Recognition of RNAs by the S9.6 antibody creates pervasive artefacts when imaging RNA:DNA hybrids

John Smolka, Lionel Sanz, Stella Hartono, and Frederic Chedin

Corresponding Author(s): Frederic Chedin, UC Davis Health System

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	Revision Received:	2021-03-05

Monitoring Editor: Roger Greenberg

Scientific Editor: Melina Casadio

Transaction Report:

May 14, 2020

Re: JCB manuscript #202004079

Dr. Frederic Chedin
UC Davis
Molecular and Cellular Biology
One Shields Avenue
Davis, CA 95616

Dear Dr. Chedin,

Thank you for submitting your manuscript entitled "Recognition of cellular RNAs by the S9.6 antibody creates pervasive imaging artefacts" to the Journal of Cell Biology. The manuscript has now been assessed by expert reviewers, whose reports are appended below. Thank you very much for your patience with the peer-review process. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that we received mixed assessments from our expert reviewers as to the definitiveness of the results and their fit for JCB. A major concern from the referees was that, although the paper clearly establishes the importance of appropriate controls for the use of the S9.6 antibody, it also somewhat oversimplifies this issue in the literature and may cast doubt on published studies that were multifaceted and didn't simply rely on S9.6 IF. We queried Reviewers #2-#3 on these points and Reviewer #2 agreed with Reviewer #1 in comments to us. In addition, at submission, we shared and discussed some of these concerns editorially as well. We were concerned that the paper seemed like an atypical submission for our Report format, which is meant for definitive, cutting-edge observations of outstanding interest to a wide readership that have the potential to open up new avenues of research. We nevertheless wanted to get more input from experts in review given our strong interest in the field.

We have discussed the reviewers' feedback in depth editorially. We agree with the reviewers that sharing these data with the R-loop community is important. We also note that there are published studies, as cited in your manuscript, that did not include appropriate controls for the staining and this is an important takeaway from your studies. However, we are concerned that the technical report that IF experiments with the S9.6 antibody need appropriate controls and should be interpreted with caution does not provide the level of definitive advance that is needed for publication as a JCB Report. Our view is that the work would be a better candidate for JCB if you could show that the S9.6 antibody isn't reliable for IF and then use a different methodology to bring forward new insights. Unfortunately, we do not have the level of reviewer support that we would need to proceed further with the paper with its current scope. We do realize that significant further work and expansion might convincingly address some of these issues, but we are hesitant to encourage you to work towards the aim of further consideration at JCB. The level of reviewer criticism makes it impossible for us to guarantee that we will be able to invite resubmission, even after revision. As it currently stands, we agree with Reviewers #1-2 that the paper is most appropriate for a technical journal. Our journal office will transfer your reviewer comments to another journal upon request. Should you be interested in extending the work to include an alternative methodology, we'd be happy to consider the paper as a new submission in our Tools

format.

Although we regret that we are not able to consider your manuscript further, we have discussed your manuscript with the editors of Life Science Alliance (http://www.life-science-alliance.org/) and they would like to offer publication of the work in Life Science Alliance, pending minor revision. The comments of the reviewers should get addressed in a point-by-point response and by changes to the manuscript text and data representation. It would be good to include the missing control (Rev#2, point 1) as well. LSA is our academic editor-led, open-access journal launched as a collaboration between RUP, EMBO Press, and Cold Spring Harbor Lab Press. You can use the link below to initiate an immediate transfer of your manuscript files and reviewer comments to LSA. Please feel free to reach out to Life Science Alliance Executive Editor Andrea Leibfried if you have any questions.

Link Not Available

We are sorry our decision is not more positive but hope that you find the reviews constructive. Of course, this decision does not imply any lack of interest in your work and we look forward to future submissions from your lab.

Thank you for your interest in the Journal of Cell Biology.

