nature portfolio

Peer Review File

Nanodynamo quantifies subcellular RNA dynamics revealing extensive coupling between steps of the RNA life cycle

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Reviewer #1 (Remarks to the Author):

Comments for Tarrero et al.,

Tarrero et al., established a new tool, named Nanodymo to estimate the rates of several important steps in gene expression and their biological links. In brief, they employed 4sU RNA labeling and direct RNA Nanopore sequencing (dRNA-seq) methods to monitor nascent transcripts in several cell fractions. They then performed computational analyses to quantify the kinetics rates of transcription, co- and post-transcriptional RNA splicing, RNA degradation, RNA export, RNA-polysome binding, and RNA degradation in the cytoplasm. They applied this approach with three different inhibitors which inhibit splicing, RNA export, and translation, respectively. Using these datasets, they also presented some steps coupling that may be biologically important for gene expression.

Pelizzola group previously established a similar method called INSPEcT. I feel Nanodymo can be a good addition and alternative method to dissect the complexity of gene expression. However, I have some concerns about this manuscript. In general, lots of critical information is missing in the texts and figures. At least to me, it will be challenging to follow their messages. The authors should make this manuscript more reader-friendly. My specific comments to improve are written below.

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- They need to mention what cell fraction shows these RNA profiles in Fig 2A.
- They may want to show a representative view (GB or IGV) of dRNA-seq for all the fractions.
10) Figure 2F: In theory, chromatin fraction does not contain polyadenylated RNAs since such mature RNAs should be released before RNA polymerase II transcription is terminated. Is this caused by a technical issue, like an RNA contamination from nucleoplasmic fraction?
11) Figure 3E: The cluster is not labeled.
12) Figure 3F: Some clusters showed up-regulated rates of post-transcriptional splicing (i.e. k5).

Post-transcriptional splicing event is not inhibited by Pladienolide B? The authors may want to discuss this. The example view (GB or IGV) of nanopore sequencing will be useful to show.
13) Figure 4B: This panel may not be mandatory since this is the same as Figure 1A except for k8 and k9.
14) Leptomycin B is an RNA export inhibitor, but it seems not to inhibit (affect) k6. This result suggests that Leptomycin B did not affect the RNA export rate. This is weird to me because mRNAs should be accumulated in the nucleus under Leptomycin B treatment, meaning the rate could not be quantified. The authors may want to discuss it.
15) Figure 4E: Co-transcriptional splicing was inhibited and post-transcriptional splicing was increased by Leptomycin B in category F. Other categories also showed a negative correlation. The authors may want to discuss it.
16) Line 309: ~ change in polyA tails length were positively correlated ~ -> negatively correlated?
17) Figure 6: The coupling of RNA cycle steps under Harringtonine treatment should be shown, even though the k8 and k9 were missing.

Reviewer #2 (Remarks to the Author):

In this work the authors propose Nanodynamo a modelling framework that involves mathematical modeling following the sequencing of native RNA from cellular fractions and polysomes. They use SUM159 triple-negative breast cancer cells as a test case to profile the kinetic rates of co-transcriptional and post-transcriptional mechanisms that define the RNA life cycle. Generally, the quantification of the kinetic rates of RNA life cycle is important for the understanding of gene regulation and could likely prove very useful to the community. However, while the authors have performed extensive work on the mathematical modeling, the work lacks orthogonal validation of the estimates and thus excitement for the work is substantially reduced.

Given enough parameters, it is not surprising that the model can fit the data. Actually, the models presented in the work appear to fit unexpectedly well, achieving correlation values of 1.0, raising concerns for model overfit. Also, it remains unclear if the fitted values correspond to the truth. The authors need to provide evidence, based on established time series labeling experiments, that show the concordance of their estimates to experimentally measured rates.

Below are some specific major points:

Line 79-80: The authors need to show their polysome profile traces for all the samples and conditions as polysome RNA-seq is an important part of the work.

Line 94: “This modified nucleotide can be detected on dRNA-seq data using the nano-ID tool”. This is incorrect. Nano-ID has been trained to detect 5EU. The authors appear to have retrained the neural network to detect 4SU using their data. Therefore, since this is a critical part of their work, they need to show that modified nucleotides can indeed be detected. Currently they only evaluate the performance of the test dataset, but no information is provided regarding the creation of the test dataset. More importantly no evaluation has been performed on a completely independent dataset. Fully connected neural networks with millions of parameters like nano-ID are prone to overfit. Given that the entire work depends on this step, a truly independent evaluation needs to be performed.

Line 99: “Following the verification of the theoretical feasibility of kinetic rates inference”. It is unclear which data support this statement and validate the theoretical feasibility of the model.

Line 103: The purpose of the section “Evaluation of Nanodynamo through simulated data” is unclear. The authors appear to have simulated data according to their model. Then have added noise based on normal distribution and then fitted the model. This seems like a trivial task that doesn’t prove much besides that the source code works. The correctness of the estimated rates remains unclear. It is also unclear how these simulations can be used to evaluate the optimal number of replicates and labelling time given that the noise added to the simulations is arbitrarily selected.

Line 134: “a single sample is sufficient to have a performance remarkably close to the optimal one”. Not requiring replicates is indeed remarkable, but also unlikely to be true in real experiments. This most probably indicates the low complexity of the simulated data.

Line 150-151: In this reviewer’s opinion correlation values of <0.5 between replicates is not acceptable to justify pooling replicates together. This indicates that either the experiments are very noisy or more likely that the model overfits.

Line 154: The use of a single read as a threshold appears to be very loose. Especially when all replicates are pooled together and there is no estimate of the variance.

Line 155-156: The authors report a median Spearman correlation between modelled and experimental values of 1.00. Biological experiments done in replicates are extremely unlikely to achieve correlation of 1.00. Yet the model seems to do so. Does this mean that the model is perfect? In this reviewer’s opinion this likely indicates that the model overfits the data.

Line 166: The authors need to provide this calculation.

Line 285: Is there any statistical significance estimation for these quantifications?

Line 289: RT-PCR differences appear modest. Do they correlate with the expected kinetic rate changes? Also, some control genes without kinetic rate changes should also be tested.

Line 610: In replicate 1 of the untreated cells the authors get approximately more than 2x poly(A) RNA compared to all the other cellular fragments. In contrast, in replicate 2 they get 2x less poly(A) RNA. Similar variations among replicates can be seen for other conditions and fragments. This raises concerns regarding experimental reproducibility, and it is unclear how this massive difference, which should be affecting calculations of the rates, can be normalized with the proposed normalization scheme.

Line 641: At which step in the experiment are the ERCC spike-ins added? It is unclear what these spike-ins control for? Do they only control for library preparation and sequencing? It would be expected that most of the variation occurs during cell counting, RNA extraction, processing, polysome isolation, all steps before library preparation. The authors need to explain how they control and normalize these steps.

Line 561-565: Metabolic labelling with 5EU of K562 cells is described in the methods but it is unclear where K562 cells were used in the work.

Line 590 and elsewhere: There are some spelling mistakes throughout the text. e.g. Cycloexamide should be cycloheximide that need to be corrected.

Line 746: Smooth density scatterplots used throughout the figures can be obscure, unless accompanied by a legend describing the point density scale. The authors should consider showing all points, perhaps with some transparency to avoid overplotting.

Reviewer #3 (Remarks to the Author):

The authors designed an interesting data-driven modeling system to explain RNA dynamics in a large-scale manner. Here are my points for the authors to consider.

1. When using the ODE model to explain RNA data, as the model is relatively simple, it did not include potential dynamic contributions from the feedback exerted by relevant transcription factors. This may be particularly important for the drug perturbation scenario as cells can activate compensatory signaling and autocrine to dynamically up/downregulate transcription factors to directly control RNA synthesis (and perhaps also RNA degradation and processing steps, if the cells happen to produce more or less of those accessory proteins). If the focus of the model is to explain short term RNA dynamics data, what about long term dynamics? This (long term dynamics) is much more meaningful in deciphering cell fate.
2. In fact in real biology, some of the major reactions described in the model, e.g. co-transcriptional processing, post-transcriptional processing, degradation, require accessory proteins, which means that while a single first-order rate description may be ok (from the modeling standpoint), it actually also overlooks the potential second/higher order protein binding and catalytic interactions (from the mechanistic standpoint). Since the levels of these accessory proteins may also vary upon drug perturbation (e.g. feedback or nonspecific drug effect), the story could become a lot more complex. In short, simply saying that some rate decreases upon drug intervention does not really offer mechanistic insight. The authors should discuss this potential limitation. Or the authors could perhaps find 1-2 genes that were shown to have reduced rates and meanwhile there were some literature evidence that can support a potential mechanistic link. Such information could be used as validation of the model insights.
3. What about the inter-cell type applicability of this model? An example would be to use 3-5 cancer cell lines (sensitive, moderate, resistant), control vs receiving the same drug (and different doses) and analyze the potentially different RNA dynamics (cell line A vs B vs C, A dose1 vs A dose2, etc.) using this framework. This would hopefully provide more meaningful information for translational research.
4. Another concern is the novelty of this study. To me the hard part is perhaps measuring the absolute RNA counts for a large number of genes in the four different cellular compartments, as the ODE modeling part is rather linear, straightforward and based on recognized mechanisms. So, have such location-specific RNA measurement experiments been done before by other studies or other groups?
5. Figure 1A. Unclear labeling. Which thing (drawing) corresponds to which variable in the equation? Reaction k3 goes to where?
6. Were the Excel files containing RNA absolute counts (of different genes) in different

compartments (in the several cell lines mentioned) provided in the supplemental files? This will be very helpful for future research.

Point by point response to Reviewers

Nanodynamo quantifies the dynamics of RNA metabolism and reveals extensive coupling between steps of the RNA life cycle

Lucia Coscujuela Tarrero, Valeria Famà, Giacomo D'Andrea, Simone Maestri, Anna de Polo, Stefano Biffo, Mattia Furlan, Mattia Pelizzola

We would like to really thank the three Reviewers for their constructive comments, which markedly contributed to improving and clarifying the manuscript and strengthening the main conclusions of our work.

Please see below for a point-by-point response to all comments. Our responses were marked in blue. Edits done in the revised main text and supplementary material were also marked in blue.

Reviewer #1

Comments for Tarrero et al.,

Tarrero et al., established a new tool, named Nanodymo to estimate the rates of several important steps in gene expression and their biological links. In brief, they employed 4sU RNA labeling and direct RNA Nanopore sequencing (dRNA-seq) methods to monitor nascent transcripts in several cell fractions. They then performed computational analyses to quantify the kinetics rates of transcription, co- and post-transcriptional RNA splicing, RNA degradation, RNA export, RNA-polysome binding, and RNA degradation in the cytoplasm. They applied this approach with three different inhibitors which inhibit splicing, RNA export, and translation, respectively. Using these datasets, they also presented some steps coupling that may be biologically important for gene expression.

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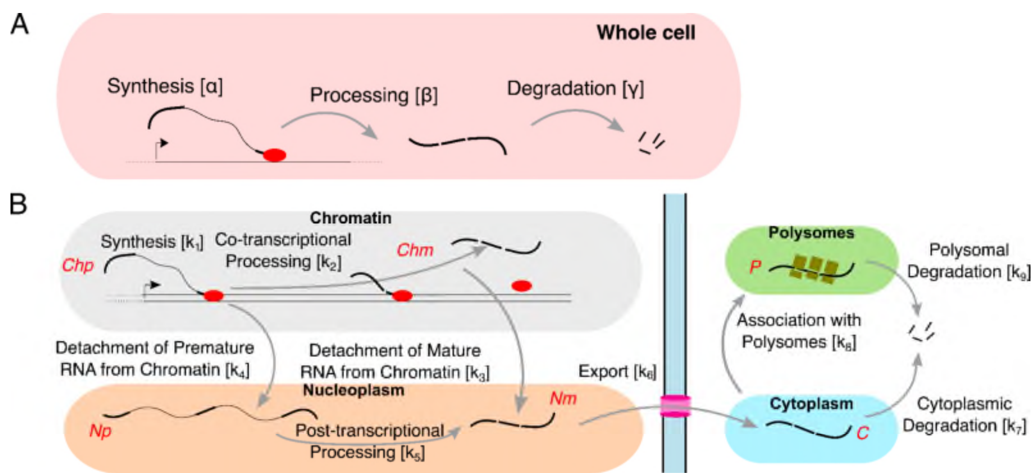
1) Some cartoons of comparison between INSPEcT and Nanodymo will help understand the impact of this study.

Following the Reviewer's suggestion, we included a new supplementary figure (Supplementary Figure 1) depicting the models of the RNA life cycle implemented in INSPEcT (panel A) and Nanodynamo (panel B).



2) Figure 1D: What are the numbers (0.33, 0.33 1, and 0.33 2) next to the box? I believe those are 20min, 60 min, and 120 min.

The Reviewer is correct, the legend in Figure 1D reports for each simulation the labeling times in hours. In the new version of the figure, we added the units of measurement.

3) Figures 1C and 1D: In the text, Figure 1D comes first. The authors should correct this.

We apologize for the mistake. We updated the figure switching the two panels, and we changed the text accordingly.

4) Figure 1F: The authors should expand the text (lines 135-139) to explain the result of Figure 1F. They especially should discuss why the rates k7-9 have a lower correlation between Expected and Inferred.

