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Cogenerating Synthetic Parts toward a Self-Replicating System

Jun Li,[†,‡] Wilhelm Haas,[§,∥] Kirsten Jackson,[⊥] Erkin Kuru,[†,‡] Michael C. Jewett,[#] Z. Hugh Fan,[⊥,▽] Steven Gygi,[∥] and George M. Church*[,†,‡]

[†]Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, United States

[‡]Wyss Harvard Institute of Biologically Inspired Engineering, 3 Blackfan Circle, Boston, Massachusetts 02115, United States

[§]Massachusetts General Hospital Cancer Center; Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, United States

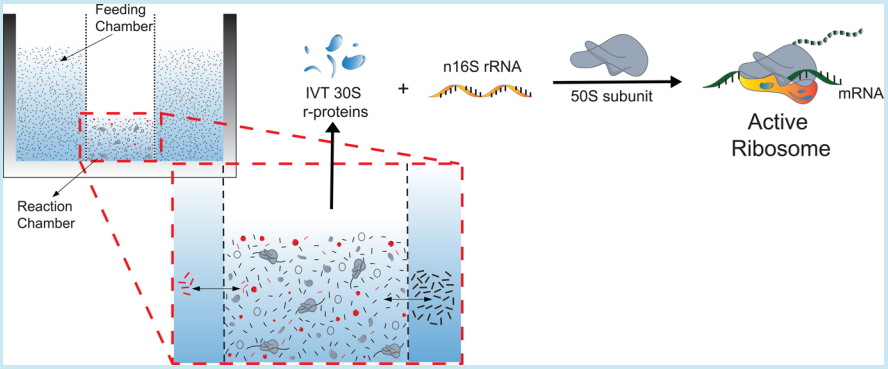
[∥]Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, United States

[⊥]J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, P.O. Box 116131, Gainesville, Florida 32611, United States

[#]Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208, United States

[▽]Department of Mechanical and Aerospace Engineering, University of Florida, P.O. Box 116250, Gainesville, Florida 32611, United States

Supporting Information



ABSTRACT: To build replicating systems with new functions, the engineering of existing biological machineries requires a sensible strategy. Protein synthesis Using Recombinant Elements (PURE) system consists of the desired components for transcription, translation, aminoacylation and energy regeneration. PURE might be the basis for a radically alterable, lifelike system after optimization. Here, we regenerated 54 *E. coli* ribosomal (r-) proteins individually from DNA templates in the PURE system. We show that using stable isotope labeling with amino acids, mass spectrometry based quantitative proteomics could detect 26 of the 33 50S and 20 of the 21 30S subunit r-proteins when coexpressed in batch format PURE system. By optimizing DNA template concentrations and adapting a miniaturized Fluid Array Device with optimized feeding solution, we were able to cogenerate and detect at least 29 of the 33 50S and all of the 21 30S subunit r-proteins in one pot. The boost on yield of a single r-protein in coexpression pool varied from ∼1.5 to 5-fold compared to the batch mode, with up to ∼2.4 μM yield for a single r-protein. Reconstituted ribosomes under physiological condition from PURE system synthesized 30S r-proteins and native 16S rRNA showed ∼13% activity of native 70S ribosomes, which increased to 21% when supplemented with GroEL/ES. This work also points to what is still needed to obtain self-replicating synthetic ribosomes *in situ* in the PURE system.

KEYWORDS: *cell-free protein synthesis, continuous-exchange, self-replicating system, proteomics, ribosome*

Construction of biochemical systems capable of autonomous replication and Darwinian evolution, *i.e.*, synthetic life, with radically new functions like novel amino acids or nucleotides represent ambitious projects.[1−9] There are two basic approaches for achieving a lifelike, self-replicating system. The "top-down" approach seeks to manipulate existing genomes from "natural" cells through genome editing and evolution.[4,5] The "bottom-up" approach seeks to assemble a synthetic cell from simple standard parts *in vitro*, such as a self-replicating system made from RNA[1] and the proposed 151 gene minimal genomic components sufficient to enable autonomous replication *in vitro*[10] currently with limited success. This has advantages over top-down approaches for enabling

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Table 1. Mass Spectrum Analysis of PURE System Cosynthesized 30S Subunit R-Proteins[a]

			CE 120 ng/μL			CE 80 ng/μL			Batch 10 ng/μL		
protein	gene name	protein size (kD)	spectral counts (L)	spectral counts (H)	H/L ratio	spectral counts (L)	spectral counts (H)	H/L ratio	spectral counts (L)	spectral counts (H)	H/L ratio
S1	rpsA	61.2	107	64	0.60	56	37	0.66	87	46	0.53
S2	rpsB	27	60	28	0.47	45	17	0.38	64	12	0.19
S3	rpsC	25.6	28	32	1.14	20	28	1.40	22	22	1.00
S4	rpsD	23.5	22	25	1.14	17	14	0.82	21	11	0.52
S5	rpsE	17.6	23	7	0.30	16	6	0.38	27	7	0.26
S6	rpsF	15.2	13	12	0.92	11	5	0.45	13	5	0.38
S7	rpsG	20	26	13	0.50	21	10	0.48	22	9	0.41
S8	rpsH	14.1	30	18	0.60	17	6	0.35	18	9	0.50
S9	rpsI	15.1	11	11	1.00	8	8	1.00	9	6	0.67
S10	rpsJ	11.7	25	13	0.52	13	7	0.54	19	11	0.58
S11	rpsK	13.8	10	12	1.20	9	10	1.11	7	6	0.86
S12	rpsL	13.7	5	6	1.20	6	6	1.00	5	3	0.60
S13	rpsM	13,1	23	15	0.65	17	11	0.65	18	7	0.39
S14	rpsN	11.6	1	1	1.00	2	2	1.00	1	0	0.00
S15	rpsO	10.3	7	1	0.14	7	1	0.14	9	1	0.11
S16	rpsP	9.2	10	12	1.20	10	10	1.00	9	4	0.44
S17	rpsQ	9.3	5	4	0.80	3	4	1.33	6	2	0.33
S18	rpsR	9	2	4	2.00	3	2	0.67	3	3	1.00
S19	rpsS	10.1	4	4	1.00	3	4	1.33	3	5	1.67
S20	rpsT	9.7	4	5	1.25	4	4	1.00	4	2	0.50
S21	rpsU	8.5	6	3	0.50	6	4	0.67	5	2	0.40

[a]Newly synthesized 30S r-proteins are labelled with ^{13}C on lysine and arginine (heavy form), while the other protein components in the PURE system are in the ^{12}C light form. Samples are first alkylated by iodoacetamide, precipitated by trichloroacetic acid, and finally digested by trypsin before subjected to mass spectrum. Spectral count data of detected peptides are presented by their encoded genes. The values for spectral counts indicate the numbers of spectra correctly assigned to the listed and grouped into L (light) and H (heavy) form.

system-level to molecular-level debugging, replacing any or many components (*e.g.*, RFs, r-proteins, tRNAs and synthetases) with radically altered versions (possibly even overall change in chirality). A suite of components (all of known function, minimizing unwanted or unknown side reactions), from the three main polymer systems of "the central dogma": DNA, RNA, and protein syntheses, is necessary.

