Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

for

Probing self-regeneration of essential protein factors required for *in vitro* translation activity by serial transfer

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Methods

DNA constructs

All plasmids used in this study are listed in Tab. S1. The plasmid pLD1, pLD2, and pLD3 were a generous gift from A. Forster (Uppsala University) and is described in more detail elsewhere.[1] Plasmids encoding for nucleotide-diphosphate kinase (pNDK, ID:124136)[2], T7-RNA polymerase (pT7RNAP, ID:124138)[2], creatine kinase m-type (pCKM, ID: 124134)[2] and adenylate kinase 1 (pAK1, ID:118977)[3] were obtained from Addgene.

Protein purification

All proteins including pLD1, pLD2, and pLD3 protein fractions but excluding RNase inhibitor and IPP were purified using an ÄKTA start HPLC system with His-Trap FF columns (both GE Healthcare). All buffers are listed in Tab. S2. Chemically competent BL21 DE3 *E. coli* (NEB) were transformed with the plasmid bearing the gene for the his-tagged protein of interest (POI). Cells were incubated over night at 37 °C in 1 L LB medium with the respective selection marker in a rotary shaker. Protein expression was auto-induced by adding a sugar-mix containing 12.5 g/L D-glucose and 50 g/L D-lactose prior to incubation. Following protein expression, cells were harvested by centrifugation. The pellet was washed twice using wash buffer and finally brought up in lysis buffer to be sonicated. The lysate was cleared by centrifugation and loaded on a His-Trap FF column (GE Healthcare) with an ÄKTA Start chromatography system (GE Healthcare). After washing with 10 column volumes of lysis buffer, the POI was eluted using 2-3 column volumes of elution buffer. Buffer-exchange to storage buffer and up-concentration were performed in a single step by four rounds of spin-concentration using 10 kD MWCO Amicon Ultraspin (Merck Millipore) columns (3kD MWCO for pLD2-protein). Protein concentration was estimated using Bradford's assay with BSA standard. The protein solutions were aliquoted, frozen in liquid nitrogen and stored at -80 °C until further use.

Protein regeneration during serial transfer

In order to test the protein synthesis viability of *de novo* synthesized POI, we employed a serial dilution of multiple IVTT reactions. 4x EM is listed in Tab. S3, 6xZM is listed in Tab. S4. Tightly coupled 70S ribosomes were obtained from strain *E. coli* D10 as reported previously[4].

At first, a fresh PURE IVTT reaction was started using 2.5 µL 4xEM, 1 µL 10xAA, 1.67 µL 6xZM, 1 µL pLD1-fraction (1 mg/mL stock), 1 µL pLD2-fraction (1 mg/mL stock), 1 µL pLD3-fraction (1 mg/mL stock), 4 nM plasmid DNA encoding the POI with a T7 promoter and 1.33 µL ddH_2O up to a total reaction volume of 10 µL. 10xAA was an all-20 amino-acids working solution which has obtained by diluting an equimolar stock (50 mM) tenfold. An aliquot of 5 µL was saved as "gen0" and stored at 4 °C until further use. The remaining 5 µL were incubated at 37 °C for 1 hour. Subsequent generations were mixed and incubated according to the description above, except for the following: The 1.33 µL ddH_2O is replaced by an aliquot from the previous IVTT reaction; and the 6xZM replaced by a 6xZMΔPOI lacking the POI. The rest of the incubated reaction was stored at 4 °C as "gen*x*", where *x* is the number of preceding incubation rounds. Control reactions were implemented using plasmids encoding proteins other than the POI (Tab. S5).

In situ BODIPY-Lys labelling

To assess POI re-synthesis expression during serial dilution, 1 µL of FluoroTect GreenLys (Promega) was added to each reaction. Prior to the addition of 2× SDS loading buffer, the samples were incubated for 30 min at 37 °C with 1 µL of RNase Cocktail (Thermo Fisher Scientific). After adding the loading buffer, the samples were incubated for 5 minutes at 55 °C. After denaturing PAGE, *de novo* synthesised proteins were visualised using a Typhoon FLA 7000 scanner. POI band volumes were quantified using *ImageQuant TL* 8.2 (Cytiva). For NDK and T7-RNAP, the fluorescence intensity of PURE-specific background bands were subtracted before quantification (Fig. S1). For visualisation in the main text, all gels were treated with the "despeckle" filter in GIMP 2.

