

Recordanelles for transcriptome-wide single-cell RNA recording in human cells

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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript by Reinkemeier et al. presents a highly creative and potentially important tool for transcriptome-wide RNA recording in mammalian cells. The idea of a mitochondria-anchored synthetic organelle ("recordanelle") that captures and preserves RNA expression histories is compelling, and the authors further demonstrated its feasibility in IFN β recording experiments, which are promising. The conceptual advance is exciting, and several parts of the paper, especially the figure legends, present the experiments clearly and make them easier to follow. However, the current version of the manuscript feels stronger in concept than in execution. To better support certain claims made in the manuscript and improve the usability of this exciting new tool, we suggest the modifications and additions outlined below. In particular, the authors' claim that transcriptome-wide RNA recruitment and recording occur "without disrupting cellular physiology" remains underdefined and under-supported. The absence of major changes in steady-state full-cell RNA levels does not exclude compensatory homeostatic responses, and the potential impact of mounting large RNA-binding condensates on the mitochondrial surface is not directly addressed. We believe that a more rigorous characterization of the cellular impact and transcript protection capabilities of the recordanelle would significantly strengthen the claims of the paper and provide valuable guidance for others interested in adopting this technology. Overall, the work is very exciting, but some of the claims would benefit from clearer presentation and more thorough biological validation.

Major Comments

1. The claim that the system functions "without disrupting cellular physiology" is central, but remains underdefined and under-supported

The authors framed the recordanelle as the recruiter and recorder of RNA without disrupting cellular physiology. This is showcased as a major design goal and a key advantage of the platform. However, the current manuscript did not clearly define what operationally counts as disrupting cellular physiology, and the evidence used to support this claim appears to rely mainly on the observation that the full cell transcriptome is not altered dramatically.

Unchanged bulk RNA abundance alone—especially given that only a subset of cells has the recordanelle—is not sufficient to support a broad conclusion. If the authors would like to retain this as a headline claim, it needs to specify more explicitly what is meant to be non-disruptive and how it was assessed. For example, does "non-disruptive" mean no detectable effect on global RNA abundance, no effect on translation, no effect on stress signaling, no effect on viability, or no effect on mitochondrial function? There are many ways as to how the authors could test these different potential effects (e.g., dyes to measure mitochondrial potential or ROS, changes in expression of inflammatory proteins, bulk translational defects). The authors also need to show that the cells that actually express the recordanelle are unchanged, rather than performing bulk assays in experiments where they used transient transfection. As currently written, this definition is too broad relative to the data the authors have shown.

As a final note, the final recordanelle design (v2) does not appear to have been characterized using RNAseq-based perturbation analysis. Given that swapping domains has been shown to impact the functionality of the recordanelle, this should be included for all lead candidate designs.

2. The manuscript should more directly address mitochondrial health and function

A central design feature of the system is that the recording platform is anchored to the mitochondrial surface. The authors also explain why mitochondria were chosen, including their large available surface area, separability, and orthogonality as a cellular platform. This rationale is understandable and technically appealing. However, mounting RNA-binding condensates on mitochondria raises an obvious biological question: does the platform itself perturb mitochondrial function?

At present, the data appears to address localization, fractionation, and RNA capture, but I did not see direct evidence addressing mitochondrial physiology itself. Given how central mitochondrial anchoring is to the platform, I think this should be discussed more carefully, and ideally tested more directly. Assays such as mitochondrial membrane potential, respiration, ATP production, ROS, or morphology/network integrity would substantially strengthen the claim that the system is biologically non-disruptive. As it stands, the manuscript demonstrates that the platform is on mitochondria, but not yet convincingly that mitochondria are unaffected by it.

3. The analysis of recordanelle's ability to protect transcripts over time would benefit from single cell experiments and a more thorough explanation of the supplementary data

Extended Data Fig. 10a provides a time course of the relative mCherry transcript abundance in the mitochondrial fraction and the full cell for different recordanelle designs. The figure shows that mCherry transcript retention can be detected in both the full cell and mitochondrial fraction, which is to be expected. However, it appears that the relative level of mCherry transcripts in the mitochondrial fraction is consistently substantially higher (~5-fold) than in the full cell fraction at Day 0. The expected and observed differences in relative transcript abundance between the two fractions should be described in greater detail in the text. Does this data suggest that, in this instance, the recordanelle is not sampling evenly from the transcriptome? All three of the designs shown (MCP, v1, and v2) can recruit mCherry::ms2 transcripts from the cytoplasm to the mitochondria, but it would be interesting to see if the elevated relative mCherry transcript abundance in the mitochondrial fraction is maintained in the absence of recruitment. Additionally, given that for the RNA to be protected, it must first be captured by the recordanelle, it would be useful to include the non-binding control data, which provides a comparison of binding with protection (recordanelle), binding without protection (MCP), and no binding at all (PCP). Currently, this comparison is only available for the full cell fraction (Extended Data Fig. 9).

Confocal imaging is used to create a compelling argument that the recordanelle recruits target RNA species in the MCP/PCP model in figures 1 and 2. However, after Fig. 3B / Extended Data Fig. 5, imaging is replaced by qPCR and RNA-seq. While there is a functional effect on RNA abundance after 24 hours (~3 to 4-fold increase over non-binding) at a population level, confocal imaging would provide substantially more information regarding the localization and abundance of a target RNA species, and reduce the risk of "contaminant" transcripts that may have been co-purified with the mitochondrial fraction (see minor comment 10). The authors cleverly use the MS2 system, which enables both fixed and live cell imaging of the target RNA. RNA FISH (as used in Fig. 1-3) would allow for comparison of mitochondrial signal to cytoplasmic signal on a per-cell basis, strengthening the case that the RNA is being protected specifically through sequestration into the condensate. Similarly, live cell imaging of MS2 localization and abundance over time in the same population of cells would provide a single cell assessment of local abundance and cell-to-cell variability, the latter being an important characteristic of the system from the perspective of a potential user (see major comment 6).

4. The IFN β experiments are a strong part of the manuscript, but they also reveal perturbation risks that should be discussed more directly

The IFN β experiments are conceptually compelling because they move beyond engineered reporters and test whether recordanelles can preserve a transient endogenous transcriptional response. This is an important and persuasive direction for the paper. The finding that the IFN-induced signature can still be detected after stimulus removal is one of the manuscript's strongest demonstrations of utility.

At the same time, these experiments also highlight a concern: some architectures appear not merely to preserve the response, but to alter its dynamics. In particular, the manuscript's interpretation of recordanelleV1 suggests that the system may amplify or prolong IFN signaling through feedback effects, whereas recordanelleV2 is presented as a less perturbing alternative. This is important because it means the work itself demonstrates that some recordanelle designs can actively influence the biological process they are intended to record. The comparison between V1 and V2 is interesting, but it does not state the perturbation issue clearly, where V2 may reduce the effect.

The authors should clearly assess if the recordanelle alters translation of the interferon-induced RNAs over time (e.g., via Western blot). Does the recordanelle alter the inflammatory response in any way (i.e., dampen or extend)?

5. Is it possible to evaluate the recording capacity of the recordanelle system?

As with all new technologies, detailed characterization of the practical limits of the technology is important for enabling widespread use by the community. The authors show the ability to record for up to 48 hours and sample almost 90% of transcripts. However, it would be useful to explore, either via further experiments or discussion in the text, the practical limits

of the system, both in terms of recording time (i.e., the timeframe over which transcripts can be protected) and recording volume (i.e., the number of transcripts that can be held in the recordanella at one time), to help guide others who intent to apply this technology to different cell systems or new applications. For example, is the timeframe over which a transcript can be protected dependent on the protein turnover of the recordanella, the number of transcripts already bound by the recordanella, the half-life of that specific transcript, etc.? Exploring, or at the very least discussing, some of the practical considerations will be a substantial help to those looking to adapt the system in the future.

Minor Comments

1. The figure legends are often clearer than the main-text narrative

This is meant as constructive feedback. In multiple places, especially in the screening and IFN β experiments, I found the figure legends easier to follow than the main text. The paper would benefit from revising the Results narrative so that the logic of each experiment is as explicit in the body text as it is in the legends.

2. The RNA “recording” effect is interesting, but the practical significance of the recovery metric could be clarified more carefully

In the dox-inducible reporter experiments, the manuscript uses a “transcript recovery” metric that reflects the amount of reporter RNA remaining after Dox withdrawal relative to day 0. This is a reasonable way to measure persistence, but the term may be easy to misinterpret on first reading. It does not represent the absolute fraction of all transcript molecules physically preserved by the system, but rather a relative residual signal over time. The reported effect is still meaningful and supports partial stabilization and extension of the detection window. However, the absolute retained fraction appears relatively modest, even when improved over controls. I therefore think the manuscript would benefit from more careful contextualization of what this metric means biologically and practically. In particular, wording that implies especially strong or highly efficient long-term retention may overstate the present effect size.

3. The manuscript should more clearly distinguish transient co-transfection experiments from later stable-cell-line experiments

In the early recruitment/localization experiments, the wording in the main text can be confusing with respect to what was transiently transfected versus what was stably integrated. Based on the Methods, the early reporter recruitment and imaging experiments appear to rely on transient co-transfection of both the recordanella construct and the reporter plasmid, whereas stable engineered cells are introduced later for the doxycycline-inducible recording assays. This distinction should be stated more explicitly in the Results, because it affects how readers interpret reproducibility, expression variability, and the maturity of the system at different stages of the paper. As written, a first-time reader can easily misread these experimental configurations.

4. The logic connecting construct screening, optimization, and lead-candidate selection is often difficult to follow

I found the optimization pipeline in the Results section harder to follow than necessary. The rationale connecting the three sections was not explicitly clear in the main text. For example, the screening logic in the early figures sometimes appears to mix recruitment efficiency, specificity, nonspecific binding, and minimal perturbation without clearly stating which criteria are being prioritized at each step.

Relatedly, phrases such as “top-performing” are used, but the basis for prioritizing particular construct families is not always fully transparent. It would help to more explicitly distinguish: (1) small-scale screening for enrichment/selectivity, (2) transcriptome-scale validation of selected constructs, and (3) final nomination of architectures suitable for long-term recording.

5. When weak construct performance is attributed to localization or expression, more quantitative normalization would strengthen the interpretation

In the endogenous mRNA recruitment section, the authors suggest that weak or absent enrichment for some architectures may be due in part to poor expression or incorrect localization. This makes sense, but more systematic quantification is needed to explain construct-to-construct differences. Does RNA enrichment align with total construct abundance in the cell, or the mitochondrial/cytoplasmic abundance? Without such normalization, it remains difficult to determine whether poor performance reflects intrinsic limitations of a given design or simply reduced effective protein levels at the relevant cellular location.

6. The meaning of “up to 90% of endogenous mRNAs” should be stated very carefully

This is a striking claim, but it is easy to over-interpret. As I understand it, the statement refers to the fraction of detected transcripts that are significantly enriched in the recordanella fraction under the authors’ analytical framework, not to literal physical recovery of 90% of all mRNA molecules in the cell. A clarification is needed to prevent audiences’ misunderstanding.

7. Terms such as “representative” and “faithful” should be used with care

The authors provide encouraging correlation analyses suggesting that captured RNA populations reflect the broader whole-cell transcriptome, especially for the stronger PABPC-based designs. However, the same data also indicate expected

biases, such as reduced recovery of nuclear and mitochondrial transcripts. I think the manuscript should maintain some caution in how broadly it describes the system as representative of the full transcriptome.

8. The manuscript would benefit from a more explicit upfront statement of what the technology is and is not designed to record

It would help readers if the Introduction or early Results more clearly stated that the system is especially suited to capturing cytoplasmic RNA histories and transient signals that would otherwise decay, rather than being interpreted as a universal recorder of all RNA states across all compartments.

9. Several mechanistic interpretations are interesting, but remain speculative

The manuscript proposes that interactions between the assembler and the RNA-binding domain may influence condensation behavior and thereby affect performance. This is a plausible and interesting mechanistic explanation, especially in light of prior literature and the observed context dependence of which architecture performs best. However, in the current manuscript, these interpretations remain somewhat speculative.

I do not think the paper necessarily needs to become a fully mechanistic study, but when these hypotheses are used to explain key result differences, the manuscript would benefit from either deeper discussion or more explicit acknowledgement that these explanations are provisional. As written, some mechanistic suggestions read more definitively than the evidence fully justifies.

Additionally, the authors state: “We further observed that at the day 0 timepoint, when the reporter is maximally induced, mCherry::ms2 expression was higher in recordanelleV1 (but not recordanelleV2) compared to the control recordanelle. This suggests that whereas recordanelleV1 may globally stabilize mRNAs, thus altering the homeostatic state of the cell, recordanelleV2 does not and thus represents the superior architecture. Taken together, we confirm mCherry::ms2 recording and protection without altering the cellular state with recordanelleV2.” This is an interesting point that needs to be explained in more depth. For example, how do you explain global stabilization of RNAs without impacting the transcriptome, as described in Extended Data Fig. 9?

10. RNA-seq methods should be described in greater detail

Given the importance of RNA sequencing data to the claims of the paper, it would benefit the reader to understand the specifics of how the RNA sequencing data were processed. For example, what sort of filtering, if any, was carried out? How were potential contaminant RNA transcripts (e.g., those that may not be housed inside the recordanelle but instead were co-purified with the mitochondrial fraction due to interaction or proximity) thought about and handled?

Figure 3C and Extended Data Fig. 8 make the case that the recordanelle does not impact global gene expression patterns and evenly samples the cytoplasmic RNA content. While the correlation values indicate that the results are consistent across replicates and the full recordanelle system shows excellent agreement between the full cell and mitochondrial transcriptomes, the visualization masks this point. While a correlation of ~0.7 (mock and non-binding controls) is clearly much lower than ~0.98 (recordanelle v1), there is still significant overlap in the transcripts identified in the mitochondrial and full cell fractions. The authors should address this point in greater detail, as it brings up important questions about whether this is “contaminant” RNA that was co-purified during mitochondria extraction or a genuine representation of the transcripts found in mitochondria. An awareness of the expected overlap may also help improve the visualization of recordanelle content, which, as currently presented in Extended Data Fig. 8, appear largely indistinguishable across the four recordanelle conditions. For example, perhaps it may be useful to generate plots showing the mitochondrial vs full cell correlation for transcripts that correlate poorly in the control samples to avoid crowding the figure with potential “contaminant” transcripts that may have been co-purified along with the mitochondrial fraction. Additionally, would the true effect of the recordanelle be clearer with a set of plots showing only RNA transcripts that are expected to be found in the cytoplasm?

