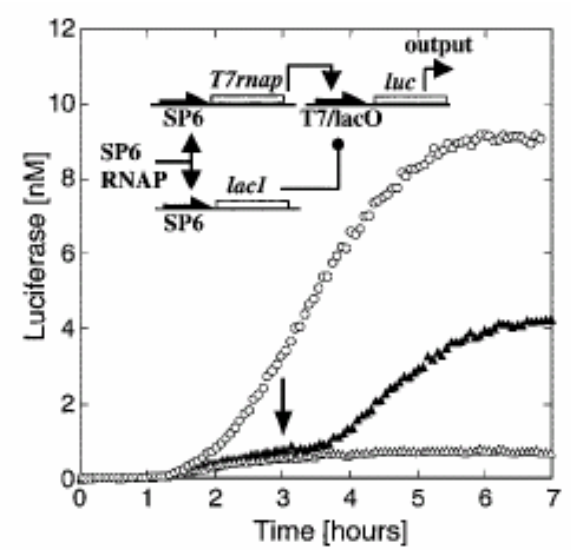
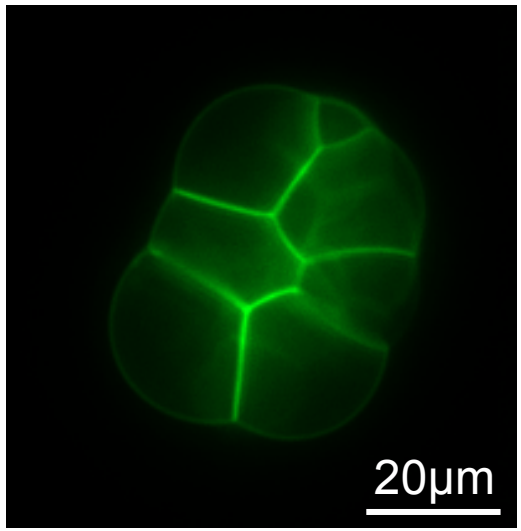
Cell-free expression: application to networks and synthetic vesicles.

Vincent Noireaux, University of Minnesota
UMN, 09 October 09

- information and gene expression
- in vitro elementary gene circuits
- expression in vesicles: protocell system
- diffusion study



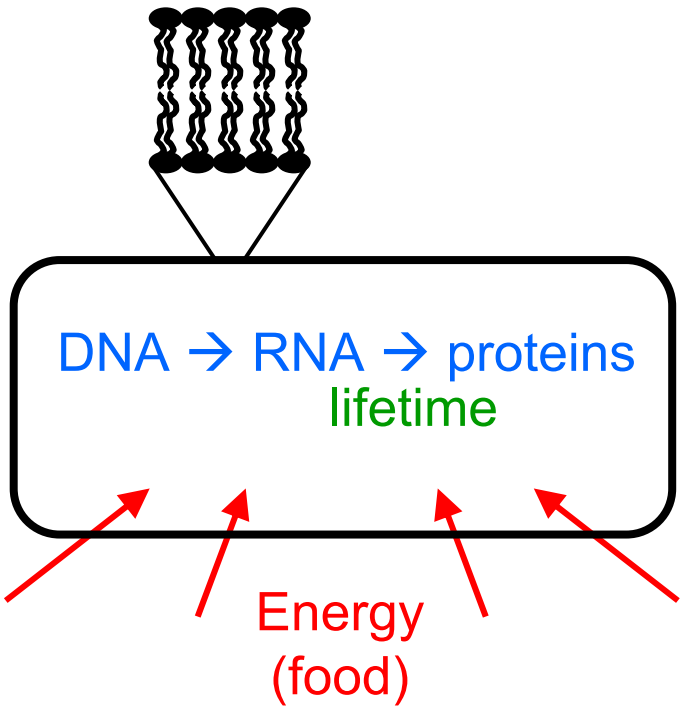
Cell-free circuits



AlphaHemolysin-eGFP
(cell-free extract encapsulated in a synthetic phospholipid vesicle)

