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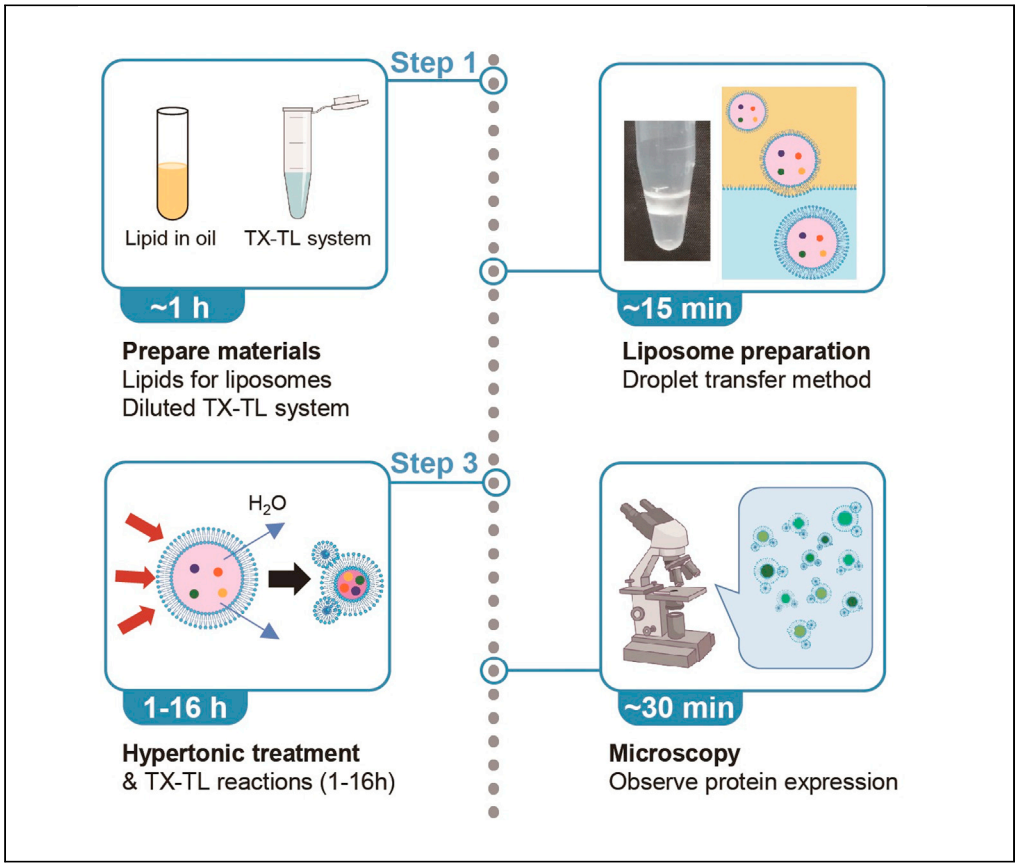
Protocol

Activation of a diluted *E. coli* cell-free transcription-translation system within liposomes by hypertonic concentration



We present a protocol for activating protein synthesis in liposomes encapsulating a diluted *E. coli* cell extract-based TX-TL (transcription-translation) system by hypertonic concentration. Protein expression is turned on in the liposome-encapsulated TX-TL system by simple treatment with a concentrated external solution. The expression of sfGFP is demonstrated here, but it can be applied to other proteins. This protocol can be applied to the development of artificial cells utilizing the switch-on mechanism to activate protein expression, responding to the outer environment.

Yukino Matsui, Toshiki Akui, Nobuhide Doi, Kei Fujiwara

fujiwara@bio.keio.ac.jp

Highlights

A diluted cell extract-based transcription translation system encapsulated in liposomes

Protein synthesis is activated inside liposomes by hypertonic treatment

Protocol can be applied to developing artificial cells utilizing the switch-on mechanism

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Activation of a diluted *E. coli* cell-free transcription-translation system within liposomes by hypertonic concentration

Yukino Matsui,[1] Toshiki Akui,[1] Nobuhide Doi,[1] and Kei Fujiwara[1,2,3,*]

[1]Department of Biosciences & Informatics, Keio University, 3-14-1 Hiyoshi, Kohokuku, Yokohama, Kanagawa 223-8522, Japan

[2]Technical contact

[3]Lead contact

*Correspondence: fujiwara@bio.keio.ac.jp
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SUMMARY

We present a protocol for activating protein synthesis in liposomes encapsulating a diluted *E. coli* cell extract-based TX-TL (transcription-translation) system by hypertonic concentration. Protein expression is turned on in the liposome-encapsulated TX-TL system by simple treatment with a concentrated external solution. The expression of sfGFP is demonstrated here, but it can be applied to other proteins. This protocol can be applied to the development of artificial cells utilizing the switch-on mechanism to activate protein expression, responding to the outer environment.

For complete details on the use and execution of this protocol, please refer to Akui et al. (2021).

BEFORE YOU BEGIN

Prepare S30 cell extract for cell-free TX-TL system

The TX-TL system required for this protocol can either be prepared from an *E. coli* cell-extract (Fujiwara and Doi, 2016) or reconstituted from individual components (PURE system, Shimizu et al., 2001). This section describes detailed steps for preparing an E coli cell extract-based TX-TL system.

Day 1

1. Prepare and autoclave the buffers and media as described below.
   a. 1 L of 2× YTPG media [Yeast Extract 10 g, Tryptone 16 g, NaCl 5 g, K_2HPO_4 7 g, KH_2PO_4 3 g, 15 mL of 1 M KOH to adjust pH7.2, and then, filled up with distilled water to 950 mL]. After autoclave, add 50 mL of 2 M filter-sterilized glucose.

