Supplementary Information

Bringing light into cell-free expression

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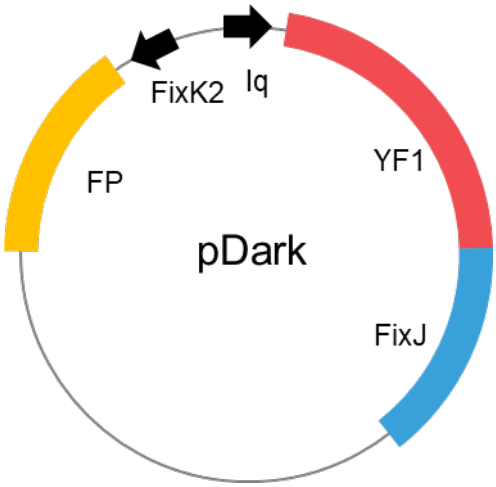
DNA part sequences

pDark



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Note: The underlined part is the *yf1* gene sequence, and the bold part is *fixj* gene sequence.

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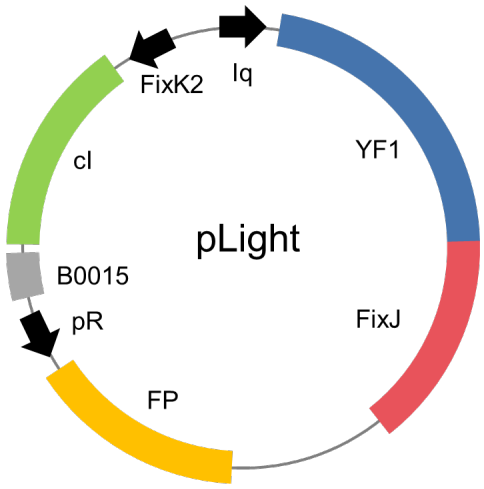

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Note: The dotted line part is the *cI* gene sequence, the solid line part is the *yfl* gene sequence, and the bold part is *fixj* gene sequence.

Table S1 Characteristics of two blue-light sensor systems

Optogenetic system	Cofactor/ Chromophore	Wavelength λ on/off	Time of activation	Induction times	Mechanism
YF1/FixJ	FMN	dark/470nm	Seconds	20	Phosphorylation
EL222	FMN	450nm/dark	Seconds	<5	Homodimerization

Table S2 The feature of cell extracts

Cell Extract	Feature
BL21 (DE3)	BL21 (DE3) contains the DE3 region of λ phage that contains T7 phage RNA polymerase. It is suitable for expressing non-toxic proteins.
BL21 Star (DE3)	BL21 Star (DE3) contains *rne131* gene mutant, which could enhance the stability of mRNA.

Table S3 Information of promoter, RBS and part gene sequence

Name	Part type	Sequence
T7	Promoter	CCTATAGTGAGTCGTATTA
J23100	Promoter	GCTAGCACTGTACCTAGGACTGAGCTAGCCGTCAA
J23101	Promoter	GCTAGCATAATACCAAGGACTGAGCTAGCTGTAAA
J23107	Promoter	GCTAGCATAATACCTAGGGCTGAGCTAGCCGTAAA
J23115	Promoter	GCTAGCATTGTACCAAGGGCTGAGCTAGCTATAAA
UTR1	RBS	TCTCCTTCTTAAAGTTAAACAAA
gcoyf1	Gene	ATGGCCAGCTTTCAGAGCTTTGGTATCCCGGGTCAGCTGGAAGTTATCAAGAAGGCTTTAGACCA TGTGCGTGTGGGCGTTGTGATCACCGATCCGGCTTTAGAAGATAATCCGATTGTGTACGTGAATC AAGGTTTCGTTCAGATGACCGGTTACGAAACCGAGGAGATTTTAGGTAAGAATTGCCGCTTTCTG CAAGGTAAGCATACCGACCCGGCCGAAGTTGATAACATCCGCACCGCACTGCAGAACAAGGAAC CGGTGACCGTGCAGATCCAAAACTACAAAAAAGATGGCACAATGTTTTGGAACGAACTGAACAT CGACCCGATGGAGATCGAGGATAAGACCTATTTTGTTGGCATTCAGAACGATATTACCGAACATC AGCAGACACAAGCTCGTCTGCAAGAACTGCAGAGCGAACTGGTGCACGTTAGCCGTTTAAGTGC AATGGGTGAAATGGCAAGTGCACTGGCCCATGAACTGAATCAGCCGCTGGCCGCCATCAGCAAC TATATGAAAGGTAGCCGCCGTTTACTGGCTGGTAGTAGTGATCCGAACACCCCGAAAGTTGAGA GCGCACTGGATCGTGCCGCAGAGCAAGCTTTACGTGCTGGTCAGATTATTCGCCGTTTACGTGAT TTTGTGGCACGCGGCGAAAGTGAGAAACGCGTGGAATCTTTAAGCAAGCTGATTGAAGAGGCTG GTGCATTAGGTCTGGCTGGTGCCCGCGAACAGAATGTGCAGCTGCGCTTTAGTCTGGATCCGGGT GCAGATCTGGTGCTGGCCGACCGCGTTCAGATCCAGCAAGTTCTGGTTAATTTATTTCGCAATGC TTTAGAAGCCATGGCCCAGAGTCAGCGCCGTGAACTGGTGGTTACCAACACCCCGGCAGCCGAT GATATGATCGAAGTGGAGGTGAGCGATACTGGTAGCGGCTTTCAAGATGATGTGATTCCTAATCT GTTTCAGACCTTCTTTACCACCAAAGACACCGGCATGGGCGTTGGTTTAAGCATTAGCCGCAGCA TTATTGAAGCCCATGGCGGCCGTATGTGGGCCGAAAGTAATGCCAGCGGCGGCGCCACATTTCG CTTTACTTTACCCGCTGCCGATGAAATGATTGGTGGTTTAGCCTAA
msfeoyf1	Gene	ATGGCAAGCTTTCAATCATTTGGGATACCAGGACAGCTGGAAGTCATCAAAAAAGCACTTGATC ACGTGCGAGTCGGTGTGGTAATTACAGATCCCGCACTTGAAGATAATCCTATTGTCTACGTAAAT CAAGGCTTTGTTCAAATGACCGGCTACGAGACCGAGGAAATTTTAGGAAAGAACTGTCGCTTCTT ACAGGGGAAACACACAGATCCTGCAGAAGTGGACAACATCAGAACCGCTTTACAAAATAAAGA ACCGGTCACCGTTCAGATCCAAAACTACAAAAAAGACGGAACGATGTTCTGGAATGAATTAAAT ATTGATCCAATGGAAATAGAGGATAAAACGTATTTTGTCGGAATTCAGAATGATATCACCGAGC ACCAGCAGACCCAGGCGCGCCTCCAGGAACTGCAATCCGAGCTCGTCCACGTCTCCAGGCTGAG CGCCATGGGCGAAATGGCGTCCGCGCTCGCGCACGAGCTCAACCAGCCGCTGGCGGCGATCAGC AACTACATGAAGGGCTCGCGGCGGCTGCTTGCCGGCAGCAGTGATCCGAACACACCGAAGGTCG AAAGCGCCCTGGACCGCGCCGCCGAGCAGGCGCTGCGCGCCGGCCAGATCATCCGGCGCCTGCG CGACTTCGTTGCCCGCGGCGAATCGGAGAAGCGGGTCGAGAGTCTCTCCAAGCTGATCGAGGAG GCCGGCGCGCTCGGGCTTGCCGGCGCGCGCGAGCAGAACGTGCAGCTCCGCTTCAGTCTCGATC CGGGCGCCGATCTCGTTCTCGCCGACCGGGTGCAGATCCAGCAGGTCCTGGTCAACCTGTTCCGC AACGCGCTGGAAGCGATGGCTCAGTCGCAGCGACGCGAGCTCGTCGTCACCAACACCCCCGCCG CCGACGACATGATCGAGGTCGAAGTGTCCGACACCGGCAGCGGTTTCCAGGACGACGTCATTCC GAACCTGTTTCAGACTTTCTTCACCACCAAGGACACCGGCATGGGCGTGGGACTGTCCATCAGCC GCTCGATCATCGAAGCTCACGGCGGGCGCATGTGGGCCGAGAGCAACGCATCGGGCGGGGCGAC CTTCCGCTTCACCCTCCCGGCAGCCGACGAGATGATAGGAGGTCTAGCATGA

