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Protein synthesis in cell free systems

Olivier Borkowski

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artificial cells

shape
energy
turnover
information

1st master and PhD course on the making of living systems

Every wednesday, 5:15 to 7:15 pm

October to December 2024

ENS Biology Department, 46 rue d'Ulm, 75005 Paris

Biomolecule synthesis: Part I

more information

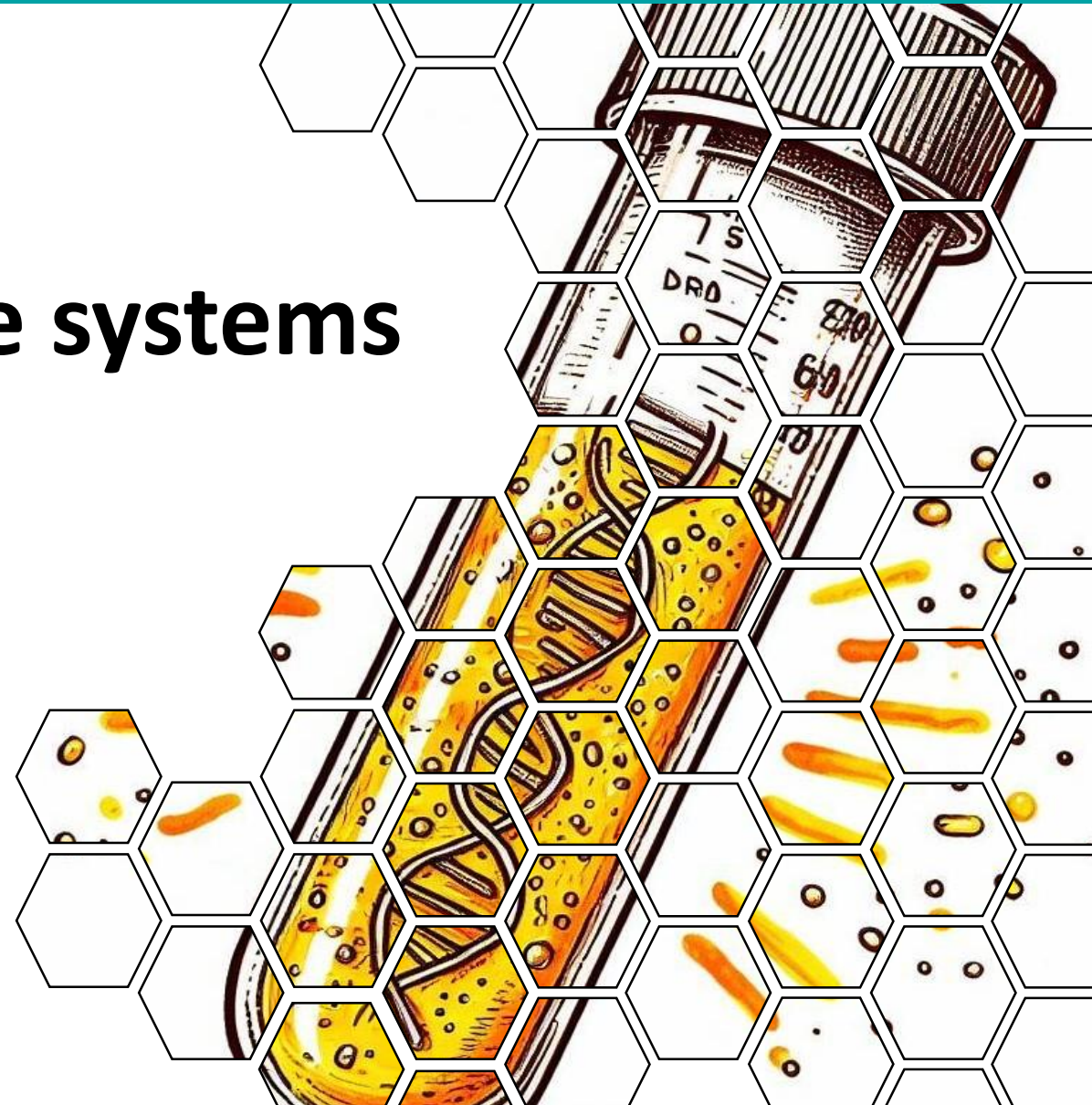


<https://www.edu.bio.ens.psl.eu/spip.php?article289>

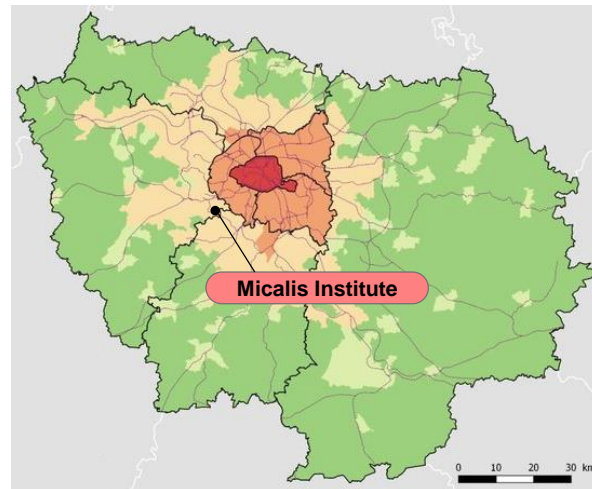
Protein synthesis in cell-free systems

Olivier Borkowski, PhD

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SyBER team
Email: olivier.borkowski@inrae.fr



Micalis Institute (INRAE-AgroParisTech)



21 Research Teams

- 340 people
- 130 scientists and engineers
- 140 PhD students and post-docs
- 70 scientific and administrative personnel

Themes

- (1) Emergence, control, and adaptation of pathogenic microorganisms
- (2) Microbial food and intestinal ecosystems
- (3) Synthetic and systems biology

Understand bacterial physiology

Bacterial physiology is a branch of biology that aims to understand overarching principles of cellular reproduction:

What are the fundamental principles underlying the regulation and coordination of biosynthesis in a given growth environment?

-> Bacterial growth law

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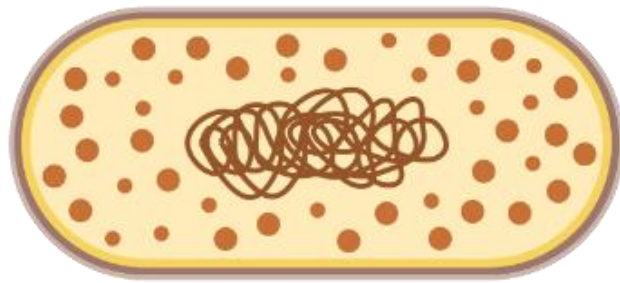
Some major principles studied in bacterial physiology:

- Link between bacterial growth and nutrient concentrations in culture media (Monod, 1942)
- Link between the macromolecular composition and cell growth rate (Schaechter et al., 1958)
- Linear relationships between RNA/DNA, RNA/protein, protein/DNA and growth rate (Bremer & Dennis, 1974)
- Increasing allocation of cellular resources to ribosome synthesis with increasing growth rate (Hwa & Scott, 2010)

-> What is happening at the gene level?

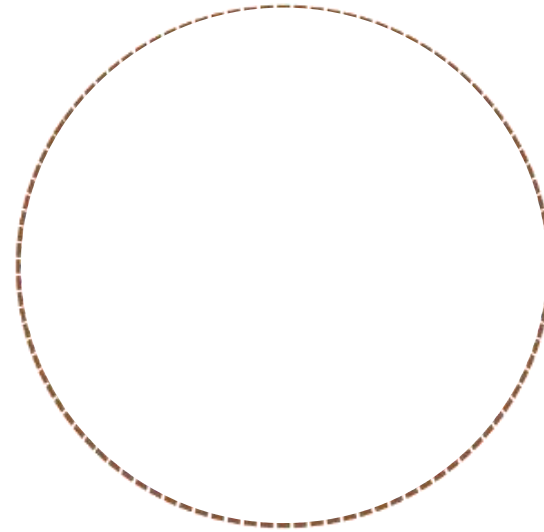
Bacterial Physiology

Experimental setup



E. coli living cells

Genomic regulations

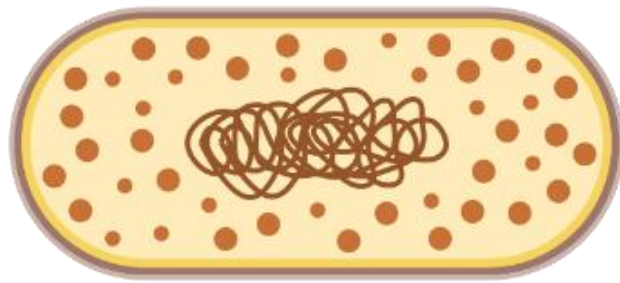


More than 4000 genes

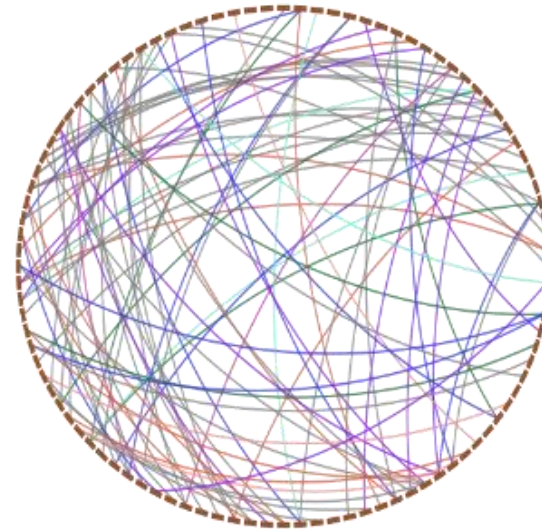
Bacterial Physiology

Experimental setup

Genomic regulations



E. coli living cells



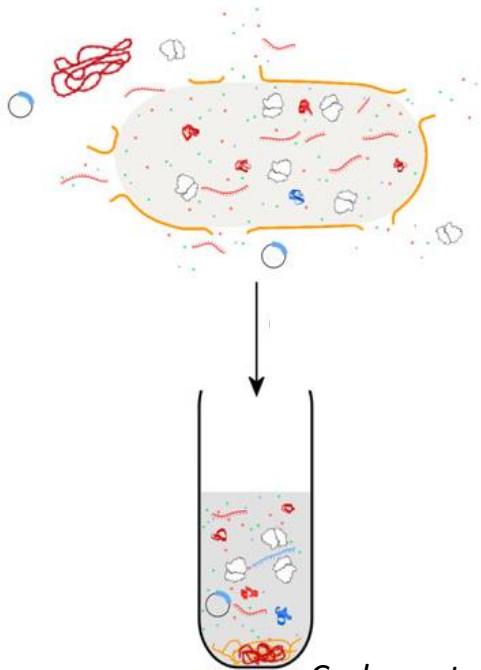
Intertwined Effects

-> New technology to decouple the many effects observed in living cells

Cell-free reaction

Gene expression in a simpler and open system

A simpler environment to measure direct effect on gene regulation (no cascade of reaction due to living organism adaptation)
A direct manipulation of the environment because it is an open system



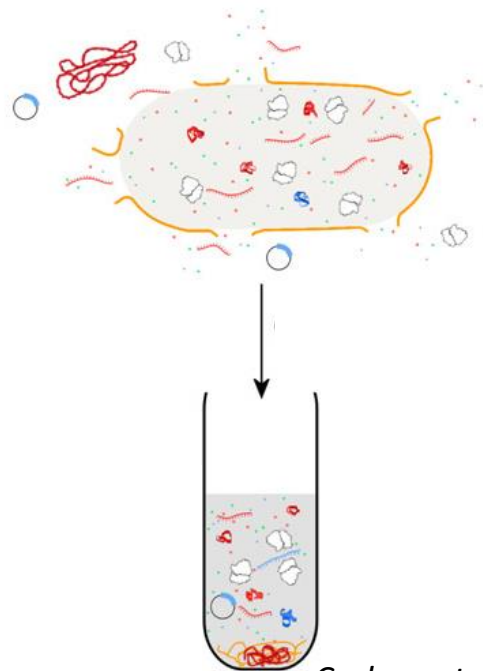
Carlson et al. Biotechnology advances (2012)

Cell-free reaction

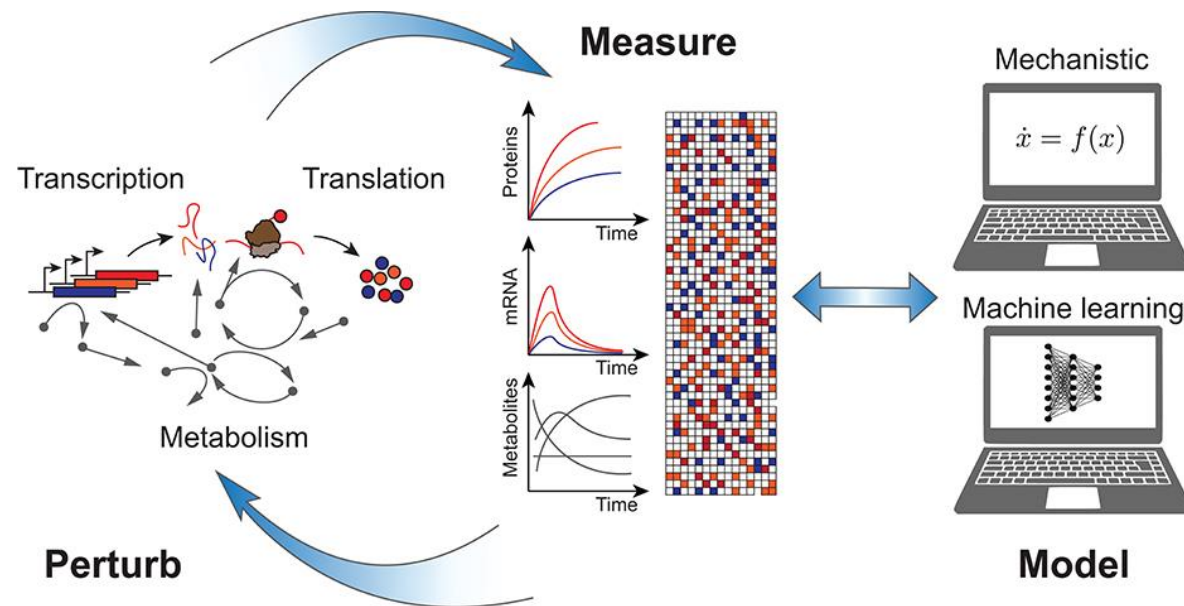
Gene expression in a simpler and open system

A simpler environment to measure direct effect on gene regulation (no cascade of reaction due to living organism adaptation)
A direct manipulation of the environment because it is an open system

- An alternative technology to study central process observed in living cells (replication, transcription, translation, degradation, metabolism)
- A versatile technology for bioengineering (Use in Synthetic Biology): life simulation system



Carlson et al. *Biotechnology advances* (2012)



Nadanai, L. *Frontiers in Bioengineering and Biotechnology* (2020)

1. Description of Cell-Free Systems: **An open system** to study biology
2. Understand living organisms: **functions preserved** in cell-free systems
3. Building artificial cells: **reconstruct functions** defective in cell-free systems

First major use of cell-free: genetic code

Cell-free was first used in the 1960's by Nirenberg and Matthaei to understand the genetic code

American & German biochemist

Johannes Heinrich
Matthaei

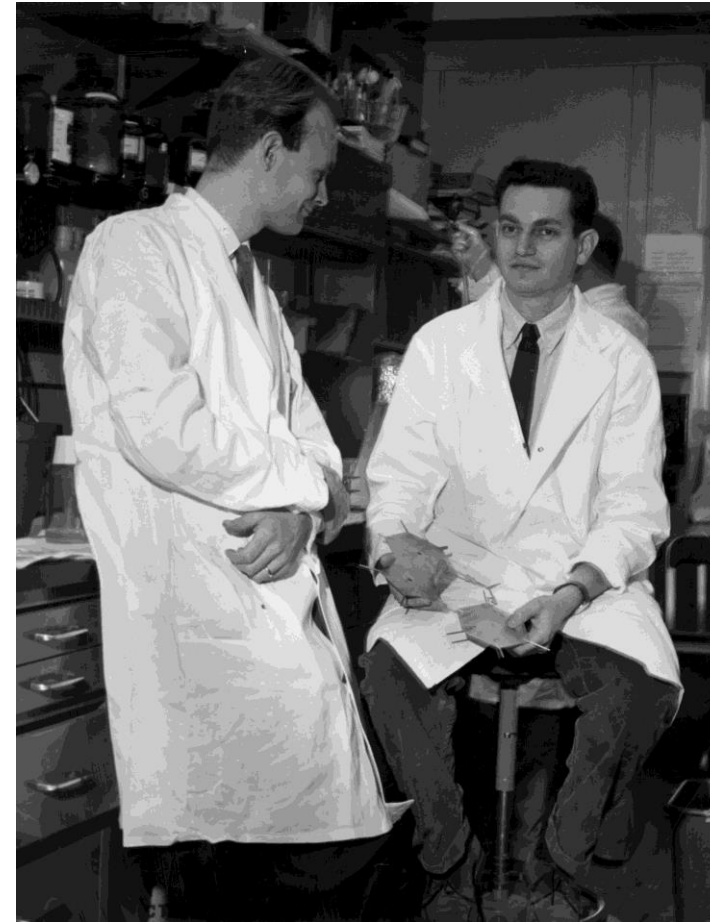
*THE DEPENDENCE OF CELL-FREE PROTEIN SYNTHESIS IN E. COLI
UPON NATURALLY OCCURRING OR SYNTHETIC
POLYRIBONUCLEOTIDES*

BY MARSHALL W. NIRENBERG AND J. HEINRICH MATTHAEI*

NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

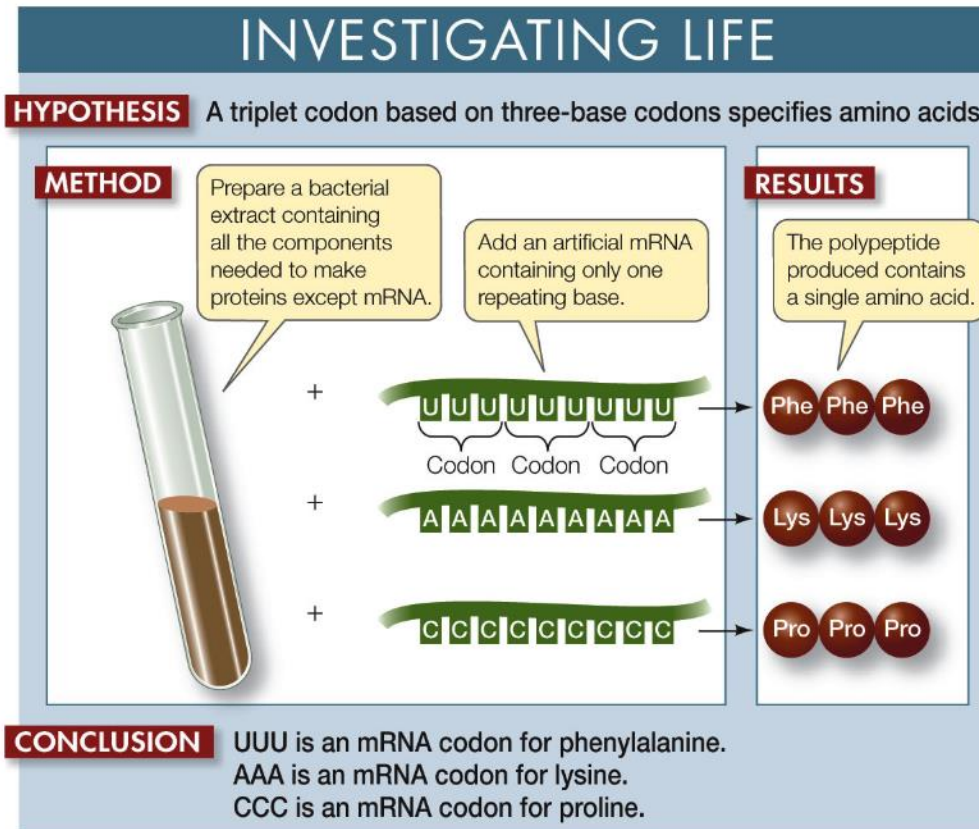
Communicated by Joseph E. Smadel, August 3, 1961

A stable cell-free system has been obtained from *E. coli* which incorporates C¹⁴-valine into protein at a rapid rate. It was shown that this apparent protein synthesis was energy-dependent, was stimulated by a mixture of L-amino acids, and was markedly inhibited by RNAase, puromycin, and chloramphenicol.¹ The present communication describes a novel characteristic of the system, that is, a requirement for template RNA, needed for amino acid incorporation even in the



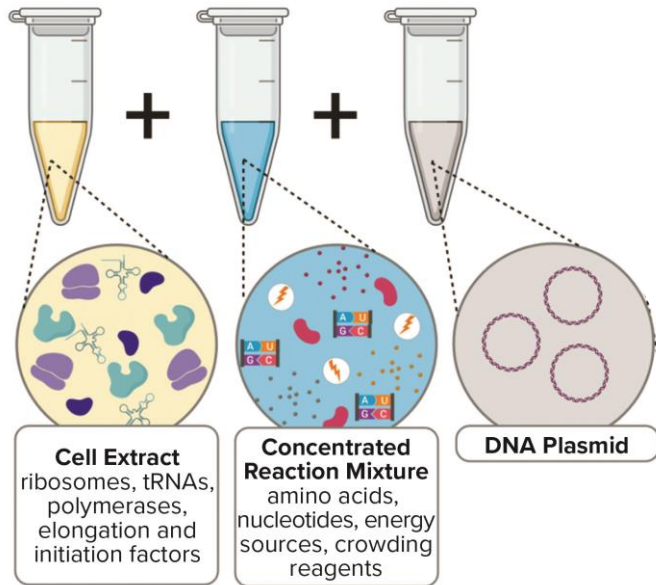
Marshall Warren
Nirenberg

Nobel prize for the interpretation of the genetic code and its function in protein synthesis



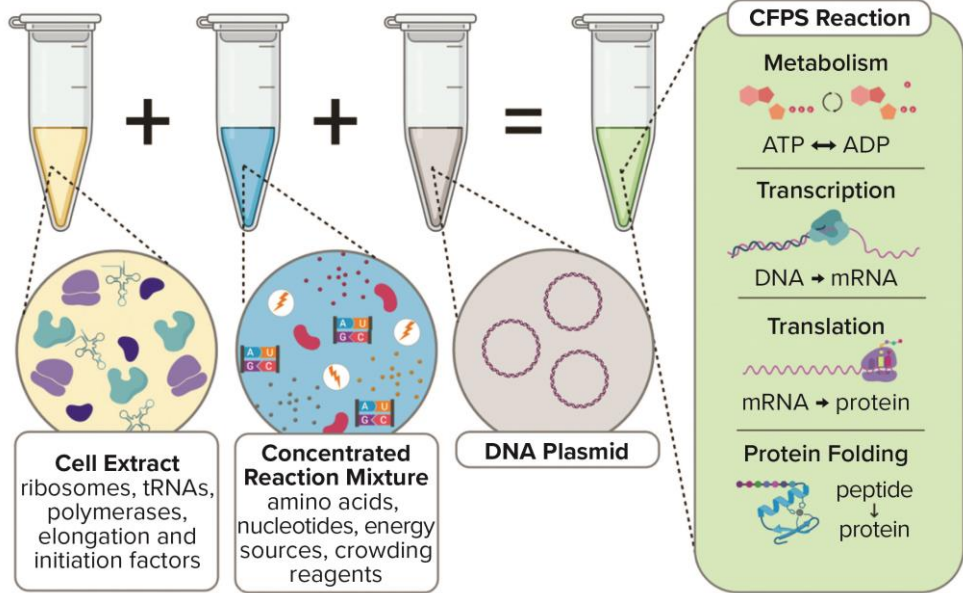
They made a synthetic RNA only with the base uracil (poly U – codon UUU) and tested it in 20 tubes, each one with a different radioactive amino acid.

