nature portfolio

Peer Review File

Autonomous Ribosome biogenesis in vitro

Corresponding Author: Professor Wataru Aoki

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

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Kozaka et al.'s paper claims that functional reconstitution of the ribosome was achieved in vitro using E. coli S150 extract. Although the conclusions of this paper are significant and interesting, the following points need to be addressed for publication in Nature Communications:

Major points:

1. In Fig S8A and FigS12, the authors claim that newly synthesized bL35 and bL36 could not be identified by MS when LSU was reconstituted. However, the authors only mention that MS detection is difficult without providing sufficient explanation for the cause. To prove that reconstitution of LSU is successful, it is necessary to show how the activity of LSU changes when bL35, bL36, or both ribosomal protein genes are omitted, compared to when all 33 genes are included, and to analyze and discuss these results. The important points are whether bL35 and bL36 are sufficiently expressed in S150, and whether bL35 and/or bL36 are necessary for the LSU activity.

Minor points:

2. P2, L48: The term “r-protein” is not a very common abbreviation, so an explanation is needed.

3. P3 L80: It is necessary to explain more specifically what is included in E. coli S150.

4. P4 L124 and Fig S1A: Fig S1A appears to be an experiment conducted in the absence of Spectinomycin, but this is not explained, making the difference from Fig 1F unclear.

5. P4 L134: The meanings of "w" and "w/o" in Fig S1D should be indicated not only in the legend but also within the figure.

6. P6 L204, L206: Fig S6C and Fig S6D both appear to be standard curves used to estimate the concentration of synthesized SSUs. Why do the standard curves differ depending on the conditions?

7. P9 L288: No signal is detected under double-antibiotic conditions. Is this for the bulk assay or the droplet assay? If it is for the bulk assay, is it also difficult to detect in the droplet assay?

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
In their manuscript "Reconstitution of ribosome biogenesis in vitro", Kosaka et al. demonstrate that autonomous ribosome biogenesis, including complete rRNA and ribosomal protein synthesis, can be established in bacterial extracts. The authors achieve this by designing a translation system using an orthogonal SD/ASD pair on the mRNA and 16S rRNA, respectively, and subjecting this system to an in vitro translation assay within femtoliter droplets coupled to deep learning-based automated femtoliter droplet assays. By thoroughly evaluating this system under a wide range of conditions, Kosaka et al.

comprehensively and conclusively demonstrate that both large and small ribosomal subunits can be functionally generated in vitro, individually and in combination. This is the first time that complete biogenesis from self-synthesised components has been achieved in a cell-free system and is an important contribution to the field. I recommend acceptance of the manuscript subject to the authors addressing the following comments:

1. lines 108-111:
Please mention that sfGFP expression was initially used as a reporter assay. It is not mentioned in the main text, only in the figure caption.

2. lines 122-124 & Fig. 1E/F:
Based on the text and data presented in Fig. 1F, it seems like that the WT-ASD 16S rRNA also contains the SpcR mutation. However, in Fig. 1E, this mutation is only depicted on the SSU with the oASD 16S rRNA. In combination, this might be easily misunderstood and confuse some readers, as to where the SpcR mutation is present, or respectively, not.

3. lines 130 – 132
Please be a bit more specific on what exactly has been additionally tested in Fig. S1C.

4. Fig. S3:
The brightness setting for the bright-field images, and the font size in the black boxes, make it somewhat difficult to see the droplets and the text in this figure, respectively.

5. Fig. S6C/D and S7C:
Some form of indication as to where the values for the in vitro reconstituted ribosomes would be on the standard curve would be appreciated. Ideally, by also showing time-resolved fluorescence changes for these reactions as individual panels.

6. lines 197 – 198: Maybe the authors could elaborate a bit more as to why they think that a decrease in DNA concentration rather than an increase improves SSU biogenesis.

