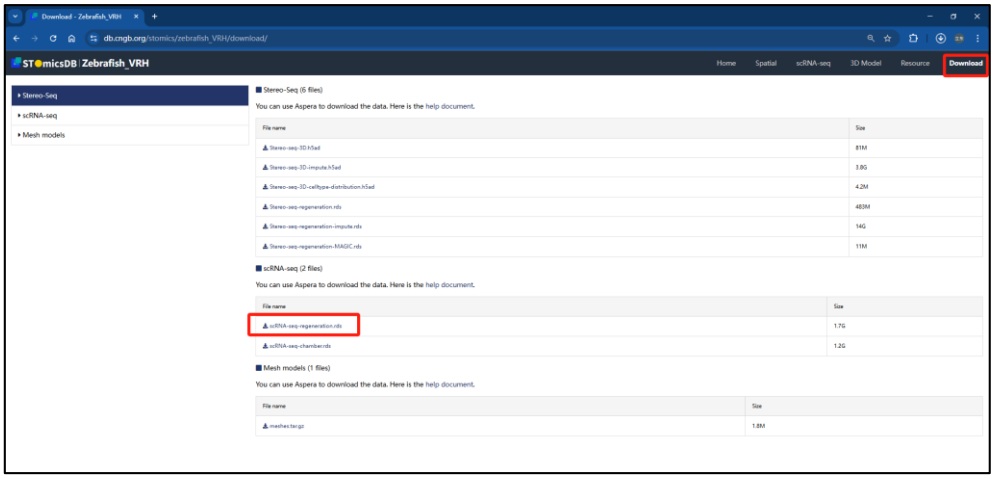
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-12hpa.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-regeneration.rds”



3) More information

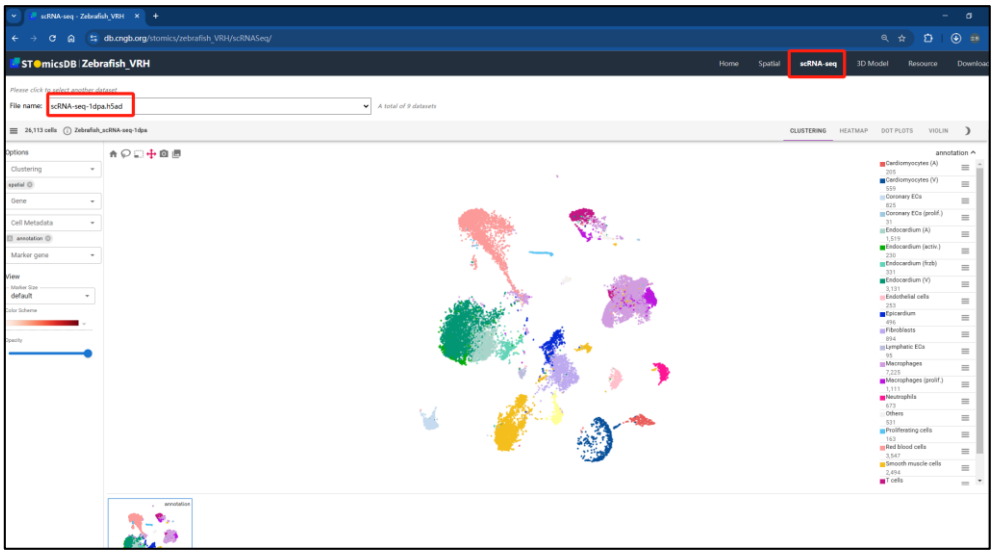
a) We use the log-normed expression for data visualization.

b) Please subset this dataset by “12 hpa”.