What is the composition of a cell-free reaction?

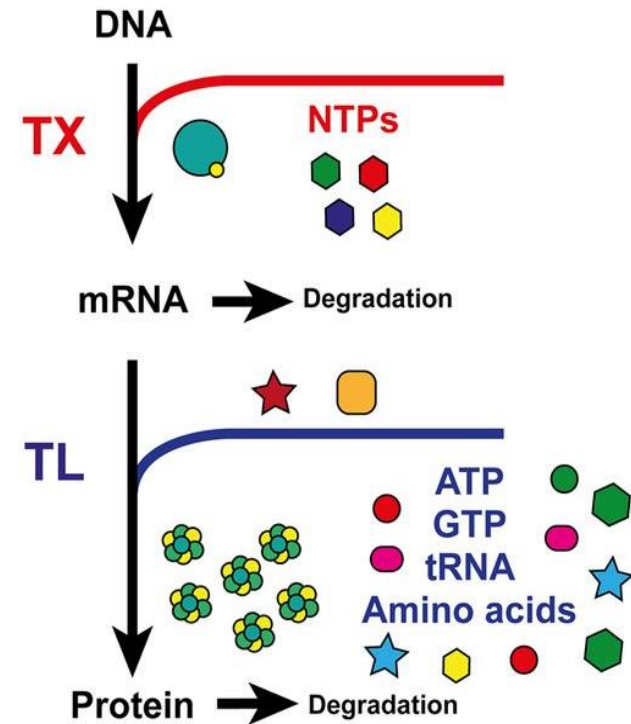
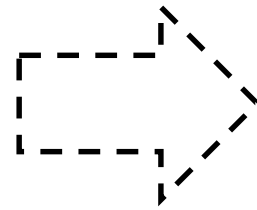


Melinek B, et al. BioProcess International (2020)

What is the composition of a cell-free reaction?

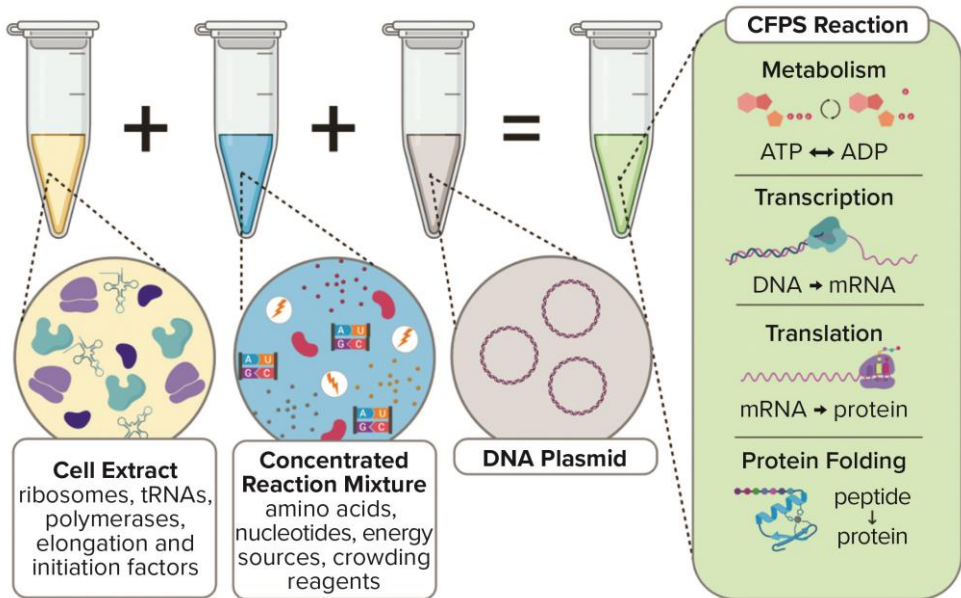


Melinek B, et al. *BioProcess International* (2020)



Seok Hoon Hong et al. *Frontiers in Chemistry* 2014

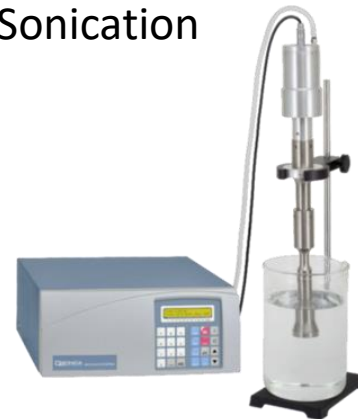
Cell-extract preparation



Melinek B, et al. *BioProcess International* (2020)

Physical disruption

Sonication

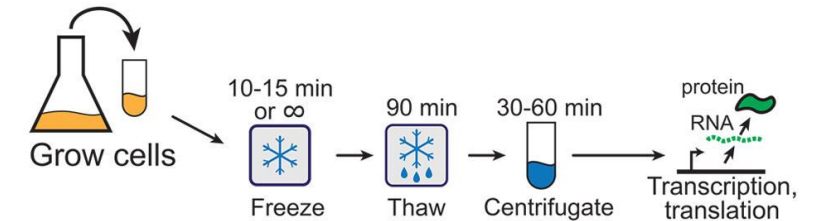


French press

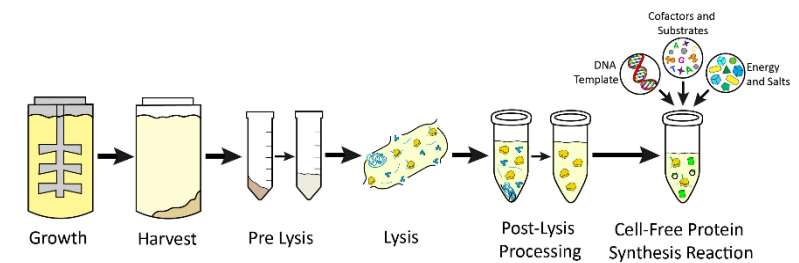


Chemical disruption

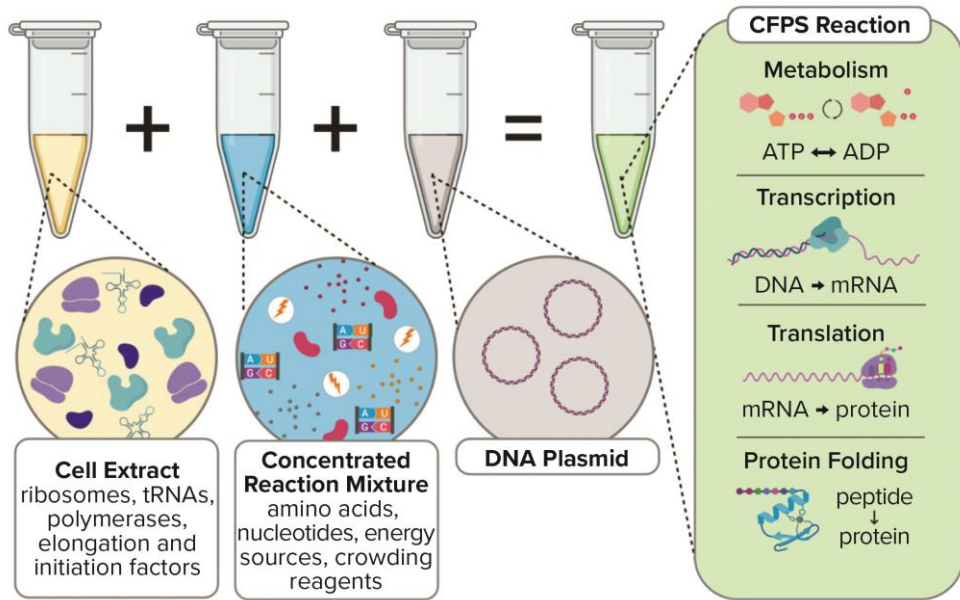
Autolysis



Lysozyme



Buffer composition

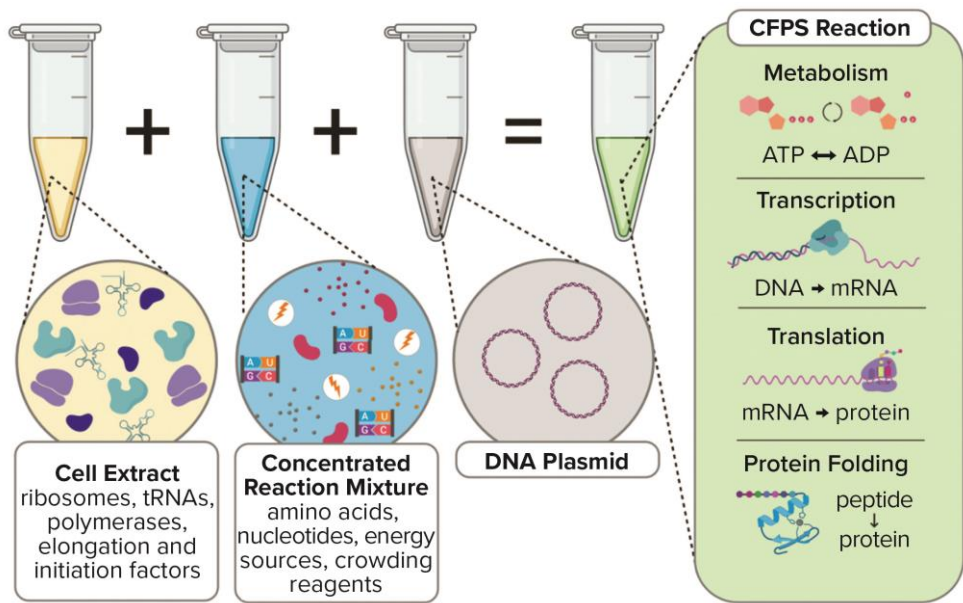


Melinek B, et al. *BioProcess International* (2020)

Amino Acid
tRNA
Mg-glutamate
K-glutamate
Folnic Acid
Spermidine
Coenzyme A
NAD
3-PGA
AMPC
Nucleoside triphosphate (NTP)
HEPES

Translation
Translation
Ribosome maintenance
Ribosome stability
A precursor for formylmethionine (fMet)
Ribosome activity /RNA stability
Regenerate ATP via glycolysis
Regenerate ATP via glycolysis
Regenerate ATP
regulating glycolysis
Transcription
PH Buffer

Buffer composition



Melinek B, et al. *BioProcess International* (2020)

Amino Acid

Mg-glutamate
K-glutamate

Spermidine

3-PGA

Nucleoside triphosphate (NTP)
HEPES

Translation

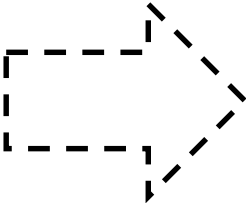
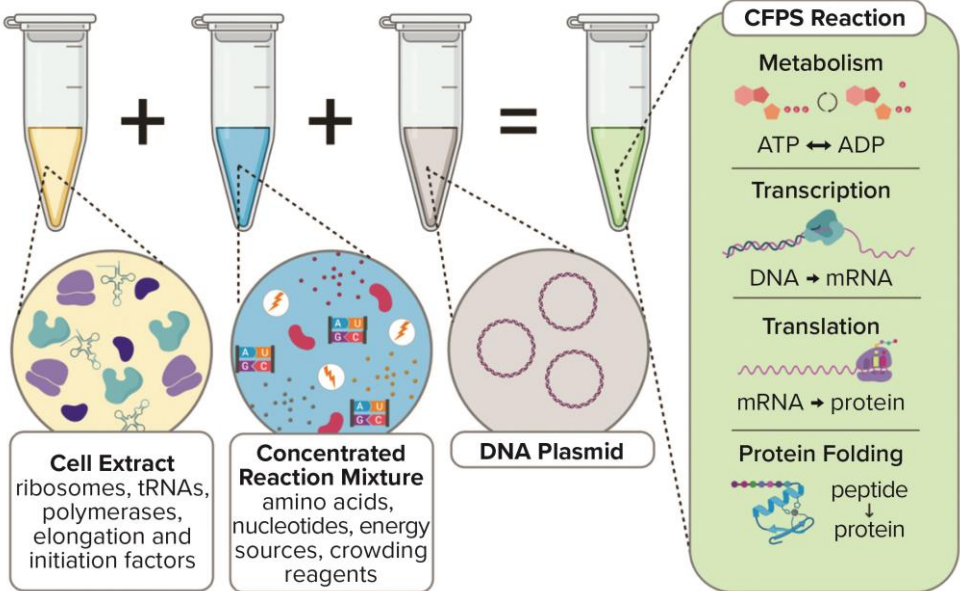
Ribosome maintenance
Ribosome stability

Ribosome activity /RNA stability

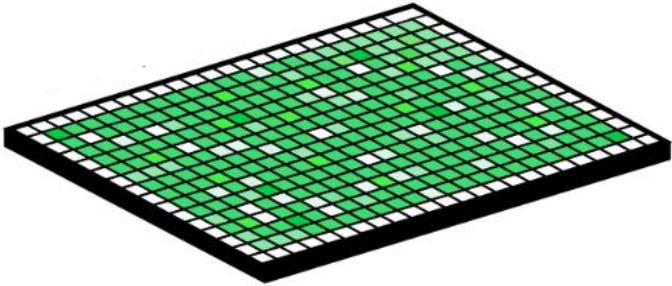
Regenerate ATP

Transcription
PH Buffer

Measurements in 384-well plate

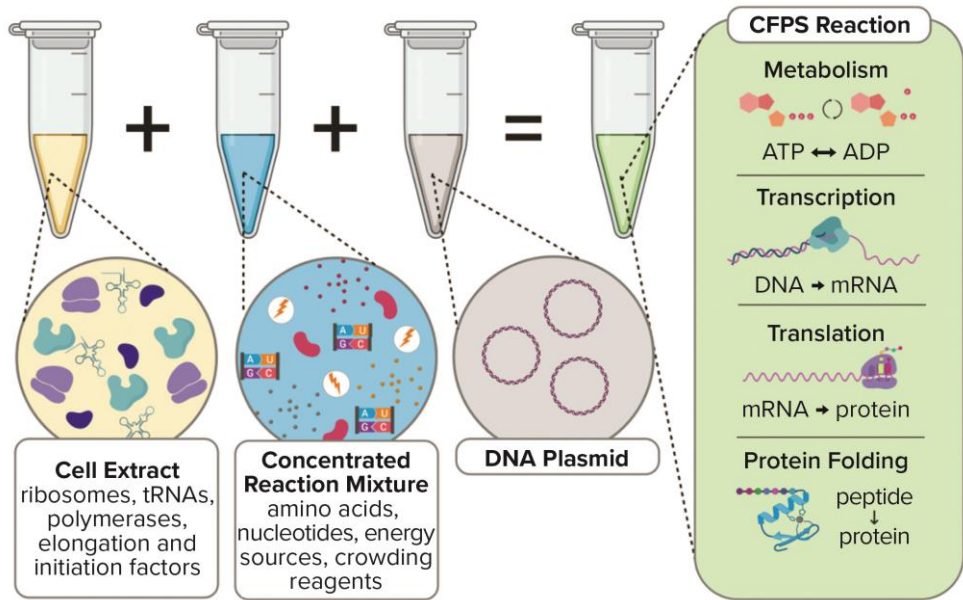


1 to 20 μ l reactions
(in 384-well)

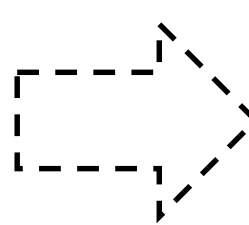


Melinek B, et al. *BioProcess International* (2020)

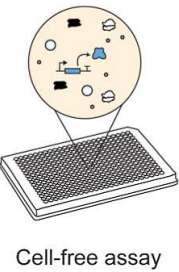
Measurements in 1536-well plate



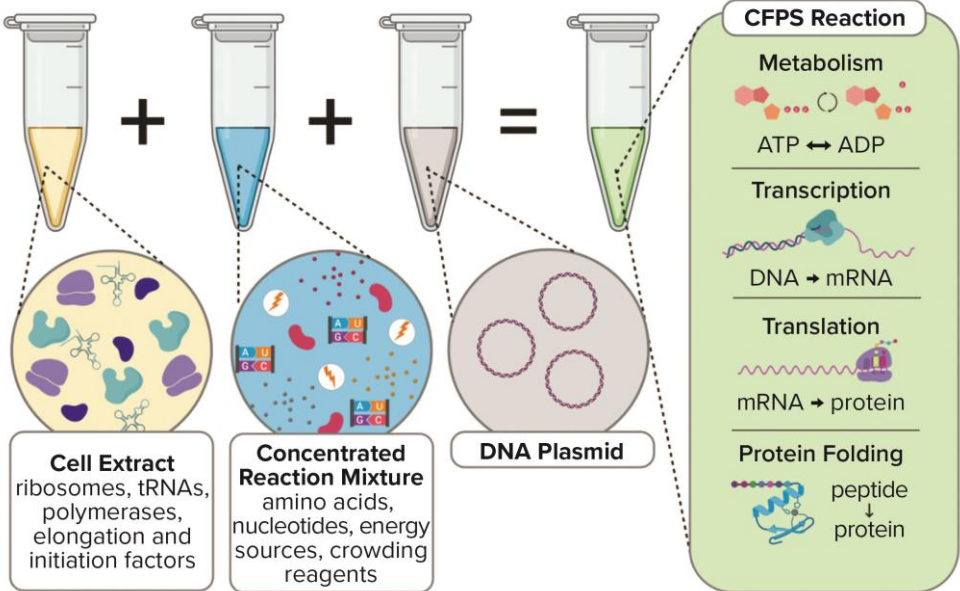
Melinek B, et al. *BioProcess International* (2020)



1 μ l reactions
(in 1536-well)

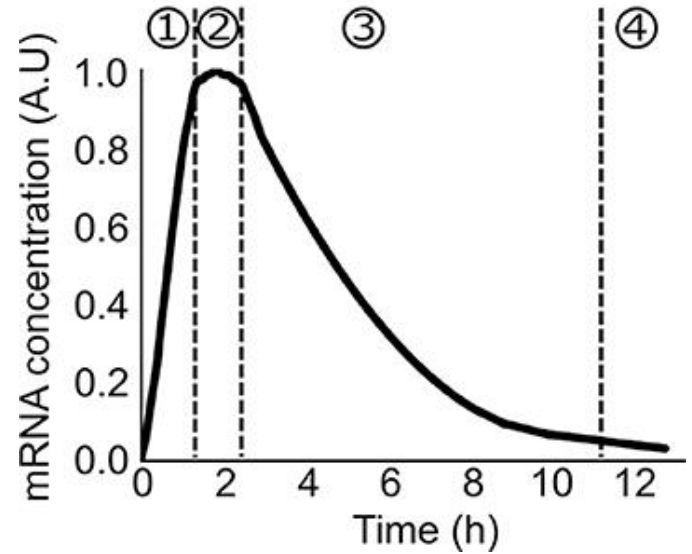
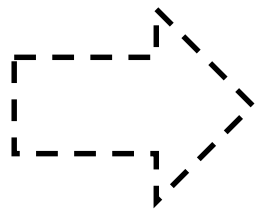