Name	Part type	Sequence
NbCEA5-His	Gene	ATGCAAGTTCAGCTGGTTGAAAGCGGTGGTGGTAGCGTTCAAGCCGGTGGCAGTCTGC GTCTGAGCTGTGCCGCGAGCGGTGATACCTATGGCAGCTACTGGATGGGTTGGTTTCG CCAAGCCCCGGGTAAAGAACGCGAAGGCGTTGCGGCCATCAATCGCGGTGGTGGCTA CACCGTTTACGCGGATAGCGTGAAAGGCCGCTTTACCATCAGTCGCGATACCGCGAAG AACACGGTGTATCTGCAGATGAACAGCCTCCGTCCGGATGATACCGCCGACTACTACT GCGCCGCCAGTGGTGTTCTGGGTGGTCTGCACGAAGACTGGTTCAATTACTGGGGTCA AGGCACCCAAGTTACCGTGAGCAGCACGAGCCATCATCATCATCACCATTAA
NbmCherry-His	Gene	ATGGCACAAGTTCAGCTGGTTGAAAGCGGTGGTAGTCTGGTTCAGCCGGGCGGTAGTC TGCGTCTGAGTTGTGCCGCGAGCGGTCGCTTTGCGGAAAGCAGCAGCATGGGTTGGTT CCGCCAAGCCCCGGGCAAAGAACGCGAATTCGTGGCCGCCATTAGCTGGAGCGGTGG TGCGACCAATTACGCCGATAGCGCCAAAGGTCGCTTCACGCTGAGCCGCGACAACACC AAGAACACCGTGTATCTGCAGATGAACAGTCTGAAGCCGGATGATACCGCGGTGTACT ATTGCGCGGCGAATCTGGGCAACTACATCAGCAGCAACCAGCGTCTGTACGGCTATTG GGGCCAAGGCACCCAAGTTACCGTTAGCAGTCCGTTCACCACCAGCCATCATCATCAT CACCATTAA
NbRota3B2-His	Gene	ATGGCCGACGTACAGCTACAAGCCAGTGGTGGTGGTCTGGCCCAAGCCGGTGATAGTC TGACGCTGAGTTGCGCCGCCAGTGGTCGCACCTTTAGCGGCTACGTGGTTGGCTGGTT CCGTCAAGCCCCGGGCGCCGAACGCGAATTTGTTGGCGCGATCCGCTGGAGTGAAGA CAGCACGTGGTATGGCGATAGCATGAAAGGCCGCATTCTGATCAGCCGCAACAACAT CAAGAACACCGTGAATCTGCAGATGTTCAATCTGAAGCCGGAGGATACCGCCGTTTAT GTTTGCGCCGCGGGTGCCGGTGATATCGTGACCACGGAGACGAGCTACAACTACTGGG GCCGCGGCACGCAAGTTACCGTTAGCAGCCGCGGCCGTACGAGCCATCATCATCATCA CCATTAA

Table S4 Introduction of all plasmids used in the research

Plasmid	Introduction
pDark-deGFP pDark-mCherry pDark-YPet	deGFP, mCherry and YPet were inserted into pDusk as reporter proteins
pLight-deGFP pLight-mCherry pLight-YPet	deGFP, mCherry and YPet were inserted into pDawn as reporter proteins
pDark-YPet(5263g-a) pDark-mCherry(5263g-a) pLight-YPet(5263g-a) pLight-mCherry(5263g-a)	The start codon GTG of *yf1* gene in plasmid was replaced with ATG
pDark-T7-YPet（5263g-a）	The promoter lq of *yf1* gene and *fixj* gene was replace with T7 promoter
pDark-T7-mCherry（5263g-a）	
pDark-J23100-YPet（5263g-a）	The promoter lq of *yf1* gene and *fixj* gene was replace with J23100 promoter
pDark-J23100-mCherry（5263g-a）	
pDark-J23101-YPet（5263g-a）	The promoter lq of *yf1* gene and *fixj* gene was replace with J23101 promoter
pDark-J23101-mCherry（5263g-a）	
pDark-J23107-YPet（5263g-a）	The promoter lq of *yf1* gene and *fixj* gene was replace with J23107 promoter
pDark-J23107-mCherry（5263g-a）	
pDark-J23115-YPet（5263g-a）	The promoter lq of *yf1* gene and *fixj* gene was replace with J23115 promoter
pDark-J23115-mCherry（5263g-a）	
pDark-YPet△YF1 pDark-mCherry△YF1	The *yf1* gene in pDark was knockouted
pDark-YPet△YF1FixJ pDark-mCherry△YF1FixJ	The *yf1* and *fixj* genes in pDark were knockouted
pLight-YPet△YF1FixJ pLight-mCherry△YF1FixJ	The *yf1* and *fixj* genes in pLight were knockouted
pET23a-YF1	*yf1* gene was assembled into pET23a vector
pET23a-FixJ	*fixj* gene was assembled into pET23a vector
pET23a-T7tag-YF1	There was a T7 tag on the YF1 protein
pET23a-T7tag-FixJ	There was a T7 tag on the FixJ protein
pET23a-gcoYF1	*gcoyf1* gene was assembled into pET23a vector
pET23a-msfeoYF1	*msfeoyf1* gene was assembled into pET23a vector
pLight-NbCEA5△YF1FixJ	*NbCEA5* gene was assembled into pLight△YF1FixJ
pLight-NbmCherry△YF1FixJ	*NbmCherry* gene was assembled into pLight△YF1FixJ
pLight-NbRota3B2△YF1FixJ	*NbRota3B2* gene was assembled into pLight△YF1FixJ
pET23a-deGFP	*deGFP* gene was assembled into pET23a vector
pET23a-YPet	*YPet* gene was assembled into pET23a vector

Table S5 Information of optimized *yf1* sequence

name	sequence	Minimum free energy	Central minimum free energy
yf1	**ATG**GCTAGTTTTCAATCATTT	-7.02 kcal/mol	-1.47 kcal/mol
	ATGGCCAGTTTTCAATCATTT	-4.09 kcal/mol	0.00 kcal/mol.
	ATGGCCAGCTTTCAATCATTT	-4.26 kcal/mol;	0.00 kcal/mol.
	ATGGCAAGTTTTCAATCATTT	-4.09 kcal/mol	0.00 kcal/mol.
msfeoyf1	**ATG**GCAAGCTTTCAATCATTT	-4.04 kcal/mol;	0.00 kcal/mol.
	ATGGCAAGCTTCCAATCATTT	-4.30 kcal/mol	0.00 kcal/mol.
	ATGGCAAGCTTTCAGTCATTT	-5.82 kcal/mol	-3.50 kcal/mol
	ATGGCAAGCTTTCAATCATTC	-4.04 kcal/mol;	-0.00 kcal/mol

Note: The bold font indicates the start codon, the horizontal line indicates the changed base.

Supplementary Methods

The optimization of *yf1* genetic codon

The expression of YF1 protein was increased by optimizing the codons of *yf1* gene without changing the induction condition. There are two ways to optimize genetic codons. One way was optimizing the overall codon, and the guiding principle was the codon usage bias of *E. coli*. This method was operated by GENEWIZ company, and the obtained gene was named *gcoyf1*. The other method was optimizing the structural free energy of *yf1* mRNA. Generally. The structural free energy of mRNA was smaller, and the ability of binding ribosomes was stronger. Research had proven that the ability of an mRNA chain combined with ribosomes primarily depended on 43 nucleotide sequences before the start codon and 18 nucleotide sequences after the start codon[1]. The 18 nucleotides of *yf1* gene were changed by synonymous substitution. The structural free energy of these sequences was calculated on RNAfold website (Table S5). The gene, which had the smallest structural free energy, was named *msfeoyf1* (Figure S18).

Cell rest test

The *E. coli* cells, which contained pDark-deGFP, pDark-mCherry, pLight-deGFP and pLight-mCherry plasmids, grew in 5 mL of 2×YT (1.6% wt Trytone, 1% wt Yeast Extract, 0.5% wt NaCl) medium and cultured overnight at 37 °C, 220 rpm. Next morning, these strains were transferred to fresh LB medium at a dilution of 1:20. The pDark-deGFP and pDark-mCherry strains were cultured under blue light, and the pLight-deGFP and pLight-mCherry strains were cultured under

dark. After 6 h, 2 mL of medium was transferred to new tubes and centrifuged. Then 2 mL Tris-HCl (pH 7.4) buffer was added into the tube and mixed. The solution was taken to measure the fluorescence and the OD_{600} value. The remaining medium was centrifuged and poured off, and the same volume of Tris-HCl buffer was added. The pDark-deGFP and pDark-mCherry strains were then cultured under blue light, and the pLight-deGFP and pLight-mCherry strains were cultured under dark. The fluorescence and OD_{600} value of these strains were measured after 6 h.

Preparation of cell extract

Extracts were prepared as described by Wen *et al.* with several modifications[2]. An overnight culture of *E. coli* was used to inoculate 200 mL of 2×YT+P media in 1-L flasks at a dilution of 1:20 for 3 hours. The culture was added to 4-L fermenter with the cultivation at 37 °C and 500 rpm for 3.5 hours. The culture was spun down at 10000×g at 4°C for 10 minutes. Cell pellets were washed twice with 100 mL ice-cold S30A buffer (14 mM Mg-glutamate, 60 mM K-glutamate, 50 mM Tris, pH 7.7) and centrifuged afterward at 10000×g at 4°C for 10 minutes. Cell pellets were then resuspended in 30-mL ice-cold S30A buffer and transferred to pre-weighed 50 mL Falcon conical tubes where they were centrifuged at 10000×g at 4°C for 10 minutes. Finally, the tubes were reweighed and flash-frozen in liquid nitrogen before storing at -80°C.

The next day, cell pellets were thawed on ice and re-suspended in 1-mL S30A buffer per gram cell pellet. Cell suspensions were lysed via twice pass through a high-pressure crusher at 15000-20000 psi and then centrifuged at 12000×g at 4°C for 10 minutes to separate cellular cytoplasm. After centrifugation, the supernatant was carefully transferred to new tubes, and 3 μL of 1M DTT

was added per 1 ml of lysate. The sample was incubated at 37°C with 120 rpm shaking for 80 minutes to digest the remaining mRNA with endogenous nucleases. Subsequently, the extract was centrifuged at 12000×g at 4°C for 10 minutes, and the supernatant was transferred to 6-8 kDa MWCO dialysis bag and dialyzed in 1 L of ice-cold S30B buffer (14 mM Mg-glutamate, 60 mM K-glutamate, ~5 mM Tris, pH 8.2) at 4°C for 3 hours. The extract was re-centrifuged at 12000×g at 4°C for 10 minutes. The supernatant (cell-free extract) was collected and aliquoted into new tubes and flash-frozen in liquid nitrogen before storing at -80°C.

Lipid solution preparation for artificial cell system

Lipid solution was prepared using a modified protocol[3]. POPC was dissolved in chloroform at a concentration of 100 μg/μL, to facilitate dispensing. Liquid paraffin at a volumetric proportion of 5:1 to chloroform was added to the solution, vortexed vigorously and incubated at 80°C for 30 minutes for complete evaporation of chloroform. The solution was then left to cool down to room temperature.

