Chimeric 3D gastruloids - a versatile tool for studies of mammalian peri-gastrulation development

Alexandra E. Wehmeyer, Katrin M. Schuele, Alexandra Conrad, Chiara M. Schröder, Simone Probst and Sebastian J. Arnold
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MS TITLE: Chimeric 3D-gastruloids - a versatile tool for studies of mammalian peri-gastrulation development

AUTHORS: Alexandra E Wehmeyer, Katrin M Schuele, Alexandra Conrad, Chiara M Schroeder, Simone Probst, and Sebastian J Arnold

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Wehmeyer and colleagues present an experimental adjustment to the mouse gastruloid protocol that enables them to explore the effect of cell mixing, or chimerism, on the formation and development of gastruloids. This is an idea that has been previously raised as a benefit of such in vitro approaches, but to my knowledge, is the first experimental validation of the approach. The authors use this technique to explore the role of Eomes overexpression on cardiac differentiation, and Brachyury knockout on morphological elongation and germ layer specification. In my view, this is an interesting article that presents some novel findings, but is rather preliminary in many respects, and would benefit from broad revision.

Comments for the author

Major Comments:
- The description of chimeric gastruloids, either by merging or mixing (Fig 1), could do with more detail about what the normal clonal behaviour is like, in purely wildtype populations. For instance, if gastruloids are made from 50:50 mixes, does this ratio change over time at all? What is the variability between gastruloids?
Is there any evidence of cell competition?
- Likewise, there seems to be some degree of spatial organisation in the chimeric gastruloids. How is this distribution normally found (with wildtype mixing) – in small local clones, or randomly dispersed? In general these sorts of statistically robust baseline data points would be helpful on which to build conclusions from the subsequent mutant lines.
- In Fig 2, the TRE.Eo mixed gastruloids seem to be less elongated than their wildtype counterparts. The authors should quantify and comment on these morphological differences.
- It was my understanding that the beating domain in cardiac gastruloids occurs in the anterior portion of the gastruloid (Rossi et al), yet in Fig 2C, it seems that the TRE.Eo cells are in the narrower, presumably posterior, end of the gastruloid. Is this the case across gastruloids? If so, how do the authors explain this?
- In Lines 183-187, the authors claim that the 90:10 mix gastruloids do not have Bra expression in the WT cells, while the 50:50 ones do. How is this affected by time? Since both elongate, presumably there has been BRA expression in both gastruloids at some point in the experiment, so perhaps this timepoint it just too late and the domain has become exhausted. Temporal data, and quantification, would be necessary here to support their claim to 'non-autonomous' effects.
- Additionally, the authors go on to claim that this might be described by the interaction of Wnt3a and Brachyury. This is testable, so the authors should examine the expression of Wnt3a and ideally, also what the impact is on the b-catenin/downstream wnt signalling cascade along the AP axis.
- Finally, the observation that the BRA-/- cells in mixed gastruloids contribute to either the endodermal tract or neuroectoderm (Fig 4) is an extremely interesting one. However, the authors do not mention how often one or other result is obtained. And how correlated is the cell type divergence matched by the morphology (ie an narrow tract vs a broader domain)? Finally, the authors should comment on the observation that this effect seems to be all-or-nothing: they either go completely DE or completely NE, why is this the case?

Minor Comments:
- It took me a while to determine that the authors were discussing chimeric mouse gastruloids, and not human ones. Indeed, this point is not currently raised in the title, abstract or introduction and indeed, was only apparent from reading the materials and methods section. The authors should state clearly, and early that they are describing a murine model.
- Fig 2D, individual data points should be shown, and ideally also coloured by biological replicate, so that the underlying variability in the data can be assessed.
- Likewise, was the '% beating gastruloids' metric determined using automation or manually? Was this quantification blinded? I can see no description of this in their methods.
- The authors should clarify in the Results whether their lines are homozygous/heterozygous
- Line 121: "normally only difficult to achieve" could be rephrased
- Line 165-6: State exact mean values rather than 'more than 30%' and 'only very rarely'