11. Statements of novelty require clarification

Claims of novelty need to be better supported and explained. For example, in the discussion, the authors state: “Here, we introduce the recordanelle, the first transcriptome-wide RNA recording technology for mammalian cells that continuously samples the cellular transcriptome”. However, it is this reviewer’s understanding that the new TimeVault technology (described later in the discussion) represents a transcriptome-wide, continuous RNA recording technology, where the time window of recording can be controlled via the addition of doxycycline. As currently presented, the novel features of recordanelle over existing technologies require clarification. Additionally, it might be worth referencing Felix Horns and colleagues’ 2023 Cell paper (PMID: 37437570) in which they developed a system for programmable RNA export for continuous, non-destructive monitoring of cell populations.

12. Add values and axes labels to figures

To improve the readability and clarity of the manuscript, I would recommend:

- reporting the mean expression value or the ratio in the plots (e.g., Fig. 4, Extended Data Fig. 9, and Extended Data Fig. 10)
- Defining the significance limits for stars (e.g., Extended Data Fig. 10)
- Including units on all axes (e.g., Extended Data Fig. 10 – counts per million)

Overall, this is a very exciting work with great potential. The idea and most of the experiments were clearly executed and

compelling. The current reservation is not about the concept but whether the current work fully supports the strong claims being made. With clearer presentation and more detailed perturbation work, I think the manuscript would be significantly strengthened.

Reviewer #2

(Remarks to the Author)

This work describes a new approach for preserving RNAs within cells and localizing them to mitochondria as a way to sequence transcriptional responses beyond the window where they would naturally return to baseline. The approach is interesting and unique among the new technologies for transcriptional recording. However, there are substantial constraints that may limit its practical utility. More importantly, the approach is likely to be substantially perturbative to the cell biology in ways that are downplayed in the manuscript and experimentally unexplored. Finally, the terminology around the technology is misleading and imprecise.

Major points:

(1) Impact of the approach on the cell biology. At multiple points the authors state that the recordanelle approach is not perturbative, e.g.: “does not perturb the cell” (page 4, line 12); “can capture a large fraction of the cytoplasmic transcriptome without altering global RNA levels (page 5, line 1); “without altering the cellular state” (page 7, line 2). These statements are based on narrow examinations of transcriptional levels, often with RNA binding domains that should only interact with synthetic transcripts. In fact, the core idea of the approach essentially guarantees that it will perturb the cell biology. Transcripts are sequestered to an unnatural location and protected over time. There are two potential ramifications: either the transcripts could be translationally repressed by this sequestration and protection, reducing protein expression; or the transcripts could be translationally active with an increased lifetime, increasing cumulative protein expression. These perturbations to the proteome of the cell could even both be true, in a manner that is dependent on each particular transcript.

Despite claiming that the approach enables “enabling transient and complex transcriptional events to be archived within the same population of cells without altering their physiology” (page 8, line 16), the work shows evidence of exactly these perturbations. Page 7:

“Intriguingly, we observed that with recordanelleV1 but not recordanelleMCP, ISG detection was not only maintained, but increased from day 0 to 2 after IFNB removal... One likely explanation for this is the well-described type-I IFN signaling positive feedback loop^{38,39}, whereby under control conditions IFNB is rapidly degraded but with the recordanelle IFNB response persists and drives the feedback loop. This could occur under conditions where the recorded ISGs mRNAs are translated. Indeed, compared to recordanelleV1, recordanelleV2 — utilizing PABPC4ΔC161A, which is supposed to abrogate translation of the bound mRNA^{40,41} — the feedback loop is prevented and ISG levels stay around day 0 levels. For recording mRNA without disrupting cell physiology, recordanelleV2 has clear advantages because it can prevent translation of RNAs within the recordanelle, and we therefore proceeded with this recordanelle architecture.”

Thus, the V1 enables translation and therefore creates a synthetic feedback that prolongs the transcriptional response. The authors state that V2 prevents translation and therefore should blunt the response (although not experimentally tested).

To fully test the extent to which the approach perturbs the biology of the cell, the authors should look at the proteome for evidence of disruption and contextualize these disruptions in a way that makes them clear to readers and potential adopters of the technology. Specifically, proteome-wide measurements should be made with the V1 and V2 version (not MCP or PCP) at multiple timepoints with and without stimulation. Moreover, another likely way that the approach will perturb the cells is via disruption of mitochondrial function due to encasement in RNA and disordered protein domains. Cells expressing the V1 and V2 versions should be profiled by Seahorse or other similar assay for mitochondrial function compared to control cells.

(2) Testing maintenance over time. The maximum time period after an event that the authors tested was 2 days. It is hard to imagine real biological questions where a two-day extension of transcripts makes the difference between being able to study a phenomenon or not. If the authors can think of one, they should point it out. Either way, it would be useful to extend the timeline of experimentation to see how rapidly the signal fades over time. The authors should continue this timecourse until the signal returns to baseline.

(3) The terminology. The authors state that they created a synthetic organelle, but lack justification for that claim. A less hyperbolic description would be that they decorated a natural organelle, the mitochondria, with RNA binding proteins. Potentially the authors wish to suggest that the disordered domains create phase separation and compartmentalization. However, that separation was never tested and the inclusion of the disordered regions was not necessary for the effect. Even if there were condensate formation around the mitochondrial that would not reach the bar for defining a new synthetic organelle. This terminology also leads to confusion where the anchored RNA binding protein construct is referred to as the recordanelle and other times the recordanelle encompasses the mitochondrial fraction. The authors should be more explicit in their language to enable understanding rather than branding.

Minor Points:

(1) Multiple times conclusions are made from comparisons that are not statistically tested. All conclusions regarding Figure 2c are a good example. For instance, the statement “While we observed enrichment of target RNAs when using a control construct without an assembler protein (Δ AP), several other constructs outperformed this condition (e.g., FUSQG, FUSopto), indicating that RNA recruitment might be aided by the biomolecular condensation properties of the recordanelle.” This conclusion is accompanied by no statistical testing (which should be corrected for multiple comparisons). Moreover, only a mean is shown for each condition, with no distribution of underlying data and N is reported inexactly as “at least three experiments per condition.”

(2) Figure 2b, 3c: One of the stains is identified as “recordanelle” but the figures, text, and legend do not identify what is being stained, nor is it obvious from the methods.

(3) The authors consistently ignore differences in the magnitude of enrichment for different transcripts. Figure 3c shows a clear difference in the magnitude of enrichment of mCherry, GFP, and the endogenous YWHAZ, for instance that the authors gloss over and similarly do not mention the enormous spread of enrichment in 3e. Some exploration of what dictates the degree of enrichment would be useful (length, abundance, structure, half-life). Additionally, this should be discussed as a major caveat of the approach for potential users who would be interested in discovery biology with a tool that may have a substantial bias.

(4) The authors should remove the line containing, “the first transcriptome-wide RNA recording technology for mammalian cells that continuously samples the cellular transcriptome,” given the recent vault work that is discussed.

Author Rebuttal letter:

We would like to thank both reviewers for their encouraging and helpful feedback and hope that with the revision experiments we addressed their concerns. In the point-by-point reply below our responses are indicated in blue.

As both reviewers expressed similar concerns on a few topics, we provide aggregated responses to these here:

Response to Reviewer 1 and Reviewer 2 on the non-disruptive nature of recordanelles.

We thank the reviewers for their important feedback. In the original manuscript we based the claim that recordanelles did not disrupt cellular physiology primarily on the absence of transcriptomic alterations. We agree with the reviewers that this is a narrow and incomplete definition of cellular physiology. In the revised manuscript we therefore include several new experiments to broaden our investigation of cellular physiology disruption from the perspective of cellular viability, mitochondrial function, and proteomic changes. We additionally include the RNA-seq enrichment experiment including our final recordanelle version as suggested by reviewer 1. To make this section easy to navigate, the different sections focus on a distinct facet of cellular physiology and the new experiment types are underlined.

Before describing the results, it is important to note that a few of these new assays were performed at the single-cell level (by microscopy), but most were performed in bulk. Under our transfection conditions we achieved a transfection efficiency of 80% (see Extended Data Fig. 9o) and we therefore expect that any perturbation can be detected with bulk assays.

Regarding the transcriptomic effect, Fig. 3 and Extended Data Fig. 4 show no substantial change in the whole-cell transcriptome relative to mock when expressing recordanelle (MCP, V1) constructs. We repeated this RNA-seq experiment to include recordanelleV2 (Extended Data Fig. 4). RecordanelleV1 and recordanelleV2 showed similar binding profiles, with recordanelleV2 slightly outperforming recordanelle V1 in a direct head-to-head comparison. However, we detected no substantial transcript up- or down-regulation beyond a few long noncoding RNAs and one histone gene, which we attribute to technical noise. We also do not observe a generalized transcriptome amplification, which the ERCC spike-in normalization would have captured. Taken together, recordanelles do not disrupt the transcriptome.

Regarding viability, in the new Extended Data Fig. 9a-c we measured viability with two orthogonal assays. At the bulk level, we use the CellTiter-Glo assay 24 and 48 hours post transfection to measure viability (inferred from ATP production). Compared to expressing mCherry, neither recordanelleMCP, recordanelleV1 or recordanelleV2 show a reduction in viability. At the single-cell level, we performed live-cell imaging of apoptosis in an Incucyte assay using the marker Annexin V. Here, we did not see an increase in apoptosis for recordanelle V1 or recordanelleV2 compared to expressing a fluorescent protein. Taken together, recordanelles do not disrupt cell viability.

Regarding mitochondrial function, we now show in Extended Data Fig. 9d-n two

different assays to measure mitochondrial function. At the bulk level, we performed a mito-stress test using the Seahorse assay, the gold standard in the field. Here, we did not observe any reduction in coupling efficiency, basal respiration, ATP production, maximal respiration or spare respiratory capacity for any of the recordanelles (tested here: recordanelleMCP, recordanelleV1 or recordanelleV2) compared to a mock or mCherry transfection. At the single-cell level, we performed live-cell imaging of mitochondrial membrane potential to continuously track recordanelle impact in an Incucyte. Also here, we did not observe a difference between recordanelleMCP, recordanelleV1 or recordanelleV2 and mock. Taken together, recordanelles do not disrupt mitochondrial function.

Regarding the proteome, we performed quantitative proteomics (see Fig. 5, Extended Data Fig. 8 and Supplementary Data Fig. 5). First, we detected no major difference in expression of the mitochondrial respiratory complex proteins compared to mock transfection. Second, although we detected more than 9'000 proteins in each condition, comparing mCherry, recordanelleV1 and recordanelleV2 with mock yielded fewer than 1% downregulated proteins (most detected with low confidence), and no pathway was substantially enriched in this set. The 1% change we are observing across random sets of proteins is well under the typical variation seen in a proteomics experiment, so we concluded that this is likely due to technical noise. Taken together, recordanelles either negligibly or do not disrupt the proteome (see Supplementary Figure 5).

In summary, these new data demonstrate that recordanelles do not disrupt the transcriptome, proteome, cellular viability, or mitochondrial function. This strongly supports our original claim that recordanelles do not alter cellular physiology.

Response to Reviewer 1 and Reviewer 2 on long-term recording.

We thank the reviewers for their important feedback. To address their concern, we performed new long-term IFNB stimulus recording experiments (see Extended Data Fig. 7 and Supplementary Fig. 2 & 3). We performed this experiment in two formats: First, we treated cells with IFNB and split them after day 2 at appropriate dilutions so that we could harvest cells after 3, 4 and 5 days. We also split the cells once more to analyze days 6 and 7. If cells continue growing, we can still detect ISGs (IFI44L or IFIT1) for up to 4 days, although the signal decays with time. This is likely due to the dilution of the old recorded mRNA with new recorded mRNA under non-IFNB stimulus conditions. Second, we treated cells with IFNB followed by harvesting them 2, 3, 4 or 5 days after removing IFNB without splitting them in between (just changing medium to replenish nutrients). With our transfection efficiency here, we would expect that 80% of cells express the recordanelle and that all cells have been exposed to IFNB. As they reach confluency after about 2 days, the cells substantially reduced their growth and transcriptional activity. In this case, while the control cells (recordanelle MCP) lose the IFNB signature after 2 days, we could still detect ISGs at the latest experimental timepoint (5 days) with both recordanelleV1 and recordanelleV2.

Taken together, this indicates that the recordanelles once formed are comparatively stable and only exhibit a low decay, driven by factors involving cell division, high transcription rates, and continuous recording of new mRNAs that dilute old signals.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript by Reinkemeier et al. presents a highly creative and potentially important tool for transcriptome-wide RNA recording in mammalian cells. The idea of a mitochondria-anchored synthetic organelle ("recordanelle") that captures and preserves RNA expression histories is compelling, and the authors further demonstrated its feasibility in IFN β recording experiments, which are promising. The conceptual advance is exciting, and several parts of the paper, especially the figure legends, present the experiments clearly and make them easier to follow. However, the current version of the manuscript feels stronger in concept than in execution. To better support certain claims made in the manuscript and improve the usability of this exciting new tool, we suggest the modifications and additions outlined below. In particular, the authors' claim that transcriptome-wide RNA recruitment and recording occur "without disrupting cellular physiology" remains underdefined and under-supported. The absence of major changes in steady-state full-cell RNA levels does not exclude compensatory homeostatic responses, and the potential impact of mounting large RNA-binding condensates on the mitochondrial surface is not directly addressed. We believe that a more rigorous characterization of the cellular impact and transcript protection capabilities of the recordanelle would significantly strengthen the claims of the paper and provide valuable guidance for others interested in adopting this

technology. Overall, the work is very exciting, but some of the claims would benefit from clearer presentation and more thorough biological validation.

We thank the reviewer for their praise of the creativity and importance of our work as well as the helpful feedback.

We performed several new experiments to thoroughly address the impact of recordanelles on cellular physiology from multiple angles as detailed below. We hope that this new data convinces the reviewer that our technology in fact has minimal impact on cellular physiology and is thus a useful non-disruptive RNA recorder.

Major Comments

1. The claim that the system functions “without disrupting cellular physiology” is central, but remains underdefined and under-supported

The authors framed the recordanelle as the recruiter and recorder of RNA without disrupting cellular physiology. This is showcased as a major design goal and a key advantage of the platform. However, the current manuscript did not clearly define what operationally counts as disrupting cellular physiology, and the evidence used to support this claim appears to rely mainly on the observation that the full cell transcriptome is not altered dramatically.

Unchanged bulk RNA abundance alone—especially given that only a subset of cells has the recordanelle—is not sufficient to support a broad conclusion. If the authors would like to retain this as a headline claim, it needs to specify more explicitly what is meant to be non-disruptive and how it was assessed. For example, does “non-disruptive” mean no detectable effect on global RNA abundance, no effect on translation, no effect on stress signaling, no effect on viability, or no effect on mitochondrial function? There are many ways as to how the authors could test these different potential effects (e.g., dyes to measure mitochondrial potential or ROS, changes in expression of inflammatory proteins, bulk translational defects). The authors also need to show that the cells that actually express the recordanelle are unchanged, rather than performing bulk assays in experiments where they used transient transfection. As currently written, this definition is too broad relative to the data the authors have shown

As a final note, the final recordanelle design (v2) does not appear to have been characterized using RNAseq-based perturbation analysis. Given that swapping domains has been shown to impact the functionality of the recordanelle, this should be included for all lead candidate designs.

