Endogenous CRISPR/Cas9 arrays for scalable whole organism lineage tracing.
James Cotterell, Marta Vila Cejudo, Laura Batlle Morera and James Sharpe
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First decision letter

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MS TITLE: Endogenous CRISPR/Cas9 arrays for scalable whole organism lineage tracing.

AUTHORS: James Cotterell, Marta Vila Cejudo, Laura Batlle Morera, and James Sharpe

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 revision of your manuscript. Both referees have concerns about the methods for lineage reconstruction and the robustness of the resulting dendrograms. Although I realise that computational methods for reconstruction of lineage trees is not the main focus of your study, being able to reconstruct accurate trees is obviously important and I think you will want to address the issues the referees raise.

Referee 2 also has several concerns about the strength of the evidence to support some of the conclusions and requests more information about some of the experiments. The concerns about the validity of the sequence alignment of the edited alleles is important to address. I would also ask that you provide more details of the mouse lineage experiments and develop the explanation for how to use the two alleles to build two lineage dendograms. The referee is also unsure how useful the technique will really be in non-model organisms. Extending your discussion of how your approach could address open questions in pre-implantation human embryos and/or organoids derived from human tissue might be helpful.

If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. 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.
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**

Recently, several groups have used CRISPR-Cas9 to generate mutations throughout the development of an organism and used them as lineage markers. One approach is to introduce a CRISPR-Cas9 "recorder" via transgenesis, which consists of several CRISPR-Cas9 targets. With the recorder in place, gRNAs and the Cas9 need to be added so mutations can begin to accumulate.

Cotterel et al. present a clever alternative approach: instead of introducing the CRISPR targets via transgenesis, they identify endogenous genome sequences that can serve as CRISPR targets. The main advantage of this approach is that it would avoid the generation of transgenic animals, which could be especially useful in non-model organisms where transgenesis has not been established. For the identification of endogenous arrays they set-up a bioinformatic pipeline which they used to analyse the mouse, zebrafish and human genomes. Then, they set a series of filters to ensure the quality of the arrays and to reduce potential off-targeting. They validate in vitro and/or in vivo the use of an endogenous array en each species. This manuscript has an important improvement compared to the one previously uploaded to bioRxiv, as now the authors include a filter that identifies arrays with Single Nucleotide Polymorphisms (SNPs). This can solve the problem of potentially sequencing two different alleles of a single cell.

Although I have some concerns, especially with their method of lineage reconstruction, I think the work presented by Cotterel et al. is an important addition to this growing field.

**Comments for the author**

My major concern with the manuscript presented by Cotterel et al is their chosen method for lineage reconstruction (see comments 1-3). Although the search for the best reconstruction algorithm is research under progress and this is allegedly not the major point of the manuscript, their in house program seems to have important flaws that need to be corrected.

**Major comments:**

1. Their algorithm uses as first step the unmutated array and searches for alleles that are 1 mutation away. These are used for the second round and so on. As the authors explicitly mention, this means that the internal nodes (just as terminal nodes) in the tree are sequenced reads. The idea of a reconstructed cell lineage is to infer the topology of the tree creating internal nodes that represent ancestral cells that by definition no longer exist when the cells are sequenced. Having a cell lineage where cells sampled at the same time are both internal and terminal nodes cannot be correct. The authors should use an alternative reconstruction method.

2. I dont think their “catch” method, even if the problem with the internal nodes is solved, would work well with saturated arrays. Having saturated arrays means that most of them will be at a similar distance from the unmutated array. Also their approach doesn't take into account the possibility of having homoplasy (different arrays acquiring same mutation in same target independently).

3 In Suppl Fig 7, if I understand correctly the tree in e) has been built with the same array shown separately in both dendrograms in g), but in e) the tree shows the arrays coming from both genomic alleles. If the lineage reconstruction is robust enough, we shouldn't see clones that are made of both alleles as each has an independent mutational history. In the figure, it seems like some clones in e) are made of a mix of both allelic versions of the array. Is this the case? In any case, for easy comparison of the figures e-g and h-j, the authors should add an array index at the right side o the figures.
4 In second to last paragraph of the results the authors say they should they can consistently generate "deep dendrograms" referring to Suppl Fig 5. I do not agree with the authors on this, as most dendrograms shown have a tree depth of 3. Also, in the same paragraph the authors say they explore the consistency of their approach by comparing the dendrograms of 9 Zebrafish embryos. Although I think this result is important as they show they can consistently generate mutations in the chosen endogenous array, I don’t think it is possible to judge the adequacy of the lineage reconstruction by only visually inspecting the topology of the tree.

Minor comments:

5 Can the authors elaborate where their low number of inter-target deletions reside (as compared with GESTALT)? Have they done anything special to avoid them or their low occurrence is only explained by low editing frequency?

6 In the first paragraph of the Discussion, the authors mention that one advantage of their approach is that it does not require single-cell sequencing. This is the case, as they demonstrate, when sequencing one array only. I think one great advantage of their approach is the possibility of multiplexing by targeting more than one endogenous array. This would mean, however, that some sort of single cell indexing or single cell sequencing would be necessary. I think it would be useful if the authors discuss how they envision multiplexing their arrays.

7 In the 4th paragraph of the Body the authors mention that 33 targets could be generate allele diversity to perform lineage tracing in a mouse of 12 billion cells. Even when in theory 33 targets could generate such diversity, this doesn't mean it would be possible to accurately reconstruct this massive lineage tree. Simulations of CRISPR Cas9 lineage recorders have shown that more than 100 targets would be required to reconstruct accurately a ~50K cells tree (Salvador-Martinez et al., 2019).

Additional comments/suggestions:

1 In the 6th paragraph of the results the authors say they "validated 3 suggested target arrays from each of the Zebrafish, Mouse and Human". I think this should be changed to "validated 1 target arrays in each species".

2 In the second paragraph of the discussion, it is mentioned that sequencing both alleles of a cell is likely to be rare. Can the authors elaborate on why this is the case?

3 In Fig 3, I think it would be more useful to have the editing frequencies per target instead of per nucleotide site. Also the colours of the m) could be changed as they are not easily discernible from each other.