Sincerely,

Roger Greenberg, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, the authors characterized the specificity of the S9.6 antibody to detect DNA:RNA hybrids in immunofluorescence analysis. They found that both the cytoplasmic and nuclear S9.6 staining cannot be eliminated by pretreatment of cells with RNase H. In contrast, pre-treatment of cells with RNase T and RNase III reduced S9.6 staining. Moreover, using Cy5-labeled DNA:RNA hybrids, they showed that S9.6 can detect exogenous DNA:RNA hybrids in cells, and the S9.6 signals are sensitive to RNase H1. Based on these results, they suggest that S9.6 can only reliably detect exogenous but not endogenous DNA:RNA hybrids in immunofluorescence analysis. As they reported before, the S9.6 antibody shows high specificity in the DRIP-seq analysis, as the R-loops detected by DRIP-seq are all sensitive to RNase H1. I completely agree with the authors that the specificity of S9.6 for DNA:RNA hybrids in immunofluorescence studies must be carefully controlled. Although the experiments in this study are quite informative for future studies on R-loops, they cannot generally disprove the previous immunofluorescence studies using S9.6. I think that these experiments should be published as a technical caution to the R-loop field. However, it is more suitable to a technical journal rather than a high-impact journal like JCB.

1. The authors cited a list of previous papers that used S9.6 and stated that they did not use RNase H1 to demonstrate the specificity of S9.6 staining. This is definitely incorrect. In fact, RNase H1 was used in many of the studies.

2. It should also be emphasized that many previous studies involved S9.6 immunofluorescence analyses used protocols that were different from what was tested in this study. It is difficult for me to understand how one can use one protocol to argue against other protocols. Conditions for cell fixation, extraction, and staining may all affect the specificity of S9.6. For example, a lot of the non-specific S9.6 signals detected in this study can be removed or reduced by optimized cell extractions.

3. It is also important note that many previous studies have expression of RNase H1 in cells suppressed S9.6 staining. Given that R-loops are embedded in chromatin in cells, it is very likely that RNase H1 cannot get access to endogenous R-loops in fixed cells. In contrast, transfected Cy5-labeled DNA:RNA hybrids should be easily accessed by RNase H1. When RNase H1 is expressed in cells, it should have a much better chance to get access to endogenous R-loops. The results in this study only suggest that "pretreatment of fixed cells with RNase H1" is not the way to go, but these negative results on RNase H1 should not be over interpreted.

4. The sensitivity of cytoplasmic and nuclear S9.6 signals to RNase III and RNase T is informative. However, these background signals from ssRNA and dsRNA could be substantially removed or reduced by cell extractions. The specific detection of chromatin-bound DNA:RNA hybrids by S9.6 is still technical feasible. Cell extractions are commonly used to distinguish chromatin-bound and free fractions of the same proteins in immunofluorescence studies. They are certainly helpful to reduce the backgrounds from free ssRNA and dsRNA.

5. I cannot follow the logic to use RNase H1 to compare the specificities of S9.6 in immunofluorescence and DRIP-seq. These are two very different assays. In immunofluorescence experiments, endogenous R-loops are embedded in chromatin in fixed cells, and exogenous RNase H1 may not have the access to these R-loops. However, in DRIP-seq experiments, sheared DNA was digested with RNase H1. Of course RNase H1 works much better on sheared DNA than in fixed cells. Why is this surprising?

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, Smolka et al. studied the tendency of the S9.6 antibody, used widely in literature to detect RNA:DNA hybrids, to bind single-stranded and double-stranded RNA species non-specifically under immunofluoresence conditions. They find that S9.6 antibody detects mostly cellular RNA, rather than RNA:DNA hybrids, during immunofluoresence, as its signal is most clearly diminished by application of RNase targeting RNA, but not by RNase H targeting RNA:DNA. They further find that in contrast to these immunofluoresence imaging artefacts, S9.6 signal from genome-wide sequence-based mapping remains Rnase H-sensitive, indicating that methods such as DRIP-seq are accurate for measuring RNA:DNA hybrid occupancy. The authors' results therefore represent an important consideration for future experimenters who wish to probe RNA:DNA hybrid presence by imaging.
Major:
1. While cy3 hybrids are clearly reduced on Rnase H treatment, the authors should show that total cellular levels of R-loops are reduced in Xlinked cells using Dot blots. This is a critical control to see whether Rnase H has reduced total cellular levels of R-loops in fixed cells and not just by using labeled oligos as a proxy for hyrbids.
Minor:

2. Various figures - Please include scale bars for all images.
3. Discussion - The authors suggest using controls to validate previous S9.6 IF observations. It might be helpful to clarify this refers to RNase T1/III pre-treatment, rather than Cy5-labeled synthetic hybrids.