13, scRNA-1dpa

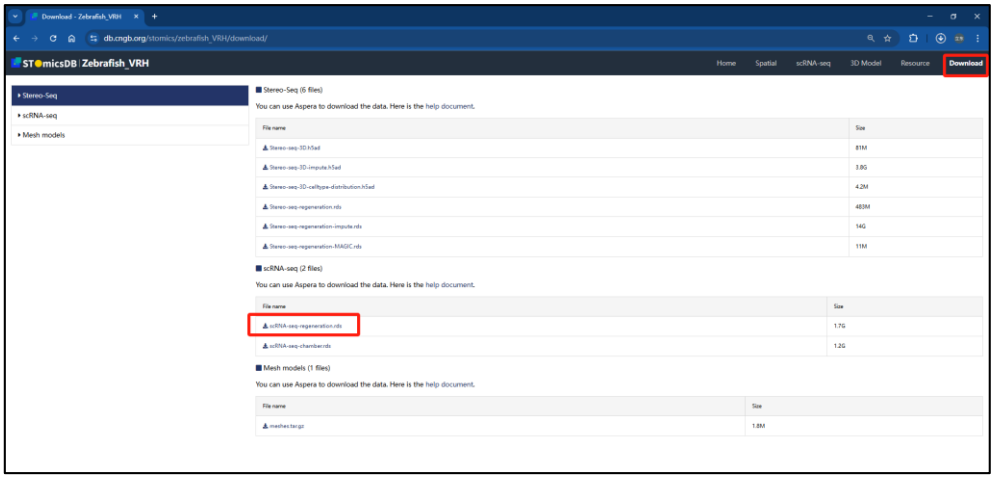
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-1dpa.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-regeneration.rds”



3) More information

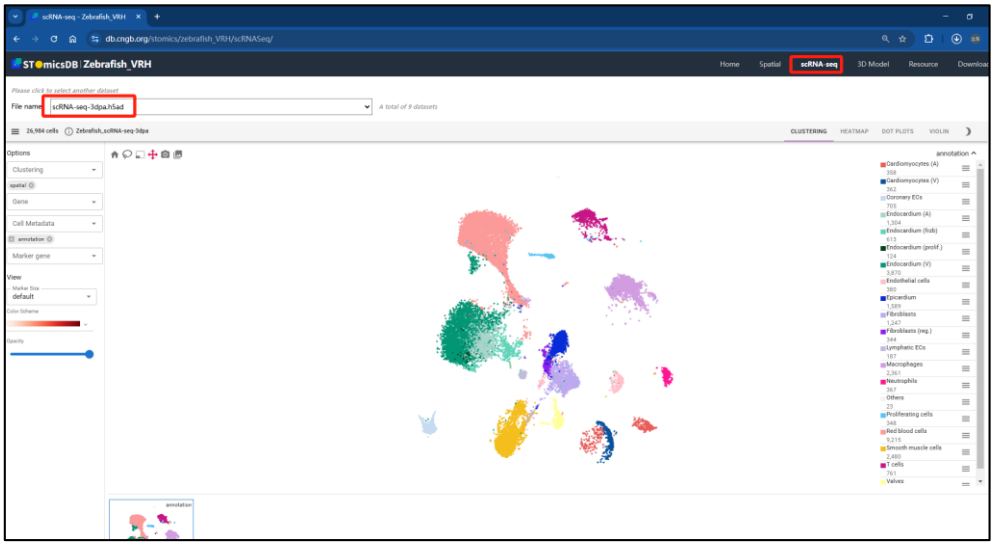
a) We use the log-normed expression for data visualization.

b) Please subset this dataset by “1 dpa”.