7. The authors should briefly mention in their conclusions that a huge increase in ribosome self-synthesis will be required to achieve self-replication in a synthetic system such as an artificial cell. At present, ribosome yields are in the low nM range, which is probably 2-3 orders of magnitude below the concentrations required for autocatalytic replication.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
Kosaka et al. describe the development of a procedure to assemble "artificial" ribosomal subunits in vitro using purified E. coli ribosomes. In their cell-free assay, they produce ribosomal proteins for the small and large subunits, which interact with ribosomal RNA produced in the same reaction by T7 RNA polymerase. By reengineering the anti-Shine-Dalgarno sequence of their "artificial" small subunit, a reporter mRNA with a corresponding Shine-Dalgarno sequence can only engage with the "artificial" small subunit. Additionally, the authors develop a highly sensitive femtoliter translation assay, encapsulating the reaction solution in micron-sized oil droplets, allowing for quick fluorescence-based assessment of the yield of "artificial ribosomes."

Major Points:
1. Lack of Clear Motivation and Goal: The manuscript does not clearly convey the motivation and goals of the study. It is unclear what this system is needed for and what advantages it offers over existing methods. The authors need to articulate the unique value of their approach.
2. Neglect of Ribosome Assembly History: The manuscript fails to acknowledge over 50 years of ribosome assembly research:
o Nomura and Nierhaus established the in vitro reconstitution of E. coli small (Traub and Nomura, PNAS, 1968) and large ribosomal
subunits (Nierhaus and Dohme, PNAS, 1974).
o Jewett et al. developed the Integrated Synthesis, Assembly, and Translation (IsAT) system, utilizing ribosomal proteins present in
cell extracts with transcribed rRNA (Jewett et al., MolSystems Biology, 2013).
o The Mankin group generated RiboT, an orthogonal translation system with engineered rRNA, that results in an rRNA where 16S and
23S and 5S rRNA are covalently linked ensuring full orthogonality (Orelle et al., Nature, 2015; Aleksashin et al., Nat Comms, 2019).

Although the authors cite some of these studies, they do not sufficiently acknowledge that powerful in vitro ribosome assembly assays already exist and have provided deep insights into ribosome assembly, some with near-atomic resolution, especially when combined with cryo-EM analyses (Nikolay et al., Mol Cell, 2018; Duss et al., Cell, 2019; Rodgers et al.,

Cell, 2019; Qin et al., Nat Comms, 2023; Dong et al., NAR, 2023; Dong et al., NAR, 2024).

3. Problematic Use of "Reconstitution": The term "reconstitution" is problematic because it has been traditionally used to describe the successful assembly of small and large ribosomal subunits using purified RNA and protein components from cells, as done by Nomura and Nierhaus. The authors should consider rephrasing (e.g., "autonomous" or "integrated") and clearly describe how their method is superior to classical in vitro ribosome assembly assays.

4. Unclear Purpose of In Vitro Transcription/Translation: While the authors aim to establish a system where both rRNA and all 54 ribosomal proteins are synthesized in the same in vitro reaction, it is unclear why this is necessary and what advantages it provides over other methods. The manuscript should explain the purpose and benefits of in vitro transcription/translation of all ribosomal proteins.

5. Concerns About Using T7 RNA Polymerase: If the goal is to create a system that mimics in vivo conditions more closely, the use of T7 RNA polymerase is problematic. The Nierhaus group (Lewicki et al., JMB, 1993; Iskakova et al., NAR, 2006) and others have shown that the transcription speed of T7 RNA polymerase is at least six times higher than that of E. coli RNA polymerase, which could have a significant artificial impact on the ribosome assembly process.