Reviewer #3 (Comments to the Authors (Required)):

This paper carefully analyzes the specificity of the S9.6 monoclonal antibody to detect R-loops. The study is very important as this antibody has been used in numerous studies. The work demonstrates that while the S9.6 antibody is very powerful to detect R-loops upon immunoprecipitation of nucleic acids, it recognizes RNA that is not in an R-loop configuration during immunofluorescence. The study outlines important controls that should be conducted when using this antibody to distinguish signal from noise. Overall, this paper will be very useful for researchers working on R-loops.

Comments:

1. Please indicate the cell type that was used for the data in figure 2.

2. Please indicate in the figure legends if n refers to number of cells, number of images or something else.

3. RNase III is supposed to cleave dsRNA into 12-15 bp dsRNA fragments. S9.6 recognizes hybrids of a minimal length of 6-8 nt. Why does S9.6 recognize long dsRNA, but not 12-15 bp long dsRNA fragments?

4. The same reference is stated twice as 2019a and 2019b: "Sanz, L.A., and F. Chedin. 2019. High-resolution, strand-specific R-loop mapping via S9.6-based DNARNA immunoprecipitation and high-throughput sequencing. Nat Protoc.".

Response letter to the reviewers.

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, the authors characterized the specificity of the S9.6 antibody to detect DNA:RNA hybrids in immunofluorescence analysis. They found that both the cytoplasmic and nuclear S9.6 staining cannot be eliminated by pretreatment of cells with RNase H. In contrast, pre-treatment of cells with RNase T and RNase III reduced S9.6 staining. Moreover, using Cy5-labeled DNA:RNA hybrids, they showed that S9.6 can detect exogenous DNA:RNA hybrids in cells, and the S9.6 signals are sensitive to RNase H1. Based on these results, they suggest that S9.6 can only reliably detect exogenous but not endogenous DNA:RNA hybrids in immunofluorescence analysis. As they reported before, the S9.6 antibody shows high specificity in the DRIP-seq analysis, as the R-loops detected by DRIP-seq are all sensitive to RNase H1. I completely agree with the authors that the specificity of S9.6 for DNA:RNA hybrids in immunofluorescence studies must be carefully controlled. Although the experiments in this study are quite informative for future studies on R-loops, they cannot generally disprove the previous immunofluorescence studies using S9.6. I think that these experiments should be published as a technical caution to the R-loop field. However, it is more suitable to a technical journal rather than a high-impact journal like JCB.

We thank the reviewer for finding our study informative and agreeing with the need to carefully control the results of immunofluorescence (IF) studies using the S9.6 antibody. As a clarification, our goal was not to "disprove" previous IF results but to raise awareness regarding critical shortcomings surrounding the use of S9.6 in IF studies and to offer users a set of rigorous, easy to adopt controls that will enable the validation of past and future findings.

1. The authors cited a list of previous papers that used S9.6 and stated that they did not use RNase H1 to demonstrate the specificity of S9.6 staining. This is definitely incorrect. In fact, RNase H1 was used in many of the studies.

In response to the reviewer's comment, we have carefully reviewed our statements and citations. One point of clarification concerns whether RNase H1 was used in an exogenous pre-treatment or expressed endogenously in cells. We view these situations as quite distinct. We have clarified that our statement in the Introduction referred to the addition of RNase H1 exogenously to fixed cells and ensured that every following reference was appropriately mentioned.

On the topic of exogenous vs. endogenous RNase H1, we refer the reviewer to a recent publication in which this was extensively covered (Chedin et al., 2021). This issue is also further discussed below in answer to comment #3.

2. It should also be emphasized that many previous studies involved S9.6 immunofluorescence analyses used protocols that were different from what was tested in this study. It is difficult for me to understand how one can use one protocol to argue against other protocols. Conditions for

cell fixation, extraction, and staining may all affect the specificity of S9.6. For example, a lot of the non-specific S9.6 signals detected in this study can be removed or reduced by optimized cell extractions.

