nature research

Peer Review Information

Journal: Nature Structural & Molecular Biology
Manuscript Title: Precise and scalable self-organization in mammalian pseudo-embryos
Corresponding author name(s): Thomas Gregor

Reviewer Comments & Decisions:

Decision Letter, initial version:

Message: Dear Dr. Gregor,

Thank you again for submitting your manuscript "Precise and scalable self-organization in mammalian pseudo-embryos". I apologize for the delay in responding, which resulted from delay in receiving the referee reports. Nevertheless, we now have comments (below) from the 2 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that both reviewers appreciate the results and find the conclusions timely and of wide interest. There are, however, several comments and suggestions that should be addressed in a revision. Specifically, reviewer #1 notes that the work requires contextualization in the literature and toning down of some statements. In addition, the referee suggests integration of the model with available gene regulatory network data, some additional analysis, as well as raising other technical concerns that need addressing. Reviewer #2 suggests that more information and analysis be provided on the number of starting cells needed for scaling. Please be sure to address and respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

Editor can outline here which reviewer concerns they are willing to overrule, and which must be addressed

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within 6 weeks. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision,

provided that no similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

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When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure item. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the final revision, as source data, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below: https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Carolina Perdigoto, PhD
Chief Editor
Nature Structural & Molecular Biology
orcid.org/0000-0002-5783-7106

Referee expertise:

Referee #1: biophysics, biophysical modelling of development

Referee #2: developmental biology, gastruloids

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Review for Precise and scalable self-organization in mammalian pseudo-embryos

In this work, Merle et al., investigate the precision of gene expression boundaries in mouse gastruloids. They implement a pipeline to record the expression profiles across the gastruloid at different time points. They use the resulting quantitative data to argue that the self-organized patterns scale with gastruloid size. They use this as an argument that gene expression boundary precision and scaling may well be emergent properties of the self-organization network.

The quantitative data collected is particularly impressive. It was especially good to see the data collected across multiple different experiments and compared rigorously (Fig. S2F). The data analysis is solid (with an exception highlighted below) and the results potentially very interesting. The data presentation is clear, and the manuscript is generally well written.

Below, I outline suggestions to improve the manuscript.

Major Concerns:

1. My main concern lies in the framing of the paper. In the introduction, the authors state "High fidelity of the underlying processes is most crucial during the earliest stages when the body plan and the future asymmetric body axes emerge at gastrulation". Yet, one could argue this is a Drosophila-centric view. For example, in vertebrates, different growth rates have been shown to drive scaled patterning of the neural tube, rather than precise scaled patterning (PMID 25258086). Further, cell sorting can lead to precise boundaries from initially noisy specification (PMID 33004519). Therefore, even if precision is seen early on in a gastruloid, it does not necessarily follow that this underlies the precise scaling and precision seen in vivo later in development. The vertebrate embryo may only need early patterning to be "good enough", and then later mechanisms (e.g., differential growth rates and plasticity in cell fate) can correct for errors. The introduction should more fairly reflect such differences from invertebrate to vertebrate – i.e., it is not even clear that the question being posed is relevant and this should at least be recognized.

2. Relatedly to (1), the Discussion needs toning down in parts. For example, the claim "Our findings point to underlying principles that govern the self-organization processes in multicellular systems, reaching across half a billion years of evolutionary change" needs

more qualification. The authors may be correct, but the evidence presented here – from one species, at one specific stage of development – hardly backs up this statement.

3. Though not essential to do, it seems a missed opportunity that the results aren't mapped onto a model of the gene regulatory network. These interactions have been explored intensively (e.g., PMID 37209681), with modelling papers exploring the network interactions (e.g., PMID 31613879). How well do the GRN models map onto the quantitative data collected here?

4. The discussion of scaling is very limited in the introduction. A single paper is cited – to a five-year-old Arxiv paper. There has been a lot of work in this area, e.g., PMIDs: 21613328, 21873045, 32048988, 32209485, 32251432, 34937053. Given the central importance of scaling to the authors conclusions, a more balanced overview of the current understanding of gene expression boundary scaling is suitable.

5. Related to the above point, r.e., scaling, the manuscript does not clearly define what "close to perfect scaling" means. This (and similar) phrasing is used multiple times. Yet, somewhat surprisingly, no quantitative measure is shown to back up this statement, beyond the linear relationships shown in, for example, Fig. 1D. In the scaling literature, there are a number of different approaches used to test claims of scaling and put a limit on its accuracy. At the moment, bold statements are made regarding the scaling, but a rigorous, unbiased test of such claims is currently lacking.