Measurements profiles

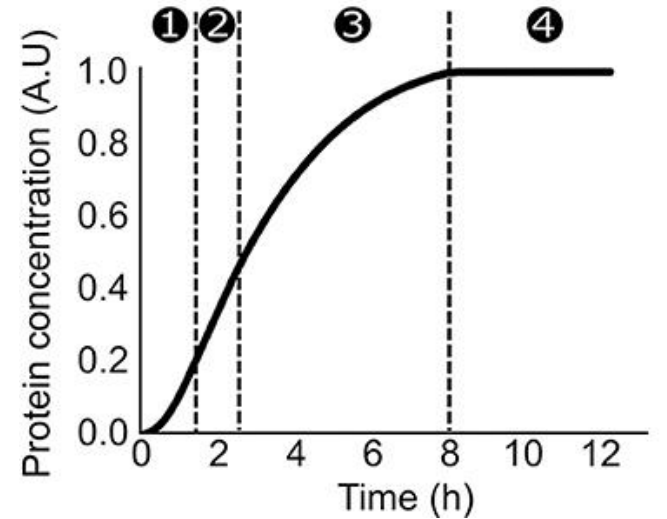


Melinek B, et al. *BioProcess International* (2020)

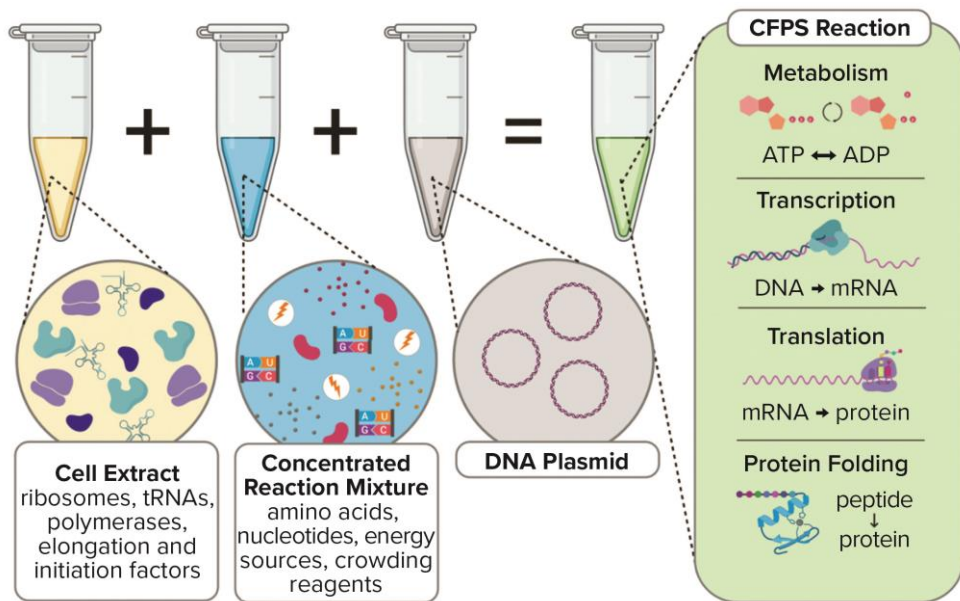
Transcription



Translation



Current existing protocol to produce bacterial cell extract



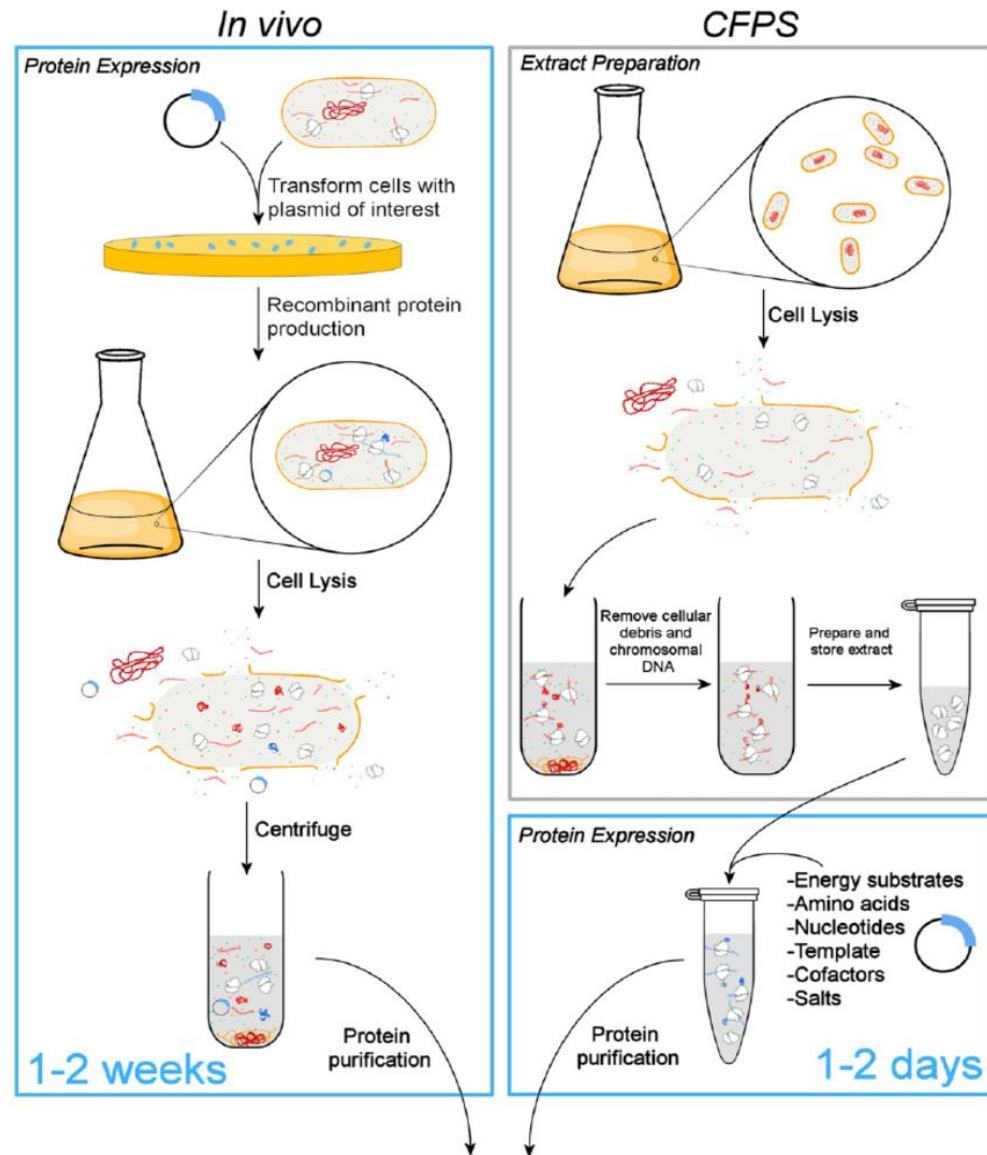
Melinek B, et al. *BioProcess International* (2020)

Table 1. Current existing protocols to produce cell-free lysates from bacteria cells

Organism	Bacteria Phylum
<i>E. coli</i>	Proteobacteria
<i>B. subtilis</i>	Firmicutes
<i>L. lactis</i>	Firmicutes
<i>B. megaterium</i>	Firmicutes
<i>C. autoethanogenum</i>	Firmicutes
<i>V. natriegens</i>	Proteobacteria
<i>P. putida</i>	Proteobacteria
<i>E. fergusonii</i>	Proteobacteria
<i>S. enterica</i>	Proteobacteria
<i>K. oxytoca</i>	Proteobacteria
<i>C. glutamicum</i>	Actinobacteria
<i>S. venezuelae</i>	Actinobacteria

Advantages of producing protein outside of the cell

- Fast reaction to produce protein
- No need for transformation
- No GMO
- No interaction with a living host
(No mutation, No burden)
- Decoupling effect
(e.g. Metabolic burden vs production burden)
- Low volume of reaction
- Higher level of control: Amount of DNA,
Composition of the reaction.
- Work with Linear DNA



2. Understand living organisms: **functions preserved** in cell-free systems

2. Understand living organisms: **functions preserved** in cell-free systems

The central dogma: from DNA to protein

- DNA replication

- Transcription and transcriptional regulation

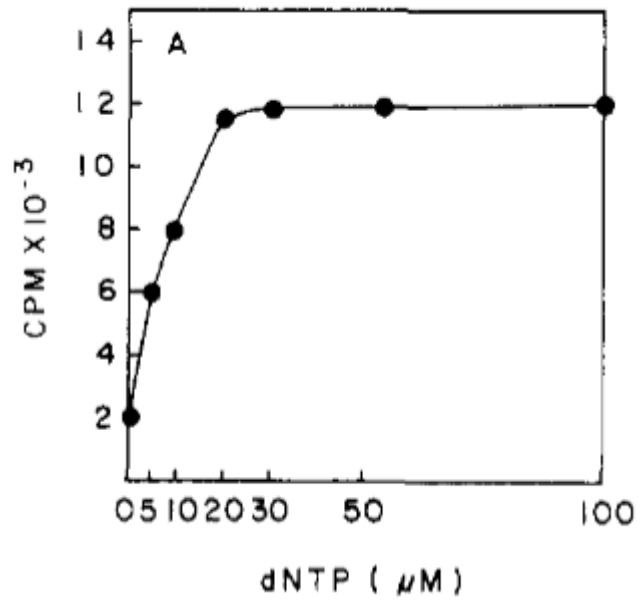
- Translation and translational regulation

Metabolism and energy production

Resources utilization

DNA replication: from the lysate

Cell-free used to understand plasmid replication (measurement using ^{32}P radioactive isotope)



DNA replication: from the lysate

Cell-free used to understand plasmid replication (measurement using ^{32}P radioactive isotope)

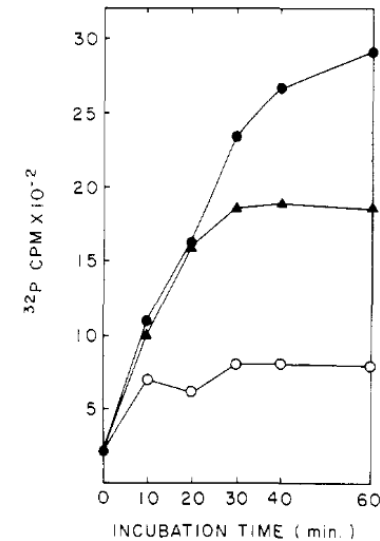
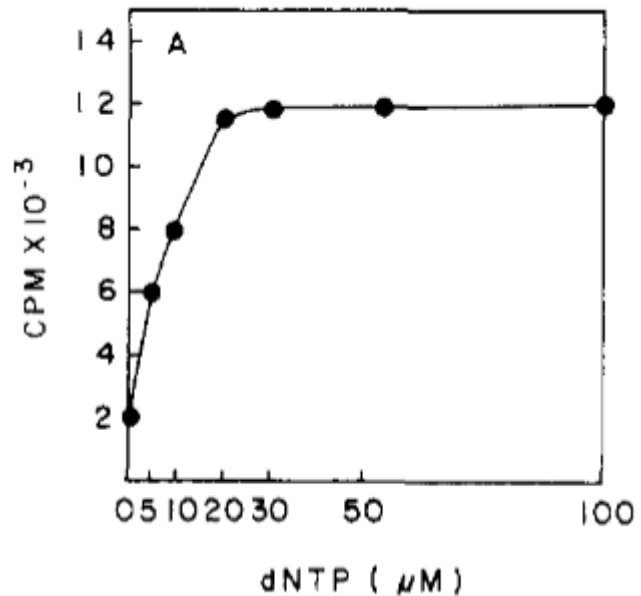


FIGURE 4: Effect of rifampin on R6K DNA synthesis. The reaction conditions were as described in the legend to Figure 2 except that $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ was used instead of $[^3\text{H}]\text{dTTP}$. Rifampin ($20 \mu\text{g}/\text{mL}$) was either not added (●) or added at 0 (○) and 10 min (▲) after incubation.

“DNA synthesis is dependent on the four deoxyribo- and ribonucleotide triphosphates and is sensitive to rifampin and streptolydigin, inhibitors of DNA-dependent RNA polymerase.”

DNA replication

Cell-free used to understand plasmid replication (measurement using ^{32}P radioactive isotope)

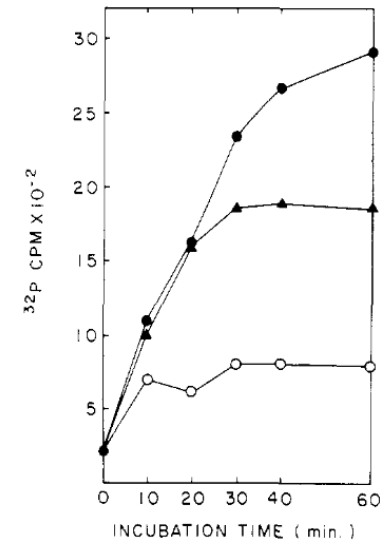
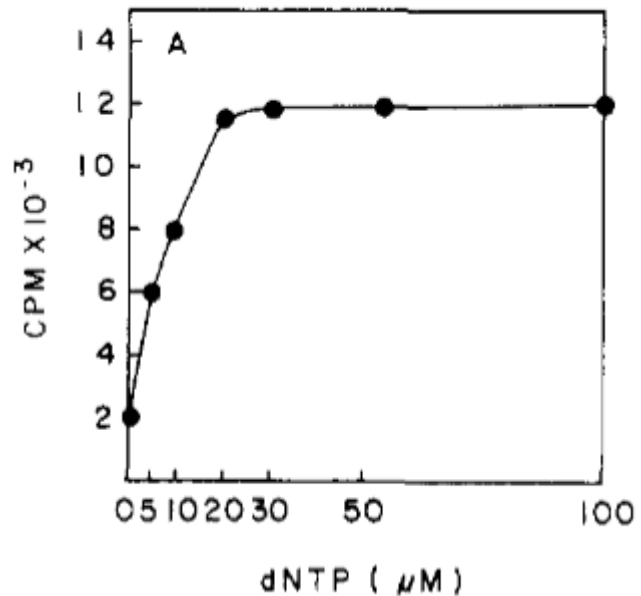
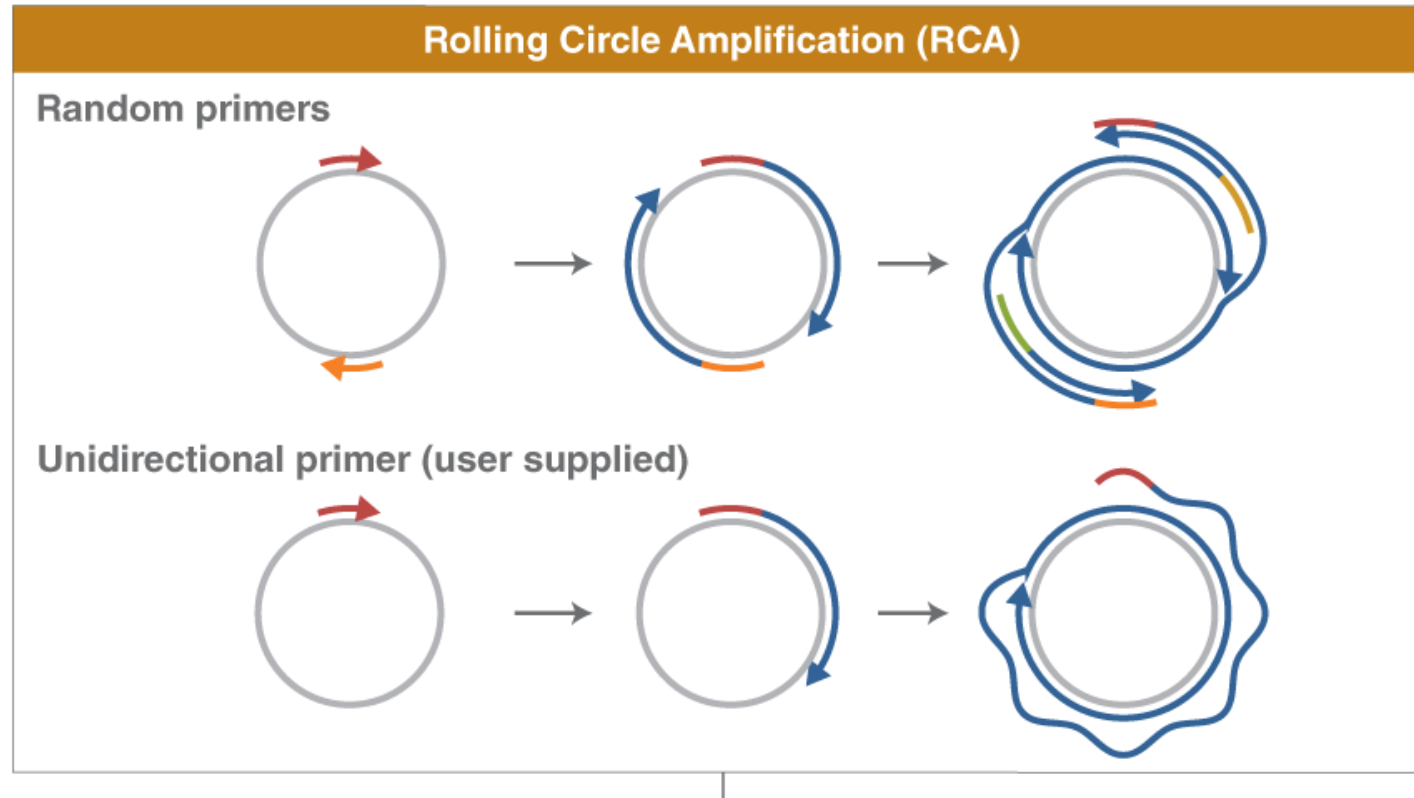


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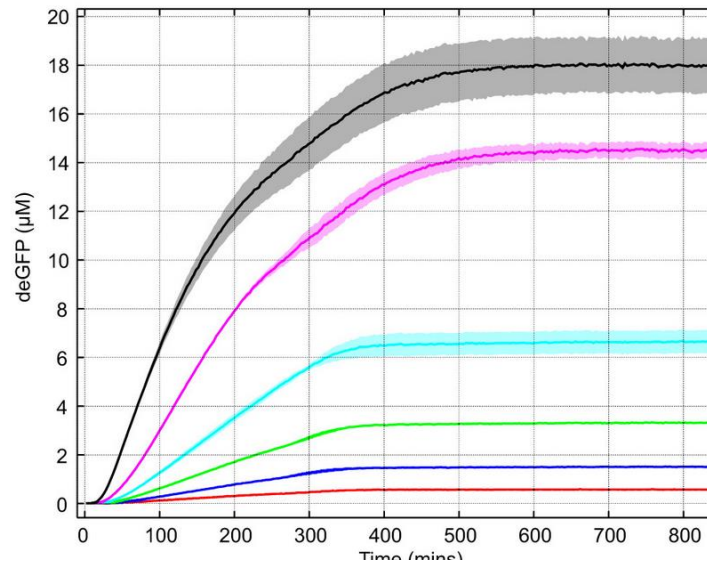
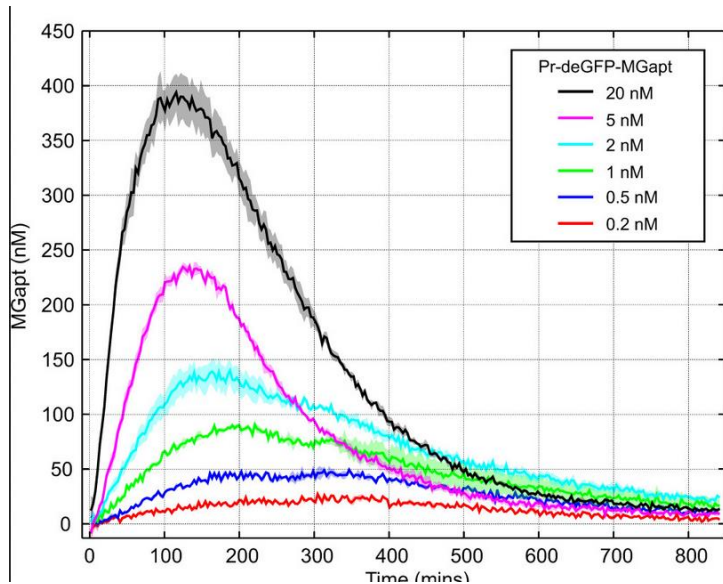
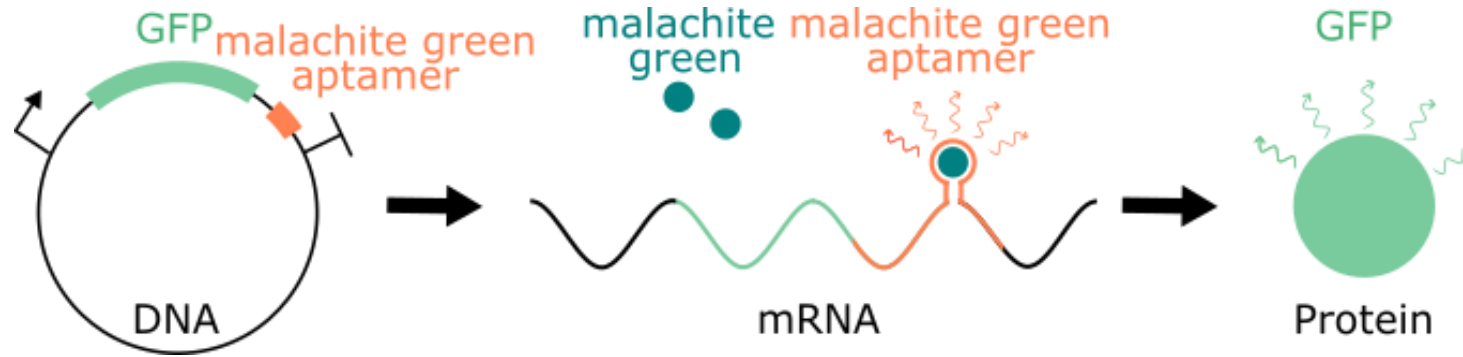
DNA replication to amplify DNA

DNA replication system reconstructed by expressing 1 gene of the bacteriophage phi 29 DNA replication .