The manuscript readily acknowledged that pre-extractions etc… have been used by various laboratories to limit "non-specific signals". In response to the reviewer's comment, we revised the Discussion to clarify that pre-extractions can be used "to reduce the amount of contaminating cellular RNAs". This should make it clear that we are not arguing "against these other protocols". Our work, instead, focused on revealing the source of the prominent cytoplasmic and nucleolar signals observed by many S9.6 imaging studies.

In this revised version, we show that S9.6 doesn't just engage in low affinity multivalent binding interactions with a variety of cellular RNAs. We provide key evidence that S9.6 possesses a strong and specific affinity for the highly abundant ribosomal RNAs. We therefore conclude that S9.6 is NOT strictly specific for RNA:DNA hybrids and that off-target binding to ribosomal RNAs is an inherent biochemical property of the S9.6 antibody. This realization is a fundamental point of our work.

Given this, it is reasonable to infer that all immunofluorescence studies relying on S9.6, regardless of whether they used pre-extractions or not, will be vulnerable to issues of non-specific binding. In that context, we note that pre-extractions may not fully or specifically remove highly abundant rRNAs and may also affect the underlying R-loop signals in ways that are hard to control. In addition, pre-extractions have the potential to affect sample integrity, possibly altering the cytological distribution of the signal. These uncertainties informed our choice of a straightforward (and often used) staining protocol devoid of any pre-extraction so we could reveal and study all S9.6 signals accessible in situ without added variables. We then devised stringent and specific ribonuclease controls to assess the impact of cellular RNAs on the staining patterns. Ultimately, our work supports that the S9.6 IF signal observed in normally cultured human cells is resistant to RNase H1 treatments not because of insufficiencies in RNase H1 activity, but because S9.6 is predominantly labeling RNase T1-sensitive RNA species, predominantly ribosomal RNAs.

These new findings further elevate concerns about the origin of the S9.6 IF signals and reinforce the need to develop rigorous specificity controls. As now discussed in the revised manuscript, the well-known fact that ribosome pools are highly responsive to a variety of cellular stresses increases the odds that changes in S9.6 signals could have been misinterpreted as changes in RNA:DNA hybrids, when they reflected changes in ribosome homeostasis. We hope these points were effectively clarified and conveyed in our revised Discussion.

3. It is also important note that many previous studies have expression of RNase H1 in cells suppressed S9.6 staining. Given that R-loops are embedded in chromatin in cells, it is very likely that RNase H1 cannot get access to endogenous R-loops in fixed cells. In contrast, transfected Cy5-labeled DNA:RNA hybrids should be easily accessed by RNase H1. When RNase H1 is expressed in cells, it should have a much better chance to get access to endogenous R-loops. The results in this study only suggest that "pretreatment of fixed cells with

RNase H1" is not the way to go, but these negative results on RNase H1 should not be over interpreted.

The issues surrounding the targeting and function of endogenous RNase H1 in cells are quite a bit more complex than portrayed here. We refer the reviewer to our recent publication (Chedin et al., 2021) discussing these issues. In particular we note that there are wide discrepancies between RNase H1 binding sites mapped via ChIP-seq and R-loops mapped via DRIP-seq experiments. We therefore view the reviewer's statement that "when RNase H1 is expressed in cells, it should have a much better chance to get access to endogenous R-loops" as currently unsupported. Instead, evidence suggests that it is most likely that cellular RNase H1 can only access a subset of short R-loops that occur in conjunction with promoter-proximal paused RNA polymerase complexes (Chedin et al., 2021). Likewise, we are not aware of data that supports the reviewer's statement that exogenous "RNase H1 cannot get access to endogenous R-loops in fixed cells" "given that R-loops are embedded in chromatin".

