nature portfolio

Peer Review File

Artificial cells with all-aqueous droplet-in-droplet structures for spatially separated transcription and translation

Corresponding Author: Professor Hiroyuki Noji

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I think this is an excellent manuscript. The authors built a mimic of eukaryotic cells through a combination of coacervates and aqueous phase separation. As with all systems, there are limitations, but in my opinion this is significant step forward in building cellular mimics. The nucleus was built with an intrinsically disordered protein (IDP), the cytoplasm was enriched in dextran and the extracellular space was enriched in PEG. Pegylated, heat-denatured beta-lactoglobulin was used to stabilize the separation of dextran and PEG.

The described system is highly contrived. T7 RNA Pol and DNA are both targeted to the IDP/nucleus phase through interactions with engineered tags (DNA was actually with a LacR-lacO system). The ribosome naturally partitioned to the dextran/cytoplasm phase. Transcription in the nucleus leads to the generation of RNA, which then equilibrates to the dextran/cytoplasm where it can be translated by the ribosome. The produced protein also possess a tag that keeps the protein stuck in the cytoplasm.

The data are clear. I see no problems with the interpretation. I am surprised that transcription proceeds efficiently in the IDP/nucleus. I would not have expected that immobilized T7RNA Pol and immobilized DNA would work well together, but the data show that they do.

I don't see the data in Figure 3e represented by the green line (-) DNA, w/ IDP droplet. The legend should indicate if that line is beneath another plotted line.

Also in Figure 3e, the authors should explain why protein expression was observed in the absence of the IDP/nucleus phase (orange line). This does make sense, of course. Expression would be expected, but I think it's worth explaining to the reader.

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I would suggest to the authors to include in the discussion section the limitations of the approach taken. Almost everything had to be specifically tagged, for example. Is that a limitation going forward? Would a lipid membrane between the dextran and PEG phases be useful or detrimental? I think this is an excellent manuscript. In my opinion, discussing the limitations would be valuable to the field.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This paper describes the construction of droplet-in-droplet compartments able to spatially segregate DNA transcription and RNA translation. These compartments are composed of an intrinsically-disordered proteins (IDP)-based condensate within a

dextran-in-PEG droplet. DNA transcription selectively occurs in the IDP condensate based on the rational addressing of enzymes using specific recognition tags. RNA translation is then performed in the outer dextran phase. These sequential events are compared to the segregation of DNA and RNA processing in cells.

The design of the system to ensure spatial segregation of DNA and RNA processing using recognition tags is well thought-out and elegant. However, I am less convinced by the extensive reliance on microscopy to characterize the transcription and translation steps. I would suggest additional experiments to be performed to back up the conclusions before the work can be accepted for Nature Communications.

1/ Characterization of transcription and translation
a) The authors extensively rely on confocal fluorescence microscopy to quantify the extent of DNA transcription and RNA translation. Since fluorescence signals can be affected both by the local environment of the fluorescent molecules and their partitioning in the different phases, complementary biochemical techniques should be implemented to characterize the yields, kinetics and efficiencies of the reactions in the different environments, e.g., RT-qPCR, western blotting…

b/ I would also suggest extracting line fluorescence intensity profiles from confocal fluorescence images to better characterize the partitioning of the different species and the reactions. Images on Fig. 4 appear to be saturated so the fluorescence intensities cannot be quantified.

c/ It would be informative to extract the kinetic traces for all the reactions until a plateau is reached. For instance, the transcription step is monitored over ca. 500 min (Figure 2g), reaching a plateau value. Yet, the translation step is only followed over 250 min (Figure 3e) without reaching a final plateau.

d/ About the localized translation in the outer DEX phase, the “calculated enrichment factor” is actually an exclusion factor since it is below 1.

e/ About the cross-talk assessment among droplets, how long do they maintain orthogonality?

2/About the experimental section:
a) It is not clear if the concentrations provided in the sample preparation are the final concentrations or the stock concentrations.

b) There is a lack of experimental details on the bench-top experiments. Did the centrifugation destabilized the microgel-stabilized DEX droplets? Where does the IDP phase end up? It there any experimental proof that the IDP phase is the denser phase?