The reviewer is correct, the rates associated with the final steps of the RNA life cycle tend to be less correlated with their expected counterparts. The reason is that $k_{7\text{-}9}$ are inferred according to the expression levels of cytoplasmic and polysomal RNA which, however, also depend on $k_{1\text{-}6}$ complicating the determination of $k_{7\text{-}9}$. This growth in complexity clearly emerges from the solution of the ODEs system. Each RNA species is indeed expressed as a combination of exponential terms whose number increases moving away from RNA synthesis. Moreover, k_7 generates a branching in the model which, again, increases the complexity of the equations and results in a more difficult inference. Indeed, the simplification of the model through the removal of this branching point - neglecting polysomal RNA and the corresponding $k_{8\text{-}9}$ rates - increases k_7 correlation (Spearman correlation 0.96). Also k_4, one of the less correlated rates, generates a branching in the model; see the new version of Figure 1E which was updated after the extensive review of our data simulation and inference routines (next points of the rebuttal). We included these observations in the discussion of Figure 1E. Finally, we wanted to highlight that rates k_7 and k_8 were switched in the first version of Figure 1E and were amended in the current version.

5) Figures 2A and 2B: These panels may be suitable for supplementary figures since they are not biologically important. Chp and Np are not well reproduced. The author should explain this.

We moved the former panels 2A and 2B to supplementary figures as suggested. The reason for the low correlations between replicates for premature species in chromatin (Chp) and nucleoplasm (Np) is very likely due to the low expression of these species, which are consequently those being more affected by experimental noise. We mentioned this point in the revised version of our manuscript discussing Supplementary Figure 7.

6) Figure 1D: What is the x-axis for? The authors should label the information on the figure.

We believe that the Reviewer was referring to Figure 2D, reporting the distribution of the various kinetic rates inferred by Nanodynamo. The rate name and the corresponding unit were displayed in the title of each histogram. However, the information about the unit of measurement was moved within the x-axis in the new Figure 2C.

7) Figure 1E: The normalized score should be located on the top of the panel. The coloring of the clusters should be changed since it is not easy to distinguish each category.

We believe that the Reviewer was referring to Figure 2E. We updated the figure (Figure 2D in the revised manuscript) according to the Reviewer's suggestions. Regarding the clusters colors, we opted to denote them with letters instead of colors to facilitate their identification.

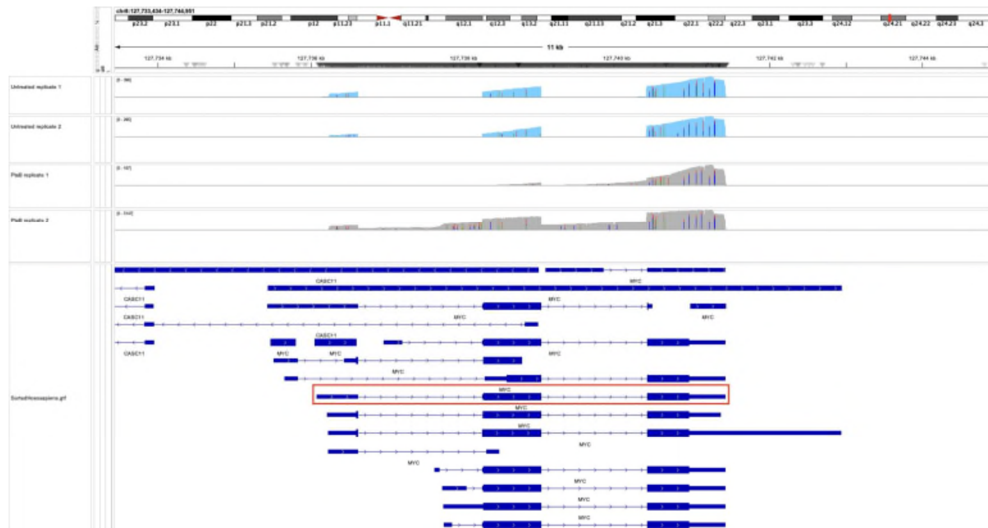
8) Figure 1G: I recommend to move this panel to supplementary information.

We believe that the Reviewer was referring to Figure 2G (renamed 2F in the revised version). The ability of Nanodynamo in processing datasets of variable complexity is important to accommodate experimental choices (e.g. avoid polysomal profiling) as well as to allow the study of the RNA life cycle of transcripts which do not fit the complete model. Some of these gene types are highly relevant species, such as intronless or noncoding RNAs, and being able to cope with their peculiar life cycle is in our opinion a quite important feature of our method. Since we believe that this Figure is essential to convey this message to the reader, we would prefer keeping this panel in the main text.

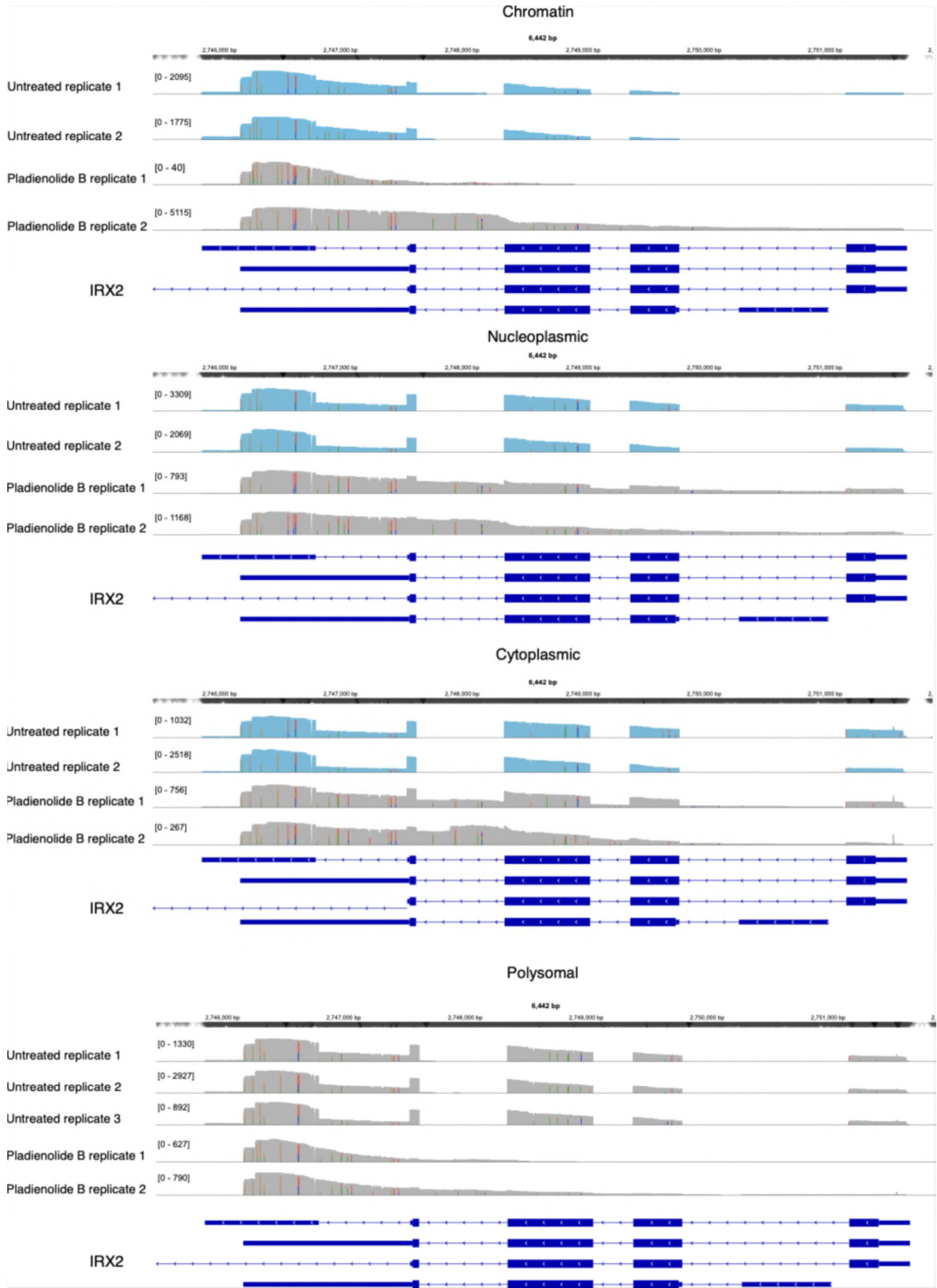
9) Figure 2A: - Two replicates of Pladienolide B treated samples look so different. Rep 1 shows premature termination rather than splicing inhibition? In fact, Pladienolide B causes premature termination in large fractions of pc genes in K562, HeLa, and HCT116 cells (Caizzi et al., Molecular Cell 2021 and Sousa-Luis et al., Molecular Cell 2021). The intensity of the y axis of rep 2 is much higher than rep 1. The author may want to explain this. Please also add the information shown below as well.
- They need to mention what cell fraction shows these RNA profiles in Fig 2A.
- They may want to show a representative view (GB or IGV) of dRNA-seq for all the fractions.

The Reviewer is right regarding the intensity of y axis of the second replicate of Pladienolide B treatment in Figure 3A: there is a marked difference in coverage between the first and the second replicate due to the fact that the first run of sequencing produced fewer reads and, additionally, the second replicate is the pooling of two sequencing runs.
Regarding the abrupt end of sequencing signal within the 3'UTR, it is due to our unfortunate choice of which MYC isoforms to report in the figure. Indeed, various MYC isoforms exist whose 3' end is perfectly

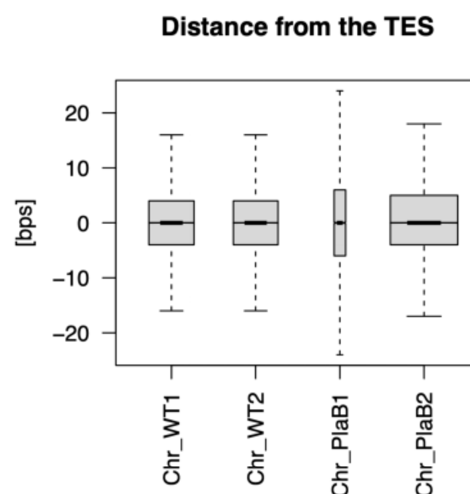
aligned with the abrupt end of the sequencing coverage. In particular, the Nanopore signal seems to have specifically captured one of these isoforms having a shorter 5’UTR, highlighted by a red box in the figure below.



Regarding the previously described effect of Pladienolide B treatment leading to premature termination, which we did not intend to deny, in our set up of dose and treatment length we could not see evidence of it - this might also be due to the small half life of prematurely terminated products. In fact, in addition to MYC, discussed above, we report here an additional example (IRX2) being well expressed in all fractions. IRX2 showed the expected accumulation of intronic signal, and the signal drops corresponded with the 3’ end of specific reported isoforms in all fractions.


Chromatin
6,442 bp
Untreated replicate 1
[0 - 2095]
Untreated replicate 2
[0 - 1775]
Pladienolide B replicate 1
[0 - 40]
Pladienolide B replicate 2
[0 - 5115]
IRX2
Nucleoplasmic
6,442 bp
Untreated replicate 1
[0 - 3309]
Untreated replicate 2
[0 - 2069]
Pladienolide B replicate 1
[0 - 793]
Pladienolide B replicate 2
[0 - 1168]
IRX2
Cytoplasmic
6,442 bp
Untreated replicate 1
[0 - 1032]
Untreated replicate 2
[0 - 2518]
Pladienolide B replicate 1
[0 - 756]
Pladienolide B replicate 2
[0 - 267]
IRX2
Polysomal
6,442 bp
Untreated replicate 1
[0 - 1330]
Untreated replicate 2
[0 - 2927]
Untreated replicate 3
[0 - 892]
Pladienolide B replicate 1
[0 - 627]
Pladienolide B replicate 2
[0 - 790]
IRX2

To further characterize this aspect, we focused on chromatin associated RNA samples for untreated and Pladienolide B treated cells and we computed, for each mapped read from these libraries, the distance between their 3' ends and the closest annotated transcription end site (TES). For both conditions, most of the distances ranged between 30 and 50 bases. In addition, we obtained distributions centered on zero, meaning that that reads were equally likely to finish up- or down-stream the annotated TES (see the figure below). The Pladienolide B distributions were only slightly broader than the untreated counterparts (especially for the replicate 1). However, the absence of a trend for reads ending upstream the expected TES, and the limited magnitude of these differences, did not support a systematic and severe premature termination in response to the drug.



10) Figure 2F: In theory, chromatin fraction does not contain polyadenylated RNAs since such mature RNAs should be released before RNA polymerase II transcription is terminated. Is this caused by a technical issue, like an RNA contamination from nucleoplasmic fraction?

Several studies support the notion that chromatin associated RNA is enriched with polyadenylated transcripts. For example, Brody et al (PLoS Biology, 2011) used live cell microscopy to study, in untreated and splicing inhibited cells, the coupling between RNA Pol2 elongation and splicing. Among other things, they found the pervasive retention of polyadenylated transcripts within chromatin. Drexler et al (Molecular Cell, 2020) profiled nascent chromatin-associated RNA through Nanopore direct RNA sequencing. In their setup, the enrichment for nascent transcripts (8 minutes of 4sU labeling) strongly reduces the chance of contamination with nucleoplasmic RNA. Yet, they observed the widespread presence of endogenous polyA tails for transcripts whose 3'ends are in proximity of polyadenylation sites. In a more recent report, Cotè et al (eLife 2023) improved the analysis of single RNA molecules through FISH to study at high resolution where splicing was occurring. They found that often unspliced transcripts, still containing introns, resided closely to the transcriptional unit encoding them, yet their transcription was already completed and they were already detached from the RNA Pol2 complex. Finally, the good correlations between replicates we observed for chromatin and nucleoplasmic RNA species (Supplementary Figure 7), together with the

validation of our protocol by western blots (Supplementary Figure 6E), reassured us about the limited extent of contamination between the fractions.

