

In vivo structure profiling reveals human cytosolic and mitochondrial tRNA structurome and interactome in response to stress

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer 1: My main concern remains with my Comment 3. Reviewer 3 shares my concern (see their comments 2,3,7.) The authors' write in response to my Comment 3, "The key here is to normalize the DMS signal at each position to the overall DMS reactivity under in vivo or in vitro conditions so that we could reasonably compare the relative DMS signal of each nucleotide under two conditions. For example, one of the major conclusions from mRNA DMS mapping papers is that mRNAs are largely denatured in vivo (i.e., higher DMS signal) due to energy-driven processes like translation and unwinding, despite the fact that DMS concentrations would generally be lower in vivo. This is because the in vivo DMS signal has already been normalized to nearby residues under the same in vivo conditions, which is similar to what we are doing here with the τ score analysis in Figure 3."

However, their τ score only normalizes the differences between DMS reactivity in vitro and in vivo to the largest (and constant) difference between DMS reactivity in vitro and in vivo; in other words, it tells us what fraction of protection happened at a given nucleotide. Other methods of normalization, normalize each condition to its own reactivity rather than to a constant reactivity, which the authors themselves desire when they write "The key here is to normalize the DMS signal at each position to the overall DMS reactivity under in vivo OR in vitro conditions so that we could reasonably compare the relative DMS signal of each nucleotide under two conditions." The OR (my capitalization) is key in that each data set should be normalized by itself, not a constant that is the average of the two. The authors are advised to look at the dataset and SI from Su et al., PNAS, 115, 12170-12175 (2018). Finally, it is unclear why the DMS reactivity of tRNA in vivo is so low given that other recent studies obtained adequate DMS reactivities of tRNA in vivo, see Yamagami et al., PNAS 119, (2022). The authors should comment on this.

Reviewer #2

(Remarks to the Author)

The authors have addressed my comments and critiques in the revised manuscript.

Reviewer #4

(Remarks to the Author)

In this manuscript, Peña, Hou, Watkins et al. develop DM-DMS-MaPseq, which couples demethylase treatment with high-throughput RNA chemical probing so that mutation signatures from naturally occurring tRNA modifications are eliminated. The authors use DM-DMS-MaPseq to characterize the human tRNA structurome and assess how tRNA structure changes in response to oxidative stress. Overall, the authors' approach is creative and will likely be useful for RNA structure probing applications in which transcripts of interest are heavily methylated. However, I share several concerns that other reviews have expressed.

Comments:

1. I share the other reviewers' concern regarding the normalization strategy. In principle, it should be possible to compare data that has been normalized using a more typical approach, e.g. the approach described by Mitchell et al., 2023 (PMID: 37334863), in which the normalization factor for each nucleotide type is the maximum of the mean of the 90th-95th percentile modification rates and the 75th percentile of modification rates >0.001 . If this approach (or another similar approach) is not possible for some reason and cross-sample normalization is necessary, the τ score has two weaknesses: First, it is not clear why it is necessary to normalize each transcript individually. Ideally normalization should be applied uniformly across all transcripts within a data set. Second, even if an individual normalization approach is appropriate, the τ score is prone to effects caused by outliers since the denominator is the difference between the most reactive position on each isodecoder. Is there a reason not to use a normalization approach that excludes outlier reactivity values?

2. I also share the other reviewers' concern regarding low in cell DMS reactivity. Mitchell et al recently performed in cell DMS probing in HEK293 cells and obtained a mean modification rates of ~ 0.015 and ~ 0.05 for A and C nucleotides respectively. Although different conditions were used, it is still concerning that the modification rates observed in the current manuscript are so low.

3. The comparison of mt-tRNA-Lys in Figure S5a should include a plot in which traces for current and previous data are overlaid and can be compared directly. The current figure in which reactivity bins are compared is fine to include, but does not facilitate a clear comparison of the data.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)

The new analysis of mRNA reactivity that the authors have performed shows that the low reactivity observed for tRNAs in vivo is biologically meaningful. The authors have also provided a reasonable justification and evidence for the use of their τ score for normalizing tRNA reactivity. The authors have therefore addressed my concerns, and I support publication.

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Referee #1 (Remarks to the Author):

In this study, Peña and coworkers study the structure of human tRNAs of chromosomal and mitochondrial origins using DMS probing, specifically their DM-DMS-Map-seq method. They find that tRNAs have stable structures *in vivo*, and that *in vivo* and *in vitro* profiles are different. One of the more compelling parts of the study are the differences in the folds of ch. tRNAs and mt tRNAs during arsenite stress where the weaker folding mt tRNA suffer greater changes in folding. Moreover, I believe that the experiments were made stronger including a polysome enriched and depleted variable. This confirmed potential tRNA events that could be occurring to stall translation in the presence of oxidative stress.

Response: We thank the reviewer for the encouraging and insightful comments.

This is an ambitious paper that has many interesting findings. However, at the same time, the data in this study seem to be confounded by the reliance in decreased DMS signal *in vivo* vs. *in vitro*. The authors use the same DMS concentration under both conditions of 5% and the same reaction time of 5 min. They even use a higher temperature *in vivo* than *in vitro* (37 vs 30), which favors *in vivo* reactivity slightly. Nonetheless, DMS *in vivo* has to contend with getting into the cell (much of it will not) and then reacting with myriad other amines and imines in all the RNA, DNA and metabolites, which effectively leads to loss in DMS concentration and thus signal. In addition, it is hard to tell where proteins bind. In some cases the tau score is zero, but we cannot tell if there is differential (i.e. uneven) protection on the RNA, as expected for protein binding, or if there is uniform loss in DMS reactivity. The large number of tRNA-interacting proteins and RNPs in the cell confounds direct interpretation. This combined with the relatively small statistical significance makes it hard to draw strong conclusions.

Major Comments:

1. Introduction should focus more on current holes in tRNA structural knowledge.

Response: We added this description in lines 74-84:

*“tRNAs have highly conserved secondary and tertiary structures among the three kingdoms of life. They are often described as the classic cloverleaf secondary structures and “L-shaped” tertiary structures. Despite abundant studies on tRNA *in vitro* structures with X-ray crystallography and various chemical probing methods, the *in vivo* structures of tRNAs have been largely taken for granted to resemble their *in vitro* forms. However, tRNAs have many well-characterized protein interactions and modifications *in vivo*, which led us to hypothesize that their *in vivo* structural states could be more diverse and dynamic.”*

2. I don't understand how the DM method is any better than comparing + and – DMS. This needs to be made clearer to the reader. Mathematically, this is equivalent to asking how is mutation rate +DM +DMS different than mutation rate +DM -DMS? If DM removes all the methylations on A and C, then the two should subtract out. Is it because sometimes DMS is modifying bases other than A and C, or at positions on the sugar phosphate backbone?

Response: The DMS analysis for tRNA is confounded by the fact that the abundant tRNA modifications (average 13 per molecule for human tRNA) and strong structure significantly increases the background mutation rate in sequencing, making the direct comparison of just + versus -DMS difficult. To demonstrate this, we added a **new Fig. S1c** showing the mutation rate of each nucleotide of tRNA^{MET} with and without DMS or demethylase treatment, with the description in lines 146-150:

“This is because compared to mRNA, DMS signals for tRNAs are substantially weaker, and that many endogenous modifications present in tRNAs leave mutation signatures (**Supplemental Figure S1c**). Additionally, some tRNA modifications contribute to the background mutation rates of nearby residues; therefore, enzyme treatment to remove these modifications is necessary.”

3. Line 150, “The *in vivo* DMS signal is drastically lower than the *in vitro* DMS signal, consistent with 5S rRNA being protected as part of the ribosome *in vivo*.” But there are many other reasons why the *in vivo* DMS signal could be lower. For instance, not all the DMS goes into the cell and the DMS could be reacting with all the other imino and amino nitrogens in the cell, reducing the effective concentration of DMS. Moreover, why would a protein protect the entire RNA (every position on the RNA is much lower in counts *in vivo*).

a. That this same effect (of *in vitro* being much more reactive at all nt positions than *in vivo*) in Fig 1f, further makes this point.

b. The same point carries on in Fig 2b. And many conclusions are made from the apparent lower *in vivo* signal. e.g. the 3’NCCA binding proteins and ribosome.