Inner and outer solution preparation for artificial cell system

The inner solution consisted of the cell-free reagents with 200 mM of sucrose added and was prepared in a vial and left on ice in order to prevent the reaction from happening. The outer solution was prepared using the same components and concentrations as the inner solution with the omission of macromolecules, and glucose was used instead of sucrose at the same molar concentration. Inner and outer solutions concentration should be kept the same. If the outer

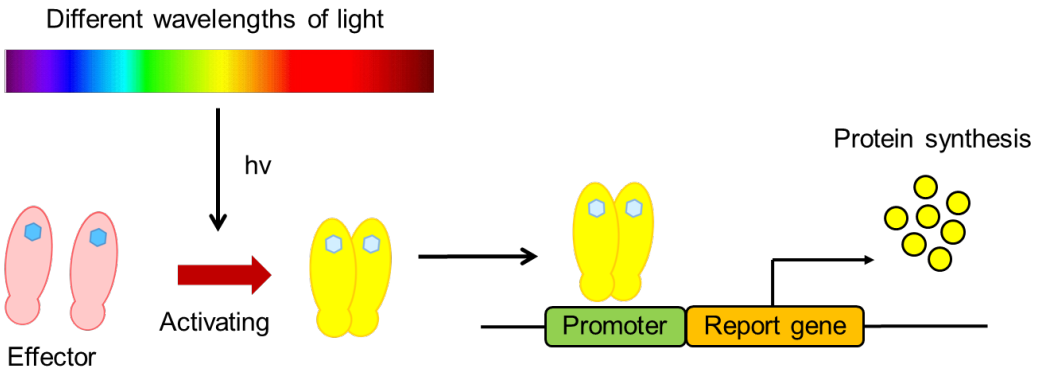
solution concentration were less, it would result in the leak of internal small molecular weight components from inside to outside of the GUV.

Curriculum

Name:_____ **Date:**_____

Bring light into cell-free expression

Introduction



Optogenetics is a technique that uses light to remotely control the modified genes. In recent years, optogenetics technique has made rapid development in life science. Traditional small-molecule ligands and antagonists are often unable to achieve precise controlling of targeted cells, and there is also a problem that cannot be precisely controlled in terms of time. In addition, chemical substances may bring unknown toxic and side effects. Optical signals can be rapidly and accurately delivered to target genes in time and space, and optogenetic techniques can be applied to precisely control the activity of cells or *in vitro* expression systems. The response of the modified cell or in *vitro* expression system to light is clear, and side effects are few. Also, light is low-cost and easy to operate.

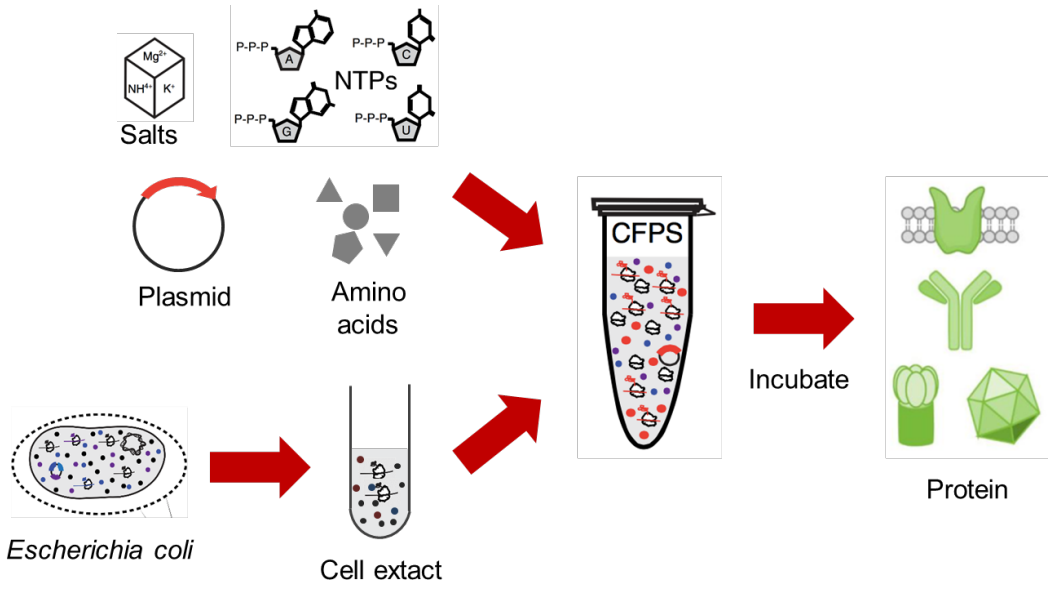
Purpose:

Students will be able to...

- Design the pattern according to the hole plate, and make the aluminum foil used in the experiment.
- Design the gradient of the amount of added plasmids, and calculate the volume of added plasmid solution.
- Experimental operation: mix the plasmid solution with the cell-free system solution, and distribute the mixed solution to the pore plate.
- Visually observe protein concentration by fluorescent intensity using fluorescence protein mCherry as the reporter

Background:

Cell-free protein synthesis (CFPS)system is currently a promising method in synthetic biology. What does cell-free mean? That is, after leaving the cell, the system can function like a cell in the artificially constructed system. In CFPS, DNA or mRNA acts as a template, inorganic salts, substrates and energy materials are supplemented to the system, and proteins are synthesized by a variety of enzymes provided by the cell extract. CFPS was first developed in 1961, when it was used to crack the code that converts mRNA to proteins. Recently, CFPS has been adopted for synthetic biology.



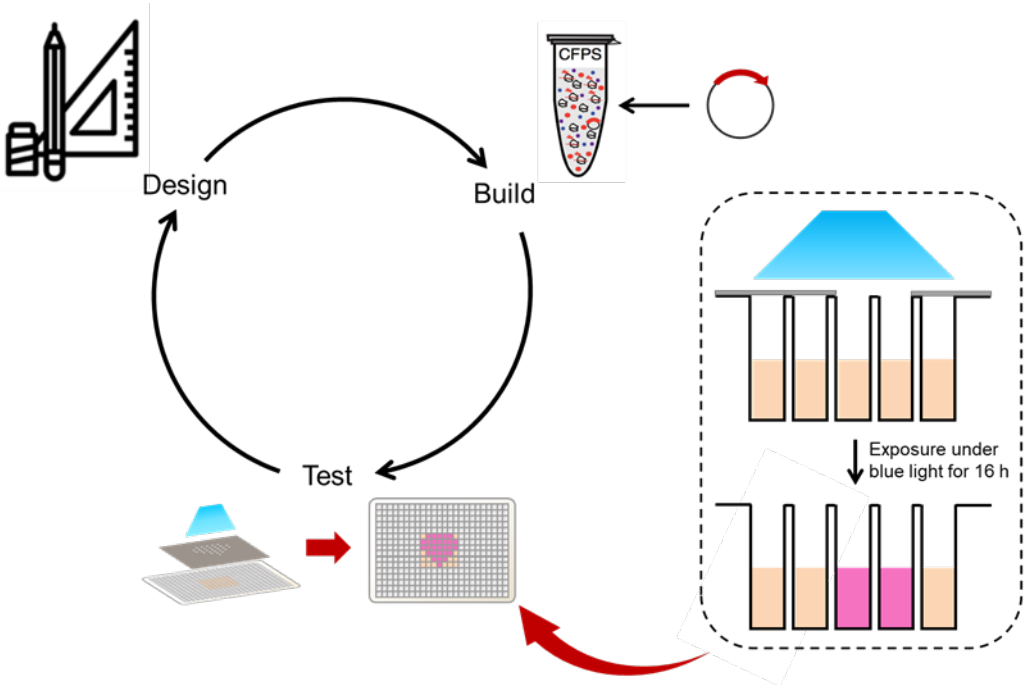
Compared with a traditional cell system, the CFPS has many advantages: Since there is no membrane barrier, exogenous substances can be directly added to CFPS. CFPS can be used to express proteins that are difficult to be expressed in the cell system, such as antimicrobial peptides and ion channel proteins that are toxic to cells. The CFPS can also produce proteins more quickly and produce more products because the reaction does not require the production of any other proteins as in living systems.

We can use CFPS to manufacture proteins of interest, such as medical proteins and enzyme preparation. In the field of biomanufacturing, it is important to find effective control methods to realize the controllable synthesis of proteins. The current control method is to add small molecule chemicals to achieve the control effect. However, the control effect of the chemical method will be restricted by the instability and toxicity of chemical substances, as well as the control rate. In order to avoid the restriction of chemical regulation, physical signals come into people's field of

vision. Physical signals, such as light, temperature, and electricity, are common elements in life. As control signals, they will not bring toxic effects and other side effects.

When design plasmids, scientists often attach their protein of interest to a reporter protein. Reporter proteins often have color or fluorescence that can be seen by eye, to indicate that the specific protein has been expressed, which proves that your reaction works. The fluorescent proteins are often used as reporter protein, such as GFP (green fluorescent protein) and YFP (yellow fluorescent protein), which can be used in CFPS.

In this study, we will control the expression of the fluorescent protein mCherry in CFPS using optical control elements. The optical elements can activate the promoter to transcribe and translate under blue light, expressing mCherry fluorescent protein. First, we will design the pattern we desire according to the suit of the plate. Second, we will mix the plasmid with the cell-free solution and express the mCherry protein under blue light. After a period of protein expression, we will observe the pattern formed finally. The picture below shows the general steps of the experiment:



Pre-lab activity

The mechanism of protein synthesis in the cell-free system is consistent with that of protein synthesis in the cell. They both follow the concept of central law in biology. The central law contains important processes such as transcription and translation, and it is necessary to recall them.

Please draw the diagram of the central law:

Recall the processes of transcription and translation, and write down the organelles and other components involved in these processes:

1. Transcription:_____

2. Translation:_____

From the background, we know that as control methods, physical signals have many advantages compared with chemicals. It is necessary for us to find what the advantages are.