3/ About the droplet-in-droplet formation:
a) Is there a relationship between the IDP droplet size and the DEX droplet size? Why some DEX droplets do not contain any IDP droplet?

b) On Figure 1, TRITC-dextran appears to be accumulated at the IDP surface. Why is so? Can this perturb the exchange of species between the IDP droplet and the DEX phase?

c) What is the permeability cut-off of the membrane at the DEX droplet surface? This is not characterized but yet critical to ensure nutrient supply.

4/ Minor points
a/ The last paragraph of the discussion is highly speculative.

b/ English should be revised throughout the manuscript.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
Review report for: Artificial cells with all-aqueous droplet-in-droplet structures for spatially separated transcription and translation

The study by Kanji Tomohara et al. designs artificial cells that mimic the compartmentalization of biochemical processes in biological cells. By creating droplet-in-droplet structures, they segregate transcription and translation processes, similar to the nucleus and cytosol in eukaryotic cells. These structures use intrinsically disordered proteins (IDPs) for inner coacervate droplets and aqueous two-phase systems (ATPS) for outer compartments, stabilized by colloidal emulsifiers. This setup enriches DNA and RNA polymerase for transcription inside the inner droplets, with translation occurring in the outer

droplets, enabling independent yet coupled cascade reactions. The modular and easily manufacturable design can be adapted for other enzymatic reactions, providing a practical tool for exploring complex artificial cell systems.

I enjoyed reading the manuscript and have only minor corrections needed before acceptance:

The fluorescence intensity of a dye measured with a confocal microscope is very compartment-dependent because of the partitioning and changes in the fluorescence quantum yield. The authors should correct for the changes in quantum yield by calibrating the fluorescence intensity of the dyes in the various compartments. For example, a known concentration of GFP in dextran, PEG, and the IDP-phases independently

"The addition of mRNA to express GFP (mStayGold QC2-6 FIQ36) resulted in comparable signal increases in both the whole mixture (IDP and Dex phases) and the supernatant Dex phase, suggesting that translation preferentially proceeds at the Dex phase rather than the IDP phase (Supplementary Figure 4e)."
I disagree with this statement. Given that the total volume fraction of the IDP phase is 1/1000 of the total volume, even if the activity would be 10X higher in the IDP phase, it would give no noticeable difference. The authors even stated earlier that the ribosomes do partition in the IDP, albeit poorly. Surely, there must be some activity.
This claim should be toned down. Ideally, the authors measure the activity in the spun-down phase.

Minor corrections:

Some spelling mistakes here and there:
"However, Dex droplets is prone to coalescence" should be: "However, Dex droplets are prone to coalescence"
"as assessed bay labeling either SZ2-tagged T7RNAP or non-tagged "
"as assessed by labeling either SZ2-tagged T7RNAP or non-tagged "

"cell-free translation components (i.e., 144 PURE frex 2.0 ΔT7RNAP). "
This requires a citation or clarification.

"the ratio of fluorescence in the"
"the ratio of fluorescence intensity in the

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I'm happy with the response to my comments and continue to find this manuscript to be of high quality. In my opinion, this manuscript will make a valuable contribution to the field. No further edits are requested.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
In their revised mnauscript, the authors have satisfactorily addressed my comments, significantly improving the robustness of their conclusions. I therefore support publication in Nature Communications.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have addressed all my concerns and I support publication of the current manuscript.

(Remarks on code availability)

Response to Reviewer comments [NCOMMS-24-29552]

We sincerely appreciate the Reviewers for their careful reading and constructive comments. We have addressed the referee's comments (***blue text***) with point-by-point responses (***black text***) and revised the manuscript. We believe that all suggestions from the reviewers have been adequately addressed and that these changes have significantly improved the novelty, readability, and clarity of our manuscript. Changes in the manuscript (main text and supplementary information) are highlighted in ***orange text***.

Reviewer #1 (Remarks to the Author):

I think this is an excellent manuscript. The authors built a mimic of eukaryotic cells through a combination of coacervates and aqueous phase separation. As with all systems, there are limitations, but in my opinion this is significant step forward in building cellular mimics. The nucleus was built with an intrinsically disordered protein (IDP), the cytoplasm was enriched in dextran and the extracellular space was enriched in PEG. Pegylated, heat-denatured beta-lactoglobulin was used to stabilize the separation of dextran and PEG.