Response: We agree that the overall lower signals can also be in part attributed to the lower reactivity of DMS *in vivo*. Indeed, the absolute reactivity is derived from a combination of *in vivo* protection by cellular proteins/ribosome and lower DMS reactivity. We edited the description in lines 169-172:

“The *in vivo* DMS signal is drastically lower than the *in vitro* DMS signal (**Figure 1c**), which could be due to the overall lower effective DMS concentration *in vivo* caused by inefficient diffusion and/or the fact that 5S rRNAs are protected as part of the ribosome *in vivo*.”

And in lines 225-228:

“Given that tRNAs form highly stable structures *in vitro*, lower DMS signal *in vivo* may be attributed to lower DMS reactivity *in vivo*, as DMS diffusion into cells may result in lower *in cellulo* DMS concentration. On the other hand, tRNAs interact extensively with proteins and ribosomes in cells, which can protect them from reacting with DMS.”

DMS mapping of mRNAs (multiple papers in the literature) also has the same issue when comparing *in vivo* and *in vitro* DMS signals. (We used the same DMS treatment conditions as mRNA studies.) The key here is to normalize the DMS signal at each position to the overall DMS reactivity under *in vivo* or *in vitro* conditions so that we could reasonably compare the relative DMS signal of each nucleotide under two conditions. For example, one of the major conclusions from mRNA DMS mapping papers is that mRNAs are largely denatured *in vivo* (i.e., higher DMS signal) due to energy-driven processes like translation and unwinding, despite the fact that DMS concentrations would generally be lower *in vivo*. This is because the *in vivo* DMS signal has already been normalized to nearby residues under the same *in vivo* conditions, which is similar to what we are doing here with the τ score analysis in Figure 3.

4. Fig. 1D. The authors point to residue DMS signal being increases with DMS concentration, but this is just one point. What about the other positions? We can see from the small snippet in this figure that residue 37 saturates at 2% DMS. This holds also in Fig. 1H where nt 33 is increases with DMS concentration, but 37 is not. So 2 of 4 points visible do not fit the trend the authors are trying to make a point of.

Response: Fig. 1d shows 3 biological replicates of DMS reactivity. The average of the three replicates at position 37 and 42 are both lower at 2% versus 5% DMS.

For Fig. 1h, we do not understand why position A37 saturated at both 2% and 5% DMS, but all other anticodon loop positions C31, C32, C33, A34, A36 show intermediary reactivity at 2% and higher DMS signal at 5% DMS (35 is U, not reactive with DMS), so this trends holds for most residues. Specifically, tRNA_i^{Met} has an N6-isopentenyladenosine (i⁶A) modification at A37 which may introduce unforeseen effects. We edited the description in lines 188-192:

“Zooming into the anticodon loop region of the *in vivo* samples again shows the expected no DMS signal for untreated, high DMS signal for high DMS concentration, and intermediate DMS signal for low DMS concentration (**Figure 1h**). The DMS signal at A37 is saturated at intermediate DMS treatment which may be related to tRNA_i^{Met} A37 being modified to N6-isopentenyladenosine (i⁶A).”

5. Conclusions are made regarding reactivity of SerAGA-4 relative to SerAGA-1 being associated to specific nt changes. But since there are multiple changes (three) and single variants aren't made. Thus, how can one be sure which of these three changes are responsible for which features opening up in the structure.

Response: We cannot be sure exactly which of the 3 residue changes dominates the structural effects. Our description is deduced from the common structural knowledge of RNA structures. We rewrote this portion to make it clear in lines 240-245:

“The conversion of A31-U39 in Ser(AGA)-1 to A31-C39 in Ser(AGA)-4 likely weakens the anticodon stem and leads to markedly increased DMS signals in the anticodon loop. The G10-U/C25 pair at the end of D stem is located ~15 Å away in the 3D structure to the end of the variable loop region. Thus the conversion of G10-C25 to G10-U25 may be responsible to the DMS signal increase in the variable loop (**Figure 2d**), perhaps transmitting long-range effects in structural change through the tertiary interaction network of tRNA.”

6. It is stated that higher-scoring isodecoders have lower DMS signals (and thus fold better). What might the lower scoring isodecoder be doing then?

Response: Both isodecoders still fold like tRNA, so in principle they are useful for protein synthesis. We added a **new Figure S2c** to show their similar proportion in the input RNA and in the polysome. We rewrote this portion in lines 254-257 to make it clear:

“On the other hand, the difference in structural accessibilities between these tRNA isodecoders does not translate to varied abilities to engage with the ribosome. Both tRNA isodecoders still fold like tRNA, as suggested by the participation of both isodecoders in translation at comparable ratios in the input and in the polysome (**Supplemental Figure S2c**).”

7. Fig 3A. It is not possible to tell if any of the low tau scores came from highly reactive positions *in vitro* and *in vivo* since they would subtract to zero in the same way that low reactive position *in vitro* and *in vivo* would. It is important to know this because high MINUS high implies an exposed region of tRNA that does NOT interact with proteins. If there are no such regions, then again it implies that that perhaps *in vivo* is just reducing the amount of DMS available to react. Perhaps Gini plots could be used to argue for protein binding, which should give an uneven protection (i.e. high Gini score). An argument is made to this effect in Fig 2b through different aaRS classes, but the difference is barely significant with a *p* of just 0.044, which is likely dominated by the one outlier in the “others” (Fig 3b) which could be excluded based on the fact that it has an unusual D loop, as the authors show in Fig S3D.

Response: As suggested, we made a Gini plot as [new Figure S1f](#) to compare the distribution of *in vitro* and *in vivo* DMS signals, and description in lines 194-197.

“To further demonstrate that the *in vivo* DMS signals are not just a reduced reactivity version of the *in vitro* signals, we made a Gini plot comparing the *in vitro* and *in vivo* DMS signals profiles of the same tRNAs (**Supplemental Figure 1f**). The *in vitro* and *in vivo* profiles have drastically different shapes, consistent with different factors contributing to these profiles.”

As suggested, we excluded the outlier ArgACG in Figure 3b and updated the *p*-value to be 0.021 (including ArgACG had a *p*-value of 0.044, outlier increases *p*-value). We also changed the figure legend in lines 771-772:

“The *p*-value is calculated with a standard t-test, excluding the outlier tRNA^{Arg}(ACG) (*p* = 0.044 including tRNA^{Arg}(ACG)).”

8. The study in Fig 3C that the energetic contribution of the tRNA body and amino acid are negatively correlated depends on the assumption that C75 tau as a proxy for binding. But this is just an end point assay since *in vivo* concentrations of all things are not known, and tight and weak binders can have the same endpoint, or even weak binders could end up with a more protected end point.

Response: We reason that the interaction between tRNAs and EF1A are highly dynamic *in vivo*. Therefore, the τ score more likely reflects the average interaction strength of the tRNA with EF1A among a dynamic ensemble of *in vivo* tRNAs. Hence, our results indicate that the average interaction strength of a tRNA with EF1A positively correlates with the energetic contribution of tRNA body to EF1A binding. We edited the description in lines 520-524:

“Given that the τ score reflects the average interaction strength of the tRNA with EF1A among a dynamic ensemble of *in vivo* tRNAs, this result suggests that the average interaction strength at the tRNA 3' end also inversely correlates with the amino acid binding affinity and the tRNA body sequence binding affinity to EF1A.”

9. Page 8◇ why is m1G9 reverse by demethylase treatment, when alkB is specific to m1A and m3C?

Response: We actually used a mixture of WT AlkB and AlkB D135S mutant which demethylates m1G. This mixture is more efficient in generating longer tRNA sequencing reads. M¹G also

potentially contributes to the background mutation rates of nearby residues. This information is already in line 660 in the method section. We also include it in the main text in lines 153-155:

“In addition to the wild-type AlkB, we also included the *E. coli* AlkB D135S mutant during DM treatment, which demethylates m¹G²⁵, to further lower background mutation rates and reduce RT stops.”

10. Fig 4C. It isn't clear how many amino acids are in each column nor how much scatter there was between replicates. The change in DMS score is small too, with medians in the 10⁻⁴ range. If there were atomic models to help understand the position 37 outcomes, they should be included.