4 I would suggest to have the intertarget deletions as a main FIG as it is one of the major points of the paper.

5 I would change 3a and 3b as in the next figures Zebrafish comes first

(signed)
Irepan Salvador-Martinez

Reviewer 2

Advance summary and potential significance to field

The manuscript submitted by Cotterell et al. represents an admirable attempt to use endogenous arrays for CRISPR lineage tracing in zebrafish and mice. Given related methods, this approach would have greatest applicability to an organism for which transgenesis is not possible. However, 1)
the authors only apply this to systems where transgenesis is already easy and widely available, 2) the manuscript lacks essential details of the experiments and data, and 3) the approach is compromised by several limitations, as described below. In summary the authors do not directly demonstrate how or why this approach will be useful for other labs, as existing methods have clear advantages and avoid the caveats inherent to this method.

Comments for the author

Below I detail my concerns with this manuscript as currently presented.

1. Statements about lineaging in mouse, are not supported by the main figures, only a single supplemental figure. Since this is the only section that attempts to demonstrate SNP discrimination of arrays, and endogenous tracing in mouse, this reviewer believes that these data need to be in a main figure. However, this section is exceedingly brief and lacks essential descriptions of the experiment and data. Did development proceed normally? How many embryos were sequenced? How many alleles are generated per embryo? How many edits per allele? How to compare these statistics to previous fish data? The text references Figure S5, which does not describe mouse data. The supplemental text “Lineaging in Mouse embryos” references Figure S6, which also does not describe mouse data. S7 appears to be the only SNP and mouse data, which is minimally described and somewhat confusing. Why do the two arrays within a single embryo contain similar edits? Many of these array edits look very small, and are scattered randomly across the array instead of being near the predicted cut sites. Perhaps they are sequencing errors instead of CRISPR edits? Yet it appears that the dendrogram still uses these low confidence mutations.

2. It’s not clear how the authors propose to use the two dendrograms generated by the SNP arrays. The authors state that “Furthermore, by using a SNP to split the reads into 2 alleles, one can build 2 dendrograms for each individual organism. These serve as internal experimental repeats which can be used to improve and validate the accuracy of the resulting dendrogram.” How do the authors propose to “improve and validate the accuracy”, without cell type information or other ways to anchor the trees together? Even with SNPs, this reviewer is struggling to understand how two dendrograms, unlikely to be sampled from the same cells, could be used together to describe the true lineage.

3. Many edits that the algorithm calls as different appear to be the same, just staggered a little. This can be seen in many of the edit plots across panels. What is the source of this? Many aligners are context dependent when deciding to open or extend a gap, which can lead to these types of errors. If this is the source of errors, it could alter relationships within the dendrogram. Are the authors certain that alignment issues are not mistakenly generating different edit outcomes from the same missing bases? The authors do not provide the parameters they used for bowtie2 alignment, nor any evidence that each array mutation is called in a context independent manner, so it is difficult to judge the confidence in alignment. How robust are these alleles to variation in the alignment parameters or algorithm?

4. “This result demonstrates that we can consistently generate deep dendrograms with a similar gross structure using the Z4 array of endogenous CRISPR/Cas9 target sites.” What led the authors to conclude that the trees have a similar gross structure? The authors only discuss the “best” tree in the text. However, Figure S5 shows very different dendrograms across multiple embryos - orders of magnitude differences in # alleles, very different dendrogram depths. No statistics or details about the nature of these CRISPR arrays, edits, alleles, trends toward local edits vs larger deletions are provided. From visual inspection, it seems that some embryos only had 100 alleles, but others had thousands. Why are they so different than the tree shown in the main figure? The authors do not provide diagrams of the alleles they find in other embryos, just the dendrograms. Depending on these data the authors should caveat their claims regarding tree depth, # alleles, and superiority of this method over others, given the variability shown in the other trees.

5. For arrays described in 1d,e,f and 2a,b,c, the authors are not clear about what set of filters were used to identify these arrays. Is this filter 1 or 2? SNP arrays only?
6. “Our method described here is the first to use only endogenous CRISPR/Cas9 sequences from the wildtype genome, thus dramatically simplifying the procedure, and perhaps more importantly opening up the field of whole organism lineage tracing to non-model species that for which it is hard to generate transgenic animals” This reviewer would not agree. This methodology is only applied to fish and mouse, where transgenesis is already widely available. The authors do not demonstrate that they opened up lineage tracing to non-model species. You would still need genome sequence, access to embryos, and ability to microinject. If a researcher had all those things the wet lab experiment may be easier, but the interpretation of the resulting dendrograms is substantially more difficult. This reviewer would argue that lineage tracing alone is not particularly useful without other information about the cells that contain the labels. The approach described here is not amenable to single cell approaches, nor did the authors do any dissection/sorting/etc to show that their lineage sort into cells/tissues/organs, as would be expected.

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First revision

**Author response to reviewers' comments**

**Rebuttal:**

We would like to start by thanking the reviewers for their time in reviewing this manuscript. We believe that the work is now considerably more robust thanks to their comments/suggestions. To reiterate how and why this approach will be useful to other laboratories:

1) Firstly, it can be used in species for which it is difficult or impossible to generate transgenic animal lines. In particular we envision that this technique could be effectively used with human pre-embryos and human iP/iPS cell derived organoids (with our in vitro validated H5 array and others). For example, recently single cell resolution gene expression time-courses have been elucidated for human pre-implantation embryos (Stirparo et al., 2018). Though this data allows one to generate a pseudolineage, defining which cell type populations are the genuine progenies of prior cell type populations requires a lineaging tool such as ours described here.

2) Even for laboratories working on species where transgenics is straightforward our tool offers a pragmatic solution for doing lineage tracing. One does not need to spend time and money importing or generating a transgenic line. They can simply order the oligos that we have provided in the supporting information and start lineage tracing almost immediately. Furthermore this approach can be used on any transgenic line, saving the time required to cross a ‘lineaging’ line to one transgenic line of interest. Many groups are attempting to model disease by generating patient specific organoids derived from iP/iPS cells. One may wish to explore if lineaging decisions have been perturbed in such models. Our tool offers the ability to use the same tool for every patient without having to generate a new transgenic iP/iPS line for every patient. Other examples include species found in zoological parks, where the combination of cost, resources and generation time obviously make generating and maintaining a transgenic line of such species totally unfeasible. However many groups are exploring organoid models of these species (See Miki Ebisuya laboratory for example) and tools such as ours would be very suitable for lineaging in such organoids again without having to generate a transgenic ES or iP/iPS cell line every time a new species is explored.