In conclusion, while the study presents a potentially interesting approach to method development, it suffers from several critical issues. These include a lack of clarity regarding its motivation and goals, an insufficient acknowledgment of prior research, and concerns about the methodology. Given these shortcomings, the manuscript may require substantial editing and be more suited to a journal focused on method development rather than Nature Communications.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Kozaka et al claims that functional in vitro biogenesis of the ribosome was achieved using E. coli S150 extract. The revised manuscript appropriately addresses the reviewers' comments and is now suitable for publication in Nature Communications.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my comments and I am in favour of publishing the manuscript in its current form. This is a very nice paper.
However, I would like to add that the new title is a bit short in my opinion and that it would be clearer to distinguish it from iSAT if it read something like "Complete autonomous ribosome biogenesis" in vitro or similar. But I guess that is more a matter of personal opinion.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have thoroughly addressed my previous concerns. By thoughtfully incorporating the constructive feedback from all reviewers, they have produced a revised version of the manuscript, with substantial improvements in clarity, depth, and methodological robustness.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

RESPONSE LETTER

We sincerely thank you for your valuable comments, which have helped us improve our manuscript. We also appreciate the time and effort you invested in providing insightful feedback. The manuscript has been carefully revised to incorporate your suggestions.

Referee #1:

Comment 1

Kozaka et al.'s paper claims that functional reconstitution of the ribosome was achieved in vitro using E. coli S150 extract. Although the conclusions of this paper are significant and interesting, the following points need to be addressed for publication in Nature Communications:

Response: We are grateful for your positive comments regarding our research. We have reviewed your comments and revised the manuscript accordingly. Our point-by-point responses are provided below.

Comment 2

In Fig S8A and FigS12, the authors claim that newly synthesized bL35 and bL36 could not be identified by MS when LSU was reconstituted. However, the authors only mention that MS detection is difficult without providing sufficient explanation for the cause. To prove that reconstitution of LSU is successful, it is necessary to show how the activity of LSU changes when bL35, bL36, or both ribosomal protein genes are omitted, compared to when all 33 genes are included, and to analyze and discuss these results. The important points are whether bL35 and bL36 are sufficiently expressed in S150, and whether bL35 and/or bL36 are necessary for the LSU activity.

Response: Thank you for emphasizing the need to explain in more detail why bL35 and bL36 are challenging targets for proteomics and for suggesting the investigation of their expression levels in the S150 cell extract.

The difficultly in identifying bL35 and bL36 via mass spectrometry stems from their small size (65 and 38 amino acids, respectively) and their basic nature. During mass spectroscopy pretreatment, proteins are purified by precipitation, and small proteins tend to precipitate with lower efficiency. Additionally, mass spectrometry relies on peptides produced via trypsin digestion, and small, basic proteins often contain fewer peptides with high ionization efficiency. Notably, the George Church group also reported that bL35 and bL36 are difficult targets for mass spectrometry (PMID: 28330337).

Regarding your comment on whether bL35 and bL36 are sufficiently expressed in the S150 cell extract, we cannot use ribosomal translational activity as an indicator because bL35 and bL36 are not required for ribosome function (PMID: 16738554). Instead, we used the FluoroTect™ GreenLys *in vitro* Translation Labeling System (Promega), which contains lysine-charged tRNA labeled with the fluorophore BODIPY for specific labeling of nascent proteins. Using this system, we found that bL35 and bL36 were efficiently expressed in the S150 extract.

Changes: We revised the manuscript as follows:

- Revised the main text to explain why bL35 and bL36 are challenging targets for mass spectrometry **(lines 264–270 in the revised manuscript)**
- Revised the main text and **Fig. S8** to show that bL35 and bL36 were efficiently expressed in the S150 extract **(lines 271–273, 491–494, and 1301–1308 in the revised manuscript)**

Comment 3

P2, L48: The term “r-protein” is not a very common abbreviation, so an explanation is needed.

Response: Thank you for highlighting the missing explanation for this abbreviation. We have revised the main text to clarify that r-proteins are ribosomal proteins.

Changes: The revised sentence now reads: “In *Escherichia coli*, three rRNAs (16S, 23S,

and 5S) are transcribed by RNA polymerase, and 54 ribosomal proteins (r-proteins) are recursively synthesized by preexisting ribosomes as structural components [3,4].” **(lines 55–57 in the revised manuscript)**

Comment 4

P3 L80: It is necessary to explain more specifically what is included in E. coli S150.