Part 1: gene expression

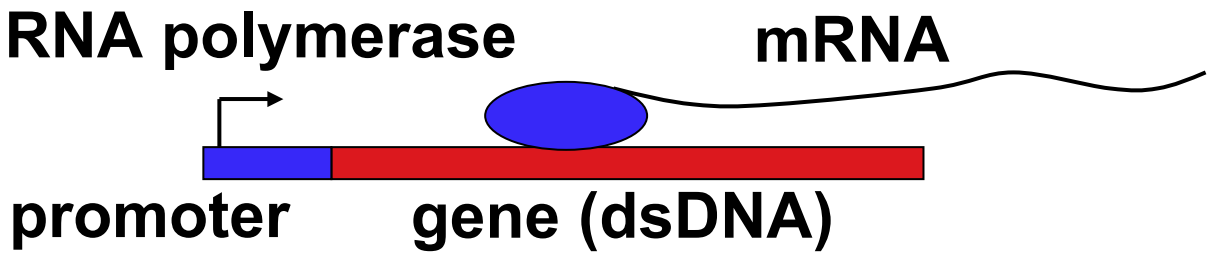
Simplified view of a cell



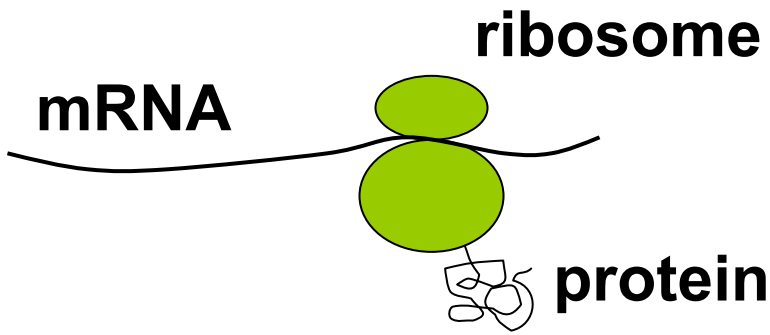
- Membrane
- DNA → RNA → proteins
- Genetics networks
- Replication-cell division
- Evolution