The described system is highly contrived. T7 RNA Pol and DNA are both targeted to the IDP/nucleus phase through interactions with engineered tags (DNA was actually with a LacR-lacO system). The ribosome naturally partitioned to the dextran/cytoplasm phase. Transcription in the nucleus leads to the generation of RNA, which then equilibrates to the dextran/cytoplasm where it can be translated by the ribosome. The produced protein also possess a tag that keeps the protein stuck in the cytoplasm.

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1.1

I don't see the data in Figure 3e represented by the green line (-) DNA, w/ IDP droplet. The legend should indicate if that line is beneath another plotted line.

We appreciate your attention to detail. We have used distinctive marker styles to differentiate between overlapping lines: thin diamond markers for the green line ((-) DNA, w/ IDP), which lies behind, and small point markers for the red line ((-) DNA, w/o IDP) in front.

1.2

Also in Figure 3e, the authors should explain why protein expression was observed in the absence of the IDP/nucleus phase (orange line). This does make sense, of course. Expression would be expected, but I think it's worth explaining to the reader.

Thank you for the advice. We added the sentence below to explain this.
"The GFP signal from Dex droplets without the inner IDP droplets originated from gene expression from DNA in the Dex phase." (Lines 213-214)

1.3

In the text, it's stated "Before transcription-translation experiments, to confirm the partitioning of ribosomes, we labeled them with dye and observed their locations." I suggest indicating here how the ribosomes were labeled to make things easier for the reader.

We added the information on how we labeled ribosomes to the original sentence. "Before transcription-translation experiments, to confirm the partitioning of ribosomes, we labeled them using FL-NHS dye targeting primary amines in ribosomal proteins and observed their locations." (Lines 175-176)

1.4

I would suggest to the authors to include in the discussion section the limitations of the approach taken. Almost everything had to be specifically tagged, for example. Is that a limitation going forward? Would a lipid membrane between the dextran and PEG phases be useful or detrimental? I think this is an excellent manuscript. In my opinion, discussing the limitations would be valuable to the field.

Thank you for this valuable suggestion. There would indeed be trade-offs for introducing a lipid membrane between the dextran and PEG phases. While the lipid membrane could help ensure genotype-phenotype correlation without the need for tagging systems, it would compromise the simple droplet formation that is a key feature of this research.
A more critical limitation of this study, however, is the reduced protein yield in the cell-free expression system within this droplet-in-droplet structure compared to standard conditions. We discussed this point in a paragraph in the discussion section starting from Line 277.

Reviewer #2 (Remarks to the Author):

This paper describes the construction of droplet-in-droplet compartments able to spatially segregate DNA transcription and RNA translation. These compartments are composed of an intrinsically-disordered proteins (IDP)-based condensate within a dextran-in-PEG droplet. DNA transcription selectively occurs in the IDP condensate based on the rational addressing of enzymes using specific recognition tags. RNA translation is then performed in the outer dextran phase. These sequential events are compared to the segregation of DNA and RNA processing in cells.

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2.1a

1/ Characterization of transcription and translation

a) The authors extensively rely on confocal fluorescence microscopy to quantify the extent of DNA transcription and RNA translation. Since fluorescence signals can be affected both by the local environment of the fluorescent molecules and their partitioning in the different phases, complementary biochemical techniques should be implemented to characterize the yields, kinetics and efficiencies of the reactions in the different environments, e.g., RT-qPCR, western blotting…

We appreciate this valuable feedback regarding potential artifacts in fluorescence microscopy.

The quantification of fluorescence signals can be affected by both non-uniform dye distribution and variations in quantum yield across different phases. These factors must be considered when comparing fluorescence intensities between distinct phases. Since three transcription/translation components (RNAP in Figure 2c, DNA in Figure 2e, ribosome in Figure 3c) were labeled by fluorescein (FL) and compared for their localization in the inner IDP-rich phase and the outer Dex-rich phase, we have included a new section in the supplementary discussions titled "Fluorescence intensity of fluorescein in polymer-rich solutions." The accompanying Supplementary Figure 2 demonstrates that fluorescein's fluorescence intensity remains constant across different polymer concentrations, validating our comparative analyses.