14, scRNA-3dpa

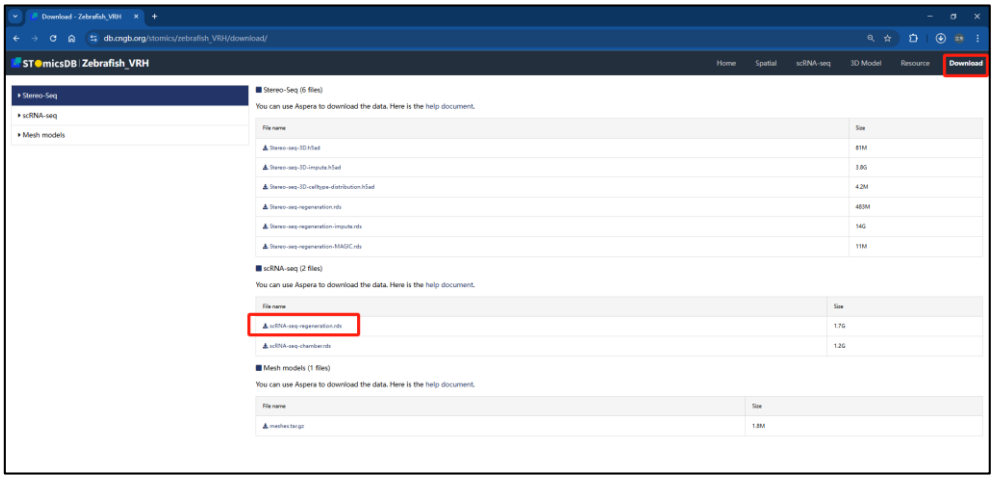
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-3dpa.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-regeneration.rds”



3) More information

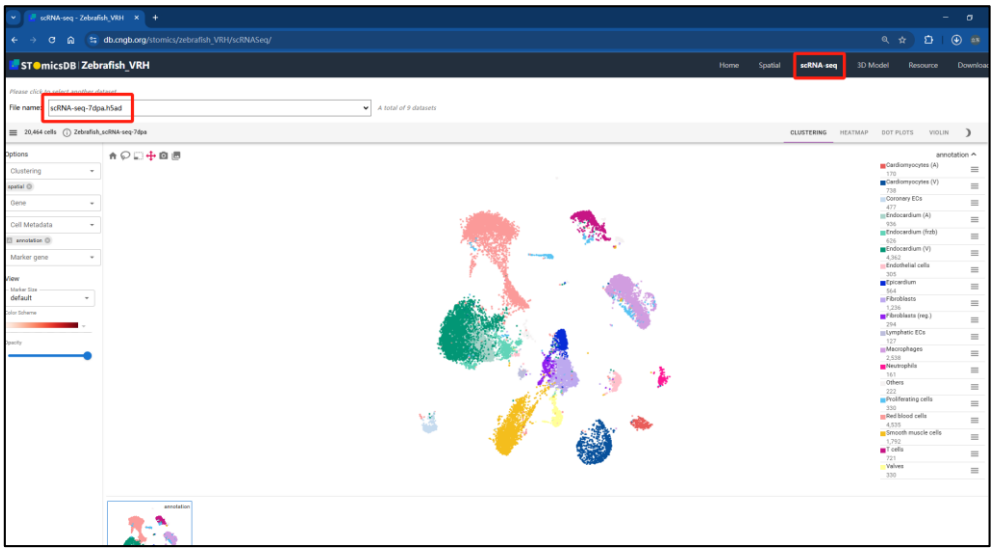
a) We use the log-normed expression for data visualization.

b) Please subset this dataset by “3 dpa”.