Reviewer 2

Advance summary and potential significance to field

This manuscript illustrates an experimental approach combining the 3D-gastruloid model system with genetically modified ESCs and fluorescent imaging approachs.
More specifically, it focuses on the generation of chimeric gastruloids composed of cells with different genetic backgrounds that can be readily traced by microscopic observation using fluorescent membrane labels. As a proof of principle, they mix gain-of-function and loss-of-function cells with WT cells to trace their ability to contribute to specific cell types which emerge during gastruloid development. The authors thus claim that the system allows researchers to generate complex genetic settings that are difficult to study by genetics in embryos: cell-autonomous and cell non-autonomous gene-functions reporter-containing cells for signalling pathway or protein-localization, to name a few. All in all, the system is described as a powerful tool to study gastrulation stage embryogenesis with increased experimental scalability observability and accessibility.

Comments for the author

Major Comments
1. The paper is very well written and experiments have been explained adequately.
However my major criticism of the work stems from the fact that as a technique use of chimaeras is not a new concept and the paper gives the contrary impression to the uninitiated. There are two aspects to this criticism Use of chimaeras has been an established approach in developmental biology. In this regard the authors should reference the landmark papers by Halpern et al (1993) and Wilson et al (1993, 1995) where chimeric zebrafish and mouse embryos were used to study Brachyury function. It is therefore important to put their current findings in the context of the classic experiments.
Even in the context of in vitro systems, mixing cells in different proportions has been tried, particularly from the perspective of imaging (He, Maynard, and Jain et al (2022)) or use of cells from different species.
It is therefore not clear that beyond the specific lines they have generated, how is the authors' approach conceptually different from such studies and thus demands a publication as a "technique and resources" paper. It is therefore very important to discuss similarities and differences of the chimeric approach in vivo and in vitro. The paper would benefit from a critical comparison of these new chimeric gastruloids with the results from the old in vivo studies.

References:
- Halpern, M. E., Ho, R. K., Walker, C. and Kimmel, C. B. (1993). Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. Cell 75, 99-111.
- V. Wilson, P. Rashbass, R.S. Beddington; Chimeric analysis of T (Brachyury) gene function. Development 1 April 1993; 117 (4): 1321–1331. doi: https://doi.org/10.1242/dev.117.4.1321
- Wilson, V., Manson, L. Skarnes, W. C. and Beddington, R. S. P. (1995). The T gene is necessary for normal mesodermal morphogenetic cell movements during gastrulation. Development 121, 877-886.
- He, Z., Maynard, A., Jain, A. et al. Lineage recording in human cerebral organoids. Nat Methods 19, 90–99 (2022). https://doi.org/10.1038/s41592-021-01344-8

2. In the discussion, the authors mention further application of the chimeric gastruloid system. However, it is not quite clear to me why it would be desirable to have reporter cells only in a chimeric fashion in the gastruloid. It would be good if the authors could elaborate.
3. In general, keeping in mind the "technique and resources" aspect of the paper it will be better to have a small table or a paragraph highlighting challenges in generating chimeric systems and possible troubleshooting strategies. A few examples that come to mind are:
Cell lines with different membrane labels can undergo unmixing leading to potential misinterpretation.
Optimization of cell numbers is often needed to make gastruloids as different cell lines often require different numbers of starting cells (Turner et al 2017, Supp Info), so what should a new researcher be careful of?

How should one deal with differences in media composition of different cells lines, for example some lines grow well only with 2i and if they need to be mixed with a line grown in ESLif, what should be done?
Therefore a list of problems, how to spot them and troubleshooting strategies will therefore add value to the manuscript.

Minor Comments
1. Line 156: The correct abbreviation in the parenthesis should be TRE.EomesGFP
2. Figure 2D is supposed to represent 33.3% ± 3.01 (+DOX) and 1.5% ± 2.31 (-DOX) but the error bars are shifted in +DOX. Also the mean of -DOX seems to be 0% rather than 1.5% and the SEM seems too big (more like 4%). Can the authors please check the graph?
3. In Figure 3B it is impossible to distinguish the red channel from the magenta channel. Can the authors please show in addition the unmerged channels, preferably in grayscale.
4. In general adjusting the figures to color-blind friendly panel will be a good practice.