Because the PURE system is a cell-free protein synthesis system that is constructed in a bottom-up approach based on well-characterized *E. coli* biochemistry,[11,12] it is the most prominent technology for a comprehensive self-renewing cell-like system. In the PURE system, various components (Table S1), responsible for transcription, translation, aminoacylation and energy regeneration, are individually prepared and reconstituted.[13] This potentially allows expression of cellular genes with known factors and avoids cellular contaminants such as proteases and nucleases inhibitory to protein synthesis.

A synthetic cell needs to be able to duplicate all components of itself to sustain its system. As the number and variety of synthesized components increase, balancing and regulating the protein synthesis become crucial. Previous models propose that ∼42 000 peptide bonds per ribosome need to be synthesized in order to produce all the components of a self-replicating system.[10] Even though classical PURE system contains wild-type ribosomes, it is still ∼1−2 orders of magnitude less efficient than this predicted necessary efficiency.[14] Therefore, it is necessary to improve protein expression efficiency in order to fulfill the self-reproduction. Different approaches have been applied to improve PURE system productivity. Although increasing the amount of input materials such as ribosomes and other translation enzymes can boost the yield by various fold,[15,16] it is not considered as a true improvement of the efficiency because the increase of input material is much more than the increase of output (reporter proteins synthesized) when counted by peptide bonds. Recent studies have improved the translation yield of the PURE system at continuous-exchange (CE) mode.[17,18] Compared to the traditional batch format, CE mode increases protein expression by enabling continuous supply of nutrients and energy molecules from a feeding solution and removal of accumulating inorganic phosphate byproducts from the reaction solution. For example, Jackson *et al.* developed a miniaturized Fluid Array Device (mFAD) with an optimized feeding solution to the PURE system and yielded 0.18 mg/mL of GFP, which was around 72.5 times higher than the GFP production with the conventional PURE system[17] significantly approaching to the proposed peptide bond formation efficiency necessary for a synthetic life.

Yet, to construct a lifelike entity with PURE system, the biggest challenge remains to enable the self-replication of ribosomes, the key component of protein synthesis. Construction of *E. coli* ribosomes requires the synthesis and assembly of 21 small subunit r-proteins (designated S1−S21, Table 1), 33 large subunit r-proteins (designated L1−L36, Table 2), and 3 rRNAs (23S, 16S, and 5S rRNA). As a step toward this long-term goal, in this study, we reconstructed the 54 r-protein genes into circular protein expression vectors and successfully expressed them individually in the PURE system reconstituted in-house. We adopted mFAD with the optimized feeding solution described in previous study[17] to individual expression of r-proteins in the PURE system and found the yields increased by ∼2 to 5-fold. For batch and CE mode PURE system, increasing the DNA template concentration to 20 ng/μL boosted the yield of S1 by ∼1.5-fold compared to 10

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Table 2. Mass Spectrum Analysis of PURE System Cosynthesized 50S Subunit R-Proteins[a]

			CE 120 ng/μL			CE 80 ng/μL			Batch 10 ng/μL		
protein	gene name	protein size (kD)	spectral counts (L)	spectral counts (H)	H/L ratio	spectral counts (L)	spectral counts (H)	H/L ratio	spectral counts (L)	spectral counts (H)	H/L ratio
L1	rplA	24.7	45	21	0.47	34	22	0.65	35	13	0.37
L2	rplB	30	18	20	1.11	19	17	0.89	18	11	0.61
L3	rplC	22.3	13	15	1.15	12	13	1.08	15	9	0.60
L4	rplD	22.1	27	10	0.37	24	11	0.46	22	6	0.27
L5	rplE	20.3	39	5	0.13	39	6	0.15	38	4	0.11
L6	rplF	18.9	19	4	0.21	14	3	0.21	19	3	0.16
L9	rplI	15.8	40	18	0.45	34	13	0.38	33	11	0.33
L10	rplJ	17.7	14	7	0.50	11	5	0.45	12	2	0.17
L11	rplK	14.9	10	4	0.40	10	2	0.20	12	2	0.17
L12	rplL	12.3	25	3	0.12	26	3	0.12	19	3	0.16
L13	rplM	16	10	10	1.00	8	11	1.38	9	3	0.33
L14	rplN	13.5	12	7	0.58	11	7	0.64	12	3	0.25
L15	rplO	15	16	9	0.56	16	8	0.50	17	8	0.47
L16	rplP	15	6	10	1.67	4	6	1.50	3	3	1.00
L17	rplQ	14.4	14	8	0.57	9	5	0.56	11	5	0.45
L18	rplR	12.8	5	7	1.40	6	4	0.67	3	3	1.00
L19	rplS	13.1	20	5	0.25	25	3	0.12	20	0	0.00
L20	rplT	13.5	10	4	0.40	9	3	0.33	6	3	0.50
L21	rplU	11.6	6	1	0.17	7	1	0.14	3	1	0.33
L22	rplV	12.1	21	13	0.62	22	9	0.41	16	5	0.31
L23	rplW	11.2	3	3	1.00	3	1	0.33	3	1	0.33
L24	rplX	11.3	20	15	0.75	13	7	0.54	19	5	0.26
L25	rplY	10.7	13	6	0.46	10	3	0.30	10	1	0.10
L27	rpmA	9.1	7	2	0.29	5	1	0.20	4	0	0.00
L28	rpmB	9	3	3	1.00	5	1	0.20	2	1	0.50
L29	rpmC	7	5	2	0.40	2	2	1.00	4	1	0.25
L30	rpmD	6.5	3	0	0.00	4	0	0.00	3	0	0.00
L31	rpmE	7.9	5	5	1.00	3	4	1.33	5	2	0.40
L32	rpmF	6.4	4	7	1.75	2	4	2.00	0	1	-------
L33	rpmG	6.4	2	1	0.50	2	1	0.50	2	0	0.00
L34	rpmH	5.4	0	0	0.00	0	0	0.00	0	0	0.00
L35	rpmI	7.3	1	0	0.00	1	0	0.00	0	0	0.00
L36	rpmJ	4.4	0	0	0.00	0	0	0.00	0	0	0.00