3) Our approach is amenable to be used in combination with single cell transcriptomics if for example the G&Tseq pipeline is applied (Macaulay et al., 2015 - see rebuttal points for further details). approach can be used in combination with single cell transcriptomics if the G&T pipeline is applied. This could potentially result in all transcriptome sequenced cells having a corresponding lineage barcode since one could limit the single cell transcriptomics to cells which have first been confirmed to have their lineage bar code(s) recovered. In current approaches due to sampling issues many cells that have had their transcriptome sequenced do not also have their transcriptome encoded lineage barcode sequenced.

4) Our approach is easily scalable. When cells are sorted and processed, it should be possible to use multiple arrays at the same time to get deeper lineage information. It is trivial with our approach to keep simply adding more arrays in this manner. Furthermore as read lengths increase we can simply use arrays with more targets to start with.
5) The allelic sequencing allows the generation of multiple dendrograms for each individual embryo which promises to increase the amount of information and/or improve the accuracy of lineaging when used in combination with other information such as cell type or cell spatial position.

In summary, we believe that our tool offers enough advantages that other laboratories should find it useful. Indeed, we have already been approached by several groups at the European Developmental Biology Society meeting 2019 that said they would like to try it some of which are awaiting publication of this manuscript.

Major changes:
1) We have now totally revised our lineaging algorithm and instead use neighbor joining algorithm implemented through PAUP*.
2) We have brought the mouse lineaging data into the main manuscript and enhanced the explanation and discussion of this data.
3) We now use 3 different sequence aligners, bowtie2, bwa mem and needleall, and explore the results with different parameters for these aligners. For the zebrafish data we ensure that we only construct lineage trees with reads where all 3 aligners call the same CIGAR string (indel spectrum) to produce a high-confidence data set.
4) We have expanded the discussion on the uses of our technique in organoids and human pre-embryos.

New figures and tables:
1) New table 1 that describes the number and percentage of mice embryos that survive to blastocyst stage and number and percentage of embryos recovered after embryo transfer.
2) New figure 5, which is an updated version of the previous figure S7 describing the mouse lineage results.
3) New table S4 and S5. We now list the feature regions of the Z4 and M7 arrays in tables to help the reader. This is part of our improved explanation in this section.
4) New table S6 listing the % of reads that map to the most common allele in the mouse embryos.
5) New figure S4 showing detected locations of indels over the Z4 array with different aligners and parameter sets.
6) New figures S5-7 which describes the indel sizes and the proportion of deletions that span adjacent sites in the array for each of the zebrafish embryos. Constructed only using reads where the CIGAR string (indel spectrum) is determined to be the same for all 3 aligners. Each figure corresponds to a different parameter set for the aligners.
7) New figures S9-11 which replaces the old figure S5. This now fully shows all the different zebrafish embryo lineage trees with the corresponding indel diagrams and histograms of mutations per array. Constructed only using reads where the CIGAR string (indel spectrum) is determined to be the same for all 3 aligners. Each figure corresponds to a different parameter set for the aligners.
8) New figure S12 which shows all the different Mouse embryo lineage trees with the corresponding indel diagrams and histograms of mutations per array.

Note that we have made many other minor changes to accommodate the major changes and to fix any errors throughout the manuscript.

Reviewer 1 Advance summary and potential significance to field
Recently, several groups have used CRISPR-Cas9 to generate mutations throughout the development of an organism and used them as lineage markers. One approach is to introduce a CRISPR-Cas9 “recorder” via transgenesis, which consists of several CRISPR-Cas9 targets. With the recorder in place, gRNAs and the Cas9 need to be added so mutations can begin to accumulate. Cotterel et al. present a clever alternative approach: instead of introducing the CRISPR targets via transgenesis, they identify endogenous genome sequences that can serve as CRISPR targets. The main advantage of this approach is that it would avoid the generation of transgenic animals, which could be especially useful in non-model organisms where transgenesis has not been established.

For the identification of endogenous arrays they set-up a bioinformatic pipeline, which they used to analyse the mouse, zebrafish and human genomes. Then, they set a series of filters to ensure the quality of the arrays and to reduce potential off-targeting. They validate in vitro and/or in vivo the use of an endogenous array en each species. This manuscript has an important improvement compared to the one previously uploaded to bioRxiv, as now the authors include a filter that
identifies arrays with Single Nucleotide Polymorphisms (SNPs). This can solve the problem of potentially sequencing two different alleles of a single cell. Although I have some concerns, especially with their method of lineage reconstruction, I think the work presented by Cotterel et al. is an important addition to this growing field.

Reviewer 1 Comments for the author
My major concern with the manuscript presented by Cotterel et al is their chosen method for lineage reconstruction (see comments 1-3). Although the search for the best reconstruction algorithm is research under progress and this is allegedly not the major point of the manuscript, their in house program seems to have important flaws that need to be corrected.

Major comments:
1. Their algorithm uses as first step the unmutated array and searches for alleles that are 1 mutation away. These are used for the second round and so on. As the authors explicitly mention, this means that the internal nodes (just as terminal nodes) in the tree are sequenced reads. The idea of a reconstructed cell lineage is to infer the topology of the tree creating internal nodes that represent ancestral cells that by definition no longer exist when the cells are sequenced. Having a cell lineage where cells sampled at the same time are both internal and terminal nodes cannot be correct. The authors should use an alternative reconstruction method.

We agree with the reviewer and instead we now construct our trees using an alternative approach using the Neighbour-joining method for dendrogram reconstruction. Though it is less accurate than other methods of lineage reconstruction it is less computationally intensive. We feel that this is appropriate since the main point of our manuscript is the method of generating lineage information with endogenous CRISPR/Cas9 arrays and not about the exact computational method to reconstruct the lineage or specific lineage tree generated. Furthermore, the simplicity of the Neighbour joining algorithm makes comparison between trees more meaningfulful (Comparison of maximum parsimony trees for example would depend on what part of tree-space the algorithm had found for different data sets and thus depend on how tree-space was searched). We have noted this in the manuscript with the text:

“Since the goal of our work here was to demonstrate a novel a lineaging tool and not define any specific lineage we used paup in Neighbour-joining mode. Neighbour Joining is not as accurate as other methods such as Maximum parsimony or Maximum-likelihood but it is less computationally intensive and comparison between data sets is more meaningful due to its simplicity.”

Our method is described in more detail in the manuscript. Briefly, we used the PAUP* software (version 4.0a build 166; Swofford, 2017) and implemented the substitution matrix distance function as in Salvador-martinez et al., 2019. However, because each of our target sites in the array are highly distinct we instead built a separate substitution matrix for each individual site. Furthermore, we increased the number of character states per site to 36 (all available alphanumeric characters). All dendrograms and descriptions have been changed in the manuscript and supporting information appropriately.