First revision

Author response to reviewers' comments

Reply to the Reviewers Comments
We thank both reviewers for their constructive criticism that significantly improved the revised version of the manuscript. The initial intention of this manuscript was to demonstrate the experimental feasibility of presented chimeric gastruloid approach. Following the reviewers' suggestions we now also included additional experimental data that further address aspects of the significance of Eomes and Brachyury embryonic functions and observed phenotypes of chimeric gastruloids. Thus, the manuscript text and abstract was adapted to cover these additional aspects as proposed by reviewers, and figures were adapted so that the manuscript now contains 4 main Figures and 4 Supplementary Figures.
Please find our response to the comments below, including referenced changes in the manuscript text and Figures indicated in bold. Additionally, we also provide a manuscript version with changes highlighted.

Reviewer 1
Wehmeyer and colleagues present an experimental adjustment to the mouse gastruloid protocol that enables them to explore the effect of cell mixing, or chimerism, on the formation and development of gastruloids. This is an idea that has been previously raised as a benefit of such in vitro approaches, but to my knowledge, is the first experimental validation of the approach. The authors use this technique to explore the role of Eomes overexpression on cardiac differentiation, and Brachyury knockout on morphological elongation and germ layer specification. In my view, this is an interesting article that presents some novel findings, but is rather preliminary in many respects, and would benefit from broad revision.

Major Comments:
-The description of chimeric gastruloids, either by merging or mixing (Fig 1), could do with more detail about what the normal clonal behaviour is like, in purely wildtype populations. For instance, if gastruloids are made from 50:50 mixes, does this ratio change over time at all? What is the variability between gastruloids? Is there any evidence of cell competition? We appreciate the reviewer pointing out to include some baseline description of cell lines used and their behaviour in chimeric gastruloids. As new Supplementary Fig. 1 we now include gastruloids generated entirely from all cell lines used in the study. To analyze normal clonal behaviour, we have also performed mixing and merging experiments using chimeras generated with mG-WT and mT-WT mESCs. As shown in the new Supplementary Figs. 2A and 4A wt cells mix extensively among each other both in merged and mixed chimeras. Mixing or merging genetically modified ESCs with wt cells leads to less mixing as described in the text in more detail (Lines 200ff, Lines 254ff). In histological analyses of WT:WT chimeras (Supplementary Fig. 4B) we did not see any indications of cell sorting, nor cell competition. In general, the variability of gastruloids is surprisingly little and results are highly reproducible as also reported in the numerous previous studies on gastruloids.