15, scRNA-7dpa

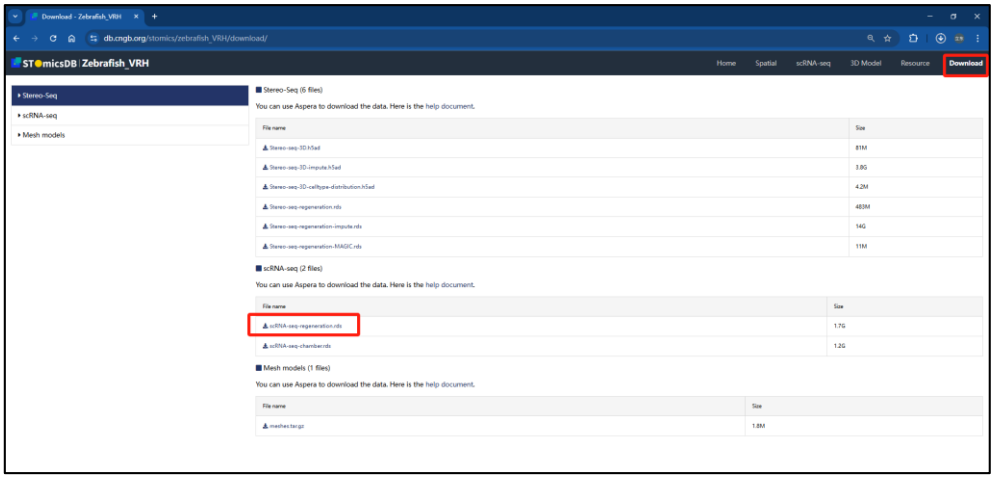
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-7dpa.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-regeneration.rds”



3) More information

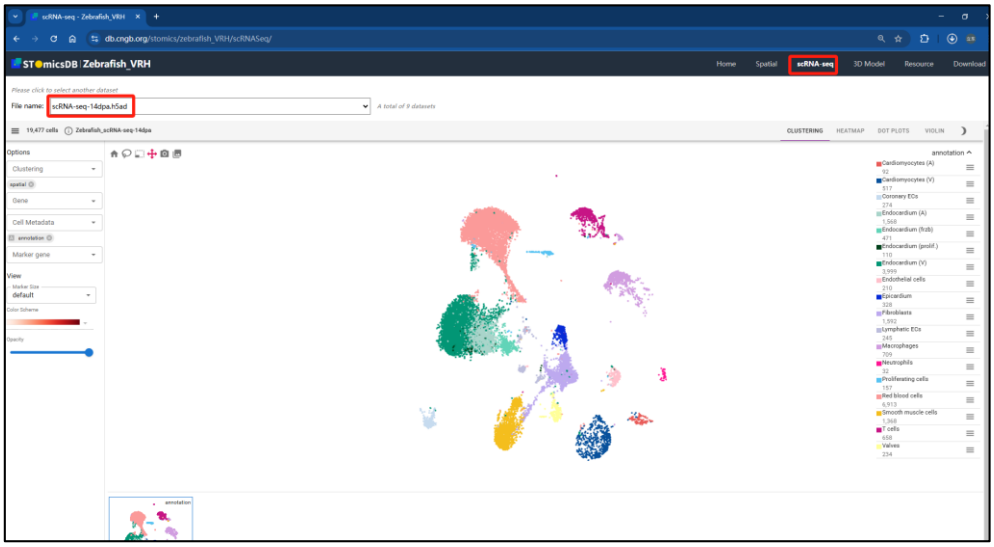
a) We use the log-normed expression for data visualization.

b) Please subset this dataset by “7 dpa”.

16, scRNA-14dpa

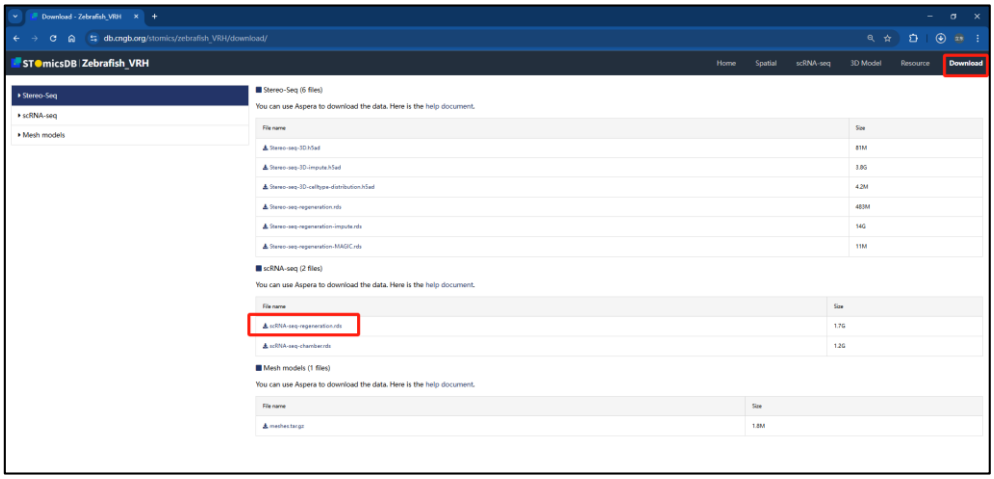
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-14dpa.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-regeneration.rds”