Related to this, the scaling data in Fig. 4 is intriguing, but needs more careful analysis. For example, the profile of FOXC1 shows clear differences at larger gastruloids. Likewise, BRA appears to change the steepness of its slope in larger gastruloids. While close to scaling, there do seem to be differences that emerge, which distinguish this from, say, the early fly embryo. Currently, there is insufficient evidence that "average profiles of all sizes collapse".

6. In Fig. 2, somewhat surprisingly the relative error appears to remain small in regions of low expression (e.g., Sox2 in region x/L = 0.1). This is not what would be expected, as the intensity gets lower, the relative error typically increases. This should be clarified – is the associated error also so low that the relative error stays small? This seems surprising, especially given the authors admit there are still experimental errors in the system so we should take their error estimates as upper bounds.

7. In the measurements of cell size, was potential extracellular space accounted for? It should be straightforward to quantify how much extracellular space there is during gastruloid formation. It may be that the cells are all tightly packed throughout, but this should be confirmed.

Other points:

1. The four chosen targets are given with little explanation. A brief summary of the four genes and why they are selected would be helpful (e.g., mesoderm-specific)
2. The % of successful gastruloids (and total number) should be clearly in the main text, not in the legend of Fig. S1.
3. Looking at Fig. S2A, the error is typically 20% variation. I agree with the authors that the observed scaling is quite remarkable, yet this is also clearly significantly larger than observations in Drosophila – where sample-sample variation is more on the order of 10%.

It is confusing that the gastruloids are claimed to have similar accuracy to Drosophila, when the data seems to suggest the error is consistently larger. Relatedly, the results of Fig. 3 are very interesting – but the error is also clearly larger than what is seen in the gap genes. So, the conclusion that it is comparable seems too bold at this point.
4. As stated at the start, I am pleased that the authors have openly shown the experiment-to-experiment variability (e.g., Fig. S8). This gives me a lot more confidence in their results. I do note that in late 2022 (Fig. S2F), there is a large increase in the gastruloid size. Given that this seemed to repeat itself over a number of experiments, was a cause identified that distinguished this batch of experiments from the other ones? This may be of broader interest to know what experimental variations can affect gastruloid size.
5. What does "quasi-perfect" mean? This seems ill-defined currently.

Reviewer #2:
Remarks to the Author:
This is an important piece of work that reveals a very important attribute of multicellular structures: their ability to produce reproducible patterns independently of boundary conditions, that scale. It is a technically exceptional and intellectually sound piece of work that provides a sound foundation for a new and important new experimental system.

There are important features of biological systems that are at the root of why we have used and learnt from model systems over the years. One example is the robustness and reproducibility of their patterns and organization, particularly during development, that allow the identification of deviations, for example through mutations, that have led to much understanding of underlying molecular mechanisms. Most of these studies are qualitative but over the last twenty years, quantitative analysis has revealed surprising new layers in termd of precision in molecular and cellular activities, that pose new questions about the way biological systems develop.
A good example of this can be found in work on the Drosophila embryo where studies over the last 30 years have shown that the pattern of the larva is laid down begore gastrulation in the form of interactions between the Bicoid gradient and a cascade of downstream genes coding for transcription factors. Quantitative analysis of this process, led by the group of Thomas Gregor, has shown that these events are extremely precise with spatial registrations happening to the level of one cell diameter. Furthermore, the patterning process scales i.e. the relative proportions of the patterns of gene expression remain constant regardless of the total size of the embryo. This is remarkable but could be argued that the basis is 'simple' since the Drosophila embryo develops within a rigid case that can provide a ruler for this precision, constraining the range and activity of the transcription factors.
It is not clear that this precision exists in vertebrates or mammals which develop in different ways. In particular their embryos create their boundary conditions as they grow. One of the reasons for our ignorance lies in the experimental difficulty, particularly in mammals which develop inside the mother and produce few embryos. Mammals are important, not only because we are mammals and therefore their development is our development but also because their embryos represent an extreme form of self organization.
Over the last ten years models based on embryonic stem cells have created experimental opportunities that can be used to address fundamental experimental questions and it is this that is being addressed in this manuscript. The authors make us of gastruloids,