[a]Newly synthesized 50S r-proteins are labelled with ^{13}C on lysine and arginine (heavy form), while the other protein components in the PURE system are in the ^{12}C light form. Samples are first alkylated by iodoacetamide, precipitated by trichloroacetic acid, and finally digested by trypsin before subjected to mass spectrum. Spectral count data of detected peptides are presented by their encoded genes. The values for spectral counts indicate the numbers of spectra correctly assigned to the listed and grouped into L (light) and H (heavy) form.

ng/μL. Further increase in DNA template concentration did not increase the yield. Toward reconstituting a self-replicating ribosome, we also coexpressed the 21 30S r-proteins or 33 50S r-proteins in the PURE system with ^{13}C-labeled lysine and arginine and probed the coexpression by Mass Spectrometry with a method similar to stable isotope labeling with amino acids in cell culture (SILAC),[19] termed stable isotope labeling with amino acids in cell-free protein synthesis (SILACFPS) (Figure 1). In CE format PURE system, we detected all of the 21 30S r-proteins and at least 29 of the 33 50S r-proteins indicating successful coexpression. The coexpression efficiency in CE format PURE system was significantly higher than the batch format base on the paired *t* test of the ratios of newly synthesized protein to initially added protein of PURE system ribosomes. To test the functional of these proteins, we reconstituted 30S subunits from purified PURE system synthesized 30S r-proteins and native 16S rRNA under physiological condition and measured reconstituted ribosome activity by synthesizing firefly luciferase. This study extends the technical capabilities of the PURE system toward achieving a lifelike system by showing that 30S and 50S r-proteins can be cosynthesized in one pot, that was previously limited to cosynthesis and assembly of the five *E. coli* RNA polymerase subunits.[20]

RESULTS

Individual Expression of 30S and 50S R-Proteins in Batch Format PURE System. PURE system contains T7 RNA polymerase to couple transcription to translation from a double-stranded DNA template containing a T7 promoter. Therefore, we cloned a total of 54 genes from *E. coli* MG1655 strain genome, the 21 30S r-protein and 33 50S r-protein genes, each to a pET-24b vector (except for rplB, see Methods) between upstream T7 promoter and downstream T7 terminator. We reconstituted PURE system in-house based on previous study[12] with 10 ng/μL plasmid template concentrations encoding each of the r-protein. All of the r-proteins, namely 21 30S (Figure S1) and 33 50S (Figure S2) were successfully expressed in the PURE system individually, when the reactions were incubated at 37 °C for 2 h.

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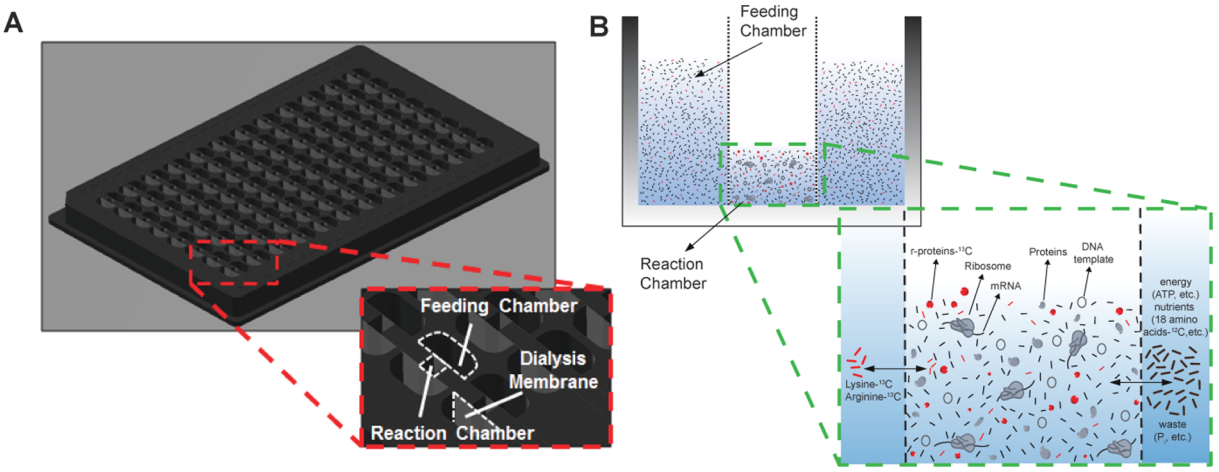

Figure 1. Illustration of the CE device mFAD and the SILACFPS r-protein coexpression strategy. (A) Three dimensional rendering of the 96-well mFAD. An expanded view of several protein expression units. As labeled, the reaction chamber is isolated from the two feeding chambers by vertically oriented dialysis membranes. (B) Cross-sectional illustration of one protein expression unit of the mFAD. The reaction chamber (central chamber) contains the PURE system capable of protein synthesis with ^{13}C-labeled arginine and lysine, r-protein DNA templates along with the synthesized r-proteins in heavy form. The feeding solution consists of the energy and nutrient molecules including ^{13}C-labeled arginine and lysine needed to sustain protein expression. An expanded view shows the different components for the reaction and feeding solutions.

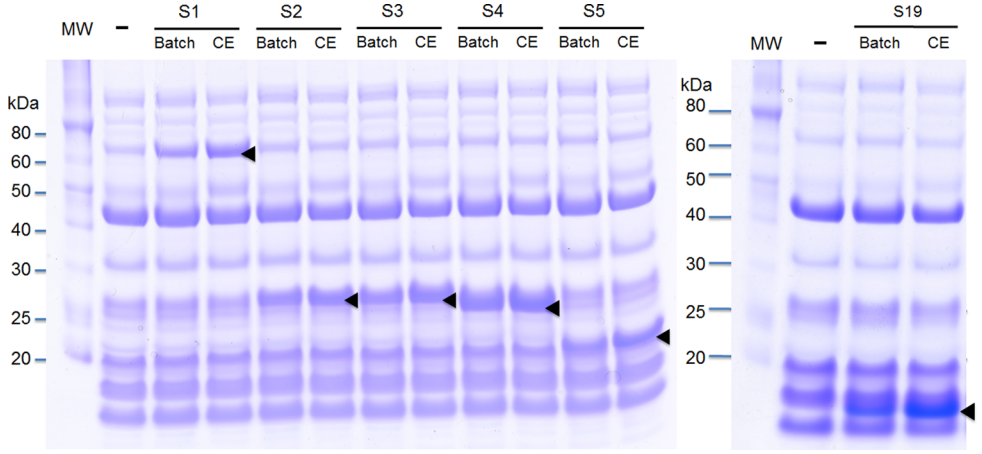

Figure 2. SDS PAGE analysis of sample 30S r-proteins S1, S2, S3, S4, S5 and S19 expressed in batch and CE mode PURE system by Coomassie Blue staining. The expected migration of each protein is marked on the gel. DNA template concentration of 10 ng/μL was used for the expression reaction for both batch and CE format. The resulting protein expression yields were quantified by the band intensity acquired by a typhoon scanner on SDS-PAGE.