Escherichia coli: 4500 genes
Minimal cell: ~ 400 genes

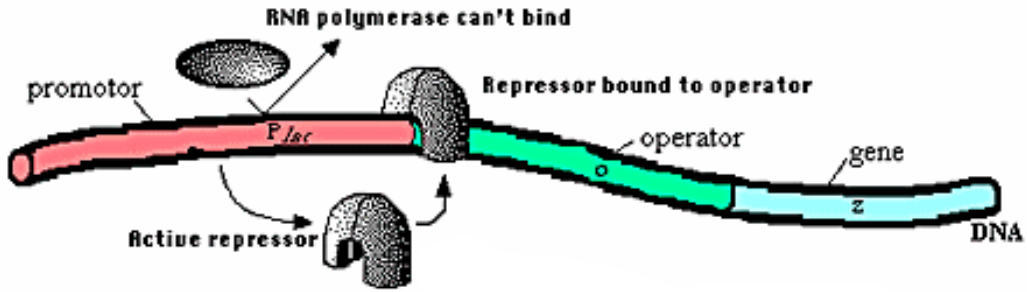
Transcription: DNA → RNA

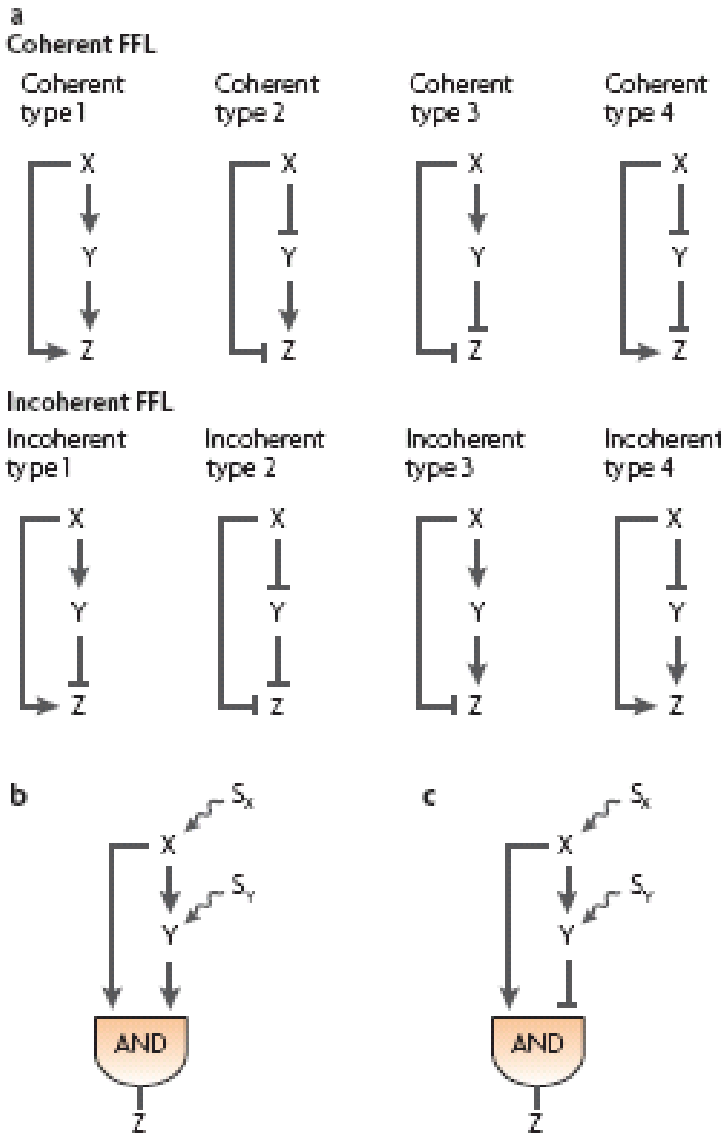


Translation: RNA → proteins



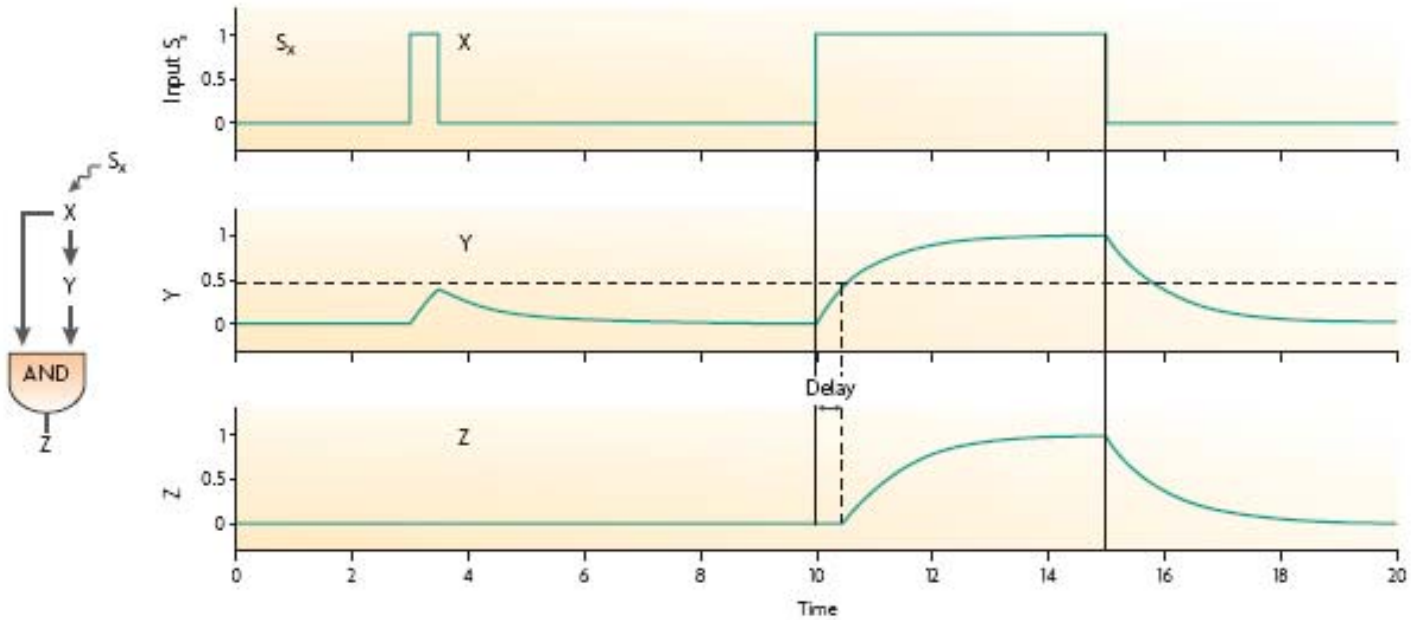
1) + GLUCOSE - LACTOSE	Operon off





Gene network motifs

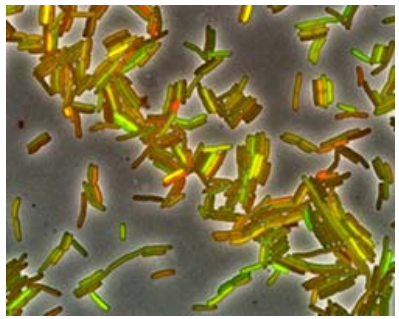
Alon U. 2007


S_x
X
Y
AND
Z
Input S_x
1
0.5
0
S_x
X
Y
Y
Delay
Z
Z
0
2
4
6
8
10
12
14
16
18
20
Time

Alon U. 2007

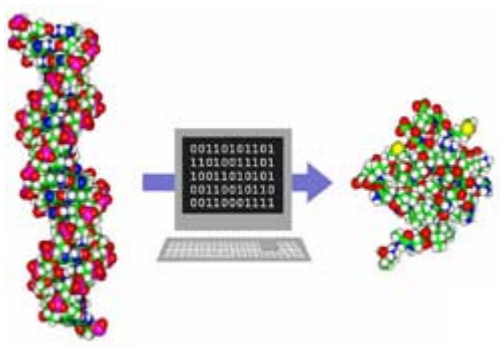
Synthetic approach to gene expression

in vivo



Elowitz et al - 2002

in silico



in vitro

In vitro approach to information

We use a cell-free approach to information in biology:

- reductive
- constructive
- quantitative approach

applied to:
(1) elementary gene networks.
(2) search problem and diffusion in biological medium.
(3) protocell system.