2. I dont think their “catch” method, even if the problem with the internal nodes is solved, would work well with saturated arrays. Having saturated arrays means that most of them will be at a similar distance from the unmutated array. Also, their approach doesn't take into account the possibility of having homoplasy (different arrays acquiring same mutation in same target independently).

We agree with the reviewer. Please see point 1 above where we have totally changed our dendrogram construction approach to one using neighbor joining implemented via PAUP*.

3 In Suppl Fig 7, if I understand correctly the tree in e) has been built with the same array shown separately in both dendrograms in g), but in e) the tree shows the arrays coming from both genomic alleles. If the lineage reconstruction is robust enough, we shouldn't see clones that are made of both alleles as each has an independent mutational history. In the figure, it seems like some clones in e) are made of a mix of both allelic versions of the array. Is this the case? In any case, for easy comparison of the figures e-g and h-j, the authors should add an array index at the right side of the figures.
We agree with the reviewer here that two large subclones should form in the full tree that represent arrays deriving from allele due to their independent mutational history. The issue is simply that the mouse data as it currently stands is too sparse to get a robust lineage reconstruction for mice. There are not enough edited alleles or a high enough edits-per-array to be informative at this point. The edits present in both alleles tend to be the common mutations rather than the more informative less common mutations. We had previously noted in the discussion that we only have “we have basic proof-of-principle in mouse producing dendrogram depth on a par with that of Kalhor et al., 201830”. However to expand on this caveat we now state: “As a consequence the lineaging dendograms are clearly less complex for mouse than for zebrafish, and homoplasy can be observed since there are some edits shared between different alleles and different sub-branches of the same tree. Therefore, improvements in the Mouse system are required in order to increase the amount of edits reported per cell and increase the amount of lineage information.” We then describe the various improvements in the technique that should remedy these problems.

In any case we have removed the combined dendrogram from this figure since it is not relevant for the subsequent lineaging. Furthermore we have now constructed these Mouse embryo dendrograms using reads mapped with the bwa mem aligner since it identifies more edits that bowtie2 (note that because of this we have not added the array index to the right side of the arrays in this figure as the font would end up very small).

4 In second to last paragraph of the results the authors say they should they can consistently generate “deep dendrograms” refering to Suppl Fig 5. I do not agree with the authors on this, as most dendrograms shown have a tree depth of 3. Firstly, as stated in point 1 and 2 we have now changed our dendrogram building algorithm, and we now refer to the new dendrograms built with this algorithm. We agree with the reviewer that we have overstated our claims with regard to consistency of dendrogram depth. Therefore have now removed the claim and instead state that “This result demonstrates that we can consistently generate edits across the endogenous Z4 CRISPR/Cas9 array”, in-line with what the reviewer mentions. Furthermore we have expanded our former supplementary figure S5 to three new supplementary figure S9-S11 that include the histograms of number-of-edits per array using indel data generated by different parameter sets for 3 different aligners (a read must have the same CIGAR string reported for all 3 aligners to be included in this dataset).

Also, in the same paragraph the authors say they explore the consistency of their approach by comparing the dendrograms of 9 Zebrafish embryos. Although I think this result is important as they show they can consistently generate mutations in the chosen endogenous array, I don't think it is possible to judge the adequacy of the lineage reconstruction by only visually inspecting the topology of the tree.

We of course agree with the reviewer that it is difficult/futile to judge the adequacy of the lineage reconstruction using only the tree topology. Labels (cell type or spatial information for example) would be required in order to employ something like the robinson-foulds metric. Therefore we have removed the discussion about exploring the consistency of the approach and instead we state “Furthermore, in order to fully explore the consistency of the lineage reconstruction between individuals, labels (cell type of spatial information for example) for the leaf nodes are required which can be added in the future by adapting our approach to a single cell sequencing pipeline (see below).”.

Minor comments:
5 Can the authors elaborate where their low number of inter-target deletions reside (as compared with GESTALT)? Have they done anything special to avoid them, or their low occurrence is only explained by low editing frequency?

We sought to compare our data more directly to the data from Mckenna et al., 2016 in order to answer this question. Mckenna et al., however used the needleall aligner with reads mapped against the amplicon. Therefore we used needleall in exactly the same way as they did with identical parameters to that stated in Mckenna et al., 2016. When we do this we find that needleall tends to identify larger deletions than bowtie2. We have explored a course comparison between
our data and the data from Mckenna et al., 2016 (by making a histogram of indel sizes for the largest data set - 48hpf illumina index 4. Note the other embryos have a similar distribution). This suggests that in fact our system is on par with the Mckenna et al., 2016 data rather than having smaller inter-target deletions. The effect simply comes from the specific aligner and parameters used and that needleall tends to report larger deletions. Hence we have removed the claim that our system generates less inter-target deletions that theirs from our manuscript. We feel that though important it is beyond the scope of the current manuscript to go into details of the different type and sized indels reported using different aligners.

6. In the first paragraph of the Discussion, the authors mention that one advantage of their approach is that it does not require single-cell sequencing. This is the case, as they demonstrate, when sequencing one array only. I think one great advantage of their approach is the possibility of multiplexing by targeting more than one endogenous array. This would mean, however, that some sort of single cell indexing or single cell sequencing would be necessary. I think it would be useful if the authors discuss how they envision multiplexing their arrays.

We agree with the reviewer. We have now described how easily scalable our approach is by multiplexing using more than one array using a single cell sequencing pipeline in the discussion. Furthermore we discuss how adapting the approach even further using the G&Tseq pipeline allows us to get cell type information and lineage information in parallel. We also believe that this point has an important relationship to point 7 below made by the reviewer. Since many more than 10 sites would be needed to accurately reconstruct an embryo with millions of cells the ability to scale these techniques is essential.

7. In the 4th paragraph of the Body the authors mention that 33 targets could be generate allele diversity to perform lineage tracing in a mouse of 12 billion cells. Even when in theory 33 targets could generate such diversity, this doesn't mean it would be possible to accurately reconstruct this massive lineage tree. Simulations of CRISPR Cas9 lineage recorders have shown that more than >100 targets would be required to reconstruct accurately a ~50K cells tree (Salvador-Martinez et al., 2019).