We also quantified RNA levels in the bench-top experiment using RT-qPCR (Supplementary Figure 5e) by collecting samples at multiple time points (Lines 167-168). These results aligned with our real-time transcription measurements obtained using molecular beacons (MB) (Supplementary Figure 5c vs. 5e). We switched from the fluorescent aptamer system to the MB method since MB allows for more accurate measurements.

To assess translation activity under polymer-free conditions, we initially considered using Western blotting or similar gel electrophoresis-based methods for protein quantification. However, these techniques lacked sufficient precision and reproducibility to measure the low concentrations of expressed proteins. Instead, we drew a calibration curve by adding known concentrations of purified GFP to bench-top experiments, comparing samples containing IDP + Dex versus Dex alone (Supplementary Figure 5f). The calibration curves for both conditions showed excellent linearity (R^2 = 0.999) and were nearly identical. By converting GFP expression levels from relative fluorescence units (RFU) to absolute concentrations (nM) through this calibration, we confirmed that the presence of IDP continued to influence transcription-translation activity when DNA was added (blue lines in Supplementary Figure 5d). In contrast, when RNA was added (orange lines in Supplementary Figure 5d), expression levels were similar between both sampling conditions, suggesting that the Dex phase produced most of the GFP.

2.1b

b/ I would also suggest extracting line fluorescence intensity profiles from confocal fluorescence images to better characterize the partitioning of the different species and the reactions. Images on Fig. 4 appear to be saturated so the fluorescence intensities cannot be quantified.

We have expanded our analysis by incorporating line profiles to demonstrate the spatial distribution of three key components: RNAP (Figure 2b), DNA (Figure 2d), and ribosomes (Figure 3b). Additional line profiles showing the distribution of transcription and translation products are provided in Supplementary Figure 7. Furthermore, we have addressed the image quality issues in the previous Figure 4, where improper brightness and contrast adjustments had resulted in pixel saturation. We have replaced these data with new measurements collected over an extended time period, which also addresses the subsequent points raised below.

2.1c

c/ It would be informative to extract the kinetic traces for all the reactions until a plateau is

reached. For instance, the transcription step is monitored over ca. 500 min (Figure 2g), reaching a plateau value. Yet, the translation step is only followed over 250 min (Figure 3e) without reaching a final plateau.

In response to this feedback, we extended our imaging of transcription-translation activity to over 700 minutes (Figure 3e). The reaction reached a plateau between 400 and 500 minutes, providing a complete view of the kinetics through the endpoint. Accordingly, we added an explanation about this point: “Figure 3e shows that the fluorescence signal from SNAP-GFP continuously increased at the Dex phase until reaching the plateau at around 400 min, while no signal increase was confirmed if the template DNA was not added.” (Lines 206-208)

2.1d

d/ About the localized translation in the outer DEX phase, the “calculated enrichment factor” is actually an exclusion factor since it is below 1.

We appreciate this accurate observation regarding terminology. Indeed, since the ratio is less than 1, "enrichment factor" was a misnomer. We have revised the plot label to "x-fold signal from Dex to IDP phase" to more accurately represent the signal ratio between phases.

2.1e

e/ About the cross-talk assessment among droplets, how long do they maintain orthogonality?

To assess the stability of droplet orthogonality, we continued monitoring the system after transcription-translation activity reached its plateau. The orthogonality remained stable throughout our extended observation period of 800 minutes, showing no significant cross-talk between droplets (Supplementary Figures 8b, 8c). This finding is also discussed in the manuscript (Lines 246-248). Thank you for your detailed comments. Your suggestions have helped improve the quality of our manuscript significantly.

2.2a

2/About the experimental section:

a) It is not clear if the concentrations provided in the sample preparation are the final concentrations or the stock concentrations.