models of early mammalian development based on pluripotent stem cells (PSCs). When aggregates of defined numbers of cells are placed under controlled culture conditions, they recapitulate the main events of gastrulation. After 5 days the result is an elongated structure that displays a range of tissue and organ primordia arranged with regard to three orthogonal axes mimicking the embryo. In the case of mouse PSCs, the structures resemble embryos about mid gestation. Gastruloids can be produced in large numbers and are being used to explore mechanisms and interactions of early mammalian development. Here, Merle et al use them to ascertain precision in patterning of gene expression along their anteroposterior axis.
In this manuscript Merle et al show that by controlling experimental conditions they can make gastruloids with nearly 100% frequency which allows them to make large numbers of independent measurements with confidence. They find a remarkable degree of precision in the dimensions, growth rate and cell numbers of gastruloids, with errors that overlap measurement errors. The work is done with a high degree of rigor and the conclusions are based, often, on the overlap of different methods. It is possible that a physicist or a mathematician could find some nuances to argue with but from the perspective of quantitative biology, it is difficult to falter the authors.
After establishing the reproducibility of the growth variables and cell numbers they address patterning using four genes that, together, span the anteroposterior axis of the gastruloid. The measurements reveal a very high degree of precision in their distribution of gene expression from one gastruloid to another. Significantly they find that the patterns scale. In essence they find that the mechanisms that pattern gene expression in gastruloids are comparable to those operating in Drosophila, despite the large differences in underlying developmental strategies. This is important because it reveals, in a quantitative manner, the existence of a general principle of pattern formation that acts at the multicellular scale independent of boundary conditions. By doing this it lays down a foundation to look for its mechanism which is more striking in a mammalian system than in Drosophila because of the fact that, in this system, the boundary conditions are created by the growth of the system. At a practical level this work is foundational in that it establishes gastruloids as a quantitative system of developmental biology to the level of embryos.

The ms has very little to question conceptually and technically, however from the biological point of view there are two issues which the authors could address to increase the clarity and scope of the message.

An important one refers to the range of starting cells within which the reported scaling occurs. Are there upper and lower bounds where it breaks down? The original publication on gastruloids reports, albeit in a qualitative and anecdotal manner that proper gastruloid formation only occurs within a certain range. Do the authors observe the same thing? If so, could they specify the range? This would, will make the argument striking.

Starting on line 99, the sentence “Gastruloid size scaling with the initial seed number suggests that contrary to mouse embryos [25–28], there seems to be no control over an absolute target size for the system”, requires a bit of a clarification. I am not clear what they mean.

The authors may want to make a small change in Figure 3c as the order of the genes seems to be backwards from other figures since anterior should be to the left thus, all they have to do is start with FoxC3 on the left and place Bra on the right.

Alfonso Martinez Arias

Author Rebuttal to Initial comments

Detailed response to reviewer comments for NSMB-A47810
(Responses to all individual points are in green.)

Reviewer #1:
Remarks to the Author:
Review for Precise and scalable self-organization in mammalian pseudo-embryos

In this work, Merle et al., investigate the precision of gene expression boundaries in mouse gastruloids. They implement a pipeline to record the expression profiles across the gastruloid at different time points. They use the resulting quantitative data to argue that the self-organized patterns scale with gastruloid size. They use this as an argument that gene expression boundary precision and scaling may well be emergent properties of the self-organization network.

The quantitative data collected is particularly impressive. It was especially good to see the data collected across multiple different experiments and compared rigorously (Fig. S2F). The data analysis is solid (with an exception highlighted below) and the results potentially very interesting. The data presentation is clear, and the manuscript is generally well written.

We thank the reviewer for their kind words pertaining to our data and analysis, as well as about the potential impact of our manuscript.

Below, I outline suggestions to improve the manuscript.

Major Concerns:

1. My main concern lies in the framing of the paper. In the introduction, the authors state “High fidelity of the underlying processes is most crucial during the earliest stages when the body plan and the future asymmetric body axes emerge at gastrulation”. Yet, one could argue this is a Drosophila-centric view. For example, in vertebrates, different growth rates have been shown to drive scaled patterning of the neural tube, rather than precise scaled patterning (PMID 25258086). Further, cell sorting can lead to precise boundaries from initially noisy specification (PMID 33004519). Therefore, even if precision is seen early on in a gastruloid, it does not necessarily follow that this underlies the precise scaling and precision seen in vivo later in development. The vertebrate embryo may only need early patterning to be “good enough”, and then later mechanisms (e.g., differential growth rates and plasticity in cell fate) can correct for errors. The introduction should more fairly reflect such differences from invertebrate to vertebrate – i.e., it is not even clear that the question being posed is relevant and this should at least be recognized.