3) More information

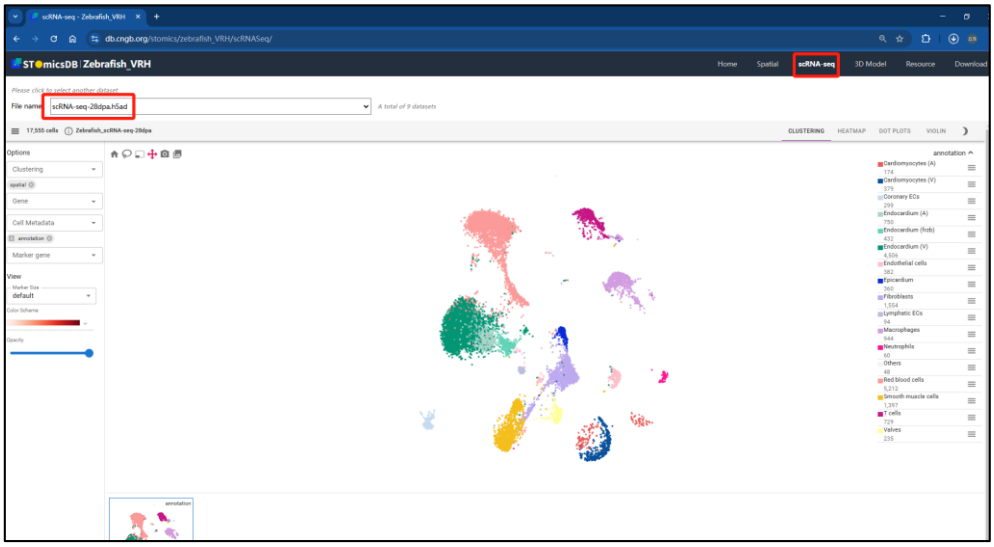
a) We use the log-normed expression for data visualization.

b) Please subset this dataset by “14 dpa”.

17, scRNA-28dpa

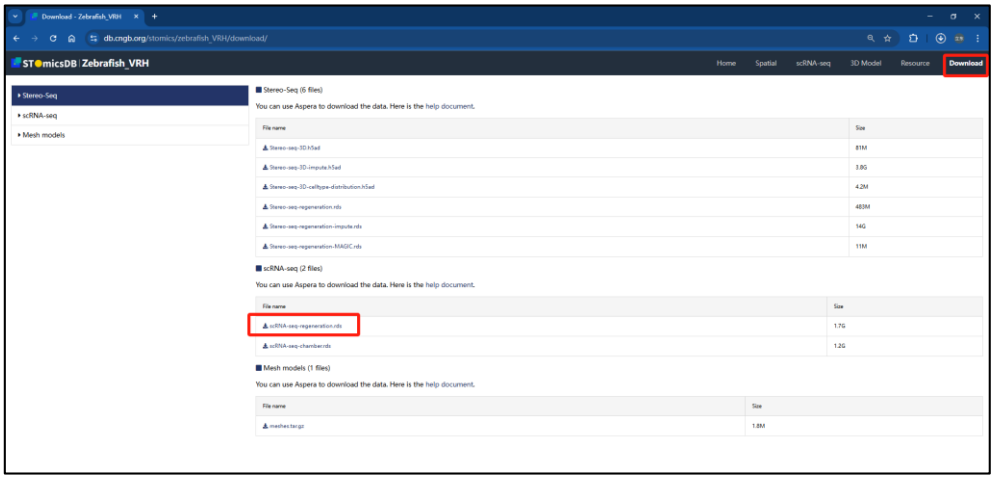
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-28dpa.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-regeneration.rds”