Thank you for bringing this point to our attention. To clarify the concentration reporting, we

have added the following statement: " Unless otherwise noted, concentrations are given in parentheses as final concentrations." (Lines 532-533)

2.2b

b) There is a lack of experimental details on the bench-top experiments. Did the centrifugation destabilized the microgel-stabilized DEX droplets? Where does the IDP phase end up? It there any experimental proof that the IDP phase is the denser phase?

Thank you for these important questions about the bench-top experimental setup. We have revised the paragraph to clarify the purpose and experimental details in the manuscript (Lines 156-164). We have also provided detailed information about the reaction composition in the supplementary methods (Lines 76-81 in the supplementary information). To address the specific questions about our bench-top experiments, we would like to clarify the key experimental details:

For bench-top experiments, we simplified the system to include only Dex and IDP phases, omitting PEG and colloidal emulsifiers. This modification was necessary because in the complete PEG-Dex-IDP three-phase system, both the Dex phase and the smaller IDP phase become too minute for reliable sampling after centrifugation-induced macro phase separation. While scaling up the reaction volume to 3-10 mL could theoretically address this issue, the cost of the PURE system at such scales would be prohibitive for conducting all necessary conditions in triplicate.

To verify the density-dependent sedimentation of the IDP phase, we conducted visualization experiments using BFP-fused IDP. As demonstrated in Supplementary Figure 5a (rightmost image), blue light illumination revealed a distinct bright blue spot at the tube bottom, confirming that the IDP phase indeed sediments due to its higher density.

2.3a

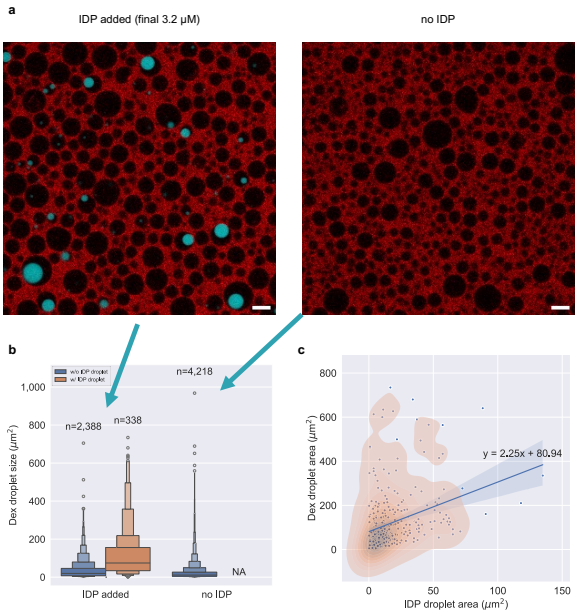
3/ About the droplet-in-droplet formation:

a) Is there a relationship between the IDP droplet size and the DEX droplet size? Why some DEX droplets do not contain any IDP droplet?

Thank you for this insightful question about the droplet-in-droplet architecture. Let us explain both the formation process and resulting size distributions:

The droplet-in-droplet structures form through a sequential process: first, IDP condensates are generated by mixing IDP with PEG. Subsequently, Dex is added to create outer droplets that can encapsulate these pre-formed IDP condensates. Given that we observe more Dex

droplets (n=2,388) than IDP condensates (n=338), the encapsulation follows a stochastic distribution, naturally resulting in some Dex droplets remaining empty (panel **b** in the figure below). Our analysis revealed two key size relationships. First, Dex droplets containing IDP condensates were consistently larger than empty Dex droplets. Second, we observed a positive correlation between IDP condensate size and their surrounding Dex droplet size (panel **c** in the figure). These findings suggest that larger Dex droplets have a higher probability of encapsulating IDP condensates, likely due to their larger volume.



2.3b

b) On Figure 1, TRITC-dextran appears to be accumulated at the IDP surface. Why is so? Can this perturb the exchange of species between the IDP droplet and the DEX phase?

Thank you for this astute observation regarding TRITC-dextran accumulation at the IDP surface. We initially hypothesized that this accumulation resulted from an affinity between IDP and TRITC, as similar rhodamine-based dyes have been reported to enrich within IDPs[1]. This affinity could potentially arise from pi-pi stacking or hydrophobic interactions between the dye and the IDP.