We agree with the reviewer and are thankful for this opportunity to improve our introduction about the noisiness underlying the gene expression, the error-correction view of development, and the difference between non vertebrate and vertebrate embryos. We have modified two formerly lengthy introductory paragraphs into six more succinct ones, including multiple new references with the reviewer’s suggestions among them. We now clearly raise the question of whether the precision at the single-cell level observed in flies also exists in mammalian pseudo embryos. We have also added a paragraph about scaling with more references in vertebrates

and non vertebrates. In parallel, the discussion has been adapted in regards to the modification of the introduction.

2. Relatedly to (1), the Discussion needs toning down in parts. For example, the claim “Our findings point to underlying principles that govern the self-organization processes in multicellular systems, reaching across half a billion years of evolutionary change” needs more qualification. The authors may be correct, but the evidence presented here – from one species, at one specific stage of development – hardly backs up this statement.

We agree with the reviewer's assessment regarding our language and have added qualifications at several places in the manuscript to tone down our statements.

3. Though not essential to do, it seems a missed opportunity that the results aren’t mapped onto a model of the gene regulatory network. These interactions have been explored intensively (e.g., PMID 37209681), with modeling papers exploring the network interactions (e.g., PMID 31613879). How well do the GRN models map onto the quantitative data collected here?

We thank the reviewer for this suggestion! Of course, we have been trying modeling efforts in the fly system with the much better established segmentation gene network. Even there it is a challenging task on which we’re still working after 20 years of experiment and theory interaction. We agree that the properties we observed, especially the scaling, imposes stringent constraints on any gene expression patterning models of the gastruloid self-organization process involving information acquisition during the symmetry breaking process. This is in fact part of an ongoing project with our theory collaborators and it turns out the task at hand is indeed very complex. We therefore believe that this project deserves its own publication and goes beyond the scope of the current manuscript.

4. The discussion of scaling is very limited in the introduction. A single paper is cited – to a five-year-old Arxiv paper. There has been a lot of work in this area, e.g., PMIDs: 21613328, 21873045, 32048988, 32209485, 32251432, 34937053. Given the central importance of scaling to the authors conclusions, a more balanced overview of the current understanding of gene expression boundary scaling is suitable.

We agree with the reviewer and have added a new paragraph in our revised introduction including a number of additional references: (PMIDs 29769221, 27093082, 30061678, 32251432, 21613328, 21873045, 32048988, 32209485, 34937053, 31862792, 32829684).

5. Related to the above point, r.e., scaling, the manuscript does not clearly define what “close to perfect scaling” means. This (and similar) phrasing is used multiple times. Yet, somewhat surprisingly, no quantitative measure is shown to back up this statement, beyond the linear relationships shown in, for example, Fig. 1D. In the scaling literature, there are a number of different approaches used to test claims of scaling and put a limit on its accuracy. At the moment, bold statements are made regarding the scaling, but a rigorous, unbiased test of such claims is currently lacking.

We apologize for the lack of precision in our quantification of scaling. We now give a definition of perfect scaling in the main text and a quantitative deviation from perfect scaling when

necessary. We define clear bounds on perfect scaling based on initial seeding number count errors and doubling time uncertainty. In addition, we present the results for each timepoint in the SI (updated Fig. S2G), illustrating how we estimated that in a certain range of N0 scaling is achieved.

“Perfect scaling, in this context, denotes a linear relationship between N(t) and N0. When we represent these values with respect to the reference seeding number N0=300 (Fig. 1D), perfect scaling is achieved by a slope=1 (black line). It signifies that starting with twice as many cells (in units of N0=300) results in precisely twice as many cells at any given time point.”

“We utilized the measured errors associated with both the initial seeding number and the doubling time to estimate the expected error at 120 hours.”

“For the case N0=300 (Fig.~1D, inset), the slope is statistically indistinguishable from 1 at all time points.”

Related to this, the scaling data in Fig. 4 is intriguing, but needs more careful analysis. For example, the profile of FOXC1 shows clear differences at larger gastruloids. Likewise, BRA appears to change the steepness of its slope in larger gastruloids. While close to scaling, there do seem to be differences that emerge, which distinguish this from, say, the early fly embryo. Currently, there is insufficient evidence that “average profiles of all sizes collapse”.

To address this important point, we provide extensive additional evidence for our scaling relationships, including new figure panels in Fig. 4, a modified Fig. S10 and a new Fig. S11.