GFP assay

The potential of *in vitro* synthesized POI to feedback into IVTT activity was estimated using expression of the reporter gene sfGFP. To this end, 10 µL IVTT reactions were set up using 100 ng of pIVEX-sfGFP plasmid, 2.5 µL 4xEM, 1 µL 10xAA, 1.67 µL 6xZM, 1 µL pLD1-fraction (1 mg/mL stock), 1 µL pLD2-fraction (1 mg/mL stock), 1 µL pLD3-fraction (1 mg/mL stock), and 1.33 µL aliquot from the respective IVTT reaction of interest, which had been stored at 4 °C after 1h of regeneration until further use. All reactions were prepared in MicroAmp Fast 8-Tube

Strips (Thermo Fisher Scientific) and incubated at 37°C in a StepOne Real-Time PCR System (Thermo Fisher Scientific). Fluorescence signals were recorded every 60 s. Each biological triplicate was derived from one gen1 master mix. Each series included also a "gen0" control samples, in which the gen1 PURE master mix was tested for overall sfGFP expression performance before POI expression. To compensate for varying sfGFP yields of different PURE batches, fluorescence end-points F (recorded value at 100 min) of each generation within an independent series of dilution experiment was normalised against all fluorescence endpoints of the same series according to the equation

$$f_{genx} = \frac{F_{genx}}{F_{gen0} + F_{gen1} + F_{gen2} + F_{gen2c} + F_{gen3} + F_{gen3c}}$$

After recording time traces, 2× SDS loading buffer was added to the respective mixtures, and samples were incubated for 5 min at 55 °C to preserve sfGFP fluorescence. 10 µL of each sample was subsequently loaded on a 12% polyacrylamide SDS-Gel. Fluorescent bands were visualised using a Typhoon FLA 7000 (Fig. S1).