To address your concern about the potential perturbation of species exchange, we conducted additional observations in the presence of the PURE system, which more accurately represents the conditions during transcription and translation. Interestingly, under these conditions, we no longer observed TRITC-Dex accumulation around the IDP phase. We speculate that components of the PURE system, such as aromatic or hydrophobic amino acids, may preferentially interact with the IDP, displacing TRITC-Dex.

Given these findings, we have updated Figure 1b to show images acquired in the presence

of the PURE system, which we believe more accurately represents the experimental conditions relevant to our transcription and translation studies.

2.3c

c) What is the permeability cut-off of the membrane at the DEX droplet surface? This is not characterized but yet critical to ensure nutrient supply.

Unlike classical membranes formed by amphiphilic molecules, colloidal emulsifiers used in this study create interfaces that allow macromolecules to pass through. Douliez *et al.*[2] demonstrated that Dex droplets surrounded by colloidal emulsifiers could uptake 150 kDa polymers. Similarly, our results in Supplementary Figure 6 show that SNAP-GFP (50 kDa) diffused from the Dex phase to the continuous PEG phase in the absence of BG-Dex.

The majority of PURE components (nutrients to be supplied) are less than 150 kDa (cf. Figure 1c in Mizuuchi *et al.*[3]). The ribosome (4 MDa) represents the only exception, which we demonstrated to be enriched in the Dex droplet.

For the continuous transcription and translation enabled by the supplement of nutrients (PURE components), we would prioritize exchanging the energy source (creatine phosphates, nucleotide triphosphates, and amino acids) to extend the lifespan of the system. This is because these small molecules (<1 kDa) are consumable, while enzymatic components remain reusable. These energy sources should exchange more efficiently than larger components due to their size. We have added this perspective to our discussion section (Lines 288-295), though detailed experimental verification remains beyond the scope of this paper. We plan to investigate this aspect in future studies. We appreciate the reviewer's insightful suggestion of investigating nutrient exchange dynamics, which we look forward to exploring in our future studies.

2.4a

4/ Minor points

a/ The last paragraph of the discussion is highly speculative.

We thank the reviewer for the comment. We have addressed this concern by removing speculative content about spatiotemporal control. The revised paragraph now focuses on the potential for studying transcription-translation interactions, interference, and optimization in our system.

2.4b

b/ English should be revised throughout the manuscript.

Thank you for your feedback regarding the language in our manuscript. We have carefully reviewed and revised the text to improve clarity and readability throughout.
If you have identified any specific areas that require further attention, we would greatly appreciate your detailed feedback.

Reviewer #3 (Remarks to the Author):

Review report for: Artificial cells with all-aqueous droplet-in-droplet structures for spatially separated transcription and translation

The study by Kanji Tomohara et al. designs artificial cells that mimic the compartmentalization of biochemical processes in biological cells. By creating droplet-in-droplet structures, they segregate transcription and translation processes, similar to the nucleus and cytosol in eukaryotic cells. These structures use intrinsically disordered proteins (IDPs) for inner coacervate droplets and aqueous two-phase systems (ATPS) for outer compartments, stabilized by colloidal emulsifiers. This setup enriches DNA and RNA polymerase for transcription inside the inner droplets, with translation occurring in the outer droplets, enabling independent yet coupled cascade reactions. The modular and easily manufacturable design can be adapted for other enzymatic reactions, providing a practical tool for exploring complex artificial cell systems.

I enjoyed reading the manuscript and have only minor corrections needed before acceptance:

3.1

The fluorescence intensity of a dye measured with a confocal microscope is very compartment-dependent because of the partitioning and changes in the fluorescence quantum yield. The authors should correct for the changes in quantum yield by calibrating the fluorescence intensity of the dyes in the various compartments. For example, a known concentration of GFP in dextran, PEG, and the IDP-phases independently

Thank you for this crucial observation regarding the compartment-dependent nature of fluorescence intensity measurements in confocal microscopy. We fully agree that both partitioning and changes in fluorescence quantum yield can significantly impact these measurements.