Response: Thank you for identifying where our explanation was insufficient. We have added details regarding the components in the optimized *E. coli* S150 cell extract.

Changes: The revised sentence now reads: “Specifically, the optimized *E. coli* S150 cell extract contains the soluble *E. coli* proteome, including dozens of accessory factors for ribosome biogenesis [3,4], physiological ions abundant in the cytoplasm (magnesium glutamate and potassium glutamate) [28,38], polyamines important for transcription and translation (spermidine and putrescine) [39], and a reducing agent (DTT).” **(lines 92–97 in the revised manuscript)**

Comment 5

P4 L124 and Fig S1A: Fig S1A appears to be an experiment conducted in the absence of Spectinomycin, but this is not explained, making the difference from Fig 1F unclear.

Response: Thank you for pointing out that the insufficient method description. As you pointed out, **Fig. S1A** shows an experiment without spectinomycin to confirm the functionality of the prepared cell extracts.

Changes: The revised sentence now reads: “The experiment was conducted without spectinomycin to assess the functionality of the cell extracts.” **(lines 1137–1138 in the revised manuscript)**

Comment 6

P4 L134: The meanings of "w" and "w/o" in Fig S1D should be indicated not only in the legend but also within the figure.

Response: Thanks for noting the unclear part of the figure.

Changes: We revised **Fig. S1D** to clarify the meanings of "w" and "w/o" in the figure. **(Fig. S1D in the revised manuscript)**

Comment 7

P6 L204, L206: Fig S6C and Fig S6D both appear to be standard curves used to estimate the concentration of synthesized SSUs. Why do the standard curves differ depending on the conditions?

Response: Thank you for indicating the insufficient explanation of the figures. The figures depict two types of assembly experiments: the autonomous assembly reported in this study and the nonautonomous iSAT assembly (PMID: 23799452). These experiments differ in key ways. In the autonomous assembly, r-protein genes are expressed in the cell extract, whereas in the nonautonomous iSAT assembly, purified r-proteins are added exogenously. Therefore, we generated two standard curves to reflect these procedural differences.

Changes: We have revised the manuscript to explain why two standard curves were generated. **(lines 1240–1242 and 1252–1254 in the revised manuscript)**

Comment 8

P9 L288: No signal is detected under double-antibiotic conditions. Is this for the bulk assay or the droplet assay? If it is for the bulk assay, is it also difficult to detect in the droplet assay?

Response: Thank you for raising this important point. We used the bulk assay and did not attempt to detect nascent artificial ribosome translational activity via the droplet assay in **Fig. 5B**. Notably, detecting nascent artificial ribosome translational activity is challenging, even with the droplet assay. Given that SSU and LSU interact randomly, and that several nM of nascent artificial SSU and LSU are produced in the presence of 80 nM native ribosomes, the estimated concentration of nascent artificial ribosomes is less than single-digit pM, below the droplet assay's detection limit **(Fig. 2B)**.

Changes: We revised our manuscript to reflect the above discussion as follows:

- Clarified in the figure legend that the bulk assay results are shown in **Fig. 5B**. **(lines 1110–1111 in the revised manuscript)**
- Revised the main text to reflect the above discussion. **(lines 328–333 in the revised manuscript)**

Finally, we thank you again for your time, effort, and insightful comments. Revising the manuscript based on your feedback has strengthened the clarity and logic of our research.