We agree with the reviewer and we have now adjusted the end of this paragraph to state: “To perform lineage tracing in the whole mouse embryo of 12 billion cells, 33 CRISPR target sites in an array (or multiple arrays) is the theoretical minimum would be required to provide enough diversity.”. Furthermore, we have expanded the exploration of this point with an adapted paragraph in the discussion since this point also relates to point 6 above. As mentioned by the reviewer one of the powers of our approach lies in the fact that multiple arrays could be used simultaneously in a single cell sequencing pipeline. This allows us to pragmatically increase the number of target sites per cell to gain more lineage information for more accurate tree reconstruction. This new paragraph reads:

“An important property of any CRISPR based whole-organism lineaging system is that it must be capable of generating enough information (bits) to uniquely identify different cells. Here we show that our system is indeed capable of generating such diversity. The dendrogram shown in figure 4c consists of 1,572 alleles demonstrating that we can generate significant diversity on par with GESTALT. Previously, we mentioned that 33 CRISPR target sites would be the theoretical minimum required to provide enough diversity to perform lineage tracing in the whole mouse embryo of 12 billion cells. However, in reality many more sites are required due to factors such as homoplasy, target site drop-out and the fact that the editing is not synchronized with cell division. Indeed, simulations of CRISPR/Cas9 lineage tree recorders has demonstrated that >100 targets would be required to accurately reconstruct even a ~50K cells tree47. The fact that the system described here is easily scalable when used in a single cell sequencing pipeline (multiple arrays and both alleles can potentially be sequenced for each cell) to generate more lineage information is thus important.”

Additional comments/suggestions:

1. In the 6th paragraph of the results the authors say they “validated 3 suggested target arrays from each of the Zebrafish, Mouse and Human”. I think this should be changed to “validated 1 target arrays in each species”.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).
2. In the second paragraph of the discussion, it is mentioned that sequencing both alleles of a cell is likely to be rare. Can the authors elaborate on why this is the case?

When the number of cells of the organism is high relative to the number of reads sequenced then we would expect that the probability of sequencing two alleles from the same cell to be low. However, obviously this depends on how deep one is sequencing their organism of choice. Therefore, we have removed this statement since it is ambiguous and unnecessary in any case.

3. In Fig 3, I think it would be more useful to have the editing frequencies per target instead of per nucleotide site. Also, the colours of the m) could be changed as they are not easily discernible from each other.

Though we can see the reviewers' point, we personally think it is more useful to have the editing per nucleotide because the spatial resolution is higher allowing one to better judge whether the edit is occurring where we expect it to occur (dashed lines). Hence we have left these figure panels as they are. We have however changed the colours of figure 3m as suggested by the reviewer.

4. I would suggest to have the intertarget deletions as a main FIG as it is one of the major points of the paper.

We have kept this figure in supporting information. As we describe earlier in point 5, the exact aligner and parameters used have an important impact on the indel sizes detected. We feel it is beyond the scope of the current paper to go into details of the different sized indels reported using different aligners.

5. I would change 3a and 3b as in the next figures Zebrafish comes first

These panels have been reversed as suggested by the reviewer. We have also expanded these panels to fill white-space.

(signed)
Irepan Salvador-Martinez

Reviewer 2 Advance summary and potential significance to field

The manuscript submitted by Cotterell et al. represents an admirable attempt to use endogenous arrays for CRISPR lineage tracing in zebrafish and mice. Given related methods, this approach would have greatest applicability to an organism for which transgenesis is not possible. However, 1) the authors only apply this to systems where transgenesis is already easy and widely available, 2) the manuscript lacks essential details of the experiments and data, and 3) the approach is compromised by several limitations, as described below. In summary, the authors do not directly demonstrate how or why this approach will be useful for other labs, as existing methods have clear advantages and avoid the caveats inherent to this method.

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Below I detail my concerns with this manuscript as currently presented.

1. Statements about lineaging in mouse, are not supported by the main figures, only a single supplemental figure. Since this is the only section that attempts to demonstrate SNP discrimination of arrays, and endogenous tracing in mouse, this reviewer believes that these data need to be in a main figure. However, this section is exceedingly brief and lacks essential descriptions of the experiment and data.

We agree with the reviewer that this section should be in the main manuscript and the description of the methods and data greatly improved. Therefore, we have done exactly this (see new figure 5) and the details described below can now be found in the main manuscript text, methods and new table 1. Furthermore, we have converted the former supplementary figure to use data from bwa
mem alignment rather than bowtie2 since this resulted in a higher number of alleles. We have also included a new paragraph in the discussion that describes the results from the mouse lineaging and compares them to the zebrafish data.

Did development proceed normally?

Most blastocysts and embryos that implanted upon embryo transfer developed normally as judged by gross morphology. We performed two rounds of microinjection at the 1-cell stage. We found that on average 90% of injected 1-cell stage embryos reached the blastocyst stage (90 of 96 in batch 1 and 49 of 59 in batch 2). This compares to typically 80-85% of embryos that reach the blastocyst stage under other microinjection scenarios (Wang et al., 2013). From batch 1, 28 blastocysts were transferred to a single pseudopregnant female (14 embryos transferred to each uterine horn) and of these 15 implanted and 12 of them (12/28=43%) were collected (3 had strange morphology). From batch 2, all 49 embryos were transferred of which 17 implanted and were recovered (35%) with normal morphology. Typically this procedure results in about half of the embryos implanting and being recovered. Overall, therefore our numbers of morphologically normal embryos recovered are similar to that of other groups. The mouse microinjection and embryo transfer were carried out by the Centre for Genomic Regulations Tissue engineering Unit (https://www.crg.eu/en/programmes-groups/tissue-engineering-unit-0), who are highly experienced with these procedures and they claim the procedure does not perturb the embryos anymore than any other typical microinjection/embryo transfer would do. This data is now included in the main manuscript including the new table 1.

How many embryos were sequenced?

A pool of 46 M7 microinjected blastocysts was sequenced along with a pool of 5 uninjected control blastocysts. 6 embryos from batch 1 and 12 embryos from batch 2 were sequenced (both batch embryos were retrieved and genomic DNA isolated at the E9-E10 stage). 7 of these embryos were determined to be heterozygote and thus used for lineage reconstruction. This data is now given in the main manuscript, methods and supporting information (Table S6).

How many alleles are generated per embryo?

We have now included those numbers of leaf nodes (alleles) in the figures when the lineaging tree appears (for zebrafish see figures S9-11, for mouse see figure S12).

How many edits per allele?