As Reviewer 2 had a similar comment, we provide one aggregated response above: Response to Reviewer 1 and Reviewer 2 on the non-disruptive nature of recordanelles.

2. The manuscript should more directly address mitochondrial health and function

A central design feature of the system is that the recording platform is anchored to the mitochondrial surface. The authors also explain why mitochondria were chosen, including their large available surface area, separability, and orthogonality as a cellular platform. This rationale is understandable and technically appealing. However, mounting RNA-binding condensates on mitochondria raises an obvious biological question: does the platform itself perturb mitochondrial function?

At present, the data appears to address localization, fractionation, and RNA capture, but I did not see direct evidence addressing mitochondrial physiology itself. Given how central mitochondrial anchoring is to the platform, I think this should be discussed more carefully, and ideally tested more directly. Assays such as mitochondrial membrane potential, respiration, ATP production, ROS, or morphology/network integrity would substantially strengthen the claim that the system is biologically non-disruptive. As it stands, the manuscript demonstrates that the platform is on mitochondria, but not yet convincingly that mitochondria are unaffected by it.

We thank the reviewer for this insightful comment. As mentioned above in our response to Major Comment 1, we have now comprehensively analyzed the impact of several recordanelle constructs on mitochondrial physiology and function using the Seahorse assay, quantitative proteomics, and fluorescent dye-based assays. All these assays clearly indicated that recordanelles (V1, V2 or MCP) do not alter mitochondrial function (see Extended Data Fig. 8 & 9 and Supplementary Fig. 5). One interesting finding from this experiment was that transient transfection with a mock

plasmid and Dox induction by themselves altered mitochondrial function. And yet, these methods are widely used in biology. Anchoring the recordanelles to the mitochondrial surface does not perturb mitochondrial function more than transiently transfecting cells in general, and less than treating cells with Dox. We have incorporated these results (see Extended Data Fig. 8) and updated the discussion accordingly in the revised manuscript.

3. The analysis of recordanelle's ability to protect transcripts over time would benefit from single cell experiments and a more thorough explanation of the supplementary data

Extended Data Fig. 10a provides a time course of the relative mCherry transcript abundance in the mitochondrial fraction and the full cell for different recordanelle designs. The figure shows that mCherry transcript retention can be detected in both the full cell and mitochondrial fraction, which is to be expected. However, it appears that the relative level of mCherry transcripts in the mitochondrial fraction is consistently substantially higher (~5-fold) than in the full cell fraction at Day 0. The expected and observed differences in relative transcript abundance between the two fractions should be described in greater detail in the text. Does this data suggest that, in this instance, the recordanelle is not sampling evenly from the transcriptome? All three of the designs shown (MCP, v1, and v2) can recruit mCherry::ms2 transcripts from the cytoplasm to the mitochondria, but it would be interesting to see if the elevated relative mCherry transcript abundance in the mitochondrial fraction is maintained in the absence of recruitment. Additionally, given that for the RNA to be protected, it must first be captured by the recordanelle, it would be useful to include the non-binding control data, which provides a comparison of binding with protection (recordanelle), binding without protection (MCP), and no binding at all (PCP). Currently, this comparison is only available for the full cell fraction (Extended Data Fig. 9).

We thank the reviewer for this comment. To address their concern, we performed new mCherry::ms2 RNA protection experiments with non-binding controls (see Extended Data Fig. 6d).

First, we would like to point out that all our qPCR data are normalized to the mitochondrially-encoded gene MT-ATP6. Therefore, for all binding systems, it is expected that the ratio in the mitochondrial fraction increases over the full-cell level. As we have shown in Extended Data Fig. 5, the concordance between transcript abundance at the full-cell level and at the mitochondrial level in the recordanelles is very high, demonstrating that the recordanelles sample homogeneously.

We repeated the mCherry::ms2 RNA protection experiment, including the non-binding control recordanellePCP, and measured mCherry::ms2 RNA in both the mitochondrial and full-cell fraction (see Extended Data Fig. 6d). We observed that mCherry is not significantly different between recordanelleMCP and recordanellePCP at the full-cell level but, as expected, recordanelleMCP significantly enriches mCherry::ms2 RNA in the mitochondrial fraction over recordanellePCP. On day 2, only recordanelleV2 showed a significant difference compared to uninduced cells, indicating that recordanelle MCP and recordanellePCP are unable to record mCherry::ms2 RNA.

While we again observed that the relative level of mCherry::ms2 transcripts in the mitochondrial fraction is consistently higher than in the full-cell fraction for all tested recordanelles, we also observed that the relative level of mCherry::ms2 is slightly higher for recordanellePCP in the mitochondrial fraction. To further investigate this, we performed the same experiment using a mock transfection as an additional control. Here, we observed that even under mock conditions, mCherry is enriched in the mitochondrial fraction (see Figure PBP 1).

Previously, it has been shown that naked mRNAs can bind to mitochondria in the absence of ribosomes *in vitro* (PMID: 24252184). Further, it has been shown that several RNA binding proteins can be found at mitochondria (see PMID: 38448244 for a review article). We speculate that this could point towards a minor binding of mCherry::ms2 RNA to mitochondria, which is visible at the low expression levels achieved by the mild Dox treatment, but not at the high expression level in the previous reporter experiments (see Fig. 2 and 3), where we normalize the data to a mock condition that has also been transfected with the same reporter. In the experiments with the mCherry::ms2 reporter cell line, we normalize to uninduced cells which exhibit a low expression level, and it can be envisioned that this special case reveals the minor differences.

Nevertheless, the most important observation is that, even here, the recordanelles with the matching RBDs exhibit significantly higher binding (recordanelleMCP, recordanelleV1 and recordanelleV2; Extended Data Fig. 6d). Further, only recordanelleV1 and recordanelleV2 maintain mCherry::ms2 RNA two days after Dox removal. Thus, we can conclude that despite this background binding, our assay is sensitive enough to

measure RBD driven binding on top of this.

20 hours after Dox treatment cells were harvested and mCherry RNA level was analyzed at the full cell level and in the mitochondrial fraction. While recordanelleMCP and recordanelleV2 enrich substantially more mCherry RNA in the mitochondrial fraction, contrasting to the full cell fraction also recordanellePCP and mock enrich mCherry::ms2 relative to MT-ATP6. This indicates that mCherry::ms2 RNA at this low expression level seems to be bound to mitochondria to some extent.

Confocal imaging is used to create a compelling argument that the recordanelle recruits target RNA species in the MCP/PCP model in figures 1 and 2. However, after Fig. 3B / Extended Data Fig. 5, imaging is replaced by qPCR and RNA-seq. While there is a functional effect on RNA abundance after 24 hours (~3 to 4-fold increase over non-binding) at a population level, confocal imaging would provide substantially more information regarding the localization and abundance of a target RNA species, and reduce the risk of “contaminant” transcripts that may have been co-purified with the mitochondrial fraction (see minor comment 10). The authors cleverly use the MS2 system, which enables both fixed and live cell imaging of the target RNA. RNA FISH (as used in Fig. 1-3) would allow for comparison of mitochondrial signal to cytoplasmic signal on a per-cell basis, strengthening the case that the RNA is being protected specifically through sequestration into the condensate. Similarly, live cell imaging of MS2 localization and abundance over time in the same population of cells would provide a single cell assessment of local abundance and cell-to-cell variability, the latter being an important characteristic of the system from the perspective of a potential user (see major comment 6).

We thank the reviewer for this comment. To address their concern, we performed FISH and immunofluorescence experiments to show that recordanelleV1 and recordanelleV2 bind RNAs in cells (see new Supplementary Figure 4). While recordanelleMCP only recruits mCherry::ms2 and recordanellePCP only mCherry::pp7, recordanelleV1 and recordanelleV2 enrich both mCherry::ms2 and mCherry::pp7, thus demonstrating efficient mRNA binding directly in living cells.

4. The IFN β experiments are a strong part of the manuscript, but they also reveal perturbation risks that should be discussed more directly

The IFN β experiments are conceptually compelling because they move beyond engineered reporters and test whether recordanelles can preserve a transient endogenous transcriptional response. This is an important and persuasive direction for the paper. The finding that the IFN-induced signature can still be detected after stimulus removal is one of the manuscript's strongest demonstrations of utility.

At the same time, these experiments also highlight a concern: some architectures appear not merely to preserve the response, but to alter its dynamics. In particular, the manuscript's interpretation of recordanelleV1 suggests that the system may amplify or prolong IFN signaling through feedback effects, whereas recordanelleV2 is presented as a less perturbing alternative. This is important because it means the work itself demonstrates that some recordanelle designs can actively influence the biological process they are intended to record. The comparison between V1 and V2 is interesting, but it does not state the perturbation issue clearly, where V2 may reduce the effect.

The authors should clearly assess if the recordanelle alters translation of the interferon-induced RNAs over time (e.g., via Western blot). Does the recordanelle alter the inflammatory response in any way (i.e., dampen or extend)?

We thank the reviewer for this insightful comment. To address their concern, we performed quantitative proteomics (see Fig. 5f, Extended Data Fig. 8 and Supplementary Fig. 5).

Specifically, we performed the same IFN β stimulation and recording experiment, in which we transiently transfected mock and mCherry (as negative controls), as well as recordanelleMCP, recordanelleV1 and recordanelleV2. We then analyzed the proteome at the full-cell level after 20 hours of IFN β stimulation and 2 days after removing IFN β . These results showed that we could detect a clear upregulation of 8 proteins corresponding to known ISGs at timepoint 0 (note that a few ISG proteins are difficult to detect via proteomics as they are comparatively short and expressed at low levels under homeostatic conditions). Importantly, for all three recordanelle constructs, these proteins were initially induced to the same level as in the mock transfection (Fig. 5f and Extended Data Fig. 8a and b).

2 days after IFNB removal, the expression level of all studied ISG proteins was identical for all negative controls (Mock, mCherry and recordanelle MCP, see Extended Data Fig. 8a), with only 2 not fully returning to baseline (RIGI, ISG15).

While both recordanelleV1 and recordanelleV2 stabilize ISG mRNAs (as measured via qPCR or RNA-seq), we observed that the ISG proteins do not return to control levels with recordanelleV1 2 days after IFNB removal (IFIT1, PARP9, DTX3L & OAS3; Extended data Fig. 8a). However, when using recordanelleV2, which is expected to have diminished protein translation due to the M161A mutation, ISG proteins return to control levels. This demonstrates that, despite the increased mRNA protection, no substantial higher output at the protein level is observed for recordanelleV2. Furthermore, we did not observe any significant difference between the initial interferon response for any of the induced conditions when compared to mock (see Extended Data Fig. 8b), indicating that the initial binding of endogenous mRNAs by recordanelles does not lead to a dampened response to the IFNB stimulus. Potentially, this could be due to the capture of only a small fraction of mRNAs from the cell that is sufficient for RNA recording but not substantial enough to alter the downstream response. In summary, recordanelleV2 does not alter translation of the interferon-induced RNAs over time or the inflammatory response.

5. Is it possible to evaluate the recording capacity of the recordanelle system?

As with all new technologies, detailed characterization of the practical limits of the technology is important for enabling widespread use by the community. The authors show the ability to record for up to 48 hours and sample almost 90% of transcripts. However, it would be useful to explore, either via further experiments or discussion in the text, the practical limits of the system, both in terms of recording time (i.e., the timeframe over which transcripts can be protected) and recording volume (i.e., the number of transcripts that can be held in the recordanelle at one time), to help guide others who intent to apply this technology to different cell systems or new applications. For example, is the timeframe over which a transcript can be protected dependent on the protein turnover of the recordanelle, the number of transcripts already bound by the recordanelle, the half-life of that specific transcript, etc.? Exploring, or at the very least discussing, some of the practical considerations will be a substantial help to those looking to adapt the system in the future.

As Reviewer 2 had a similar comment, we provide one aggregated response above:
Response to Reviewer 1 and Reviewer 2 on long-term recording.

Minor Comments

1. The figure legends are often clearer than the main-text narrative

This is meant as constructive feedback. In multiple places, especially in the screening and IFN β experiments, I found the figure legends easier to follow than the main text. The paper would benefit from revising the Results narrative so that the logic of each experiment is as explicit in the body text as it is in the legends.

We thank the reviewer for this constructive suggestion. We originally wrote the manuscript for a more general scientific audience by avoiding overly technical language in the main text body. However, to maintain technical depth, we included all the key technical information in the figure legends and methods. Nonetheless, we appreciate the reviewer's perspective and reworked the main text to include more technical information.

2. The RNA "recording" effect is interesting, but the practical significance of the recovery metric could be clarified more carefully

In the dox-inducible reporter experiments, the manuscript uses a "transcript recovery" metric that reflects the amount of reporter RNA remaining after Dox withdrawal relative to day 0. This is a reasonable way to measure persistence, but the term may be easy to misinterpret on first reading. It does not represent the absolute fraction of all transcript molecules physically preserved by the system, but rather a relative residual signal over time. The reported effect is still meaningful and supports partial stabilization and extension of the detection window. However, the absolute retained fraction appears relatively modest, even when improved over controls. I therefore think the manuscript would benefit from more careful contextualization of what this metric means biologically and practically. In particular, wording that implies especially strong or highly efficient long-term retention may overstate the present effect size.

We thank the reviewer for this helpful comment. To address this, we now explain this metric more specifically here and in the main text:

“We then quantified the mCherry::ms2 mRNA “transcript recovery” by comparing the ratio between values at days 1 and 0. A high ratio indicates that reporter mRNA levels are similar at days 0 and 1, indicating that the transiently expressed transcript was stabilized. Inversely, a low ratio signifies that most reporter transcripts have disappeared”

3. The manuscript should more clearly distinguish transient co-transfection experiments from later stable-cell-line experiments

In the early recruitment/localization experiments, the wording in the main text can be confusing with respect to what was transiently transfected versus what was stably integrated. Based on the Methods, the early reporter recruitment and imaging experiments appear to rely on transient co-transfection of both the recordanelle construct and the reporter plasmid, whereas stable engineered cells are introduced later for the doxycycline-inducible recording assays. This distinction should be stated more explicitly in the Results, because it affects how readers interpret reproducibility, expression variability, and the maturity of the system at different stages of the paper. As written, a first-time reader can easily misread these experimental configurations.

We thank the reviewer for this comment. In the manuscript, we never worked with a stable recordanelle cell line. We only established a reporter cell line to characterize inducible transgene expression (mCherry::ms2). In all experiments, the recordanelle constructs were transiently transfected. We updated the figure legends to clarify this for each relevant panel.