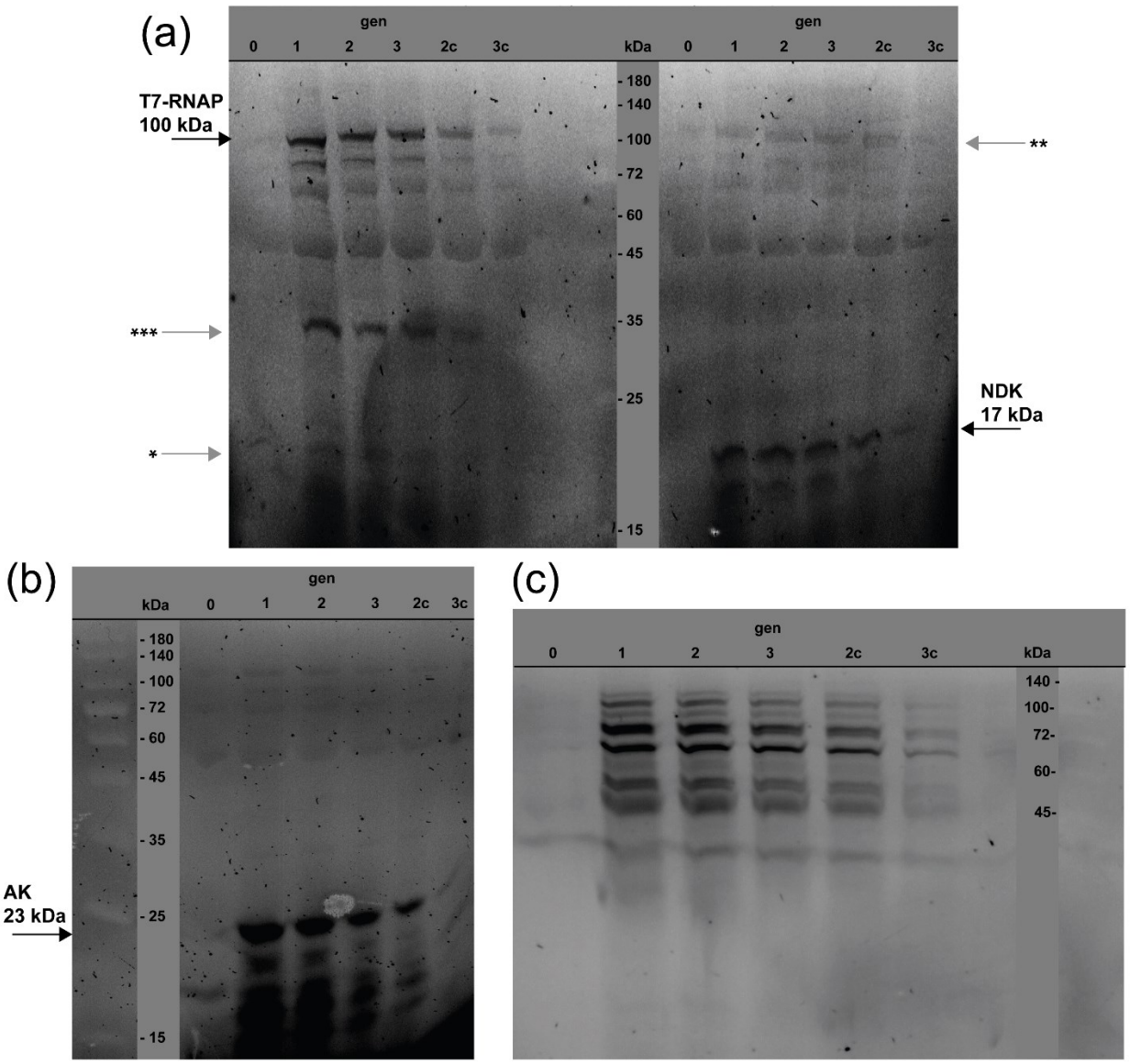

Fig. S1. Representative raw gel images of the *in situ* BODIPY-Lys labelling experiments in Fig 2. (a) Labelling of T7-RNA Polymerase (T7 Pol, left) and nucleoside-diphosphate kinase (NDK, right) in serial transfers with pT7 in PUREΔT7-RNAP or pNDK in PUREΔNDK. Control plasmids for each gen2c and gen3c are listed in Tab. S5. In both experiment, PURE background bands were observed that overlap with the detected protein of interest (* for NDK and ** for T7 Pol). The band intensities of * (for NDK) and ** (for T7 Pol) were subtracted from each generation during quantification of both POIs. Note that the background bands were less significant in the other gels due to a generally increased POI-fluorescence signal.. pT7 produced a side product (***) at ~35 kDa, which was not included in the analysis. (b) *In situ* labelling during serial transfer of PUREΔAK1 systems containing pAK1 in gen1-3 or pT7 in gen2c and 3c. (c) *In situ* labelling during serial transfer of PUREΔAK1 systems containing pLD1 in gen1-3 and pLD2 in gen2c and gen3c. In all experiments, prestained markers were visible as shadows and used to verify the molecular weight of each POI. Gen0 represents the initial PURE boot-up system before incubation at 37 °C (see Methods).