We have now presented histograms of edits-per-allele everytime we show a lineage tree for both mouse (figure S12) and zebrafish (Figures S9-11).

How to compare these statistics to previous fish data?

The only statistics that can realistically be compared between the mouse and the fish data are the number of edited alleles and the number of edits per allele at this point. As mentioned below and responding to reviewer 1, we agree with both reviewers that it is not meaningful to compare lineage trees (nor are they particularly useful) without labels on the leaves which we have discussed in the manuscript (also see responses to other points).

Regarding the number of edited alleles and the number of edits per allele it can be clearly seen in our new data that the fish data contains many more alleles than the mouse data. The number of edits per allele is also much higher in the fish data than in the mouse data. Every deep sequenced fish embryo have at least one allele with 5 edits with some having as many as 8 edits as for zebrafish index 4 (48hpf). The mouse transferred embryos by contrast at E9-10 only contain up to 4 edits per allele. We believe the difference between the two species is due to the speed of development, which is much faster in fish than in mouse. We have now discussed this in the main manuscript.

The text references Figure S5, which does not describe mouse data. The supplemental text “Lineaging in Mouse embryos” references Figure S6, which also does not describe mouse data. S7 appears to be the only SNP and mouse data, which is minimally described and somewhat confusing.
This has been corrected and now explained in much more detail in the main manuscript, methods, new figure 5 and S12.

Why do the two arrays within a single embryo contain similar edits?

We apologize but we are not entirely sure what the reviewer means by this point since we only used one array per embryo. We assume the reviewer means why does each copy of chromosome 7 (where the M7 array is located) contain similar edits. If so then we presume this is because in general with this approach some edits are simply much more common than others. These are unfortunately the low information edits since they occur so frequently and can lead to homoplasy. In our lineaging approach, like other groups we now give more prominence to lower frequency mutations since these contain more lineage information. However it is clear that more sites are needed for mice in order to allow for accurate lineaging. We have now caveated the mouse data in the discussion and describe how we think this can be improved.

Many of these array edits look very small, and are scattered randomly across the array instead of being near the predicted cut sites. Perhaps they are sequencing errors instead of CRISPR edits? Yet it appears that the dendrogram still uses these low confidence mutations.

There is a mistake here in that mutations of only 1bp were being allowed through our filter for the mouse data when they shouldn’t have been. This has been corrected to >1bp edits (as is used for the fish data). Now edits appear more or less the correct locations of the array.

2. It’s not clear how the authors propose to use the two dendrograms generated by the SNP arrays. The authors state that “Furthermore, by using a SNP to split the reads into 2 alleles, one can build 2 dendrograms for each individual organism. These serve as internal experimental repeats which can be used to improve and validate the accuracy of the resulting dendrogram.” How do the authors propose to “improve and validate the accuracy”, without cell type information or other ways to anchor the trees together? Even with SNPs, this reviewer is struggling to understand how two dendrograms, unlikely to be sampled from the same cells, could be used together to describe the true lineage.

We agree with the reviewer that we have overstated our claims here. Of course we would need additional label data (such as single cell transcriptomics or spatial information) in order to anchor the trees. Our approach is amenable to obtain this data (See final rebuttal point 6 for more detail on single cell transcriptomics with our endogenous array approach) but clearly, we have not shown such data in the current manuscript. We have now adjusted these claims to instead state “Furthermore, by using a SNP to split the reads into 2 alleles, one can build 2 dendrograms for each individual organism. These serve as internal experimental repeats which could be used to improve and validate the accuracy of the resulting dendrogram if the approach is amended to include additional label information such as single cell transcriptomic or spatial information allowing one to anchor the data sets”

3. Many edits that the algorithm calls as different appear to be the same, just staggered a little. This can be seen in many of the edit plots across panels. What is the source of this? Many aligners are context dependent when deciding to open or extend a gap, which can lead to these types of errors. If this is the source of errors, it could alter relationships within the dendrogram. Are the authors certain that alignment issues are not mistakenly generating different edit outcomes from the same missing bases?

We manually inspected some of these situations with the staggered edits to get an insight into what was causing them. In every case we inspected there was a mismatch (between the read and the reference sequence) around the indel call. This suggests that the cause of the staggered indels are alignment errors in the way that the reviewer suggests. However, alignment issues was the reason we originally designed our pipeline to first define a feature array from mapped sequence data before dendrogram construction. In the feature array the same but staggered edit appears as the same feature and therefore should not cause problems with the dendrogram construction. This is also why when indels are the same but very slightly staggered they are typically adjacent in the lineage tree as the algorithm detects them as the same mutations. We clearly did not explain our
feature array approach well enough so we have improved the description in the manuscript and supporting information.

The authors do not provide the parameters they used for bowtie2 alignment, nor any evidence that each array mutation is called in a context independent manner, so it is difficult to judge the confidence in alignment. How robust are these alleles to variation in the alignment parameters or algorithm?

To give the reviewer more confidence that our result is not simply due to the alignment parameters or specific aligner used we have now included a number of changes:
1) We generate the dendrograms of the zebrafish embryo lineage only using reads where 3 aligners, bowtie2, bwa mem and needleall, all report the same CIGAR string (indel spectrum). This is described in greater detail in the manuscript, methods and supporting information.
2) We have performed this analysis for 3 different alignment parameters sets. See supporting figures S4-7 and S9-11 that show the results are similar between the parameter sets in terms of number of alleles and edits per allele distribution.

Note that we have only done this for the zebrafish data where we have a lot of alleles. The data is very scarce for the Mouse embryos that very few alleles result if we restrict ourselves only to the reads where all 3 aligners agree on the CIGAR string (indel spectrum). Therefore we have instead used only bwa mem for the mouse data (as it results in the most alleles) and we have caveated the reliability of the Mouse data in the discussion.

4. “This result demonstrates that we can consistently generate deep dendrograms with a similar gross structure using the Z4 array of endogenous CRISPR/Cas9 target sites.” What led the authors to conclude that the trees have a similar gross structure?

As also mentioned in response to reviewer 1, we feel we have overstated our claims here. In general it is difficult/futile to compare the structure of unlabeled dendrograms. Labels (cell type or spatial information for example) would be required in order to employ something like the robinson-foulds metric for tree comparison. Therefore we have removed the discussion about exploring the consistency of the approach and instead we state “In order to fully explore the consistency of the lineage reconstruction between individuals, labels (cell type of spatial information for example) for the leaf nodes are required which can be added in the future by adapting our approach to a single cell sequencing pipeline (see below).”.

