Investigating cell cycle-dependent gene expression in the context of nuclear architecture at a single-allele resolution

Shivnarayan Dhuppar and Aprotim Mazumder
DOI: 10.1242/jcs.246330

Editor: Maria Carmo-Fonseca

Review timeline
Original submission: 13 March 2020
Editorial decision: 3 April 2020
First revision received: 20 April 2020
Accepted: 10 May 2020

Original submission

First decision letter

MS ID#: JOCES/2020/246330

MS TITLE: Investigating cell cycle-dependent gene expression in the context of nuclear architecture at a single allele resolution

AUTHORS: Shivnarayan Dhuppar and Aprotim Mazumder
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

I have now received comments on your manuscript from one expert referee. Unfortunately, the other two reviewers withdrew due to the current pandemic situation. I have carefully read the paper and in principle, I agree with reviewer #1 that your approach is potentially interesting. However, I also agree with the serious concerns raised by this reviewer. If you think you can review the manuscript by 1) citing the relevant literature, as indicated; and 2) performing quantitative analysis for colocalization of DNA and RNA signals, I would be pleased to see a revised version of your paper. I would then return it to the reviewer.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please 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. Please attend to

all of the reviewers' comments. 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

Dhuppar and Mazmder present a new protocol to investigate the transcription of individual genes within the cell nucleus at a single-cell level. The authors report a “simple” way to combine DNA FISH with smFISH for RNA and immunofluorescence, while preserving the 3D nuclear architecture of a cell. The new approach is used to analyze the expression of the Cyclin A2 gene, which is known for its cell cycle dependent expression.
They observed that most of the cells had three copies of this gene and chose for their analysis only those with exactly three copies. From their analysis the authors conclude that the expression of a respective gene copy is stochastic and does not depend on its subnuclear position within the nucleus nor on the expression of the other copies. This result is not discussed in the context of the existing – very rich – literature (see, e.g. Buxbaum, Haimovich and Singer, 2015)

The presented approach is potentially interesting, however, it is not clear for this reviewer, in which sense the approach is new or allows new experiments. Techniques for combining DNA FISH with FISH for RNA have been carried out since 25 years – and the authors completely fail to cite the relevant literature: e.g. publications by the Singer group, Lawrence group, Tyagi group, and so on. The combination of DNA FISH smFISH with immunofluorescence is not extremely exciting. Finally, the article contains an important technical flaw: The colocalization precision of DNA and RNA signals has not been evaluated in a quantitative manner.
Therefore, the validity of the conclusions drawn cannot be judged.

Positive: An easy method to investigate gene regulation in 3D on a single-cell level.

Comments for the author

Further, questions remain after reading the article:
• The discussion and evaluation of the dynamic extrusion-based model is incomplete. The colocalization precision of DNA and RNA signals has not been evaluated. In this context: how far can the looping out of genes be expected? Could it adequately be measured given the colocalization precision achieved in the experiments? Furthermore: a putative extrusion would possibly not only occur only towards the centroid/lamina. In case it would occur in a perpendicular direction with regards to the centroid/lamina direction - how would this affect the interpretation?

• It should be discussed, how long the mRNA does remain at a gene while or after being transcribed?
Assuming the duration time is short and the cells are fixed at an mRNA-free moment false-negative results can be drawn regarding the activity of a gene.

Minor comments:

• Scale bars missing in: Figure 1B, 2B

• Could an additional fixation step with glutaraldehyde help to improve the structural preservation of the genome?

• In the section “3D nuclear segmentation” it should be stressed that the quantification of DNA content is used to define the cell cycle state

First revision

Author response to reviewers' comments

Reviewer 1

Reviewer comment

Advance Summary and Potential Significance to Field:

Dhuppar and Mazmder present a new protocol to investigate the transcription of individual genes within the cell nucleus at a single-cell level. The authors report a “simple” way to combine DNA FISH with smFISH for RNA and immunofluorescence, while preserving the 3D nuclear architecture of a cell. The new approach is used to analyze the expression of the Cyclin A2 gene, which is known for its cell cycle dependent expression. They observed that most of the cells had three copies of this gene and chose for their analysis only those with exactly three copies. From their analysis the authors conclude that the expression of a respective gene copy is stochastic and does not depend on its subnuclear position within the nucleus nor on the expression of the other copies. This result is not discussed in the context of the existing – very rich – literature (see, e.g. Buxbaum, Haimovich and Singer, 2015)

Author response

We thank the reviewer for pointing out these relevant references. We have further modified the text to discuss our results in the light of the above-mentioned study in addition to other studies as required.