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-Likewise, there seems to be some degree of spatial organisation in the chimeric gastruloids. How is this distribution normally found (with wildtype mixing) – in small local clones, or randomly dispersed? In general, these sorts of statistically robust baseline data points would be helpful on which to build conclusions from the subsequent mutant lines.
This point relates to the first. We now included WT:WT chimeras to compare results of genetically altered : WT chimeras (Supplementary Fig. 2A; Supplementary Fig. 3A, B; Supplementary Fig. 4C, D). The observed cell distribution and cell sorting phenotypes were consistently and robustly found using genetically altered cells, but were absent in WT:WT chimeras.
-In Fig 2, the TRE.Eo mixed gastruloids seem to be less elongated than their wildtype counterparts. The authors should quantify and comment on these morphological differences.
-It was my understanding that the beating domain in cardiac gastruloids occurs in the anterior portion of the gastruloid (Rossi et al.), yet in Fig 2C, it seems that the TRE.Eo cells are in the narrower, presumably posterior, end of the gastruloid. Is this the case across gastruloids? If so, how do the authors explain this?
Indeed, the gastruloids of Fig. 2 that were generated by merging WT and TRE.Eomes cells after 24 hrs of aggregation are less elongated compared to merged gastruloids of non-Eomes-expressing cells which is now quantified in Supplementary Fig. 2C. In addition, we show BRACHYURY and CDX2 IF stainings (Fig. 2 G, H) that indicate the posterior pole of merged gastruloids showing that cardiogenic regions are forming along the side of the gastruloids. To reflect this positioning more clearly, we also exchanged the image of Fig. 2C and the corresponding Supplementary Movie 2.
-In Lines 183-187, the authors claim that the 90:10 mix gastruloids do not have Bra expression in the WT cells, while the 50:50 ones do. How is this affected by time? Since both elongate, presumably there has been BRA expression in both gastruloids at some point in the experiment, so perhaps this timepoint it just too late and the domain has become exhausted. Temporal data, and quantification, would be necessary here to support their claim to 'non-autonomous' effects.
Many thanks for pointing out this inconsistency in our explanation. To address temporal aspects of reduced Brachyury expression we now included comparative antiBRACHYURY Immunofluorescent analysis of 80:20 and 50:50 mixed gastruloids at 72, 96, and 120 h showing the gradual loss and mislocalisation of BRACHYURY over time (Fig. 3C, D).
-Additionally, the authors go on to claim that this might be described by the interaction of Wnt3a and Brachyury. This is testable, so the authors should examine the expression of Wnt3a and ideally, also what the impact is on the b-catenin/downstream wnt signalling cascade along the AP axis.
This highly relevant aspect is now experimentally addressed using in situ hybridization analysis for Wnt3a and the putative Brachyury-regulated Wnt co-ligand Rspo3 at 72, 96, and 120 h (Fig. 3E). Comparative WISH analysis in WT, Bra-/-, and in 80:20 and 50:50 mixed Bra-/-
:WT gastruloids shows that expression of Wnt3a, that is normally confined to the posterior pole of gastruloids, is entirely absent in Bra-deficient gastruloids. Also 80:20
3
and 50:50 mixed Bra-/-
:WT gastruloids are mostly devoid of Wnt3a expression at 120 h.
Instead, we find some Wnt3a expression at intermediate levels of mixed gastruloids, most likely in WT cells that are displaced from the most posterior region by Bra-/-. This aspect is further discussed (Lines 233ff). Similarly, Rspo3 is prematurely lost in mixed gastruloids as an indication of "exhausted" Brachyury expression (Fig. 3E).
-Finally, the observation that the BRA-/- cells in mixed gastruloids contribute to either the endodermal tract or neuroectoderm (Fig 4) is an extremely interesting one. However, the authors do not mention how often one or other result is obtained. And how correlated is the cell type divergence matched by the morphology (ie an narrow tract vs a broader domain)?
Finally, the authors should comment on the observation that this effect seems to be all-ornothing: they either go completely DE or completely NE, why is this the case?
We apologize for the obviously misleading description of the observation that Bra-/- cells contribute predominantly to SOX2+ regions and the DE of the primary gut tube in mixed gastruloids. This cell sorting is not exclusive but can be seen within the same gastruloids.
We now used antibody co-staining against FOXA2 and SOX2 in mixed Bra-/-
:WTgastruloids at 120hrs showing that Bra-/- cells contribute to both tissues in the same gastruloids (Fig. 4G). We now included numbers of how often shown results were observed in the Figure legend of Fig. 4. As mention above, the experimental outcome using gastruloids is highly reproducible and consistently generates similar appearing gastruloids.