Response: We added more detailed description in the figure legend in lines 794-800 (legend of Fig. 4c):

“Only the most abundant isodecoders from each isoacceptor family are included. tRNAs are grouped as follows: A37 (*n*=14): Asp, GluCTC, GluTTC, GlyCCC, GlyGCC, GlyTCC, GlnCTC, GlnTTC, ThrAGT, ThrCGT, ThrTGT, ValAAC, ValCAC, ValTAC; t6/i6A37 (*n*=12): IleAAT, IleTAT, LysCTT, LysTTT, Met, iMet, Asn, SerAGA, SerCGA, SerGCT, SerTGA, ArgCCT, ArgTCT; m1G/m1I/W37 (*n*=19): AlaAGC, AlaCGC, AlaTGC, ArgACG, ArgCCG, ArgTCG, Cys, His, LeuAAG, LeuCAG, LeuTAG, LeuCAA, LeuTAA, Phe, ProAGG, ProCGG, ProTGG, Trp, Tyr.”

We also added a Cryo-EM structure from the literature (**new Figure S4d**) highlighting position 37 at the ribosome A site and corresponding description in lines 379-381:

“The purine at tRNA position 37 closely interacts with the 18S rRNA in the ribosome which stabilizes codon-anticodon interaction at the A site (**Supplemental Figure 4d**)^{65,66}.”

11. Fig 3B and 4C,D. Sample sizes can also directly be included in the bar plots to get a sense of the number data points used to perform statistical analysis.

Response: We added the number of data points to the figure legends:

Fig. 3b: lines 770-771: “Charging of tRNA^{Leu}, tRNA^{Ser}, and tRNA^{Ala} (*n*=13) does not require anticodon recognition, while others (*n*=34) do.”

Fig. 4c: please see comment #10 above.

Fig. 4d: lines 804-805: “Average *n*=42 for the depleted group and *n*=14 for the enriched group for each region.”

12. Fig S5. There are bases missing in many of the structures with no explanation for it in the legend.

Response: Sorry about this omission. We added dashed line connection and explanation in the figure legend:

Fig. 5d, S5b, c: “The dashed lines connect the residues in this schematic for mt-tRNAs. Mt-tRNA positions are according to reference 28.”

13. The reason for focusing on oxidative stress wasn't clear, unless a molecular interpretation is possible. Is it? Could the changes seen be related to G oxidation? Sugar oxidations?

Response: Arsenite stress is known to have large effects on global translation and mitochondrial activity. We have explained the rationale in lines 349-355.

“In cells, global translation regulation is tightly linked to cellular metabolism and stress. As the critical components of the translation system, tRNAs undergo significant reprogramming in their expression, modification, charging, and fragmentation under stress^{24,60,61}. To understand the dynamics of cellular tRNA structure and interaction in response to stress, we treated cells with sodium arsenite to induce oxidative stress which is a common stress condition for the studies of translational stress response. Arsenite exposure induces strong phosphorylation of eIF2 α , which leads to significant downregulation of translation initiation⁶².”

G oxidation mostly leads to 8-oxo-G which is not detectable in our sequencing data.

Minor Comments

1. Ref 13 is out of order.

Response: fixed.

2. line 50. It would be more accurate to say that DMS reacts with unpaired RNAs rather than single-stranded RNAs since nearly all RNAs are single stranded. (we made the change.)

Response: fixed.

3. line 192. Is a reference missing?

Response: we added an explanation in lines 216-218: “The anticodon loop region shows the highest DMS signals which is consistent with this region forming the least extent of tertiary interactions among all loops: D, variable and T loops form the tertiary structural core in the canonical tRNA tertiary structure.”

4. line 468. end with a period after tRNA and start a new sentence with perhaps.

Response: fixed.

5. Fig 1b. The resolution isn't high enough to see the snoRNA signals clearly.

Response: We think the reviewer is referring to Fig. S1b. the snoRNA read fractions are <0.5% in these sequencing data which is just hard to see. We added this info in the figure legend.

6. Fig 1g. A structure capturing the differences would be helpful.

Response: Unfortunately, there is no high resolution human tRNA_i^{Met} structure on its own (all human tRNA_i^{Met} structures are in the ribosome).

Referee #2 (Remarks to the Author):

Peña et al. report on the development and use of a strategy to map the human tRNA “structurome”, based on chemical probing with dimethyl sulfate (DMS) coupled to mutational profiling (MaPseq) and RNA demethylation. The authors are pioneers of the tRNA sequencing methodology, and have presented this general approach in prior excellent manuscripts. The coupling of DMS and MaPseq (DMS-MapSeq), as well as its use to map tRNA structures in a high-throughput manner, have been previously described. Therefore, at its core, this study presents the addition of the demethylation step to those strategies. The authors should emphasize and make this clearer in the introduction, especially expand on lines 73-74, where they vaguely mention the mapping of tRNA structurome for E. coli. Overall, this is a well performed study demonstrating the power of their approach for *in vivo* investigations of RNAs. The results of the manuscript per se however are incremental and perhaps more appropriate for a specialized journal. That said, here are some comments and critiques to help the authors improve the work.

Response: we thank the reviewer for the encouraging and insightful comments.

We expanded the description of tRNA structure-seq (reference 20) in lines 82-84: “tRNA structure-seq results significantly improved tRNA structure prediction under *in vivo* conditions and revealed tRNA structural dynamics under heat stress which also correlated with changes in tRNA abundance.”

Major comments

- The authors explain their finding on the light of tRNA-protein interactions, whereby known tRNA binding proteins shield the RNA from reacting with DMS. They profoundly discuss this in the case of CCA tails, where they propose for the differential DMS profiles to be caused by interaction with EF1A. Furthermore, they use this as a proxy for EF1A affinity, which is used to explain the energetic contributions in aa-tRNA – EF1A binding (see the correlation in Fig 3c, from E. coli data of Uhlenbeck). Although this is a sound hypothesis, no validation/proof is offered. For this, it would be necessary to at least see the DMS changes upon EF1A knockdown, otherwise the discussion of those results it’s more suited for the Discussion section of the manuscript.

Response: EF-1A is an essential house keeping protein, so we are reluctant to do the knockdown, as interpretation of the results could involve multiple factors.

We agree with the reviewer that our result only infers a similarity between tRNA interaction with EF-1A and the established EF-Tu work. We moved the interpretative description from Results to the Discussion section in lines 519-526:

“Our analysis of tRNA C75 τ scores supports the same EF1A-tRNA uniform binding paradigm originally identified from *in vitro* prokaryotic EF-Tu-tRNA studies. Given that the τ score reflects the average interaction strength of the tRNA with EF1A among a dynamic ensemble of *in vivo* tRNAs, our result suggests that the average interaction strength at the tRNA 3' end also inversely correlates with the amino acid binding affinity and the tRNA body sequence binding affinity to EF1A. Since tRNAs are generally highly charged *in vivo*⁵³ and C75 is located closely to the charged amino acid, this result provides evidence for a similar EF1A-aa-tRNA interaction paradigm in human cells as previously described for *E. coli* EF-Tu-aa-tRNA.”

- The authors emphasize the high number of tRNA species in humans. However, the main results

are derived from the data of the most abundant ones. Could the authors discuss the DMS differences/similarities between abundant and rare isodecoders? A supplemental figure of those comparisons would be beneficial for the text and will further showcase the strength of the sequencing method.

Response: We added **new Figures S2d-e** and corresponding description in lines 258-262:

“DM-DMS-MaPseq can characterize the *in vivo* tRNA structures for “rare” tRNA isodecoders as well, define here as present at <10% levels of the most abundant isodecoder tRNA of the same anticodon family (**Supplemental Figure S2d**). As an exemplar, we present the *in vivo* DMS profiles of seven isodecoders in the tRNA^{Ala}(AGC) family together with their tRNAScan scores to showcase the diverse structural accessibilities among them (**Supplemental Figure S2e**).”

-Page 16. The effects on iMet observed upon oxidative stress are discussed under the light of its interaction with eIF2. However, no supporting evidence is given. This should be limited to stating the results and the speculation on possible explanations moved to the discussion.