3) More information

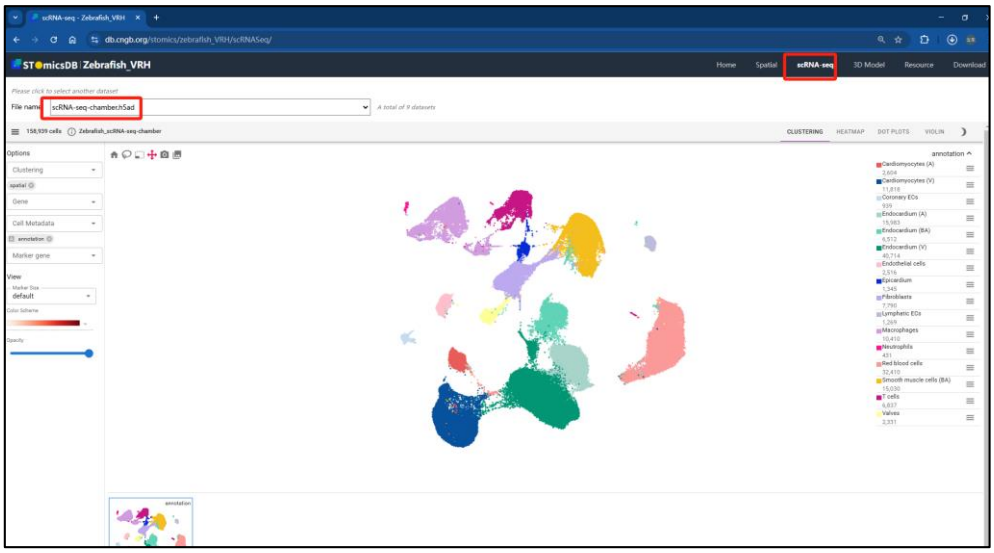
a) We use the log-normed expression for data visualization.

b) Please subset this dataset by “28 dpa”.

18, scRNA-chamber

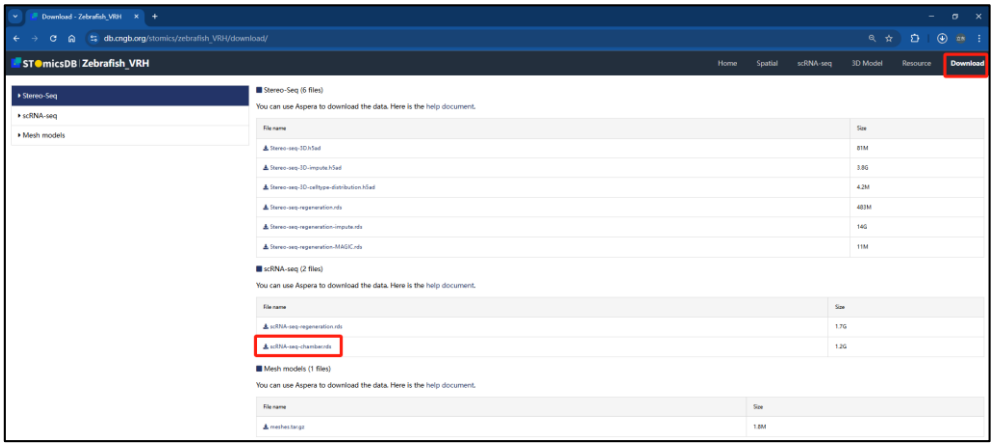
1) Visualization in 2D view

Click “scRNA-seq” tab → Choose “scRNA-seq-chamber.h5ad”