4. The logic connecting construct screening, optimization, and lead-candidate selection is often difficult to follow

I found the optimization pipeline in the Results section harder to follow than necessary. The rationale connecting the three sections was not explicitly clear in the main text. For example, the screening logic in the early figures sometimes appears to mix recruitment efficiency, specificity, nonspecific binding, and minimal perturbation without clearly stating which criteria are being prioritized at each step.

Relatedly, phrases such as “top-performing” are used, but the basis for prioritizing particular construct families is not always fully transparent. It would help to more explicitly distinguish: (1) small-scale screening for enrichment/selectivity, (2) transcriptome-scale validation of selected constructs, and (3) final nomination of architectures suitable for long-term recording.

We thank the reviewer for this comment. In the revised manuscript we made numerous small changes to explicitly state which parameters we used to select specific constructs for further characterization.

5. When weak construct performance is attributed to localization or expression, more quantitative normalization would strengthen the interpretation

In the endogenous mRNA recruitment section, the authors suggest that weak or absent enrichment for some architectures may be due in part to poor expression or incorrect localization. This makes sense, but more systematic quantification is needed to explain construct-to-construct differences. Does RNA enrichment align with total construct abundance in the cell, or the mitochondrial/cytoplasmic abundance? Without such normalization, it remains difficult to determine whether poor performance reflects intrinsic limitations of a given design or simply reduced effective protein levels at the relevant cellular location.

We thank the reviewer for this comment. We acknowledge that in the original manuscript we overly generalized the results without quantification. As this speculation and quantification of protein localization aren't critical for the manuscript or identifying the best functional binder/recorder, we addressed the reviewer's concern by removing this generalization/speculation from the text. The text now reads:

“In several cases, the recordanelle constructs either weakly or did not enrich mRNA within the mitochondrial fraction and we did not characterize these further.”

6. The meaning of “up to 90% of endogenous mRNAs” should be stated very carefully

This is a striking claim, but it is easy to over-interpret. As I understand it, the statement refers to the fraction of detected transcripts that are significantly enriched in the recordanelle fraction under the authors' analytical framework, not to literal physical recovery of 90% of all mRNA molecules in the cell. A clarification is needed to prevent audiences' misunderstanding.

We thank the reviewer for pointing out this ambiguity. We now more precisely state: “To quantify the global enrichment in RNAs within the recordanelle, we set a stringent threshold corresponding to a fold-change greater than 1.5 and a false discovery rate (FDR) less than 0.01, which revealed that 64% (FUSQG) and 90% (TAF15N) of genes expressed within HEK293T cells were enriched within the recordanelles compared to a non-binding control. Importantly, this does not indicate that the recordanelles are bound to 64-90% of all RNA in the cell, but rather that 64-90% of transcripts are represented in the recordanelles. In contrast, control constructs without an RBD showed an enrichment less than 0.1%. An analysis of DEGs further confirms transcriptome-scale enrichment of mRNAs within the recordanelle (Fig. 3e and Extended Data Fig. 4). Given that TAF15N-based recordanelles showed superior transcriptome-scale binding, we selected them as our lead constructs in subsequent experiments.”

7. Terms such as “representative” and “faithful” should be used with care

The authors provide encouraging correlation analyses suggesting that captured RNA populations reflect the broader whole-cell transcriptome, especially for the stronger PABPC-based designs. However, the same data also indicate expected biases, such as reduced recovery of nuclear and mitochondrial transcripts. I think the manuscript should maintain some caution in how broadly it describes the system as representative of the full transcriptome.

We thank the reviewer for pointing out this potential misunderstanding. In the revised manuscript we avoid these terms and are more technically precise: “In the presence of a PABPC4 or PABPC4ΔC-containing recordanelle, we observe a high concordance between the whole cell and mitochondrial fraction (Extended Data Fig. 5), indicating that the enriched RNAs reflect the cytoplasmic RNA content of a cell. The few depleted or enriched transcripts within the mitochondrial fraction compared to whole cell correspond to nuclear (e.g., the long non-coding RNA Xist) or mitochondrial RNAs, respectively, which can be expected from a recorder enriching cytoplasmic RNAs.”

8. The manuscript would benefit from a more explicit upfront statement of what the technology is and is not designed to record

It would help readers if the Introduction or early Results more clearly stated that the system is especially suited to capturing cytoplasmic RNA histories and transient signals that would otherwise decay, rather than being interpreted as a universal recorder of all RNA states across all compartments.

We thank the reviewer for pointing out this omission. We now state at the beginning of the manuscript that the recorder is designed for cytoplasmic RNAs: “We conceived of a solution meeting these demands which we term “recordanelle” (RNA recording organelle) — a genetically-encoded system that is inspired by the principles of biomolecular condensation^{10–12} to drive the unbiased and scalable storage of endogenous cytoplasmic RNAs within an orthogonal and heritable organelle (Fig. 1).”

9. Several mechanistic interpretations are interesting, but remain speculative

The manuscript proposes that interactions between the assembler and the RNA-binding domain may influence condensation behavior and thereby affect performance. This is a plausible and interesting mechanistic explanation, especially in light of prior literature and the observed context dependence of which architecture performs best. However, in the current manuscript, these interpretations remain somewhat speculative.

I do not think the paper necessarily needs to become a fully mechanistic study, but when these hypotheses are used to explain key result differences, the manuscript would benefit from either deeper discussion or more explicit acknowledgement that these explanations are provisional. As written, some mechanistic suggestions read more definitively than the evidence fully justifies.

Additionally, the authors state: “We further observed that at the day 0 timepoint, when

the reporter is maximally induced, mCherry::ms2 expression was higher in recordanelleV1 (but not recordanelleV2) compared to the control recordanelle. This suggests that whereas recordanelleV1 may globally stabilize mRNAs, thus altering the homeostatic state of the cell, recordanelleV2 does not and thus represents the superior architecture. Taken together, we confirm mCherry::ms2 recording and protection without altering the cellular state with recordanelleV2." This is an interesting point that needs to be explained in more depth. For example, how do you explain global stabilization of RNAs without impacting the transcriptome, as described in Extended Data Fig. 9?

We thank the reviewer for this comment. To mark clearly that we do not have a mechanistic insight into the PABP/IDR interaction we now state:

"We speculate that this could be due to the RBD influencing the biomolecular condensation behavior of the assembler, which has been shown previously in the context of PABPC137. This suggests that recordanelle performance depends on interactions between their component parts, and thus combinatorial testing of recordanelle architectures could yield emergent properties."

Regarding the difference for mCherry::ms2 recording in the case of recordanelleV1 vs recordanelleV2. We work with a low concentration of Dox, leading to a small induction of the mCherry transcript. Thus, it is possible that the difference for recordanelle V1 is more pronounced but does not come to the fore at the global transcriptome, which is likely more stable. However, as we do not have an accurate mechanistic insight into this difference, we removed our speculative statement and more simply state:

"At day 0, when the reporter was maximally induced, mCherry::ms2 expression was higher in recordanelleV1, but not recordanelleV2, compared to the control recordanelleMCP (Extended Data Fig. 6). Because recordanelleV2 records this transiently expressed RNA without altering its initial expression level, it represents the superior architecture."

10. RNA-seq methods should be described in greater detail

Given the importance of RNA sequencing data to the claims of the paper, it would benefit the reader to understand the specifics of how the RNA sequencing data were processed. For example, what sort of filtering, if any, was carried out? How were potential contaminant RNA transcripts (e.g., those that may not be housed inside the recordanelle but instead were co-purified with the mitochondrial fraction due to interaction or proximity) thought about and handled?

Figure 3C and Extended Data Fig. 8 make the case that the recordanelle does not impact global gene expression patterns and evenly samples the cytoplasmic RNA content. While the correlation values indicate that the results are consistent across replicates and the full recordanelle system shows excellent agreement between the full cell and mitochondrial transcriptomes, the visualization masks this point. While a correlation of ~0.7 (mock and non-binding controls) is clearly much lower than ~0.98 (recordanelle v1), there is still significant overlap in the transcripts identified in the mitochondrial and full cell fractions. The authors should address this point in greater detail, as it brings up important questions about whether this is "contaminant" RNA that was co-purified during mitochondria extraction or a genuine representation of the transcripts found in mitochondria. An awareness of the expected overlap may also help improve the visualization of recordanelle content, which, as currently presented in Extended Data Fig. 8, appear largely indistinguishable across the four recordanelle conditions. For example, perhaps it may be useful to generate plots showing the mitochondrial vs full cell correlation for transcripts that correlate poorly in the control samples to avoid crowding the figure with potential "contaminant" transcripts that may have been co-purified along with the mitochondrial fraction. Additionally, would the true effect of the recordanelle be clearer with a set of plots showing only RNA transcripts that are expected to be found in the cytoplasm?

We thank the reviewer for this comment. To clarify, we deliberately chose a fast mitochondrial isolation protocol for this work to accelerate technology development. This method however comes with the limitation that not all of the cytoplasmic fraction is removed from the mitochondrial fraction.

Therefore, even in the mock control we observe correlation. Reanalyzing the data we realized that this difference is apparent only when the correlation is calculated on the linear CPMs. This is mostly driven by highly expressed transcripts (mitochondrial genes in the mitochondrial fraction). When we analyze the correlation on log transformed CPMs excluding mitochondrial genes, we observe a similar correlation between the mitochondrial fraction and the full-cell level for all constructs.

This indicates that the cytosolic background in the mock condition is reflective of the

cellular transcriptome. However, we demonstrated that PABPC4-based recordanelles, enrich a substantial fraction of the transcriptome (see Fig. 3e and Extended Data Fig. 4). As here the correlation between the full-cell level and the mitochondrial fraction is similarly high, this is indicative of the fact that the enriched transcripts are representative of the cytosolic transcriptome.

For the analysis of bulk RNA-seq data, we only filtered lowly expressed genes to enable accurate quantification of enrichment and differential expression. The specific metrics for filtering “genes with low expression were filtered out if they failed to meet the criteria of maximum CPM ≥ 7 in at least one condition and mean CPM ≥ 5 across samples” are specified in the methods. To facilitate the understanding of bulk RNA-seq, we have extended the description of the analysis in the methods section.

When we assessed enrichment in the mitochondrial fraction between several recordanelles compared to a mock condition for recordanelles containing MCP and PCP we only observe differential enrichment of mCherry::ms2 and EGFP::pp7 respectively, while poly-A binding variants enrich a large fraction of the transcriptome. At the same time, the control without an RBD does not significantly enrich any transcript.

This indicates that we have a consistent cytoplasmic background, but we are nevertheless able to detect specific binding on top of this in our assay.

11. Statements of novelty require clarification

Claims of novelty need to be better supported and explained. For example, in the discussion, the authors state: “Here, we introduce the recordanelle, the first transcriptome-wide RNA recording technology for mammalian cells that continuously samples the cellular transcriptome”. However, it is this reviewer’s understanding that the new TimeVault technology (described later in the discussion) represents a transcriptome-wide, continuous RNA recording technology, where the time window of recording can be controlled via the addition of doxycycline. As currently presented, the novel features of recordanelle over existing technologies require clarification. Additionally, it might be worth referencing Felix Horns and colleagues’ 2023 Cell paper (PMID: 37437570) in which they developed a system for programmable RNA export for continuous, non-destructive monitoring of cell populations.

We thank the reviewer for this comment, which we address in three parts:

1. Our technology differs from the most closely related recording approach, TimeVault, in two key respects. First, TimeVault captures discrete, defined snapshots of the transcriptome, whereas the recordanelle remains open to the cell and samples continuously. Second, TimeVault shows clear signal loss below 1’000 cells, making it incompatible with single-cell RNA sequencing; by contrast, the recordanelle captures IFNB signaling efficiently at the single-cell level. We now include this clarification in the Discussion section.

2. In the revised manuscript, we also added a reference to the work by Felix Horns.

3. With our new single-cell data, we additionally clarify the technical advantage of recordanelles:

“Here, we introduce the recordanelle, the first transcriptome-wide RNA recording technology for mammalian cells that continuously samples the cellular transcriptome in single cells.”

12. Add values and axes labels to figures

To improve the readability and clarity of the manuscript, I would recommend:

- reporting the mean expression value or the ratio in the plots (e.g., Fig. 4, Extended Data Fig. 9, and Extended Data Fig. 10)
- Defining the significance limits for stars (e.g., Extended Data Fig. 10)
- Including units on all axes (e.g., Extended Data Fig. 10 – counts per million)

We thank the reviewer for this comment.

To clarify the meaning of the statistical analysis we now include a statement in the methods:

“Statistical analysis of qPCR data, CellTiter-Glo and Seahorse assays was performed using GraphPad Prism software, the used statistical tests are indicated in the figure legends significant differences are displayed as using asterisks in the figures (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$).”

To improve readability of the heatmaps we now also add bar graphs in Extended Data Fig. 2 and 3.

Overall, this is a very exciting work with great potential. The idea and most of the

experiments were clearly executed and compelling. The current reservation is not about the concept but whether the current work fully supports the strong claims being made. With clearer presentation and more detailed perturbation work, I think the manuscript would be significantly strengthened.

We thank the reviewer for this positive overall assessment and hope that our revised manuscript address all their concerns.
Reviewer #2 (Remarks to the Author):

This work describes a new approach for preserving RNAs within cells and localizing them to mitochondria as a way to sequence transcriptional responses beyond the window where they would naturally return to baseline. The approach is interesting and unique among the new technologies for transcriptional recording. However, there are substantial constraints that may limit its practical utility. More importantly, the approach is likely to be substantially perturbative to the cell biology in ways that are downplayed in the manuscript and experimentally unexplored. Finally, the terminology around the technology is misleading and imprecise.

We thank the reviewer for their enthusiasm and pointing out the uniqueness of our recording approach. We addressed all their concerns regarding the constraints of the technology with our revision experiments and hope that these convince the reviewer of the utility of our approach.

Major points:

(1) Impact of the approach on the cell biology. At multiple points the authors state that the recordanelle approach is not perturbative, e.g.: “does not perturb the cell” (page 4, line 12); “can capture a large fraction of the cytoplasmic transcriptome without altering global RNA levels (page 5, line 1); “without altering the cellular state” (page 7, line 2). These statements are based on narrow examinations of transcriptional levels, often with RNA binding domains that should only interact with synthetic transcripts. In fact, the core idea of the approach essentially guarantees that it will perturb the cell biology. Transcripts are sequestered to an unnatural location and protected over time. There are two potential ramifications: either the transcripts could be translationally repressed by this sequestration and protection, reducing protein expression; or the transcripts could be translationally active with an increased lifetime, increasing cumulative protein expression. These perturbations to the proteome of the cell could even both be true, in a manner that is dependent on each particular transcript.