Response: We agree with the reviewer on the nature of conclusion. We moved the interpretative description from Results to the Discussion section in lines 532-536:

“Intriguingly, tRNA_i^{Met} exhibits a high *in vivo* signal of C71 (tRNA nomenclature; C70 in the tRNA_i^{Met} position, **Figure 1f**) in the acceptor stem, which base pairs with G2 in its structure. This DMS signal is absent *in vitro*, suggesting that the C71-G2 base pair is weakened *in vivo*, potentially through its interaction with the eIF2B complex or the 48S scanning ribosome⁶³.”

-The discussion, in general, is a summary of the results section with little actual discussion of the what implications the described findings have. It needs to be reworked.

Response: We reworked the Discussion section to be more concise and to reflect more implications.

-Perhaps the coolest result is the difference in mito tRNA structure *in vivo* and *in vitro*. This is probably the major new discovery of this work and should be emphasized more in a revised manuscript.

Response: Thanks very much for pointing this out. We added “cytosolic and mitochondrial” tRNA in the title, the abstract, and multiple places in the text to emphasize our results on both types of tRNAs.

Minor comments:

- Given the dimensionality of the DMS quantities used in the figures, it would be ideal to visualize those numbers in the log scale. Especially for figure 1, where plots seem to be truncated (1f, 1c).

Response: We tried to replot e.g. Figures 1f, 1c in log scale, and decided that it is not ideal because these emphasize the smaller differences, so we left the figures as presented. Hope this is ok.

-The various metrics used to describe the different types of DMS differences are confusing. Perhaps a schematic of them atop the main figures could help. Or a succinct explanation of the estimate in the main text, instead of just the methods.

Response: We added the metric description used in each figure at the beginning of figure legends.

-References needed in lines 240-41

Response: References added (lines 275-276 in revision).

Referee #3 (Remarks to the Author):

The study by Pena et al. presents an interesting study on tRNA structurome in response to stress, but I have several major concerns, particularly regarding the success and interpretation of the structural probing experiments and the arguments about protein binding protection.

Response: we thank the reviewer for the critical comments which improved our paper.

Major points:

1. The pipeline includes a protocol of library construction of MSR-seq. Although the method has been published, it is crucial to briefly mention how it works. Does it enrich tRNAs? How about the effectiveness?

In fact, I have objections to give this pipeline a new name “DM-DMS-MaPSeq”. It appears to be an application of DMS-MaPSeq to study tRNA structurome, with an additional step of tRNA enrichment.

Response: We believe that this comment was from a misunderstanding of how our sequencing method works. There is no tRNA enrichment step experimentally, we use total RNA as input for sequencing library construction. DM stands for “demethylase” which is an enzyme treatment step during library preparation, no relationship to tRNA enrichment. We provided a detailed description to make it clear in lines 123-133.

“For *in vivo* structure profiling, the protocol started with DMS treatment on live human HEK293T cells. Total RNAs were extracted and used directly as the input for library preparation. For *in vitro* structure profiling, DMS treatment was performed on extracted, deproteinated total RNA. Following the MSR-seq protocol²⁴, total RNAs, including deacylated tRNAs, were ligated to barcoded, biotinylated multiplexing adapters. Multiple barcoded samples were combined and immobilized on streptavidin beads. The combined samples were split into two, one mock and the other treated with two recombinant *E. coli* AlkB demethylases (DM), which selectively demethylated m¹A and m³C^{26,29}. Library construction proceeded with reverse transcription using the thermostable Superscript IV reverse transcriptase and an optimized reaction condition, followed by on-bead second adapter ligation and PCR to generate DNA libraries for Illumina sequencing.”

2. A critical concern is the very low DMS reactivity scores observed, which raises doubts about

the success of the *in vivo* probing experiments. The strong dose-dependent effects shown in Figures 1d and 1h suggest the DMS treatment may not be sufficient. The authors should thoroughly assess the quality of the DMS probing by comparing the obtained RNA structures to known structures and ensuring agreement with previous studies.

Response: Compared to mRNA DMS signals, DMS signals for tRNA are substantially weaker, and the many modifications present in tRNA also increases the background mutation rate in sequencing read mapping. This is why we employed the demethylase treatment (DM) step to ascertain the DMS signals.

The DMS reactivity is derived from a combination of *in vivo* protection by cellular proteins/ribosome and lower DMS reactivity. We edited the description in lines 169-172:

“The *in vivo* DMS signal is drastically lower than the *in vitro* DMS signal (**Figure 1c**), which could be due to the overall lower effective DMS concentration *in vivo* caused by inefficient diffusion and/or the fact that 5S rRNAs are protected as part of the ribosome *in vivo*.”

And in lines 225-228:

“Given that tRNAs form highly stable structures *in vitro*, lower DMS signal *in vivo* may be attributed to lower DMS reactivity *in vivo*, as DMS diffusion into cells may result in lower *in cellulo* DMS concentration. On the other hand, tRNAs interact extensively with proteins and ribosomes in cells, which can protect them from reacting with DMS.”

We cannot find *in vivo* DMS data for human 5S rRNA or tRNA_i^{Met} in the literature for comparison. The single literature data of *in vivo* DMS data for human tRNA is for mitochondrial tRNA^{Lys}, which we do compare in response to comment #6 below.

3. The central argument about proteins protecting bound regions from DMS probing requires very careful re-evaluation. Previous studies have shown that RNA structural probing can effectively detect protein-bound regions, so the "protection" observed here may be an artifact of inadequate DMS treatment. The authors need to extensively revisit this fundamental premise of their study.

Response: The effective concentration of DMS is lower *in vivo* and leads to generally lower global DMS reactivity. DMS mapping of mRNAs (multiple papers in the literature) also has the same issue when comparing *in vivo* and *in vitro* DMS signals. (We used the same DMS treatment conditions as mRNA studies.) The key here is to normalize the DMS signal at each position to the overall DMS reactivity under *in vivo* or *in vitro* conditions so that we could reasonably compare the relative DMS signal of each nucleotide under two conditions. For example, one of the major conclusions from mRNA DMS mapping papers is that mRNAs are largely denatured *in vivo* (i.e., higher DMS signal) due to energy-driven processes like translation and unwinding, despite the fact that DMS concentrations would generally be lower *in vivo*. This is because their *in vivo* DMS signal has already been normalized to nearby residues under the same *in vivo* conditions, which is similar to what we are doing here with the τ score analysis in Figure 3. We clarified this issue in lines 282-288:

“To assess the *in vivo* and *in vitro* differential DMS signals in individual tRNA regions and infer information on tRNA-protein interaction, we introduce the τ score, which is the normalized difference between *in vitro* and *in vivo* DMS signals at each nucleotide relative to the largest delta across the entire molecule for each tRNA isodecoder (**Supplemental Figure S3a**).

In part due to the inefficient diffusion of DMS into cells which can lower the overall DMS reactivity *in vivo*, the normalization of τ score is critical to compensate for the global DMS reactivity differences between the *in vivo* and *in vitro* conditions.”

4. The claim that known natural modifications are largely reversed by DMS treatment is not sufficiently clear from the data in Figures 1b-d and 1h.

Response: We think the reviewer meant DM (demethylase) treatment. The removal of natural modifications is >95%. We added a **new Figure S1d** to show the effect of DM treatment in DMS-treated *in vitro* and *in vivo* samples and added a description in lines 155-157:

“Globally, the removal of natural modifications with DM is >95%, as shown by the largely reduced mutation rates of the native m¹A modifications from a mean of ~70% to <1% with DM treatment (**Supplemental Figure S1d**).”

5. The small size of Figures 1b-d and 1h makes them very difficult to read. Figures showing tRNA structures should be labeled with nucleotide indices to facilitate interpretation. In particular, the correspondence between Figures 1f and 1g is unclear.

Response: We increased the size of labels in Fig. 1b-d, h, and we added residue numbers in the tRNA diagrams in Figs. 1g, 2c, 2e, 5b, 5c, 5d, 6d. We edited the figure legend of figure 1g in lines 733-736 to make its relationship with 1f more clear:

“(g) Secondary structure of tRNA^{iMet} overlaid with *in vitro* and *in vivo* DMS signals (average of three biological replicates for each group). Each A or C nucleotide is colored based on DMS signal values: no color: < 0.00125; light blue: 0.00125-0.0025; blue: 0.0025-0.005; yellow: 0.005- 0.01; orange: 0.01-0.02; red: > 0.02.”