11) Figure 3E: The cluster is not labeled.

The clusters labels were on the left of the panel, yet not in an ideal position. We now decided to remove, here and elsewhere, the association of clusters with colors. Instead, we marked the clusters directly with the corresponding letters beside the heatmap(s) (see Figure 3G in the revised manuscript).

12) Figure 3F: Some clusters showed up-regulated rates of post-transcriptional splicing (i.e. k5). Post-transcriptional splicing event is not inhibited by Pladienolide B? The authors may want to discuss this. The example view (GB or IGV) of nanopore sequencing will be useful to show.

Both co- ($k_{2\text{-}3}$) and post-transcriptional ($k_{4\text{-}5}$) processing rates were impacted by Pladienolide B treatment (see clusters A and C in Figure 3G). In the revised manuscript we better discussed the potential regulatory responses behind the repression of both mRNA processing branches.
The application of Nanodynamo to untreated cells revealed that co- and post-transcriptional processing are largely mutually exclusive, meaning that either a gene is very efficient in one of these or in the other one (Figure 2D, clusters A,B,E,F vs C,D). This suggests that the spliceosomal machinery is active within chromatin and within the nucleoplasm for different sets of genes. It is important to this regard to stress that major biases due to contamination between the fractions are unlikely (as discussed in the main text), due to: (i) the validation of fractionation with markers expected for each fraction (Supplementary Figure 6E), (ii) the reproducibility between replicates of the RNA species abundance in the various fractions (Supplementary Figure 7), and (iii) the lack of specific structural features (transcripts length, introns length and number, sequence features) between genes associated to co- vs post-transcriptional processing (Supplementary Figure 14).
Following the treatment with Pladienolide B, specific sets of genes are repressed in processing, markedly those within clusters A and C in Figure 3G. Specifically, genes in cluster C are repressed in co-transcriptional processing, while those in cluster A are repressed in post-transcriptional processing. Strikingly, genes in cluster C are, in untreated cells, the least expressed (low k_1, Figure 3I) among the most efficiently co-transcriptionally spliced (high k_2, Figure 3I). Similarly, genes in cluster A are, in untreated cells, the most efficiently post-transcriptionally spliced (high k_5, Figure 3I) and are also particularly low expressed (low k_1, Figure 3I). Thus Pladienolide B seems to affect specifically low expressed, efficiently spliced genes. We explained this observation by reasoning that, assuming a uniform concentration of the drug within a given cell, poorly expressed genes are those reached by the highest proportion of drug molecules per transcript.
At this point, the switch of genes repressed in co-transcriptional processing to increasing post-transcriptional processing, and vice-versa, occurs. This could be due to coupling mechanisms which could be actively controlled, perhaps mediated by specific RNA binding proteins coordinating changes among the two processing branches, or coordinating changes in the rates of diffusion of RNAs between chromatin and nucleoplasm ($k_{3\text{-}4}$). This might represent an active attempt of the cells to compensate for the drug impact. Alternatively, the coupling could originate from the release of spliceosomal resources passively promoting a re-equilibrium between the two mutually exclusive processing pathways. Importantly, the increase in co-transcriptional processing as a consequence of switching is facilitated by the low magnitude

of the co-transcriptional rate for genes in cluster A - the same applying to genes in cluster C for the switch to post-transcriptional processing. All these considerations were added in the corresponding Results and Discussion sections of the revised manuscript.

13) Figure 4B: This panel may not be mandatory since this is the same as Figure 1A except for k8 and k9.

We believe that the Reviewer was referring to the schema of the simplified model in Figure 5B. This was moved to the supplementary figures as the Reviewer was suggesting.

14) Leptomycin B is an RNA export inhibitor, but it seems not to inhibit (affect) k6. This result suggests that Leptomycin B did not affect the RNA export rate. This is weird to me because mRNAs should be accumulated in the nucleus under Leptomycin B treatment, meaning the rate could not be quantified. The authors may want to discuss it.

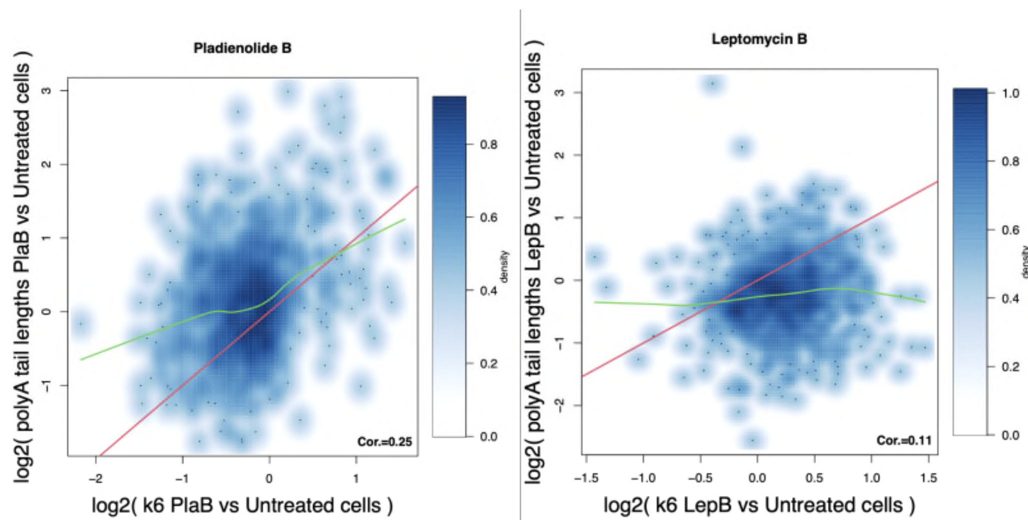
Leptomycin B is commonly used to block the export of proteins and transcripts to the cytoplasm. This drug acts on CMR1, which is a selective and mainly protein export factor (Kudo N et al, Exper Cell Res 1998, Kudo N et al, PNAS 1999). A subset of RNAs were also retained in the nucleus following the treatment with this drug (Jang BC, J Biol Chem 2003). This was confirmed by a more recent study (Engel et al, Nucleic Acid Res 2022) where they reported 105 transcripts being reduced and 74 being increased in their nuclear localization following 15h Leptomycin B treatment in HeLa cells. The overlap between these genes and the ones reported by us on SUM159 cells is small (8 genes), potentially due to the different cell types. Yet, the number of genes affected in RNA export is similar. We validated 6 of these genes by PCR following the repeated cell fractionation (now in Figure 4D). We additionally successfully included in the validation 2 genes that were not previously reported as affected in RNA export. Finally, we successfully included 2 genes that we deemed not affected in RNA export, as requested by the Reviewer (Supplementary Figure 25).
As discussed in the main text, several of the genes that we found significantly perturbed in export are involved in the regulation of key steps of the RNA life cycle including: chromatin dynamics/transcription (MED6 [Mediator Complex Component], CREM [Foulkes et al Cell 1991], XRN2 [Skourti-Stathaki et al Mol Cell 2011], CCNDBP1 [https://www.ncbi.nlm.nih.gov/gene/23582 15/04/2024], PPHLN1 [HUSH Complex Component - Kurita et al Biochem Biophys Res Commun 2007], HMGN3 [https://www.ncbi.nlm.nih.gov/gene/9324 15/04/2024], CHD1L [Wang et al Nat Com 2021], TARDBP [Bhardwaj et al NAR 2013], DOT1L [Min et al Cell 2003], UFL1 [Qin et al Nat Com 2019]), splicing (TARDBP [Bhardwaj et al NAR 2013]), export (LRPPRC [Volpon et al RNA 2017]), RNA stability (CSDE1 [Chang Genes Dev 2004]), translation (LARS1 [Liu et al NAR 2020], RPL23A [Large Ribosomal Subunit Component], CSDE1 [Chang Genes Dev 2004]]), and protein folding/stability (SERPINB1 [Choi et al Nat Immunol 2019], HSPB1 [Almeida-Souza et al J Biol Chem 2010], SPCS3 [Signal Peptidase Complex component - Liaci et al Mol Cell 2021], HERPUD1 [Schulz et al J Cell Sci 2017). Consequently, as discussed in the main text, the broad impact of this prolonged treatment on RNA metabolism is likely an indirect effect of the perturbation of factors that impinge on other key steps of the RNA life cycle. We believe that this is important for the field, since this drug is typically used in prolonged treatments.

15) Figure 4E: Co-transcriptional splicing was inhibited and post-transcriptional splicing was increased by Leptomycin B in category F. Other categories also showed a negative correlation. The authors may want to discuss it.

The modulations mentioned by the Reviewer likely resulted from coupling mechanisms. Specifically, the Leptomycin B treatment impacted several steps of the RNA life cycle (see Figure 4F, clusters A and C in the revised manuscript), which we proposed to be likely due to the impairment in export of selected transcripts coding for proteins involved in these biological processes. These modulations directly or indirectly involved the rates of co- and post-transcriptional splicing, which emerged as strongly coupled from all our analyses (i.e. upon the various drug treatments). These considerations were extensively discussed in the main section dedicated to the coupling of rates.

16) Line 309: ~ change in polyA tails length were positively correlated ~ -> negatively correlated?

We verified that, as previously reported in the text, changes in polyA tails lengths for both Pladienolide B and Leptomycin B treated cells compared to untreated cells were positively correlated to changes in the rate of export. Please see the following scatter plots.



17) Figure 6: The coupling of RNA cycle steps under Harringtonine treatment should be shown, even though the k8 and k9 were missing.

We followed the Reviewer's suggestion and included the coupling network for the Harringtonine treatment in Figure 6A.

Reviewer #2

In this work the authors propose Nanodynamo a modelling framework that involves mathematical modeling following the sequencing of native RNA from cellular fractions and polysomes. They use SUM159 triple-negative breast cancer cells as a test case to profile the kinetic rates of co-transcriptional and post-

transcriptional mechanisms that define the RNA life cycle. Generally, the quantification of the kinetic rates of RNA life cycle is important for the understanding of gene regulation and could likely prove very useful to the community. However, while the authors have performed extensive work on the mathematical modeling, the work lacks orthogonal validation of the estimates and thus excitement for the work is substantially reduced.

Given enough parameters, it is not surprising that the model can fit the data. Actually, the models presented in the work appear to fit unexpectedly well, achieving correlation values of 1.0, raising concerns for model overfit. Also, it remains unclear if the fitted values correspond to the truth. The authors need to provide evidence, based on established time series labeling experiments, that show the concordance of their estimates to experimentally measured rates.

Below are some specific major points:

Line 79-80: The authors need to show their polysome profile traces for all the samples and conditions as polysome RNA-seq is an important part of the work.

We thank the Reviewer for the suggestion. We now included polysome profile traces in the main figures for each condition (untreated and drug-treated cells). Additional replicates for those are reported in Supplementary Figure 6.

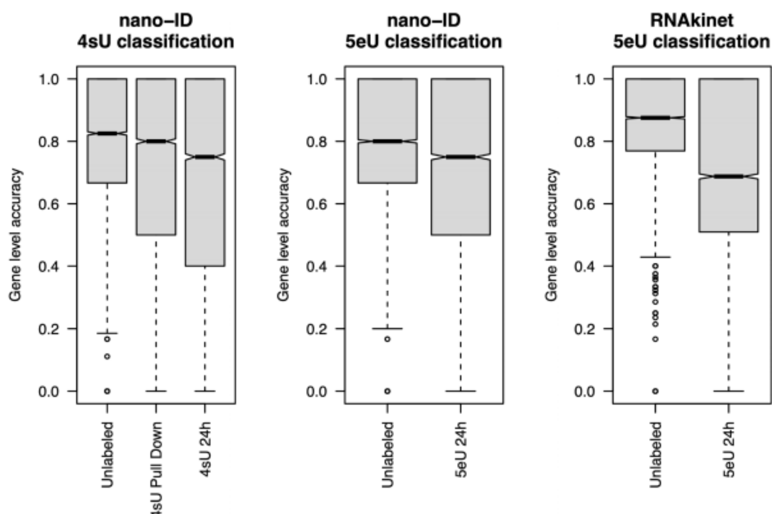
Line 94: "This modified nucleotide can be detected on dRNA-seq data using the nano-ID tool". This is incorrect. Nano-ID has been trained to detect 5EU. The authors appear to have retrained the neural network to detect 4SU using their data. Therefore, since this is a critical part of their work, they need to show that modified nucleotides can indeed be detected. Currently they only evaluate the performance of the test dataset, but no information is provided regarding the creation of the test dataset. More importantly no evaluation has been performed on a completely independent dataset. Fully connected neural networks with millions of parameters like nano-ID are prone to overfit. Given that the entire work depends on this step, a truly independent evaluation needs to be performed.

The Reviewer is correct, nano-ID has been developed to classify nanopore dRNA-seq reads according to the presence/absence of 5eU, and we retrained the algorithm on our data to detect 4sU. Specifically, we sequenced unlabeled and fully-labeled RNA from SUM159 cells, the latter obtained after 8 hours of 4sU metabolic labeling (500 nM) followed by the pull-down of labeled RNA. Then, we extracted all the features required by nano-ID with a custom nextflow pipeline which performs reads alignment (minimap2 -ax splice -k14), BAM sorting and filtering (samtools view -F 2308 -q 20), and executes all the R scripts provided by Maier and colleagues in the original nano-ID publication. The latter required a substantial effort, since the code released in that study was not sufficiently tidy to be directly executed. The dataset was then sub-sampled to balance the amount of labeled and unlabeled reads per gene, and splitted in training and test sets (70% and 30% of the reads, respectively) which were finally used for nano-ID training and evaluation.
We validated our pipeline using it on the original nano-ID dataset, re-basecalled with Guppy6, obtaining a classification performance in agreement with the one presented in the nano-ID publication (Accuracy 0.86, AUROC 0.95).