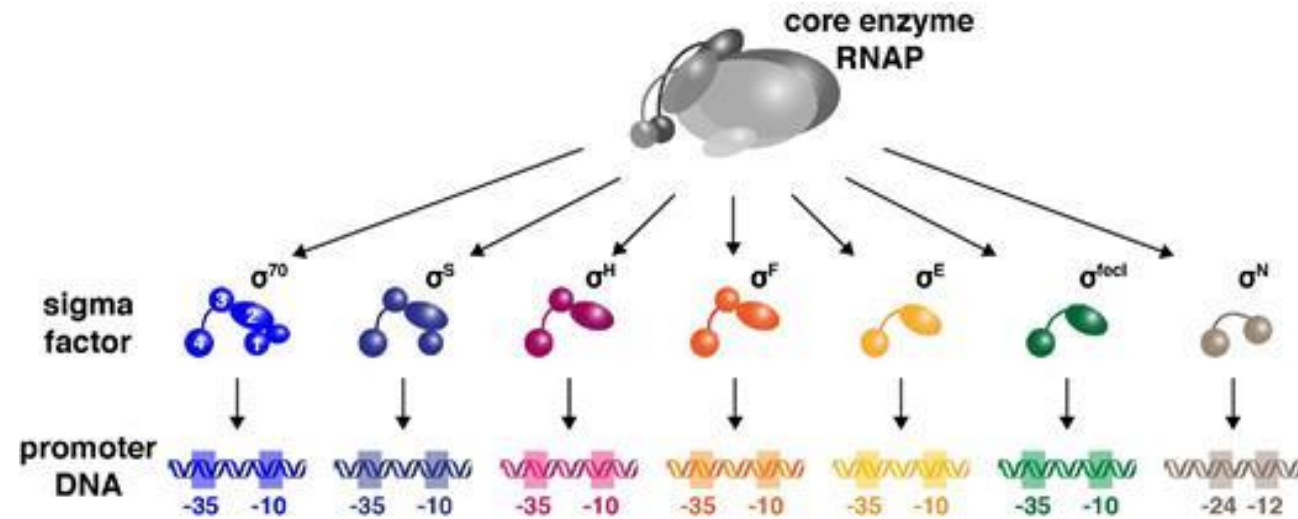
Transcription and transcriptional regulation

The core RNA polymerase remains present and active in cell-free



Siegal-Gaskins, D. ACS synthetic biology (2014)
Wagner L et al. (in progress)

Transcription and transcriptional regulation



σ -factor	Function	Group
σ^D/σ^{70}	Housekeeping genes	1
σ^S/σ^{38}	Stationary phase	2
σ^{H1}/σ^{32}	Heat shock response	3
σ^F/σ^{28}	Motility genes	3
σ^E/σ^{24}	Extracytoplasmic function	4
$\sigma^{fectl}/\sigma^{19}$	Extracytoplasmic function	4
σ^N/σ^{54}	Nitrogen metabolism	

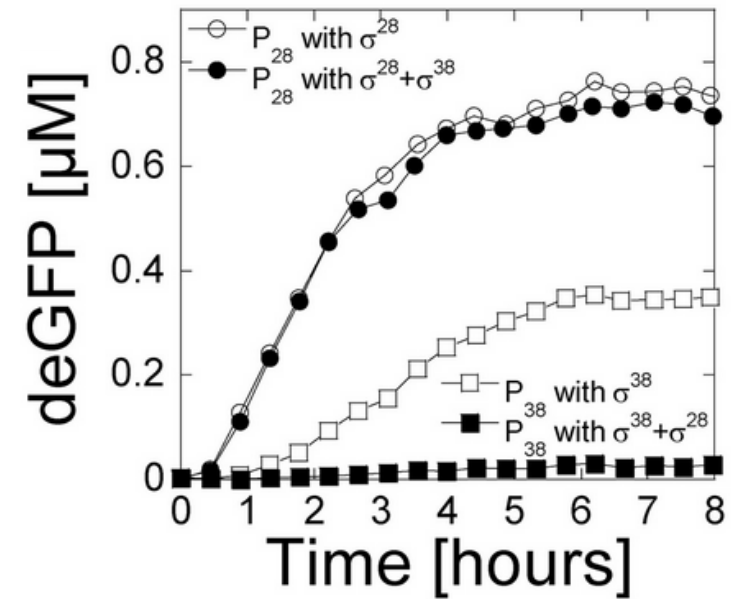
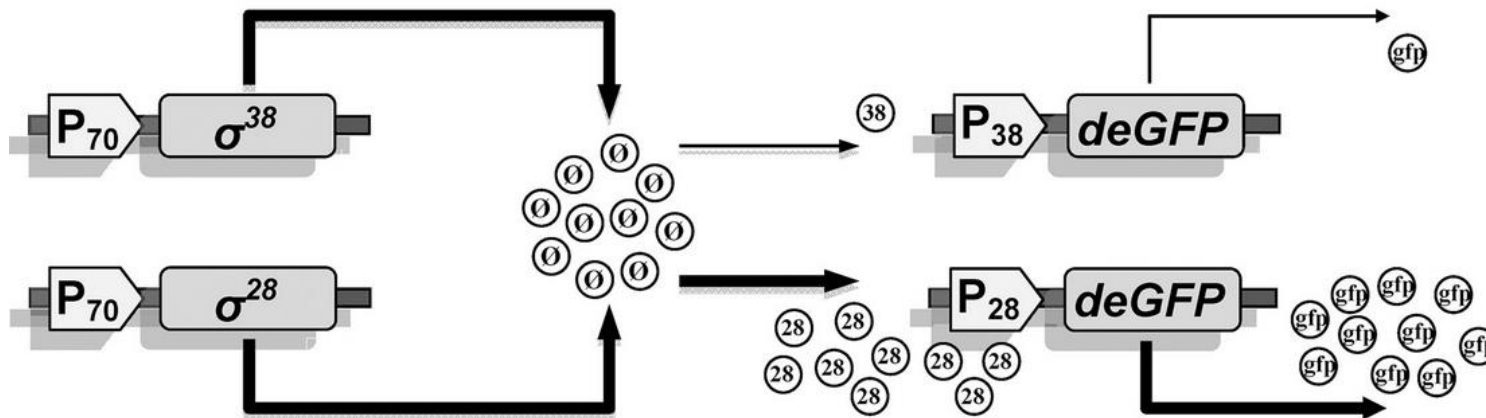
Transcription and transcriptional regulation

Promoter selection and characterization Orthogonality of *E. coli* sigma factors

deGFP [μ M]		Transcription factor expressed							
		$(\sigma^{70})^*$	σ^{19}	σ^{24}	σ^{28}	σ^{32}	σ^{38}	T3RNAP	T7RNAP
Promoters	P ₇₀	6.40	5.89	5.45	3.96	4.74	6.01	6.06	5.76
	P ₁₉	< 0.01	0.89	0.01	0.02	< 0.01	0.01	< 0.01	< 0.01
	P ₂₄	0.01	0.03	0.74	< 0.01	0.01	0.03	0.01	0.01
	P ₂₈	< 0.01	0.02	< 0.01	8.06	< 0.01	< 0.01	< 0.01	< 0.01
	P ₃₂	0.20	0.21	0.20	0.12	5.44	0.20	0.20	0.22
	P ₃₈	< 0.01	0.13	0.03	0.01	< 0.01	0.83	0.01	< 0.01
	P _{T3}	0.02	0.02	0.03	< 0.01	0.02	0.03	13.44	0.02
	P _{T7}	0.02	0.03	0.02	< 0.01	< 0.01	0.02	0.02	17.91
Rate of deGFP synthesis [nM/min]		Transcription factor expressed							
		$(\sigma^{70})^*$	σ^{19}	σ^{24}	σ^{28}	σ^{32}	σ^{38}	T3RNAP	T7RNAP
Promoters	P ₇₀	23.91	23.53	25.68	20.16	23.08	26.98	23.15	22.98
	P ₁₉	< 0.01	3.67	0.04	0.01	< 0.01	0.05	< 0.01	< 0.01
	P ₂₄	0.02	0.02	2.35	< 0.01	< 0.01	0.08	0.03	0.03
	P ₂₈	< 0.01	< 0.01	< 0.01	35.28	< 0.01	< 0.01	< 0.01	< 0.01
	P ₃₂	0.53	0.55	0.76	0.43	24.30	0.81	0.73	0.78
	P ₃₈	< 0.01	0.27	0.08	0.02	< 0.01	5.38	0.04	< 0.01
	P _{T3}	0.07	0.05	0.10	< 0.01	0.18	0.1	75.33	0.16
	P _{T7}	0.04	0.06	0.05	< 0.01	< 0.01	0.08	0.83	93.87

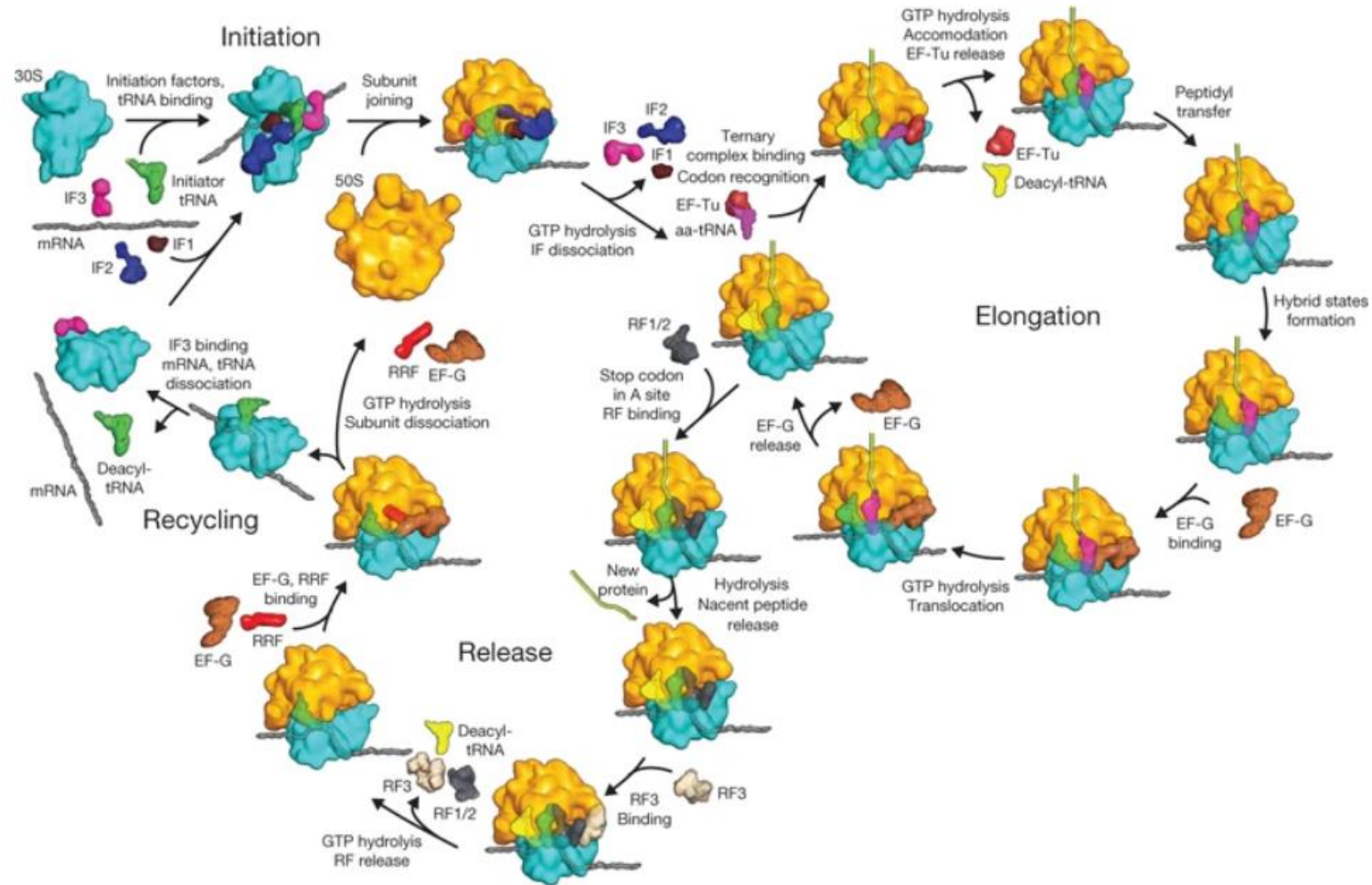
Transcription and transcriptional regulation

Passive transcription regulation by competition between sigma factors



Translation and translation regulation

Overview of bacterial translation:



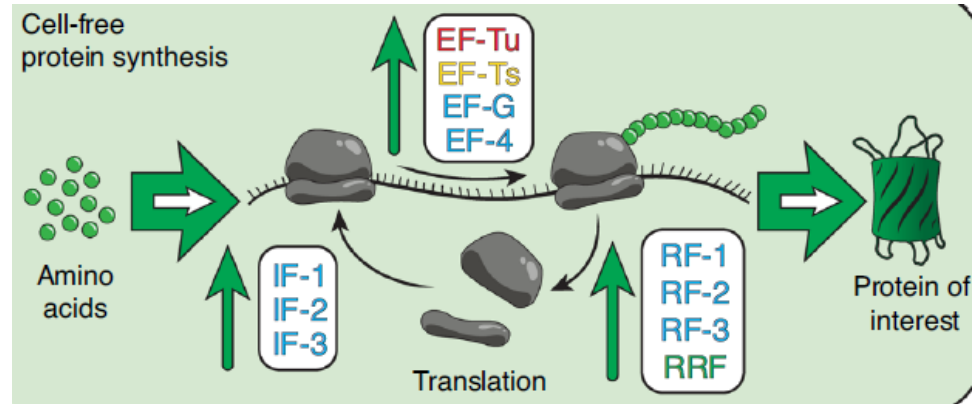
Translation and translation regulation

All ribosomal proteins + TL factors were identified by LC/MS in cell-free lysate

#	Identified proteins	Access. No.	MW
Subunit S30			
REF	EF-Tu	WP_000031784.1	43 kDa
1	30S ribosomal protein S1	WP_000140327.1	61 kDa
2	30S ribosomal protein S2	WP_000246882.1	27 kDa
3	30S ribosomal protein S3	WP_000529945.1	26 kDa
4	30S ribosomal protein S5	WP_000940120.1	18 kDa
5	30S ribosomal protein S4	WP_000135224.1	23 kDa
6	30S ribosomal protein S7	WP_001138043.1	18 kDa
7	30S ribosomal protein S11	WP_001029684.1	14 kDa
8	30S ribosomal protein S12	WP_000246815.1	14 kDa
9	30S ribosomal protein S13	WP_000090775.1	13 kDa
10	30S ribosomal protein S19	WP_001138117.1	10 kDa
11	30S ribosomal protein S10	WP_001181004.1	12 kDa
12	30S ribosomal protein S6	WP_001216675.1	15 kDa
13	30S ribosomal protein S9	WP_000829818.1	15 kDa
14	30S ribosomal protein S21	WP_001144069.1	9 kDa
15	30S ribosomal protein S15	WP_000059466.1	10 kDa
16	30S ribosomal protein S16	WP_000256450.1	9 kDa
17	30S ribosomal protein S20	WP_001274021.1	10 kDa
18	30S ribosomal protein S8	WP_000062611.1	14 kDa
19	30S ribosomal protein S18	WP_000135199.1	9 kDa
20	30S ribosomal protein S14	WP_001118930.1	12 kDa
21	30S ribosomal protein S17	WP_000130100.1	10 kDa
22	30S ribosomal protein S12 RimO	WP_000049367.1	50 kDa
23	30S ribosomal protein S12 YcaO	WP_001295344.1	66 kDa
TL initiation			
1	Translation initiation factor IF-2	WP_000133044.1	97 kDa
2	Translation initiation factor IF-3	WP_001700733.1	21 kDa
3	Translation initiation factor IF-1	WP_001040187.1	8 kDa
TL elongation			
1	Elongation factor G	WP_000124700.1	78 kDa
2	Elongation factor Ts	WP_000818114.1	30 kDa
3	Elongation factor P	WP_000257278.1	21 kDa
4	Elongation factor 4	WP_000790168.1	67 kDa
5	Elongation factor P-like protein YeiP	WP_001136827.1	22 kDa
TL termination			
1	Peptide chain release factor 3 prfC	WP_000175940.1	60 kDa
2	Peptide chain release factor 1 prfA	WP_000804726.1	41 kDa

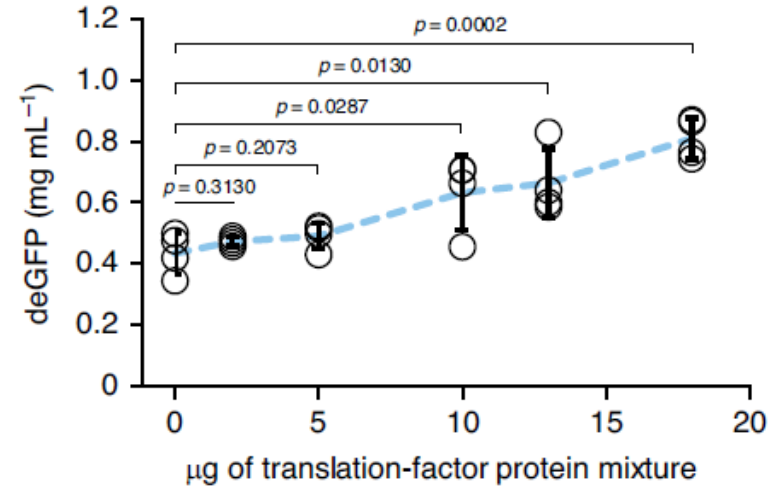
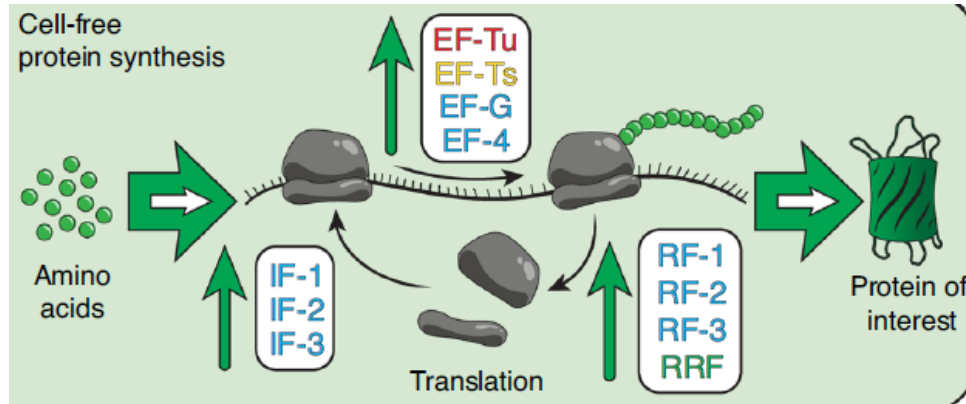
#	Identified proteins	Access. No.	MW
Subunit S50			
REF	EF-Tu	WP_000031784.1	43 kDa
1	50S ribosomal protein L2	WP_000301864.1	30 kDa
2	50S ribosomal protein L17	WP_001216368.1	14 kDa
3	50S ribosomal protein L9	WP_001196062.1	16 kDa
4	50S ribosomal protein L5	WP_001096200.1	20 kDa
5	50S ribosomal protein L6	WP_000091945.1	19 kDa
6	50S ribosomal protein L1	WP_001096684.1	25 kDa
7	50S ribosomal protein L24	WP_000729185.1	11 kDa
8	50S ribosomal protein L3	WP_000579833.1	22 kDa
9	50S ribosomal protein L7/L12	WP_000028878.1	12 kDa
10	50S ribosomal protein L28	WP_000091955.1	9 kDa
11	50S ribosomal protein L19	WP_000065253.1	13 kDa
12	50S ribosomal protein L25	WP_000494183.1	11 kDa
13	50S ribosomal protein L10	WP_001207201.1	18 kDa
14	50S ribosomal protein L13	WP_000847559.1	16 kDa
15	50S ribosomal protein L23	WP_000617544.1	11 kDa
16	50S ribosomal protein L22	WP_000447529.1	12 kDa
17	50S ribosomal protein L4	WP_000424395.1	22 kDa
18	50S ribosomal protein L15	WP_001238917.1	15 kDa
19	50S ribosomal protein L14	WP_000613955.1	14 kDa
20	50S ribosomal protein L11	WP_001085926.1	15 kDa
21	50S ribosomal protein L18	WP_000358960.1	13 kDa
22	50S ribosomal protein L21	WP_000271401.1	12 kDa
23	50S ribosomal protein L30	WP_001140433.1	7 kDa
24	50S ribosomal protein L29	WP_000644741.1	7 kDa
25	50S ribosomal protein L20	WP_000124850.1	13 kDa
26	50S ribosomal protein L32	WP_000290727.1	6 kDa
27	50S ribosomal protein L16	WP_000941212.1	15 kDa
28	50S ribosomal protein L33	WP_001051798.1	6 kDa
29	50S ribosomal protein L27	WP_000940595.1	9 kDa
30	50S ribosomal protein L31	WP_000710769.1	8 kDa
31	50S ribosomal protein L3 N(5)-GM	WP_001295704.1	35 kDa
Other TL proteins			
1	Ribosome-recycling factor (RFF, RF4)	WP_000622418.1	21 kDa
2	Ribosome-associated inhibitor A (YfiA)	WP_000178456.1	13 kDa
3	Ribosome-binding factor A	WP_001040205.1	15 kDa
4	Ribosome hibernation promoting factor	WP_001176599.1	11 kDa
5	Ribosome maturation factor	WP_001300397.1	17 kDa
6	Ribosome silencing factor RsfS	WP_001161664.1	12 kDa
7	Ribosome assembly protein YhbY	WP_001054420.1	11 kDa
8	Ribosome maturation factor RimM	WP_000043335.1	21 kDa