Despite claiming that the approach enables “enabling transient and complex transcriptional events to be archived within the same population of cells without altering their physiology” (page 8, line 16), the work shows evidence of exactly these perturbations. Page 7:

“Intriguingly, we observed that with recordanelleV1 but not recordanelleMCP, ISG detection was not only maintained, but increased from day 0 to 2 after IFNB removal... One likely explanation for this is the well-described type-I IFN signaling positive feedback loop^{38,39}, whereby under control conditions IFNB is rapidly degraded but with the recordanelle IFNB response persists and drives the feedback loop. This could occur under conditions where the recorded ISGs mRNAs are translated. Indeed, compared to recordanelleV1, recordanelleV2 — utilizing PABPC4ΔC161A, which is supposed to abrogate translation of the bound mRNA^{40,41} — the feedback loop is prevented and ISG levels stay around day 0 levels. For recording mRNA without disrupting cell physiology, recordanelleV2 has clear advantages because it can prevent translation of RNAs within the recordanelle, and we therefore proceeded with this recordanelle architecture.”

Thus, the V1 enables translation and therefore creates a synthetic feedback that prolongs the transcriptional response. The authors state that V2 prevents translation and therefore should blunt the response (although not experimentally tested).

To fully test the extent to which the approach perturbs the biology of the cell, the authors should look at the proteome for evidence of disruption and contextualize these disruptions in a way that makes them clear to readers and potential adopters of the technology. Specifically, proteome-wide measurements should be made with the V1 and V2 version (not MCP or PCP) at multiple timepoints with and without stimulation. Moreover, another likely way that the approach will perturb the cells is via disruption of mitochondrial function due to encasement in RNA and disordered protein domains. Cells expressing the V1 and V2 versions should be profiled by Seahorse or other similar

assay for mitochondrial function compared to control cells.

As Reviewer 1 had a similar comment, we provide one aggregated response above:
Response to Reviewer 1 and Reviewer 2 on the non-disruptive nature of recordanelles.

(2) Testing maintenance over time. The maximum time period after an event that the authors tested was 2 days. It is hard to imagine real biological questions where a two-day extension of transcripts makes the difference between being able to study a phenomenon or not. If the authors can think of one, they should point it out. Either way, it would be useful to extend the timeline of experimentation to see how rapidly the signal fades over time. The authors should continue this timecourse until the signal returns to baseline.

We thank the reviewer for this comment. While we disagree with the reviewer on the impact of transcriptome-wide recording over 2 days, we agree that longer-term recording would have an even bigger impact and open up the technology to a wider set of biological phenomenon and questions. We therefore set out to demonstrate longer-term recording. As Reviewer 1 had a similar comment on long-term recording, we provide one aggregated response above: Response to Reviewer 1 and Reviewer 2 on long-term recording.

One other facet that we would like to point out is how recordanelles are differentiated from the current leading (and only) transcriptome-wide recording tool: TimeVaults. Whereas TimeVaults record a 24-hour time window, we originally showed that Recordanelles could record a 2-day time window and we now show that this can be extended to at least 5 days. For this reason, we claim that recordanelles are continuous recorders. Moreover, Recordanelles are compatible with single-cell RNA sequencing while TimeVaults are not. By extending recording to at least 5 days and in single cells this extends the utility of recordanelles to a greater number of biological phenomenon and questions. For instance, murine pre-implantation development occurs over 5 days.

(3) The terminology. The authors state that they created a synthetic organelle, but lack justification for that claim. A less hyperbolic description would be that they decorated a natural organelle, the mitochondria, with RNA binding proteins. Potentially the authors wish to suggest that the disordered domains create phase separation and compartmentalization. However, that separation was never tested and the inclusion of the disordered regions was not necessary for the effect. Even if there were condensate formation around the mitochondrial that would not reach the bar for defining a new synthetic organelle. This terminology also leads to confusion where the anchored RNA binding protein construct is referred to as the recordanelle and other times the recordanelle encompasses the mitochondrial fraction. The authors should be more explicit in their language to enable understanding rather than branding.

We thank the reviewer for this comment. We believe that the terminology of calling this a synthetic organelle is justified and corresponds to the literature standard. Similar systems for other applications have been termed synthetic organelles/condensates by us and others before:

- Garabedian et al. 2021 (PMID: 34341589)
- Scharrel et al. 2024 (PMID: 39448590)
- Hastings & Boeynaems 2021 (PMID: 33539874)
- Wan et al. 2024 (PMID: 38588907)
- Lasker et al. 2022 (PMID: 36163138)
- Shapiro et al. 2025 (PMID: 39929988)
- Reinkemeier et al. 2019, 2021 (PMID: 30923194, 34433013)

Nevertheless, to demonstrate the importance of the IDR component for recording we performed additional experiments (See Extended Data Fig. 7i-j). Here, we show that the TAF15 IDR aids recording. We transfected HEK293T cells transiently with constructs encoding the recordanelleMCP, recordanelleV2 and recordanelleV2,ΔIDR and treated them with IFNβ (20 h, 60 U/mL). Subsequently, cells were harvested right at the time of IFNβ removal or 2 days later. The RNA content was analyzed via qPCR both at the full-cell level (i) and in the mitochondrial fraction (j). At day 0, recordanelleV2,ΔIDR exhibits similar transcript enrichment as recordanelleV2. However, when contrasting recordanelleV2 and recordanelleV2,ΔIDR at day 2, we observe significantly stronger recording with recordanelleV2. We thus conclude that each component of the recordanelle is important for its recording performance and thus we believe that the terminology is warranted.

Minor Points:

(1) Multiple times conclusions are made from comparisons that are not statistically tested. All conclusions regarding Figure 2c are a good example. For instance, the statement “While we observed enrichment of target RNAs when using a control construct without an assembler protein (Δ AP), several other constructs outperformed this condition (e.g., FUSQG, FUSopto), indicating that RNA recruitment might be aided by the biomolecular condensation properties of the recordanelle.” This conclusion is accompanied by no statistical testing (which should be corrected for multiple comparisons). Moreover, only a mean is shown for each condition, with no distribution of underlying data and N is reported inexactly as “at least three experiments per condition.”

We thank the reviewer for this comment. Whenever we apply statistical testing we correct for multiple comparisons, and we state the exact test used in the respective figure legend.

As the initial screening was designed to identify candidates of interest, we decided to show them on a heat map to account for the high dimensionality. We now additionally show bar graphs (with error bars) corresponding to the heat maps in the Extended Data Figures 2 and 3 respectively. In these figures we also show the exact number of experiments per condition. With our mitochondrial extraction method, we can only process 12-18 samples per experiment. Therefore, to test all conditions, we performed a total of 24 experiments for the two bar graphs, always including mock as a normalization control. We also add TAF15N:: Δ RBD, TAF15N::MCP and TAF15N::PCP as additional controls whenever possible; this is why the exact sample number varies. We do not make statistical claims here, as we test multiple variants for recording later on in the manuscript: See e.g. Fig 4c, Fig 5 b, c, f; Extended Data Fig. 6, Extended Data Fig. 7, Supplementary Figure 2 & 3.

(2) Figure 2b, 3c: One of the stains is identified as “recordanelle” but the figures, text, and legend do not identify what is being stained, nor is it obvious from the methods.

We thank the reviewer for catching this omission. We now explicitly state that the recordanelles are epitope tagged (HA) and stained accordingly.

(3) The authors consistently ignore differences in the magnitude of enrichment for different transcripts. Figure 3c shows a clear difference in the magnitude of enrichment of mCherry, GFP, and the endogenous YWHAZ, for instance that the authors gloss over and similarly do not mention the enormous spread of enrichment in 3e. Some exploration of what dictates the degree of enrichment would be useful (length, abundance, structure, half-life). Additionally, this should be discussed as a major caveat of the approach for potential users who would be interested in discovery biology with a tool that may have a substantial bias.

We thank the reviewer for this comment. First, in Fig. 3e, data are normalized to ERCC spike-ins and mitochondrial genes, contrasting to mock. While we observe some spread in the data, the spread on the log2FC is relatively minor (normal distributed centered around approximately 1.7-fold, as can be seen in Fig. 3d). We do observe a large spread on FDRs, as this is related to the statistical power on the transcripts (expression level, dispersion between replicates, etc.); however, this is not indicative of unequal sampling. If there would be a major bias, we would expect a skewed distribution or the appearance of bimodality. As this is not the case and we further observe a strong correlation between the CPMs at the full-cell level and in the mitochondrial fraction (see Extended Data Fig. 5). We can therefore conclude that the recordanelle overall samples the cytoplasmic transcriptome without substantial bias.

(4) The authors should remove the line containing, “the first transcriptome-wide RNA recording technology for mammalian cells that continuously samples the cellular transcriptome,” given the recent vault work that is discussed.

We thank the reviewer for this comment. With our original comment we were trying to differentiate recordanelles from TimeVaults. To reiterate what we mentioned above: TimeVaults record a 24-hour time window, whereas recordanelles record continuously for at least 5 days. Thus, we can differentiate recordanelles from TimeVaults by saying that they are continuous recorders (compared to TimeVaults which are 24-hour window recorders). We can also differentiate recordanelles from TimeVaults in that they are

compatible with single-cell RNA sequencing (TimeVaults require more than 1k cells to capture a transient signal). To make these two key differentiators clear to the readers we think it is important to state it plainly:

“Here, we introduce the recordanelle, the first transcriptome-wide RNA recording technology for mammalian cells that continuously samples the cellular transcriptome in single cells.”

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

We thank the authors for the thorough and detailed response. The revised manuscript includes multiple new experiments that directly address the questions and concerns raised during peer review. These include transcript- and protein-level analyses, mitochondrial function assays, and cellular viability assays, together demonstrating that the lead candidate recordanellev2 design is minimally perturbative. Additionally, the inclusion of longer-term studies, imaging data, and extensive controls bolster the claims that the recordanellev2 is a safe, sensitive, and scalable transcriptional recording device.

I have only two minor points of clarification:

1. Although the new set of data strengthens the utility claim of the study, I would still recommend carefully framing the conclusion as “no-detectable perturbation under the conditions tested in this study” rather than saying the system is non-disruptive; all experiments conducted to date have covered a handful of important dimensions, but they cannot exclude all context-dependent effects.

2. 5 days experiments are informative but also suggest that recording persistency relies on cell status (proliferation, transcriptional state). The authors have also stated that the signals could be diluted by division and confluence - Thus, this dependence should be stated clearly so that there will not be confusion for the “at least five days” as a condition-independent recording duration.

Overall, the authors have responded to the major concerns raised by this reviewer. The new experiments substantially strengthen the manuscript by providing evidence that the recordanelle is physiologically tolerable and providing control experiments to ensure that this tool can be adapted with confidence by others in the field. I strongly support the publication of this exciting work, which constitutes a breakthrough for the field and undoubtedly will have multiple applications.

Reviewer #2

(Remarks to the Author)

In my initial review of this manuscript, I found an interesting new approach that had some rough edges in the description – missing some fixable experimental characterization of the limits and pitfalls of the approach and overstated in places. Unfortunately, although this revision adds useful new experiments – like a longer timecourse showing the perseverance of the recording up to 3-5 days – the authors have chosen to persist in unreasonable claims about the cellular implications of recording and deal with data in a way that gives me deep misgivings about the scientific integrity of the work.

My major issue with this version is unchanged from the original submission, the authors claim repeatedly that this approach does not have any impact on the cell physiology, transcriptome, or proteome. Before even getting into the data, it is important to point out that ALL transcriptional recorders have an impact on cells. Even the most efficient, optimized recorders will have a thermodynamic cost if information is stored, and the field is still nascent, so current recorders, the recordanelle included, are likely to be quite perturbative due to direct interactions with the molecules that they are intended to track.

In order to advance the field, the standard for publication should not be to prove that there is no cellular impact of recording (impossible), but rather to transparently show the potential impact so that users of the technology are appropriately informed and technology developers can fruitfully build on the concept. That is what I was asking the authors for, but this version just repeats the same impossible statements that there are no impacts or alterations to the biology of the cell.

To get into the specifics, the recordanelle approach increases the longevity of mRNA. In the authors' own words, “recordanelle architectures can both recruit and protect RNA for at least 48 hours.” With just logic, one should be able to conclude that under conditions of change (such as IFN β stimulation, differentiation, or other conditions where transcriptional recording is interesting), increasing the longevity of a naturally transient transcript must have some impact on the resulting biology. How big that impact is and whether it matters may vary by the exact context of the experiment, but there is no way there is no impact.

In this revision, the authors go out of their way to avoid comparisons that would show these impacts and minimize them when they do occur. They add a transcriptional comparison (Extended Data Fig. 4), but without stimulation where there is unlikely to be an effect since the preserved and new transcript are identical. Similarly, we are only given a full proteome

volcano plot at day 0 (Supplementary Fig. 5), even though I explicitly asked for it at multiple timepoints with stimulation. They collected the data at day 2, it's just only shown in small parts (Extended Data Fig. 8). Not only is new data not presented transparently to quantify the impact as requested, but the authors have even decided to remove statements from the text that previously pointed to exactly these effects:

Original submission: "Intriguingly, we observed that with recordanelleV1 but not recordanelleMCP, ISG detection was not only maintained, but increased from day 0 to 2 after IFNB removal (Fig 5b and Extended Data Fig. 12). This increase in detection was not observed for the YWHAZ housekeeping gene, suggesting that the increase was due to a feature of IFNB signaling. One likely explanation for this is the well-described type-I IFN signaling positive feedback loop, whereby under control conditions IFNB is rapidly degraded but with the recordanelle IFNB response persists and drives the feedback loop. This could occur under conditions where the recorded ISGs mRNAs are translated."

Revised submission: "Under certain conditions we observed that in recordanelleV1-containing cells, ISG detection was not only maintained but increased from day 0 to 2 after IFNB removal (Fig 5b and Extended Data Fig. 7a,b). This increase appeared specific to IFNB signaling as it was absent for the YWHAZ housekeeping gene. As we did not observe this behavior for recordanelleV2, it seems that this version is superior as an RNA recording tool and we therefore proceeded with this recordanelle architecture."

Yet, the evidence is there, the authors just ignore it:

In Figure 5: The authors only show the mitochondrial fraction in Figure 5c on day 2, not the full cell (which would be where an impact of prolonging transcript longevity would be seen). There is a clear signature of transiently expressed, IFNB responsive genes in the mitochondrial fraction at day 2. However, in 5e this is compared with the full cell and we can see that those same genes that should have declined by day 2 are also enriched at day 2 in the full cell with the V2 recordanelle. This is direct evidence that the recordanelle affects the transcriptome and will have some impact on the cell.