The choice of applying the nano-ID framework to a different modified nucleoside was motivated by our observation that a prolonged exposure of SUM159 cells to 5eU (500 nM - 24h labeling) led to cells suffering with potential impact on RNA metabolism and/or 5eU incorporation (observations based on Trypan Blue Cell counting). Rather, we could not detect any suffering in cells exposed to 4sU, prompting us to switch to that modified nucleoside. To further minimize the impact of metabolic labeling - when cells were exposed for prolonged time as in the case of the fully labeled condition - we also reduced the labeling time to 8h, followed by pull-down of the labeled RNA to minimize the potential presence of unlabeled transcripts. This strategy led to a slight improvement in the nano-ID classification (Accuracy 0.75, AUROC 0.83) compared to 24h labeling with 5eU (Accuracy 0.72, AUROC 0.80) and compared to 24h labeling with 4sU (Accuracy 0.73, AUROC 0.82).

As suggested by the Reviewer we quantified the performance of nano-ID on a completely independent sample, which was not involved in the training step. To this end, we exploited the 4sU 24h labeled sample introduced above. The per-gene accuracy was only mildly impacted (see attached figure). Furthermore, the per-gene accuracy was in line with the that obtained with 5eU 24h labeling, and also with the performance of an independent tool - RNAkinet (Martinek V et al, bioRxiv 2023) - recently developed to detect 5eU labeled reads (Accuracy 0.70, AUROC 0.78). Importantly, RNAkinet was not retrained on our data, nor the code for its training has been released yet. RNAkinet was specifically developed for reducing overfit compared to fully connected neural networks. Altogether, these analyses reassured us about the possibility to apply nano-ID to efficiently identify 4sU-labeled reads. We anticipate that, once RNAkinet will be fully released, the Nanodynamo framework could be easily adapted to use it and shall benefit from the improved classification of labeled reads.

The methods section was edited to better describe how we used nano-ID to classify newly synthetized transcripts through 4sU metabolic labeling.



Nascent RNA profiling Accuracy in SUM159. *Accuracy distributions, at single gene resolution, for unlabeled and fully labeled reads. (Left) Performance of a nano-ID instance for 4sU containing reads detection. The unlabeled and 4sU Pull Down samples were used for training while the 4sU 24h sample was not. (Center) Performance of a nano-ID instance for 5eU containing reads detection on its training samples. (Right) Performance of RNAkinet, a piece of software developed to detect 5eU containing reads, on samples not involved in its training.*

Line 99: "Following the verification of the theoretical feasibility of kinetic rates inference". It is unclear which data support this statement and validate the theoretical feasibility of the model.

The theoretical feasibility of kinetic rates inference has been verified testing the global structural identifiability of all the parameters of the model, i.e. evaluating whether the value of each parameter can be recovered uniquely given continuous and noise-free data. We performed this analysis with a Julia implementation of the SIAN software (Structural Identifiability ANalyser) which, to the best of our knowledge, can be considered the state of the art for Ordinary Differential Equations models. This algorithm leverages differential algebra and Taylor series expansion to provide, for any suitable input, a classification of each parameter of the model as: not identifiable, locally identifiable, or globally identifiable. Noticeably, this classification is by design correct with a probability of 0.99.
To present the concept of global structural identifiability more clearly in the revised version of our manuscript, we now mention the corresponding methods session in the main text.

Line 103: The purpose of the section "Evaluation of Nanodynamo through simulated data" is unclear. The authors appear to have simulated data according to their model. Then have added noise based on normal distribution and then fitted the model. This seems like a trivial task that doesn't prove much besides that the source code works. The correctness of the estimated rates remains unclear. It is also unclear how these simulations can be used to evaluate the optimal number of replicates and labelling time given that the noise added to the simulations is arbitrarily selected.

The validation of parameters global structural identifiability guarantees the theoretical feasibility of kinetic rates inference, nevertheless, this analysis involves noise-free data and disregards potential numerical issues.
For this reason, it is essential to test the goodness of our inference framework on simulated data generated according to a known set of rates and incorporating a reasonable amount of noise. In the first version of our manuscript, we used a variation coefficient (CV) of 0.35 for all the RNA species involved in the model. Importantly, this value was not arbitrarily selected. It was determined from a large RNA-seq dataset that we collected in a previous study where we profiled premature and mature RNA within both total and nascent RNA pools (using 10 minutes of 4sU metabolic labeling), over time (11 time points) following Myc induction in 3T9 mouse fibroblasts (de Pretis S et al, Genome Res 2017). We determined the gene-level CV for each RNA species based on the entire time series, i.e. also accounting for the biological variability, and we used the CVs distributions to select 0.35 as a conservative variation coefficient - this value was larger than 90% of the estimated CVs.
We now improved the noise estimation by avoiding using a unique CV for all RNA species. To this end, the CV was determined independently for each RNA species based on the raw counts of untreated cells - taking the average of all gene-level CVs for that species - and relied on these to generate the simulated data. The obtained results were in agreement with those presented in the first version of the manuscript both in terms of goodness of fit and impact of replicates (Figure 1D-E and Supplementary Figures 4-5).
We would like to stress that the inference of simulated rates is far from being trivial given a reasonable amount of noise which significantly impacts the simulated gene expression values. Indeed, we used this information to evaluate whether or not considering two potential extensions of our model, which involved the degradation of either premature or mature nuclear RNA. Eventually, we decided to ignore these steps of the RNA life cycle because the corresponding rates could not be inferred (Spearman correlation coefficients based on simulated data 0.05 and 0.08, respectively) despite their global structural identifiability.

Line 134: "a single sample is sufficient to have a performance remarkably close to the optimal one". Not requiring replicates is indeed remarkable, but also unlikely to be true in real experiments. This most probably indicates the low complexity of the simulated data.

Regarding the quality and usefulness of the simulated data we refer to the response to the previous comment above.
The Reviewer is right, replicates are crucial for any measurement to estimate the associated variability, and this applies also to RNA sequencing. Nevertheless, the Nanodynamo framework is based on the joint analysis of several RNA pools which are profiled through independent experiments, in the real case scenario, or derive from the independent sampling of multiple distributions for the simulated data analysis. Therefore, multiple measurements simultaneously contribute to the definition of the optimum set of rate, even if a single value is provided for each RNA species, and this allows to mitigate the detrimental effect of having few or even only one replicate.
Eventually, the inference routine was updated in the new version of the manuscript (see next point), so that it takes full advantage of replicated measurements for the abundance of the various required RNA species. Indeed, the single replicate configuration is not recommended anymore, and the individual replicates are not pooled anymore.

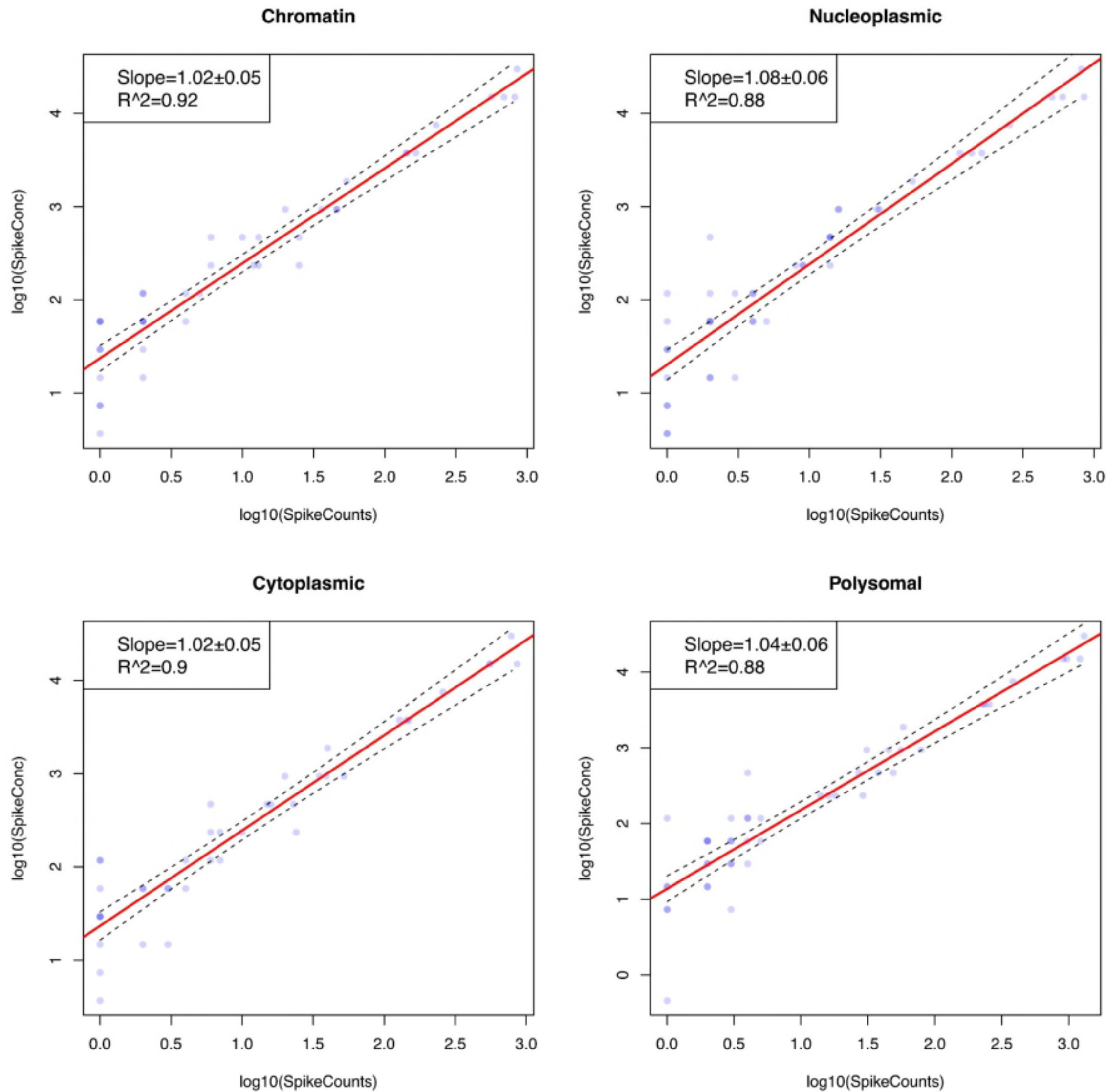
Line 150-151: In this reviewer's opinion correlation values of <0.5 between replicates is not acceptable to justify pooling replicates together. This indicates that either the experiments are very noisy or more likely that the model overfits.

As discussed in the response to the previous comment, following the Reviewer's suggestion, we extensively revised our inference approach to avoid replicates pooling. Specifically, for a given gene, we now estimate the optimal rates on the RNA species expression levels from both replicates simultaneously. This approach allows to process datasets with an arbitrary number of replicates fully exploiting the available information.

Line 154: The use of a single read as a threshold appears to be very loose. Especially when all replicates are pooled together and there is no estimate of the variance.

As discussed in the response to the previous comment, following the Reviewer's suggestion, we extensively revised our inference approach to avoid the replicates pooling.
Regarding how we introduced the estimation of gene-level variance and used it to detect differential expression across treatments, please refer to the response to the "*Line 285*" Reviewer's comment below.
To evaluate the goodness and usefulness of single-count reads, we took advantage of the spike-ins added for each sample. We performed, for each fraction of the untreated cells, a linear regression between the expected spike-ins concentration and the spike-in counts. Slopes ranged between 1.02 and 1.08, and R^2 between 0.88 and 0.92. This indicated that our Nanopore profiling was highly quantitative on the full spectrum of spike-ins concentration, which covers a broad range of values. Notably, this included the spike-ins with the lowest concentrations, often quantified by individual reads. Even though the noise was moderately increasing at low concentrations, the goodness of the linear fit and the displayed 95th confidence intervals reassured about the use of one count as a minimal threshold for gene expression level. Furthermore, a one-tailed Wilcoxon-test was performed to assess the statistical significance of the difference between the mean concentration of the group of spike-ins with 1 count versus the mean of the