The reviewer is nonetheless correct to point out that some (not many) studies have shown a reduction in S9.6 staining upon endogenous RNase H1 expression. The significance of that observation is quite unclear given the major concerns raised here about S9.6 staining. Furthermore, we and others have shown that over-expressing RNase H1 may cause a variety of indirect effects on cells, including alterations to gene expression programs that may artificially cause staining patterns to change. Interestingly, genes involved in ribosome biogenesis and nucleolar function were the most responsive to the manipulation of endogenous RNase H levels in *S. pombe* (Hartono et al., 2018). Similarly, RNase H1 over-expression in human cells was reported to lead to a dramatic increase in S9.6 nucleolar signals (Sollier et al., 2014). Given these caveats, which we recently reviewed (Chedin et al., 2021), we respectfully suggest that the biological significance of observed reductions of S9.6 IF signal upon RNase H1 expression in cells remains murky at this point. We continue to believe that exogenous RNase H1 treatments, combined with the use of transfected, labeled, RNA:DNA hybrids, represent the best approach for validating the specificity of the S9.6 signal in IF studies. We revised the manuscript to include a brief discussion of exogenous vs. endogenous RNase H1 expression and thank the reviewer for raising this point.

4. The sensitivity of cytoplasmic and nuclear S9.6 signals to RNase III and RNase T is informative. However, these background signals from ssRNA and dsRNA could be substantially removed or reduced by cell extractions. The specific detection of chromatin-bound DNA:RNA hybrids by S9.6 is still technical feasible. Cell extractions are commonly used to distinguish chromatin-bound and free fractions of the same proteins in immunofluorescence studies. They are certainly helpful to reduce the backgrounds from free ssRNA and dsRNA.

As mentioned above, the revised manuscript contains new data showing that the S9.6 IF signal is indistinguishable in both its distribution and nuclease sensitivity from that of ribosomal RNAs. In addition, we demonstrate that S9.6 directly binds with high affinity and specificity to purified native human ribosomes. Thus, the affinity of S9.6 for rRNAs is an intrinsic property of this antibody. While washes and cell extractions may help to reduce some of this signal, they will not change the fact that S9.6 is not RNA:DNA hybrid specific. This is particularly problematic given that rRNAs are the most abundant RNA species in cells and that rRNAs are found both throughout the cytoplasm, in the nucleus, and in nucleoli. It remains to be established whether

extractions and/or washes can specifically do away with the issue of rRNA recognition to enable quantitative S9.6 imaging.

As a result, we unfortunately do not share the reviewer's declaration that "The specific detection of chromatin-bound DNA:RNA hybrids by S9.6 is still technical feasible". Indeed, we tried to determine whether RNase H-sensitive IF signal can be detected after cellular RNAs have been removed by RNase T1/III treatment. Our data so far (Figure S2E) shows that this is not the case, at least for normally cultured human cells. Thus, our work suggests that RNA:DNA hybrids or R-loops are cytologically undetectable in normally cultured human cells using S9.6 as an imaging tool.

5. I cannot follow the logic to use RNase H1 to compare the specificities of S9.6 in immunofluorescence and DRIP-seq. These are two very different assays. In immunofluorescence experiments, endogenous R-loops are embedded in chromatin in fixed cells, and exogenous RNase H1 may not have the access to these R-loops. However, in DRIP-seq experiments, sheared DNA was digested with RNase H1. Of course RNase H1 works much better on sheared DNA than in fixed cells. Why is this surprising?

We apologize if the logic wasn't clear. The idea is that the identification of RNA binding as a predominant contributor to S9.6 signal in imaging assays raises broader concerns about the utilization of S9.6 in other assays. As highlighted in the revised discussion, over 100 proteins including almost all top interactors identified in an S9.6 proteomics screen published earlier by the Gromak lab correspond to ribosomal proteins most likely identified due to the affinity of S9.6 for rRNAs. Given that the Chedin lab has contributed numerous genomic R-loop mapping datasets, we wished to evaluate the validity of S9.6 as a genomics tool. This need is reinforced by prior observations that S9.6-based R-loop mapping results are liable to significant contamination by RNA species when sequencing libraries are built from RNA (Hartono et al., 2018; Chedin et al., 2021), as in the high-resolution, strand-specific method DRIPc-seq (Sanz et al., 2016). To address this concern, we optimized a novel version of the DRIP assay that permits high-resolution, strand-specific R-loop mapping that we demonstrate using the controls we established for IF, is not vulnerable to RNA contamination. Thus, this method offers an excellent alternative to DRIPc-seq when high-resolution strand-specific maps are desired. Note that the ability of S9.6 to perform well as a genomic tool does not entail a change in its specificity, but instead reflects the fact that sequencing libraries are built from immunoprecipitated DNA materials. We have significantly edited the manuscript to clarify our logic and better present these results and thank the reviewer for raising this point.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, Smolka et al. studied the tendency of the S9.6 antibody, used widely in literature to detect RNA:DNA hybrids, to bind single-stranded and double-stranded RNA species non-specifically under immunofluoresence conditions. They find that S9.6 antibody detects mostly cellular RNA, rather than RNA:DNA hybrids, during immunofluoresence, as its signal is most clearly diminished by application of RNase targeting RNA, but not by RNase H targeting RNA:DNA. They further find that in contrast to these immunofluoresence imaging artefacts, S9.6 signal from genome-wide sequence-based mapping remains Rnase H-sensitive, indicating that methods such as DRIP-seq are accurate for measuring RNA:DNA hybrid occupancy. The