Translation and translation regulation



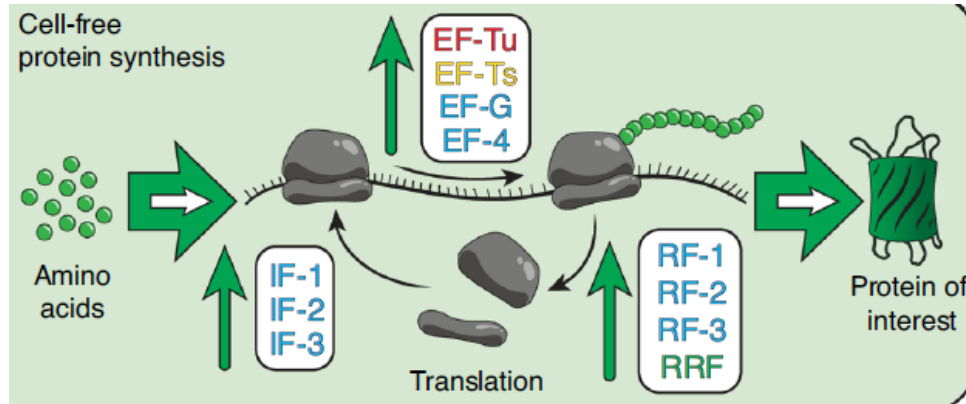
Add purified proteins of the translation machinery

Translation and translation regulation

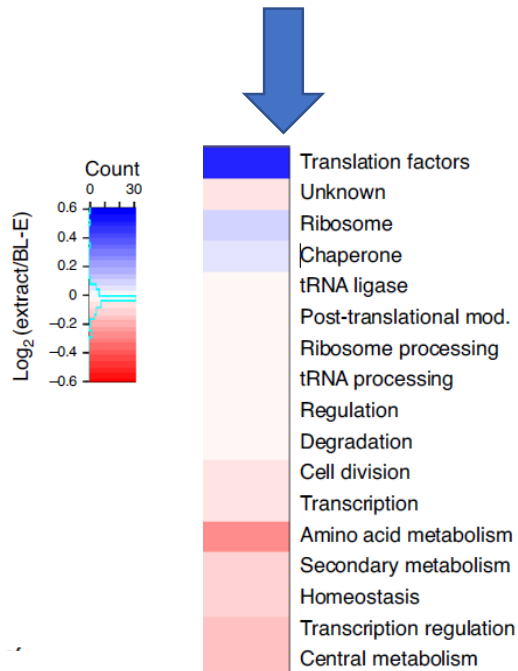
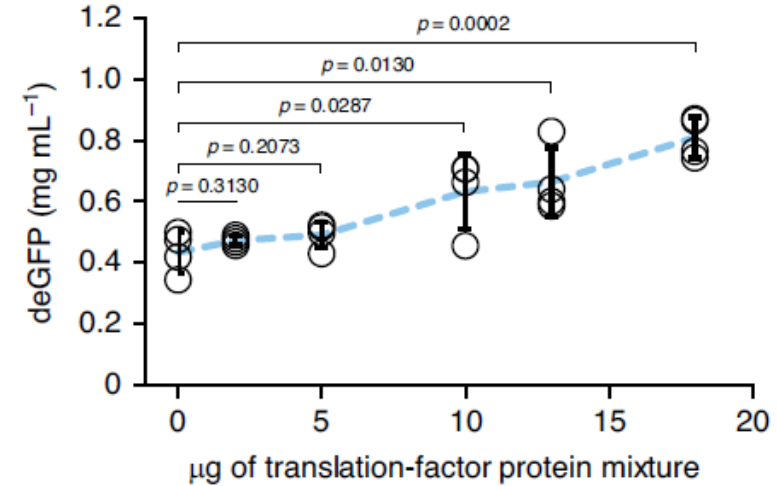


Increase protein production with purified translation machinery

Translation and translation regulation

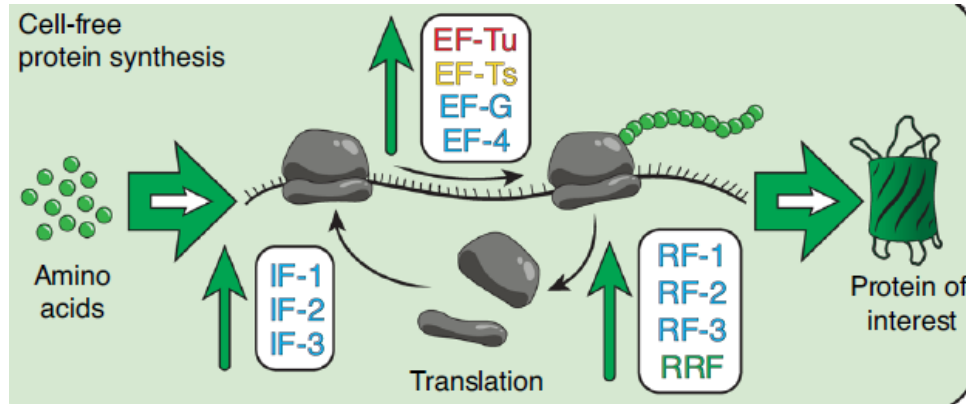


Increase protein production with purified translation machinery

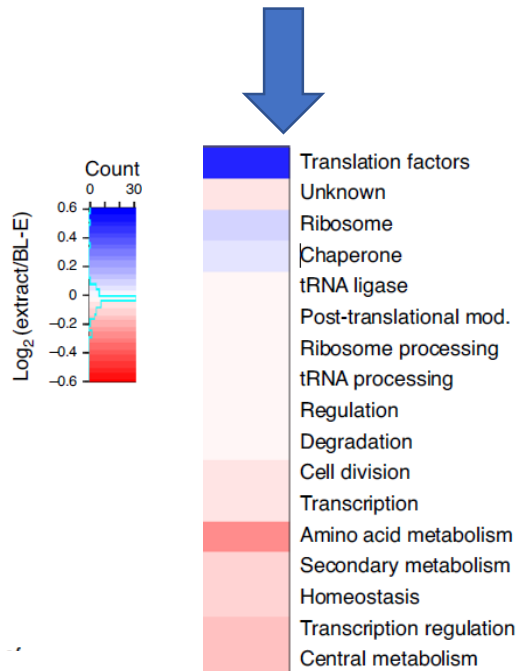
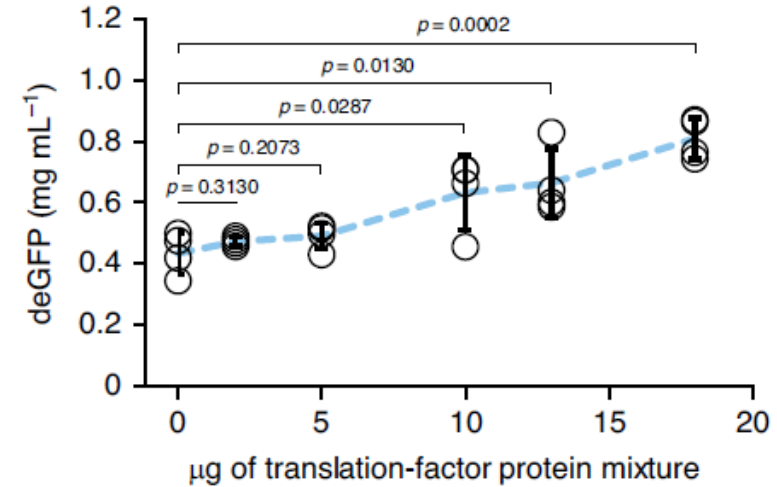


Proteome reprogramming: Mimic some bacterial law in living cell

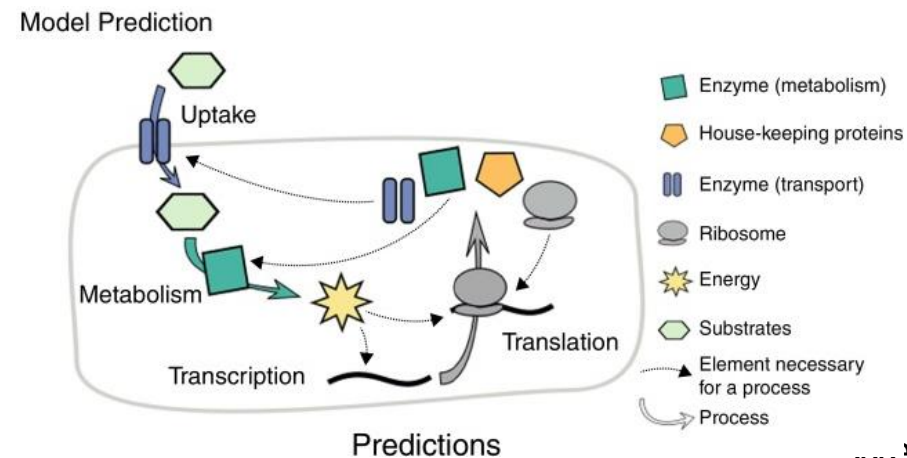
Translation and translation regulation



Increase protein production with purified translation machinery



Proteome reprogramming: Mimic some bacterial law in living cell

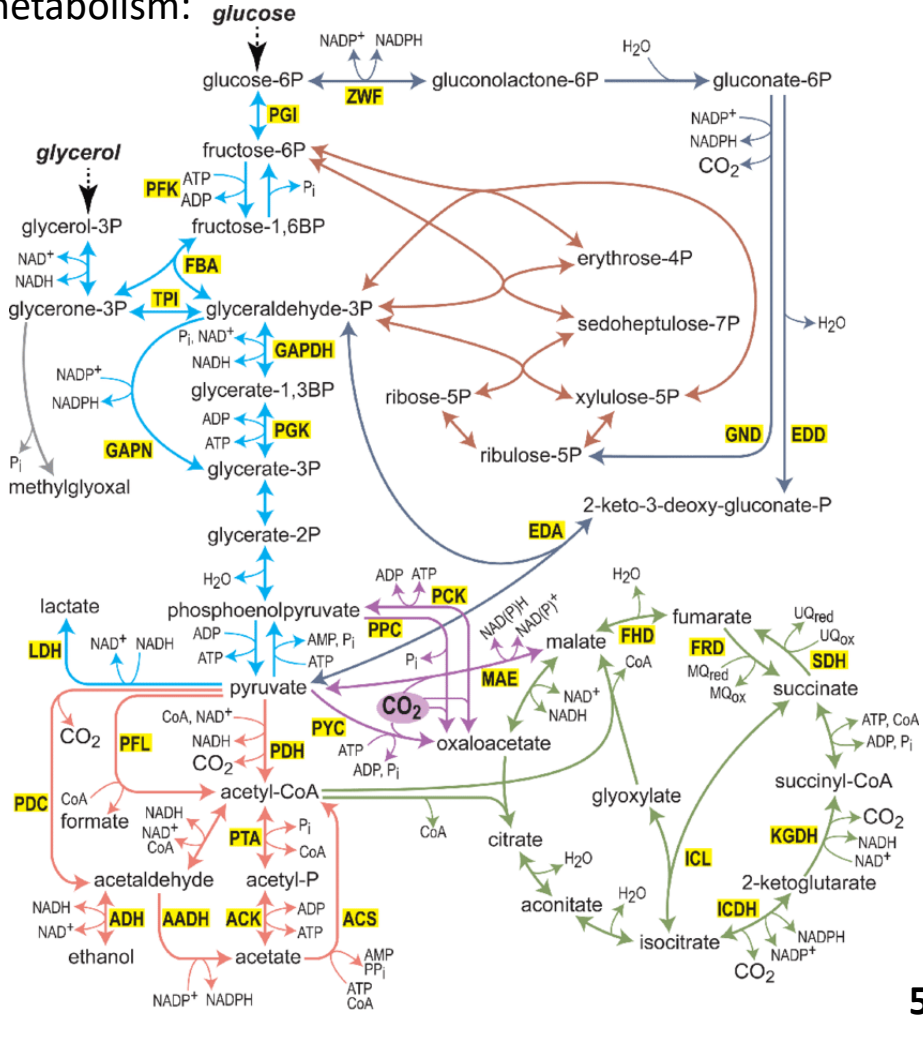


2. Understand living organisms: **functions preserved** in cell-free systems

Metabolism and energy production

Metabolism: ATP regeneration system

Overview of bacterial central metabolism:



Aslan S et al. *Biochemical journal* 2017

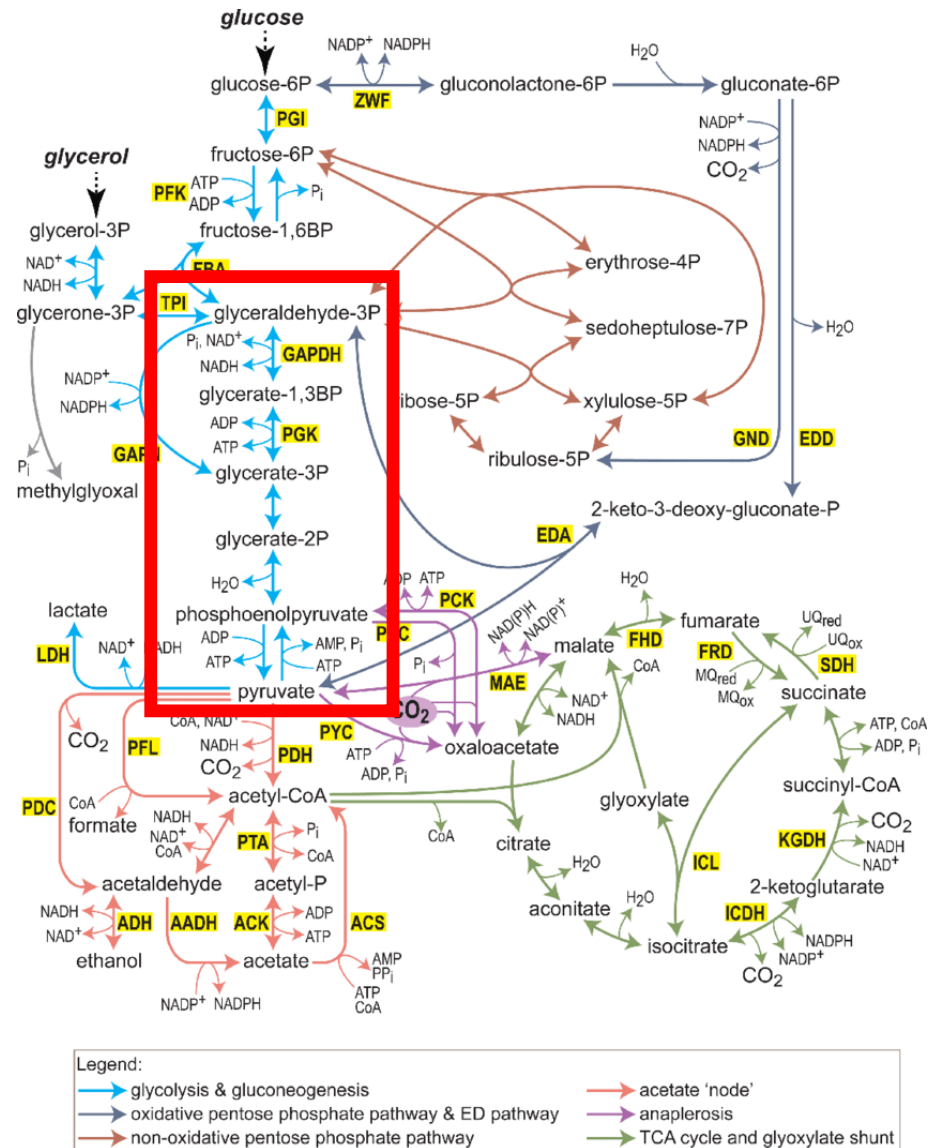
500–800 proteins were identified by LC/MS in cell-free lysate



Garenne D et al. *Rapid Commun Mass Spectrom.* 2019

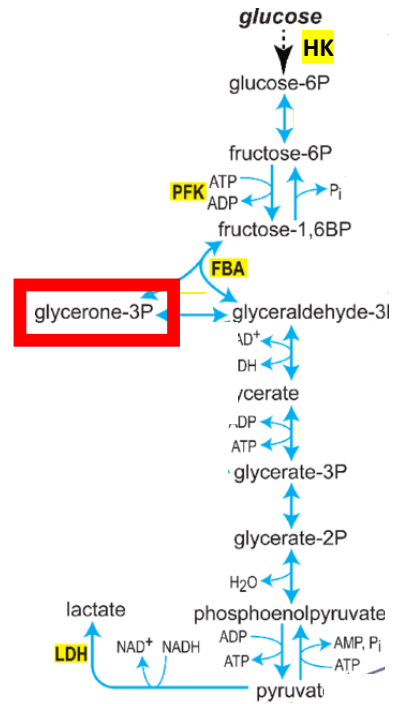
Metabolism: ATP regeneration system

ATP regeneration: **3-PGA**



Metabolism: ATP regeneration system

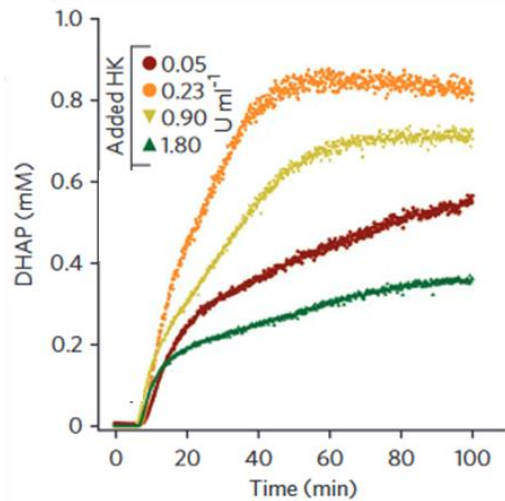
Pathway optimization for glycerone-3p production



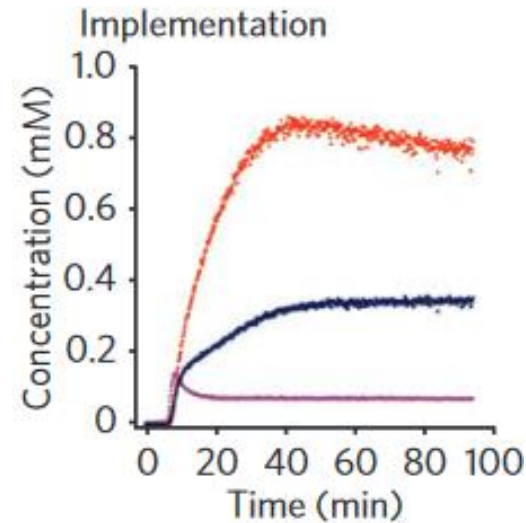
Metabolism: Optimization of in vitro glycolysis

Pathway optimization for glycerone-3p production

In cell-free the system properties can be monitored directly for real-time analysis

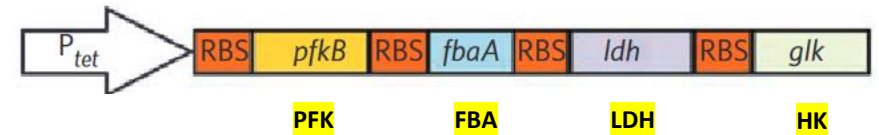


Genetic implementation
as an operon *in vitro*



DHaP (red), G6P (purple)
and FBP (blue)