Referee #2:

Comment 1

In their manuscript "Reconstitution of ribosome biogenesis in vitro", Kosaka et al. demonstrate that autonomous ribosome biogenesis, including complete rRNA and ribosomal protein synthesis, can be established in bacterial extracts. The authors achieve this by designing a translation system using an orthogonal SD/ASD pair on the mRNA and 16S rRNA, respectively, and subjecting this system to an in vitro translation assay within femtoliter droplets coupled to deep learning-based automated femtoliter droplet assays. By thoroughly evaluating this system under a wide range of conditions, Kosaka et al. comprehensively and conclusively demonstrate that both large and small ribosomal subunits can be functionally generated in vitro, individually and in combination. This is the first time that complete biogenesis from self-synthesised components has been achieved in a cell-free system and is an important contribution to the field. I recommend acceptance of the manuscript subject to the authors addressing the following comments:

Response: We thank you for your positive comments on our research. We have carefully reviewed your feedback and revised the manuscript accordingly. Below are our point-by-point responses.

Comment 2

lines 108-111:
Please mention that sfGFP expression was initially used as a reporter assay. It is not mentioned in the main text, only in the figure caption.

Response: Thank you for noting the insufficient description. We have revised the main text to clarify that sfGFP was initially used as a reporter.

Changes: The revised sentence now reads: “Using oSD–sfGFP reporters, we observed that six oSDs (b, c, d, or1, or4, and j) did not show any functional interaction with the

native ribosomes **(Fig. 1C)**." **(lines 126–127 in the revised manuscript)**

Comment 3

lines 122-124 & Fig. 1E/F: Based on the text and data presented in Fig. 1F, it seems like that the WT-ASD 16S rRNA also contains the SpcR mutation. However, in Fig. 1E, this mutation is only depicted on the SSU with the oASD 16S rRNA. In combination, this might be easily misunderstood and confuse some readers, as to where the SpcR mutation is present, or respectively, not.

Response: Thank you for highlighting the confusing part of our description. In **Fig. 1EF**, we prepared four types of cell extracts using *E. coli* expressing an artificial rRNA operon with WT-ASD or oASD (b, or1, or or4). As you pointed out, all artificial rRNAs contain the SpcR mutation.

Changes: We have revised **Fig. 1E** to show that all artificial ribosomes used in **Fig. 1EFG** contain the SpcR mutation.

Comment 4

lines 130 – 132 Please be a bit more specific on what exactly has been additionally tested in Fig. S1C.

Response: In **Fig. 1**, we showed that the artificial ribosome with or1-oASD and SpcR mutations exhibited two-sided orthogonality in cell extracts. However, as this ribosome has both or1-oASD and SpcR mutations, we could not rule out that SpcR may affect orthogonal translation. Therefore, as shown in **Fig. S1C**, we confirmed that SpcR does not influence orthologous translation. We have revised the main text to clarify the tests depicted in **Fig. S1C**.

Changes: The revised sentence now reads: "We verified in a follow-up control

experiment that the reporter signals originated from the or1-oSD·oASD pairing, not from SpcR **(Fig. S1C)**." **(lines 145–147 in the revised manuscript)**

Comment 5

Fig. S3: The brightness setting for the bright-field images, and the font size in the black boxes, make it somewhat difficult to see the droplets and the text in this figure, respectively.

Response: Thank you for your comment. Accordingly, we revised **Fig. S3** to improve visibility.

Changes: In Fig. S3, we adjusted the brightness for the bright-field images and improved the font size and color settings.

Comment 6

Fig. S6C/D and S7C: Some form of indication as to where the values for the in vitro reconstituted ribosomes would be on the standard curve would be appreciated. Ideally, by also showing time-resolved fluorescence changes for these reactions as individual panels.

Response: Thank you for pointing out the need for better graph descriptions. We have revised **Fig. S6CD** and **S7C** according to your comments. During this revision, we also found a calculation error. Specifically, we overlooked that the second reaction solution for detecting nascent artificial ribosome translational activity was diluted by a factor of 2 from the first reaction solution. To account for this, we doubled the concentrations of the nascent artificial ribosomes.

Changes: The manuscript was revised as follows:

- Time-resolved fluorescence changes for the standard curves were added, and the values for the *in vitro* reconstituted ribosomes were included in the standard curves.