The authors only discuss the “best” tree in the text. However, Figure S5 shows very different dendrograms across multiple embryos - orders of magnitude differences in # alleles, very different dendrogram depths. No statistics or details about the nature of these CRISPR arrays, edits, alleles, trends toward local edits vs larger deletions are provided.

Firstly, note we have now changed our dendrogram building in response to reasonable criticism from reviewer 1 algorithm (we now use neighbor joining with PAUP*). We have now changed all dendrograms using this approach and now refer to the new dendrograms built with this algorithm. We now state the number of alleles for each embryo, show a histogram of the number of edits per array (like in figure 4), and show the histograms of indel size and deletions spanning multiple sites for each embryo in figures S5-7 and S9-11. Note this data was generated using the reads where CIGAR strings are reported the same by all 3 aligners like used to generate the dendrograms.

From visual inspection, it seems that some embryos only had 100 alleles, but others had thousands. Why are they so different than the tree shown in the main figure?

The principal cause of this is likely to be variability in injection of the Cas9/CRISPR components into the zebrafish egg. Though it is straight-forward to control the consistency of the concentration of the components and injection pressure there is often variation in the bore size of the injection needle or exact angle which we inject which likely leads to different exact amounts and distribution of CRISPR/Cas9 machinery in the egg. Note that we see similar variability for the mouse embryos. Hence to get multiple deep trees many alleles it is typically just a case of injecting more embryos. Nevertheless, we have added the following text in the discussion when we summarise/compare the mouse and zebrafish data “Nevertheless, when we compare the mouse and
zebrafish data sets we find that variability in the number of edited alleles and edits-per-allele is evident in both species which likely arises from variability in microinjection parameters.”.

The authors do not provide diagrams of the alleles they find in other embryos, just the dendrograms.

We have now included the allele diagrams for every dendrogram presented.

Depending on these data, the authors should caveat their claims regarding tree depth, # alleles, and superiority of this method over others, given the variability shown in the other trees.

We have now adjusted our claims with regard to consistency of dendrogram depth. Specifically, we have removed the claim that we can consistently generate deep dendrograms and instead state that “This result demonstrates that we can consistently generate edits across the endogenous Z4 CRISPR/Cas9 array”, which more accurately reflects the results presented. We have also removed the claims about the drop-out improvement of our technique compared to that of McKenna et al., 2016 (see point 5 reviewer 1) as the differences seem to be due to the exact aligner used.

5. For arrays described in 1d,e,f and 2a,b,c, the authors are not clear about what set of filters were used to identify these arrays. Is this filter 1 or 2? SNP arrays only?

We apologize to the reviewer, as we have clearly not explained ourselves well enough here. For the data in figure 1, neither filter set version 1 or 2 was used. It is simply scanning the genome with a 350bp window and quantifying how many non-overlapping PAM/spacer sequences appear in that window. It was meant as our justification as to why the endogenous array approach was practical. In figure 2, we show the identified arrays using version 2 of the pipeline. All version 2 arrays are suggested to harbor SNPS, but there is no guarantee there will be a SNP in these arrays. This entirely depends on the particular line of the species (and the level of inbreeding) and requires some trial and error to confirm whether these sites indeed harbor a SNP. We have now clarified the description of this data in the respective figure legends.

6. “Our method described here is the first to use only endogenous CRISPR/Cas9 sequences from the wildtype genome, thus dramatically simplifying the procedure, and perhaps more importantly opening up the field of whole organism lineage tracing to non-model species that for which it is hard to generate transgenic animals” This reviewer would not agree. This methodology is only applied to fish and mouse, where transgenesis is already widely available. The authors do not demonstrate that they opened up lineage tracing to non-model species. You would still need genome sequence, access to embryos, and ability to microinject.

We agree with the reviewer that we have overstated our claims here. We have thus adjusted our claims to support more accurately what we have demonstrated in the manuscript. We have now restated the last part of this claim as “and perhaps more importantly making whole organism lineage tracing more feasible in non-model species that for which it is hard to generate transgenic animals”. Note we have also expanded upon our discussion of the advantages of our technique which relates to organoids as well as embryos.

If a researcher had all those things, the wet lab experiment may be easier, but the interpretation of the resulting dendrograms is substantially more difficult. This reviewer would argue that lineage tracing alone is not particularly useful without other information about the cells that contain the labels. The approach described here is not amenable to single cell approaches, nor did the authors do any dissection/sorting/etc to show that their lineage sort into cells/tissues/organs, as would be expected.

We totally agree with the reviewer that lineage information alone is not particularly useful without label information such as cell type or spatial origin of the cell of interest and hence that is precisely why the field as a whole is moving in the direction of adding this information. However, we would disagree that the interpretation of the resulting dendrogram would be more difficult because our approach is not amenable to single cell transcriptomic sequencing (or other way of labelling the leaf nodes). There are multiple approaches to use our endogenous lineaging tracing approach in combination with single cell transcriptomics.
G&Tseq (https://www.nature.com/articles/nmeth.3370) for example allows one to perform a smart-seq type pipeline and sequence both the genome and transcriptome of individually sorted cells in parallel. It only requires a single additional step to separate the genomic DNA from mRNA with magnetic beads conjugated to a polyT oligo. One could perform G&Tseq with our arrays and get single cell transcriptomic data together with parallel lineage data. Alternatively, after single cell sorting have been using a couple of rounds of PCR with a primer containing a T7 transcription start site to generate an amplicon that is transcribed in vitro allowing a piece of genome to be read out in the sequence of the transcriptome (Philip Junker personal communication). We have now adjusted our discussion to include the sentence “The endogenous array approach described here is also amenable to parallel lineage tracing and single cell transcriptomics if one applies the G&Tseq pipeline to first separate genomic DNA from mRNA for sorted single cells.” In the section that describes single cell transcriptomics in combination with lineage tracing. We also include the new reference to G&Tseq.

Second decision letter
MS ID#: DEVELOP/2019/184481
MS TITLE: Endogenous CRISPR/Cas9 arrays for scalable whole organism lineage tracing.
AUTHORS: James Cotterell, Marta Vila Cejudo, Laura Batlle Morera, and James Sharpe

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the few remaining referees' comments are dealt with. Please attend to all of the reviewers' comments in your revised manuscript.