Reviewer comment

The presented approach is potentially interesting, however, it is not clear for this reviewer, in which sense the approach is new or allows new experiments.

Author response

We thank the reviewer for appreciating the potential applications of the technique presented here. We take this opportunity to point out salient features of our technique:

1) The technique developed here combines 3D DNA FISH with both smFISH and immunofluorescence to study the comparative effects of nuclear architecture and cell cycle on gene expression at a single cell resolution. As the reviewer is conscious, smFISH yields absolute RNA counts in a cell, and not relative intensities as in standard in situ hybridization procedures. This allows access to very low levels of expression too.

2) We have also standardized the experimental technique and removed some of the more involved steps found in conventional 3D DNA FISH such as nitrogen free-thaw cycle and acid denaturation. Moreover, we have also done away with serial alcohol dehydration which is known to affect mostly the cell nucleus, thus preserving the 3D architecture of the cells. Most older DNA FISH methods cause flattening of the cell nucleus and loss of three dimensional information. Compared to other 3D DNA FISH techniques, our method is simpler, and allows for multiplexing with smFISH for RNA and immunofluorescence for proteins. While undoubtedly each of these methods have been performed many times in isolation by many groups, combining all the three in the same cell brings new insight into nuclear architecture-dependent gene expression.

3) We have combined the above experimental techniques with completely-automated image analysis modules for high throughput, high resolution quantitative analysis of gene regulation in the context of cell cycle and nuclear architecture at a single cell, single allele resolution. These allow both for assigning of cell cycle stage to a given cell, and also quantitatively determining the position for an allele in the three dimensional context of the cell nucleus. These custom programmes are made available publicly.

Ours will be the first report, so far as our knowledge goes, to have combined 3D DNA FISH with smFISH

and immunofluorescence with microscopy-based cell cycle staging while at the same time quantifying nuclear architecture-related information in 3D. Furthermore we should emphasize that most of these analyses techniques require minimum human interference and are easy to implement, and the analysis routines have been made available in the public domain.

Reviewer comment

Techniques for combining DNA FISH with FISH for RNA have been carried out since 25 years – and the authors completely fail to cite the relevant literature: e.g. publications by the Singer group, Lawrence group, Tyagi group, and so on. The combination of DNA FISH, smFISH with immunofluorescence is not extremely exciting.

Author response

We apologize for this has not been a wilful neglect. We take this opportunity to emphasize that we had made every effort to give credits to previous studies by citing relevant studies by the Tyagi group (Raj et al., 2006; Raj et al., 2008), the Lawrence group (Clemson et al., 2009), the Singer group (Zenklusen et al., 2008), or the Axel group (Lomvardas et al., 2006) and others.

But we agree in that we might not have been comprehensive in our citations given the large volume of excellent studies on gene expression produced by these groups and we hope that now we have been able to address this concern to some extent in our revised manuscript by adding additional references.

Reviewer comment

Finally, the article contains an important technical flaw: The colocalization precision of DNA and RNA signals has not been evaluated in a quantitative manner. Therefore, the validity of the conclusions drawn cannot be judged.

Author response

We are glad that the reviewer raised this concern. We take this opportunity to further demonstrate the efficacy and precision of our 3D colocalization metric with new quantifications as follows:

There are two things to be considered: 1) The precision of colocalization from the metric defined and 2) the quantitation of colocalization for the gene spots with nascent mRNA in our experiments. To measure the precision of our colocalization metric we validate the metric against synthetic 3D images of spots. Here we assume that two spots can either colocalize or not and generate two random images Image 1 and Image 2. We then looked at the colocalization of spots in the two images pair-wise. In our study we quantitate the colocalization of gene spots and mRNA for all the three copies of CCNA2 gene using a similar algorithm, which too is presented here (Figure R1). This figure has now been included in the Supplementary Information File.