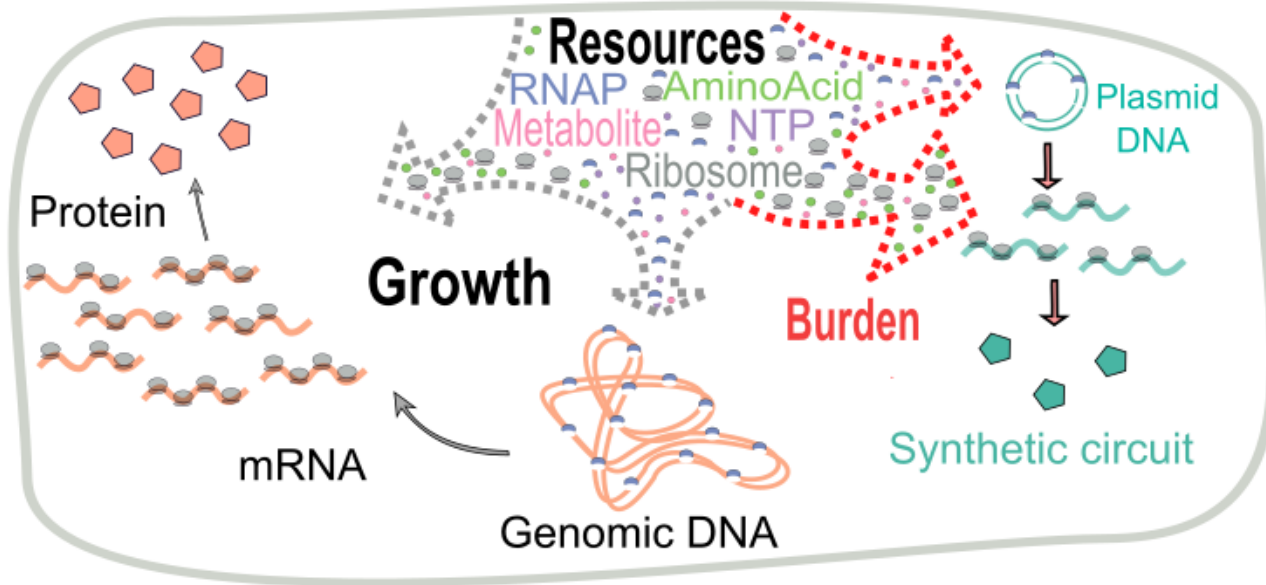
4 enzymes: test 16 combinations of purified
enzyme additions (4*4 concentrations)



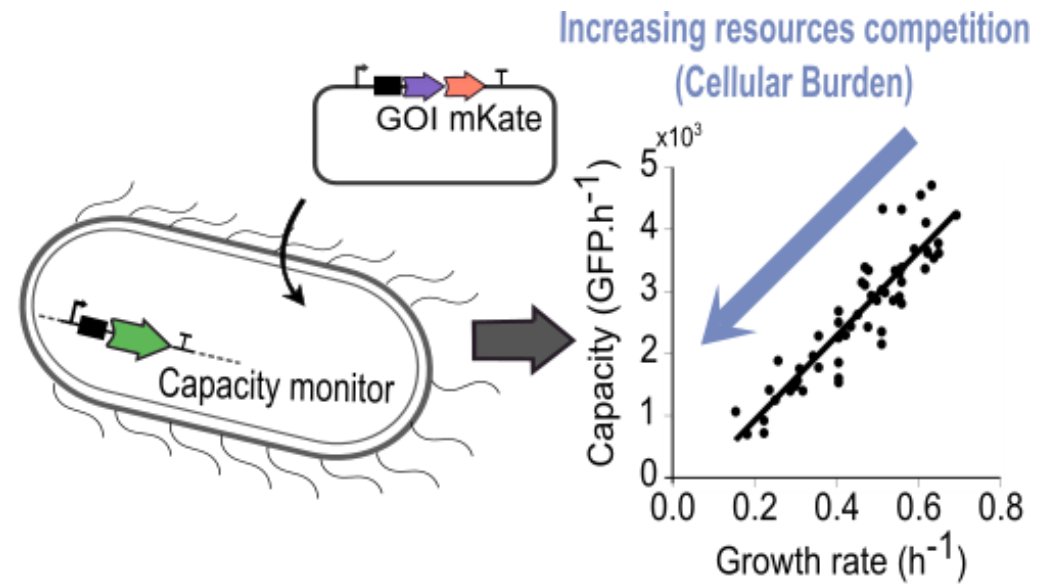
2. Understand living organisms: **functions preserved** in cell-free systems

Burden and resources competition

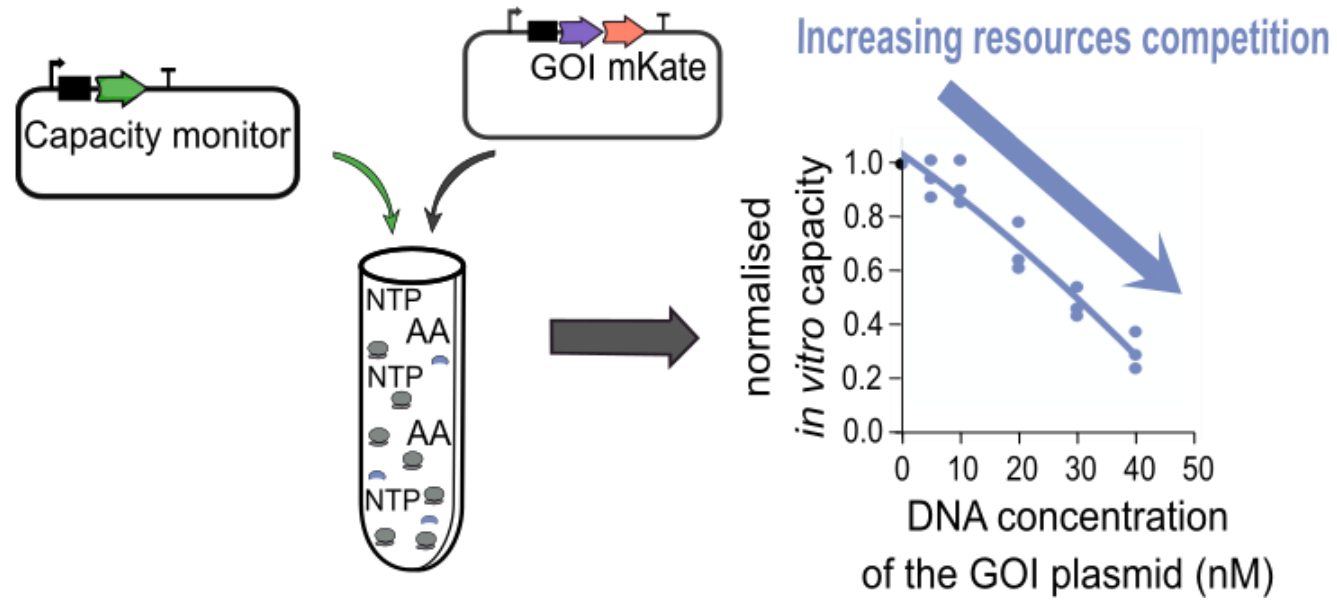
Exploring Resource Competition



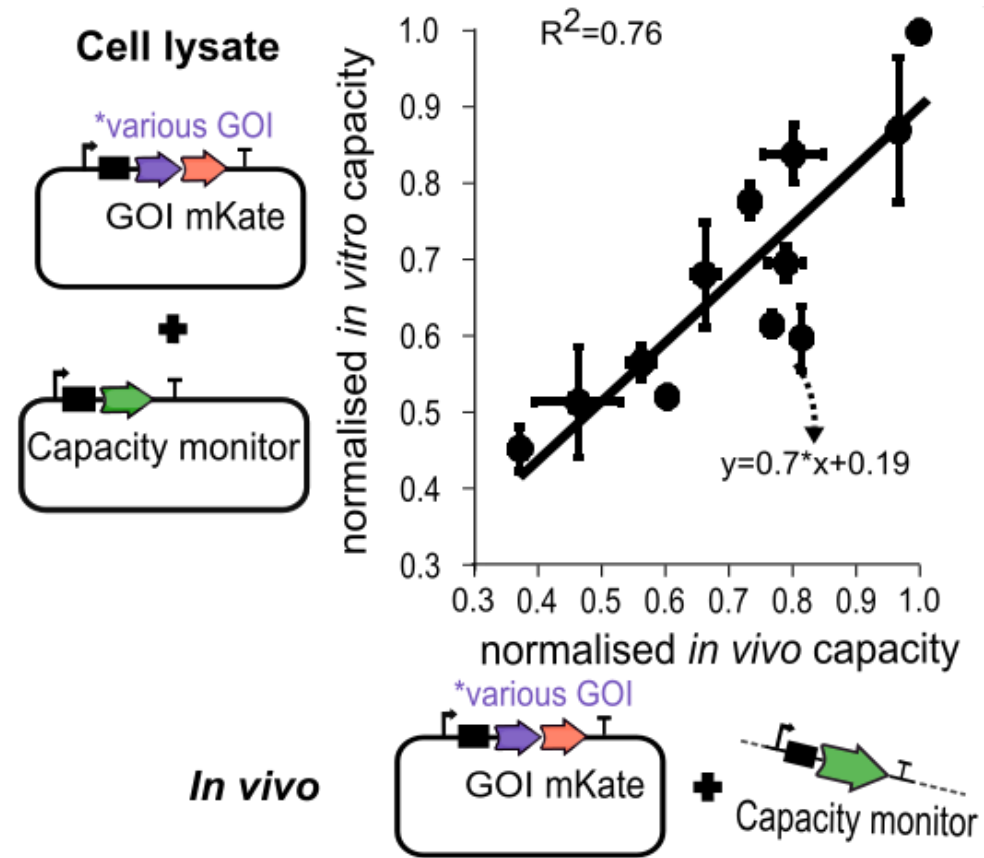
Exploring Resource Competition



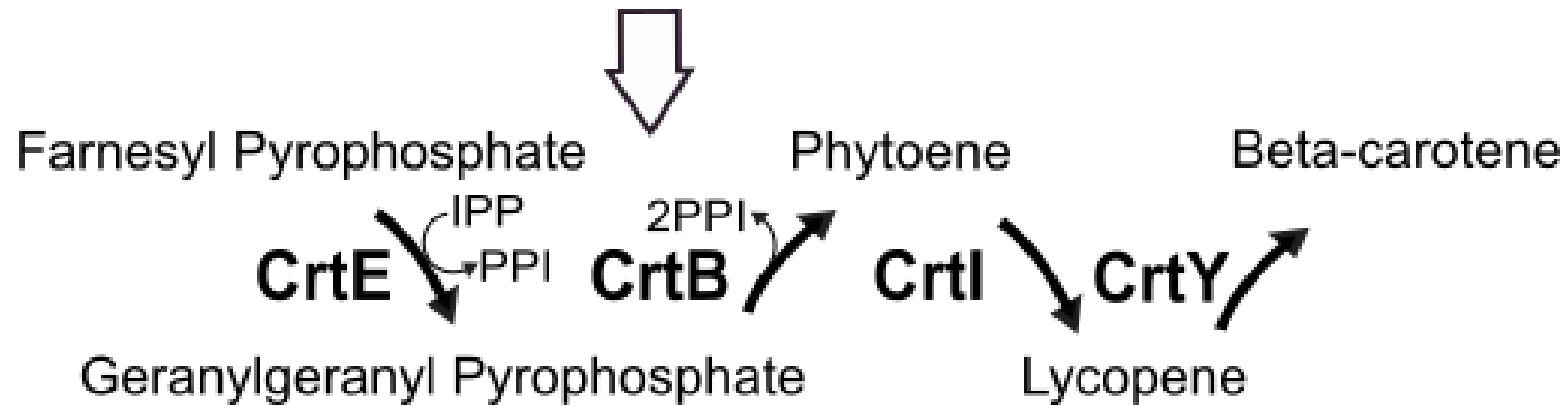
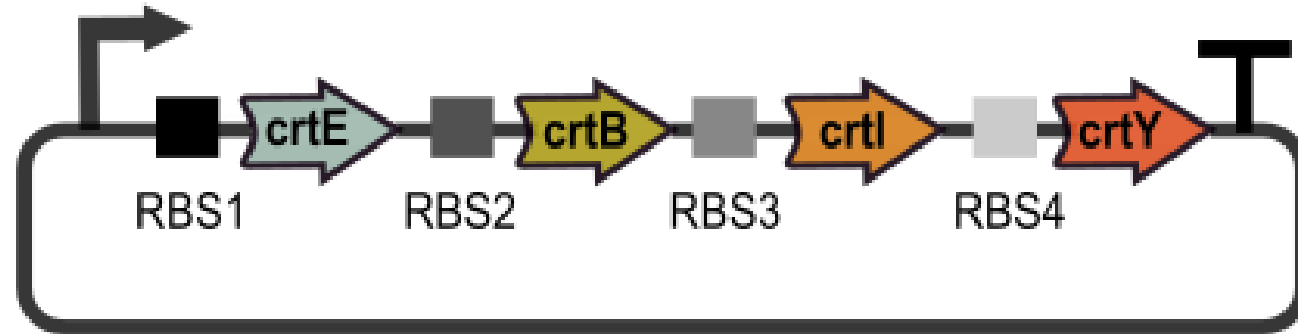
Exploring Resource Competition in cell-free



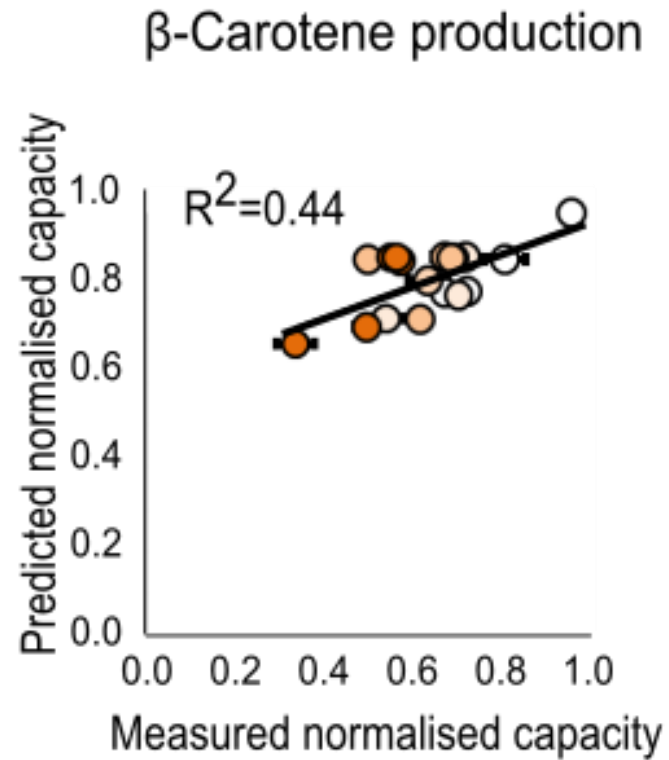
Exploring Resource Competition in cell-free



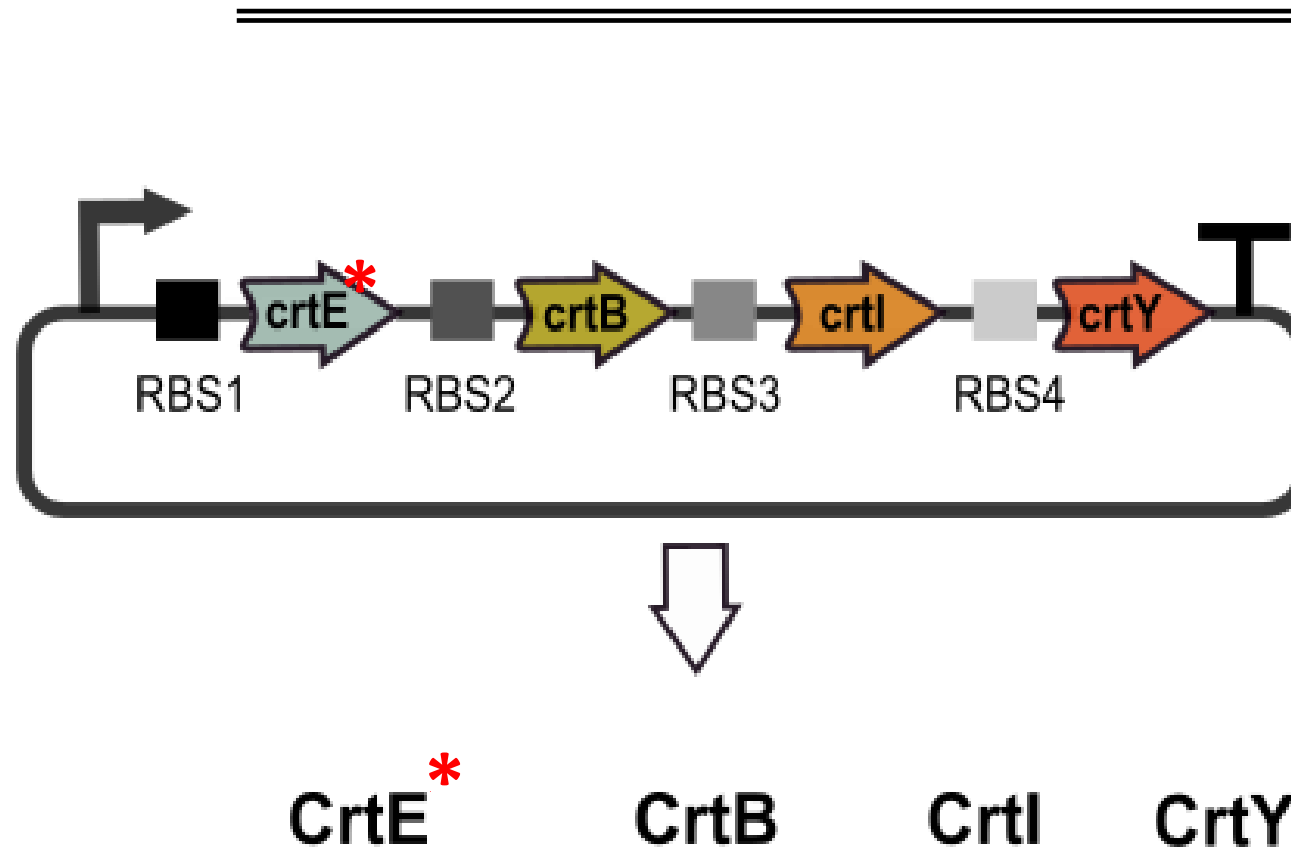
Exploring Resource Competition in cell-free: Metabolic burden



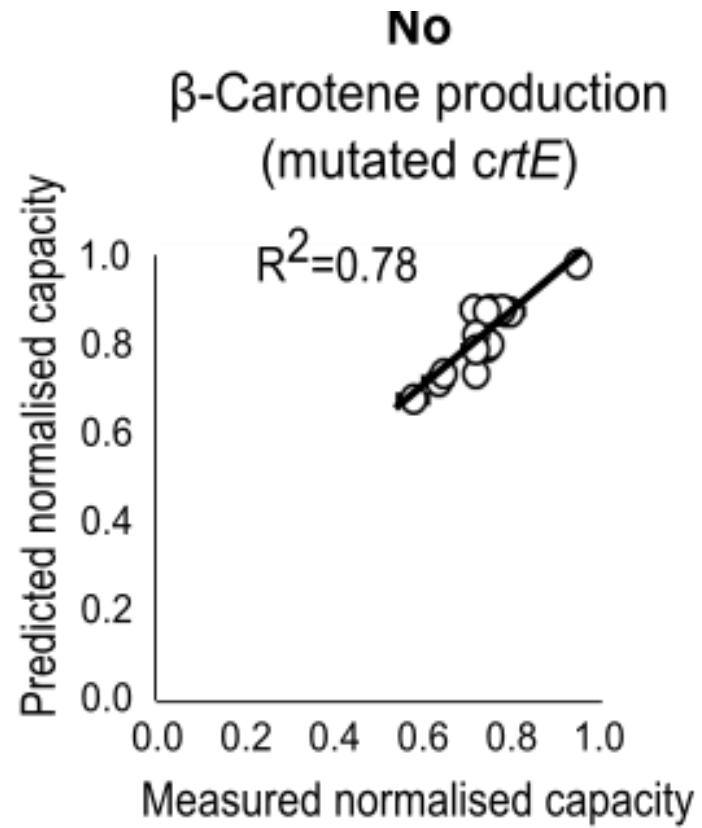
Exploring Resource Competition in cell-free: Metabolic burden