6. A visual inspection of Figures 2a, 2c, and 2e does not reveal good agreement between the structural models and the DMS scores, for example, for SerGCT. The authors should perform AUC calculations and benchmark them against previous studies.

Response: We believe that the comment on Fig. 2a, 2c, and 2e was a misunderstanding on what is presented. Fig. 2a compares *in vitro* and *in vivo* results of just the most abundant tRNA isodecoder from each anticodon family. Fig. 2c, 2e compares only the *in vivo* data for two tRNA isodecoders from SerAGA or ValUAC families. Fig. 2a is unrelated to Fig. 2c and 2e. We rewrote this sentence (lines 232-234) to make this clear:

“To further explore how sequence variations between high confidence isodecoders impact tRNA folding *in vivo*, we compared only the *in vivo* DMS signal profiles of two pairs of tRNA isodecoders with different tRNAScan scores.”

There are no previous DMS studies on human tRNA structures *in vivo*. We only found human mitochondrial tRNA^{Lys} DMS mapping data *in organello* from a recently published paper (reference 23) whereas our results were *in cellulo*. Nevertheless, we made a direct comparison to the published and our data on mt-tRNA^{Lys} in a **new Figure S5a**, revealing highly comparable results.

7. The near-absence of *in vivo* DMS signals in Figure 2b is concerning. The authors should report the average score for all transcripts and nucleotides.

Response: The original Fig. 2b data included the stem residues and U and G nucleotides that do not have DMS signals. Since the purpose of this figure is to compare *in vitro* versus *in vivo* data, we remade Fig. 2b to include only DMS reactive A and C residues in the respective loop regions for each tRNA residue pair ($x - \text{in vitro}$, where $x = \text{in vitro}$ or *in vivo*) in Figure 2b with new legend in lines 747-750:

“(b) Box-whisker plot of DMS signal differences for each A and C residues in every tRNA using the formula ($x - \text{in vitro}$) where $x = \text{in vitro}$ (1), or *in vivo* (2) in D, T, anticodon, variable loops, and the 3' unpaired CCA regions. *P*-values are calculated with standard Wilcoxon t-test; ****: $p < 10^{-4}$.”

8. The authors should provide more details on the statistical tests used and their *p*-values. Moreover, the differences in DMS signals between *in vivo* and *in vitro* conditions may be explained not only as structural differences, but also by differential protein binding levels for different transcripts and regions. This is an argument that may shake the ground of the whole story and many conclusions/discussions.

Response: We provide more descriptions of statistics in the figure legends where shown, 2b, 3b, 3d, 3e, 4c, 4d, 6e.

We already point out that the *in vivo* and *in vitro* difference is a combination of both structural differences and protein/ribosome binding. As the reviewer points out, this is why we use τ score to remove DMS reactivity bias and focus on the protein/ribosome interaction difference. Please also see the detailed response to comment #3.

9. The subsection from page 11 to 15 is overly long and explanatory. This is also a severe problem of the entire study. Many results and discussions are very subjective.

Response: We rewrote this section “*In vivo-in vitro* difference in DMS signals reflects cytosolic tRNA-protein/ribosome interactions” to be more clear and less subjective, and reduced the description from 4.5 pages to 3.7 pages.

10. Given the low *in vivo* DMS signals, the authors should perform a quality check on the Tao score analysis by performing it for other RNA types and comparing with previous studies.

Response: As mentioned under response to comments #2 and #6, we could not find previous human rRNA or tRNA DMS mapping data from mRNA structural mapping papers. The only one we can find is for mitochondrial tRNA^{Lys} for which we made a [new Fig. S5a](#) for comparison (comment #6).

11. Without clear nucleotide indices in the structural charts, it is very challenging to follow the discussions in the subsection on page 16.

Response: We added nucleotide indices related to the description above the heatmap in Fig. 4a.

12. The small DMS signal differences of 0.001-0.002 reported in Figures 4 and 5 raise concerns about their biological relevance.

Response: The original Fig. 4d data include the stem residues and U and G nucleotides that do not have DMS signals. We remade Fig. 4d using only DMS reactive A and C in the respective loop regions with the corresponding figure legends in lines 802-806:

“(d) Box-whisker plot of DMS signal differences for each A and C residues in every tRNA in D, T, anticodon, variable loops, and the 3’ unpaired CCA regions. Depleted (= 1) or enriched (= 2): tRNAs that are significantly decreased or increased in the polysomes with arsenite, respectively. Average $n=42$ for the depleted group and $n=14$ for the enriched group for each region. P -values are calculated with standard Wilcoxon t-test; n.s.: not significant, **: $p<10^{-2}$.”

We also updated the description for Figure 4d in lines 391-394:

“The Δ DMS signals at A and C residues in the anticodon loop and 3’ CCA are higher in the polysome-depleted tRNAs compared to the polysome-enriched tRNAs, consistent with the loss of tRNA-ribosome interactions due to the reduced translation usage for polysome-depleted tRNAs under arsenite stress.”

No such data is presented in Fig. 5, we think the reviewer meant Fig. 6. The original Fig. 6e data include the stem residues and U and G nucleotides that do not have DMS signals. We remade Fig. 6e using only DMS reactive A and C in the respective loop regions with the corresponding figure legends in lines 846-850:

“(e) Box-whisker plot of DMS signal differences for each A and C residues in every tRNA in D, T, anticodon, variable loops, and the 3’ unpaired CCA regions. Depleted (= 1) or enriched (= 2): tRNAs that are significantly decreased or increased in the polysome fraction with arsenite, respectively. P -values are calculated with standard Wilcoxon t-test; n.s.: not significant, *: $p<0.05$, **: $p<0.01$, ****: $p<10^{-4}$.”

13. Line 374, you mean figure 4d? And appropriate controls and analyses are needed to substantiate these findings. What about the structural changes at other (stem) regions?

Response: Yes, we meant Fig. 4d, sorry about the typo. We have replotted them to show only the DMS reactive A and C residues in the respective loop regions. There is no DMS reactivity for the stem residues of the cytosolic tRNAs. Please also see the response to comments #12.

14. Figure 5, again, please label the nucleotides and their indices, and calculate the AUC scores to assess the probing data quality.

Response: We labeled the residue numbers in Fig. 5. Please also see response to comment #8. For comparing to literature data (i.e. AUC curves), please see response to comment #6.

15. Line 408-410, how to tell the “in vivo DMS signals confirm its mostly stable stems”? Line 411, how to tell it is the most unstable? Again, the authors' interpretations of stable and unstable

regions based on DMS signals are problematic, as they may be influenced by differential protein binding rather than intrinsic RNA structure.

Response: We rewrote this part of the text to be more clear in lines 418-423:

“Mt-tRNA^{Leu}(UAA) is considered to be one of the most stable mt-tRNAs, and the lack of *in vivo* DMS signals confirm its mostly stable stems. Despite that, we see DMS signals for the unpaired A12:C23 at the D stem and A31:C39 at the anticodon stem *in vitro* (**Figure 5b**). Type III mt-tRNA^{Ser}(GCU) is believed to be the least stable among all mt-tRNAs *in vitro*. However, its structure is largely stabilized *in vivo*, as shown by the reduced number of DMS reactive nucleotides in all three stems (**Figure 5c**).”

16. Line 412, I have difficulty to see the “reduced DMS reactive nucleotides”

Response: We marked the acceptor stem, anticodon stem, and the T stem in Fig. 5c and in the figure legend. The color coded DMS reactivity scale bar is also shown directly in Fig. 5c.

17. Line 418, “show strong signature”, what does this “strong signature” mean?

Response: We rewrote this part to be more clear in lines 428-430:

“The DMS signals for mt-tRNA^{Val} have much higher values throughout this tRNA *in vitro* but were largely reduced and more restricted in the D and anticodon loops *in vivo* (**Figure 5e**).”

18. Line 447, where is the data supporting the “acute loss of their interactions”?

Response: We rewrote this to be more clear in lines 458-460 and added references on loss of mitochondrial translation under arsenite stress:

“This mitochondrial versus cytosolic tRNA change may be exacerbated by the severe loss of mitochondrial translation under arsenite stress⁷⁷⁻⁷⁹.”