(Fig. S6CD, Fig. S7C, and lines 1242–1258 and 1278–1287 in the revised manuscript)

- Concentrations of nascent artificial ribosomes were corrected. **(lines 218, 220, and 258 in the revised manuscript)**

Comment 7

lines 197 – 198: Maybe the authors could elaborate a bit more as to why they think that a decrease in DNA concentration rather than an increase improves SSU biogenesis.

Response: Thank you for raising this important point. Although the underlying mechanism explaining the importance of reducing DNA concentrations remains unclear, two main possibilities exist. First, energy depletion in cell-free transcription and translation systems may occur, with transcription consuming too much energy, leaving insufficient energy for translation. Second, high mRNA concentrations could inhibit translation, as suggested in a previous study (PMID: 30625117). An imbalance between mRNA and translation components, such as formyl-Met-tRNA and initiation factors, could lead to the formation of inactive translation complexes.

Changes: We have revised the manuscript to reflect the above discussion. **(lines 225–233 in the revised manuscript)**

Comment 8

The authors should briefly mention in their conclusions that a huge increase in ribosome self-synthesis will be required to achieve self-replication in a synthetic system such as an artificial cell. At present, ribosome yields are in the low nM range, which is probably 2-3 orders of magnitude below the concentrations required for autocatalytic replication.

Response: We appreciate your comment. Accordingly, we calculated the improvement required for continuous self-replication. In this study, 9.8 nM SSU and 3 nM LSU were synthesized from 80 nM native ribosomes, yielding reproduction ratios of 12 % (9.8/80

× 100) and 3.8 % (3/80 × 100), respectively. Given that one ribosome must synthesize one or more ribosomes to achieve continuous self-replication, a minimum 26-fold improvement is necessary.

Changes: We have revised the manuscript to incorporate the above discussion. **(lines 363–368 in the revised manuscript)**

We once again thank you for your time, effort, and pertinent comments. Having revised the manuscript based on your suggestions, the clarity and logic of our research has been greatly strengthened.

Referee #3:

Comment 1

Kosaka et al. describe the development of a procedure to assemble "artificial" ribosomal subunits in vitro using purified E. coli ribosomes. In their cell-free assay, they produce ribosomal proteins for the small and large subunits, which interact with ribosomal RNA produced in the same reaction by T7 RNA polymerase. By reengineering the anti-Shine-Dalgarno sequence of their "artificial" small subunit, a reporter mRNA with a corresponding Shine-Dalgarno sequence can only engage with the "artificial" small subunit. Additionally, the authors develop a highly sensitive femtoliter translation assay, encapsulating the reaction solution in micron-sized oil droplets, allowing for quick fluorescence-based assessment of the yield of "artificial ribosomes."

Response: We thank you for your valuable comments, which have helped us improve our manuscript. We have carefully reviewed your feedback and revised the manuscript accordingly. Below are our point-by-point responses.

Comment 2

Lack of Clear Motivation and Goal: The manuscript does not clearly convey the motivation and goals of the study. It is unclear what this system is needed for and what advantages it offers over existing methods. The authors need to articulate the unique value of their approach.

Comment 5

Unclear Purpose of In Vitro Transcription/Translation: While the authors aim to establish a system where both rRNA and all 54 ribosomal proteins are synthesized in the same in vitro reaction, it is unclear why this is necessary and what advantages it provides over other methods. The manuscript should explain the purpose and benefits of in vitro transcription/translation of all ribosomal proteins.

Response: Thank you for pointing out the insufficient explanation of our motivation

and goal. We addressed Comments 2 and 5 together, as they are closely related.

Our study was driven by the goal to build "self-replicating artificial cells," or a self-replicating central dogma, a long-standing pursuit in synthetic biology that promises profound scientific advances. Building artificial cells requires attaining a deep understanding of cellular components and their organization, which would greatly enhance our comprehension of this complex, dynamic system. Furthermore, the ability to build artificial cells would enable scientists to create novel cell types beyond those found in nature, allowing them to explore the vast possibilities of life beyond its current form.