Looking up the materials, then answer: what are the possible advantages of using the following physical signals as control switches?

1. Light: _____

_____

2. Temperature: _____

_____

3. Electricity: _____

_____

4. Magnetic: _____

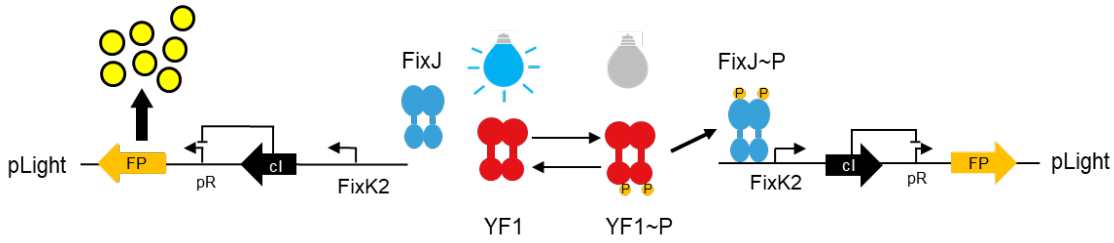
_____

Procedure:

Mechanism

Based on the YF1/FixJ system, the plasmid pLight that can achieve blue light control was designed. YF1 autophosphorylates in the dark and transfers phosphate to the cognate response regulator FixJ. Under blue light, YF1 dimer is dephosphorylated and converted to phosphatase for FixJ~P. The YF1/FixJ system drives the expression of the λ phage repressor cI from the FixK2

promoter, which, in turn, represses the expression from the strong λ promoter pR, so pLight enables gene expression under blue light.



Materials

- Cell-free system solution (prepared by the instructor)
- Plasmid DNA
- Deionized water
- Sterile pipet tips
- Microcentrifuge tubes for DNA dilution
- 96-well plate and 384-well plate
- LED light

Attention

- Be careful to avoid contamination by using a new pipet tip for each solution! Do not touch your pipet tips to any surface other than the inside of the tubes while you are working.
- Remember to keep all of your tubes on ice when mix and distribute the solution.

Experimental protocol

The solution of cell-free system was prepared by the instructor, and the plasmid is added in the solution by students.

Day 1

1. Design the pattern according to the shape of the hole plate, and then punch holes on the aluminum foil according to the design.

2. Calculate the total volume of the cell-free solution distributed to each hole in the pore plate. The concentration of plasmids in the final mixture was guaranteed to be a gradient, for example, 2.5, 5, 7.5, 10 nmol in the solution. Calculate the volumes of the different components in the system. Make sure that the volume of the final solution exceeds 10% of the calculated total value to avoid running out of solution during the distribution.

Plasmid Concentration	Volume			
	Plasmid	Cell-free system	Deionized water	Total

3. Before mix these components, label the empty tubes with different concentration. Prepare the solution in corresponding labeled 15 mL BD tube according to the calculation results in the above table. Use the pipette to blow the solution several times before taking it out.

4. Distribute the cell-free solution in the 15 mL BD tube to each hole of the 96-well plate or 384-well plate. Pipette the solution up and down before distribute to every hole of the plate.

5. Cover the hole plate with the prepared aluminum foil and ensure that the hole position matches the plate hole. The rest of the plate hole cannot pass through the light.

6. Place the plate in the 30℃ incubator, and turn on the blue LED lamp installed in the incubator for 16 h.

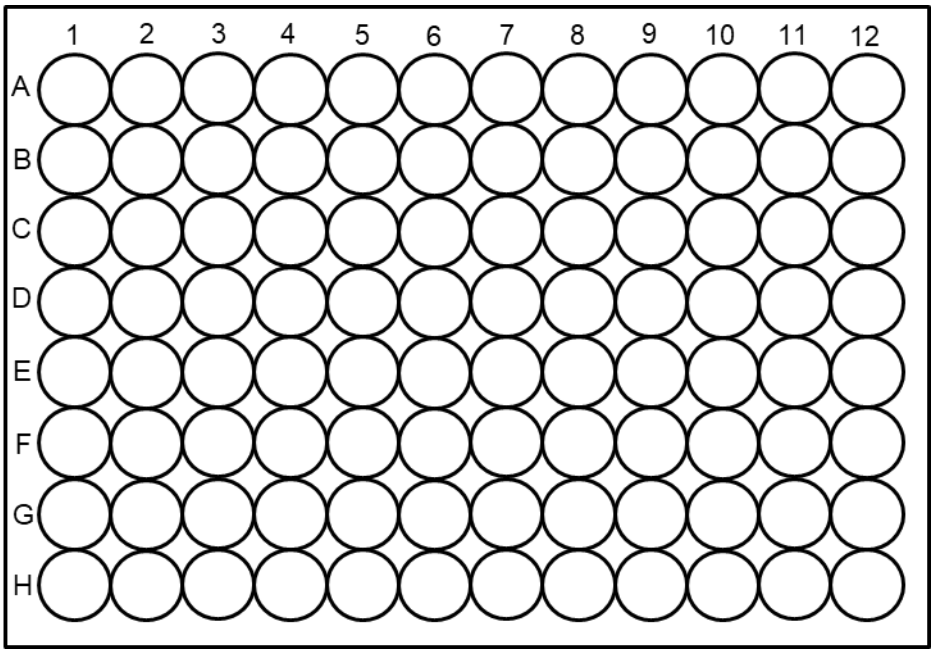
Day 2

1. After 16 hours, take out the hole plate, open the wrapped aluminum foil, and observe the fluorescence protein expression with different plasmid concentrations and patterns. Take a picture of your reactions.

2. Measure the fluorescent intensity in the hole plate under the guidance of instructor. Record your results in an appropriately labeled data table that you should create.

Data:

Use the 96-well plate below to design a pattern:



Recording the fluorescent intensity in the table below:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Post-lab Analysis

1. Generate a graph of your results. Include appropriate scale, labels, units, and a descriptive title.

2. Use the results to make a claim about the relationship between plasmid concentration and fluorescent intensity. Use the evidence you collected to support your claim. Explain your reasoning.

3. Propose an explanation for why this pattern exists using your own understanding of transcription and translation. What is happening in the cell-free system that causes this to occur?

4. If there are any data points that seem to be outliers that do not fit the pattern, propose an

explanation for why this may have happened.

5. Whether all the hole exposed to blue light have a high expression of fluorescent protein compared? If not, give a possible explanation.

6. Consider the lab procedures. Do you see any sources of error? Propose one change that could be made to improve these procedures.

Inquiry Lab

As a class, we need to explore "The feasibility of blue light control imaging and the optimal plasmid concentration." Your group needs to give a pattern that you would like to create and a plasmid concentration gradient, then designs a procedure for testing that condition.

1. Your teacher has a list of available supplies that you can use. Before writing your procedure, check with your teacher to make sure that you have the needed equipment/supplies available.
2. Write out your plan, which should include:
    a. The question you decide to answer
    b. Your hypothesis (prediction and reason for that prediction)
    c. Experiment materials
    d. Detailed procedures, including amounts of each of the supplies
    e. Experimental control
    f. Data tables and a plan for collecting/analyzing your results
3. Show your teacher your completed plan and experiment design for approval.
4. Implement your plan, collect your data, and analyze your results!
5. Submit your data to your teacher.
6. You will be given a summary of data as collected from several groups of students. Use all of the data sets to make a claim about the optimal plasmid concentration for the transcription and translation process in this cell-free system. Support your claim using evidence, and provide reasoning for every piece of evidence.
7. Using your understanding of biochemical reactions, discuss the possible ways to improve the

blue light control imaging.

8. If you were to do this lab again, what changes or improvements would you make? Discuss ways to improve your procedure for testing the same question.

For the inquiry portion of the lab, we suggest that students can consider varying other parameters: the amount of specific ions, temperature and pH. Magnesium is important to transcribe and translate, and adding extra magnesium is a viable option. You can also further discuss with teachers how to improve the blue light imaging effect by changing other parameters. Design further experiments based on the results obtained from the discussion to verify your guess.