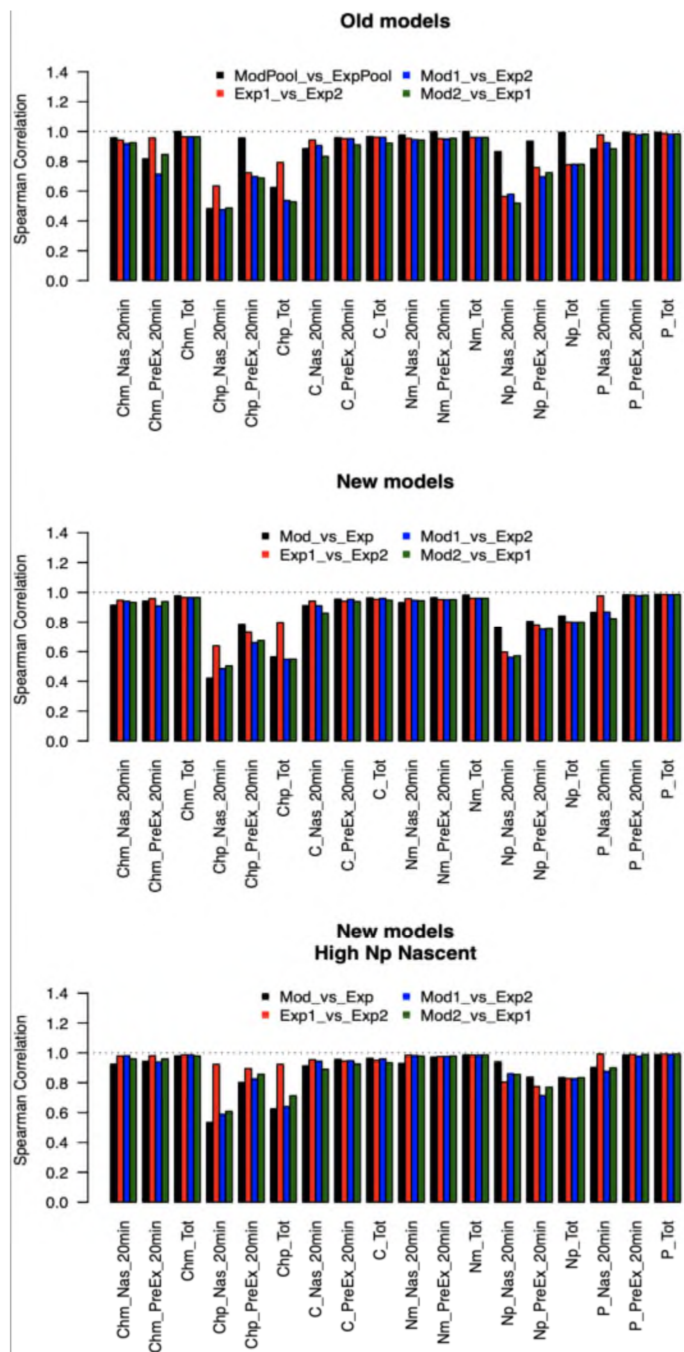
group of spike-ins with 2 counts. The resulting -log10(pvalue) are: 1.5 for the chromatin fraction, 1.47 for the nucleoplasmic one, 0.41 for the cytoplasmic one, and 1.3 for the polysomal one. Thus, the difference between the two groups is significant in every fraction except for the cytoplasmic one, which can be justified by the scarcity of data points in the second group (2 counts) for this fraction, together with the fact that the first group spans a wider range.



Line 155-156: The authors report a median Spearman correlation between modelled and experimental values of 1.00. Biological experiments done in replicates are extremely unlikely to achieve correlation of 1.00. Yet the model seems to do so. Does this mean that the model is perfect? In this reviewer's opinion this likely indicates that the model overfits the data.

The median Spearman correlation between modeled and experimental values was 0.96 while 1.00 was the maximum; we apologize for the misleading message.

Nevertheless, the observation of the Reviewer is important because our first analyses reported a high number of species very well correlated. To test the overfit hypothesis, we determined the correlations between replicates by comparing different RNA expression levels: (i) experimental vs inferred data following replicates pooling (*ModPool_vs_ExpPool* in the figure below), (ii) experimental data replicate 1 vs 2 (*Exp1_vs_Exp2* in figure), (iii) inferred data for replicate 1 vs experimental data for replicate 2 (*Mod1_vs_Exp2* in figure), and (iv) inferred data for replicate 2 vs experimental data for replicate 1



(*Mod2_vs_Exp1* in figure). As displayed in the figure below ("Old models"), we noticed that chromatin premature pre-existing (*Chp_PreEx* in figure) and nucleoplasmic premature (*Np_Nas, Np_PreEx, and Np_Tot*) RNA species showed high correlations between pooled experimental and inferred data (i), compared to the correlation between replicates (ii) or against independent data (iii and iv), suggesting a certain amount of overfit.

We reasoned that the primary origin of overfit was probably the complexity of our model compared to the dimensionality of the dataset. Therefore, we decided to estimate the optimal rates on the RNA species expression levels from both replicates simultaneously ("New models" in the figure). This new modeling framework improved the performance limiting the overfit to the nascent nucleoplasmic premature RNA. Noticeably, this is less evident for genes with a high level of this RNA species (top 10%, see "New models, High Np Nascent" in the figure), suggesting that increasing sequencing depth could remove the mild residual overfit. We discussed this point in the Discussion session of our reviewed manuscript, the corresponding data were reported in Supplementary Figure 40.

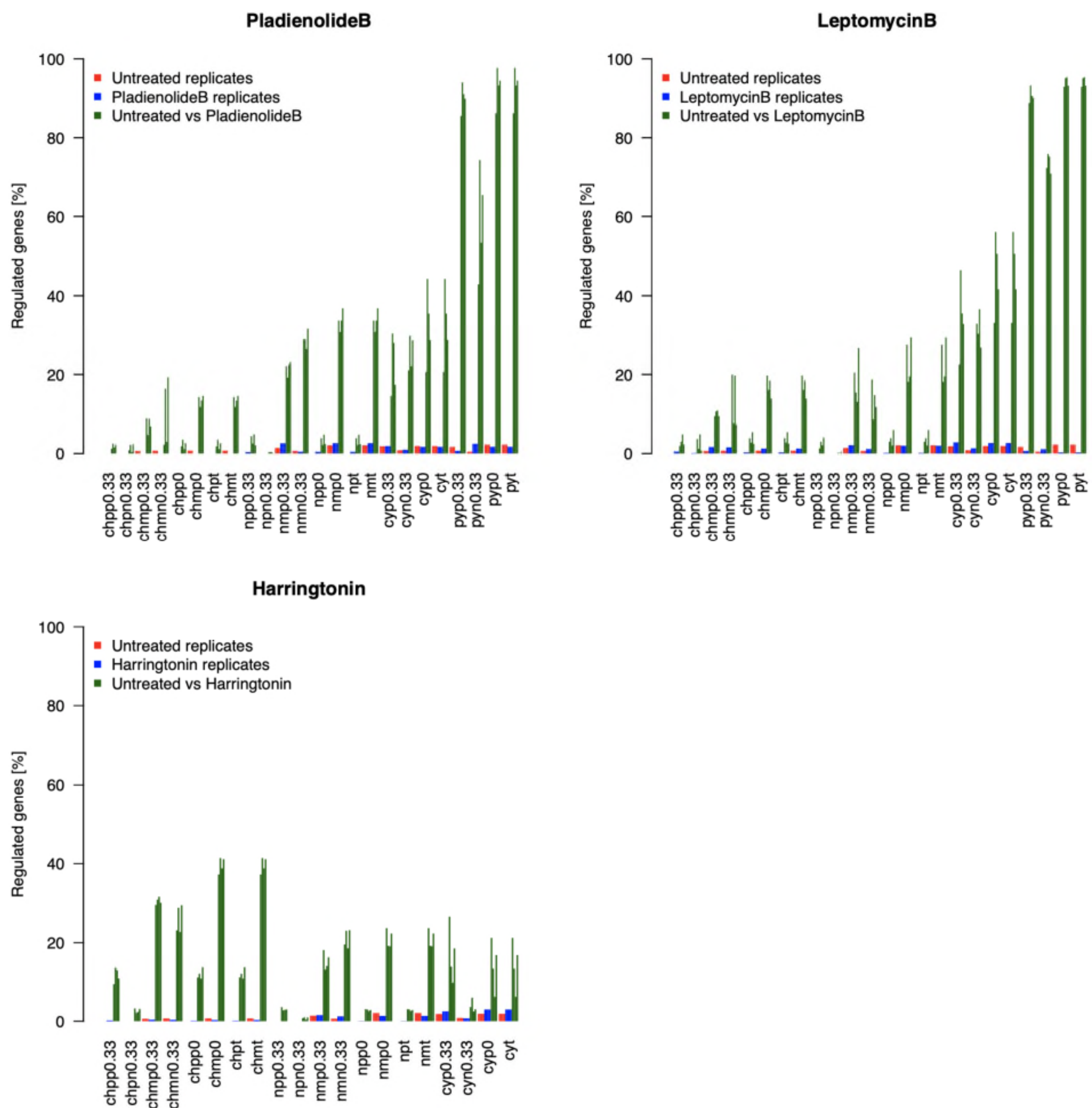
Line 166: The authors need to provide this calculation.

According to the work of Biasini and colleagues, after 15 minutes of labeling 1.5% of the RNA is expected to be nascent. According to our quantifications of RNA yield in untreated SUM159 cells (see the methods session *Fractionation and mRNA extraction*), this correspond to ~**11 ng / (Million of cells * h)** considering 180 ng of RNA from the fractions and 6% of nascent RNA. This value is well recapitulated by Nanodynamo, which returned a median rate of RNA synthesis per gene of 15 pg / (Million of cells * h). Assuming 10^3-10^4 expressed genes per cell, this sums up to 15-150*10^3 pg / (Million of cells * h), which corresponds to **15-150 ng/(Million of cells * h)**. Notably, this analysis was conducted using a limited set of highly expressed genes, which may account for the slight overestimation observed.

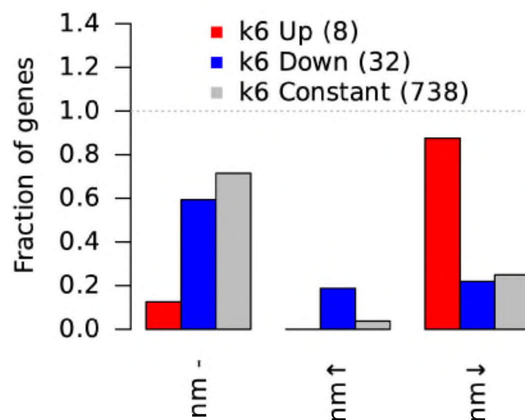
Line 285: Is there any statistical significance estimation for these quantifications?

Following the Reviewer's comment, we extensively revised our framework to estimate the variability of the normalized expression level of each profiled RNA species at the single gene resolution.
Briefly, for each sequenced sample, we determined the share of reads counts per RNA species, by comparing the total number of reads counts of a given RNA species against the total number of counts. This information is then used to split the yield of the corresponding fraction. For example, given 100 fg/Milion of cells of chromatin RNA, if 7% of the reads are annotated as nascent premature RNA, the corresponding yield would be 7 fg/Milion of cells. Then, we applied DESeq2 independently to each RNA species to estimate the parameters of gene-specific negative binomial distributions: dispersion values, and replicate-specific mean values. After that, we randomly sampled each distribution and we used the resulting counts to split the yield of each RNA species across genes (same principle applied above for fractions). Iterating this sampling scheme 1000 times, we obtained a normalized expression level distribution for each RNA species at the single-gene and single-replicate resolution.
The comparison of normalized expression levels distributions between conditions (untreated cells and drug treatments) allowed us to identify RNA species modulations. For a gene to be classified as upregulated, the mean of the treated distribution was required to be larger than the 97.5th percentile of the untreated one, while the mean of the untreated distribution was required to be lower than the 2.5th percentile of the treated

one. Vice-versa, for a gene to be classified as downregulated the mean of the untreated distribution was required to be larger than the 97.5th percentile of the treated one, while the mean of the treated distribution was required to be lower than the 2.5th percentile of the untreated one.
Importantly, the fraction of genes classified as up- or down-regulated comparing replicates from the same treatment was remarkably low, reassuring about the precision of our procedure (see figure below included in the revised version as Supplementary Figure 41). Since this analysis can be repeated for each couple of untreated and treated samples replicates, we decided to classify a gene as up- or down- regulated if it were coherently modulated in at least 2 of the 4 possible combinations (configurations characterized by the same number of opposite regulations were discarded).



We adopted this framework to assess the significance of the changes in RNA export (k_6) resulting from Leptomycin B treatment, as required by the Reviewer. We reasoned that nuclear mature RNA steady state level (Nm) is equal to k_1/k_6, therefore, a modulation of the latter could result in the differential expression of this RNA species. Indeed, genes up-regulated in k_6 were enriched in transcriptional units down-regulated in Nm (Fisher test p-value $< 4e-4$), while genes down-regulated in k_6 were enriched in transcriptional units up-regulated in Nm (Fisher test p-value $< 1.5e-3$), see the figure below. Interestingly, 88% of the genes up-regulated in k_6 were differentially expressed in Nm while the percentage was remarkably lower for the down-regulated counterparts (19%). The reason is that the rate of synthesis contributes to the definition of Nm expression level, consequently, a modulation in k_1 can either expose or conceal the footprint left by k_6 on Nm. Since k_1 is globally down-regulated in response to the treatment (see Figure 4F), this made the identification of k6 down-regulations harder to be detected. Nevertheless, this issue affected all the transcriptional units involved in the analysis, therefore, the enrichment significance clearly emerged despite the modest number of differential genes detected. All the technical aspects presented above are detailed in the *Quantification of gene expression levels* section of the reviewed manuscript, while the results obtained for k_6 modulated genes are mentioned in the main text.