We added subsets of individual profiles for each gene and each N0 (Fig.S10 C-D) to show that scaling goes beyond the level of average profiles and is verified at the level of individual profiles. This is further documented in Fig. 4C, where we show for each studied gene, the absolute distance of the pattern boundary from the anterior pole as a function of the total length of each gastruloid. We assess scaling in these data by a linear fit and inspecting R2. Perfect scaling would imply R2=1; for the genes SOX2, CDX2, and BRA, the scaling relationship with respect to gastruloid length explains 96–97% of the boundary position variance. We also analyzed two additional positions of the boundary for each gene: the 25% and 75% levels of maximum gene expression in individual gastruloids in Fig. S11A-B.

We further analyzed the relative positions of the 25%, 50% and 75% maximum intensity levels as a function of L for each gastruloid. Perfect scaling predicts a statistical independence of the relative boundary positions from absolute gastruloid length. We determined a slight deviation from independence (Fig. S11C). For three genes (Sox2, Cdx2 and FoxC1) we observe that a decrease or an increase of 300 μm around the case of N0 = 300 cells leads to a shift of the positional marker of ∼1% along the AP midline, i.e. ∼6 μm (i.e. a small fraction of the cell diameter). For the gene BRA we observe that a decrease or an increase of 300 μm around the N0=300 cells case leads to a more significant shift of the positional marker of ∼10% along the AP midline, i.e. ∼60 μm or roughly four cell diameters.

Finally, we assessed the positional error for the three boundary markers (25%, 50%, 75%) for the rescaled pattern boundaries (Fig. S11D). When converted to cell diameter units, the positional error remains between 1–2 cell diameters for all genes and all markers within a certain length range (up to 600 μm for FOXC1, up to 800 μm for the other genes). This range

corresponds to the mean length of gastruloids in a range $100 \leq N0 \leq 500$ for each experiment (Fig. S10B).

6. In Fig. 2, somewhat surprisingly the relative error appears to remain small in regions of low expression (e.g., Sox2 in region x/L = 0.1). This is not what would be expected, as the intensity gets lower, the relative error typically increases. This should be clarified – is the associated error also so low that the relative error stays small? This seems surprising, especially given the authors admit there are still experimental errors in the system so we should take their error estimates as upper bounds.

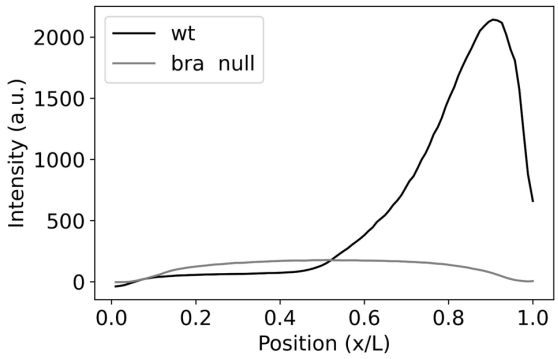
We thank the reviewer again for raising another very important point and for the opportunity to address it here. The region x/L=0.1 for Sox2 is a region where Sox2 is not expressed. Yet, the mean value of intensity in this region is non-zero as there is a fluorescent background due to non-specific interactions of the antibodies and gastruloid auto-fluorescence. Measuring this background value is non-trivial, and thus correct for it quite challenging. Here we argue that in the regions that matter to our analysis and scientific assessments, the corresponding error-correction due to this background level of fluorescence is very low and has almost no effect, which is why we omit it in our analyses.

To evaluate the fluorescence background and the impact of the corrections on our measure of variability, we performed an additional control experiment where we dual-labeled gastruloids for SOX2 and CDX2 in two batches, with and without the primary antibodies. We present the results in a new supplementary figure S4. We show that the background intensity is low (Fig. S4A-C) and that correcting the mean profile by subtracting this background intensity profile has almost no impact on the variability of the profiles as soon as the profile intensity exceeds 5% of the maximal expression levels (Fig. S4D). We therefore choose to work directly with the raw profiles, knowing that we underestimate the standard deviation in the region of lowest gene expression.

Working with the raw profiles is not impacting any of our major results: precision is only a function of the intensity gradient of the mean profile and standard deviation around this mean profile but not the absolute value; for scaling, all our profiles are normalized between 0 and 1. We believe that our ability to measure gene expression profiles will continue to improve in the next few years, and with henceforth improved measurements of gene expression we will be able to go deeper into the question of reproducibility.

Finally, to also account for non-specificities of the primary antibody, we compared the wild-type BRA expression profile side-by-side with the fluorescence levels obtained during the same procedure from gastruloids generated from a BRA-null mutant mESC line that is still unpublished, which is why we only show these results here (see figure below).