Minor points:

1. The low quality of the Figure 1 line charts makes them difficult to evaluate.

Response: Fig. 1 line charts shows the biological triplicate data to demonstrate the sequencing data quality. Most sequencing papers only show the averages, not individual sequencing samples. In our experience, our data is of superior quality for tRNA sequencing.

2. The authors should highlight the specific residues mentioned in the text (line 440).

Response: Done.

Point-by-point response to the reviewer's comments

Reviewer #1

My main concern remains with my Comment 3. Reviewer 3 shares my concern (see their comments 2,3,7.) The authors' write in response to my Comment 3, "The key here is to normalize the DMS signal at each position to the overall DMS reactivity under *in vivo* or *in vitro* conditions so that we could reasonably compare the relative DMS signal of each nucleotide under two conditions. For example, one of the major conclusions from mRNA DMS mapping papers is that mRNAs are largely denatured *in vivo* (i.e., higher DMS signal) due to energy-driven processes like translation and unwinding, despite the fact that DMS concentrations would generally be lower *in vivo*. This is because the *in vivo* DMS signal has already been normalized to nearby residues under the same *in vivo* conditions, which is similar to what we are doing here with the τ score analysis in Figure 3."

However, their τ score only normalizes the differences between DMS reactivity *in vitro* and *in vivo* to the largest (and constant) difference between DMS reactivity *in vitro* and *in vivo*; in other words, it tells us what fraction of protection happened at a given nucleotide. Other methods of normalization, normalize each condition to its own reactivity rather than to a constant reactivity, which the authors themselves desire when they write "The key here is to normalize the DMS signal at each position to the overall DMS reactivity under *in vivo* OR *in vitro* conditions so that we could reasonably compare the relative DMS signal of each nucleotide under two conditions." The OR (my capitalization) is key in that each data set should be normalized by itself, not a constant that is the average of the two. The authors are advised to look at the dataset and SI from Su et al., PNAS, 115, 12170-12175 (2018).

Response: We thank the reviewer for the continued discussion that makes our manuscript stronger.

We thank the reviewer for suggesting Su et al. 2018. It used an internal global normalization method, which applies a single normalization factor for each experimental condition that is the mean of 90th-98th percentile range. This method is generally effective when the data are more consistent internally and differ significantly under different conditions. In our tRNA study, however, such normalization methods can have two caveats: (i) they do not consider the heterogeneity between different tRNAs, which is far greater than mRNAs and other non-coding RNAs. (ii) They select a percentile range that is close to the maximum mutation rate in tRNAs which are typically from the non-DMS-derived, heavily modified residues whose signals cannot be removed through demethylation, thus irrelevant to "real" DMS signals.

tRNAs vary greatly in cells in their abundance, variety, and density of modifications (on average 13 modifications per human tRNA molecule). When choosing *in vivo* versus *in vitro* normalization methods, we strive to balance between minimizing internal bias and cross-condition bias. In essence, the question comes down to whether (i) a specific tRNA is more comparable between *in vitro* and *in vivo* conditions or (ii) under either condition, different tRNAs are more comparable to each other. Given our interest in unveiling biological insights into the differences among tRNAs, we prioritized minimizing internal bias. Our τ score first calculates the positional DMS signal differences between *in vitro* and *in vivo* conditions. Next, for each tRNA transcript, we normalized the positional DMS signal difference to the maximum

difference. The advantage of our approach is that it compares tRNA by tRNA and position by position. For heavily modified tRNA positions, mutation signals tend to be very high both *in vivo* and *in vitro*, and the subtraction can partially reduce the gap between positions. The normalization to the maximum difference simply brings the scale of the τ score between 0-1.

In any case, we did experiment with the suggested normalization method from Su et al 2018. The calculated normalization factor is 0.013 for *in vivo* and 0.033 for *in vitro*, which are both way out of the range of true DMS signals in our tRNA datasets and likely reflect the outliers of naturally occurring, heavily modified residues.

As a side note: In the Su et al., 2018 paper, the normalization strategy comes from a heuristic rule based on general experience in their laboratory. There does not seem to be a standardized normalization method in the RNA structural mapping field. Due to the confounding effects of the pervasive natural tRNA modifications and the associated complications in tRNA structural mapping, we believe that the typical normalization methods for mRNA and other non-coding RNAs may not be more appropriate for our study.

We now cite Su et al. (reference 43) and include these discussions in the manuscript regarding these issues of normalization and DMS reaction conditions on pages 14-15.

“Our τ score normalization differs from other internal global normalization methods used in the literature^{43,44} which apply a single normalization factor for each experimental condition that is the mean of a certain percentile range (e.g. 90-98th or 90-95th). These methods are generally very effective when the data are more consistent internally and differ significantly under different conditions. In our tRNA study, however, such normalization methods can have two caveats: (i) they do not consider the heterogeneity between different tRNAs, which is far greater than mRNAs and other non-coding RNAs. (ii) They select a percentile range that is close to the maximum mutation rate in tRNAs which are typically from the non-DMS-derived, heavily modified residues whose signals cannot be removed through demethylation, thus not relevant to “real” DMS signals.

The high tRNA variability in abundance, variety and modification density necessitates choosing a balance between minimizing internal bias and cross-conditional bias in choosing *in vivo* versus *in vitro* normalization methods. In essence, the question comes down to whether (i) a specific tRNA is more comparable between *in vitro* and *in vivo* conditions or (ii) under either condition, different tRNAs are more comparable to each other. Given our interest in unveiling biological insights into the differences among tRNAs, we prioritized minimizing internal bias. τ score first calculates the positional DMS signal differences between *in vitro* and *in vivo* conditions. Next, for each tRNA transcript, we normalized the positional DMS signal difference to the maximum difference. The advantage of our approach is that it compares tRNA by tRNA and position by position. For heavily modified tRNA positions, mutation signals tend to be very high both *in vivo* and *in vitro*, and the subtraction can partially reduce the gap between positions. The normalization to the maximum difference simply brings the scale of the τ score between 0-1.”

Finally, it is unclear why the DMS reactivity of tRNA *in vivo* is so low given that other recent studies obtained adequate DMS reactivities of tRNA *in vivo*, see Yamagami et al., PNAS 119, (2022). The authors should comment on this.

Response: (A) Our understanding of the main concern of the reviewer's comment is that the *in vivo* and *in vitro* difference may be derived from a systematically low DMS reactivity *in vivo* for the multiple reasons outlined by the reviewer. To further address this we did the following analysis:

(i) We compared the *in vivo* DMS signals for the cytosolic tRNA and the mitochondrial tRNAs (Fig. R1 = new Fig. S1f). Because of the low structural stability, the mitochondrial tRNAs are expected to have higher DMS reactivity which is exactly what we observe.

However, this result is the opposite to the prediction of a systematical low reactivity. In cells, mitochondrial tRNAs are enclosed in mitochondria with an extra membrane protection against DMS diffusing in. Therefore, intracellular DMS concentration should be lower in mitochondria compared to the cytosol so that we would expect a lower DMS reactivity for mitochondrial tRNAs. Clearly, our result shows the opposite.

(ii) We also mapped the DMS reactivity in mRNA reads in our sequencing data. Our MSR-seq uses total RNA as input so 0.5-1 million mRNA reads are still present together with the tRNA reads. We found that the mRNA median Adenosine mutation rates were ~10-times higher than those in tRNAs (Fig. R2 = new Fig. S1g).

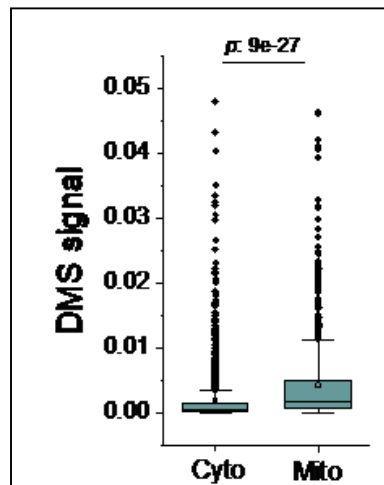


Figure R1 (new Fig. S1f). DMS signals for cytosolic tRNAs and mitochondrial tRNAs showing higher reactivity of mito-tRNAs. This result is the opposite of the expectation for systematically low DMS reactivity *in vivo*.