   Note: Glucose should be added to the media after autoclave.

   Note: We typically use 2 L baffled culture flasks with foil for cell culture. It is no problem to use different sizes of baffled flasks or Sakaguchi flasks.

   b. S30A Buffer [10 mM BisTris, 60 mM GluK (potassium-glutamate), 10 mM $Mg(oAc)_2$]

   Note: Prepare 10 mL per 1 L culture.

   c. S30B Buffer [10 mM BisTris, 60 mM GluK, 14 mM $Mg(oAc)_2$]

Note: The amount needed varies according to the buffer exchange protocol. In the case of the Amicon filter method, prepare 100 mL per 1 L culture. In the case of dialysis protocol, prepare 1 L or more (it depends on the volume of cell extract).

d. 400 mM Sucrose

Note: Prepare 200 mL per 1 L culture. Store in the refrigerator and cool it on ice prior to use.

2. Cell Culture
   a. Inoculate *Escherichia coli* BL21(DE3) in 10 mL of 2× YTPG or LB.

Note: This protocol is not suitable for some *Escherichia coli* strains. It may be possible to obtain cell extract from other *E. coli* strains. For example, we confirmed that it was possible with MG1655 and BL21-CodonPlus(DE3) strains. However, some strains, such as BL21 Star (DE3) (it has a mutation in RNaseE gene) are not suitable. In most cases, we recommend using BL21(DE3) or BL21-CodonPlus(DE3) strains.

   b. Cultivate the cells for overnight (16–20 h) at 37°C with shaking (120–200 rpm).

Day 2

3. Cell Culture, Harvest
   a. Inoculate 1 L of 2×YTPG media with 10 mL of the o/n culture.
   b. Grow the culture at 37°C with shaking at 170 rpm until OD_{600} reaches 1.5–1.8, which requires 2–3 h cultivation.

Optional: When your template for TX-TL uses T7 promoter for gene expression, express T7 RNA polymerase encoded in DE3 gene by adding IPTG (0.1 mM final) at OD_{600} = 0.1.

⚠ CRITICAL: Collect cells at late log phase. OD_{600} should be measured after dilution of the cell culture with water or medium to ensure linear correlation between OD_{600} and cell number.

   c. Before harvesting, leave the culture for 15 min at room temperature (20°C–30°C). During the process, pre-cool the centrifuge and rotor for cell collection.
   d. Collect cells by centrifugation. For example, 15,000 × *g* centrifugation for 5 min at 4°C. Centrifugation speed is not critical.
   e. Remove the supernatant and keep the cell pellet on ice.

⚠ CRITICAL: Keep the sample on ice all the time after this step.

4. Lysozyme treatment
   a. Wash a paint brush with 5–10 mL of 400 mM sucrose in a 50 mL tube (Figure 1).
   b. Add 20 mL of 400 mM ice-cold sucrose to the cell pellet and resuspend softly using the brush. If you have more than one pellet, you can pour this cell suspension into the other pellet to gather cells in a single tube (Methods video S1).

Note: Resuspend all the cell pellets as homogenous as possible.

   c. After resuspending, pour the total cell suspension to a single 50 mL tube and centrifuge at 15,000 × *g* for 2 min at 4°C.
   d. Remove the supernatant and resuspend the cell pellet with 20 mL sucrose using the brush. Centrifuge again.
   e. Remove the supernatant and resuspend the cell pellet in 30 mL sucrose.

f. Add 150 μL of 20 mg/mL Lysozyme to the cell suspension and mix the cell suspension with moderate shakes until the lysozyme solution is homogeneously diffused. Repeat this step 3 times (in total you will add 600 μL lysozyme solution).
g. Keep the sample on ice for 1 h (lysozyme treatment).
h. Centrifuge at 15,000 × *g* for 2 min at 4°C.
i. Wash the cell pellet twice by repetitively resuspending in 30 mL sucrose and centrifuging to remove the supernatant.
j. After the final centrifugation step, remove the supernatant and measure the wet cell weight (typically 2 g/1 L culture).

5. Freeze and thaw
   a. Resuspend the cell pellet with the cold S30A Buffer (1.5 mL per gram wet cell pellet weight).
   b. Aliquot the cell suspension into 1.7 mL tubes (0.5–1 mL for each tube).
   c. Dip the tubes into liquid nitrogen for 5 min.
   d. Transfer the tubes to ice water until fully thawed (Use a floating tube rack and take care not to immerse the lid of the tube in ice water). To facilitate thawing, any ice that has formed around the tubes should be removed at about 3, 6, 9 min after the transfer. This step would take around 30 min.
6. Centrifuge for 1 h at 30,000 × *g* at 4°C.

7. Run-off reaction
   a. Dispense the supernatant into 1.7 mL tubes (1 mL for each tube).
   b. Incubate the tubes for 1 h at 37°C with shaking at 200 rpm.

   Note: The supernatant should become milky white during this step.

   Note: Pre-warm centrifuge to 37°C for the next step (7c).

   c. Centrifuge at 10,000 × *g* for 20 min at 4°C.
   d. Collect the supernatants in a single 15 mL tube or multiple 1.7 mL tubes (The merit of collection in multiple 1.7 mL tubes is faster thawing in step 8a).
   e. Store the collected supernatants as S30 cell extract at −20°C or below (this step can be skipped if the next step is going to be performed on the same day).

   Note: Flash-freezing is not required.