The fluorescence in the null mutant should account for the non-specificity of both the primary and the secondary antibody, as well as the overall measurement noise. However, the BRA-null cell line represents a different clone than our regular cell line with which we performed all our gastruloid measurements. Thus, gastruloids from these two cell lines have different average morphology (gastruloids made with Bra null mutant don't elongate so much) and different auto-fluorescence backgrounds. We observe a similar background in these mutant gastruloids than when we omit primary antibodies in the staining protocol (Fig. S4).



7. In the measurements of cell size, was potential extracellular space accounted for? It should be straightforward to quantify how much extracellular space there is during gastruloid formation. It may be that the cells are all tightly packed throughout, but this should be confirmed.

Again, we are thankful to be able to address this very pertinent point. Gastruloid cells are densely packed, making 3D segmentation challenging (Fig. S7A). Importantly, our cell size measurements with the 3D segmentation method are independent of potential extracellular space: we extract cell size from the size distribution of segmented cells only. We estimate the extracellular space inside gastruloids to represent on average less than 1 % (0.7%) of the total gastruloids volume. While we observe lumen-like structures in some gastruloids, their size is negligible compared to the overall size of the gastruloid.

In contrast, our gastruloid dissociation method cannot recover any value for extracellular space as we use the measured total gastruloid volume (estimated from reconstructed 2D image stacks) to calculate the average effective cell size (Fig. S1E). There is a significant difference between our estimated volume measures for the 2D and the 3D methods (Fig. S7B), and we account for that difference in the error estimation for the 2D reconstructed volumes.

We have added a paragraph at the end of Methods Section L detailing this account.

Other points:

1. The four chosen targets are given with little explanation. A brief summary of the four genes and why they are selected would be helpful (e.g., mesoderm-specific)

These genes, involved in the antero-posterior patterning during gastrulation, were chosen for their role in the establishment of the body plan and the cell fate determination, in which the Wnt signaling pathway (activated in gastruloid protocol by adding Chiron at 48h) plays a critical role (Neijts et al, 2014). Cdx2 (endoderm marker) and Bra (mesoderm marker) co-activate genes involved in the Wnt signaling pathways and are both essential for posterior axis elongation (Amin et al, 2016). Sox2 (neuroectoderm marker) controls the response of cells to Wnt signaling by regulating the chromatin occupancy of WNT mediator, depending on its level of expression (Blassberg et al, 2022). FoxC1 (mesoderm marker) is a key early mesodermal regulator (Mittnenzweig et al, 2021). We have added this information (incl. references) in the main text when these genes are first introduced in the results section “Reproducible gene expression patterning.”

2. The % of successful gastruloids (and total number) should be clearly in the main text, not in the legend of Fig. S1.

Following the reviewers suggestion we have added a sentence in the first paragraph of results pertaining to that effect: “Within the confines of the original protocol (Beccari et al), we first achieved a 97% success rate in inducing the elongation of gastruloids along a single axis (Fig. S1).”

3. Looking at Fig. S2A, the error is typically 20% variation. I agree with the authors that the observed scaling is quite remarkable, yet this is also clearly significantly larger than observations in Drosophila – where sample-sample variation is more on the order of 10%. It is confusing that the gastruloids are claimed to have similar accuracy to Drosophila, when the data seems to suggest the error is consistently larger. Relatedly, the results of Fig. 3 are very interesting – but the error is also clearly larger than what is seen in the gap genes. So, the conclusion that it is comparable seems too bold at this point.

We agree with the reviewer’s comment and apologize for the confusion concerning the results in Fig.3. What we meant to be similar is that the precision is close to one cell diameter, which is essentially what we found in Drosophila. However, we fully agree that our text was confusing, and we have corrected this in the revised version of the manuscript:

“This finding demonstrates that mammalian gastruloids exhibit patterning precision on par with patterning systems in fly embryos [Gregor2007], worms [Moore2013], and ascidians [Guignard2020]. In all these systems, the positional error resulting from gene expression fluctuations allows for distinguishing neighboring cells.”

Fig.S2A presents the results on the variability of gastruloid’s *volume* over time for the case N0=300, which is typically around 20% indeed. In Drosophila, egg length variabilities of up to ~7% is observed (PMID 18423206), which is on the same order of magnitude than what we observe in gastruloids for midline *length* variation (Fig. 1A). Moreover, we showed that variability in volume comes from both variability in effective doubling time and initial number of seeded cells (Fig.S2B-C), whereas in a Drosophila embryo both the number of nuclei (there are no cells yet) and the doubling times are under very stringent control.