In response to your suggestion, we have conducted a comprehensive calibration of fluorescein (FL) fluorescence intensity across the various compartments in our system. We focused on FL as it was used for quantitative comparisons of transcription and translation component localization (T7RNAP, DNA, and ribosomes) between phases.

Our results, presented in Supplementary Figure 2a, demonstrate that neither 10 wt% Dex nor 8 wt% PEG significantly alters FL fluorescence intensity. Similarly, Supplementary Figure 2b shows that 13.6 μM IDP does not measurably affect FL fluorescence. This IDP concentration represents the highest achievable without inducing phase separation at ambient temperature, a limitation we overcame by adding ATP, known to act as a hydrotrope in solubilizing IDP condensates[4].

These calibrations provide crucial validation for our quantitative comparisons across phases, ensuring that observed differences in fluorescence intensities accurately reflect variations in component concentrations rather than phase-specific alterations in dye behavior.

Furthermore, we conducted a calibration of GFP fluorescence for our bench-top experiments, comparing IDP + Dex and Dex-only conditions (Supplementary Figure 5f). The calibration curves for both sampling conditions were nearly identical, indicating that the GFP signal is not significantly biased between these two conditions. We utilized these calibration curves to convert GFP measurements from relative fluorescence units (RFU) to absolute concentrations (nM), enabling more precise quantitative analyses of GFP expression levels.

3.2

The addition of mRNA to express GFP (mStayGold QC2-6 FIQ36) resulted in comparable signal increases in both the whole mixture (IDP and Dex phases) and the supernatant Dex phase, suggesting that translation preferentially proceeds at the Dex phase rather than the IDP phase (Supplementary Figure 4e).”

I disagree with this statement. Given that the total volume fraction of the IDP phase is 1/1000 of the total volume, even if the activity would be 10X higher in the IDP phase, it would give no noticeable difference. The authors even stated earlier that the ribosomes do partition in the IDP, albeit poorly. Surely, there must be some activity.

This claim should be toned down. Ideally, the authors measure the activity in the spun-down phase.

Thank you for your insightful feedback. Regarding your suggestion to measure activity in the spun-down IDP phase, we face a technical limitation. As shown in the rightmost image of Supplementary Figure 5a, the IDP phase volume is extremely small, making direct sampling challenging. Scaling up the total volume to overcome this issue is economically unfeasible

due to the high cost of the PURE system components.

In light of these considerations, we have revised our main text to reflect our findings more accurately (Lines 187-189): "This result suggests that the yield of expressed GFP is predominantly from the Dex phase rather than the IDP phase (Supplementary Figure 5d)." This rephrasing acknowledges the possibility of activity in the IDP phase while still conveying our primary observation.

We appreciate your valuable critique, as it has helped us refine our interpretation and presentation of the data.

3.3

Minor corrections:

Some spelling mistakes here and there:

“However, Dex droplets is prone to coalescence” should be: “However, Dex droplets are prone to coalescence”

“as assessed bay labeling either SZ2-tagged T7RNAP or non-tagged “

“as assessed by labeling either SZ2-tagged T7RNAP or non-tagged “

“cell-free translation components (i.e., 144 PURE frex 2.0 ΔT7RNAP). “

This requires a citation or clarification.

“the ratio of fluorescence in the”

“the ratio of fluorescence intensity in the

Thank you for your feedback. We have addressed all the points you raised and made the necessary corrections in the main text. These revisions have improved the clarity and accuracy of our paper. We appreciate your thorough review.

References:

1. Schuster, B. S. *et al.* Controllable protein phase separation and modular recruitment to form responsive membraneless organelles. *Nat. Commun.* **9**, 2985 (2018).

2. Douliez, J.-P. *et al.* Preparation of swellable hydrogel-containing colloidosomes from aqueous two-phase Pickering emulsion droplets. *Angew. Chem. Weinheim Bergstr. Ger.*

130, 7906–7910 (2018).

3. Mizuuchi, R. & Ichihashi, N. Translation-coupled RNA replication and parasitic replicators in membrane-free compartments. *Chem. Commun.* **56**, 13453–13456 (2020).

4. Patel, A. *et al.* ATP as a biological hydrotrope. *Science* **356**, 753–756 (2017).