Minor Comments:
-It took me a while to determine that the authors were discussing chimeric mouse gastruloids, and not human ones. Indeed, this point is not currently raised in the title, abstract or introduction and indeed, was only apparent from reading the materials and methods section.
The authors should state clearly, and early, that they are describing a murine model.
Many thanks for pointing out this oversight. We now included the mentioning of the species mouse in the abstract and changed ESCs to mESC throughout the manuscript where appropriate to indicate the use of mouse cells more clearly.
-Fig 2D, individual data points should be shown, and ideally also coloured by biological replicate, so that the underlying variability in the data can be assessed.
Many thanks, we now changed the graph accordingly (Fig. 2D).
-Likewise, was the '% beating gastruloids' metric determined using automation or manually? Was this quantification blinded? I can see no description of this in their methods.
We now included a sentence in the methods section to state that the evaluation of beating cardiomyocytes was performed my "manual" observation in a non-blinded fashion.
Due to the unambiguous outcome of this experiment we assume that a non-blinded counting of beating vs. non-beating gastruloids is justified.
-The authors should clarify in the Results whether their lines are homozygous/heterozygous
4
We now included a more specific description of used cell lines and their allelic state in the Results and methods section.
-Line 121: "normally only difficult to achieve" could be rephrased
We rephrased this sentence to point out more clearly the meaning of this sentence (Lines 141ff).
-Line 165-6: State exact mean values rather than 'more than 30%' and 'only very rarely' We entered precise numbers in the text (Lines 190ff).

Reviewer 2
This manuscript illustrates an experimental approach combining the 3D-gastruloid model system with genetically modified ESCs and fluorescent imaging approaches. More specifically, it focuses on the generation of chimeric gastruloids composed of cells with different genetic backgrounds that can be readily traced by microscopic observation using fluorescent membrane labels. As a proof of principle, they mix gain-of-function and loss-of-function cells with WT cells to trace their ability to contribute to specific cell types which emerge during gastruloid development. The authors thus claim that the system allows researchers to generate complex genetic settings that are difficult to study by genetics in embryos: cellautonomous and cell non-autonomous gene-functions, reporter-containing cells for signalling pathway or protein-localization, to name a few. All in all, the system is described as a powerful tool to study gastrulation stage embryogenesis with increased experimental scalability, observability and accessibility.

Reviewer 2 Comments for the author

Major Comments
1. The paper is very well written and experiments have been explained adequately. However, my major criticism of the work stems from the fact that as a technique, use of chimaeras is not a new concept and the paper gives the contrary impression to the uninitiated. There are two aspects to this criticism Use of chimaeras has been an established approach in developmental biology. In this regard the authors should reference the landmark papers by Halpern et al (1993) and Wilson et al (1993, 1995) where chimeric zebrafish and mouse embryos were used to study Brachyury function. It is therefore important to put their current findings in the context of the classic experiments. Even in the context of in vitro systems, mixing cells in different proportions has been tried, particularly from the perspective of imaging (He, Maynard, and Jain, et al (2022)) or use of cells from different species. It is therefore not clear that beyond the specific lines they have generated, how is the authors' approach conceptually different from such studies and thus demands a publication as a "technique and resources" paper. It is therefore very important to discuss similarities and differences of the chimeric approach in vivo and in vitro. The paper would benefit from a critical comparison of these new chimeric gastruloids with the results from the old in vivo studies.