The single cell data in Figure 5i-k also shows this. It is the V2 recordanelle, and single cell (not mitochondrial fraction) transcriptomes carry a signature of the prior stimulation. They have to for the recording to be useful, but that fact also necessarily means that the transcriptome is perturbed and there could be cellular consequences depending on the biology being investigated. My guess is that we would also see evidence of this perturbation in the proteome if they had shown the full volcano plots at day 2 after stimulation as requested, rather than pick out a small subset of proteins (where, by the way, there is a perturbation that is downplayed).

All of this adds up to what appears to be an intentional lack of transparency. Going further, the authors repeatedly take liberties with when to use or disregard statistical tests. In my review of the original manuscript, I pointed out that they made a statement that wasn't supported by a statistical analysis of the data: "While we observed enrichment of target RNAs when using a control construct without an assembler protein (Δ AP), several other constructs exhibit a higher binding of the mCherry mRNA (e.g., FUSQG, FUSN)." The authors replied to this by adding bar graphs that seem to confirm my suspicion that this difference is likely not statistically supported, and yet say, "We do not make statistical claims here, as we test multiple variants for recording later on in the manuscript." But they leave the statement in about the higher binding when they know it's not supported by the data. I can't understand that decision, and it makes me uncomfortable with the data practices.

On the other hand, throughout the manuscript and the rebuttal they brush away statistically significant differences in the transcriptomes or proteome as technical noise when it doesn't suit their argument without any further explanation or experiments:

"We suspect that the additional 1 enriched and 2 depleted genes within the MCP-containing recordanelles were due to technical noise or MCP binding."

"variation seen in a proteomics experiment, so we concluded that this is likely due to technical noise."

Additional minor comments:

-The authors justify this statement, "Here, we introduce the recordanelle, the first transcriptome-wide RNA recording technology for mammalian cells that continuously samples the cellular transcriptome in single cells," at least partially by differentiating their approach from the TimeVaults approach based on TimeVaults being limited to 24-hour expression snapshots, which is not a feature or strict limitation of the TimeVault approach.

-I remain unconvinced that this is a 'synthetic organelle' given that it is inseparable from and embedded in the membrane of the mitochondria. The other examples of membraneless organelles that are cited as parallels are distinct compartments and in most cases some effort was put in to show that they are phase separated, which has not been done in this work.

Author Rebuttal letter:

We thank both reviewers for their comments. In the following point-by-point reply the reviewers' comments are shown in black, our replies in blue, quoted text that we have replaced in cyan, and newly added text in yellow.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

We thank the authors for the thorough and detailed response. The revised manuscript includes multiple new experiments that directly address the questions and concerns raised during peer review. These include transcript- and protein-level analyses, mitochondrial function assays, and cellular viability assays, together demonstrating that the lead candidate recordanellev2 design is minimally perturbative. Additionally, the inclusion of longer-term studies, imaging data, and extensive controls bolster the claims that the recordanellev2 is a safe, sensitive, and scalable transcriptional recording device.

We thank the reviewer for their appreciation for the new additions and insightful comments.

I have only two minor points of clarification:

1. Although the new set of data strengthens the utility claim of the study, I would still recommend carefully framing the conclusion as “no-detectable perturbation under the conditions tested in this study” rather than saying the system is non-disruptive; all experiments conducted to date have covered a handful of important dimensions, but they cannot exclude all context-dependent eNects.

In the revised manuscript we have either removed the claim or added the suggested clarifier at several points:

1) Abstract

Old text

“We demonstrate that the recordanelle can be engineered to recruit engineered RNAs or a sampling of up to 90% of all endogenous mRNAs and protect them over several days without disrupting cellular physiology.”

Second revised submission removal (page 1, line 17-19):

“We demonstrate that the recordanelle can be engineered to recruit engineered RNAs or a sampling of up to 90% of all endogenous mRNAs and protect them over several days.”

2) Results section

Old text

“Taken together, these results demonstrate that recordanelles do not impact cell viability, the cellular proteome, or mitochondrial functionality.”

Second revised submission addition (page 8, lines 3-6):

“Taken together, these results demonstrate that recordanelles do not substantially impact cell viability, the cellular proteome, or mitochondrial functionality measured at the bulk and single cell level under the conditions tested in this study. Moreover, we cannot exclude that the recordanelles have an impact beyond what was measured in these assays.”

3) Discussion section

Old text

“Our optimized recordanelles capture a subset of all mRNAs — reflecting up to 90% of the transcriptome — over multiple days and cell divisions, enabling transient and complex transcriptional events to be archived within single cells without altering their viability, mitochondrial function, transcriptome, or proteome.”

Second revised submission addition (page 8, lines 35-39):

“Our optimized recordanelles capture a subset of all mRNAs — reflecting up to 90% of the transcriptome — over multiple days and cell divisions, enabling transient and complex transcriptional events to be archived within single cells. Recordanelles achieve this without substantially altering cellular viability or mitochondrial function as well as the cellular transcriptome and proteome under the conditions tested in this study.”

2. 5 days experiments are informative but also suggest that recording persistency relies on cell status (proliferation, transcriptional state). The authors have also stated that the

signals could be diluted by division and confluence - Thus, this dependence should be stated clearly so that there will not be confusion for the "at least five days" as a condition-independent recording duration.

In the revised manuscript we have added the suggested clarifier in the discussion:

Second revised submission addition (page 8, lines 39-43):

"The degree of the apparent recording seems to be influenced by the cellular state, such that without passaging we can record IFNB signaling for at least 5 days, while with passaging the signal decays more quickly. We interpret this as proliferation increasing the turnover of the transcriptional record, whereby previously recorded transcripts can be lost, and newly transcribed mRNAs are recorded in their place."

Overall, the authors have responded to the major concerns raised by this reviewer. The new experiments substantially strengthen the manuscript by providing evidence that the recordanella is physiologically tolerable and providing control experiments to ensure that this tool can be adapted with confidence by others in the field. I strongly support the publication of this exciting work, which constitutes a breakthrough for the field and undoubtedly will have multiple applications. We thank the reviewer for their kind words and support.

Reviewer #2 (Remarks to the Author):

In my initial review of this manuscript, I found an interesting new approach that had some rough edges in the description – missing some fixable experimental characterization of the limits and pitfalls of the approach and overstated in places. Unfortunately, although this revision adds useful new experiments – like a longer timecourse showing the perseverance of the recording up to 3-5 days – the authors have chosen to persist in unreasonable claims about the cellular implications of recording and deal with data in a way that gives me deep misgivings about the scientific integrity of the work.

My major issue with this version is unchanged from the original submission, the authors claim repeatedly that this approach does not have any impact on the cell physiology, transcriptome, or proteome. Before even getting into the data, it is important to point out that ALL transcriptional recorders have an impact on cells. Even the most efficient, optimized recorders will have a thermodynamic cost if information is stored, and the field is still nascent, so current recorders, the recordanella included, are likely to be quite perturbative due to direct interactions with the molecules that they are intended to track.

In order to advance the field, the standard for publication should not be to prove that there is no cellular impact of recording (impossible), but rather to transparently show the potential impact so that users of the technology are appropriately informed and technology developers can fruitfully build on the concept. That is what I was asking the authors for, but this version just repeats the same impossible statements that there are no impacts or alterations to the biology of the cell.

To get into the specifics, the recordanella approach increases the longevity of mRNA. In the authors' own words, "recordanella architectures can both recruit and protect RNA for at least 48 hours." With just logic, one should be able to conclude that under conditions of change (such as IFNB stimulation, differentiation, or other conditions where transcriptional recording is interesting), increasing the longevity of a naturally transient transcript must have some impact on the resulting biology. How big that impact is and whether it matters may vary by the exact context of the experiment, but there is no way there is no impact.

As regular developers of molecular and transcriptional recording tools, we can fully agree with the reviewer in that detailed characterizations of cellular impact and transparent reporting of the results and implications are paramount. We strongly believe that we went to great lengths to do so. We were therefore genuinely perplexed by the reviewer's characterization of our work, which we do not feel reflects the analyses and disclosures presented in the manuscript as well as our own integrity. We take the concerns of the reviewer very seriously and believe that most are due to misunderstandings or differences in style and framing. To address this, we have systematically responded to each of the reviewer's points as well as made many new additions and alterations to the revised manuscript. We agree with the reviewer that, on first principles, all transcriptional recorders (as well as any transgene overexpression context in general) could have an impact on cells in

some way. In our work, we generally compare recordanelles to either mock transfection or expression controls (e.g. mCherry, EGFP) so we admittedly are not looking at the burden of transfection or expression itself. We also agree with the principle that there must be some thermodynamic cost and potentially direct molecular interaction eNects. While our data does not support the notion that these are strong eNects under our experimental conditions, we agree that they are theoretically likely. To make these potential limitations clearer to the reader, as well as to tone down and better specify what we are observing in terms of cellular perturbations, we have made the following changes to the revised manuscript.

1) Discussion

Second revised submission addition (page 8, line 44 – page 9, line 5):

“While the data reported here suggest that recordanelles do not substantially perturb cellular function under the tested conditions, it is important to clarify that transcriptional recorders such as recordanelles by their very nature are perturbations to cells. These perturbations include the burden of transgene expression, occupancy of the mitochondrial membrane and its immediate surroundings, unnatural interactions with the RNAs they track, depletion of RNAs from the cellular pool, and thermodynamic costs of information storage. As the field moves from building technologies to applying them to ask fundamental questions about biology, exactly how these perturbations manifest and potentially confound our interpretation of biology remain to be seen.”

2) Abstract

Old text

“We demonstrate that the recordanelle can be engineered to recruit engineered RNAs or a sampling of up to 90% of all endogenous mRNAs and protect them over several days without disrupting cellular physiology.”

Second revised submission addition (page 1, lines 17-19):

“We demonstrate that the recordanelle can be engineered to recruit engineered RNAs or a sampling of up to 90% of all endogenous mRNAs and protect them over several days.”

2) Results

Old text

“Taken together, these results demonstrate that recordanelles do not impact cell viability, the cellular proteome, or mitochondrial functionality.”

Second revised submission addition (page 8, lines 3-6):

“Taken together, these results demonstrate that recordanelles do not substantially impact cell viability, the cellular proteome, or mitochondrial functionality measured at the bulk and single cell level under the conditions tested in this study. Moreover, we cannot exclude that the recordanelles have an impact beyond what was measured in these assays.”

3) Discussion

Old text

“Our optimized recordanelles capture a subset of all mRNAs — reflecting up to 90% of the transcriptome — over multiple days and cell divisions, enabling transient and complex transcriptional events to be archived within single cells without altering their viability, mitochondrial function, transcriptome, or proteome.”

Second revised submission addition (page 8, lines 37-39):

“Our optimized recordanelles capture a subset of all mRNAs — reflecting up to 90% of the transcriptome — over multiple days and cell divisions, enabling transient and complex transcriptional events to be archived within single cells. Recordanelles achieve this without substantially altering cellular viability or mitochondrial function as well as the cellular transcriptome and proteome under the conditions tested in this study.”

In this revision, the authors go out of their way to avoid comparisons that would show these impacts and minimize them when they do occur. They add a transcriptional comparison (Extended Data Fig. 4), but without stimulation where there is unlikely to be

an eNect since the preserved and new transcript are identical. Similarly, we are only given a full proteome volcano plot at day 0 (Supplementary Fig. 5), even though I explicitly asked for it at multiple timepoints with stimulation. They collected the data at day 2, it's just only shown in small parts (Extended Data Fig. 8). Not only is new data not presented transparently to quantify the impact as requested, but the authors have even decided to remove statements from the text that previously pointed to exactly these eNects:

Original submission: "Intriguingly, we observed that with recordanelleV1 but not recordanelleMCP, ISG detection was not only maintained, but increased from day 0 to 2 after IFNB removal (Fig 5b and Extended Data Fig. 12). This increase in detection was not observed for the YWHAZ housekeeping gene, suggesting that the increase was due to a feature of IFNB signaling. One likely explanation for this is the well-described type-I IFN signaling positive feedback loop, whereby under control conditions IFNB is rapidly degraded but with the recordanelle IFNB response persists and drives the feedback loop. This could occur under conditions where the recorded ISGs mRNAs are translated."

Revised submission: "Under certain conditions we observed that in recordanelleV1-containing cells, ISG detection was not only maintained but increased from day 0 to 2 after IFNB removal (Fig 5b and Extended Data 35 Fig. 7a,b). This increase appeared specific to IFNB signaling as it was absent for the YWHAZ housekeeping gene. As we did not observe this behavior for recordanelleV2, it seems that this version is superior as an RNA recording tool and we therefore proceeded with this recordanelle architecture."

We respond to the three sub-points in the sections below.

Sub-point 1:

"They add a transcriptional comparison (Extended Data Fig. 4), but without stimulation where there is unlikely to be an eEffect since the preserved and new transcript are identical."

The data and analysis the reviewer is requesting was already shown as part of the revised manuscript in Extended Data Fig 7g-h. We did not avoid making such a comparison. We made multiple comparisons and transparently showed the full datasets as volcano plots under steady state (Extended Data Fig. 4) and stimulation (IFNB) (Extended Data Fig. 7g-h) conditions.

Extended Data Fig 7g-h asks the question to what extent recordanelles have an impact under stimulation (IFNB). Extended Data Fig 7g looks at the differentially expressed genes when contrasting IFNB +/- stimulation in either recordanelleMCP (left panel) or recordanelleV2 (right panel) conditions. Extended Data Fig 7h then specifically contrasts recordanelleMCP and recordanelleV2 under IFNB - (left panel) or + (right panel) conditions. These volcano plots transparently show the extent to which recording alters the transcriptome under IFNB +/- stimulation conditions. These volcano plots show six DEGs, five of which are not polyadenylated and/or low confidence predicted lncRNAs: 7SK, RN7SL1, and the predicted low confidence lncRNAs ENSG00000282885, ENSG00000287001, ENSG00000310376. As these RNAs are not predicted to be polyadenylated, they should not be captured by our poly(A) enrichment library preparation workflow and therefore likely represent noise. Under IFNB stimulation we only observe one moderately upregulated gene at day 0 (TXNIP). To increase transparency, we now mention these results explicitly in the revised manuscript.

Second revised submission addition (page 46, lines 31-40):

"In the absence of IFNB we observed depletion of five RNAs at day 0: 7SK (a small nuclear RNA), RN7SL1 (a noncoding RNA), and three novel lncRNAs – none of which are predicted to be polyadenylated. In the presence of IFNB three RNAs were depleted (7SK, and two lncRNAs) and TXNIP was moderately upregulated. Because our library preparation protocol relied on poly(A) enrichment, we attribute the non-polyadenylated hits to technical noise. TXNIP, a polyadenylated protein-coding gene involved in immune regulation and interferon signaling, is significantly upregulated with a low fold change ($\log_2FC$ 1.1, FDR 1.5×10^{-17}). As we only observe this transcript upregulated at day 0 (at day 2 the $\log_2FC$ is 0.3, FDR 0.08) it is possible that this is due to technical noise, however we cannot rule out that this is a bona fide impact of transcriptional recording."