4. As stated at the start, I am pleased that the authors have openly shown the experiment-to-experiment variability (e.g., Fig. S8). This gives me a lot more confidence in their results. I do note that in late 2022 (Fig. S2F), there is a large increase in the gastruloid size. Given that this seemed to repeat itself over a number of experiments, was a cause identified that distinguished this batch of experiments from the other ones? This may be of broader interest to know what experimental variations can affect gastruloid size.

In Fig. S2F, each point on the graph corresponds to the mean gastruloid size within a single plate (96 wells), some of them started in culture the same day (e.g., 1 to 3 plates seeded simultaneously in the graph), so that the same experimental variation on a single day can impact several points on the graph at once. Downward triangles are experiments seeded by multi-pipetting; upward triangles are experiments seeded using FACS. When manually seeding gastruloids, cell concentrations are measured using an automatic cell counter, which sometimes leads to shifts in the average number of cells seeded per well.

Various factors can influence the development of mouse gastruloids. We observed that the initial pluripotency state of the embryonic stem cells (also recently reported in PMID 37815089) and the quality of the N2B27 media used in the protocol are particularly important for good reproducibility. On the specific date mentioned (i.e., late 2022), we also experienced a small modification in gastruloid behavior due to the particular batches of N2 and B27 used to prepare our in-house N2B27 at that time.

5. What does “quasi-perfect” mean? This seems ill-defined currently.

We agree with the reviewer that quasi-perfect is ill-defined and have therefore removed any use of that term from the manuscript. Instead, we provide a clear definition for “perfect scaling” (see major point 5 above) and how we assessed it quantitatively in our data.

Reviewer #2:
Remarks to the Author:
This is an important piece of work that reveals a very important attribute of multicellular structures: their ability to produce reproducible patterns independently of boundary conditions, that scale. It is a technically exceptional and intellectually sound piece of work that provides a sound foundation for a new and important new experimental system.

There are important features of biological systems that are at the root of why we have used and learnt from model systems over the years. One example is the robustness and reproducibility of their patterns and organization, particularly during development, that allow the identification of deviations, for example through mutations, that have led to much understanding of underlying molecular mechanisms. Most of these studies are qualitative but over the last twenty years, quantitative analysis has revealed surprising new layers in terms of precision in molecular and cellular activities, that pose new questions about the way biological systems develop.

A good example of this can be found in work on the Drosophila embryo where studies over the last 30 years have shown that the pattern of the larva is laid down before gastrulation in the form of interactions between the Bicoid gradient and a cascade of downstream genes coding for transcription factors. Quantitative analysis of this process, led by the group of Thomas Gregor, has shown that these events are extremely precise with spatial registrations happening to the level of one cell diameter. Furthermore, the patterning process scales i.e. the relative proportions of the patterns of gene expression remain constant regardless of the total size of the embryo. This is remarkable but could be argued that the basis is ‘simple’ since the Drosophila embryo develops within a rigid case that can provide a ruler for this precision, constraining the range and activity of the transcription factors.

It is not clear that this precision exists in vertebrates or mammals which develop in different ways. In particular their embryos create their boundary conditions as they grow. One of the reasons for our ignorance lies in the experimental difficulty, particularly in mammals which develop inside the mother and produce few embryos. Mammals are important, not only because we are mammals and therefore their development is our development but also because their embryos represent an extreme form of self organization.

Over the last ten years models based on embryonic stem cells have created experimental opportunities that can be used to address fundamental experimental questions and it is this that is being addressed in this manuscript. The authors make use of gastruloids, models of early mammalian development based on pluripotent stem cells (PSCs). When aggregates of defined numbers of cells are placed under controlled culture conditions, they

recapitulate the main events of gastrulation. After 5 days the result is an elongated structure that displays a range of tissue and organ primordia arranged with regard to three orthogonal axes mimicking the embryo. In the case of mouse PSCs, the structures resemble embryos about mid gestation. Gastruloids can be produced in large numbers and are being used to explore mechanisms and interactions of early mammalian development. Here, Merle et al use them to ascertain precision in patterning of gene expression along their anteroposterior axis.

In this manuscript Merle et al show that by controlling experimental conditions they can make gastruloids with nearly 100% frequency which allows them to make large numbers of independent measurements with confidence. They find a remarkable degree of precision in the dimensions, growth rate and cell numbers of gastruloids, with errors that overlap measurement errors. The work is done with a high degree of rigor and the conclusions are based, often, on the overlap of different methods. It is possible that a physicist or a mathematician could find some nuances to argue with but from the perspective of quantitative biology, it is difficult to falter the authors.