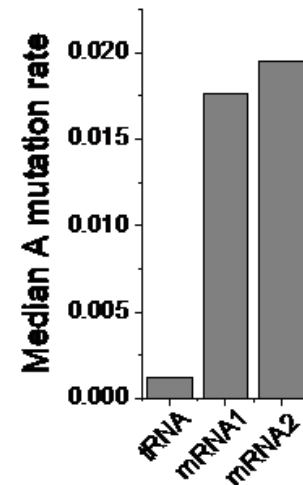


Figure R2 (new Fig. S1g). Median mutation rate for A residues in tRNAs and mRNAs to measure DMS reactivity *in vivo*. The positional read filter to calculate the mutation rate is ≥ 100 for mRNA1 and ≥ 500 for mRNA2.

We now include these results as new Fig. S1f, S1g and the associated description on p. 10, first paragraph.

“To further demonstrate that the *in vivo* DMS signals are not just a reduced reactivity version of the *in vitro* signals, we did three additional analyses. First, we compared the *in vivo* DMS signals for the cytosolic tRNA and mitochondrial tRNAs (**Supplemental Fig. S1f**). Because of the low structural stability, the mitochondrial tRNAs are expected to have higher DMS reactivity which is what we observe. However, in cells, mitochondrial tRNAs are enclosed in mitochondria with an extra membrane protection against DMS diffusing in. Therefore, higher *in vivo* reactivity of mitochondrial tRNAs contradicts a model of systematic low reactivity of *in vivo* versus *in vitro* conditions. Second, we mapped the DMS reactivity in mRNA reads in our sequencing data. MSR-seq uses total RNA as input so 0.5-1 million mRNA reads are still present together with the tRNA reads. We found that the mRNA median Adenosine mutation rates were ~10-times higher than those in tRNAs (**Supplemental Fig. S1g**), indicating that our *in vivo* DMS condition is within the

range of reactivity in other mRNA structural mapping studies. Third, we made a Gini plot comparing the *in vitro* and *in vivo* DMS signal profiles of the same tRNAs (**Supplemental Figure 1h**). The *in vitro* and *in vivo* profiles have drastically different shapes, consistent with different factors contributing to these profiles.”

(B) Yamagami et al. 2022 is the reference 20 in our manuscript. The differences in mutation rates between our manuscript and Yamagami et al. can be explained by the different experiment conditions and the biological differences between human cell lines and *E. coli* cells. We used 5% of DMS and treated HEK293T cells at 37°C for 5 min. HEK293T cells are adherent cells that grow as a thin layer attached to the bottom of the cell culture dish. Yamagami et al. applied 1% (100 mM DMS) to treat *E. coli* cell suspension at 37°C for 5 min. Although Yamagami et al. used a gentler DMS treatment, human cells with more complex cell membranes may uptake DMS from the culture solution much less efficiently than *E. coli* cells, leading to a lower intracellular DMS concentration. Furthermore, it is also possible that human tRNAs are better protected by proteins than *E. coli* due to the distinct tRNA processing and regulation between eukaryotic and prokaryotic systems.

We now include this discussion on p. 25, first paragraph.

“The tRNA structural mapping differences between *E. coli*²⁰ and our study on human cells can be attributed to both the different experimental conditions and the biological differences. HEK293T are adherent cells that grow as a thin layer attached to the bottom of the cell culture dish. *E. coli* cells were suspensions when treated with DMS. Although the *E. coli* study used a gentler DMS treatment, human cells with more complex cell membranes may uptake DMS from the culture solution at different efficiencies as *E. coli* cells, leading to a lower intracellular DMS concentration in human cells. Furthermore, it is also possible that human tRNAs are better protected by proteins than *E. coli* due to the distinct tRNA processing and biological regulation of translation between eukaryotic and prokaryotic systems.”

Reviewer #4

In this manuscript, Peña, Hou, Watkins et al. develop DM-DMS-MaPseq, which couples demethylase treatment with high-throughput RNA chemical probing so that mutation signatures from naturally occurring tRNA modifications are eliminated. The authors use DM-DMS-MaPseq to characterize the human tRNA structurome and assess how tRNA structure changes in response to oxidative stress. Overall, the authors' approach is creative and will likely be useful for RNA structure probing applications in which transcripts of interest are heavily methylated. However, I share several concerns that other reviews have expressed.

Response: We thank the reviewer for the encouraging and insightful comments.

Comments:

1. I share the other reviewers' concern regarding the normalization strategy. In principle, it should be possible to compare data that has been normalized using a more typical approach, e.g. the approach described by Mitchell et al., 2023 (PMID: 37334863), in which the normalization factor for each nucleotide type is the maximum of the mean of the 90th-95th percentile modification rates and the 75th percentile of modification rates >0.001. If this approach (or another similar approach) is not possible for some reason and cross-sample normalization is

necessary, the τ score has two weaknesses: First, it is not clear why it is necessary to normalize each transcript individually. Ideally normalization should be applied uniformly across all transcripts within a data set. Second, even if an individual normalization approach is appropriate, the τ score is prone to effects caused by outliers since the denominator is the difference between the most reactive position on each isodecoder. Is there a reason not to use a normalization approach that excludes outlier reactivity values?

Response: We appreciate the reviewers' advice regarding our normalization approach. Both reviewers 1 and 4 suggest internal global normalization methods, which apply a single normalization factor for each experimental condition that is the mean of a certain percentile range (90th-98th in Su et al., 2018 suggested by reviewer 1, and 90th-95th in Mitchell et al., 2023 suggested by reviewer 4). These methods are generally effective when the data are more consistent internally and differ significantly under different conditions. In our tRNA study, however, such normalization methods can have two caveats: (i) they do not consider the heterogeneity between different tRNAs, which is far greater than mRNAs and other non-coding RNAs. (ii) They select a percentile range that is close to the maximum mutation rate in tRNAs which are typically from the non-DMS-derived, heavily modified residues whose signals cannot be removed through demethylation, thus not relevant to "real" DMS signals.

tRNAs vary greatly in cells in their abundance, variety, and density of modifications (on average 13 modifications per human tRNA molecule). When choosing *in vivo* versus *in vitro* normalization methods, we strive to balance between minimizing internal bias and cross-condition bias. In essence, the question comes down to whether (i) a specific tRNA is more comparable between *in vitro* and *in vivo* conditions or (ii) under either condition, different tRNAs are more comparable to each other. Given our interest in unveiling biological insights into the differences among tRNAs, we prioritized minimizing internal bias. Our τ score first calculates the positional DMS signal differences between *in vitro* and *in vivo* conditions. Next, for each tRNA transcript, we normalized the positional DMS signal difference to the maximum difference. The advantage of our approach is that it compares tRNA by tRNA and position by position. For heavily modified tRNA positions, mutation signals tend to be very high both *in vivo* and *in vitro*, and the subtraction can partially reduce the gap between positions. The normalization to the maximum difference simply brings the scale of the τ score between 0-1.

In any case, we did experiment with the suggested normalization methods. We obtained a Pearson $r = 0.812$ between the Su et al. 2018 and the Mitchell et al. 2023 method, suggesting that both normalizations generate similar results. Using the Su et al., 2018 method as exemplar, the calculated normalization factor is 0.013 for *in vivo* and 0.033 for *in vitro*, which are both way out of the range of true DMS signals in our tRNA datasets and likely reflect the outliers of naturally occurring, heavily modified residues.

As a side note: In the Su et al., 2018 paper, according to the authors, the normalization strategy comes from a heuristic rule based on general experience in their laboratory. In the Mitchell et al., 2023 paper, according to the authors, their normalization is more robust for RNAs where most nucleotides are unreactive, such as the ribosome RNAs. There does not seem to be a standardized normalization method in the RNA structural mapping field. Due to the confounding effects of the pervasive natural tRNA modifications and the associated complications in tRNA

structural mapping, we believe that the typical normalization methods for mRNA and other non-coding RNAs may not be more appropriate for our study.

We now cite Mitchell et al. (reference 44) and include these discussions on pages 14-15.