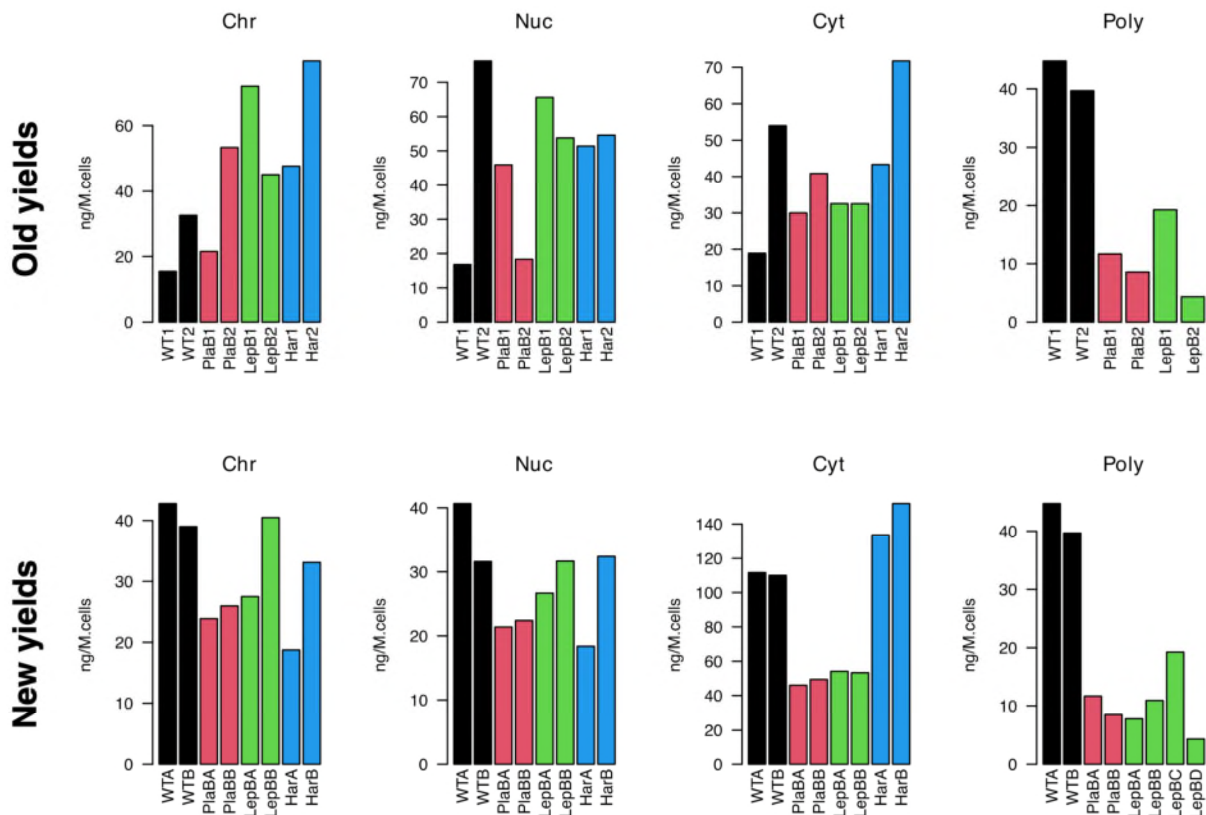
Line 289: RT-PCR differences appear modest. Do they correlate with the expected kinetic rate changes? Also, some control genes without kinetic rate changes should also be tested.

Due to a bug in the code, we were not selecting genes properly. Indeed, we selected genes that were lacking either the mature RNA species in the nucleoplasm or the cytoplasmatic RNA species and not, as required from the complete model, genes for which signal was available for all the species. Despite this, the corresponding RT-PCRs nicely recapitulated the expected trends in the species abundance, and consequently the changes in RNA export (k6).
The new RT-PCRs have been performed for genes for which modulation upon Leptomycin B treatment was previously reported (Engel KR et al, NAR 2022) or that we detected as modulated in k_6 (DOTIL and LGALS were not part of the set of genes by Engel and colleagues). The RT-PCRs for up-regulated (LGALS,ADARB1,PRKAG2,POLE4) and down-regulated genes (DOTIL,CYREN) nicely recapitulated the expected trends and magnitude of the changes (Figure 4D). Finally, we tested two genes

(SNRPB2,HTRAI) whose export rate was not reported to change. Both confirmed the lack of modulation, the second resulting in a very mild difference (15% reduction; Supplementary Figure 25).

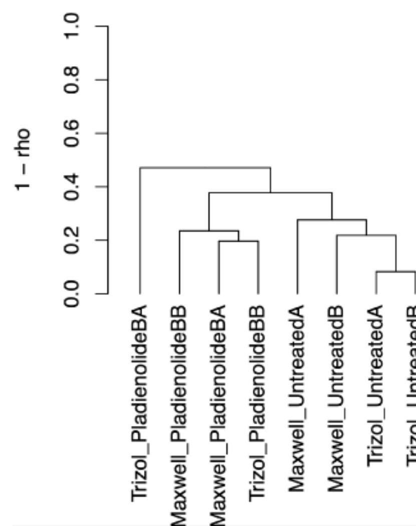
Line 610: In replicate 1 of the untreated cells the authors get approximately more than 2x poly(A) RNA compared to all the other cellular fragments. In contrast, in replicate 2 they get 2x less poly(A) RNA. Similar variations among replicates can be seen for other conditions and fragments. This raises concerns regarding experimental reproducibility, and it is unclear how this massive difference, which should be affecting calculations of the rates, can be normalized with the proposed normalization scheme.

The Reviewer is right about the substantial variability in RNA yields across replicates. To improve on this central aspect of our study, we changed the RNA extraction method from an approach based on TRIzol to the Maxwell instrument for three of the cellular fractions of interest (chromatin, nucleoplasm and cytosol). The shift to Maxwell RNA extraction markedly improved the reproducibility in the calculation of RNA yield per fraction and per replicate (see figure below and see the methods session *Fractionation and mRNA extraction*). The median CV for RNA yields decreased from 0.35 to 0.08.



The Maxwell RNA extraction approach was not applicable to the polysomal fraction because the sucrose gradient is more than 8ml per sample, which is not compatible / practical for handling with the Maxwell RNA extraction platform. For the polysomal fraction, we instead decided to collect two additional yield estimates based on TRIzol RNA extraction for the LeptomycinB treatment, which was particularly noisy in our initial quantifications.

We also decided to change the original workflow by decoupling the quantification of RNA yields from the actual library preparation and subsequent Nanopore dRNA-seq. This choice provided much higher flexibility in the design of the experiment. Moreover, it allows easily acquiring various replicated measurements of RNA yields without incurring additional sequencing costs and waiting time. To exclude that having changed the RNA extraction method introduced biases against the previously acquired sequencing data (which relied on TRIzol extraction), we sequenced chromatin RNA extracted with the Maxwell from untreated and PladienolideB treated cells (two flow-cells per condition), and we compared the resulting gene expression levels to the TRIzol based counterparts. Hierarchical clustering reported in the figure below for gene-level RNA counts using 1 - Spearman Rho as distance, nicely grouped the samples within each biological condition, confirming the absence of a systematic bias due to the RNA extraction protocol.



Line 641: At which step in the experiment are the ERCC spike-ins added? It is unclear what these spike-ins control for? Do they only control for library preparation and sequencing? It would be expected that most of the variation occurs during cell counting, RNA extraction, processing, polysome isolation, all steps before library preparation. The authors need to explain how they control and normalize these steps.

The reviewer is right, most of the variation occurs during RNA extraction and quantification. In this regard, we modified our experimental workflow to improve the reproducibility of our data. Please see above for details on how we improved our ability in quantifying RNA yield per fraction and their reproducibility (please see the response to the comment *"Line 610"* for details).
Regarding the ERCC spike ins, they are added directly to the mRNA solution just before library preparation. They are used as quality control, and for assessing the linearity of our quantifications compared to the expected amount of these species (please see the response to the comment *"Line 154"* for details).
The normalization step has changed, and we are no longer considering ERCCs for it. For each species, we compute a normalization factor that is equal to the ratio between the extracted mRNA and the number of cells from which RNA has been extracted. Next, we ran DESeq2 independently on each fraction to estimate gene counts distributions, RNA species' normalized counts and dispersions and use the normalization factor

to split the yield across genes. This has been detailed in the *Counts normalization* session of the main text methods which is also reported below.
Importantly, reassuring on the reliability of our normalization, the absolute quantification of nascent RNA was in line with what previously reported in the literature (please see the response to the comment *"Line 166"* for details).

Counts normalization *– For each sample, the following factor was computed to account for the amount of PolyA RNA extracted from each fraction; a crucial step to move from relative to absolute gene expression levels.*

$$Normalization\ factor = \frac{fg\ of\ mRNA}{Milion\ of\ cells}$$

Then, for each RNA species and sequenced sample, we estimate genes counts and we use them to split the yield of the corresponding fraction (e.g. given 100 fg/Milion of cells of chromatin RNA, if 7% of the reads were annotated as nascent premature the yield of Chpn would be 7 fg/Milion of cells - see the methods session Fractionation and mRNA extraction). Then, we applied DESeq2 independently to each RNA species to estimate the parameters of gene-specific negative binomial distributions: dispersion values, and replicate-specific mean values. After that, we randomly sampled each distribution and we used the resulting counts to split the yield of each RNA species across genes (same principle applied above for fractions). Iterating this sampling scheme 1000 times, we got a normalized expression level distribution for each RNA species at the single gene and single replicate resolution. The mean of these distributions were used as input data for rates inference. Gene expression quantification was performed in R 4.2 using the Bioconductor package DESeq2 [Love et al Genome Biology 2014] (v1.38.3).

Line 561-565: Metabolic labelling with 5EU of K562 cells is described in the methods but it is unclear where K562 cells were used in the work.

The K562 dataset was used to characterize the impact of ribo depletion and in-vitro polyadenylation in terms of premature RNA profiling; *Discussion* session of the main text. We explicitly mentioned the cell line in the revised manuscript.

Line 590 and elsewhere: There are some spelling mistakes throughout the text. e.g. Cycloexamide should be cycloheximide that need to be corrected.

Apologies for the mistakes, we carefully reviewed the text of the manuscript to address the spelling errors.

Line 746: Smooth density scatterplots used throughout the figures can be obscure, unless accompanied by a legend describing the point density scale. The authors should consider showing all points, perhaps with some transparency to avoid overplotting.

We followed the Reviewer's suggestion adding a color-scale to the smooth density scatterplots.

Reviewer #3

The authors designed an interesting data-driven modeling system to explain RNA dynamics in a large-scale manner. Here are my points for the authors to consider.

1. When using the ODE model to explain RNA data, as the model is relatively simple, it did not include potential dynamic contributions from the feedback exerted by relevant transcription factors. This may be particularly important for the drug perturbation scenario as cells can activate compensatory signaling and autocrine to dynamically up/downregulate transcription factors to directly control RNA synthesis (and perhaps also RNA degradation and processing steps, if the cells happen to produce more or less of those accessory proteins). If the focus of the model is to explain short term RNA dynamics data, what about long term dynamics? This (long term dynamics) is much more meaningful in deciphering cell fate.

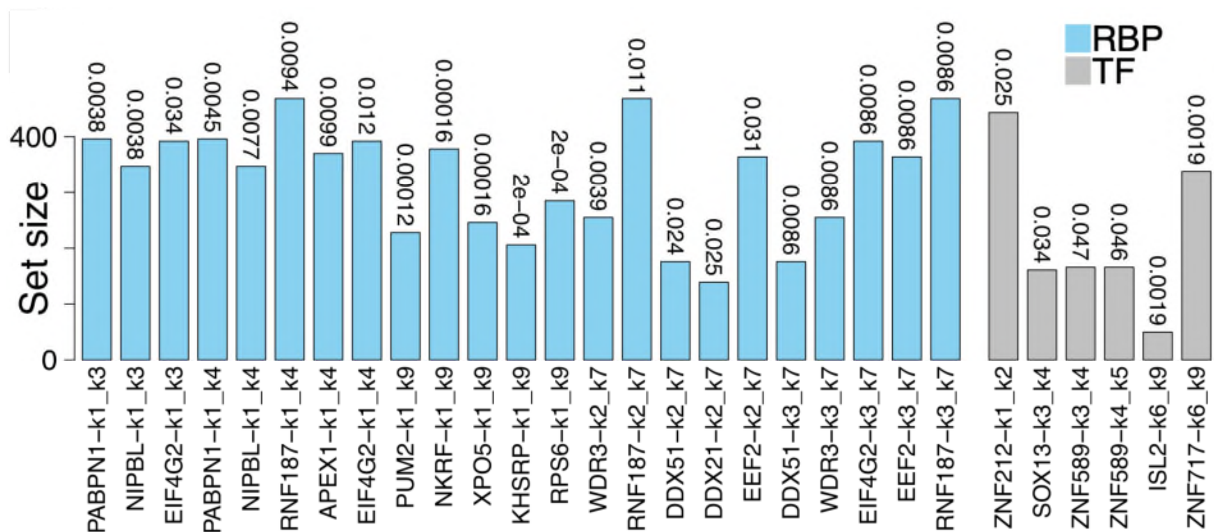
The response to this Reviewer point was combined with the response to the following comment, please see below.

2. In fact in real biology, some of the major reactions described in the model, e.g. co-transcriptional processing, post-transcriptional processing, degradation, require accessory proteins, which means that while a single first-order rate description may be ok (from the modeling standpoint), it actually also overlooks the potential second/higher order protein binding and catalytic interactions (from the mechanistic standpoint). Since the levels of these accessory proteins may also vary upon drug perturbation (e.g. feedback or nonspecific drug effect), the story could become a lot more complex. In short, simply saying that some rate decreases upon drug intervention does not really offer mechanistic insight. The authors should discuss this potential limitation. Or the authors could perhaps find 1-2 genes that were shown to have reduced rates and meanwhile there were some literature evidence that can support a potential mechanistic link. Such information could be used as validation of the model insights.