To go even further, in the revised manuscript we have now added Supplementary Fig. 6, which contains analogous volcano plot comparisons (to Extended Data Fig. 7h) for day 2 with GO-term analysis of enriched biological terms. Consistent with our previous analyses, the depleted RNAs were mostly novel transcripts that are predicted lncRNAs (ENSG0000028700) or typically non-polyadenylated RNAs (7SK). In addition, we

observed two downregulated genes with very low log2FC (TMEM135 or MIR210HG) for the IFNB treated condition. At day 2 without IFNB treatment we saw two mildly upregulated transcripts, DDIT3 and ATF5. In the IFNB treated condition the dominant GO terms were "Defense response to Virus" and "IFNB response" at day 2. This indicated that in this contrast, recordanelleV2 captured and recapitulated the IFNB response at the full cell level. Therefore, we did not observe a substantial impact on the transcriptome under the tested conditions beyond recording of the IFNB signal at day 2.

Taken together, to address sub-point 1 we increased transparency by elaborating on our findings and reasoning (via the Second revised submission addition above) and including the new Supplementary Fig. 6.

Sub-point 2:

"Similarly, we are only given a full proteome volcano plot at day 0 (Supplementary Fig. 5), even though I explicitly asked for it at multiple timepoints with stimulation. They collected the data at day 2, it's just only shown in small parts (Extended Data Fig. 8)."

As indicated by the reviewer, we had performed the exact experiment they requested: proteomics at multiple time points with and without IFNB stimulation (Fig. 5f and Extended Data Fig. 8). We chose to show the results as heatmaps of fold changes and differential expression (rather than as volcano plots) to focus the analysis on the specific concerns being mentioned by the reviewers. The first question was regarding cellular perturbation effects, and we thus performed a focused analysis on the most likely cellular process to be disrupted: mitochondrial respiratory chain. The second question was regarding whether the recorded transcripts were being translated and whether this was leading to an altered IFNB response, and we thus performed a focused analysis to address this. It was not our intention to hide any data. We wanted to show a specific, well-powered, and well-framed analysis.

To address the concerns of the reviewer and increase transparency, we looked deeper into the quantitative proteomics data and in the revised manuscript have included a much broader presentation of the data.

Specifically, we now show the following contrasts:

- Supplementary Fig. 7, analogous to previous Supplementary Fig. 5, addresses the potential impact on the proteome 24 hours after transfection.
 - o Contrasts shown: mCherry, recordanelleMCP, recordanelleV1 and recordanelleV2; without IFNB treatment at Day 0, relative to mock without IFNB treatment at Day 0.
- Supplementary Fig. 8 addresses the potential impact on the proteome 72 hours after transfection.
 - o Contrasts shown: mCherry, recordanelleMCP, recordanelleV1 and recordanelleV2; without IFNB treatment at Day 2, relative to mock without IFNB treatment at Day 2.
- Supplementary Figs. 9-11 provide an extension of the data shown in the heatmap in Extended Data Fig. 8a. They show the initial IFNB response at the protein level as well as the decay of the proteins 2 days later, relative to an untreated control. Note, the heatmap was a visualization of log2FC and significance for the set of IFNB stimulated proteins. The values were directly taken from these volcano plots for a set of ISGs to focus the visualization of stimulation and decay. For transparency, we now display these volcano plots as independent figures.
 - o Contrasts shown in Supplementary Fig. 9: mock and recordanelleMCP; with IFNB treatment at Day 0 and Day 2, relative to mock without IFNB treatment at Day 0 and Day 2.
 - o Contrasts shown in Supplementary Fig. 10: RecordanelleV1 and recordanelleV2; with IFNB treatment at Day 0 and Day 2, relative to mock without IFNB treatment at Day 0 and Day 2.
 - o Contrasts shown in Supplementary Fig. 11: mCherry; with IFNB treatment at Day 0 and Day 2, relative to mock without IFNB treatment at Day 0 and Day 2.
- Supplementary Figs. 12-13 provide an extension of the data shown in the heatmap in Extended Data Fig. 8b. They show the IFNB response relative to the IFNB treated control at the protein level as well as the decay of the proteins 2 days later. As indicated before, values of log2FC and significance displayed in Extended Data Fig. 8b were directly taken from these contrasts.
 - o Contrasts shown in Supplementary Fig. 12: mCherry, recordanelleMCP, recordanelleV1 and recordanelleV2; with IFNB treatment at Day 0, relative

to mock with IFNB treatment at Day 0.

o Contrasts shown in Supplementary Fig. 13: mCherry, recordanelleMCP, recordanelleV1 and recordanelleV2; with IFNB treatment at Day 2, relative to mock with IFNB treatment at Day 2.

We would like to point out that, in all plots, the majority of differentially expressed proteins are fully imputed in one arm of the contrast. Data imputation is a common practice in label-free quantitative proteomics. Since peptide fragmentation is a semi-stochastic process, there is always a certain fraction of the data missing at random that needs to be imputed, contributing to noise. In our experiments we need to apply comparatively loose filters (see Methods). This is necessary as the ISG proteins are typically short and lowly expressed and might not generate tryptic peptides that ionize well and can be detected reliably. This problem is aggravated in the absence of IFNB, when the expression of these proteins is particularly low. However, this loose filtering leads to an increased representation of noisy proteins with unreliable detection across samples. Even with imputation, we typically observe less than 1% up- or downregulated proteins. To make this distinction easier to appreciate for the reader, we show the proteins that are only detected in one group and fully imputed in the other, separately as a list below the volcano plots. For these proteins, the reported log₂FC and FDR are determined by the imputation model rather than by a measured abundance in the missing group and therefore do not constitute reliable quantitative estimates.

For all contrasts, we also now included a GO term analysis of biological processes which conservatively includes proteins even if they are fully imputed in one group. Only when cells are treated with IFNB can we detect the significant and consistent enrichment of meaningful processes in this analysis. For the other conditions, where most of the up or down regulated proteins are fully imputed in one group, we mostly do not see any enriched pathway. In the legends of these figures we report when specific contrasts show significant enrichment of pathways, transparently showing any impact in the proteome in conditions with and without stimulation at multiple timepoints.

Considering the inherent noise level of the imputation and the resulting low certainty in the detection of these specific proteins, we therefore interpret this as no substantial change to the cellular proteome. However, as absence of evidence is not the same as evidence of absence, we added the indicated Supplementary Figures as well as this disclaimer in the results section of the main text as mentioned above.

Second revised submission addition (page 8, lines 3-6):

“Taken together, these results demonstrate that recordanelles do not substantially impact cell viability, the cellular proteome, or mitochondrial functionality measured at the bulk and single cell level under the conditions tested in this study. Moreover, we cannot exclude that the recordanelles have an impact beyond what was measured in these assays.”

Sub-point 3:

“Not only is new data not presented transparently to quantify the impact as requested, but the authors have even decided to remove statements from the text that previously pointed to exactly these eNects:

Original submission: “Intriguingly, we observed that with recordanelleV1 but not recordanelleMCP, ISG detection was not only maintained, but increased from day 0 to 2 after IFNB removal (Fig 5b and Extended Data Fig. 12). This increase in detection was not observed for the YWHAZ housekeeping gene, suggesting that the increase was due to a feature of IFNB signaling. One likely explanation for this is the well-described type-I IFN signaling positive feedback loop, whereby under control conditions IFNB is rapidly degraded but with the recordanelle IFNB response persists and drives the feedback loop. This could occur under conditions where the recorded ISGs mRNAs are translated.”

Revised submission: “Under certain conditions we observed that in recordanelleV1-containing cells, ISG detection was not only maintained but increased from day 0 to 2 after IFNB removal (Fig 5b and Extended Data 35 Fig. 7a,b). This increase appeared specific to IFNB signaling as it was absent for the YWHAZ housekeeping gene. As we did not observe this behavior for recordanelleV2, it seems that this version is superior as an RNA recording tool and we therefore proceeded with this recordanelle architecture.”

Since the reviewer’s focus in this section is on proteomics our understanding is that the eNect under question is whether the recorded mRNAs are being translated. The

reviewer is saying we “decided to remove statements from the text that previously pointed to exactly these eNects”. They are claiming that we removed this sentence: “This could occur under conditions where the recorded ISGs mRNAs are translated.”

While it is true that that sentence is not in the block of text copied and pasted by the reviewer, we explicitly mention this exact point in the discussion and in the legend of Extended Data Fig. 8:

Original revised submission (discussion, page 9, lines 3-5):

“Furthermore, we showed that recordanelleV1 may facilitate translation of the recorded RNAs, which in the IFNB stimulation experiments led to amplified downstream signaling presumably occurring via the intrinsic feedback loop for this cytokine.”

Original revised submission (Extended Data Fig. 8 figure legend):

“However, for recordanelleV1 IFIT1, PARP9, DTX3L and OAS3 remain significantly overexpressed. This can potentially be explained by the well-described type-I IFN signaling positive feedback loop^{50,51}, whereby under control conditions IFNB is rapidly degraded but with the recordanelleV1 IFNB response persists and drives the feedback loop. This could occur under conditions where the recorded ISGs mRNAs are translated. Compared to recordanelleV1, recordanelleV2 — utilizing PABPC4ΔC161A, which is supposed to abrogate translation of the bound mRNA^{52,53} — shows a reduced signal increase and we speculate that this could be due to a diminished feedback loop.”

To avoid confusion and increase transparency further, in the revised manuscript we have re-inserted the original sentence in the results section (thus now mentioning the translational eNect three times – once in the results, once in a figure legend, and once in the discussion).

Second revised submission addition (page 6, lines 37-39):

“Under certain conditions we observed that in recordanelleV1-containing cells, ISG detection was not only maintained but increased from day 0 to 2 after IFNB removal (Fig 5b and Extended Data Fig. 7a,b), this could occur under conditions where the recorded ISG mRNAs are translated.”

Yet, the evidence is there, the authors just ignore it:

In Figure 5: The authors only show the mitochondrial fraction in Figure 5c on day 2, not the full cell (which would be where an impact of prolonging transcript longevity would be seen). There is a clear signature of transiently expressed, INFB responsive genes in the mitochondrial fraction at day 2. However, in 5e this is compared with the full cell and we can see that those same genes that should have declined by day 2 are also enriched at day 2 in the full cell with the V2 recordanelle. This is direct evidence that the recordanelle aNects the transcriptome and will have some impact on the cell.

To first clarify one misunderstanding, Fig. 5c showed the mitochondrial fraction on both day 0 (left column of volcano plots) and day 2 (right column of volcano plots), not just day 2 as mentioned. We also showed the full cell fraction data at day 0 in Extended Data Fig. 7g-h. We chose not to show the full cell fraction data at day 2 as a separate volcano plot, as this was redundant with the mitochondrial fraction data, since we already showed concordance/correlation between the mitochondrial and full cell fractions in Fig. 5e and Extended Data Fig. 7a. It is entirely expected that the mitochondrial and full cell fractions correlate because the recordanelle is part of the full cell fraction. We are unable to separate the cytoplasm from the recordanelle component and are thus unable to observe the recordanelle-free cell fraction. We thank the reviewer for drawing our attention to this potential lack of clarity. For the sake of completeness and transparently showing all the data, we now show the RNA-seq volcano plots at the full cell level analogous to Fig. 5c but at both days 0 and 2 (new Supplementary Fig. 5). This data shows equivalent transcriptional responses at the mitochondrial and full cell level as expected.

As indicated above, we further expanded on the impact of recordanelleV2 compared to recordanelleMCP on the transcriptome of cells in the presence or absence of IFNB stimulation at multiple timepoints (see Supplementary Fig. 6).

We further clarify in the manuscript that the cell fraction contains the recordanelle and report the transcriptomic impact of the recordanelle in the context of IFNB stimulation:

Original revised submission:

"From this data we can also draw three other conclusions: i) from the recorded information we can reconstruct a detailed picture of the (historical) IFNB response, as indicated by a gene ontology (GO) term analysis of DEGs retained by recordanelleV2 (+ IFNB compared to no IFNB) within the mitochondrial fraction (Fig. 5d); ii) the recorded response faithfully recapitulates whole-cell gene expression levels, as indicated by concordance between whole cell and recordanelle LFCs (Fig. 5e); and iii) recordanelle expression did not alter the cellular transcriptome, as indicated by a lack of differentially expressed genes when comparing recordanelle versus controls on day 0 in the presence of IFNB (Extended Data Fig. 7h)."

Second revised submission addition (page 7, lines 11-19):

"From this data we can also draw three other conclusions: i) from the recorded information we can reconstruct a detailed picture of the (historical) IFNB response, as indicated by a gene ontology (GO) term analysis of DEGs retained by recordanelleV2 (+IFNB compared to no IFNB) within the mitochondrial fraction (Fig. 5d); ii) the response can also be captured at the full cell level, which contains the recordanelle, as indicated by concordance between whole cell and recordanelle LFCs (Fig. 5e, and Supplementary Fig. 5); and iii) recordanelle expression did not substantially alter the cellular transcriptome under these conditions, as indicated by a lack of differentially enriched biological processes when comparing recordanelle versus controls on day 0 and day 2 in the presence and absence of IFNB (Extended Data Fig. 7h and Supplementary Fig. 6)."

The single cell data in Figure 5i-k also shows this. It is the V2 recordanelle, and single cell (not mitochondrial fraction) transcriptomes carry a signature of the prior stimulation. They have to for the recording to be useful, but that fact also necessarily means that the transcriptome is perturbed and there could be cellular consequences depending on the biology being investigated. My guess is that we would also see evidence of this perturbation in the proteome if they had shown the full volcano plots at day 2 after stimulation as requested, rather than pick out a small subset of proteins (where, by the way, there is a perturbation that is downplayed).

We have already addressed the proteome part of this comment above. Here, we will focus on the new scRNA-seq and cellular perturbation facets we have not already addressed.

As mentioned in our previous response, it is expected that the recorded RNAs are found at the full cell level. The recordanelles are found within the cell, so full cell measurements will pick them up. Strictly speaking, the reviewer is right in that this is a perturbation in the sense that recordanelle-containing cells have a compartment that normal cells do not. Whether this leads to meaningful changes in cell behavior or whether this precludes the interrogation of a biological question are the key questions. We have tried to address these key questions in our work.