Day 3

8. Buffer exchange
   a. Leave the S30 extract on ice until completely thawed if frozen.
   b. Exchange the buffer with S30B buffer by dialysis (Usage of pore membrane smaller than 14 kDa MWCO and two-rounds dialysis is recommended) or Amicon Ultra centrifugal filter (10 kDa MWCO). In the case of Amicon filter, concentrate and dilute with the S30B Buffer, and repeat this cycle until the S30A Buffer is diluted 500 to 1000 times with the S30B buffer.
   c. Using the Amicon filter, concentrate the S30 extract until it reaches less than half the volume of the original S30 extract in step a
   d. Measure the concentration of the S30 cell extract using a BCA assay kit.
   e. Dispense 10–100 μL of S30 extract into tubes and store them at −20°C or less. Typically, about 30 mg cell extract is obtained from 1 g wet cells weight.

   Note: Flash-freezing is not required. But aliquot S30 extract for storage to avoid multiple freeze-thaw cycles. (Although we have not tested how many freeze-thaw cycles could significantly decrease the TX-TL activity, we find that the activity is not affected by up to 2 freeze-thaw cycles.)

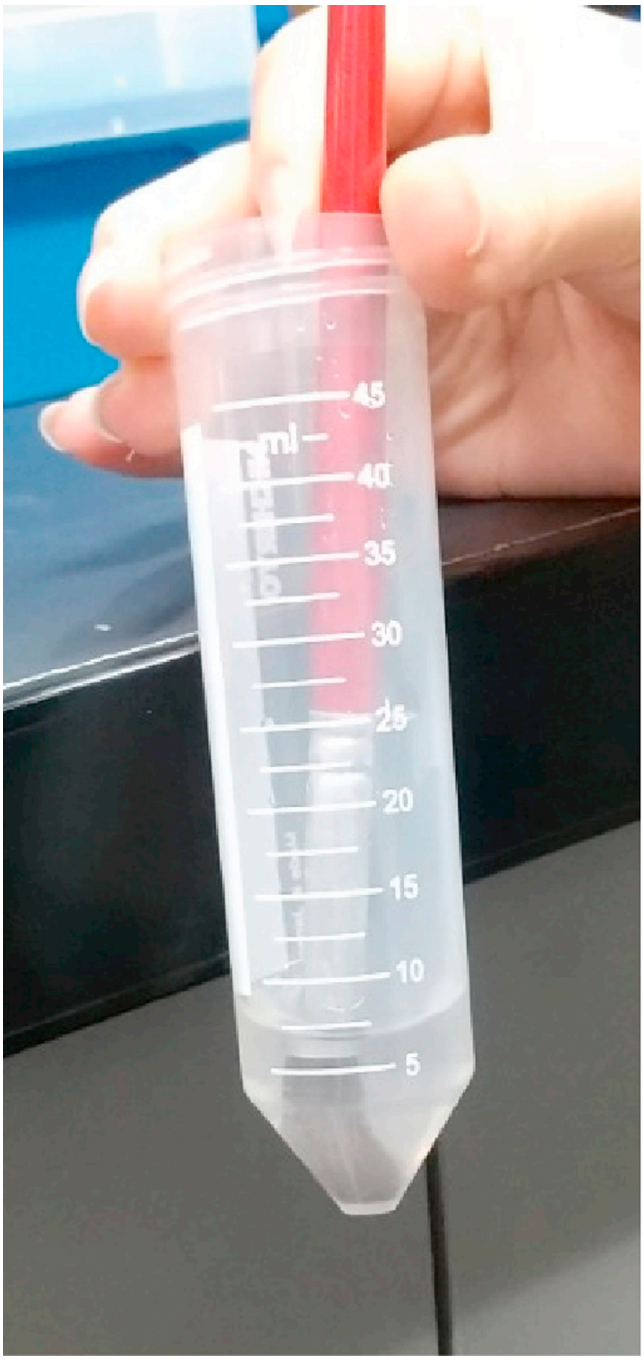

Figure 1. A paint brush washed with the sucrose solution.

Prepare an indicator for observation of liposomes

We recommend using a fluorescent indicator to aid identification of liposomes in the microscopic field; without a fluorescent indicator it is not easy to judge whether spherical objects observed in transmitted light are liposomes or not (since contaminant oil in water emulsion is also spherical).

In this section, we outline the steps for the preparation of mCherry.

Note: mCherry can be replaced with other fluorescent molecules if desired.

Day 1

9. Transform *E. coli* BL21 (DE3) with pET29-mCherry-His (Akui et al., 2021) using competent cells, and cultivate overnight (16–20 h) at 37°C on LB agar medium supplemented with 50 μg/mL kanamycin at final.

Day 2

10. In the evening, inoculate the transformant into 1 mL LB medium supplemented with 50 μg/mL kanamycin and cultivate the cells overnight (16–20 h) at 37°C with shaking at 120–200 rpm.

Day 3

11. Inoculate 1 L of LB medium containing 50 μg/mL kanamycin with 1 mL of the precultured cells.
12. Cultivate the cells at 37°C with shaking 120–200 rpm to reach OD_{600} = 0.1–0.4.
13. Add 0.1 mM IPTG and cultivate overnight (16–20 h) at 18°C with shaking at 120–200 rpm.

Day 4

14. In the morning, collect the cells and resuspend the cell pellet in 10 mL of Binding Buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 40 mM Imidazole].
15. Disrupt the cells by sonication. During sonication, the tubes should be cooled with ice water.
16. Collect the supernatant after 15,000 × *g* centrifuge for 10 min at 4°C.
17. Purify the mCherry from the supernatant by using HisTrap column (or equivalent) following the manufacturer's instructions. For wash and elution, the Binding Buffer and Elution Buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 400 mM Imidazole] are used, respectively.
18. Exchange the buffer with 20 mM Hepes-KOH (pH 7.6) by dialysis or the Amicon Filter method. Because 16 μM mCherry is required for the preparation of the TX-TL system solution below, concentrate mCherry by the Amicon Filter if its concentration is lower than 16 μM.