The Reviewer is right, gene expression is extremely complex and involves hundreds of actors which contribute to the fine tuning of the expression level of each gene. In particular, the Reviewer stresses the key role of regulatory factors, such as transcription factors (TFs) and RNA binding proteins (RBPs), which are key determinants of the RNA life cycle steps that are included in our model. However, hundreds of such factors exist and their target preferences are largely unexplored. For this reason, including their role in such mechanistic modeling efforts would be a daunting effort given the current available data in the field. Indeed, our first-order model is far from providing an accurate description of all these regulatory cues. Rather, Nanodynamo aims at providing a comprehensive and accurate picture of the outcome of all these regulatory interactions, quantifying how RNAs flow across the different steps of their life cycle. This is a crucial first step of any mechanistic study. In fact, in the Discussion section we propose that Nanodynamo could be a powerful tool for exploring the functional role of TFs and RBPs. For example, determining the consequences of knocking down or out such factors on the dynamics of RNA metabolism. This would shed light on which steps of the RNA life cycle are mostly affected and thus are likely regulated by the perturbed factors.

Finally, the Reviewer points to the coordinated action of the various machineries that regulate the RNA life cycle. We hope that our study has provided some advance in this direction, based on our comprehensive analysis of coupling between the steps of RNA metabolism across various drugs perturbations. To this regard, we extended our analyses by trying to identify the factors - TFs and RBPs - that could be involved in the implementation of these couplings. We performed a GSEA-based analysis to identify enrichments in

TFs and RBPs targets among genes supporting each given coupling. Proteins binding sites were retrieved from the ENCODE web portal [10.1038/nature11247, 10.1093/nar/gkz1062] downloading ChIP-seq and eCLIP BED files respectively (GRCh38 - K562 and HepG2 not perturbed cell lines). RBPs binding sites were annotated according to the overlap with genes exonic regions (see the methods session Premature reads profiling); and only genes with at least 25 binding sites for a given RPB were considered targets. TFs binding sites were annotated according to the overlap with promotes which were defined as regions 2000 bases upstream and 1000 bases downstream genes transcription start sites (TSSs) in a strand-aware manner. TSSs were retrieved from genes exonic regions (see the methods session Premature reads profiling) taking the lower coordinate for genes on the positive strand and the larger coordinate otherwise. The rankings for the GSEA analyses were defined according to the product of rates log2 fold changes compared to the untreated condition times the sign of their Spearman correlation. In this way, the top genes for positive or negative couplings were characterized by strong coherent or opposite modulations respectively.
Overall, we identified 93 and 149 enriched proteins for Pladienolide B and Leptomycin B respectively (GSEA adjusted p-value < 0.05), the vast majority deriving from the k_{19} edge, among which 88 were common. The 5 most significant proteins for each coupling were reported in Figure 6D for the Pladienolide B treatment, and in Supplementary Figure 36 for the Leptomycin B one. Noticeably, four proteins involved in gene expression regulation emerged as top enrichments for couplings involving RNA synthesis and processing rates (k3-5) in both the treatments: NIPBL, APEX1, EIF4G2, and PABPN1. The latter takes part in RNA polyadenylation which might suggest the involvement of this regulatory layer in mediating transcriptional couplings [https://doi.org/10.1038/cr.2012.86, https://doi.org/10.15252/embr.202357128]. This analysis was presented in the session of the manuscript: *A comprehensive analysis of coupling among RNA life cycle steps*.



Barplot showing the 5 most significant coupling factors candidates (RBPs and TFs) for the couplings identified with the Pladienolide B treatment; on the top of each bar we reported GSEA p-value.

3. What about the inter-cell type applicability of this model? An example would be to use 3-5 cancer cell lines (sensitive, moderate, resistant), control vs receiving the same drug (and different doses) and analyze the potentially different RNA dynamics (cell line A vs B vs C, A dose1 vs A dose2, etc.) using this framework. This would hopefully provide more meaningful information for translational research.

The Reviewer is suggesting an extremely interesting research project which represents a natural application of the Nanodynamo framework. Indeed, we are currently working on the application of our method to the fine characterization of transcriptional programs in a large panel of breast cancer cell lines. However, this represents an independent study which will take a remarkable amount of time, workload and resources. For these reasons, we really appreciate the suggestion, but we consider it out of the scope of the current manuscript. Instead, we believe that the application of Nanodynamo for studying the impact of various drugs against key steps of RNA metabolism represent an immediate, and hopefully timely and relevant, application for our method.

4. Another concern is the novelty of this study. To me the hard part is perhaps measuring the absolute RNA counts for a large number of genes in the four different cellular compartments, as the ODE modeling part is rather linear, straightforward and based on recognized mechanisms. So, have such location-specific RNA measurement experiments been done before by other studies or other groups?

A large part of the literature in the field of RNA dynamics, either for bulk populations or for single cells, focused on the development of approaches to quantify transcripts half-lives, thus focusing on RNA decay only. A subset of those studies, attempted to expand these analyses to the study of RNA synthesis and, an even more limited number of studies also integrated splicing efficiency. In the last decade we have been part of this effort, with a number of tools that we released in the INSPEcT R/Bioconductor package and a relatively updated review on the advances in the field that we published in 2021 (Furlan M et at, Briefings In Bioinformatics 2021).
To the best of our knowledge, a more limited literature tried to cover these aspects at the subcellular level. In particular, previous works leveraged on location-specific RNA sequencing to estimate the rate of transcripts export (Chen, Plos Gen. 2017), or the rate of association with polysomes (Li, Current Opinion in Microbiology 2015, Fang, Cell Systems 2018). Therefore, we believe that the model implemented and inferred by Nanodynamo represents a major improvement of the state of the art per se.
Nevertheless, defining an experimental and computational workflow for a routinely feasible while reliable acquisition of the key input data - mostly RNA yields across cellular fractions and the quantification of RNA species from Nanopore dRNA-seq data - was a major challenge of our research project, that required further experimental and computational work during the revision process (see the rebuttal to Reviewer 2, points about Line 610), resulting in an additional source of novelty.

5. Figure 1A. Unclear labeling. Which thing (drawing) corresponds to which variable in the equation? Reaction k3 goes to where?

In order to clarify the cartoon, we added labels corresponding to terms in the ODE system to the cartoon.

6. Were the Excel files containing RNA absolute counts (of different genes) in different compartments (in the several cell lines mentioned) provided in the supplemental files? This will be very helpful for future research.

All the raw and normalized data will be available as R objects on GitHub upon publication, including the RNA absolute counts and code for reproducing all main and supplementary analyses and plots.

Additionally, normalized abundance of all RNA species and the corresponding RNA kinetic rates are available in a supplementary Excel file.

Reviewer #1 (Remarks to the Author):

The authors greatly improved the manuscript. They appropriately answered all my comments. I strongly recommend publishing the revised manuscript as is. I am sure Nanodynamo technology will be a very useful tool for dissecting the RNA life cycle. Congratulations to the team.

Reviewer #1 (Remarks on code availability):

I cannot assess the code since I am not a specialist in bioinformatics.

Reviewer #2 (Remarks to the Author):

In the revised version the authors have addressed many of the comments and the manuscript has substantially improved. However, some major concerns remain.

1. The authors mention that they validated their 4sU model on an independent 4sU 24h labeled sample. It is unclear if they tested this sample against an independent control sample that was also not used for training. That would be important for an independent evaluation. Could they clarify?

2. In the revised version of the manuscript (ln 136) the authors mention that just 2 replicates are sufficient to have a performance remarkably close to the optimal one. In their response they mention that the use of an additional replicate (for a total of 2 replicates) reduced the correlation to the inferred measurement. In fact, for some of the rates the reduction was substantial which I agree indicates improvement of overfitting. However, these two observations seemingly contradict each other. Do the authors have a suggestion to the users regarding the number of replicates? Also, what is the rate concordance when using 1 or 2 replicates (please show the actual values instead of correlation)? Do the authors have any indication as to how a third replicate would affect the rates?

3. The authors mention that they now use the Maxwell RNA extraction approach to measure RNA yield. Were these measurements performed on the same samples used for library preparation and sequencing? Could the authors clarify? If they were not, it is unclear how these values can be used for normalization of the sequencing libraries when they are derived from different samples. Can the authors explain?

4. On the same topic of normalization. The authors mention they use the RNA yield/#cells as a crucial step to move from relative to absolute gene expression levels (ln 673) for each fraction. It is unclear to me how this methodology results in absolute gene expression levels and how it controls for the efficiency of the fraction separation. For example, assuming that the authors get 3 times more yield for the cytoplasmic fraction compared to the nuclear, does that mean that the actual RNA in the cytoplasm is 3 times more? Could the authors clarify? This could introduce substantial bias in the rate estimations downstream. Similarly, the same question applies for splitting the fraction yield to the different species. How do the authors control for the efficiency of the individual protocols e.g. polysome fractionation?

Reviewer #3 (Remarks to the Author):

The authors' response is very detailed and comprehensive. For my comments, the answers are mostly satisfactory. I still recommend that the authors discuss the potential limitations of this study in Discussion somewhere on points such as model's long-term validity, new mechanistic elements (?) that can be added to the modeling part.

Reviewer #3 (Remarks on code availability):

The code packages is quite comprehensive and should be enough for purposes such as reproducing the study.

Point by point response to Reviewers

Nanodynamo quantifies the dynamics of RNA metabolism and reveals extensive coupling between steps of the RNA life cycle

Lucia Coscujuela Tarrero, Valeria Famà, Giacomo D'Andrea, Simone Maestri, Anna de Polo, Stefano Biffo, Mattia Furlan, Mattia Pelizzola

Reviewer #1 (Remarks to the Author):

The authors greatly improved the manuscript. They appropriately answered all my comments. I strongly recommend publishing the revised manuscript as is. I am sure Nanodynamo technology will be a very useful tool for dissecting the RNA life cycle. Congratulations to the team.

We would like to thank the Reviewer for the positive assessment of our revised manuscript and for the constructive comments that markedly contributed to improving our study.

Reviewer #1 (Remarks on code availability):

I cannot assess the code since I am not a specialist in bioinformatics.

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Reviewer #2 (Remarks to the Author):

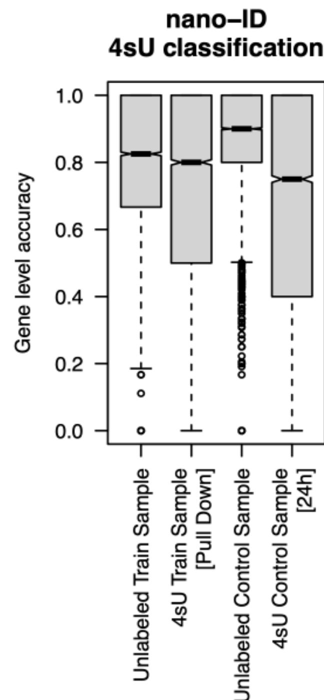
In the revised version the authors have addressed many of the comments and the manuscript has substantially improved. However, some major concerns remain.

We would like to thank the Reviewer for recognizing the improvements in the revised version of our manuscript and for the constructive feedback; a point-by-point response follows.

1. The authors mention that they validated their 4sU model on an independent 4sU 24h labeled sample. It is unclear if they tested this sample against an independent control sample that was also not used for training. That would be important for an independent evaluation. Could they clarify?

During the first stage of the manuscript revision, we tested the accuracy of our nano-ID instance on an independent 4sU fully-labelled sample, which was not used for training. This condition was chosen to directly address the Reviewer's concern regarding the applicability of the nano-ID framework in detecting 4sU instead of 5eU (the modified-base originally used by the developers of the method). However, we agree that the characterization of this neural network's performance would benefit from the analysis of an additional unlabeled dataset not involved in its training.
To address this, we generated a new sequencing run of untreated SUM159 total unlabelled RNA and processed the data using nano-ID, achieving remarkable performance (see "Unlabeled Control Sample" in the figure below). This observation, along with other pieces of

evidence presented in our first rebuttal letter and in the manuscript, further reassured us about the quality of our nascent RNA profiling.
We also agree that the quantification of nascent RNA is important for our analyses and acknowledge that there is room for improvement. As mentioned in our first rebuttal letter, we are exploring better alternatives to nano-ID to integrate into the Nanodynamo framework (e.g., RNAkinet).



Nascent RNA profiling accuracy in SUM159 cells. *Accuracy distributions, at single gene level, for unlabelled and fully labelled reads. Performance of a nano-ID instance for 4sU-containing reads detection; the "Train" samples were used for training while the others were not.*

2. In the revised version of the manuscript (ln 136) the authors mention that just 2 replicates are sufficient to have a performance remarkably close to the optimal one. In their response they mention that the use of an additional replicate (for a total of 2 replicates) reduced the correlation to the inferred measurement. In fact, for some of the rates the reduction was substantial which I agree indicates improvement of overfitting. However, these two observations seemingly contradict each other. Do the authors have a suggestion to the users regarding the number of replicates? Also, what is the rate concordance when using 1 or 2 replicates (please show the actual values instead of correlation)? Do the authors have any indication as to how a third replicate would affect the rates?