Ribosome biogenesis *in vitro* is recognized as a key milestone in achieving "self-replicating artificial cells." Indeed, leading synthetic biologists have emphasized this goal in major review articles (PMID: 38530077 and 36944355), as generating ribosomes from DNA is essential for synthetic cell construction.

Changes: We have revised the manuscript to clearly state our motivation and goal in the introduction. **(lines 33–37, 42–44, 47–53, and 69–71 in the revised manuscript)**

Comment 3

Neglect of Ribosome Assembly History: The manuscript fails to acknowledge over 50 years of ribosome assembly research:

o Nomura and Nierhaus established the in vitro reconstitution of E. coli small (Traub and Nomura, PNAS, 1968) and large ribosomal subunits (Nierhaus and Dohme, PNAS, 1974).
o Jewett et al. developed the Integrated Synthesis, Assembly, and Translation (IsAT) system, utilizing ribosomal proteins present in cell extracts with transcribed rRNA (Jewett et al., MolSystems Biology, 2013).
o The Mankin group generated RiboT, an orthogonal translation system with engineered rRNA, that results in an rRNA where 16S and 23S and 5S rRNA are covalently linked ensuring full orthogonality (Orelle et al., Nature, 2015; Aleksashin et al., Nat Comms, 2019).

Although the authors cite some of these studies, they do not sufficiently acknowledge that powerful in vitro ribosome assembly assays already exist and have provided deep insights into ribosome assembly, some with near-atomic resolution, especially when combined with cryo-EM analyses (Nikolay et al., Mol Cell, 2018; Duss et al., Cell, 2019; Rodgers et al., Cell, 2019; Qin et al., Nat Comms, 2023; Dong et al., NAR, 2023; Dong et al., NAR, 2024).

Response: We greatly appreciate your comments regarding our introduction.

The impression that we neglected prior research may stem from our writing guidelines for the introduction not being clear to readers. When writing the introduction, we followed two guidelines. First, because our goal is to realize ribosome biogenesis *in vitro*, we focused on the history of the development of *in vitro* ribosome assembly. Second, we kept the introduction concise to avoid making it too long.

Based on the first guideline, we cited the following papers on the development of *in vitro* ribosome assembly in the original version of the manuscript:

1. The Nomura group's SSU assembly map (PMID: 4912319)
2. The Nierhaus group's LSU assembly map (PMID: 7038683)
3. The Noller group's SSU reconstitution from a complete set of recombinant SSU r-proteins (PMID: 10376881)
4. The Jewett group's integrated synthesis, assembly, and translation (iSAT) (PMID: 23799452 and 24792158)
5. The Shimizu group's recombinant-based iSAT (R-iSAT) (PMID: 32214223)
6. The Shimizu group's ribosome reconstitution from a complete set of recombinant r-proteins (PMID: 34750629)
7. The Church group's trial attempting *in vitro* cogeneration of 54 r-proteins from DNA (PMID: 28330337)
8. The Bar-Ziv group's autonomous SSU assembly on a chip (PMID: 32494616)

In the original version of the manuscript, we omitted papers outside the study's main scope, including those on *in vivo* ribosome engineering and those attempting to

use cryo-EM and other methods to detail ribosome assembly. However, based on your comments, we thought it would be useful to readers if we revised the introduction to cite these studies.

Based on the second guideline, to keep the introduction concise, the original manuscript did not contain citations for some of the papers you mentioned. Specifically, regarding the Nomura group's SSU assembly map, we cited Nature 1970 rather than PNAS 1968. Regarding the Nierhaus group's LSU assembly map, we cited PNAS 1982 rather than PNAS 1974. However, based on your comments, we agree that the missing references should be included.