authors' results therefore represent an important consideration for future experimenters who wish to probe RNA:DNA hybrid presence by imaging.

We thank the reviewer for agreeing that our results represent an important consideration for the future use of S9.6 as an imaging tool.

Major:
1. While cy3 hybrids are clearly reduced on Rnase H treatment, the authors should show that total cellular levels of R-loops are reduced in Xlinked cells using Dot blots. This is a critical control to see whether Rnase H has reduced total cellular levels of R-loops in fixed cells and not just by using labeled oligos as a proxy for hyrbids.

First, let us clarify that the treatment of Cy5-labeled RNA:DNA hybrids with RNase H1 was done in the context of methanol fixed cells, which are not crosslinked (methanol induces the precipitation of cellular materials, not crosslinking). Second, we are not exactly clear that the proposed control would be informative, or necessary to support our primary conclusions. The loss of S9.6 staining over transfected Cy5-labeled hybrids clearly shows that RNase H1 is active *in situ*, a key point with which the reviewer appears to agree. The lack of sensitivity of other signals to active RNase H1, together with the strong sensitivity to RNase T1 / III clearly indicates that the signal was derived from RNA, not RNA:DNA hybrids. We are unclear how RNase H1 treatment of genomic DNA extracted from fixed and/or crosslinked cells in a totally different type of assay (dot blot) will improve our conclusions. The crux of the matter is to demonstrate that RNase H1 is active *in situ* in imaging assays, which we did. We also note that prior work showed that at least formaldehyde crosslinking may affect RNase H activity (El Hage et al., 2014).

Minor:
2. Various figures - Please include scale bars for all images.

We did – thank you.

3. Discussion - The authors suggest using controls to validate previous S9.6 IF observations. It might be helpful to clarify this refers to RNase T1/III pre-treatment, rather than Cy5-labeled synthetic hybrids.

We meant to refer to both and have now added a sentence in the Discussion to clarify this point.

Reviewer #3 (Comments to the Authors (Required)):

This paper carefully analyzes the specificity of the S9.6 monoclonal antibody to detect R-loops. The study is very important as this antibody has been used in numerous studies. The work demonstrates that while the S9.6 antibody is very powerful to detect R-loops upon immunoprecipitation of nucleic acids, it recognizes RNA that is not in an R-loop configuration during immunofluorescence. The study outlines important controls that should be conducted when using this antibody to distinguish signal from noise. Overall, this paper will be very useful

for researchers working on R-loops.

We thank the reviewer for his/her support and agree that this study *"will be very useful for researchers working on R-loops"*.

Comments:

1. Please indicate the cell type that was used for the data in figure 2.

We did – thank you.

2. Please indicate in the figure legends if n refers to number of cells, number of images or something else.

It refers to number of cells – this was clarified.

3. RNase III is supposed to cleave dsRNA into 12-15 bp dsRNA fragments. S9.6 recognizes hybrids of a minimal length of 6-8 nt. Why does S9.6 recognize long dsRNA, but not 12-15 bp long dsRNA fragments?