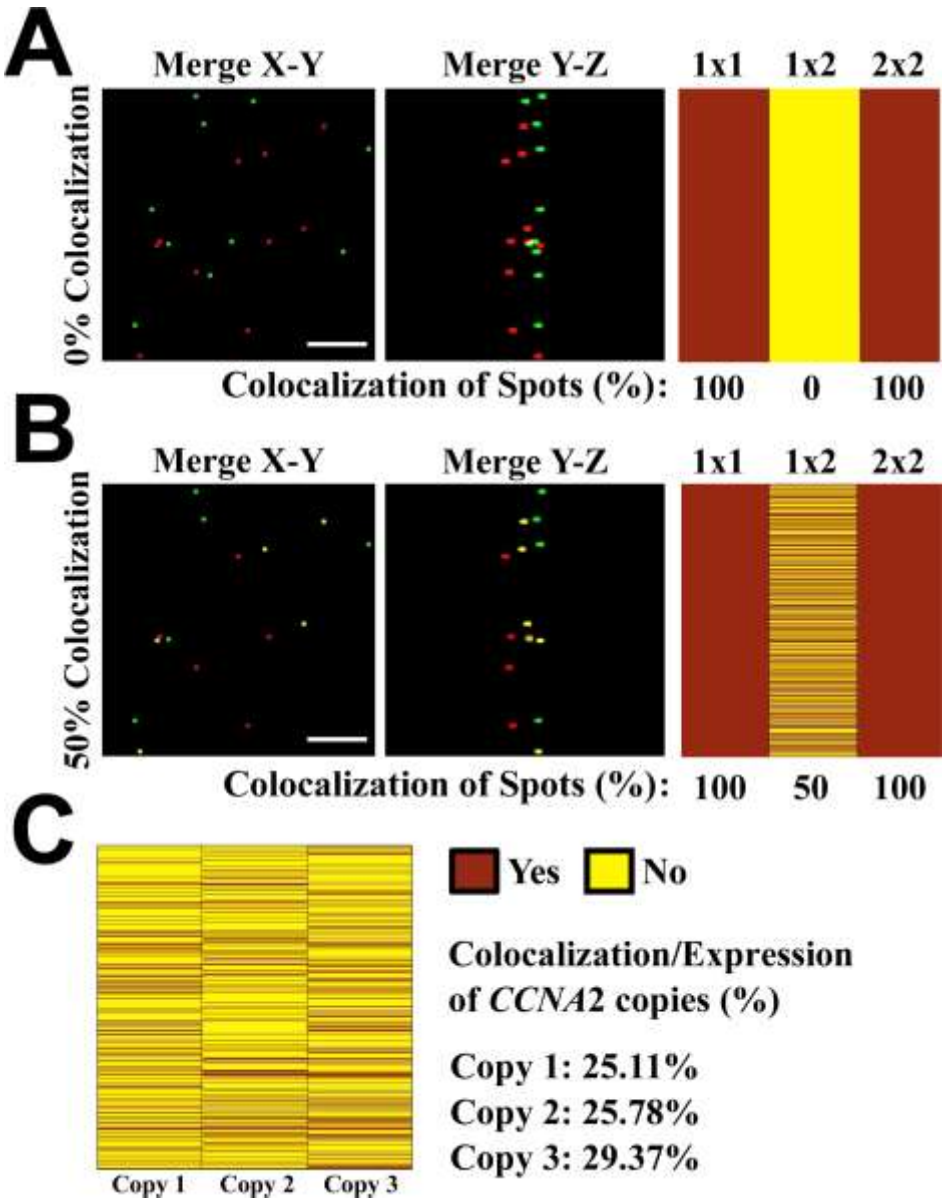

Figure R1: Benchmarking the colocalization metric between DNA FISH and RNA FISH Signals. For validation of the colocalization metric, synthetic 3D images of dimension 600x600x31 were generated with 10 randomly positioned spots which mimic the actual RNA FISH and DNA FISH signals. The stack number and pixel size were set to match with those for the actual images from the real experiments. Scale bar: 10 µm. (A) Red spots correspond to synthetic Image 1 and green to Image 2. For this panel, Image 1 has no spots colocalizing with Image 2. The first element in the panel shows X-Y projected image of the merge obtained for Image1 and Image 2 while the second element shows the Y-Z projected image of the same to visualize colocalization in the Z direction. The last panel shows the quantification for colocalization between the spots. Each line in this heat map matrix corresponds to a spot and represents 3D colocalization (yes or no) between spots from two images. 1x1 and 2x2 represent self-colocalization of the spots in Image 1 and Image 2 respectively while 1x2 represent the colocalization between the spots in Image 1 and Image 2. 'Yes' represents that the spots in the two images colocalize and vice versa for 'No'. As is observed the metric captures colocalization with 100% efficiency. More than 400 spots were analyzed in total. (B) Here images are generated such that Image 1 and Image 2 have exactly 50% of (that is 5) the spots colocalize. As seen in the second element of the panel, exactly 5 spots between Image 1 and Image 2 colocalize in 3D. Again, the metric quantifying the colocalization captures this with 100% efficiency. More than 400 spots were analyzed in total. (C) Quantification for colocalization between DNA FISH and RNA FISH signals for *CCNA2* in experimental images. Each line corresponds to a cell and represents the colocalization (yes or no) between DNA FISH and RNA FISH signals which also measures the expression

of a *CCNA2* copy in this case. More than 400 cells were analyzed in total. Copies are numbered such that Copy 1 is closest to the lamina followed by Copy 2 and then Copy 3. Copy 3, which is farthest from the periphery, shows a slightly higher percentage of expression. See Figure 1 for representative cell images.

Reviewer comment

Positive: An easy method to investigate gene regulation in 3D on a single-cell level.

Author response

We are happy for the reviewer's positive comment, especially because we have made all possible efforts to make this important technique approachable and available to anybody who wants to use it.

Reviewer comment

Further, questions remain after reading the article:

•The discussion and evaluation of the dynamic extrusion-based model is incomplete. The colocalization precision of DNA and RNA signals has not been evaluated.

Author response