References:
5
- Halpern, M. E., Ho, R. K., Walker, C. and Kimmel, C. B. (1993). Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. Cell 75, 99-111.
- V. Wilson, P. Rashbass, R.S. Beddington; Chimeric analysis of T (Brachyury) gene function. Development 1 April 1993; 117 (4): 1321–1331. doi: https://doi.org/10.1242/dev.117.4.1321
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- He, Z., Maynard, A., Jain, A. et al. Lineage recording in human cerebral organoids. Nat Methods 19, 90–99 (2022). https://doi.org/10.1038/s41592-021-01344-8.
We thank the reviewer for this valid criticism and rephrased and expanded the introductory paragraph (Line 120ff) to reflect that the use of chimeric analyses is by no means a novel approach (as we also used Eomes-chimeras previously, Arnold et al. 2008). We now included the missing references of previous studies on Brachyury-deficient cells and discuss the current approach in the light of these “classical experiment” (Line 286ff).
2. In the discussion, the authors mention further application of the chimeric gastruloid system. However, it is not quite clear to me why it would be desirable to have reporter cells only in a chimeric fashion in the gastruloid. It would be good if the authors could elaborate.
We rephrased and extended the discussion to include a discussion of previous chimera experiments (mostly performed by Val Wilson). Thus, we now omitted the mentioned aspect of the discussion since it didn’t add to the current study and would require more lengthy explanations. In brief, reporter cells in chimeric situations would allow to closely study cell behaviour, e.g. of mutant vs. wildtype cells. Often general reporter staining obliques observability, for example when membrane/actin labels are used to follow single cells. Microscopy is complicated in fully labelled cellular environments but can be more easily performed in otherwise unlabelled cellular environments.
3. In general, keeping in mind the “technique and resources” aspect of the paper, it will be better to have a small table or a paragraph highlighting challenges in generating chimeric systems and possible troubleshooting strategies. A few examples that come to mind are: Cell lines with different membrane labels can undergo unmixing leading to potential misinterpretation. Optimization of cell numbers is often needed to make gastruloids as different cell lines often require different numbers of starting cells (Turner et al 2017, Supp Info), so what should a new researcher be careful of? How should one deal with differences in media composition of different cells lines, for example some lines grow well only with 2i and if they need to be mixed with a line grown in ESLif, what should be done? Therefore, a list of problems, how to spot them and troubleshooting strategies will therefore add value to the manuscript.
We very much appreciate this suggestion. We now experimentally addressed some of mentioned challenges, such as potential impact of different membrane labels on cell behaviour in chimeric gastruloids (Supplementary Fig. 1) and included additional controls by generating WT:WT chimeras (Supplementary Fig. 2A, Supplementary Fig. 4A).
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All mESC lines used in this study were derived from the same parental clone of A2lox, E14- based mESCs and thus were always treated with same ESC culture conditions as described. In our experiments, we could not discern obvious patterns of different cell behaviour between WT:WT chimeras. Thus, at current state we cannot give additional advice on the challenges and/or pitfalls and the troubleshooting that come with it, since our mESCs came from a “homogenous source” not involving different culture conditions or similar. So, we couldn’t address these challenges further to our internal experimental controls, and beyond the previously reported challenges and their solutions, that are mentioned by the Reviewer (Turner et al. 2017). However, we added a section addressing these potential pitfalls (Lines 279ff).

Minor Comments
1. Line 156: The correct abbreviation in the parenthesis should be TRE.EomesGFP
We rephrased the sentence to indicate more clearly the genotype of the resulting cells which are TRE.EomesGFP and contain a membrane-label for Tomato (mT) (Line 151ff).

2. Figure 2D is supposed to represent 33.3% ± 3.01 (+DOX) and 1.5% ± 2.31 (-DOX) but the error bars are shifted in +DOX. Also, the mean of -DOX seems to be 0% rather than 1.5% and the SEM seems too big (more like 4%). Can the authors please check the graph?

The graph was corrected (Fig. 2D) Many thanks for the comment (as also spotted by Reviewer 1).
3. In Figure 3B it is impossible to distinguish the red channel from the magenta channel. Can the authors please show in addition the unmerged channels, preferably in grayscale.
Resulting from additional experiments this part of Figure 3 is now presented as Supplementary Fig. 3A, B. and now shows unmerged channels to indicate absence of BRACHYURY staining in Bra-/- cells.
4. In general adjusting the figures to color-blind friendly panel will be a good practice.
We appreciate this comment on an important issue. May we suggest to keep the figures red and green in accordance to the membrane labels we used for the main version of the manuscript. However, we would like to suggest to the editors that we provide for each figure, or as a combined PDF a colour-blind friendly version of the figures/the manuscript. This could be generated using software tools, such as https://forhue.herokuapp.com and could be provided as supplementary material with the online version of the paper

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Second decision letter

MS ID#: DEVELOP/2022/200812

MS TITLE: Chimeric 3D-gastruloids - a versatile tool for studies of mammalian peri-gastrulation development

AUTHORS: Alexandra E Wehmeyer, Katrin M Schuele, Alexandra Conrad, Chiara M Schroeder, Simone Probst, and Sebastian J Arnold
ARTICLE TYPE: Techniques and Resources Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all the concerns/points raised in the previous revision round.

Comments for the author

None