⚠ CRITICAL: It is important to reduce the osmotic pressure derived from the buffer for the hypertonic concentration for protein expression. NaCl and Imidazole concentration derived from mCherry purification should be less than 1 mM in the final solution for the TX-TL system. We do not recommend the addition of glycerol to the purification buffers.

19. Dispense 10–100 μL of the mCherry solution into tubes and store at −20°C or below.

Note: Flash-freezing is not required.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli BL21 (DE3)	New England BioLabs	Cat# C2527
Chemicals, peptides, and recombinant proteins		
Folinic acid	Tokyo Chemical Industry	Cat# C2235
Ultrapure water	Thermo Fisher Scientific	Cat# 10977023
Potassium glutamate	Sigma-Aldrich	Cat# G1501-1KG
Magnesium glutamate	Sigma-Aldrich	Cat# 49605-250G

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Magnesium acetate	Nacalai Tesque	Cat# 208-21
tRNA	Roche	Cat# 10109541001
Creatine kinase	Roche	Cat# 10127566001
ATP	Wako Chemicals	Cat# 014-16913
GTP	Wako Chemicals	Cat# 073-03113
CTP	Tokyo Chemical Industry	Discontinued
UTP	Affymetrix	Discontinued
IPTG	Nacalai Tesque	Cat# 19742-94
Tris(hydroxymethyl)aminomethane	Nacalai Tesque	Cat# 35434-05
NaCl	Nacalai Tesque	Cat# 31320-05
Imidazole	Nacalai Tesque	Cat# 19004-35
HEPES	Nacalai Tesque	Cat# 17546-05
HCl	Nacalai Tesque	Cat# 18321-05
KOH	Nacalai Tesque	Cat# 28616-45
Sucrose	Nacalai Tesque	Cat# 30404-45
Ala	Nacalai Tesque	Cat# 01115-22
Cys	Nacalai Tesque	Cat# 10309-12
Asp	Nacalai Tesque	Cat# 03561-12
Glu	Nacalai Tesque	Cat# 16911-22
Phe	Nacalai Tesque	Cat# 26910-22
Gly	Nacalai Tesque	Cat# 17109-35
His	Nacalai Tesque	Cat# 18116-92
Ile	Nacalai Tesque	Cat# 20330-02
Lys	Tokyo Chemical Industry	Cat# L0129
Leu	Nacalai Tesque	Cat# 20327-62
Met	Nacalai Tesque	Cat# 21719-02
Asn	Nacalai Tesque	Cat# 03427-82
Pro	Nacalai Tesque	Cat# 29001-42
Gln	Nacalai Tesque	Cat# 16919-42
Arg	Nacalai Tesque	Cat# 03321-65
Ser	Nacalai Tesque	Cat# 30608-72
Thr	Nacalai Tesque	Cat# 33820-82
Val	Nacalai Tesque	Cat# 36108-42
Trp	Wako Chemicals	Cat# 202-03383
Tyr	Nacalai Tesque	Cat# 35709-02
Spermidine	Nacalai Tesque	Cat# 32108-91
Creatine phosphate	Wako Chemicals	Cat# 030-04584
Chloroform	Nacalai Tesque	Cat# 08402-55
1-Palmitoyl-2-Oleoylphosphatidylcholine (POPC)	Avanti Polar Lipids	Cat# 850457C
Lysozyme	Nacalai Tesque	Cat# 19499-04
Ampicillin	Nacalai Tesque	Cat# 02739-32
Kanamycin	Nacalai Tesque	Cat# 19860-44
Yeast Extract	Gibco	Cat# 212750
Tryptone	Nacalai Tesque	Cat# 35640-95
NaCl	Nacalai Tesque	Cat# 31319-45
K_2HPO_4	Nacalai Tesque	Cat# 28727-95
KH_2PO_4	Nacalai Tesque	Cat# 28721-55
Mineral oil	Nacalai Tesque	Cat# 23306-84
Glucose	Nacalai Tesque	Cat# 16805-35
Critical commercial assays		
HisTrap HP	GE Healthcare	Cat# 17524701
Amicon-Ultra 15 filter 10k	Merck Millipore	Cat# UFC901096
BCA Assay kit	Thermo Fisher Scientific	Cat# 23225
Recombinant DNA		

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
pET29-mCherry-His	Akui et al. (2021)	N/A
pOR2OR1-sfGFP	Fujiwara and Doi (2016)	N/A
Others		
molecular sieve 4A	Nacalai Tesque	Cat# 04171-05
Silicone rubber sheet	TOGAWA Rubber Co. Ltd.	Cat# K-125(50)

Note: All reagents and supplies can be replaced with alternatives.

MATERIALS AND EQUIPMENT

Mineral oil

Just after opening the mineral oil tube, we always treat the oil with a molecular sieve to remove water and other small chemicals which can be contaminated from environments. Add 10 g of molecular sieve 4A to 100 mL mineral oil. [Store at room temperature (20°C–30°C)].

2× YTPG medium	
Reagent	Amount
Yeast Extract	10 g
Tryptone	16 g
NaCl	5 g
K_2HPO_4	7 g
KH_2PO_4	3 g
KOH (1M)	15 mL
Glucose (2M)	50 mL
Distilled water	935 mL
Total	**1 L**

Note: Glucose should be added after autoclaving the medium that contains all the rest.

S30A Buffer			
Reagent	Initial concentration (mM)	Final concentration (mM)	Volume (mL)
Hepes-KOH pH7.6	2000	10	0.05
GluK	4000	60	0.15
$Mg(oAc)_2$	1000	10	0.14
Ultrapure Water		n/a	9.66
Total		n/a	10

Note: Autoclave before use.