Changes to cellular behavior could manifest due to a range of possibilities, including depletion of RNA from the cellular pool, RNA being accessible and active in the recordanelle, and functional RNA being released from the recordanelle. It is also possible that none of these are true and the amount of RNA being taken from the cell is negligible, the recordanelle is fully inaccessible, and any released RNA is no longer functioning (e.g., because it was partially degraded by cellular RNases due to not being fully shielded). We did not fully resolve all these possibilities in all contexts in our work. A full mechanistic understanding of all of these is out of scope of the current manuscript. What we think the reviewer wants from us is not a conclusive answer, but more transparency about the knowns, unknowns, boundaries, and limitations. In the revised manuscript, we have done our best to elaborate on such facets so that future readers are more informed about the complexity and limitations given the current state of the technology. As indicated above we contain a specific disclaimer on this potential limitation in the discussion:

Second revised submission addition (page 8, line 44 – page 9, line 5):

"While the data reported here suggest that recordanelles do not substantially perturb cellular function under the tested conditions, it is important to clarify that transcriptional recorders such as recordanelles by their very nature are perturbations to cells. These perturbations include the burden of transgene expression, occupancy of the mitochondrial membrane and its immediate surroundings, unnatural interactions with the RNAs they track, depletion of RNAs from the cellular pool, and thermodynamic costs of information storage. As the

field moves from building technologies to applying them to ask fundamental questions about biology, exactly how these perturbations manifest and potentially confound our interpretation of biology remains to be seen.”

All of this adds up to what appears to be an intentional lack of transparency. Going further, the authors repeatedly take liberties with when to use or disregard statistical tests. In my review of the original manuscript, I pointed out that they made a statement that wasn't supported by a statistical analysis of the data: “While we observed enrichment of target RNAs when using a control construct without an assembler protein (Δ AP), several other constructs exhibit a higher binding of the mCherry mRNA (e.g., FUSQG, FUSN).” The authors replied to this by adding bar graphs that seem to confirm my suspicion that this difference is likely not statistically supported, and yet say, “We do not make statistical claims here, as we test multiple variants for recording later on in the manuscript.” But they leave the statement in about the higher binding when they know it's not supported by the data. I can't understand that decision, and it makes me uncomfortable with the data practices.

The reviewer is right that a statistical test of this difference does not survive multiple hypothesis correcting. We do not refute this and were not trying to hide this. What we were trying to do is communicate to the reader our reasoning for making decisions and selecting certain features for further development. Our grounds for selecting construct elements to progress through technology development at this stage was the extent of binding of mCherry RNA. However, we can acknowledge the reviewer's point that if we do not phrase this correctly it will appear like we are making statistical claims. We have thus revised the manuscript to try and accomplish our goal of explaining selection choices without making absolute claims about binding.

Old text: “Using cellular fractionation and qPCR, we observed an enrichment of mCherry::ms2 RNAs within the recordanelles (i.e., mitochondrial cell fraction) with an efficiency ranging from 3- to 11-fold compared to a mock control (Fig. 2c and Extended Data Fig. 2a). While we observed enrichment of target RNAs when using a control construct without an assembler protein (Δ AP), several other constructs exhibit a higher binding of the mCherry mRNA (e.g., FUSQG, FUSN).”

Second revised submission addition (page 3, lines 16-22):

“Using cellular fractionation and qPCR, we observed an enrichment of mCherry::ms2 RNAs within the recordanelles (i.e., mitochondrial cell fraction) with an efficiency ranging from 3- to 11-fold compared to a mock control (Fig. 2c and Extended Data Fig. 2a). We also observed enrichment of target RNAs when using a control construct without an assembler protein (Δ AP). Some recordanelle architectures (e.g., FUSQG and FUSN) however seem to exhibit a higher mean mCherry::ms2 enrichment (not statistically significant). Based on this small-scale screen we selected several lead candidates for subsequent analysis (FUSQG, FUSN, EWSR1, TAF15N).”

Second revised submission addition (page 7, lines 19-22):

“We further observed that while without an assembler protein specific mRNAs can be very efficiently recruited into the mitochondrial fraction (Δ AP control, Fig. 2c), general mRNA recording is diminished when TAF15N is removed from recordanelleV2 (Extended Data Fig. 7i-j).”

On the other hand, throughout the manuscript and the rebuttal they brush away statistically significant differences in the transcriptomes or proteome as technical noise when it doesn't suit their argument without any further explanation or experiments:

“We suspect that the additional 1 enriched and 2 depleted genes within the MCP-containing recordanelles were due to technical noise or MCP binding.”

In this specific case mentioned by the reviewer, we had examined this carefully and could not find compelling reasons why these three genes (in addition to the two targeted reporter RNAs) were anything but technical noise or an unexpected binding event. We thus made the conclusions we did. Nonetheless, we get the reviewer's point that our reasoning should be made more transparent to the reader.

To support this, we now show the counts per million for the five DE targets in the contrast shown in Fig. 2d (Point by Point Fig. 1).

Point by Point Figure 1. Log2 counts per million (CPM) for the five DE genes in the contrasts displayed in Fig. 2d calculated from the raw, unfiltered data. Conditions are TOM20::FUSQG::(No RBD / MCP / PCP). Shown are the CPM for the full cell (black) and mitochondrial fraction (red). Each dot corresponds to a biological replicate, and the bar indicates the mean.

ZNF350 is just below our fold-change threshold and has high dispersion for all conditions, including the full cell fraction and one outlier replicate of TOM20::FUSQG, which is driving most of the eNect. For BLOC1S5-TXNDC5, the eNect is dominated by one complete dropout in TOM20::FUSQG::MCP. It seems most likely to us that this is either noise or construct specific. ENSG00000297093 is a predicted lncRNA and like in other sections we attribute such hits to technical noise. Moreover, in the TOM20::FUSQG::MCP condition, we see that this lncRNA is enriched in the mitochondrial fraction but not in the full cell fraction. To the best of our knowledge, no binding to MCP has been reported. As this is a sole gene out of more than 11000 that is enriched besides mCherry::ms2, it is possible that this is a specific interaction between MCP and ENSG00000297093. However, without further verification we do not want to make a too definitive statement on this.

To address the reviewer's concerns on the specific sentence being quoted, we have changed the sentence to one that mentions the three DEGs without speculating on their origin.

Old text:

"We suspect that the additional 1 enriched and 2 depleted genes within the MCP-containing recordanelles were due to technical noise or MCP binding."

Second revised submission addition (page 3, lines 43-46):

"For the MCP-containing recordanelle we additionally observed one enriched predicted lncRNA and two depleted genes. While we attribute these subtle changes to technical noise, we cannot rule out that the enrichment of the lncRNA is caused by a so far undescribed binding to MCP."

"variation seen in a proteomics experiment, so we concluded that this is likely due to technical noise."

We addressed this point in our response on proteomics above.

Additional minor comments:

-The authors justify this statement, "Here, we introduce the recordanelle, the first transcriptome-wide RNA recording technology for mammalian cells that continuously samples the cellular transcriptome in single cells," at least partially by differentiating their approach from the TimeVaults approach based on TimeVaults being limited to 24-hour expression snapshots, which is not a feature or strict limitation of the TimeVault approach.

We thank the reviewer for this comment. In the TimeVault work, they themselves talk about defined recording windows (see Chao et al., 2026, p. 1343), which is distinct from our work where recording is continuous. In our manuscript we do not discuss their 24-hour observation window at all, but we would nonetheless like to stress here that in no section of their manuscript do they show acquisition windows longer than 24 hours. The more important differentiator between the two technologies is in terms of cellular input required. TimeVaults cannot distinguish signal when using inputs of below 1000 cells (see Chao et al., 2026, Fig. S5A and page 4 of 15). In contrast, recordanelles distinguish signals in single cells. We would like to leave the sentence in to articulate the two novel features that recordanelles can offer that TimeVaults cannot: continuous recording and single cell recording. TimeVaults are interesting and valuable, and we think future work will find that they can be used in a complementary way.

-I remain unconvinced that this is a 'synthetic organelle' given that it is inseparable from and embedded in the membrane of the mitochondria. The other examples of membraneless organelles that are cited as parallels are distinct compartments and in most cases some effort was put in to show that they are phase separated, which has not been done in this work.

We thank the reviewer for this comment. We still believe that the terminology of calling this a synthetic organelle is justified and corresponds to the literature standard. In 2021, it was shown that fusions of TOM20-70 and an IDR such as EWSR1 or FUS form film-like organelles on the mitochondrial membrane surface [Reinkemeier et al., 2021

(PMID: 34433013)]. In that study, STORM-microscopy has been used to demonstrate the film-like nature, see Figure 4B in that manuscript. For transparency, this prior study was also led by the first author of our manuscript.

Since the original publication above, a body of independent work has emerged that has maintained the terminology:

- Yu et al., 2023 (PMID: 37100914): “To circumvent this problem, we utilized our recently developed film-like, synthetic orthogonally translating organelles (OTOs) to form a distinct protein translational machinery on the outer mitochondrial membrane surface³⁹.”
- Ruan et al., 2025 (PMID: 41253788): “Since TCO^{*}A incorporation can occur in any protein containing a TAG codon, resulting in nonspecific background labelling, we utilised our recently developed orthogonally translating organelles (OTOs) (Supplementary Fig. 9). These film-like compartments accumulate both orthogonal aminoacyl-tRNA synthetases (aaRS) and the mRNA of the protein of interest (POI), thereby achieving high recruitment and labelling specificity for the NHA9 mRNA during translation^{55,56}.”
- Schartel et al., 2024 (PMID: 39448590) developed a system composed of a membrane anchor (LCK) and the N-terminal domain of FUS2-276, which they classified as a film-like synthetic organelle. For instance, on page 1 of their paper: “Here, we demonstrate that a film-like organelle architecture enables mRNA-selective pseudouridinylation as a proof-of-principle”.

Several reviews also use similar terminology for membrane anchored IDR/phase separation fusions:

- Dai et al., 2023 (PMID: 37359769): “A follow-up study demonstrated the integration of a membrane-binding domain that spatially localizes the synthetic condensates next to diNerent membrane-based organelles such as the Golgi body and mitochondria¹⁰⁸.”
- Dunkelmann & Chin 2024 (PMID: 39235427): “In an extension of this work, PylRS variants for distinct ncAAs were directed to distinct sub cellular compartments, and distinct mRNAs bearing amber stop codons were directed to each compartment. This enabled the incorporation of a diNerent ncAA in response to the amber codon in each message.¹⁹⁹”
- Niu & Guo 2025 (PMID: 39207844): “Using synthetic organelles represents an innovative approach to enhancing GCE in eukaryotes.^{124–126}”

Considering that the recordanelles we developed here follow a similar architecture and show the expected localization on mitochondria [analogous to Reinkemeier et al., 2021 (PMID: 34433013)], we strongly believe that this terminology falls within the literature standard and is thus appropriate here.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

In this second revision, Reinkemeier et al. have achieved what I was hoping to see in terms of transparency in the presentation of their findings. Potential limitations are appropriately discussed, conclusions have been better contextualized, and data is fully presented so it may be directly evaluated by readers. This version is a much more useful addition to the transcriptional recording field.

I only have two remaining concerns where I do not think the revision has fully fixed the earlier issues:

1. In the interpretation of transcriptome recordings, the following was changed:

Original:

“iii) recordanelle expression did not alter the cellular transcriptome, as indicated by a lack of differentially expressed genes when comparing recordanelle versus controls on day 0 in the presence of IFNB (Extended Data Fig. 7h).”

Revised:

“iii) recordanella expression did not substantially alter the cellular transcriptome under these conditions, as indicated by a lack of differentially enriched biological processes when comparing recordanella versus controls on day 0 and day 2 in the presence and absence of IFNB (Extended Data Fig. 7h and Supplementary Fig. 6).”

This remains an inaccurate conclusion as stated. As is clear in Supplementary Figure 6f, the cellular (whole cell) transcriptome is altered at day 2, with many differentially expressed genes in the recordanella V2 versus MCP, and there are differentially enriched biological processes as well with that same condition (Supplementary Figure 6h). Guessing at intention, it seems that the authors mean that the altered transcript abundance is expected based on the stimulation, and they do not see substantial altered transcripts that they cannot explain. They should be explicit, not saying that there is no alteration in cellular transcriptome, but instead saying that the transcriptional pattern that is altered is largely anticipated given the stimulation.

2. The authors have chosen to leave in this statement about TimeVaults:

“Whereas TimeVaults use free-floating vault particles to capture 24-hour expression snapshots, recordanellas use membrane-anchored biomolecular condensates to continuously record expression. While the tools are complementary, one main advantage of recordanellas is that they can continuously sample the transcriptome over arbitrary time windows and are therefore uniquely suited to biological questions that extend beyond 24 hours and where the precise time of interest is unknown. Furthermore, recordanellas are sensitive enough to capture cellular signaling events in single cells”

I will reiterate that this is a misleading contextualization. While the major biological experiments in the TimeVault manuscript used a 24-hour recording window, there is nothing in that work to indicate that 24 hours is the only window that could be recorded. Twenty-four hours was an arbitrary window that was tested, just like the arbitrary windows tested for the recordanella in this manuscript. It would be fine to say that TimeVaults have not been extensively tested for biological recordings beyond 24h (although the manuscript does demonstrate recording beyond 24h, e.g. Fig S3B), but it is inappropriate to characterize it as a property of the approach. I have no problem with the single-cell aspect of this disambiguation.

Author Rebuttal letter:

We thank the reviewer for their comments. In the following point-by-point reply, the reviewer's comments are shown in black, our replies in blue, quoted text that we have removed in magenta, replaced in cyan, and newly added text in yellow.

Reviewers' comments:

We thank the reviewer for their appreciation for the new additions and insightful comments.

I only have two remaining concerns where I do not think the revision has fully fixed the earlier issues:

1. In the interpretation of transcriptome recordings, the following was changed:

Original:

“iii) recordanella expression did not alter the cellular transcriptome, as indicated by a lack of differentially expressed genes when comparing recordanella versus controls on day 0 in the presence of IFNB (Extended Data Fig. 7h).”

Revised:

“iii) recordanella expression did not substantially alter the cellular transcriptome h and Supplementary Fig. 6).”

We thank the reviewer for pointing out this inconsistency. The statement that the transcriptome is not altered in presence of recordanellaV2 only applies in absence of IFNB. We now point this out specifically in the manuscript:

Old text:

“iii) recordanella expression did not substantially alter the cellular transcriptome
”

“iii) recordanelle expression did not substantially alter the cellular transcriptome in

”

Old text:

“ii) the response can also be captured at the full cell level, which contains the recordanelle, as indicated by concordance between whole cell and recordanelle LFCs (Fig. 5e, and Supplementary Fig. 5);”

chosen to leave in this statement about TimeVaults:

“Whereas TimeVaults use free-floating vault particles to capture 24-hour expression of interest is unknown. Furthermore, recordanelles are sensitive enough to capture cellular signaling events in single cells”

We thank the reviewer for this comment. We removed the statement that the 24-hour recording window is a property of TimeVaults and substituted it with the fact that this was the main window tested in that manuscript.

Old text:

“Whereas TimeVaults use free-floating vault particles to capture 24-hour expression snapshots,”

New text:

“Whereas TimeVaults use free-floating vault particles that have been predominately tested for capturing 24-hour expression snapshots;”

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