Exploring Resource Competition in cell-free: Metabolic burden

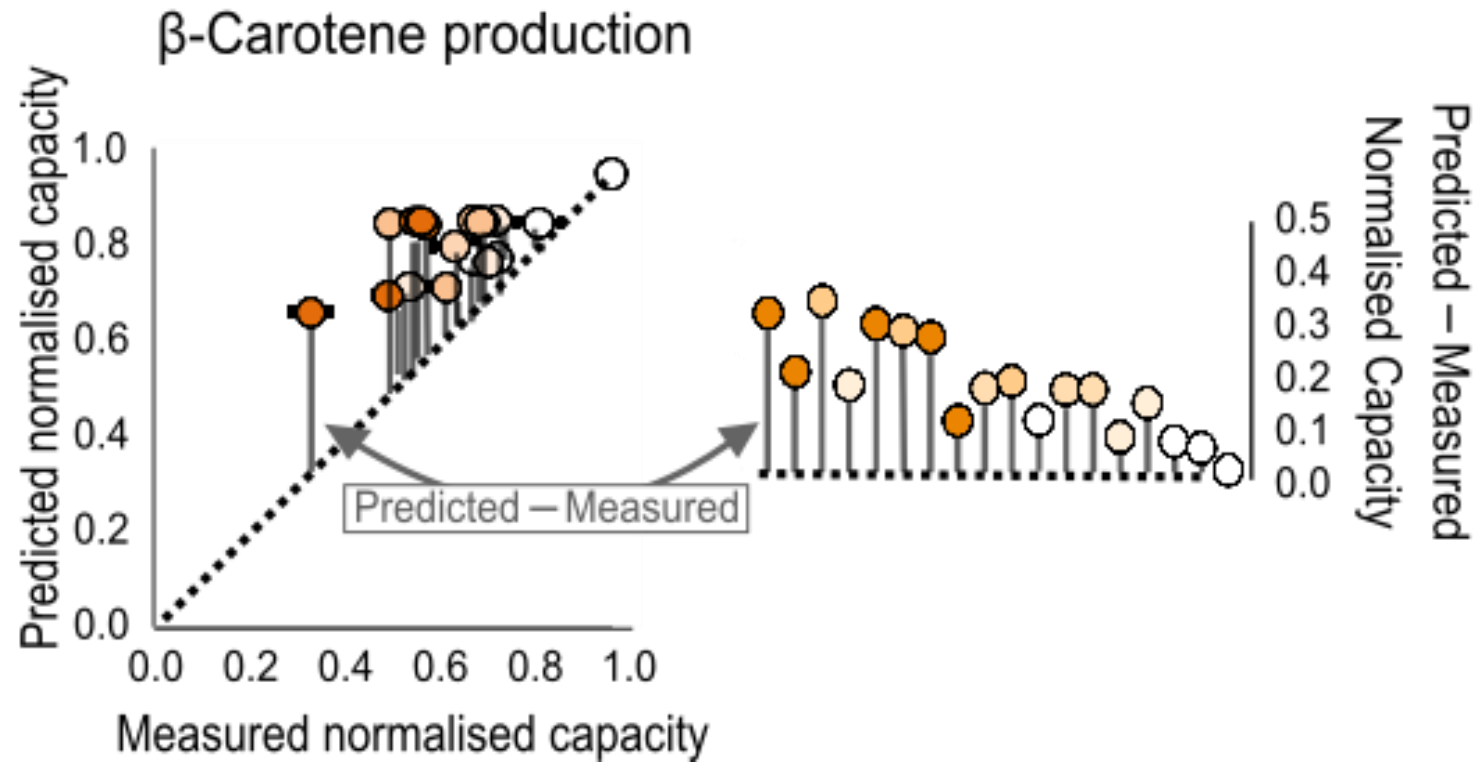


Exploring Resource Competition in cell-free: Metabolic burden



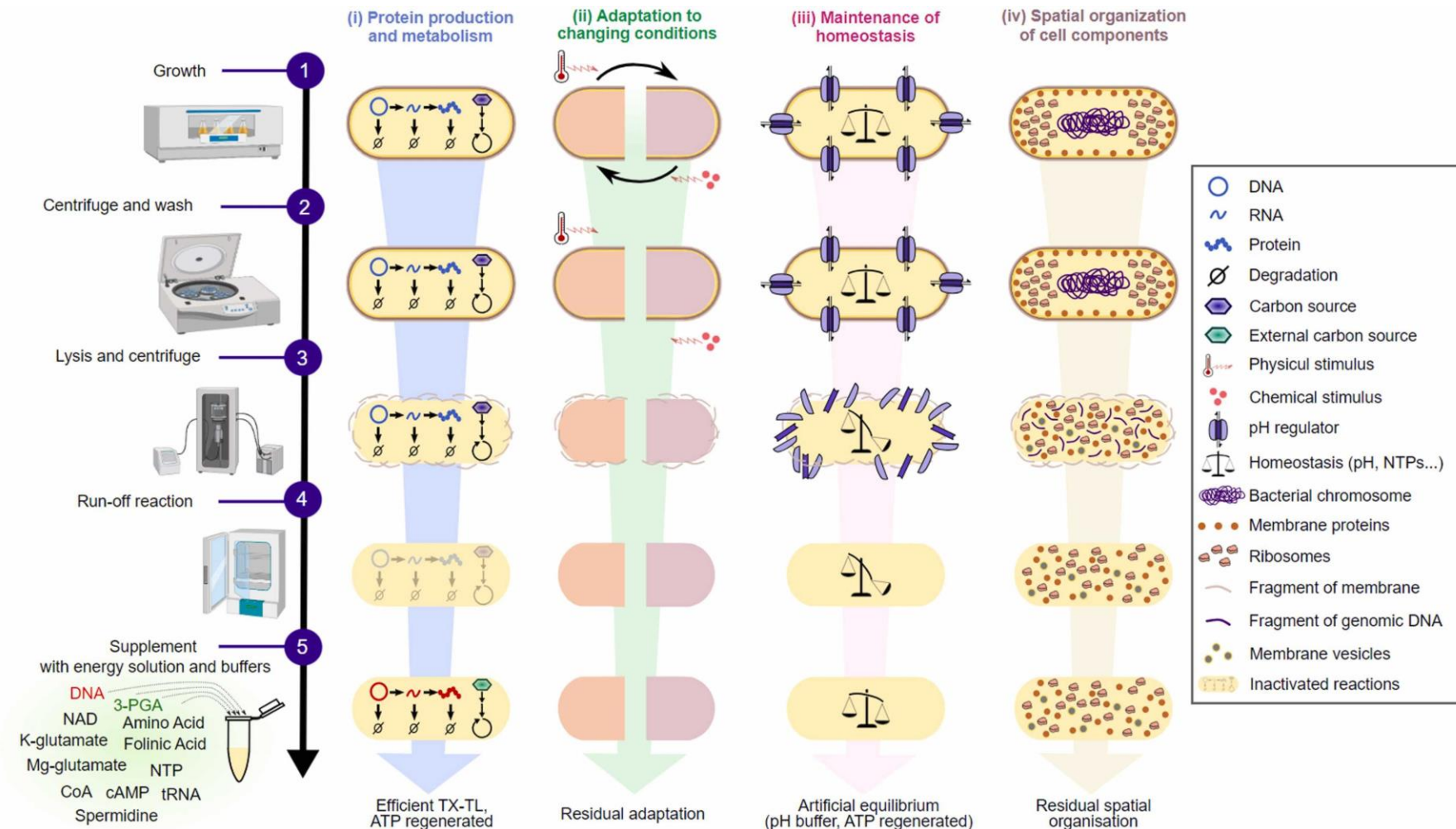
Exploring Resource Competition in cell-free: Metabolic burden

Decouple production and metabolic burden



3. Building artificial cells: **reconstruct functions** defective in cell-free systems

What remains from living cells in bacterial lysate-based cell-free systems



3. Building artificial cells: **reconstruct functions** defective in cell-free systems

Spatial organization

Homeostasis & Adaptation to changing conditions

Cell Division

Liposome (phospholipid bilayers) encapsulation

Cell-free reaction + mineral oil with dissolved phospholipids



vortex

extract-oil emulsion



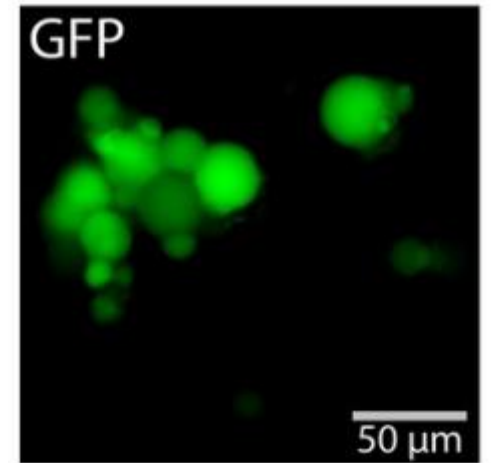
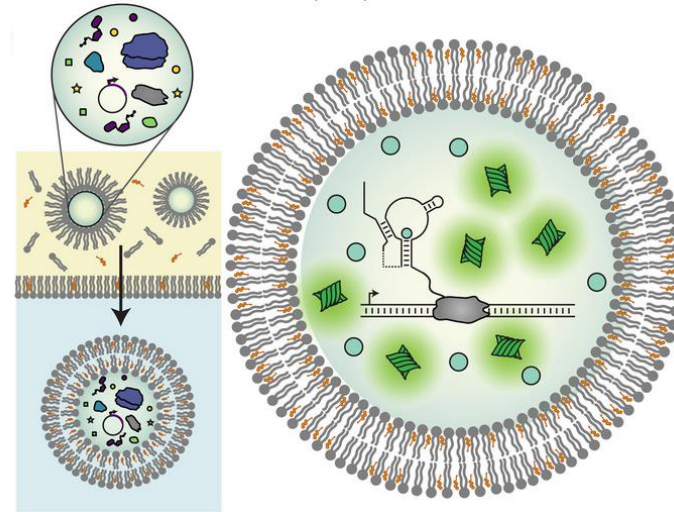
Wait a few minutes

Microdroplets stabilized by a monolayer of phospholipid



Transfer in feeding solution
(Amino acid + ribonucleotides)
+ centrifugation

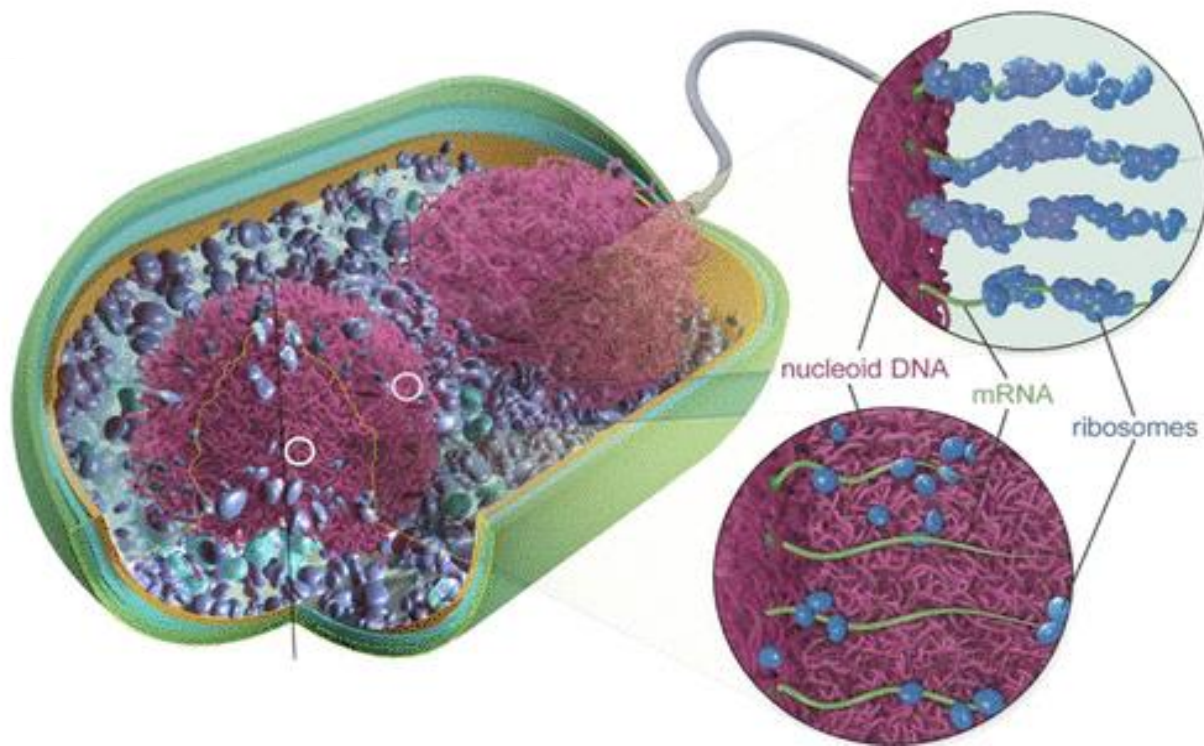
Vesicle



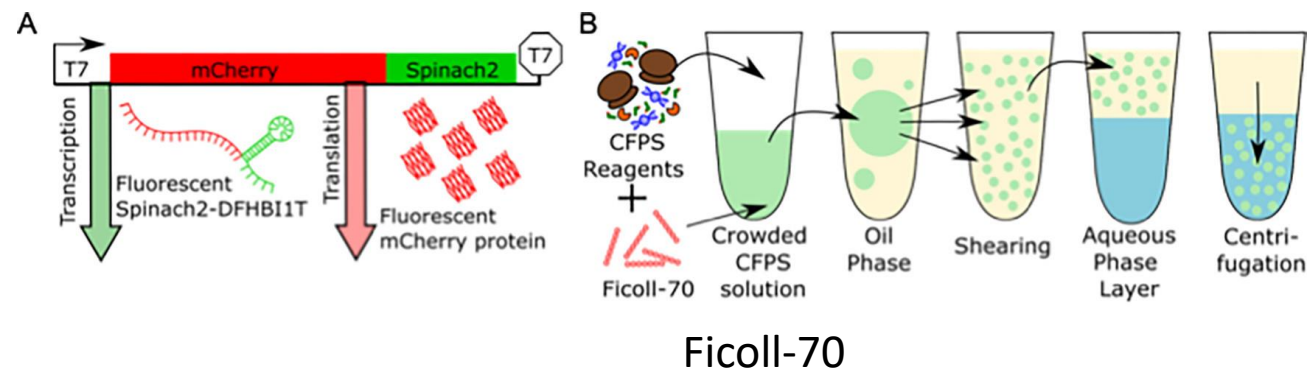
3. Building artificial cells: **reconstruct functions** defective in cell-free systems

Spatial organization

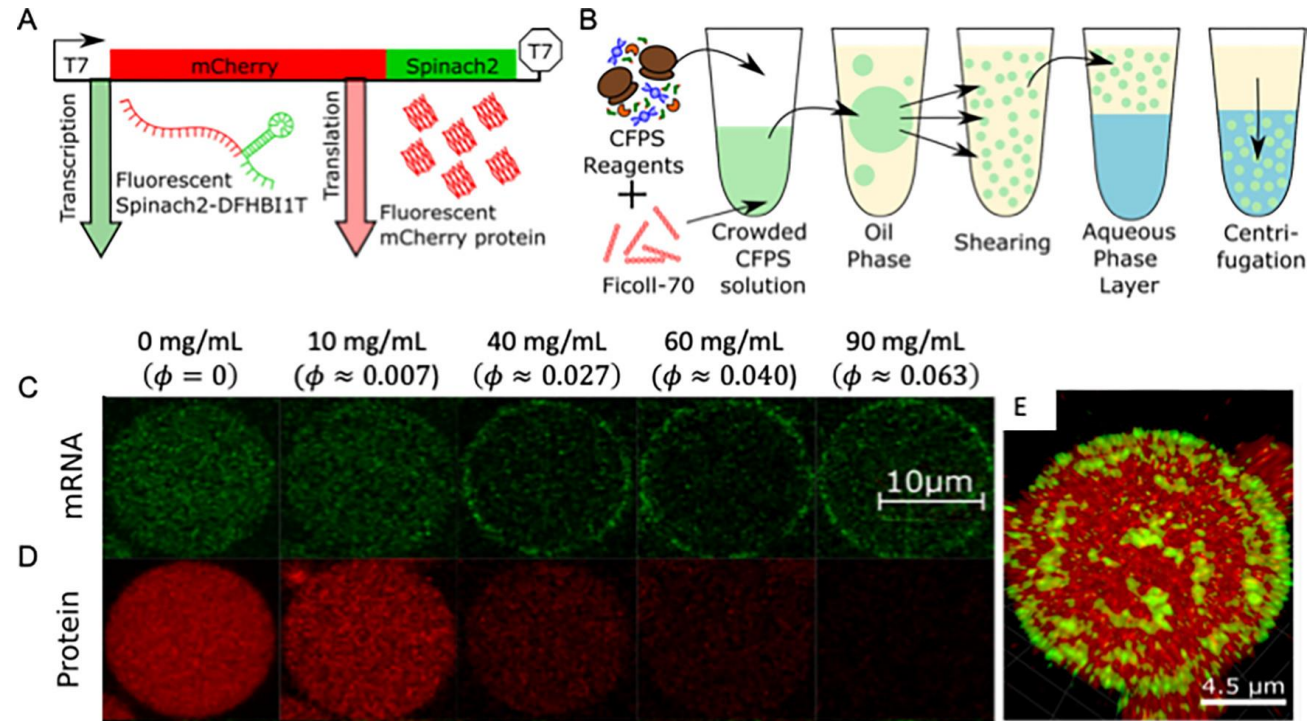
Spatial organization



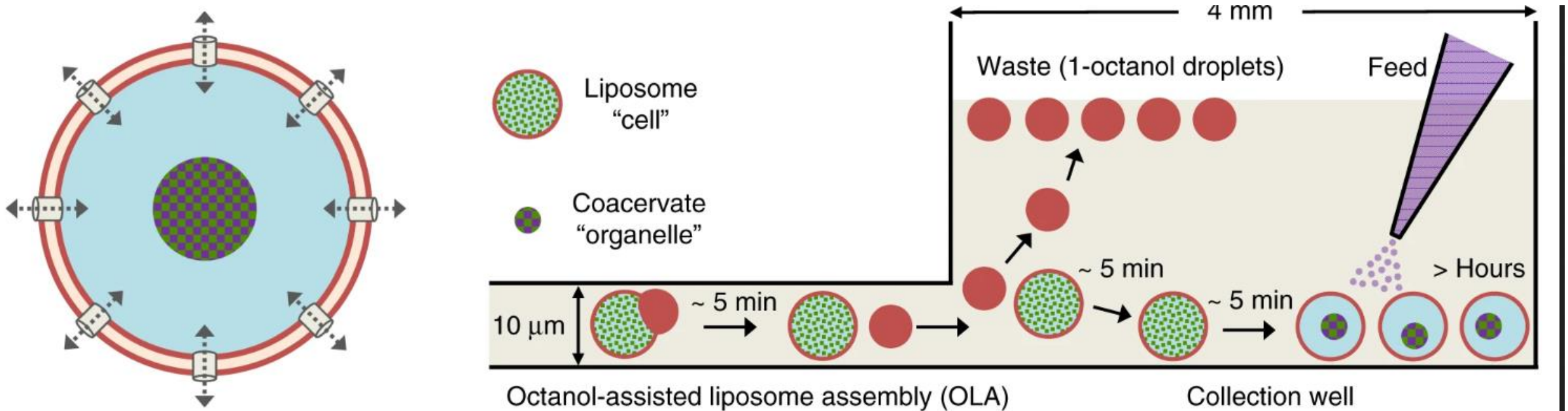
Spatial organization



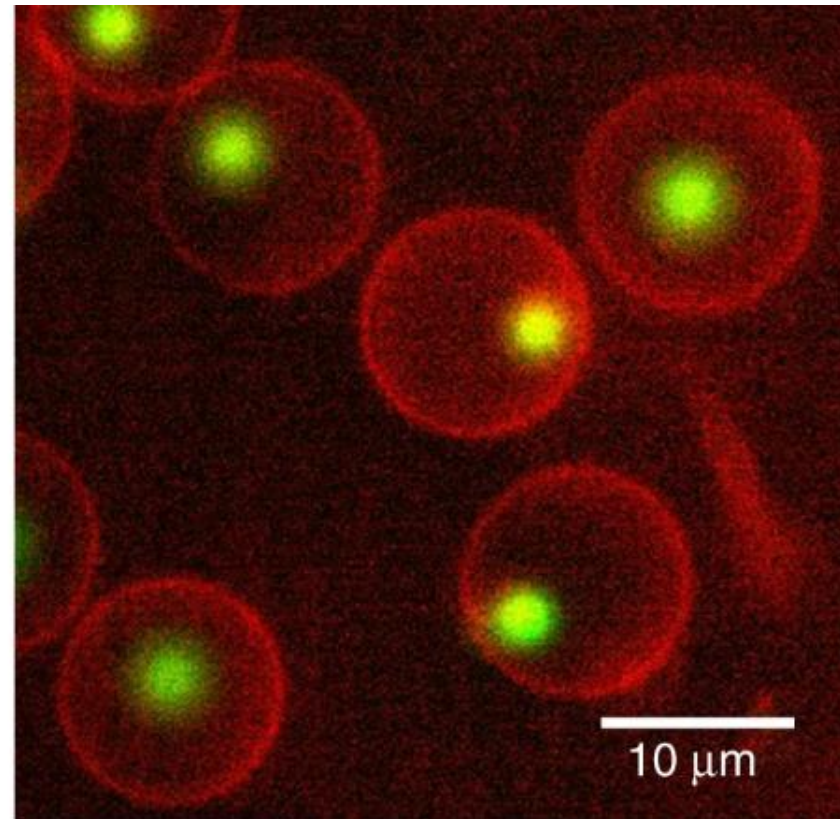
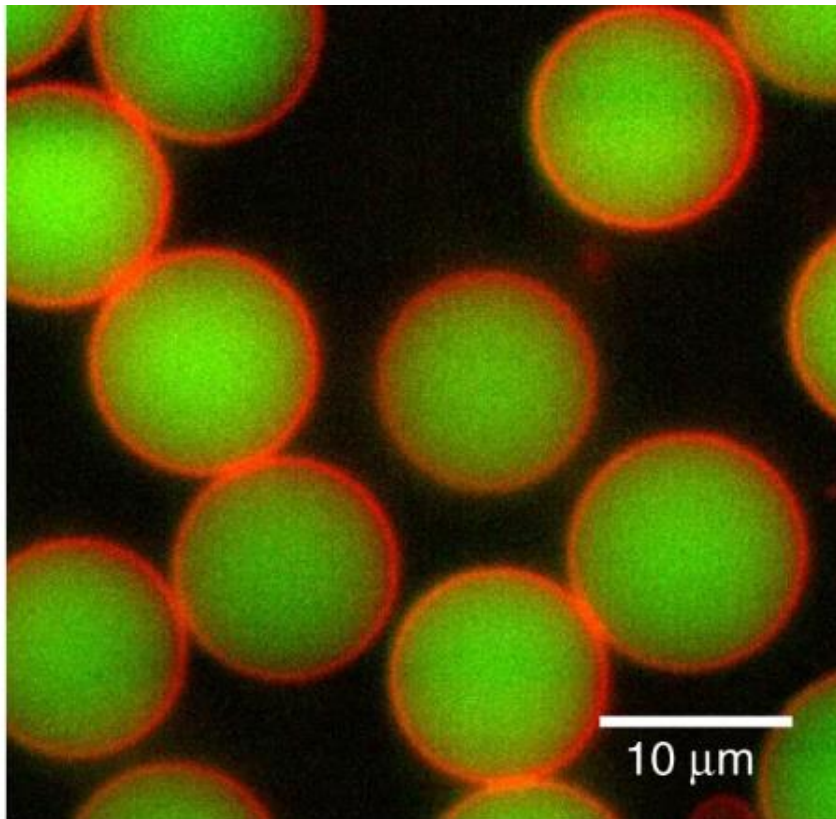
Spatial organization