Supplementary Figures

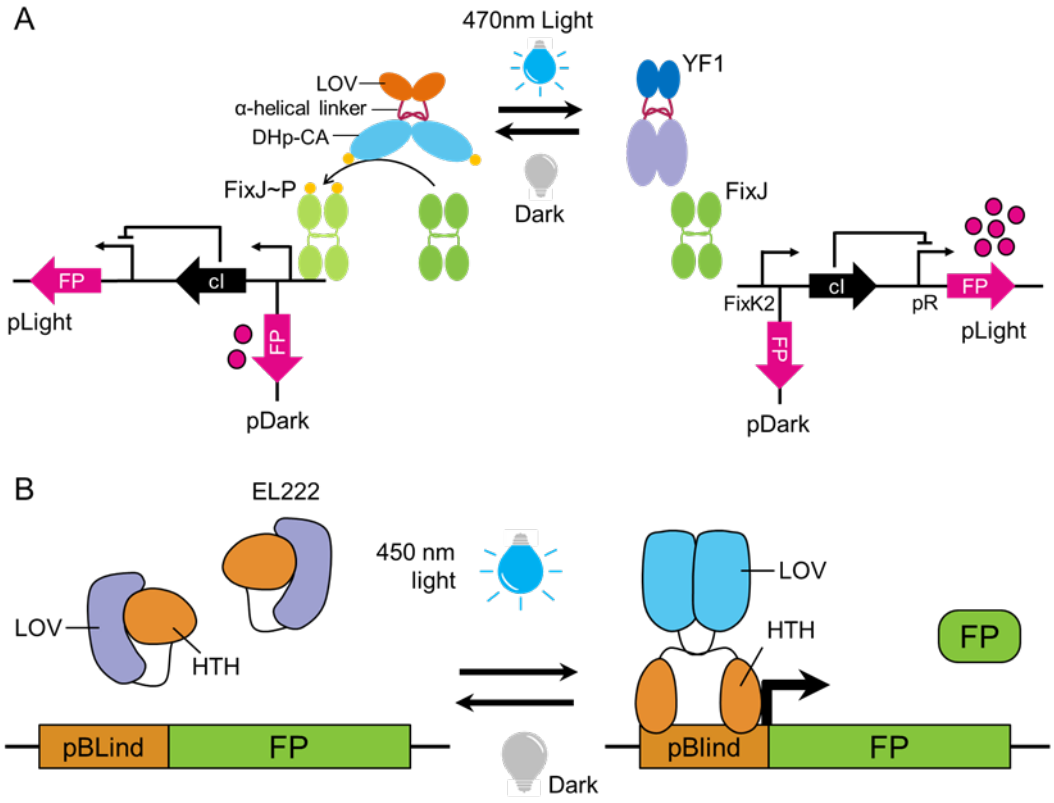


Figure S1. The mechanism of YF1/FixJ and EL222. (A) YF1/FixJ system. YF1 autophosphorylates in the dark and transfers phosphate to the cognate response regulator FixJ. Under blue light, YF1 dimer is dephosphorylated and converted to phosphatase for FixJ~P. The phosphorylation and dephosphorylation of FixJ by YF1 achieves the blue light control. (B) EL222 system. The HTH domain is bound and thereby inhibited by the LOV domain in the dark. Upon blue light illumination, the HTH domain is released, thereby enabling DNA-binding and promoting the transcription reaction.

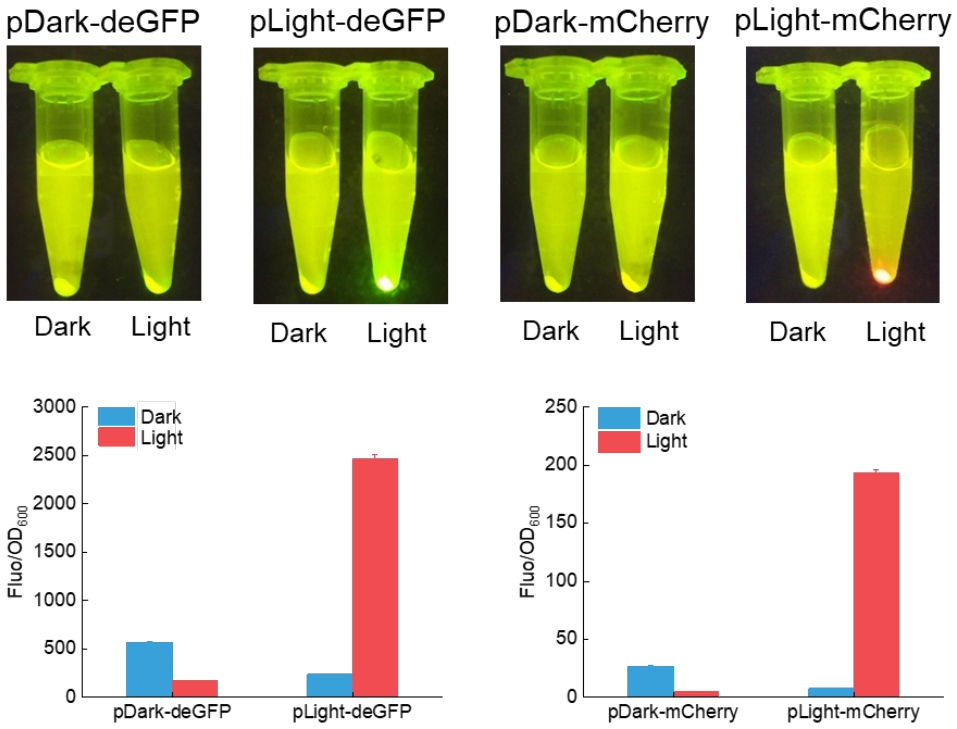


Figure S2. Blue light-regulated expression of two fluorescent proteins in cell system. *E. coli* containing the light-regulated plasmid was inoculated into LB liquid medium and cultured under blue light or dark overnight, and then the mean fluorescence and OD600 values of cells were measured. The results showed that pDark could synthesize fluorescent protein in the dark, and the production was very low under light. pLight had opposite control effects compared to pDark.

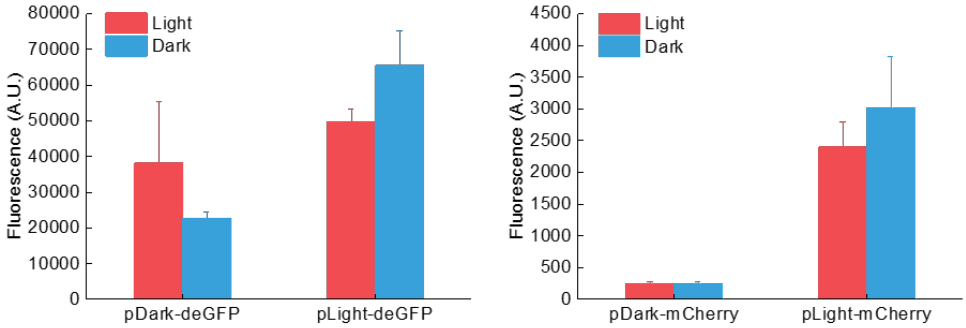


Figure S3. Mean fluorescence of deGFP and mCherry in blue light-switchable cell-free system. The cell-free synthesis system was placed under blue light or dark for 12 h, and then the mean fluorescence was tested. The result showed that pDark and pLight had no light regulation in cell-free systems.

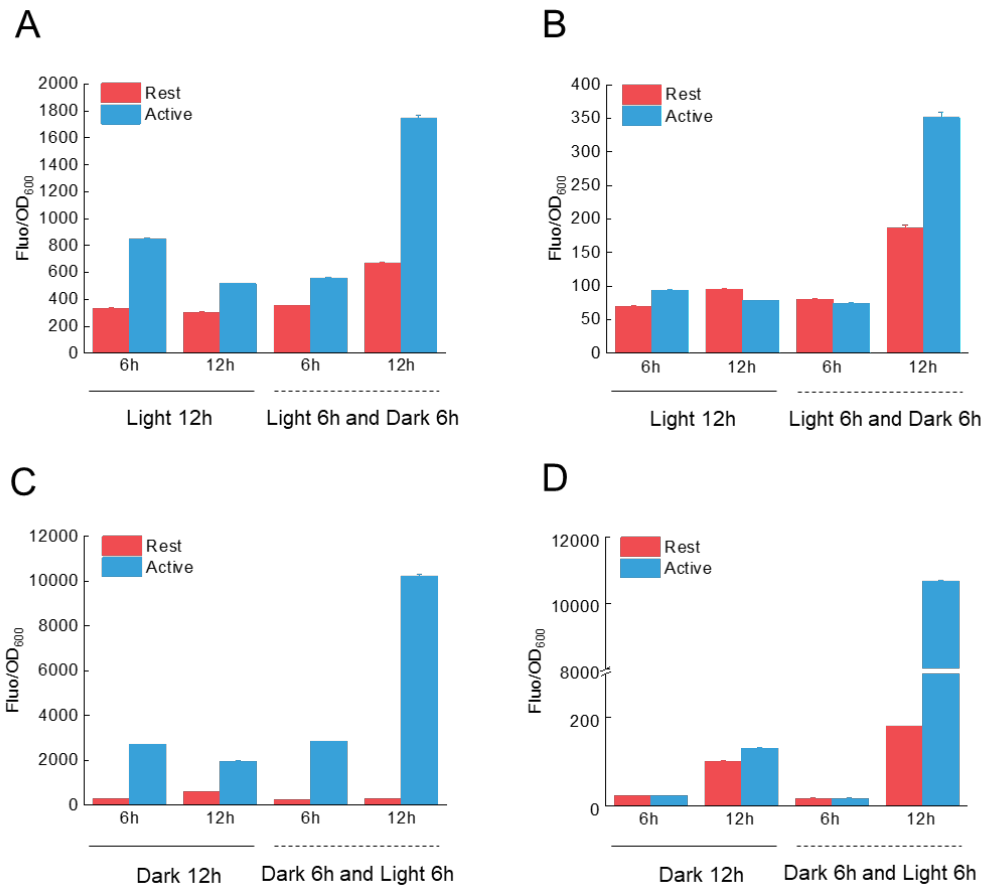


Figure S4. Exploring the effects of blue light-regulated protein expression in rest cell and living cell. (A) pDark-deGFP. (B) pDark-mCherry. (C) pLight-deGFP. (D) pLight-mCherry. Cells were cultured under blue light or dark. After 6h, the mean fluorescence and OD_{600} values of cells were tested. Some cells were processed into rest state, and continued to be cultured in blue light or dark for 6h, and then the mean fluorescence and OD_{600} values of cells in rest and active state were measured. It was shown that pDark had high fluorescence expression in dark, and pLight had high fluorescence value under blue light. However, the blue light regulation in the rest state was not as effective as that in an active state, and the mean fluorescence of deGFP under blue light was always less than that in dark conditions.