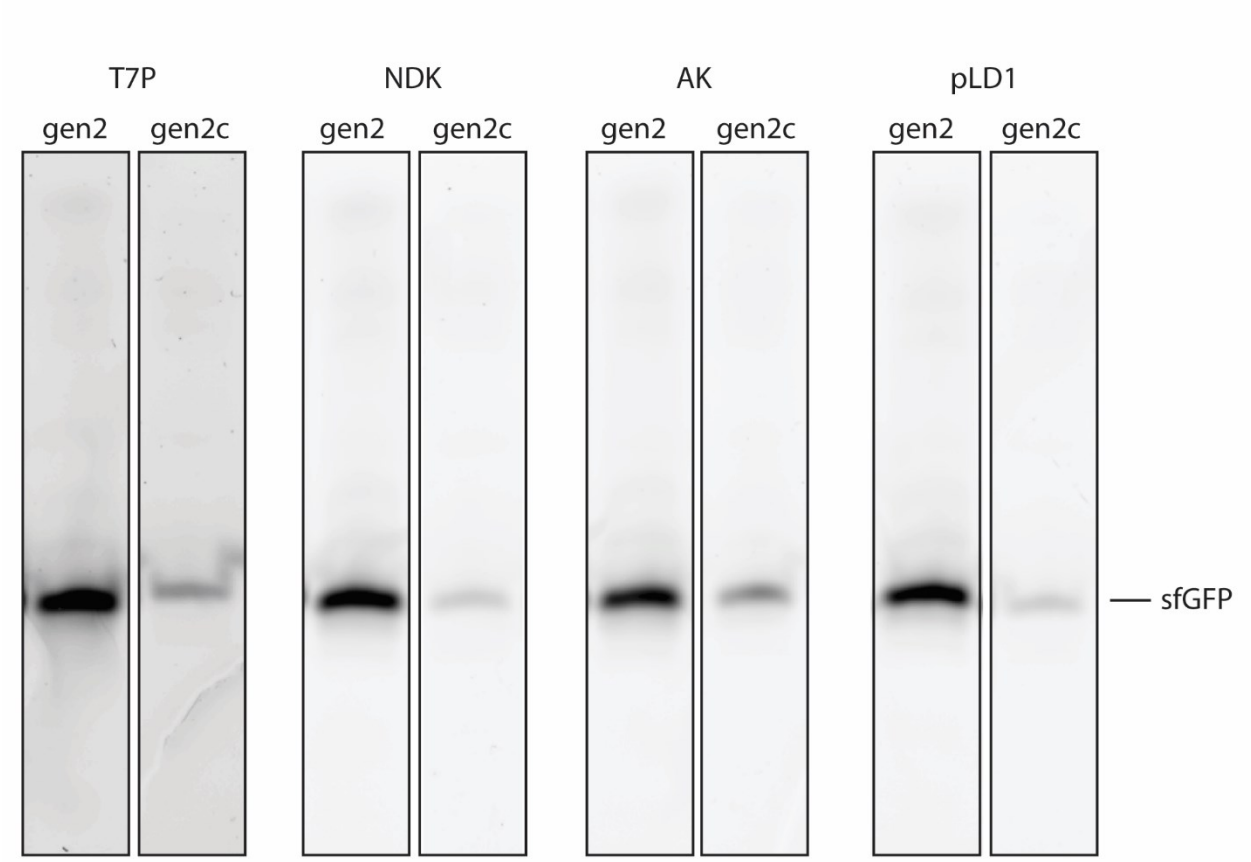

Fig. S2. In-gel visualisation of sfGFP expression for different PUREΔPOI samples after POI expression at gen2 / gen2c (Fig. 2 and Fig 3(a)). POIs were either T7-RNAP, NDK, AK, or the pLD1-protein fraction. POI(s) were expressed from their respective expression plasmid as described in the experimental section. Plasmid combinations including negative control plasmids are shown in Tab. S5.

Tab. S1: Plasmids used in this study

Plasmid	Source	Reference
pLD1, pLD2, pLD3	A. Forster, Uppsala University	[1]
pQE30-His-T7RNAP-t7p	Addgene, ID:124138	[2]
pQE30-His-CKM	Addgene, ID:124134	[2]
pQE30-His-NDK-t7p	Addgene, ID:124136	[3]
pET29b-AK1-His-t7p	Addgene, ID:118977	[3]
pEFTu	in-house	[5]
pIVEX2.3d-sfGFP	P. Schwille, MPI Biochemistry	[5]

Tab. S2: Buffers used during protein purification

Ingredient	*unit*	*lysis buffer*	*wash buffer*	*elution buffer*	*storage buffer*
HEPES·KOH pH 7.6	mM	50	-	50	50
Tris·HCl pH 7.5	mM	-	50	-	-
NH_4Cl	mM	250	-	250	-
KCl	mM	-	-	-	100
NaCl	mM	-	50	-	-
$MgCl_2$	mM	10	-	10	10
DTT	mM	5	5	5	7
imidazole	mM	-	-	300	-
glycerol	v/v %	-	-	-	30

Tab. S3: 4x energy mix composition

Compound	Concentration
K-glutamate (Sigma)	400 mM
Spermidine (Sigma)	10 mM
***E. coli* tRNA (Roche)**	216 OD_{260}/mL
ATP (NEB)	8 mM
GTP (NEB)	8 mM
CTP (NEB)	4 mM
UTP (NEB)	4 mM
sodium creatine phosphate (Sigma)	80 mM
folinic acid (Sigma)	1 mM
HEPES·KOH pH 8 (Sigma)	200 mM
Mg-glutamate (Sigma)	52 mM
DTT (Sigma)	20 mM

Tab. S4: 6x Enzyme Mix

Compound	Concentration
T7-RNAP	120 µg/mL
Myokinase	30 µg/mL
Creatine Kinase	60 µg/mL
NDK	12 µg/L
IPP (NEB)	6 U/mL
RNase Inhibitor (NEB)	1500 u/mL
EF-Tu	100 µg/mL
70S ribosomes	9 µM
Glycerol	15 (v/v) %
HEPES·KOH pH 8	50 mM
DTT (Sigma Aldrich)	5 mM

Tab. S5: POIs and their respective negative controls

POI plasmid (gen2, gen3)	control plasmid (gen2c, gen3c)
pT7	pNDK
pAK1	pT7
pNDK	pAK1
pLD1	pID2

Tab. S6: Relative band volumes, Figure 4a

band	gen1	gen2	gen3	gen2c	gen3c
ValRS	100%	84%	59%	41%	15%
LeuRS	100%	93%	70%	55%	20%
ThrRS / MetRS	100%	89%	69%	49%	12%
ArgRS / GlnRS / LysRS	100%	89%	70%	55%	15%
CysRS / SerRS / HisRS	100%	88%	68%	53%	14%
TyrRS / RF1	100%	87%	55%	50%	16%
TrpRS	100%	91%	72%	55%	22%

References

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4. Blanchard, S.C., Kim, H.D., Gonzalez, R.L. Jr, Puglisi, J.D., Chu S. tRNA dynamics on the ribosome during translation. *Proc Natl Acad Sci U S A.* **101**, 12893-8, 2004
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