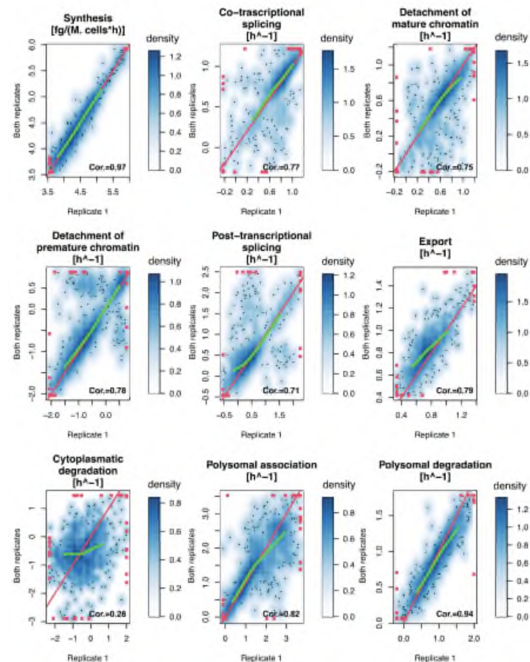
In the revised version of Nanodynamo, we followed the Reviewer's recommendations by shifting from an inference framework based on merging replicates to one that simultaneously exploits the information from both replicates to find the optimal set of rates. As the Reviewer mentioned, this approach significantly improved the overfitting, which was almost completely removed according to the analysis of our real dataset. This suggests that the joint analysis of only two replicates is sufficient to avoid the deleterious impact of overfitting.

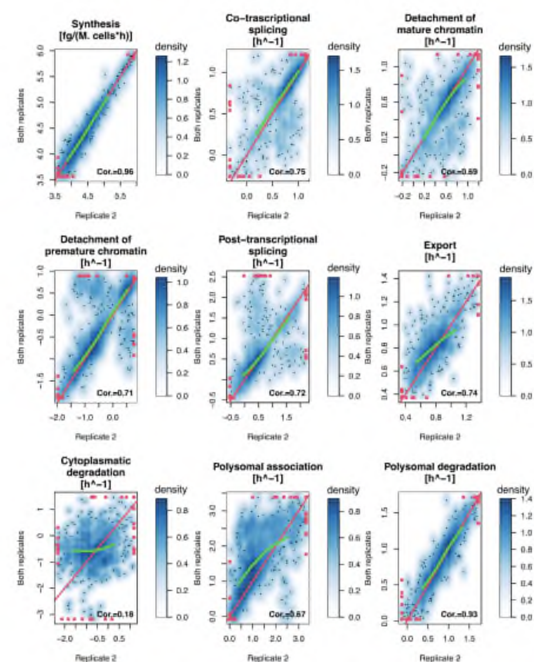
This observation does not contradict the results of our simulated data analysis which suggests a significant improvement in inference when moving from one to two replicates, and a mild improvement further increasing the number of replicates (Supplementary Figure 4B - rates k1, k3, k5, k6, and k9).
Notably, one of the rates that benefits most from a third replicate is k4, which is involved in defining nucleoplasmic premature RNA expression levels, the only RNA species still affected by overfitting. This suggests that a third replicate would specifically improve the determination of this kinetic rate, potentially reducing the residual overfitting of this RNA species.Furthermore, adding a third replicate would also slightly improve the overall inference performance, as indicated by the weak yet clear general trend observed in our simulated data.
However, given the cost and effort required to collect a third replicate for the entire dataset (which would require four additional dRNA-seq runs), our recommendation to users is that two replicates are a reasonable compromise.

This concept was stated in line 135, and is now further stressed in the *The Nanodynamo model, limitations and potential extensions* session of the manuscript (text highlighted in green here and in the revised manuscript accompanying this rebuttal).

"Similarly, the inference performance would benefit from the profiling of a higher number of replicates particularly for the rates k4, k7, and k8, as suggested by the modest yet clear trend observed in simulated datasets (Supplementary Figure 4). Clearly, the drawback of all these improvements is the significant increase in experimental costs; for this reason, we suggest the experimental design used in this study as a reasonable compromise."

Finally, in our revised manuscript, we investigated Nanodynamo reproducibility by comparing the kinetic rates obtained from the independent analysis of each replicate, and we reported both their actual values and correlations in Supplementary Figure 8. Following the Reviewer's suggestion, we extended this analysis by directly comparing these values against the rates obtained by the joint analysis of both replicates (see the figure below); the results are in line with the correlations reported in Supplementary Figure 8.





Untreated SUM159 reproducibility – Kinetic rates. *Smooth density scatterplots between the inferred kinetic rates profiled simultaneously on 2 biological replicates (Y-axes) and the counterparts profiled with either replicate 1 (left - X-axes) or replicate 2 (right - X-axes). For each scatterplot, we report the bisector line (red), the loess line (green), and the Spearman correlation coefficient. Red dots represent saturated points.*

3. The authors mention that they now use the Maxwell RNA extraction approach to measure RNA yield. Were these measurements performed on the same samples used for library preparation and sequencing? Could the authors clarify? If they were not, it is unclear how these values can be used for normalization of the sequencing libraries when they are derived from different samples. Can the authors explain?

In the revised version of the Nanodynamo framework, the quantification of RNA yield from a sample of interest is independent of the profiling of its transcriptome through dRNA-seq. However, this does not pose a problem for defining gene expression levels through sequencing data normalisation, as these two experiments characterise different yet essential aspects of the sample's transcriptional program: the absolute amount of RNA present in the cells (RNA yield), and the relative abundance of different RNA species (dRNA-seq data), respectively. The first quantity, together with the number of cells, defines the normalisation factor that allows moving from relative to absolute expression levels in the -omics data.

Importantly, the independent quantification of RNA yields from the dRNA-seq step provides greater flexibility in the experimental design, allowing for the easy acquisition of various replicated measurements of RNA yields without incurring additional sequencing costs and waiting time.
The only precaution necessary to jointly analyse these two pieces of data is ensuring that they are indeed representative of the same RNA pool. For this reason, in the previous revision round, we compared the quantification of chromatin-associated RNA (dRNA-seq;

formerly extracted with TRIzol) with the quantification of chromatin-associated RNA extracted via Maxwell (RNA used for the quantification of RNA yield), and demonstrated the absence of any systematic bias between the two data.
Notably, once this potential issue is disproved, any method for RNA yield quantification can be used to complement the collected sequencing data and improve the quantification of gene expression levels. This adaptability makes the Nanodynamo framework easily adjustable to technological advancements.

We stressed the independence of RNA yield profiling from direct RNA sequencing in the *Discussion* and *Counts normalization* sessions of the manuscript (text highlighted in green).

"All these potential extensions are feasible until the ODEs system parameters are globally identifiable, and the required RNA pools can be isolated for: dRNA-seq library preparation and RNA yield measurement. Noticeably, these two steps of the Nanodynamo framework are decoupled and they can be performed on independent samples providing more flexibility in the experimental design and allowing for the collection of various replicates of RNA yields without incurring additional sequencing costs and waiting time."

4. On the same topic of normalization. The authors mention they use the RNA yield/#cells as a crucial step to move from relative to absolute gene expression levels (ln 673) for each fraction. It is unclear to me how this methodology results in absolute gene expression levels and how it controls for the efficiency of the fraction separation. For example, assuming that the authors get 3 times more yield for the cytoplasmic fraction compared to the nuclear, does that mean that the actual RNA in the cytoplasm is 3 times more? Could the authors clarify? This could introduce substantial bias in the rate estimations downstream. Similarly, the same question applies for splitting the fraction yield to the different species. How do the authors control for the efficiency of the individual protocols e.g. polysome fractionation?

The Reviewer is correct in interpreting our normalisation approach, and in the reported example with a ratio of 3 between two nuclear fractions. We agree on the relevance of this aspect for the Nanodynamo inference framework, as well as the potential impact of RNA yield profiling across cellular fractions. Indeed, we extensively worked on RNA extraction (e.g., benchmarking different RNA extraction protocols) and gene expression profiling (e.g., decoupling RNA yield profiling from dRNA-seq, while checking their consistency through sequencing) to improve this aspect of our framework.
We also validated the efficacy of our fractionation protocol by monitoring the abundances of well-localised proteins across fractions (Supplementary Figure 6E) and the reproducibility of gene expression levels for all the RNA species profiled with Nanodynamo (Supplementary Figure 7). These tests reassured us about the quality of our experimental and computational approaches.

To the best of our knowledge, the method we followed for RNA yield profiling can be considered good practice in the field. Nevertheless, we acknowledge that a systematic bias associated with specific fractionation protocols - although not suggested by any evidence in our hands - cannot be completely excluded. This further supports the importance of Nanodynamo's flexibility in incorporating RNA yield qualifications provided by other techniques that might be developed in the future.

Importantly, such a bias would similarly affect all the investigated conditions, likely resulting in a minimal impact on our conclusions which are largely based on comparative analyses between conditions.

We mentioned this relevant point in the *Discussion* session of the manuscript (text highlighted in green):

"*All these potential extensions are feasible until the ODEs system parameters are globally identifiable, and the required RNA pools can be isolated for: dRNA-seq library preparation and RNA yield measurement. Noticeably, these two steps of the Nanodynamo framework are decoupled and they can be performed on independent samples providing more flexibility in the experimental design and allowing for the collection of various replicates of RNA yields without incurring additional sequencing costs and waiting time. This is particularly important because RNA yield quantification is crucial for inference purposes, and it can potentially introduce systematic biases affecting the absolute kinetic rates quantifications."*

Regarding the polysomal fractionation, polysomal profiles is a standard technology widely employed in our laboratories [Brina D et al, Nat Commun 2015; Oliveto S et al, Cancer Res 2018; Ricciardi S et al, Cell Metab 2018]. The preparation of libraries from polysomes is done according to quality standards that start from the analysis of the peaks [Brina D et al, Nat Commun 2015; Calamita P et al, PLoS Genet 2017], and include real time-analysis of individual mRNA targets from all fractions [Brina D et al, Nat Commun 2015; Ricciardi S et al, Cell Metab 2018; Gorrini C et al, Proc Natl Acad Sci 2005; Oberkersch RE et al, Dev Cell 2022]. Specifically, the ratio of polysomes to 80S is an indication of the degree of initiation of translation of cells. Samples in which this ratio is not consistent are always discarded. Individual fractions are then analysed for the presence of mRNAs that are not expected to change, such as actin [Gorrini C et al, Proc Natl Acad Sci 2005], although this requires prior validation that the target is not affected. All the references mentioned here are from previous studies of our collaborator Stefano Biffo, co-author in this study and expert on polysomal fractionation and RNA translation.

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Reviewer #3 (Remarks to the Author):

The authors' response is very detailed and comprehensive. For my comments, the answers are mostly satisfactory. I still recommend that the authors discuss the potential limitations of this study in Discussion somewhere on points such as model's long-term validity, new mechanistic elements (?) that can be added to the modeling part.

We appreciate the Reviewer's positive evaluation of our revised manuscript and the constructive feedback that has significantly enhanced our study.

Following the Reviewer's suggestion, we expanded our *Discussion* session with considerations about the extension of our model potentially including: a more detailed description of transcripts synthesis, additional cellular compartments, determinants of

transcriptional programs and their coordination (e.g. RNA modifications, RBPs, and TFs). We also discussed the relevance of RNA yield quantification, its potential impact on inference results, and the importance from this point of view of decoupling this step from dRNA-seq. Finally, we discussed the relevance of replicates and throughput in determining Nanodynamo inference performance with a focus on the experimental cost, an important limitation of our method, and the introduction of potential workarounds based on both Nanopore and Illumina RNA-seq technologies (text highlighted in green).

"The same perspective could be applied also to improve our characterization of RNA synthesis by explicitly modelling key steps of RNA polymerase activity[27] (e.g., initiation, pause-release, elongation, and termination). More generally, the Nanodynamo framework could be extended to include rates describing the transition of RNA molecules across a large set of states defined according to a specific feature of the transcripts (e.g., retention of intronic signal) and/or their localization. In this regard, we foresee an interesting extension of our model based on the isolation of biomolecular condensates (e.g., stress granules and P-bodies).

Moreover, we anticipate the possibility of extending the Nanodynamo framework to incorporate information about key determinants of gene expression programs and kinetic rates couplings, such as the level of RNA modifications, RBPs, and TFs. For the latter two classes of regulatory factors, gene expression levels and/or the rate of association with polysomes are potential proxies for those factors protein abundance. For example, the models of genes targeted by specific factors could be coupled with the equations describing those factors' life cycle.

All these potential extensions are feasible until the ODEs system parameters are globally identifiable, and the required RNA pools can be isolated for: dRNA-seq library preparation and RNA yield measurement. Noticeably, these two steps of the Nanodynamo framework are decoupled and they can be performed on independent samples providing more flexibility in the experimental design and allowing for the collection of various replicates of RNA yields without incurring additional sequencing costs and waiting time. This is particularly important because RNA yield quantification is crucial for inference purposes, and it can potentially introduce systematic biases affecting the absolute kinetic rates quantifications.

[...]

Similarly, the inference performance would benefit from the profiling of a higher number of replicates, as suggested by the modest yet clear trend observed in simulated datasets (Supplementary Figure 4). Clearly, the drawback of all these improvements is the significant increase in experimental costs; for this reason, we suggest the experimental design used in this study as a reasonable compromise.

We anticipate that an alternative and effective workaround to reduce the experimental cost of Nanodynamo would be shifting from the Nanopore to the Illumina RNA sequencing platform, leveraging protocols for the chemical conversion of incorporated nucleotides for nascent RNA profiling[17,21]. This would provide better control over sequencing depth (i.e., cost) and a higher ratio of detected genes per million sequenced bases. On the other hand,

this approach would not benefit from key features of long-read direct RNA-seq, such as the ability to better discriminate expressed isoforms and intronic signal, as well as the intrinsic profiling of important determinants of gene expression programs like RNA modifications and polyA tails."

Reviewer #3 (Remarks on code availability):

The code packages is quite comprehensive and should be enough for purposes such as reproducing the study.

Reviewer #2 (Remarks to the Author):

The authors have improved the manuscript and have addressed my concerns.

Reviewer #3 (Remarks to the Author):

The authors have added discussion and further improved the manuscript. I recommend acceptance of this manuscript.