This concern has now been addressed in our response to one of the previous comments (Figure R1).

Reviewer comment

In this context: how far can the looping out of genes be expected? Could it adequately be measured given the colocalization precision achieved in the experiments?

Author response

The topological model of gene regulation suggests that large mega-base pair looping happen only in undifferentiated embryonic stem cells (Chambeyron and Bickmore, 2004; Misteli, 2005; Morey et al., 2009; Osborne et al., 2004), whereas in differentiated cells, genes undergo only short excursions to the periphery of a chromosome territory to possible transcription factories (Osborne et al., 2004). Our study is limited by the diffraction limit of light, but even with such microscopic assays mega-base pair loops have been previously determined (Chambeyron and Bickmore, 2004) in ES cells. To determine the extent of looping, we have to paint the chromosome territory too, and check for relative colocalization, and our assay should be very much amenable to that. Additionally, unlike many previous studies, we will be able to follow transcript counts in the very same cells. We thank the reviewer for this very insightful suggestion and will explore it in future experiments. A discussion of this has now been included in the current manuscript.

Reviewer comment

Furthermore: a putative extrusion would possibly not only occur only towards the centroid/lamina. In case it would occur in a perpendicular direction with regards to the centroid/lamina direction - how would this affect the interpretation?

Author response

Yes, we completely agree with the reviewer that the extrusion might happen in any direction and we have taken care of that in our definition of 'Closeness' metric where absolute distances of the gene copies measured (with the accuracy that we have from the optical resolution) from the nuclear boundary and the nuclear centroid are converted into a number which grades the proximity of the copies with respect to the periphery between 0 and 1. This in fact is done in 3D and takes into account

an extrusion in an orthogonal direction.

The reviewer has again suggested a great possibility where one can use similar, slightly- modified 'Closeness' metric whereby one can measure the relative extrusion of a gene from a chromosome territory. And this is very easily addressed with the experimental and analysis tools developed in this study. We would further like to mention that indeed we are working in this direction whereby we can measure such excursions in 3D for known genes and look at the effect on the expression of those genes in the very same cells.

Reviewer comment

•It should be discussed, how long the mRNA does remain at a gene while or after being transcribed? Assuming the duration time is short and the cells are fixed at an mRNA-free moment false-negative results can be drawn regarding the activity of a gene.