Individual Expression of 30S and 50S R-Proteins in CE Format PURE System. Previous studies developed and optimized a feeding solution specific to the PURE system using a CE device mFAD[17,18] in a 96-well format (Figure 1). The GFP expression yield in the mFAD with nonoptimized feeding solution, which was based on the existing buffer and energy reagent concentrations, increased 20.9 times over the traditional batch format, while with optimized feeding solution (recipe listed in methods) increased 72.5-fold compared to the batch format.[17] Further optimization on experimental parameters, such as solution volume ratio, shaking and temperature, GFP expression increased 77.8-fold compared to the batch format in a standard microplate.[18] Here, to increase the yield of r-proteins, we applied the mFAD and the optimized feeding solution to the expression reaction. Specifically, 30S subunit r-protein S1, S2, S3, S4, S5, and S19 with various protein sizes from 10 kDa to 61 kDa were expressed in the batch format in the conventional microplate and the mFAD with optimized feeding solutions. Compared to the batch format, mFAD with the optimized feeding solution enhanced protein expression yield for all proteins (Figure 2): The CE mode increased protein expression yields of S1, S2, S3, S4, S5, and S19–3, ~3, ~3, ~2, ~2, and ~5-fold increase, respectively, over the batch format.

Previous studies have showed that the amount of synthesized protein in the PURE system is largely correlated to the amount of the encoding mRNA (within a certain concentration range) transcribed by T7 RNA polymerase.[20,21] This suggests that the amount of DNA template could also influence the amount of synthesized proteins. Indeed, increasing the DNA template concentration from 10 ng/μL to 20 ng/μL slightly increased the yield of S1 in the batch format, while further increase from 20 ng/μL to 120 ng/μL did not further enhance the yield (Figure 3). We observed a similar trend for the CE format: Yield reached maximum at a DNA template concentration of 20 ng/μL and stayed steady when template concentration exceeded 20 ng/μL (Figure 3).

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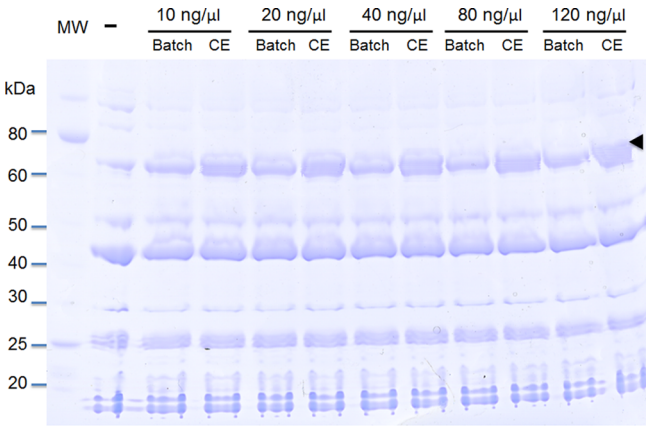

Figure 3. SDS-PAGE analysis of S1 synthesis in batch and CE format PURE system with different DNA template concentrations. The gel was stained by Coomassie Blue. The expected migration of each protein was marked on the gel. The CE format produced larger amount of S1 compared to the batch format, no matter which template concentration was used.

Coexpression of 30S and 50S R-Proteins in Batch and CE Format PURE System. To achieve a self-replicating of ribosome, synthesis of adequate amounts of 54 the r-proteins in one pot of PURE system are a curial first step. Therefore, we tried to coexpress these proteins in PURE system in batch and CE format. In order to distinguish ribosomes, *i.e.*, r-proteins, already present in the PURE system from the newly synthesized r-proteins, we employed a quantitative mass spectrometry-based proteomics approach SILACFPS.[19] In order to label the newly synthesized r-proteins, we replaced lysine and arginine amino acids of the PURE coexpression reaction mixture and feeding solution with ^{13}C-labeled lysine and arginine, so that after trypsin digestion, each digested newly synthesized peptide fragment has at least one ^{13}C-labeled lysine or arginine. These peptides therefore can be distinguished from peptides coming from ribosomes already present in the PURE system, allowing us to quantify their relative expression levels (Table 1 and 2). Other protein components in the PURE system can be taken as negative control: Only light form peptides were supposed to be detected (Table S2 and S3).

Specifically, we coexpressed 30S and 50S r-proteins in the PURE system in the batch (DNA template concentration: each r-protein 10 ng/μL) and CE format (DNA template concentration: each r-protein 80 ng/μL or 120 ng/μL) containing ^{13}C-labeled arginine and lysine. When using the batch format, we found that 20 of the 21 30S r-proteins (Table 1) as well as 26 of the 33 50S r-proteins (Table 2) were identified as carrying ^{13}C-labeled lysine or arginine, showing that most of these proteins were successfully coexpressed in our system. The ratio of detected peptides representing newly synthesized protein (Heavy form) to initially added ribosomes (Light form), H/L ratio for short, was calculated to measure the efficiency of expression (Table 1 and 2). Some H/L ratios were found to as high as 1.7 (S11) for 30S r-proteins and 1 (L16) for 50S r-proteins showing high efficiency of expression of these proteins. When using the CE format, all of the 30S r-proteins (Table 1) and 29 of the 33 50S r-proteins (Table 2) were identified as carrying ^{13}C-labeled lysine or arginine for both of the two DNA template concentration sets. The highest H/L ratio 2 was observed for S18 (30S, CE format, 120 ng/μL template concentrations) and L32 (50S, CE format, 80 ng/μL template concentrations). Paired *t* test of H/L ratios showed that CE mode with 80 ng/μL and 120 ng/μL template concentrations produced significant higher amount of r-proteins than batch mode with a *p*-value of 0.005 and 0.012, respectively, for the 30S, and a *p*-value of 0.0026 and <0.0001, respectively, for the 50S (Figure 4). According to the H/L ratios, the yield increase of a single r-protein in coexpression pool in CE mode varied from ~1.5 to 5-fold compared to the batch mode. Paired *t* test of H/L ratios between 80 ng/μL and 120 ng/μL template concentrations in CE mode showed that the difference is not significant (Figure 4). In optimal conditions (120 ng/μL template and CE mode), 10 of the 21 30S r-proteins and 9 of the 33 50S r-proteins had an H/L ratio ≥1 (Figure 4), indicating that we could generate at least as much proteins as we initially added for these proteins. Thus, we could estimate the yield of each r-protein based on the initial ribosome concentration added to the system. As the initial ribosome concentration is 1.2 μM, the yields of the proteins with H/L ratio ≥1 were estimated to be at least 1.2 μM.