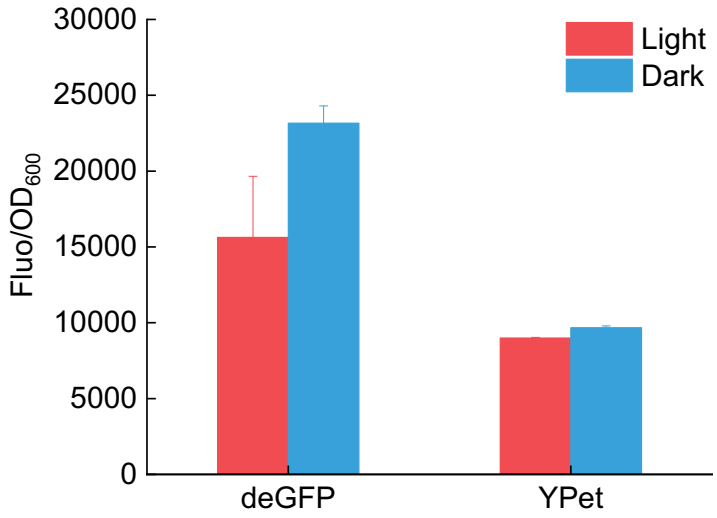


Figure S5. Mean fluorescence of deGFP and YPet in the cell under light and dark. The fluorescence value of deGFP had a significant decrease under blue light. YPet has better stability than deGFP. The deGFP was constructed by fusing a degradation domain of mouse ornithine decarboxylase with eGFP[4], which has a faster degradation rate. Blue light has a photobleaching effect on deGFP, which might be related to a complex chemical process, but the mechanisms still require to be further explored. However, YPet is the brightest yellow fluorescent protein (YFP) variant developed to date, and it appears not to be affected by the photo-instability [5].

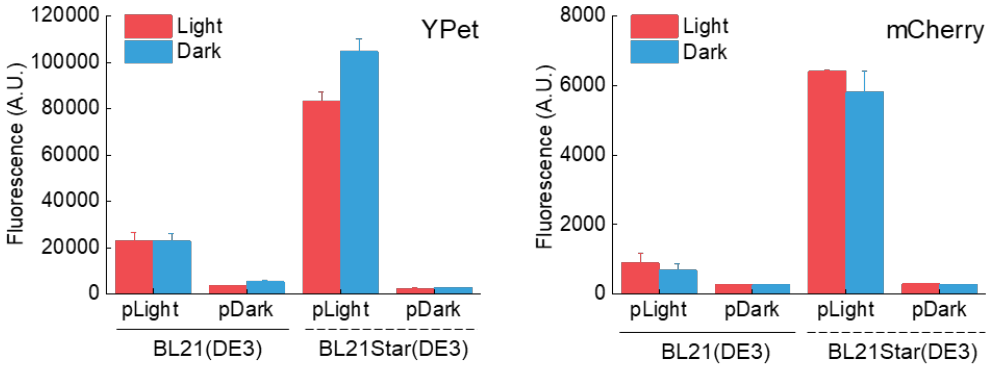


Figure S6. Mean fluorescence of YPet and mCherry in the cell-free system with two cell extracts. Cell extract was the key factor that affected cell-free protein synthesis. The fluorescence intensity value was increased after the switch of cell extract, but there was still no difference between the samples under blue light and dark. It was confirmed that cell extract was not the main reason in the case of the optical sensing of the cell-free system.

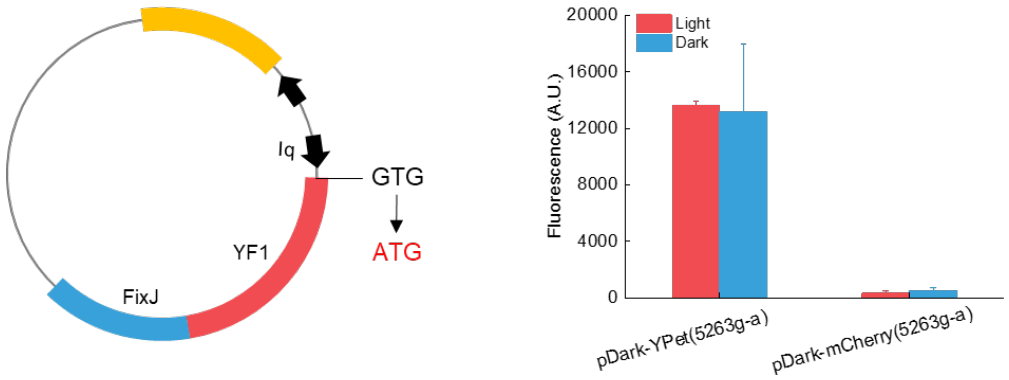


Figure S7. Changing the start codon sequence of the *yf1* gene. The ratio of two regulatory proteins might play an instrumental role in the effect of regulation in TCS. YF1/FixJ is a polycistronic system, and the start codon of *yf1* gene was a rare start codon GTG. Biological system regulated the relative yield of YF1 and FixJ through these methods to achieve better light control effects. However, there was few translation elements for start codon GTG compared to other translation elements of codons in the cell-free system, which might have resulted in no expression of YF1 and FixJ. The start codon GTG of *yf1* gene in pDark was hence replaced with ATG. However, plasmids pDark-YPet and pDark-mCherry did not realize blue light sensing with changing start codon in the cell-free system.

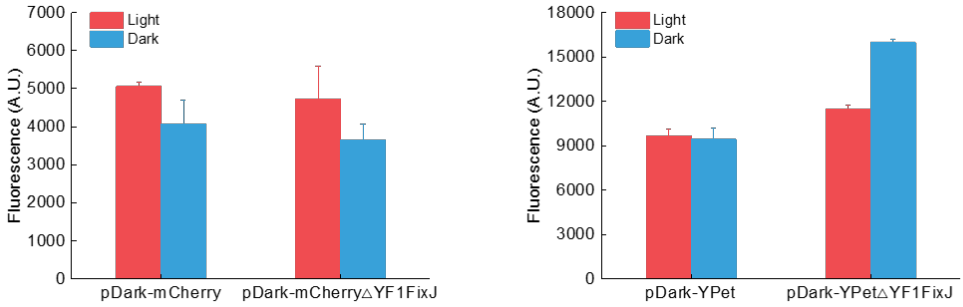


Figure S8. Knocking out *yf1* and *fixj* genes on pDark. YF1 and FixJ are essential proteins for blue light sensing. Knocking out *yf1* and *fixj* genes from the plasmid could determine whether YF1 and FixJ proteins play role in cell-free system. Plasmids pDark-mCherry△YF1FixJ and pDark-YPet△YF1FixJ could express fluorescent protein in cell-free systems.

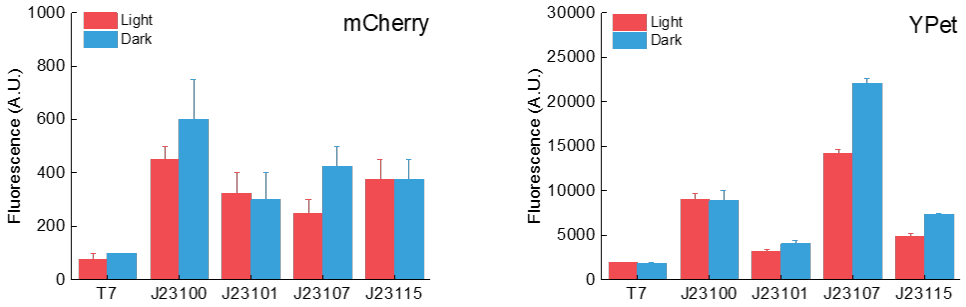


Figure S9. Changing the promoter of *yf1* and *fixj*. Promoters with different transcription strength could influence protein expression. In order to improve production of YF1 and FixJ in the cell-free system, a series of promoters with different transcription strength were selected (T7> J23101>J23101> J23107 > J23115) for cell-free blue light sensing. There was no significant difference in fluorescence value between the blue light and dark.

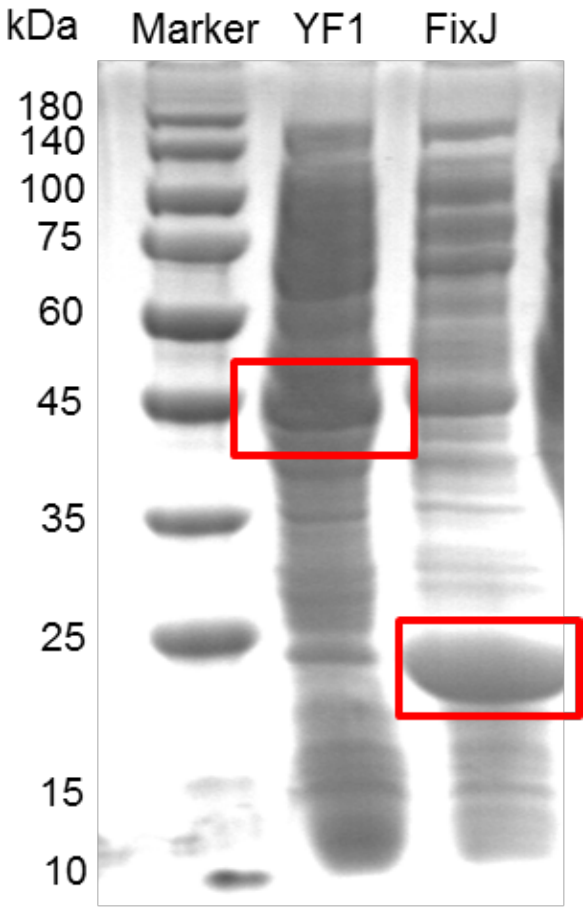


Figure S10. The SDS-PAGE analysis of cell extracts of YF1 and FixJ.