After establishing the reproducibility of the growth variables and cell numbers they address patterning using four genes that, together, span the anteroposterior axis of the gastruloid. The measurements reveal a very high degree of precision in their distribution of gene expression from one gastruloid to another. Significantly they find that the patterns scale. In essence they find that the mechanisms that pattern gene expression in gastruloids are comparable to those operating in Drosophila, despite the large differences in underlying developmental strategies. This is important because it reveals, in a quantitative manner, the existence of a general principle of pattern formation that acts at the multicellular scale independent of boundary conditions. By doing this it lays down a foundation to look for its mechanism which is more striking in a mammalian system than in Drosophila because of the fact that, in this system, the boundary conditions are created by the growth of the system. At a practical level this work is foundational in that it establishes gastruloids as a quantitative system of developmental biology to the level of embryos.

The ms has very little to question conceptually and technically, however from the biological point of view there are two issues which the authors could address to increase the clarity and scope of the message.

We thank Dr. Martinez-Arias for their kind and detailed evaluation of our manuscript.

An important one refers to the range of starting cells within which the reported scaling occurs. Are there upper and lower bounds where it breaks down? The original publication on gastruloids reports, albeit in a qualitative and anecdotal manner, that proper gastruloid formation only occurs within a certain range. Do the authors observe the same thing? If so, could they specify the range? This would, will make the argument striking.

Many thanks for raising this pertinent point; it gives us indeed the opportunity to discuss the important limits of our findings. It turns out that there is something special about the seeding range for N0 between 100 and 600 cells. It is within this range that our results for elongation, precision, reproducibility, and scaling hold best. When we lower or increase N0 beyond this range we observe quantitative deviations from our reported results in the 100 to 600 range.

We also observed that the range 100 to 600 of initial seed cells corresponds to the range for which growth scales at all timepoints up to 120h (we added detailed scaling curves in Fig. S2 and commented in this sense in the main text). The data presented in Figure 1 only contains

gastruloids that formed a single pole of elongation as it is required for our morphological analysis (length and volume).

Finally, a similar range applies to the scaling properties of gene expression patterns, which we now detail in Figures S10 and S11.

We have added a section on limitations to the discussion section of the revised manuscript.

Starting on line 99, the sentence “Gastruloid size scaling with the initial seed number suggests that contrary to mouse embryos [25–28], there seems to be no control over an absolute target size for the system”, requires a bit of a clarification. I am not clear what they mean.

In the literature it was reported that mouse embryos are able to regulate their size. For Instance, in halved embryos the onset of gastrulation is delayed until the size of the continuously growing embryo approximates the size of a full embryo at that stage (G. F. Rands, Size regulation in the mouse embryo. II). In contrast, with gastruloids we observe that the timing of elongation and gene patterning is independent of the size of the gastruloid (modulated here by changing N0). We have expanded this passage in our revised manuscript to clarify this point.

The authors may want to make a small change in Figure 3c as the order of the genes seems to be backwards from other figures since anterior should be to the left thus, all they have to do is start with FoxC3 on the left and place Bra on the right.

We apologize to the reviewer for the confusion. If we understand the reviewer correctly, he refers to the “legend” in figure 3C which is written just above the anterior-posterior axis. Here it seems confusing to have an anterior gene (FoxC1) be written at the posterior end and a posterior gene (BRA) be written at the anterior end. We have corrected the gene order in the legend of our revised Figure 3C.

Decision Letter, first revision:

Message: Our ref: NSMB-A47810A

29th Nov 2023

Dear Dr. Gregor,

Thank you for submitting your revised manuscript "Precise and scalable self-organization in mammalian pseudo-embryos" (NSMB-A47810A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, it is important that we have a copy of the main text as a word file. If you could please send along a word version of this file as soon as possible, we would greatly appreciate it; please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Carolina Perdigoto, PhD
Chief Editor
Nature Structural & Molecular Biology
orcid.org/0000-0002-5783-7106

Reviewer #1 (Remarks to the Author):

I thank the authors for the detailed response to my review. I am happy with the changes.
Timothy Saunders

Reviewer #2 (Remarks to the Author):

I am happy with the replies of the authors to the referees comments

Final Decision Letter:

Message: 8th Feb 2024

Dear Dr. Gregor,

We are now happy to accept your revised paper "Precise and scalable self-organization in mammalian pseudo-embryos" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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