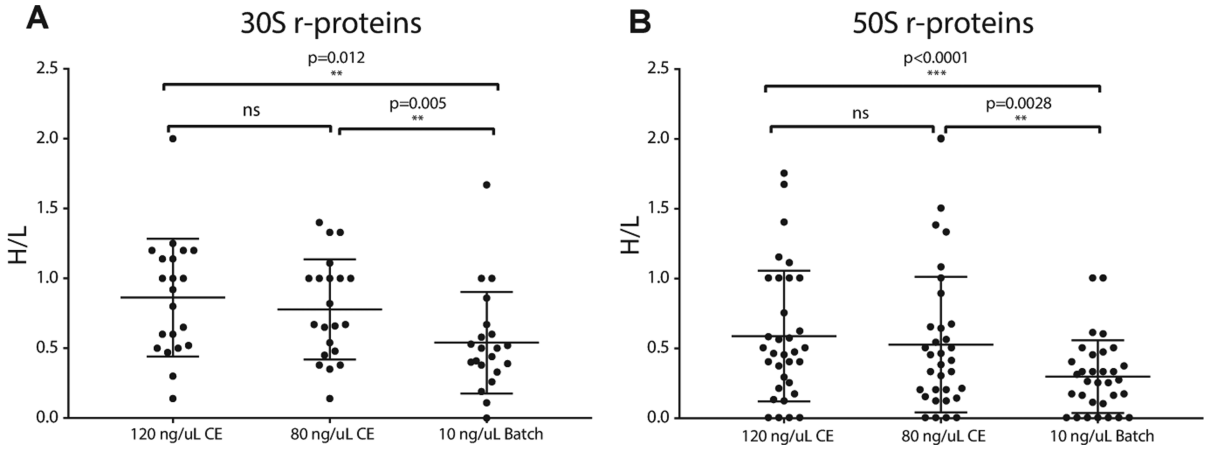

Figure 4. H/L ratio plots of r-proteins coexpressed in batch and CE format PURE system. (A) H/L ratio plots of 30S subunit r-proteins coexpressed in batch and CE format PURE system containing ^{13}C-labeled arginine and lysine. (B) H/L ratio plots of 50S subunit r-proteins coexpressed in batch and CE format PURE system containing ^{13}C-labeled arginine and lysine. DNA template concentrations of each protein in the reactions are indicated as above. *P*-values were calculated by a paired *t* test.

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30S Subunit Reconstitution with PURE System Synthesized 30S R-Proteins. To our knowledge 30S subunit reconstitution has never been done with *in vitro* synthesized 30S r-proteins instead of TP30. Thus, in an effort to make these semisynthetic 30S subunits, we purified PURE system synthesized 30S r-proteins using Reverse His-tag Purification method described in PURE system handbook. S7, S9, S11, S12, S13 and S18 failed to pass the Amicon Ultracel 0.5 mL−100 K spin concentrator and could not be purified using this method. Therefore, a strep-tagged version of S7, S9, S11, S12, S13 and S18 was constructed. Step-tagged proteins were synthesized in PURE system and purified as described in the methods. Purified proteins were analyzed by SDS-PAGE (Figure S3) and concentrations were determined by Bradford assay.

30S subunit reconstitution was conducted in PURE system with no ribosomes (PURE Δ ribosome system), serving as a platform to integrate ribosome reconstitution and protein synthesis in one compartment. Reconstituted ribosome activity was measured by synthesizing a reporter protein firefly luciferase (Fluc) here. Specifically, 30S subunit reconstitution, its coupling with native 50S subunit, Fluc transcription and translation occurred simultaneously in a 2-h 37 °C 15 μL batch reaction and Fluc was quantified by luciferase assay. Coupling native 50S and 30S subunits (50S + 30S; ratio = 1:1) exhibited ∼87% activity of intact native 70S ribosomes (70S) (Figure 5),

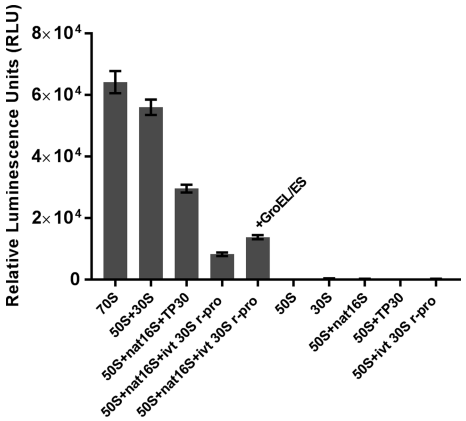

Figure 5. Reconstitution of 30S subunits in PURE Δ ribosome system under physiological condition. 70S ribosomes isolated from *E. coli* was taken as a positive control. 30S ribosome reconstitutions from components as labeled in the figure were measured by luciferase production. The last five columns were negative control reactions. For reconstitution reactions, 0.3 μM 70S, 0.3 μM 50S, 0.3 μM 30S, 0.3 μM native 16S rRNA (nat16S), 0.9 μM TP30, 0.9 μM PURE system synthesized r-proteins (ivt 30S r-pro) and 0.6 μM of GroEL/ES were added as indicated. Reactions were incubated at 37 °C for 2 h. Luciferase activities were measured in relative luminescence unit. Error bars are ± standard deviations, with $n = 5$.

indicating that the recoupling process is not a bottleneck for ribosome reconstitution in our system. On the other hand, when we reconstituted 30S subunits from native 16S rRNA (nat16S) and total r-proteins of 30S subunit (TP30) extracted from *E. coli* cells and coupled them with native 50S (nat16S + TP30 + 50S; ratio = 1:3:1), they exhibited ∼46% activity of 70S (Figure 5). This decrease in activity could be explained by the lack of assembly cofactors and/or rRNA processing functionalities. Finally, we replaced TP30 with PURE system synthesized 30S r-proteins in our integrated reconstitution reaction. Reconstituted 30S subunits from native 16S rRNA and PURE system synthesized 30S r-proteins (nat16S + ivt 30S r-pro +50S; ratio = 1:3:1) showed ∼13% activity of native 70S ribosomes, which increased to ∼21% when supplemented with chaperone system GroEL/ES (Figure 5). The activity drop from 46% to 13% caused by replacing TP30 with PURE system synthesized proteins suggested that a fraction of r-proteins synthesized in PURE was not active or properly folded, possibly due to the missing of protein factors facilitating protein folding in PURE.[22]

DISCUSSION

A cell-free protein synthesis environment that consists of the optimized concentrations of proteins, energy source, monomer substrates, cofactors and DNA with desired genes, in theory, could constitute a self-replicating dynamic system. The ribosome, as the key component for protein translation, needs to regenerate itself to ensure the self-replication of the entire system. Previously, Tian *et al.* reported that 30S r-proteins S2, S4, S5, S6, S9, S10, S12, S13, S15, S16, S17, and S21 could individually be expressed in the *E. coli* cell lysate based cell-free protein synthesis system using PCR product templates, but small subunit proteins S1, S3, S7, S8, S11, S14, S18, S19, S20 could not.[23] This might have been due to the degradation of linear DNA templates, mRNAs or proteins caused by the nucleases and proteases present in the cell lysate based system. PURE system is a defined reconstituted cell-free system with minimal components for protein transcription and translation. With no nucleases and proteases contamination present and being entirely modular in terms of content and concentrations of system components, PURE system appears to be a more attractive platform toward constructing a self-replicating entity.