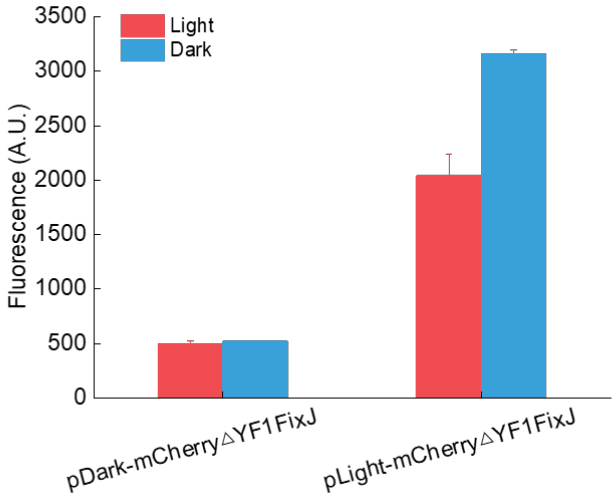


Figure S11. The effects of knocking out *yf1* and *fixj* genes on cell-free light-sensing system. In order to quantify concentrations of YF1 and FixJ in cell-free system, the *yf1* and *fixj* genes of plasmids pDark-mCherry and pLight-mCherry were knocked out. YF1 and FixJ in the cell extract were subjected to the blue light sensing. Cell-free system could not achieve blue light sensing when only cell extracts contained YF1 and FixJ.

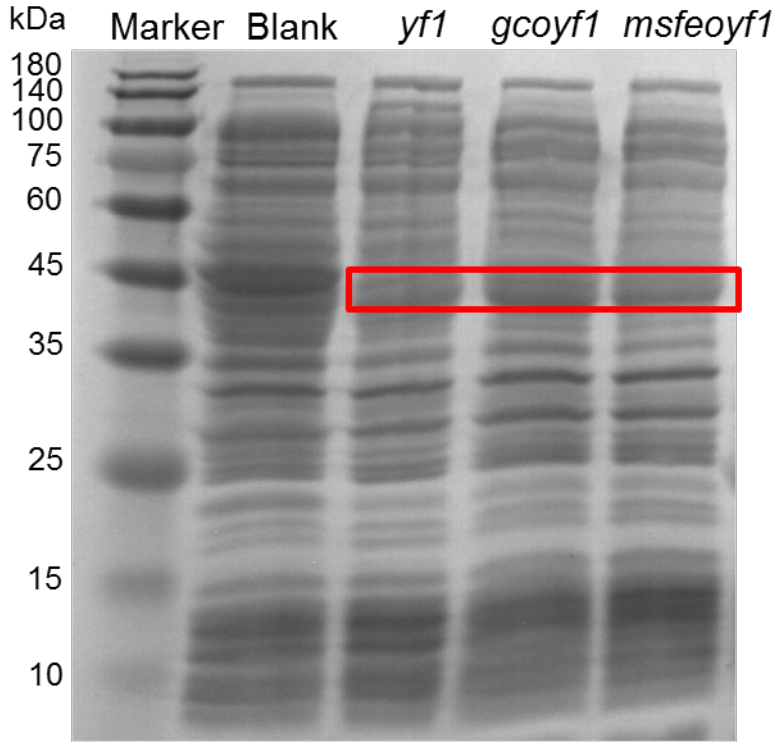


Figure S12. The SDS-PAGE analysis of three cell extracts containing YF1.

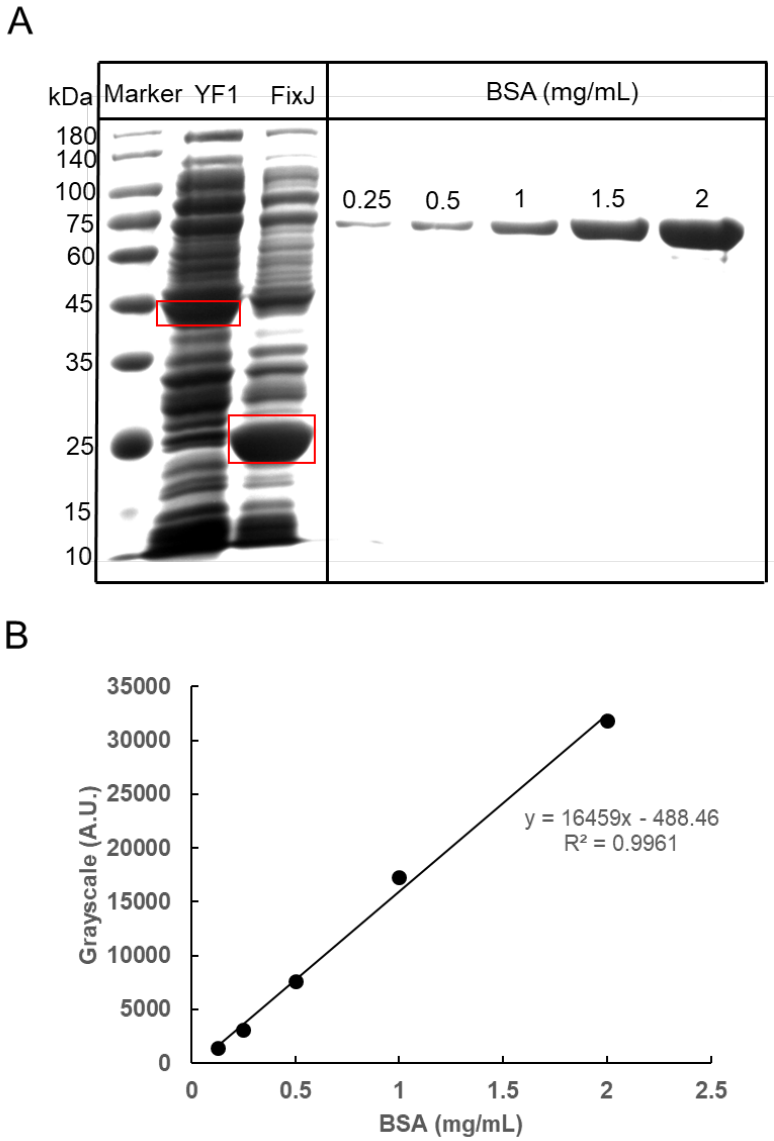


Figure S13. Determining the concentration of YF1 and FixJ in cell extracts. By determining the concentration of YF1 and FixJ in cell extracts, the ratio of YF1 and FixJ in the cell-free system could be precisely adjusted. Gray analysis was performed on the SDS-PAGE gel map. The gray value of YF1 was 8915.92, and the gray value of FixJ was 55638.95. The concentration of YF1 in cell extract was 0.69 mg/mL, and the concentration of FixJ was 4.1 mg/mL by calculating the standard curve. (A) The SDS-PAGE analysis of cell extract of YF1, cell extract of FixJ and bovine serum albumin (BSA) with different concentrations. (B) The standard curve line of BSA.

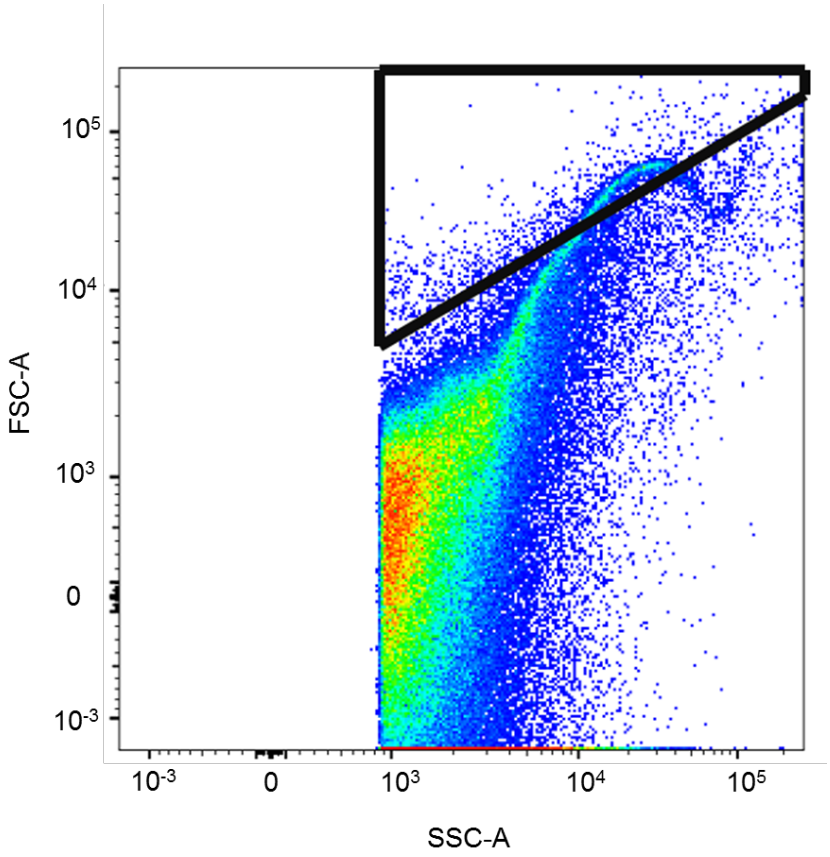


Figure S14. Gating GUV. The correlation between FS and SS[6, 7]was used to gate unilamellar vesicles for the obtained results as shown above, then the resultant samples were subsequently gated by fluorescence intensity. These two consecutive gates were applied to both mCherry and YPet flow cytometry results; and for a meaningful comparison, at least 1,000 liposomes were included within the gate. GUVs have size range from 1-100 mm, which have a size that is more typical of living cells- yet such heterogeneity in sizes was shown not to affect the yield of protein[8].