“Our τ score normalization differs from other internal global normalization methods used in the literature^{43,44} which apply a single normalization factor for each experimental condition that is the mean of a certain percentile range (e.g. 90-98th or 90-95th). These methods are generally very effective when the data are more consistent internally and differ significantly under different conditions. In our tRNA study, however, such normalization methods can have two caveats: (i) they do not consider the heterogeneity between different tRNAs, which is far greater than mRNAs and other non-coding RNAs. (ii) They select a percentile range that is close to the maximum mutation rate in tRNAs which are typically from the non-DMS-derived, heavily modified residues whose signals cannot be removed through demethylation, thus not relevant to “real” DMS signals.

The high tRNA variability in abundance, variety and modification density necessitates choosing a balance between minimizing internal bias and cross-conditional bias in choosing *in vivo* versus *in vitro* normalization methods. In essence, the question comes down to whether (i) a specific tRNA is more comparable between *in vitro* and *in vivo* conditions or (ii) under either condition, different tRNAs are more comparable to each other. Given our interest in unveiling biological insights into the differences among tRNAs, we prioritized minimizing internal bias. τ score first calculates the positional DMS signal differences between *in vitro* and *in vivo* conditions. Next, for each tRNA transcript, we normalized the positional DMS signal difference to the maximum difference. The advantage of our approach is that it compares tRNA by tRNA and position by position. For heavily modified tRNA positions, mutation signals tend to be very high both *in vivo* and *in vitro*, and the subtraction can partially reduce the gap between positions. The normalization to the maximum difference simply brings the scale of the τ score between 0-1.”

2. I also share the other reviewers' concern regarding low in cell DMS reactivity. Mitchell et al recently performed in cell DMS probing in HEK293 cells and obtained a mean modification rates of ~0.015 and ~0.05 for A and C nucleotides respectively. Although different conditions were used, it is still concerning that the modification rates observed in the current manuscript are so low.

Response: Our *in vitro* condition indeed showed the typically high DMS reactivity observed in the literature. Our understanding of the main concern of the reviewer's comment is that the *in vivo* and *in vitro* difference may be derived from a systematically low DMS reactivity *in vivo*. To further address this, we did the following analysis:

(i) We compared the *in vivo* DMS signals for the cytosolic tRNA and the mitochondrial tRNAs (Fig. R1 = new Fig. S1f). Because of the low structural stability, the mitochondrial tRNAs are expected to have higher DMS reactivity which is exactly what we observe.

However, this result is the opposite to the prediction of a systematical low reactivity. In cells, mitochondrial tRNAs are enclosed in mitochondria with extra membrane protection against DMS diffusing in. Therefore, intracellular DMS concentration should be lower in mitochondria compared to the cytosol so that we would expect a lower DMS reactivity for mitochondrial tRNAs. Clearly, our result shows the opposite.

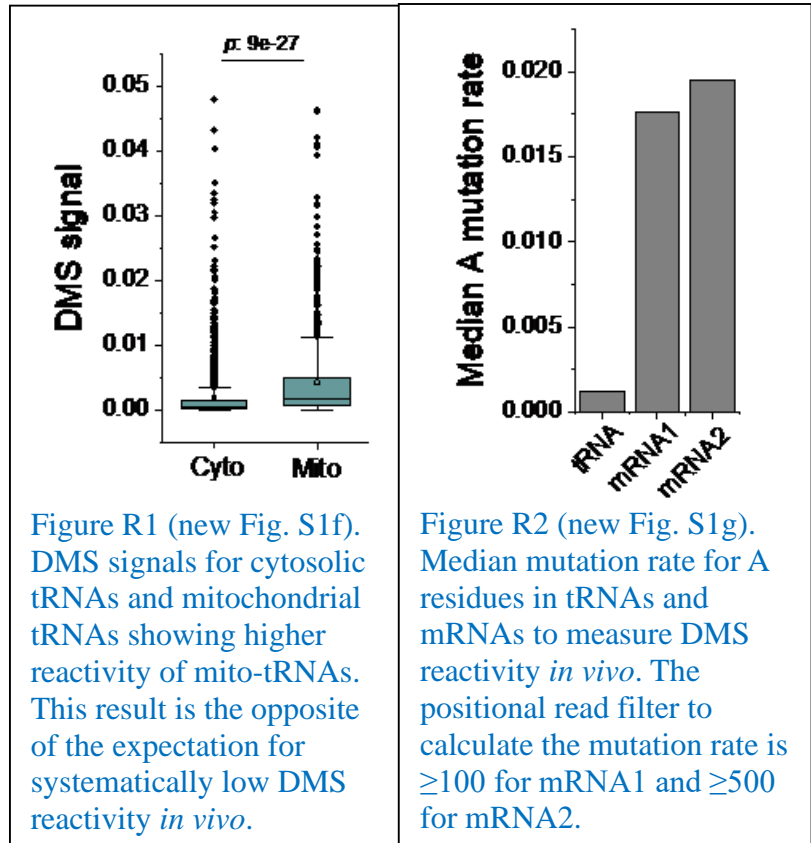
(ii) We also mapped the DMS reactivity in mRNA reads in our sequencing data. Our MSR-seq uses total RNA as input so 0.5-1 million mRNA reads are still present together with the tRNA reads. We found that the mRNA median Adenosine mutation rates were ~10 times higher than those in tRNAs (Fig. R2 = new Fig. S1g).

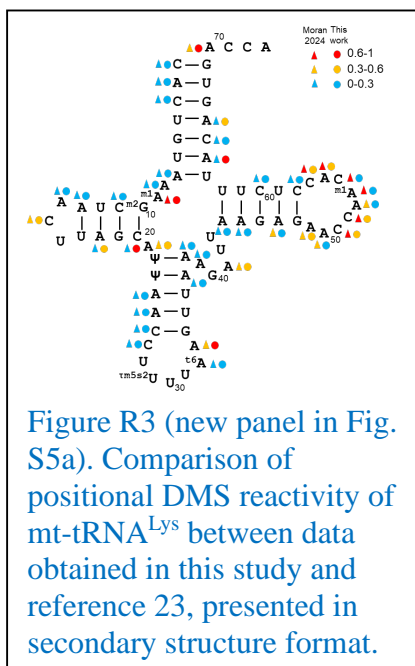
We now include these results as new Fig. S1f, S1g and the associated description on p. 10, first paragraph.

“To further demonstrate that the *in vivo* DMS signals are not just a reduced reactivity version of the *in vitro* signals, we did three additional analyses. First, we compared the *in vivo* DMS signals for the cytosolic tRNA and mitochondrial tRNAs (**Supplemental Fig. S1f**). Because of the low structural stability, the mitochondrial tRNAs are expected to have higher DMS reactivity which is what we observe. However, in cells, mitochondrial tRNAs are enclosed in mitochondria with extra membrane protection against DMS diffusing in. Therefore, higher *in vivo* reactivity of mitochondrial tRNAs contradicts a model of systematic low reactivity of *in vivo* versus *in vitro* conditions. Second, we mapped the DMS reactivity in mRNA reads in our sequencing data. MSR-seq uses total RNA as input so 0.5-1 million mRNA reads are still present together with the tRNA reads. We found that the mRNA median Adenosine mutation rates were ~10 times higher than those in tRNAs (**Supplemental Fig. S1g**), indicating that our *in vivo* DMS condition is within the range of reactivity in other mRNA structural mapping studies. Third, we made a Gini plot comparing the *in vitro* and *in vivo* DMS signal profiles of the same tRNAs (**Supplemental Figure 1h**). The *in vitro* and *in vivo* profiles have drastically different shapes, consistent with different factors contributing to these profiles.”

3. The comparison of mt-tRNA-Lys in Figure S5a should include a plot in which traces for current and previous data are overlaid and can be compared directly. The current figure in which reactivity bins are compared is fine to include, but does not facilitate a clear comparison of the data.

Response: Direct comparison in the context of mt-tRNA^{Lys} secondary structure is added as a new right panel in Figure S5a (Fig. R3).





Point-by-point response to the reviewer's comments

Reviewer #4 (Remarks to the Author):

The new analysis of mRNA reactivity that the authors have performed shows that the low reactivity observed for tRNAs in vivo is biologically meaningful. The authors have also provided a reasonable justification and evidence for the use of their τ score for normalizing tRNA reactivity. The authors have therefore addressed my concerns, and I support publication.

Response: Thanks very much!