Changes: The introduction was revised as follows:

- Clarified our motivation and goal in the introduction, as also described in the response to your Comments 2 and 5. **(lines 33–37, 42–44, 47–53, and 69–71 in the revised manuscript)**
- Cited studies on ribosome engineering and ribosome assembly using cryo-EM and other methods in the introduction. **(lines 66–68 and 75–77 in the revised manuscript)**
- Cited PNAS 1968 and PNAS 1974, authored by the Nomura and Nierhaus groups, respectively, in the introduction. **(line 73 in the revised manuscript)**

Comment 4

Problematic Use of "Reconstitution": The term "reconstitution" is problematic because it has been traditionally used to describe the successful assembly of small and large ribosomal subunits using purified RNA and protein components from cells, as done by Nomura and Nierhaus. The authors should consider rephrasing (e.g., "autonomous" or "integrated") and clearly describe how their method is superior to classical in vitro ribosome assembly assays.

Response: We appreciate your comment.

You are correct that "reconstitution" is traditionally used to describe assembling ribosomes from purified rRNAs and r-proteins. In the original version of the

manuscript, we used “reconstitution” more broadly to refer to activating biological processes *in vitro*; however, we agree that this usage could be confusing for readers.

Regarding your latter point that we should clearly describe the advantages of our study, please refer to our response to Comments 2 and 5.

Changes: We have removed the word “reconstitution” throughout the manuscript. **(lines 1, 33–37, 69, 81, 84, 87, 104, 111, 151, 154–155, 195–197, 205–206, 210, 235–236, 250–253, 261, 275, 279, 309, 313, 356, 372, 495, 506, 515, 660, 1060–1061, 1084–1085, 1109, 1168–1169, and 1295–1296 in the revised manuscript)**

Comment 6

Concerns About Using T7 RNA Polymerase: If the goal is to create a system that mimics in vivo conditions more closely, the use of T7 RNA polymerase is problematic. The Nierhaus group (Lewicki et al., JMB, 1993; Iskakova et al., NAR, 2006) and others have shown that the transcription speed of T7 RNA polymerase is at least six times higher than that of E. coli RNA polymerase, which could have a significant artificial impact on the ribosome assembly process.

Response: Thank you for raising this important point.

Our goal is not to replicate *in vivo* conditions but to achieve ribosome biogenesis *in vitro* to support the creation of artificial cells. As you mentioned, the Nierhaus group suggested that T7 RNAP may not transcribe functional rRNA (PMID: 8515441). However, the Jewett group showed that a T7 RNAP-promoted rRNA operon achieved functional ribosome synthesis (PMID: 24792158). Although the high transcription speed of T7 RNAP may have effects that differ from those of *E. coli* RNAP, many studies following the Jewett group’s discovery have used T7 RNAP for ribosome assembly because of its simplicity and effectiveness as a single-domain protein (PMID: 36864669, 29053248, and 32214223). Especially, the Williamson group found that T7 RNAP-promoted rRNA operon produced near native ribosomes (PMID: 36864669). For our objective—creating artificial cells—mimicking *in vivo* conditions exactly was not necessary, which is why we chose to use T7 RNAP in our study, following previous

studies.

Changes:

We revised our manuscript as follows:

- Clarified that our goal is to achieve ribosome biogenesis *in vitro* as part of the effort to create artificial cells (as noted in our response to Comments 2 and 5). **(lines 33–36, 42–44, 47–53, and 69–72 in the revised manuscript)**
- Revised the manuscript to explain why we used T7 RNAP in our study. **(lines 406–410 in the revised manuscript)**

Once again, we sincerely thank you for your time, effort, and thoughtful comments. We have carefully revised the manuscript based on your suggestions, clarifying that our motivation is to create self-replicating artificial cells, with ribosome biogenesis *in vitro* as a key milestone in this effort. We also revised the manuscript by adding additional references, removing the word “reconstitution,” and explaining our choice of T7 RNAP. We believe that these revisions have greatly improved the clarity and completeness of the manuscript.

Referee #4:

Comment 1

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We are grateful for your co-review of our research. We have carefully revised our manuscript to incorporate all suggestions.