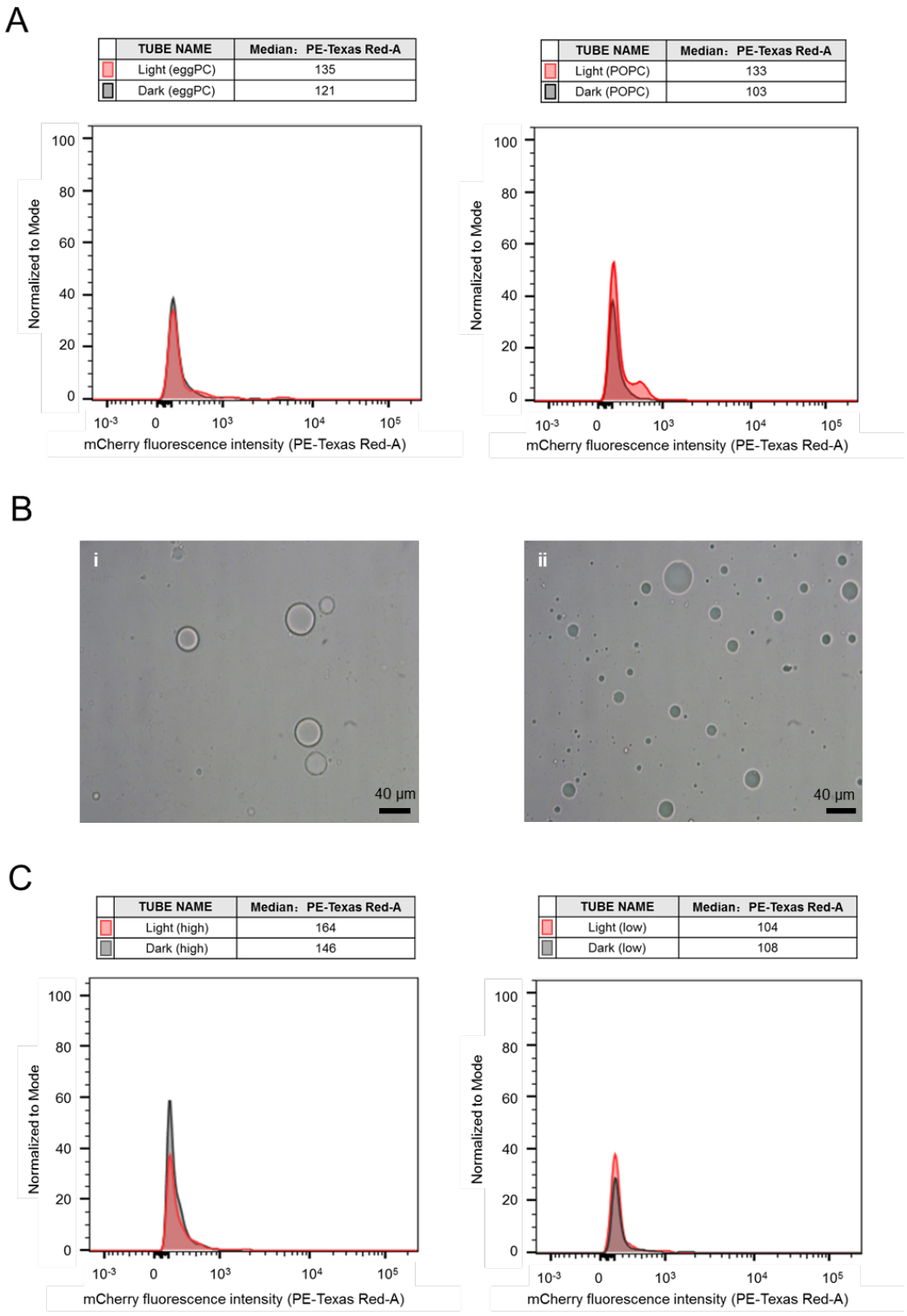


Figure S15. The optimization for the artificial cell system incorporating the cell-free system.

(A) Effect of different types of lipids on protein production in artificial cell system. egg PC and

POPC displayed almost similar results of encapsulation efficiency. (B) Microscopy images showing the effect of the use of PEG 8000 in the formation of artificial cell incorporating the cell-free system. PEG 8000 did not show a clear effect of higher protein production. **i** With PEG 8000. **ii** Without PEG 8000. (C) Concentrations of plasmid in cell-free system within the artificial cell system. Concentrations of the plasmids in the cell-free systems were also varied. Lower concentration (1.5 nM) was tested as it was hypothesized that it may reduce the overall pressure of forming the successful artificial cell system. It showed that the lipid compartments expressed the proteins successfully with the concentration the same used in the tube environment.

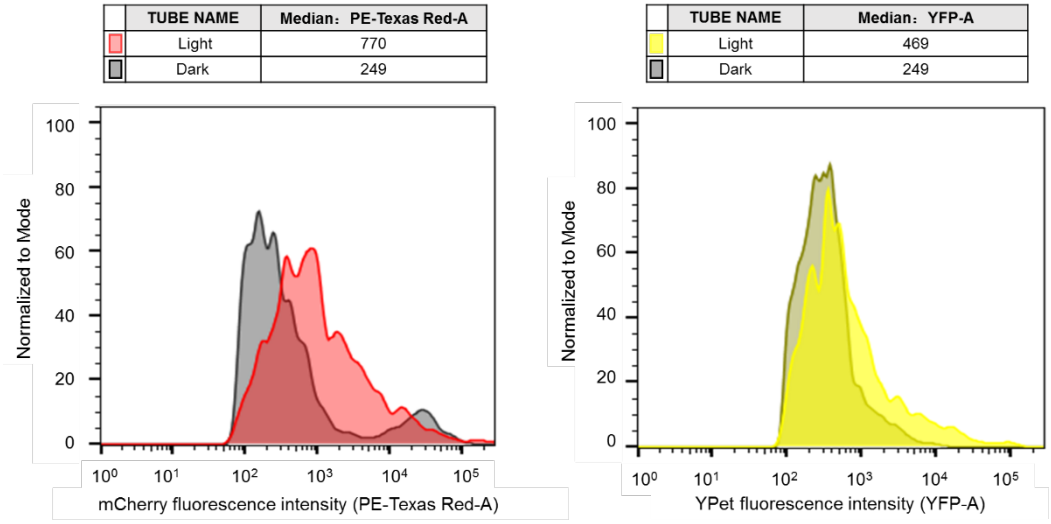


Figure S16. Flow cytometry results for artificial cell compartmentalizing the previously reacted cell-free system.

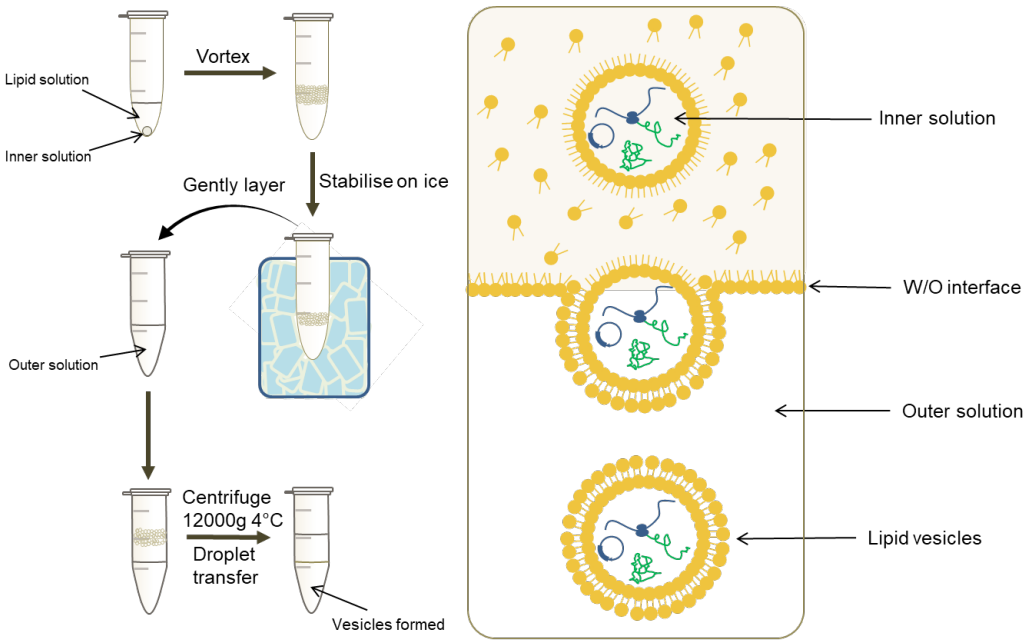


Figure S17. The operation process and schematic diagram of the artificial cell.

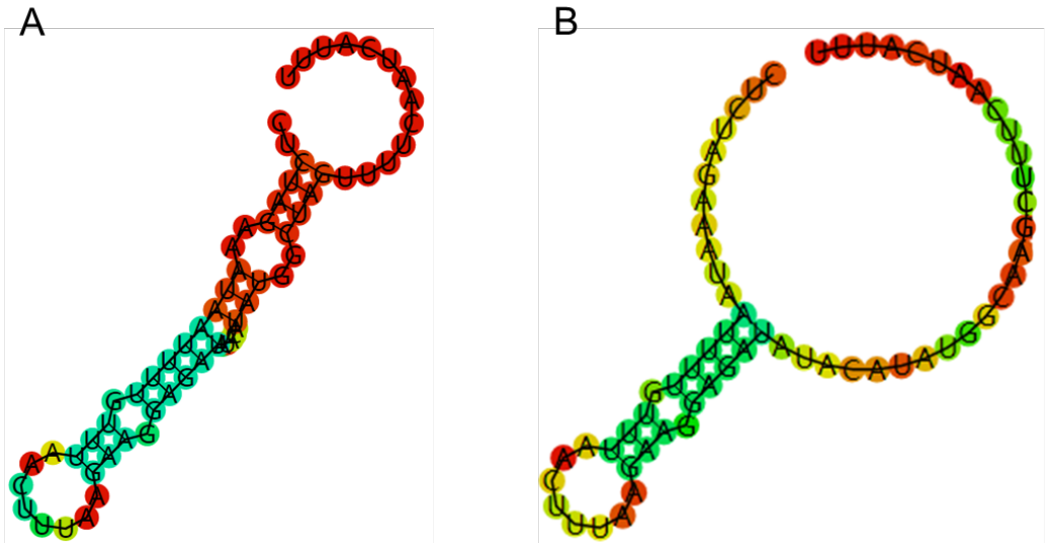


Figure S18. The maps of RNA secondary structure. (A) *yf1*. (B) *mafeoyf1*.

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