In this study, we demonstrated that PURE system can be utilized to express all of the 54 r-proteins individually in batch format with a ∼2 to 5-fold additional increase in yield when a CE device mFAD was applied. We also tried combinations of different DNA template concentrations of r-proteins to find the optimal template concentration for the highest expression levels. Increasing the DNA template concentration to 20 ng/μL boosted the yield of 30S subunit r-protein S1 in both batch and CE mode, but the yield did not increase further beyond this template concentration. By labeling the newly synthesized proteins with ^{13}C lysine and arginine, we established a Mass Spectrometry-based detection method which can distinguish the newly cosynthesized r-proteins from the original ones present in the PURE system. The mass spectrometry data showed 20 of the 21 30S r-proteins and 26 of the 33 50S r-proteins were coexpressed in batch format PURE system; while in CE format PURE system, the *de novo* coexpression was significantly higher and heavy forms of all the 30S r-proteins and 29 of the 33 50S r-proteins could be detected, for both of the two DNA template concentration sets. Notably, the four 50S subunit r-proteins (L30, L34, L35 and L36) that could not be detected are all smaller than 8 kDa and light form peptides coming from two of those r-proteins (L34 and L36) already present in the PURE system could not be detected either. The underrepresentation of these small proteins may be due to a low efficiency of precipitating these proteins by applying the TCA precipitation protocol or due to relatively small number of peptides being generated by trypsin digestion.[24]

A robust, self-replicating entity would rely on *in situ* reconstitution of the protein translation machinery, from individual components (most notably r-proteins) synthesized

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de novo. Therefore, balancing the amount of proteins synthesized for each target protein individually, so that they can stoichiometrically be assembled into ribosome complex, is our ultimate goal. The optimal ratio for the relative amounts of the templates encoding r-proteins was not determined in this study. A systematic optimization may be necessary to further balance the amount of the synthesized r-proteins. For the proteins which were not synthesized efficiently by the PURE system, several fine-tuning strategies, such as altering promoter strengths or codon optimization, will be pursued. For example, gene codons can be altered to be A + U rich for destabilizing mRNA secondary structure.[25] Genes can also be changed to have only the most frequently used codon (*e.g.*, only the triplet CAG for glutamine—present 69% of the time in *E. coli* genes—would be used). Nevertheless, our results suggest that coexpression of r-proteins in the PURE system approaches to (or exceeds) the level of r-proteins that are initially added to boot the system up and that assembling a synthetic lifelike system is not a far-fetched dream. Moreover, these *in vitro* translated 30S r-proteins, after purification, can reconstitute 70S ribosomes with native 50S subunits and native 16S rRNA with ∼13% activity of native 70S ribosomes under physiological condition in PURE. This activity increased to 21% when chaperone system GroEL/ES was supplemented in the reconstitution reaction. Future improvements of PURE system productivity and ribosome reconstitution efficiency can be possibly achieved by adding translation facilitating and protein folding factors to PURE system protein synthesis[15,22,26] or by adding ribosome assembly cofactors to reconstitution reaction.[27] Our findings also provide hope that, fully synthetic ribosomes capable of self-replicating can be achieved in the PURE system based platform by integrating rRNA transcription, r-protein cotranslation and ribosome assembly together. More ambitiously, further improvements of this system will enable a foundation to construct a DNA, RNA and protein based replicating system.[10]

METHODS

Media, Chemicals, and Reagents. Unless specified, all chemicals were obtained from Sigma-Aldrich. Tryptone and yeast extract were obtained from BD Difco. GroEL/ES were obtained from Takara.

PURE System Reconstitution. PURE system component plasmids were obtained from the Ueda group. Home-made PURE system was prepared according to refs 12, 17. Reagent concentrations: 1.2 μM ribosomes, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 0.26 μM EF-G, 0.92 μM EF-Tu, 0.66 μM EF-Ts, 0.25 μM RF1, 0.24 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 1900 U/mL AlaRS, 2500 U/mL ArgRS, 20 mg/mL AsnRs, 2500 U/mL AspRs, 630 U/mL CysRs, 1300 U/mL Gln *Rs*, 1900 U/mL GluRs, 5000 U/mL GlyRs, 630 U/mL HisRs, 2500 U/mL IleRS, 3800 U/mL LeuRS, 3800 U/mL LysRS, 6300 U/mL MetRS, 1300 U/mL PheRS, 1300 U/mL ProRS, 1900 U/mL SerRS, 1300 U/mL ThrRS, 630 U/mL TrpRS, 630 U/mL TyrRS, 3100 U/mL ValRS, 4500 U/mL MTF, 4 μg/mL creatine kinase, 3 μg/mL myokinase, 1.1 μg/mL nucleoside-diphosphate kinase, 2.0 units/mL pyrophosphatase, 10 μg/mL T7 RNA polymerase, 2 mM ATP, 2 mM GTP, 1 mM CTP, 1 mM UTP, 20 mM creatine phosphate, 50 mM HEPES-KOH (pH 7.6), 100 mM potassium glutamate, 13 mM magnesium acetate, 2 mM spermidine, 1 mM dithiothreitol (DTT), 0.3 mM 20 amino acids, 10 μg/mL 10-formyl-5,6,7,8-tetrahydrofolic acid and 56 A260 per ml tRNA mix.

Device Fabrication. Fabrication of the mFAD was described in ref 17.

Optimized Feeding Solution. Optimized feeding solution contains 50 mM HEPES-KOH (pH 7.6), 100 mM potassium glutamate, 17 mM magnesium acetate, 2 mM spermidine, 1 mM DTT, 4 mM ATP, 4 mM GTP, 1 mM CTP, 1 mM UTP, 20 mM creatine phosphate, 0.5 mM amino acids, 10 μg/mL 10-formyl-5,6,7,8-tetrahydrofolic acid as listed in ref 17.