Spatial organization: compartement



Spatial organization: compartement



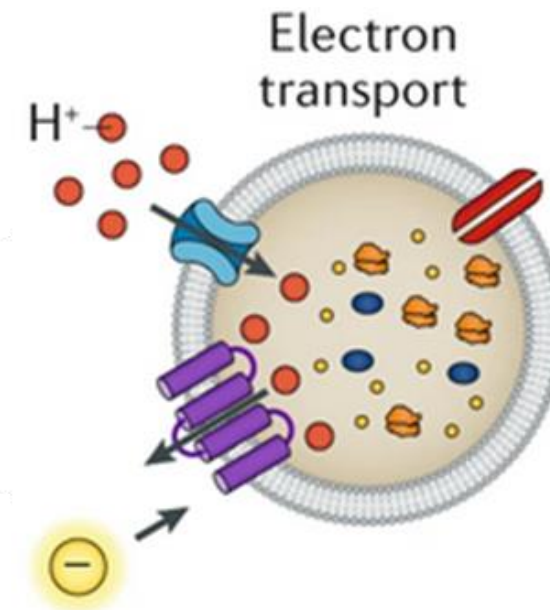
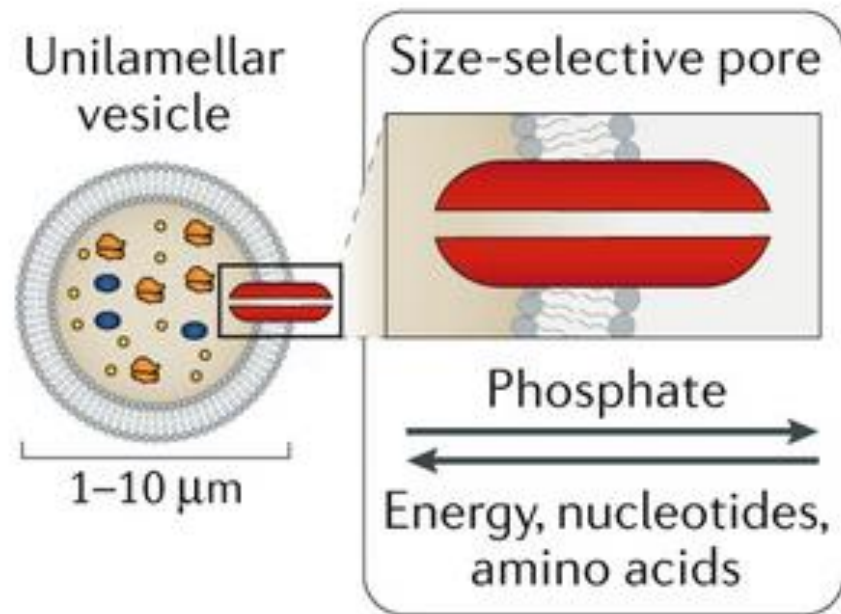
3. Building artificial cells: **reconstruct functions** defective in cell-free systems

Homeostasis & Adaptation to changing conditions

Homeostasis & Adaptation to changing conditions

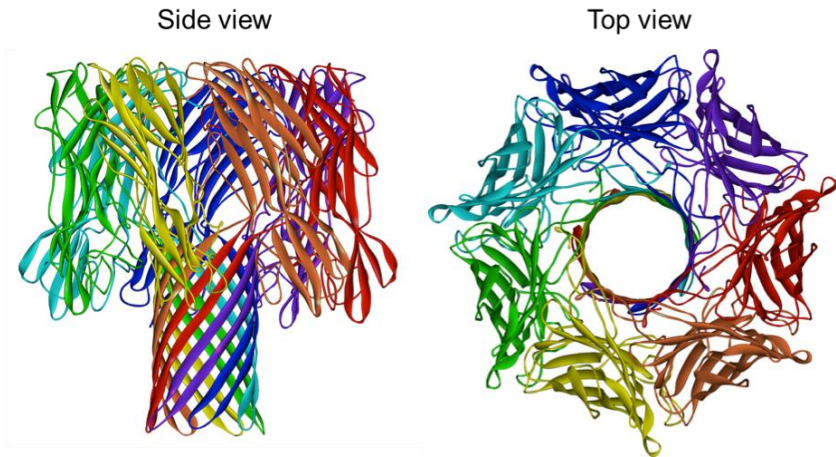
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is a commonly used buffer in CFS with a pKa of 7.5 and useful pH range of 6.8–8.2

Exchange with the environment



Homeostasie & Adaptation to changing conditions

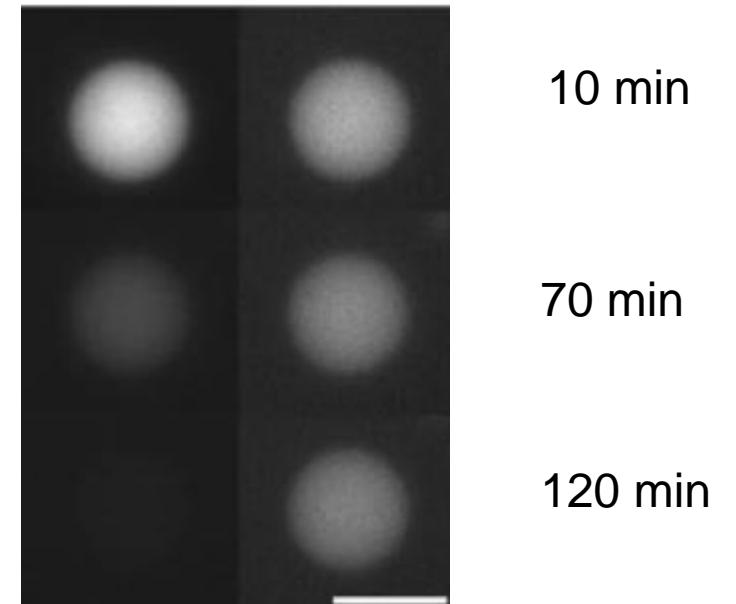
haemolysin protein pores



The extract with the plasmid (Pasmid pIVEX2.3d a hemolysin) and the fluorescent probes (BSA-rhodamine and fluorescein-UTP) are encapsulated in the vesicles

Ribonucleotide UTP
labeled with fluorescein

BSA protein
labeled with rhodamine



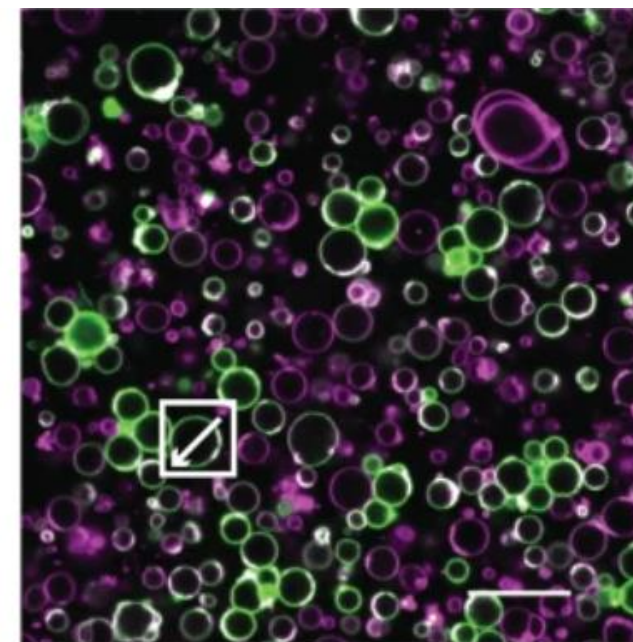
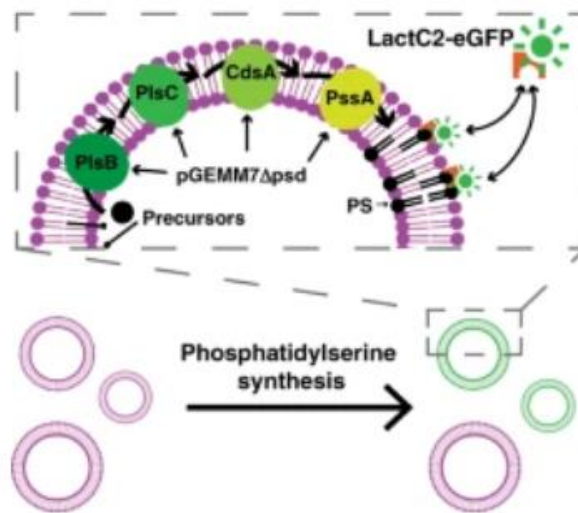
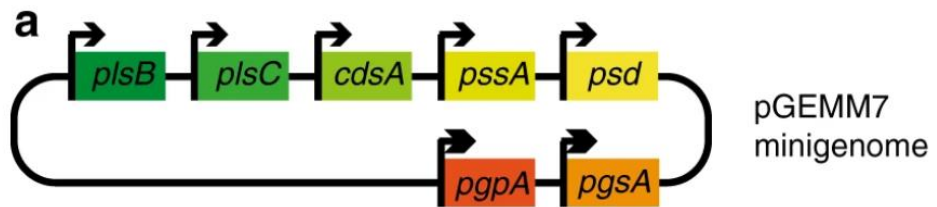
Ribonucleotides and amino acids can diffuse in and out while the proteins are kept inside the vesicle

3. Building artificial cells: **reconstruct functions** defective in cell-free systems

Cell Division

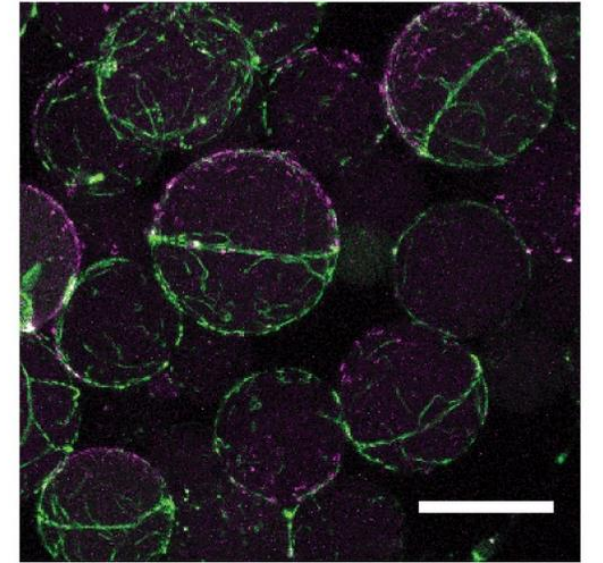
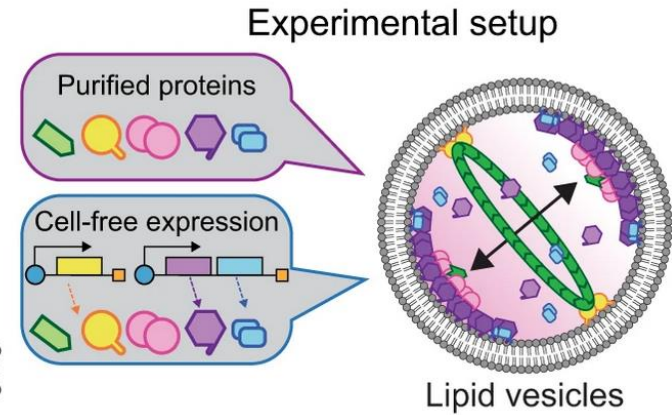
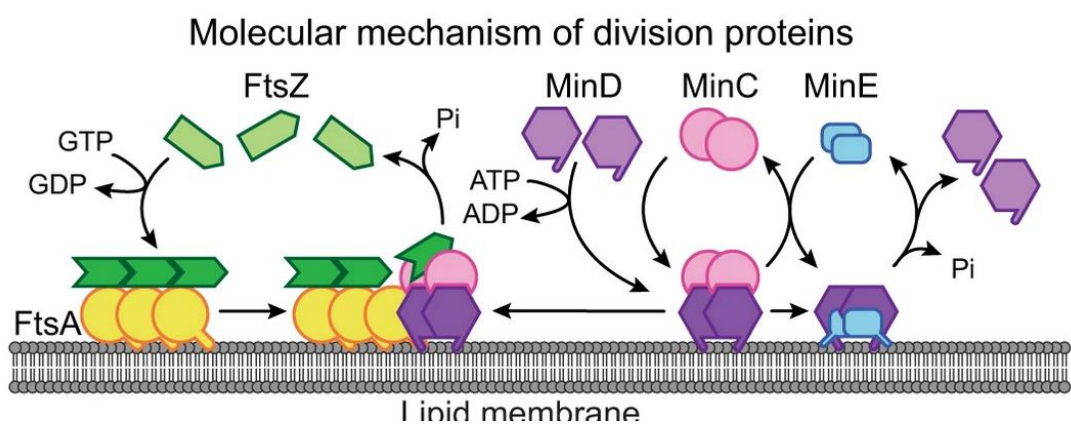
Cell Division

De novo synthesis phospholipids



Cell Division

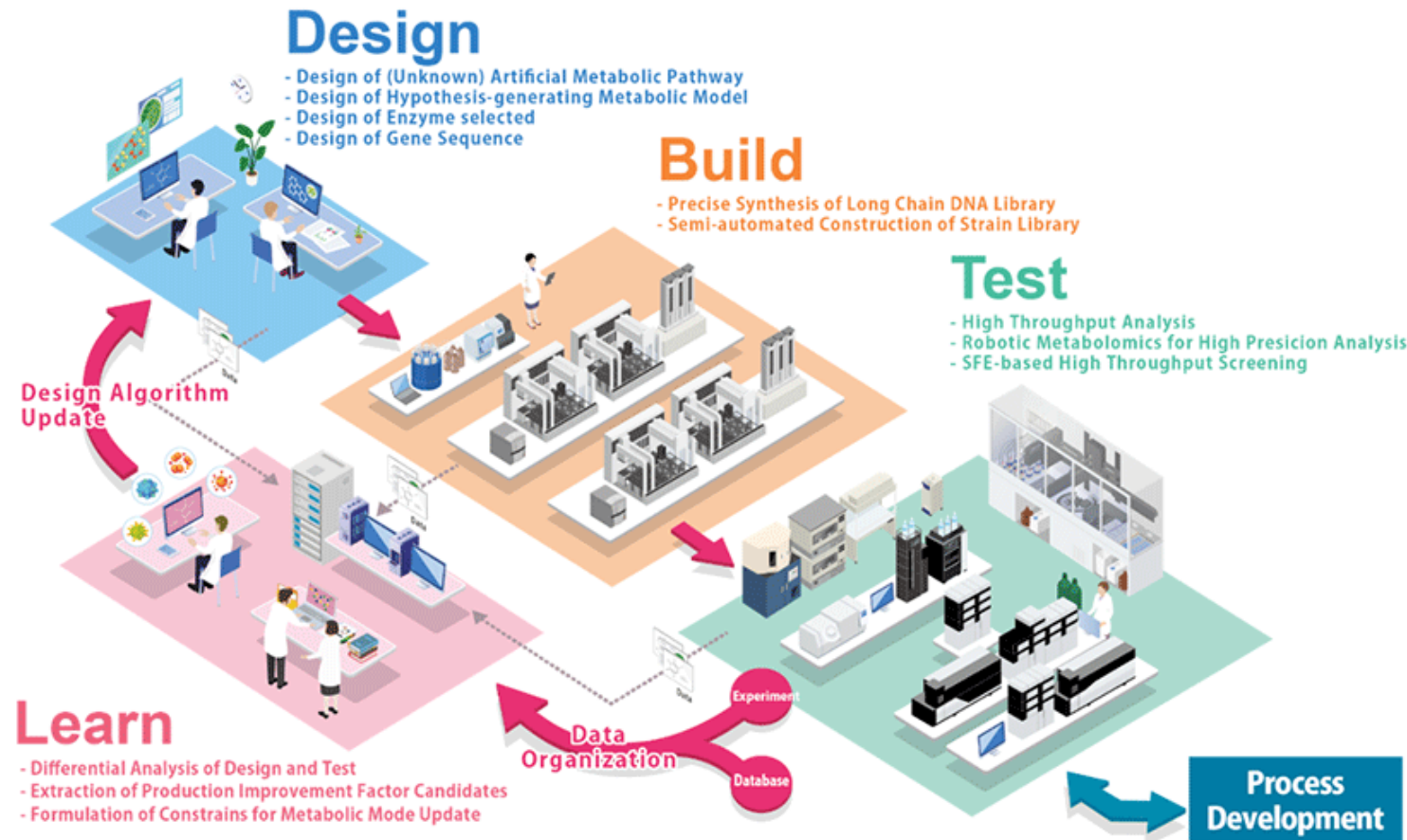
Reconstitution of proto-ring



mScarlet-I-MinC & FtsZ-Venus-mts

Cell-free Biofoundry at INRAE jouy-en-josas

Part of the **Bioconvergence Paris Biofoundry** :
INRAE/Paris-Saclay Université + Sorbonne Université + Institut Curie + Genopole Evry



BIOCONVS
Bioconvergence pour la Santé

Domaine de Recherche et d'Innovation Majeur

Région
île de France

Acknowledgements



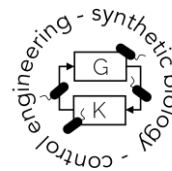
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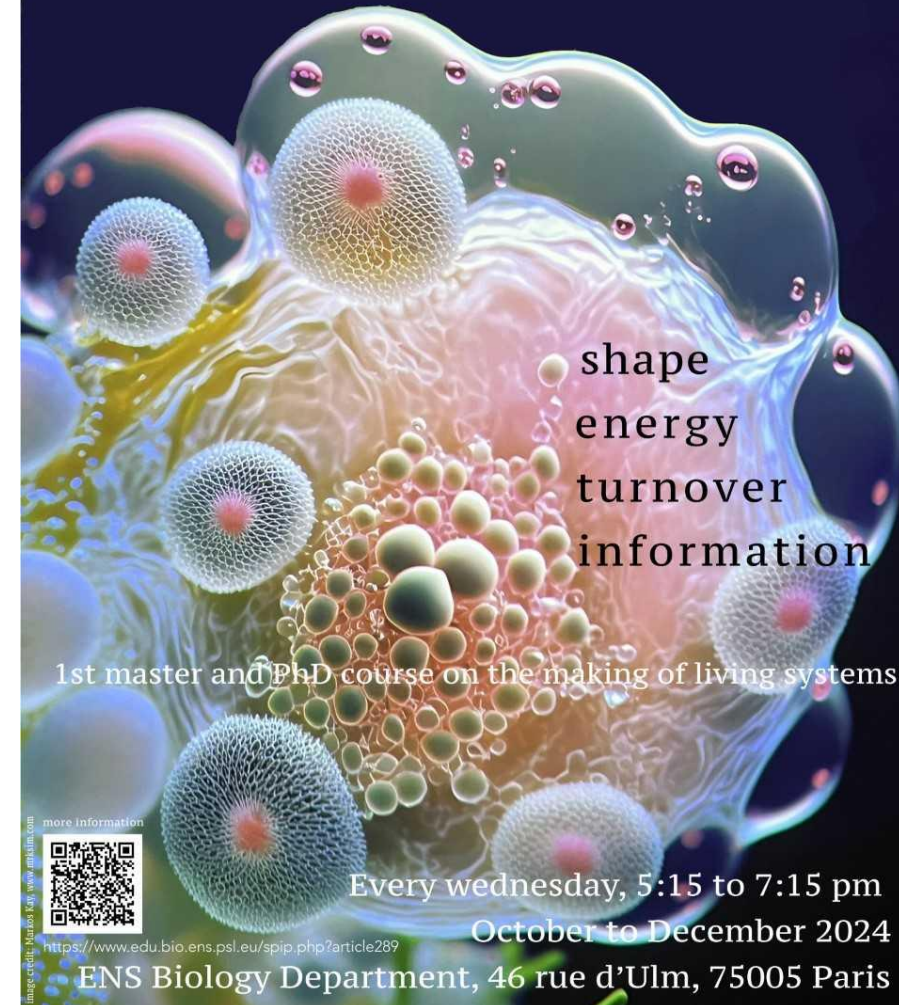
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artificial cells



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Every wednesday, 5:15 to 7:15 pm

October to December 2024

ENS Biology Department, 46 rue d'Ulm, 75005 Paris

Communication between Cell-free reaction

- <https://doi.org/10.1101/2024.01.10.575012> (Ricouvier et al 2024, biorxiv)