Our data shows that S9.6 staining is minimally sensitive to RNase III pre-treatments. How to interpret this is slightly unclear. It could be that S9.6 binding to dsRNAs, whether they are long or chopped into 12-15 bp fragments, remains strong and unchanged. It could also be that the main target for S9.6 binding correspond to partially / transiently structured dsRNAs that are not ideal RNase III targets, explaining the modest impact of RNase III. Regardless, we show that S9.6 staining is: 1) acutely sensitive to RNase T1; and 2) primarily due to binding to ribosomal RNAs. We further verified that staining of ribosomal RNAs via the anti-rRNA antibody Y10b, shows a similar pattern of nuclease sensitivity as that observed by S9.6.

4. The same reference is stated twice as 2019a and 2019b: "Sanz, L.A., and F. Chedin. 2019. High-resolution, strand-specific R-loop mapping via S9.6-based DNARNA immunoprecipitation and high-throughput sequencing. Nat Protoc.".

Fixed.

REFERENCES

Chedin, F., S.R. Hartono, L.A. Sanz, and V. Vanoosthuyse. 2021. Best practices for the visualization, mapping, and manipulation of R-loops. *EMBO J*:e106394.
El Hage, A., S. Webb, A. Kerr, and D. Tollervey. 2014. Genome-wide distribution of RNA-DNA hybrids identifies RNase H targets in tRNA genes, retrotransposons and mitochondria. *PLoS genetics*. 10:e1004716.
Hartono, S.R., A. Malapert, P. Legros, P. Bernard, F. Chedin, and V. Vanoosthuyse. 2018. The Affinity of the S9.6 Antibody for Double-Stranded RNAs Impacts the Accurate Mapping of R-Loops in Fission Yeast. *J Mol Biol*. 430:272-284.

February 25, 2021

RE: JCB Manuscript #202004079R-A

Dr. Frederic Chedin
UC Davis Health System
Molecular and Cellular Biology
One Shields Avenue
Davis, CA 95616

Dear Dr. Chedin,

Thank you for submitting your revised manuscript entitled "Recognition of cellular RNAs by the S9.6 antibody creates pervasive imaging artefacts when imaging RNA:DNA hybrids". Two of the original referees were available to re-review the paper, and we contacted a new reviewer as well. All agree that the paper has technical merit. There were concerns from these experts about the suitability of the paper for JCB. Editorially, however, we feel that the study has the potential to provide important, definitive evidence to a question of outstanding interest to the community, and therefore we are favorable to publication.

We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). Please also respond to the final reviewer comments in the text and in a point-by-point response. We will leave it to you to decide whether to add any data. New experimentation is in our view not required for publication.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
Please revise the eTOC statement to meet our preferred style: it should start with "First author name(s) et al…" (as opposed to "we").

2) JCB Reports must have a combined "Results and Discussion" section. Please remove the "Discussion" header and make edits accordingly.

3) JCB Reports can have up to 5 main and 3 supplementary figures. Could some of the supplementary data be combined to meet this limit please? We really appreciate your efforts to format the paper for acceptance. Each figure can span up to one entire page as long as all panels fit on the page.

4) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 3BC (magnifications), 4A (magnification), S2ACE, S3 Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. **Please include molecular weight with unit labels on all gel panels if markers were run.**

5) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features *even if described in other published work or gifted to you by other investigators*
- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please add one brief descriptive sentence per item.

8) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

9) Author contributions: A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Roger Greenberg, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

In this revised manuscript the authors have provided some clarification for the comments raised in the first submission. While I still think that it is important to caution researchers against using S9.6 for imaging studies, this revised manuscript still lacks sufficient content and novelty.
A couple of points to consider - Many labs still refer to DRIPc as a gold standard for strand specific R-loop identification. Because the results here suggest that DRIPc should not be recommended because of the cross reactivity of S9.6 to RNA, the authors should more strongly state the DRIPc is

an inefficient technique that has a high potential for artifacts and help steer the field away from this technique. Second, many labs already perform DRIP based methods after sonication and these results have been published (Nadel et al 2015). To generate strand specific libraries, that is also used in other R-loop technologies such as R-ChIP, does not seem enough of a technical advance. Therefore, my level of enthusiasm for this manuscript remains low.

Reviewer #3 (Comments to the Authors (Required)):

The authors have addressed all my concerns very well. I consider the findings to be important and I recommend acceptance of the paper.