Note: A month's storage at 4°C is assured.

S30B Buffer			
Reagent	Initial concentration (mM)	Final concentration (mM)	Volume (mL)
Hepes-KOH pH7.6	2000	10	0.5
GluK	4000	60	1.5
$Mg(oAc)_2$	1000	14	1.4
Ultrapure Water		n/a	96.6
Total		n/a	100

Note: Autoclave before use.

Note: A month's storage at 4°C is assured.

Binding Buffer			
Reagent	Initial concentration (mM)	Final concentration (mM)	Volume (mL)
Tris-HCl (pH8.0)	1000	50	10
NaCl	1000	500	100
Imidazole	1000	40	8
Ultrapure Water	n/a	n/a	82
Total	n/a	n/a	200

Note: Autoclave before use.

Note: Two years storage at 4°C is assured.

Elution Buffer			
Reagent	Initial concentration (mM)	Final concentration (mM)	Volume (mL)
Tris-HCl (pH8.0)	1000	50	10
NaCl	1000	500	100
Imidazole	1000	400	80
Ultrapure Water	n/a	n/a	10
Total	n/a	n/a	200

Note: Autoclave before use.

Note: Two years storage at 4°C is assured.

50× NTP mixture			
Reagent	Initial concentration (mM)	50× Final concentration (mM)	Volume (μL)
ATP	500	100	20
GTP	500	100	20
CTP	500	65	13
UTP	500	65	13
Ultrapure water	n/a	n/a	34
Total	**n/a**	**n/a**	**100**
Store at −20°C or below. Two years storage is assured.			

Note: 1× NTP indicates a mixture of 2.0 mM ATP, 2.0 mM GTP, 1.3 mM UTP, and 1.3 mM CTP.

5× reaction mixture			
Reagent	Initial concentration	5× Final concentration	Volume (μL)
Hepes-KOH pH7.6	2000 mM	250 mM	12.5
GluK	4000 mM	500 mM	12.5
tRNA mixture	51840 μg/mL	990 μg/mL	1.9
FD (folinic acid)	33.9 mM	0.34 mM	1
Creatine phosphate	1000 mM	400 mM	40

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Continued			
Reagent	Initial concentration	5× Final concentration	Volume (μL)
20 amino acids mixture	20 mM each	3 mM each	15
Spermidine	500 mM	5 mM	1
50× NTP mixture	50×	5×	10
IPTG	1000 mM	5 mM	0.5
Ultrapure water	n/a	n/a	5.6
Total	n/a	n/a	100
Store at -20°C or below. Two years storage is assured.			

Note: 1 μL of 5× reaction mixture is used to prepare a 5 μL TX-TL system. Scale up or down the total stock amount for your purpose.

Note: 1× reaction mixture indicates a mixture of 50 mM Hepes-KOH (pH7.6), 100 mM GluK, 198 μg/mL tRNA mixture, 0.068 mM FD, 80 mM Creatine phosphate, 0.6 mM 20 amino acids mixture, 1 mM Spermidine, 1× NTP mixture, and 1 mM IPTG.

Note: 20 amino acids mixture is prepared by dissolving 20 amino acids in Ultrapure water.

TX-TL system solution			
Reagent	Initial concentration	Final concentration	Volume (μL)
5× reaction mixture	5×	1×	1
Cell extract	40 mg/mL	10 mg/mL	1.25
GluMg	200 mM	11.5 mM	0.3
mCherry	16 μM	4 μM	1.25
Creatine kinase	3000 μg/mL	300 μg/mL	0.5
DNA (pOR2OR1-sfGFP)	200 nM	20 nM	0.5
Ultrapure water	n/a	n/a	0.2
Total	**n/a**	**n/a**	**5 μL**
Prepare before use.			

Note: In this protocol, the final concentration of magnesium in the TX-TL system is set as 15 mM in total. Because S30 cell extract contains 14 mM magnesium, 3.5 mM magnesium in the TX-TL system is derived from the S30 cell extract. GluMg added is to compensate for the rest.

Alternatives: 5× reaction mixture, Cell extract, GluMg and Creatine kinase can be replaced with PURE system.

External buffer			
Reagent	Initial concentration	Final concentration	Volume (μL)
5× reaction mixture	5×	1 x	20
S30B Buffer	1×	0.25×	25
GluMg	200 mM	11.5 mM	5.8
Hepes-KOH (pH 7.6)	20 mM	5 mM	25
Ultrapure water	n/a	n/a	24.3
Total	**n/a**	**n/a**	**100**
Store at −20°C or below. Two years storage is assured.			

⚠ CRITICAL: The osmotic pressure of the External buffer should be the same as TX-TL system solution. S30B Buffer is added to compensate for the small molecules that cell extract contains in the TX-TL system solution. Hepes-KOH (pH 7.6) is the replacement for mCherry.

Note: 20 μL of the External buffer is used to prepare one liposome sample. Scale up or down the total stock amount for your purpose.

2× External buffer

Reagent	Initial concentration	Final concentration	Volume (μL)
5× reaction mixture	5×	2×	40
S30B Buffer	1×	0.5×	50
GluMg	1000 mM	23 mM	2.3
Hepes-KOH (pH 7.6)	200 mM	10 mM	5
Ultrapure water	n/a	n/a	2.7
Total	**n/a**	**n/a**	**100**

Store at −20°C or below. Two years storage is assured.

Note: 20 μL of 2× External buffer is used for one hypertonic treatment. Scale up or down the total stock amount for your purpose.