2) Downloading

Click “Download” tab → Choose “scRNA-seq-chamber.rds”



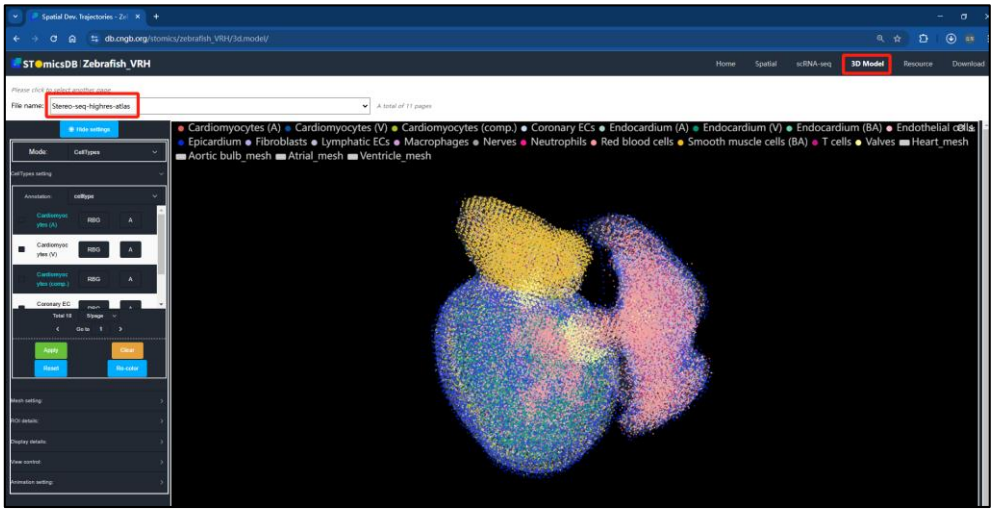
3) More information

We use the log-normed expression for data visualization.

19, Stereo-seq-high-res

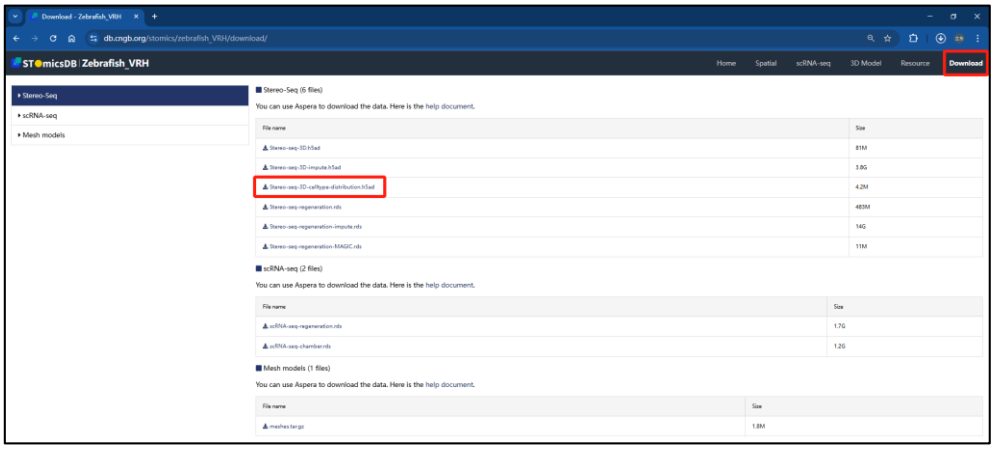
1) Visualization in 3D view

Click “3D Model” tab → Choose “Stereo-seq-highres-atlas”



2) Downloading

Click “Download” tab → Choose “Stereo-seq-3D-celltype-distribution.h5ad”



3) More information

a) We use the Seurat-impute expression from Stereo-seq.3D.impute.h5ad for gene visualization.

b) The downloading data only contain merged cell type distribution.