Reviewer #4 (Comments to the Authors (Required)):

This is a very informative and well written paper addressing several caveats associated with the use of the S9.6 antibody for cytological detection of RNA:DNA hybrids and R-loops. The weaknesses of the S9.6 antibody are known for quite some time now, however (surprisingly) this antibody is still widely used to detect hybrids by indirect immunofluorescence. The authors address this issue in a vary detailed manner using different cell lines and control treatments. I find the binding to rRNA particularly striking and informative. Overall, I think that this paper has real merit. I would have liked to see some novel mechanistic insights to feel completely comfortable in supporting publication in JCB (rather than in a more specialised journal); however, I fully recognise the importance and thoroughness of the work and agree with the points made by the authors to address the comments of the reviewers.

There is one experiment that I would suggest, which can definitely strengthen the data about rRNA recognition. The authors should deplete rRNA and probe with the S9.6 before and after depletion. While this might not be straightforward in cells, it can be performed very easily on total RNA followed by dot-blotting. I realise that this would not fully recapitulate a staining of fixed cells, however it would clearly validate that assumption that a lot of signal comes from rRNA and guide future experiments employing the antibody for hybrid detection in cells and/or nucleic acids preps.

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We agree with the reviewer that caution when using S9.6 in imaging is an important scientific issue in the R-loop field. To clarify, however, we are not arguing against the use of the antibody in imaging: we encourage its use within the framework of the controls established here and call for caution in data interpretation due to the newly described binding of S9.6 to ribosomal RNAs. We believe that addressing the longstanding issues with S9.6 imaging represents "sufficient content" and an important advance. In that regard, we view the findings described here as novel and know they will likely cause a wholesale re-interpretation of many published studies in the field.

Regarding caveats surrounding DRIPc: We explicitly acknowledged these issues and went as far as saying that the sDRIP methodology tested in this study should be favored over DRIPc, because DRIPc is vulnerable to the effects of nonspecific RNA recognition by S9.6. We note however, that DRIPc-seq, when correctly performed, remains completely valid and produces very valuable datasets.

Finally, we do not argue that sDRIP represents a major technical advance. Others have indeed used sonication previously (Nadel et al., 2015; Crossley et al., 2020), although we note that the datasets from Nadel et al. (2015) are broadly discordant from other DRIP datasets (Chedin et al., 2021). sDRIP simply represents a convenient method for R-loop mapping that is not susceptible to off-target RNA binding by S9.6, as we demonstrate here. Thus, genomic mapping of R-loops using S9.6 remains a robust methodology, which was the main point we wanted to establish here.

Reviewer #3 (Comments to the Authors (Required)):

The authors have addressed all my concerns very well. I consider the findings to be important and I recommend acceptance of the paper.

We are happy to hear that we have effectively addressed the reviewers concerns and thank the reviewer for their recommendation.

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We appreciate the reviewer's positive comments and the conclusion that the work has real merit and importance. We acknowledge that our data do not provide direct mechanistic insight into the biology of R-loops. However, the demonstration that S9.6 IF signals are primarily derived from ribosomal RNAs will likely cause a broad critical re-evaluation of previous mechanistic insights, and thus have a strong impact on the field. While not emphasized in the manuscript, our findings also suggest that changes in ribosome homeostasis are often associated with the perturbation of many RNA processing or genome maintenance factors studied previously as R-loop regulators. In that way, we believe that the work will ultimately be of significance to our understanding of the mechanisms linking RNA processing, genome stability, ribosome biogenesis, and R-loop metabolism.

We agree that the experiments suggested by the reviewer would strengthen the findings that S9.6 binds ribosomal RNA, especially if they could be performed in cells after rRNA depletion. As acknowledged by the reviewer, however, this may not be straightforward. In addition, we believe that the current data already provides very strong evidence that the large majority of the S9.6 signal originates from rRNA, including via direct biochemical assays using purified, native, functional, ribosomal complexes. Given

current constraints on time and personnel availability (the first author has moved on to a post-doctoral position and time is limited for other lab personnel with their own projects), along with our belief that the peer-reviewed form of this work needs to be available to the R-loop community as soon as possible, we respectfully request to move forward with publication without the addition of this experiment.