Silicon spacer

To make the silicon spacer for microscopic observation, cut a silicone rubber sheet (0.5 or 1 mm thick) into 1 cm squares and make a hole in it by using a hole punch.

STEP-BY-STEP METHOD DETAILS

Preparation of liposomes encapsulating a diluted *E. coli* TX-TL system

Timing: 90 min

In this step, liposomes are prepared by a modified droplet-transfer method (Fujiwara and Yanagisawa, 2014)

1. Preparation of lipid solution
   a. Dry 40 μL of 25 mg/mL POPC (dissolved in chloroform and stored at −20°C in a round glass microtube) under Ar or N_2 gas flow. We typically perform this process without using a vacuum desiccator.
   b. Add 500 μL mineral oil to the dried POPC.
   c. Incubate the lipid solution at 80°C for 1 h (e.g., in an Aluminum block incubator). Do not cap the tube in this process.
   d. Cool the tube to room temperature (20°C–30°C).
   e. After capping the tube by an attached screw cap, vortex the lipid solution for 1 min.

   Note: The lipid solution can be stored at room temperature (20°C–30°C) for at least more than several hours with the tube wrapped by aluminum foil. Vortex again before the emulsification (step 2).

2. Emulsification
   a. Prepare the TX-TL system solution on ice.
   b. Add 20 μL of ultrapure water to 5 μL of the TX-TL system to generate a 1/5-fold dilution.
   c. Add 50 μL of the lipid solution into a 0.6 mL tube and pipette 1 μL of the diluted TX-TL system to the lipid solution.

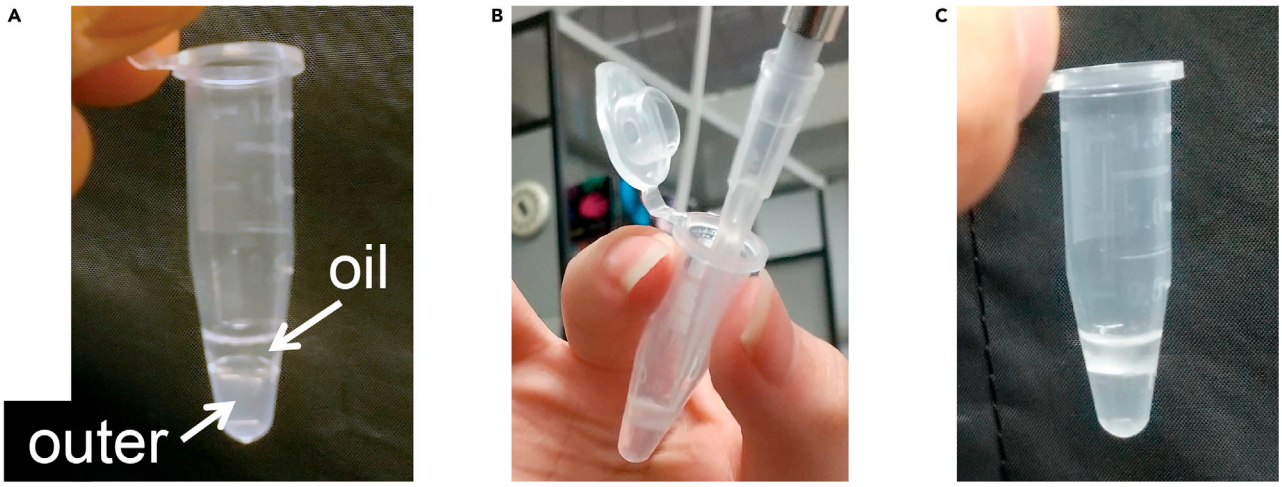

Figure 2. Pipetting emulsion onto the oil phase

(A) Lipids in oil is added on top of the diluted External buffer.

(B) Emulsion is pipetted onto the oil phase.

(C) A resultant solution with phases. The solution is centrifuged to form liposomes at the next step.

Note: From this step, prepare samples at room temperature (20°C–30°C) .

d. Tap the tube about 10 times for emulsification.

Note: The size range of liposomes depends on how strong and how many times you tap the tube in this step. We recommend preparing liposomes larger than 5 μm (diameter) for microscopic observation. You may need to optimize this step in order to get an appropriate size range.

Note: Keep the emulsified solution at room temperature (20°C–30°C) until used in step 3d. Tapping the tube might be required to form emulsions again just before step 3d.

3. Preparation of liposomes
   a. Prepare the External buffer.
   b. Add 80 μL of ultrapure water to 20 μL of the External buffer in a 1.7 mL tube to generate a 1/5-fold dilution (in order to balance the osmotic pressure between the inside and outside of the liposomes).
   c. Carefully add 50 μL of the lipid solution (prepared in step 1) on top of the diluted External buffer with pipette (Figure 2).
   d. Add 50 μL of the emulsified solution (prepared in step 2) onto the oil phase with the lipids (Figure 2).

⚠ CRITICAL: Be careful not to mix the emulsified solution with the phase of diluted External buffer (water phase).

e. Centrifuge at 20,000 × *g* for 5 min at 4°C and remove the oil phase.

Note: Remove as much of the oil phase as possible. Contaminant oil forms a liposome-like sphere structure in the solution (oil in water emulsion).

f. Recover liposomes by gently pipetting the bottom of the tube and transfer the liposome solution into a new 0.6 mL tube.