Molecular Cloning. 50S and 30S r-protein genes were PCR amplified from *E. coli* MG1655 genome (ATCC) with primers listed in Table S4 and S5 and cloned to pET-24b (Novagen) using restriction enzyme NdeI and XhoI except rplB. RplB was cloned to pIVEX 2.3d vector (Roche) with restriction enzyme NcoI and XhoI due to its internal cleavage site of NdeI. Genes were cloned in its natural format with no additional amino acid or affinity tags. PCR and standard Sanger sequencing primers were obtained from Integrated DNA Technologies with standard purification. Sanger sequencing verification of each clone was performed by Genewiz.

Individual Expression of R-Proteins in Batch Format PURE System. Each protein was expressed in a 25 μL PURE system reaction with 0.8 U/μL Murine RNase Inhibitor (New England Biolabs) at 37 °C for 2 h with a plasmid template concentration of 10 ng/μL or other concentrations as indicated in the text. Reactions were later analyzed on 4−12% Bis-Tris Protein Gels (Life Technologies) and stained by Coomassie Blue G-250.

Individual Expression of R-Proteins in CE Format PURE System. For the CE format in the mFAD, 10 μL PURE system reaction solution with 0.8 U/μL Murine RNase Inhibitor and a plasmid template concentration as indicated in the text was dispensed into the reaction chamber, while a total of 200 μL of the feeding solution was pipetted into the feeding chambers (100 μL in each of the two feeding chambers). Negative controls were prepared by replacing the DNA in the reaction solution with an equal volume of nuclease-free water. Batch format controls were carried in 10 μL PURE system reaction with 0.8 U/μL Murine RNase Inhibitor and a plasmid template concentration as indicated in the text and dispensed into a conventional microplate. To prevent evaporation, the conventional microplate and the mFAD were covered with a tape. Then the microplate and mFAD were incubated at 37 °C (optimized temperature in ref 18) for 4 h with shaking. Samples were later analyzed on 4−12% Bis-Tris Protein Gels (Life Technologies) and stained by Coomassie Blue G-250.

Coexpression of 30S and 50S R-Proteins. For batch format controls, 10 μL PURE system reaction was reconstituted with 10 ng/μL of each plasmid encoding 30S or 50S r-proteins, 0.8 U/μL Murine RNase Inhibitor, 0.3 mM ^{13}C-labeled lysine, arginine and 0.3 mM the other 18 amino acids. Reactions were dispensed into a conventional microplate. For CE format, 10 μL PURE system reaction was reconstituted with 80 ng/μL or 120 ng/μL of each plasmid encoding 30S or 50S r-proteins, 0.8 U/μL Murine RNase Inhibitor, 0.3 mM ^{13}C-labeled lysine, arginine and 0.3 mM the other 18 amino acids. Reactions were dispensed into mFAD. Negative controls were prepared by replacing the DNA in the reaction solution with an equal volume of nuclease-free water. The microplate and mFAD were sealed and incubated at 37 °C for 4 h with shaking.

Mass Spectrometry Sample Preparation. Ten μL of coexpression reaction was taken and diluted to 1 mg/mL with Alkylation buffer (8 M Urea, 25 mM Tris-HCl pH 8.0, 10 mM

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DTT) and incubated at 56 °C for 30 min, then cool down to room temperature. Iodoacetamide was added to the protein solution to a final concentration of 30 mM. The tube was wrapped with foil and incubated at room temperature for 30 min. DTT was added to 20 mM and incubated at 37 °C for 30 min to quench the reaction. 1/4 volume of 100% TCA was added dropwise to each sample and incubated for 10 min on ice. Each sample was then spun at ∼14K rpm for 5 min at 4 °C. Supernatant was discarded. Pellet was washed with 200 μL ice cold acetone per tube by vortexing and spun again at ∼14K rpm for 5 min at 4 °C. The washing step was repeated twice. Pellet was air-dried on bench. 500 μL digestion buffer (8 M Urea, 50 mM Tris-HCl pH 8.0) was added to protein pellet and incubated at 56 °C for 60 min to denature the proteins. Each sample was then cooled. 100 mM Tris-HCl (pH 8.0) was added to each sample until urea concentration was less than 1M. Twenty μg trypsin was added to protein samples and incubated at 37 °C for 6 h. 1/2 volume of 5% acetonitrile/5% formic acid was added to samples to reach a final pH < 4. Water's tc18 column was prewet with 1 mL 100% acetonitrile and 1 mL 90% acetonitrile/5% formic acid and then equilibrated with 1 mL 5% acetonitrile/5% formic acid. Acidified samples were loaded to the column slowly (<1 mL/min), washed with 1 mL 5% acetonitrile/5% formic acid and eluted with 500 μL 50% acetonitrile/5% formic acid. Samples were then dried using Speedvac.

Mass Spectrometry Data Analysis. The generated peptides were analyzed using liquid-chromatography tandem mass spectrometry (LC−MS/MS) essentially as described previously.[28] Briefly, the analysis was performed using an Orbitrap Elite mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an Accela 600 binary HPLC pump (Thermo Scientific) and a Famos autosampler (LC Packings). Peptides were fractionated over a 100 μm I.D. in-house-made microcapillary column packed first with approximately 0.5 cm of Magic C_4 resin (5 μm, 100 Å, Michrom Bioresources) followed by 20 cm of Maccel C_{18}AQ resin (3 μm, 200 Å, Nest Group). Fractionation was achieved by applying a gradient from 10% to 32% acetonitrile in 0.125% formic acid over 75 min at a flow rate of approximately 300 nl min^{-1}. The mass spectrometer was operated in a data-dependent mode collecting survey MS spectra in the Orbitrap over an m/z range of 300−1500, followed by the collection of MS2 spectra acquired in the dual pressure linear ion trap on the up to 20 most abundant ions detected in the survey MS spectrum. The settings for collecting survey MS spectra were AGC target, 1×10^6; maximum ion time, 50 ms; resolution 6×10^3. The settings for the acquisition of MS2 spectra were isolation width, 2 m/z; AGC target, 2×10^3; max. ion time, 100 ms; normalized collision energy, 35; dynamic exclusion, 40 s at 10 ppm.