Reviewer 1

Advance summary and potential significance to field

Recently, several groups have used CRISPR-Cas9 to generate mutations throughout the development of an organism and used them as lineage markers. One approach is to introduce a CRISPR-Cas9 "recorder" via transgenesis, which consists of several CRISPR-Cas9 targets. With the recorder in place, gRNAs and the Cas9 need to be added so mutations can begin to accumulate. Cotterel et al. present a clever alternative approach: instead of introducing the CRISPR targets via transgenesis, they identify endogenous genome sequences that can serve as CRISPR targets. The main advantage of this approach is that it would avoid the generation of transgenic animals, which could be especially useful in non-model organisms where transgenesis has not been established.

For the identification of endogenous arrays they set-up a bioinformatic pipeline, which they used to analyse the mouse, zebrafish and human genomes. Then, they set a series of filters to ensure the quality of the arrays and to reduce potential off-targeting. They validate in vitro and/or in vivo the use of an endogenous array en each species.
I think the work presented by Cotterel et al. is an important addition to this growing field.

Cotterel and collaborators have greatly improved their manuscript, addressing all my comments. More specifically, they have 1) changed the reconstruction algorithm used through the manuscript 2) described how the approach can be multiplexed and combined with a single cell sequencing pipeline in the discussion and 3) adjusted/removed some overstating claims.
Comments for the author

Major revisions:
NONE

Minor comments/suggestions:

I only have some additional minor suggestions to the authors:

1) In the Discussion the authors mention that in Zebrafish they can produce nearly saturated arrays after 30h. I think this is not entirely correct, as most arrays contain 2-4 mutations. I would suggest the authors describe these results in a different manner.

2) In the Results section when describing the results of the Z4 array in the paragraph that starts with “In order to generate a representative metric…”, I think it would be useful if the authors include the mean/median number of mutations per allele.

3) I think the Y-axis of Figure 4B should be in a linear scale, just as in the Suppl Figs S9-S11.

(signed)
Irepan Salvador-Martinez

Reviewer 2

Advance summary and potential significance to field

The authors have done an excellent job improving their manuscript on the use of endogenous arrays for CRISPR lineage tracing. The data justify the conclusions. I commend the authors on their extensive revisions and clarifications, which alleviated most of the concerns I raised in my previous review. This study has substantial promise for opening the door to lineage tracing in a variety of organisms, and I look forward to the authors’ future work on this (most especially the G&Tseq concept mentioned in the Discussion)!

Comments for the author

One minor suggestion is to update this sentence “Some groups have also recently used single cell sequencing alone at different developmental time points to reconstruct cellular lineage.” I feel that these scRNAseq-only studies are capable of generating developmental / cellular / gene expression trajectories but do not actually reconstruct cellular lineage (aka records of cell divisions). I would suggest updating this sentence, since this and related methods will truly enable lineage recording.

Second revision

Author response to reviewers’ comments

General points:

Acknowledgements:
We have added a small acknowledgements section reading:
“We thank Jan Philipp Junker, Irepan Salvador-Martinez, Cedric Notredame and Emilio Palumbo for helpful comments and suggestions. Furthermore, we thank Antoni Matyjaszkiewicz for proof reading. We thank the UPF Genomics Core Facility for performing the Illumina Miseq deep sequencing, Sanger sequencing and bioanalyzer analysis. We also thank the PRBB Animal facility for Zebrafish and Mouse husbandry”
Minor corrections:
Note that we had to adapt the abstract (last sentence) very slightly as it was 184 words (max is 180) and we corrected two other minor errors that we found in the manuscript.

Reviewer 1:
1) In the Discussion the authors mention that in Zebrafish they can produce nearly saturated arrays after 30h. I think this is not entirely correct, as most arrays contain 2-4 mutations. I would suggest the authors describe these results in a different manner. We have simply removed the beginning of the sentence “Since our editing is almost complete by 30hpf (see figure S9-11),” which makes the remaining sentence correct.

2) In the Results section when describing the results of the Z4 array in the paragraph that starts with “In order to generate a representative metric...”, I think it would be useful if the authors include the mean/median number of mutations per allele. We have included the mean number of mutations in the description. Furthermore, we have updated this paragraph slightly to make it reflect the data a bit better. It now reads:

“In order to generate a representative metric of the utility of the approach we explored the distribution of the number of mutations of all reads. The histograms of dendrogram depth for the Z4 array are shown in figure 4b and Figures S9-11 demonstrating that the full trees are generated by reads with up to 8 mutations (Mean number of mutations per allele is 3.03 for the zebrafish embryo in figure 4). The full dendrogram for one zebrafish embryo consisting of 1,572 alleles is shown in figure 4c and a subset of that dendrogram is shown in figure 4d. To explore the consistency of this approach we deep sequenced a total of 10 embryos microinjected with the Z4 sgRNA pool with genomic DNA extracted at either 30 or 48 hours post fertilization. The resulting dendrograms are shown in Figures S9-11 (each with a different alignment parameter set). This result demonstrates that we can consistently generate edits across the endogenous Z4 CRISPR/Cas9 array. Taken together this data demonstrates how our approach can be used effectively to perform lineage tracing in embryos.”

3) I think the Y-axis of Figure 4B should be in a linear scale, just as in the Suppl Figs S.9-S11. This figure panel has been updated as suggested by the reviewer and now the histogram in figure 4b is identical to that in supplementary figure S10. Note that the histogram shown in figure 4b was mistakenly showing the data from the O6E1 alignment parameter instead of the O5E3 alignment parameter. This has been corrected to keep the histogram consistent with the dendrogram shown in figure 4c. Anyhow, all histograms and corresponding dendrograms can be found in supplementary figures S9-11.

Reviewer 2:
One minor suggestion is to update this sentence “Some groups have also recently used single cell sequencing alone at different developmental time points to reconstruct cellular lineage.” I feel that these scRNAseq-only studies are capable of generating developmental / cellular / gene expression trajectories, but do not actually reconstruct cellular lineage (aka records of cell divisions). I would suggest updating this sentence, since this and related methods will truly enable lineage recording.

We agree with the reviewer and we have now adapted this sentence to read: “Some groups have also recently used single cell sequencing alone at different developmental time points to construct pseudo-cellular lineages”
Third decision letter

MS ID#: DEVELOP/2019/184481

MS TITLE: Endogenous CRISPR/Cas9 arrays for scalable whole organism lineage tracing.

AUTHORS: James Cotterell, Marta Vila Cejudo, Laura Batlle Morera, and James Sharpe

ARTICLE TYPE: Techniques and Resources Article

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