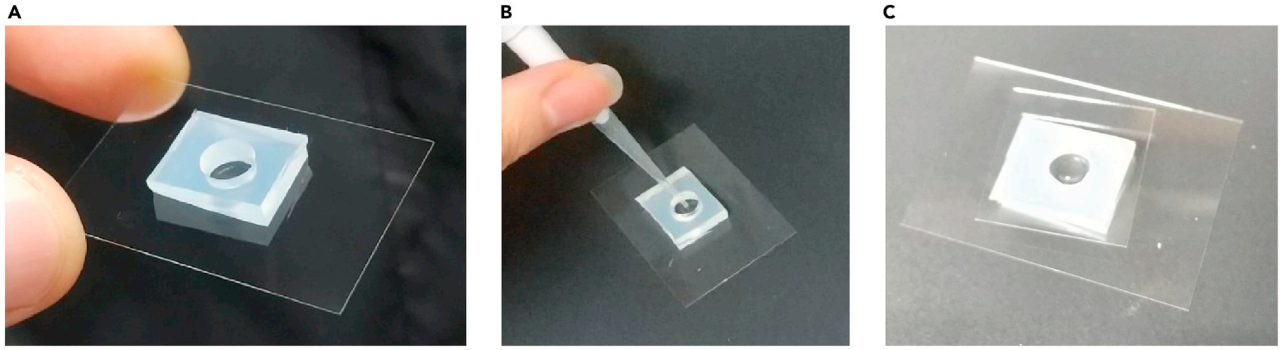

Figure 3. A unit spaced with silicon spacer for microscopic observation of liposomes
(A) A silicon spacer spotted on the cover glass.
(B) Spotting liposome solution.
(C) The silicon spacer chamber covered with another cover glass.

Activation of the TX-TL system inside liposomes by hypertonic treatment

Timing: 1–16 h. (Depending on reaction time.)

In this step, the prepared liposomes are treated by osmolytes to concentrate molecules within liposomes, and the activation of the TX-TL system is confirmed by measuring sfGFP fluorescence. A fluorescent microscope is used to obtain the fluorescent images. We use sfGFP expression as a demonstration of this protocol. Other genes should be expressed by the protocol. We have confirmed the expression of α-hemolysin by the protocol (Akui et al., 2021).

4. For the experimental condition, mix 20 μL of 2× External buffer with 20 μL of liposome solution in a 1.7 mL tube by gently pipetting. As a negative control (without hypertonic treatment), mix 20 μL 1/5-diluted External buffer with 20 μL of liposome solution in another 1.7 mL tube by gently pipetting.
5. Incubate the sample at 25°C for an appropriate time (1–16 h).

Note: The activation of the TX-TL system starts as soon as you add a 2× External buffer to the liposome solution. It may take 30 min to an hour for the sfGFP to be expressed to a detectable level. (In the case that T7 promoter is used for DNA template, the speed of protein synthesis and the final amount will be higher than when the endogenous promoter is used.)

6. Pipette the reacted sample (hypertonic treatment) into the central chamber made of silicon spacer on a slide glass (Figure 3). Do the same for the negative control on a separate glass slide.

Note: To prevent the reacted sample from drying, cover the sample with another slide glass (Figure 3), especially if the pipetted sample volume is a few microliters or the observation takes a long time.

7. Observe the fluorescence of mCherry (indicator) and sfGFP (synthesized protein), respectively, by fluorescent microscope.

EXPECTED OUTCOMES

Tens to hundreds of liposomes are collected by the protocol, and at least 72% liposomes show synthesis of sfGFP in cytosols (see also Akui et al, 2021). Typical data obtained by the protocol is shown in Figure 4.

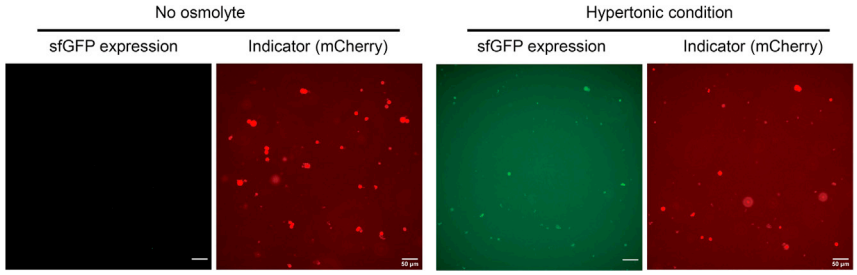

Figure 4. A representative result obtained by this protocol (16 h incubation)
The thresholds of Indicator (mCherry) images are differently adjusted among the two conditions to clearly show the position of liposomes in both cases. Thus, the fluorescent levels of indicator (mCherry) do not correlate with the actual mCherry concentrations: mCherry is concentrated by the hypertonic treatment.

LIMITATIONS

The TX-TL activity inside liposomes can't be switched on by more than 5-fold hypertonic treatment, although it was confirmed that macromolecules inside liposomes can be concentrated up to 30 times by hypertonic treatment (Akui et al, 2021). (The osmotic pressure ratio of the outer solution to the inner solution of liposomes determines the concentration factor by hypertonic treatment. if the ratio is 5, the concentration of macromolecules inside liposomes become 5-times higher by the treatment.)

TROUBLESHOOTING

Problem 1

No or low TX-TL activity of prepared S30 cell extract. (Before you begin, prepare S30 cell extract for cell-free TX-TL system)

Potential solution

(i) Check the timing of the cell collection in step 3b. The activity of the TX-TL system will significantly decrease if stationary phase cells were collected. Optimal OD_{600} for cultivation may depend on the apparatus to measure OD_{600}. Typically, less than half of the maximal OD_{600} is the best OD_{600} for collecting cells. (ii) Cell lysis and the centrifugation to collect the extract after cell lysis can be a critical step for activity. It takes at least 30 min to collect sufficient volume of the supernatant (0.3–0.7 mL per 1 mL cell suspension) by centrifugation in step 6. If cell debris were completely pelleted within a few min centrifugation, either the cell disruption failed (In this case, cell extract seems to be clear) or the timing of cell harvest was too late (In this case, cell extract shows yellow color). A small volume of supernatant (less than 0.3 mL per 1 mL cell suspension) suggests extensive cell lysis. In this case, the timing of cell harvest was too early, or lysozyme treatment was too strong. In the latter case, decrease amounts of lysozyme or shorten the reaction time. (iii) Otherwise, try other preparation methods, which use French press, sonication, or other apparatus for cell disruption. (iv) Cell extract can be replaced with a reconstituted transcription-translation system (PURE system) in the original study (Akui et al, 2021).