Author response

The reviewer has raised a very interesting possibility and yes, we agree with the reviewer this can very well happen. And while an individual allele may be turned off at a given time, we also have access to the total transcript count in the cell, giving us a clear idea about the overall transcriptional status. It should be borne in mind that we are performing the experiments on an asynchronous population of cells, and in a population, on the average, we will be able to see an overall pattern. If a gene is transcribed more when closer to the nuclear centre that one expects to see and capture this phenomenon at the population level which should happen in most of the cases if not all. If no mRNA is present at a given allele at the moment of fixation, that allele would indeed be considered to be off at that point in time. I hope the reviewer agrees with us on this.

If there are multiple RNAP II units transcribing a gene at a given time, the gene locus shows up as a brighter spot in nucleus in smFISH experiments, and comparing its intensity to the intensity of cytoplasmic mature mRNA spots, one can estimate the number of nascent transcripts as done previously (Zenklusen et al., 2008). For the CCNA2 gene we do not see such clear bright nuclear spots and only some of the alleles colocalize with an mRNA spot, indicating perhaps that the gene is only intermittently transcribed.

Reviewer minor comments and author responses:

•Scale bars missing in: Figure 1B, 2B

We thank the reviewer for pointing this out. We have now added the scale bars to the images.

•Could an additional fixation step with glutaraldehyde help to improve the structural preservation of the genome?

We thank the reviewer for asking this question. We do think that an additional fixation step with glutaraldehyde might help preserve the structure a bit better. But then, it is reported that glutaraldehyde-based fixation leads to higher background in terms of increased autofluorescence in the cell (Mazumder et al., 2013) which can adversely affect subsequent imaging steps of single RNA molecules. Which is why, in our hands, 4% paraformaldehyde was a safer option especially in cases when multiplexing was necessary.

•In the section "3D nuclear segmentation" it should be stressed that the quantification of DNA content is used to define the cell cycle state

We have modified the manuscript to make this point clearer.

References

Chambeyron, S. and Bickmore, W. A. (2004). Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. *Genes Dev.* **18**, 1119– 1130.

Clemson, C. M., Hutchinson, J. N., Sara, S. A., Ensminger, A. W., Fox, A. H., Chess, A. and Lawrence, J. B. (2009). An Architectural Role for a Nuclear Noncoding RNA: NEAT1 RNA Is Essential for the Structure of Paraspeckles. *Mol. Cell* **33**, 717–726.

Lomvardas, S., Barnea, G., Pisapia, D. J., Mendelsohn, M., Kirkland, J. and Axel, R. (2006). Interchromosomal Interactions and Olfactory Receptor Choice. *Cell* 126, 403–413.

Mazumder, A., Pesudo, L. Q., McRee, S., Bathe, M. and Samson, L. D. (2013). Genome- wide single-cell-level screen for protein abundance and localization changes in response to DNA damage in S. cerevisiae. *Nucleic Acids Res.* **41**, 9310–9324.

Misteli, T. (2005). Concepts in nuclear architecture. *BioEssays* **27**, 477–487.

Morey, C., Kress, C. and Bickmore, W. A. (2009). Lack of bystander activation shows that localization exterior to chromosome territories is not sufficient to up-regulate gene expression. *Genome Res.* **19**, 1184–94.

Osborne, C. S., Chakalova, L., Brown, K. E., Carter, D., Horton, A., Debrand, E., Goyenechea, B., Mitchell, J. A., Lopes, S., Reik, W., et al. (2004). Active genes dynamically colocalize to shared sites of ongoing transcription. *Nat. Genet.* **36**, 1065– 1071.

Raj, A., Peskin, C. S., Tranchina, D., Vargas, D. Y. and Tyagi, S. (2006). Stochastic mRNA synthesis in mammalian cells. *PLoS Biol.* **4**, 1707–1719.

Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A. and Tyagi, S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. *Nat. Methods* **5**, 877–9.

Zenklusen, D., Larson, D. R. and Singer, R. H. (2008). Single-RNA counting reveals alternative modes of gene expression in yeast. *Nat. Struct. Mol. Biol.* **15**, 1263–1271.

Second decision letter

MS ID#: JOCES/2020/246330

MS TITLE: Investigating cell cycle-dependent gene expression in the context of nuclear architecture at a single allele resolution

AUTHORS: Shivnarayan Dhuppar and Aprotim Mazumder
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed my concerns successfully. The key points became clearer and the context of the work is now appropriately described.

Comments for the author

-