Peptides were identified from the MS2 data using the Sequest algorithm[23] operated in an in-house-developed software suite environment that was also applied for filtering the search results and extracting the quantitative data. The data was searched against a protein sequence database comprised of the sequences of *E. coli* ribosomal proteins, of known contaminants such as porcine trypsin, and of 500 nonsense protein sequences derived from all *S. cerevisiae* protein sequences using a fourth order Markov chain model.[29] To this forward (target) database component we added a reversed (decoy) component including all listed protein sequences in reversed order.[30] The Markov chain model derived nonsense protein sequences were added to generate a protein sequence database size allowing an accurate estimation of the false discovery rate of assigned peptides and proteins. Searches were performed using a 50 ppm precursor ion mass tolerance and we required that both termini of the assigned peptide sequences were consistent with trypsin specificity allowing two missed cleavages. Carbamidomethylation of cysteines (+57.02146) was set as static information and full ^{13}C labeling on arginine and lysine (+6.020129) as well as oxidation on methionine (+15.99492) were set as variable modifications. A false discovery rate of less than 1% for the assignments of both peptides and proteins was achieved using the target-decoy search strategy.[30] Assignments of MS2 spectra were filtered using linear discriminant analysis to define one composite score from the following spectral and peptide sequence specific properties: mass accuracy, XCorr, ΔCn, peptide length, and the number of missed cleavages.[31] Protein identifications were filtered based on the combined probabilities of being correctly assigned for all peptides assembled into each protein.[31] Both peptide and protein assignments were filtered to a false-discovery rate of less than 1%. Relative peptide quantification was done in an automated manner by producing extracted ion chromatograms (XIC) for the light (^{12}C arginine or lysine) and heavy (^{13}C arginine or lysine) forms of a peptide ion followed by measuring the area under the chromatographic peaks.[32] A peptide ion was considered to be quantified when the sum of the signal-to-noise ratio of both light and heavy form was larger or equal to 10. The median of the log2 (heavy/light) ratios measured for all peptides assembled into a protein was defined as the protein log2 ratio. If only the ion signal of the light peptide form was observed above the noise level the log2 heavy-to-light ratio was defined as being smaller than log2 of the signal-to-noise for the light peptide ion. As orthogonal approach to determine the occurrence of the light and heavy forms of a protein we counted the number of MS2 spectra assigned to each forms of a protein.

Isolation of Tightly Coupled 70S Ribosomes and Ribosomal Subunits. Tightly coupled 70S ribosomes, 30S and 50S subunits were prepared as described by Michael C. Jewett.[33] Results from three independent ribosome preparations and subsequent rRNA and total protein preparations were used and averaged to generate the final results shown in the manuscript.

Isolation of Native rRNA and R-Proteins. TP30 and native 16S rRNA were prepared as described by Nierhaus.[34] TP30 was purified using acetone precipitation.

Preparation of PURE System Synthesized 30S R-Proteins. 30S r-proteins, except S7, S9, S11, S12, S13 and S18, were expressed in PURE system reactions and purified using reverse his-tag purification method. Specifically, after expression, 200 μL reaction was collected for each protein and then diluted with 200 μL dilution buffer (50 mM Hepes-KOH, 10 mM Magnesium acetate, 0.8 M NaCl, 0.1% Tween 20). The diluted reaction mixture was applied to Amicon Ultracel 0.5 mL-100 K spin concentrator and centrifuged for 50 min at 15000*g* at 4 °C. The permeate was transferred to a new tube and mixed with 0.25 volumes Ni-NTA magnetic beads (Qiagen) for 40 min at 4 °C. The reaction mixture slurry was applied to an empty Bio-Rad microspin column and centrifuged for 2 min at 1500*g* at 4 °C. The elute containing purified protein was concentrated by Amicon Ultracel 0.5 mL-3 K spin concentrator and exchanged to a storage buffer to a final concentration of 50 mM Tris-HCl pH 7.6, 100 mM KCl, 10 mM Magnesium acetate, 1 mM DTT and 30% glycerol. A

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strep-tag was inserted to S7 at C-terminal, S9 at N-terminal, S11 at N-terminal, S12 at C-terminal, S13 at N-terminal and S18 at N-terminal on the protein expression plasmids using QuikChange Lightening Site-Directed Mutagenesis Kits (Agilent Genomics) (Table S6). These strep-tagged proteins were expressed in PURE system. 200 μL Strep-Tactin magnetic beads (Qiagen) were washed three times with 1 mL wash buffer (50 mM Tris HCl pH 7.6, 0.5 M NaCl, 6 mM β-mercaptoethanol) and resuspended in 200 μL wash buffer. 200 μL PURE reaction for each strep-tagged protein was collected and mixed with the beads mixture on a rotator at 4 °C for 3 h. The beads were then immobilized with a magnet. Supernatant was discarded and beads were washed twice with 200 μL wash buffer. Strep-tagged r-protein was eluted by 150 μL elution buffer (50 mM Tris HCl pH 7.6, 0.5 M NaCl, 10 mM biotin, 6 mM β-mercaptoethanol), concentrated by EMD Amicon Ultracel 0.5 mL-3 K spin concentrator and exchanged to a storage buffer to a final concentration of 50 mM Tris-HCl pH 7.6, 100 mM KCl, 10 mM Magnesium acetate, 1 mM DTT and 30% glycerol. All purified r-proteins were analyzed on 4−12% Bis-Tris Gel (Life Technologies) and concentrations were determined by Bradford Assay (Bio-Rad). After concentration determination, all of the 21 30S r-proteins were mixed at an equal molar ratio and buffer exchanged to 50 mM Tris-HCl pH 7.6, 100 mM KCl, 10 mM Magnesium acetate, 1 mM DTT and concentrated by Amicon Ultracel 0.5 mL−3 K spin concentrator to a certain fold. The final concentration of the r-protein mixture stock was the original concentration times the fold number. The stock was used for the reconstitution assay.

Integrated Ribosome Assembly, *In Vitro* Transcription and Translation. Integrated assay was set up to 15 μL with PURE system factors, 0.8 U/μL Murine RNase Inhibitor, 10 ng/μL pIVEX 2.3d Fluc plasmid (expressing firefly luciferase under a T7 promoter control, obtained from[15]), 0.3 μM 50S subunit, 0.9 μM PURE system synthesized 30S r-proteins or 0.9 μM TP30 and 0.3 μM native 16S rRNA with or without 0.6 μM GroEL/ES. When TP30 or PURE system synthesized 30S r-proteins mixture were added, an equal volume of the same buffer was added to other reconstitution reactions to rule out the influence from buffers. Reactions were incubated at 37 °C for 2 h. After incubation, 7 μL reaction was mixed with 40 μL luciferase assay substrate (Promega) and incubated at room temperature for 10 min. Luminescence was measured by SpectraMaxM5 plate reader (Molecular Devices, Sunnyvale, CA). Five replicates were conducted for each condition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00342.

Supporting tables and figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: gchurch@genetics.med.harvard.edu. Tel: +1. 617.432.7562. Fax: +1. 617.432.6513.

ORCID

George M. Church: 0000-0003-3535-2076

Author Contributions

JL, MCJ and GMC designed the study. JL performed the experiments. WH and SG contributed to the mass-spec data acquisition and analysis. KJ and ZHF manufactured the CE device mFAD. JL, WH and EK wrote the manuscript. All authors helped edit the final manuscript.

Notes

The authors declare no competing financial interest.

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