Problem 2

Poor emulsification. (Step-by-step method details, preparation of liposomes encapsulating a diluted E. coli TX-TL system, step 2 Emulsification). Poor emulsification will lead to poor liposome formation. In some cases, emulsion disappears after several minutes, with water and oil phases separating.

Potential solution

(i) Minimize the time between emulsification and liposome preparation (i.e., these two steps should be performed within about 5 min of each other). Tap the tube and form emulsions again just before adding to the next step. If the time is not the cause, it is mainly attributed to the condition of lipids solution. Primarily, (ii) Vortex the lipid solution (prepared in step-by-step method details, 1.

Preparation of lipid solution) just before pipetting the diluted TX-TL system for emulsification. (iii) Try an alternative method for preparing lipids solution, such as sonication in a hot water bath (Kohyama et al., 2019, (Kohyama et al., 2020)). Finally, (iv) Water contamination in lipids should be concerned: Any water contamination should be evaporated in a vacuum desiccator for several hours after drying chloroform.

Problem 3

Poor liposome yield. (Step-by-step method details, preparation of liposomes encapsulating a diluted E. coli TX-TL system, step 3 Preparation of liposomes)

Potential solution

Primarily, check if emulsions are successfully prepared by using a microscope. (step 2 Emulsification). If emulsions are not formed, refer to Problem 2 & Potential solution. If emulsions are successfully formed, (i) check if the osmotic pressure between the inner solution (TX-TL system) and the outer solution (External Buffer) are balanced in step 3 Preparation of liposomes. Refer to the CRITICAL in materials and equipment, External buffer. (ii) Alternative solutions may be found in the condition of lipid solution. Use the lipid solution prepared on the same day. If liquid paraffin is used instead of mineral oil in step 1 Preparation of lipid solutions, we recommend to use liquid paraffin that is just after opened or stored in the freezer after it was opened.

Problem 4

No liposome was found in the microscopic field. (Step-by-step method details, activation of the TX-TL system inside liposomes by hypertonic treatment, step 7)

Potential solution

(i) Increase the concentration of fluorescent proteins or molecules, the indicator of liposome position: mCherry in this protocol. (ii) Refer to Problem 3 & Potential solution.

Problem 5

Protein expression could not be detected in liposomes after hypertonic treatment (e.g., the fluorescence intensity of GPF in the experimental condition was not significantly stronger than that in the negative control). (Step-by-step method details, activation of the TX-TL system inside liposomes by hypertonic treatment, step 7)

Potential solution

Primarily, check if the TX-TL system solution has the activity in a test tube. If TX-TL works in the case of the tube reaction, lipid membrane or semipermeability is the cause of the low TX-TL activity inside liposomes. If polar lipids or other anionic lipids are used, negative charges of lipids affect TX-TL activity. In this case, add 10 mg/mL or more BSA to the TX-TL system like in our previous study (Yoshida et al., 2019). If lipids with neutral charges are used, but the TX-TL activity is low, some essential small molecules for TX-TL may leak out from liposomes due to the semi-permeability of lipid membranes or the hypertonic treatment. In this case, please make sure that the External buffer contains the essential small molecules for TX-TL reaction.

RESOURCE AVAILABILITY

Lead contact

[Kei Fujiwara] (fujiwara@bio.keio.ac.jp).

Materials availability

Plasmids constructed in this study are available from the corresponding author on request.

Data and code availability

This paper does not report original code. For questions regarding this study, please contact the corresponding author.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101003.

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AUTHOR CONTRIBUTIONS

Y.M., T.A., and K.F. established the protocol. T.A., N.D., and K.F. contributed to the original set-up of the experimental system. Y.M. and K.F. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Akui, T., Fujiwara, K., Sato, G., Takinoue, M., Nomura, S.M., and Doi, N. (2021). System concentration shift as a regulator of transcription-translation system within liposomes. iScience *24*, 102859.

Fujiwara, K., and Doi, N. (2016). Biochemical preparation of cell extract for cell-free protein synthesis without physical disruption. PLoS One *11*, e0154614.

Fujiwara, K., and Yanagisawa, M. (2014). Generation of giant unilamellar liposomes containing biomacromolecules at physiological intracellular concentrations using hypertonic conditions. ACS Synth. Biol. *3*, 870–874.

Kohyama, S., Fujiwara, K., Yoshinaga, N., and Doi, N. (2020). Conformational equilibrium of MinE regulates the allowable concentration ranges of a protein wave for cell division. Nanoscale *12*, 11960–11970.

Kohyama, S., Yoshinaga, N., Yanagisawa, M., Fujiwara, K., and Doi, N. (2019). Cell-sized confinement controls generation and stability of a protein wave for spatiotemporal regulation in cells. eLife *8*, e44591.

Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001). Cell-free translation reconstituted with purified components. Nat. Biotechnol. *19*, 751–755.

Yoshida, A., Kohyama, S., Fujiwara, K., Nishikawa, S., and Doi, N. (2019). Regulation of spatiotemporal patterning in artificial cells by a defined protein expression system. Chem. Sci. *10*, 11064–11072.