Part 2: cell-free gene expression Round1.

Cell-free systems (1/2)

- **cytoplasmic extract to express genes in vitro:**
  - endogenous DNA and mRNA are removed.
  - the extract contains:
    - **energy**: ATP, GTP.
    - **building blocks** (nucleotides, amino acids).
    - **translation machinery** (ribosomes, transfer RNA).

- **in vitro coupled transcription/translation in test tube:**

DNA → mRNA → proteins

Cell-free systems (2/2)

advantages:

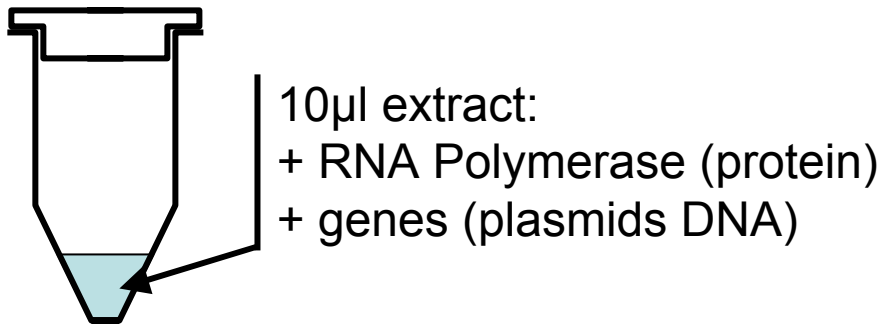
- reductive – constructive - quantitative.
- no endogenous expression activity.
- work with toxic genes is easier.

disadvantages:

- transcription limited to bacteriophage RNAP T7, T3, SP6.
- no control of mRNA and protein degradation.
- expression not as powerful as in vivo.

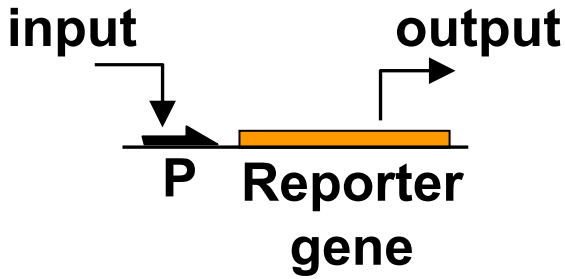
Batch mode reaction

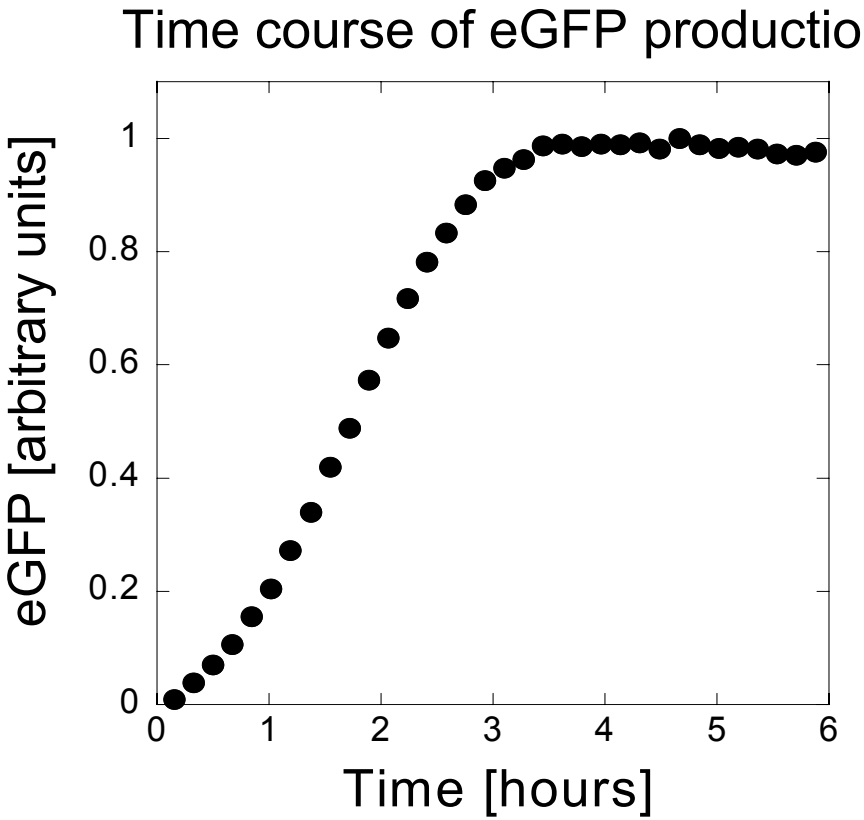
in a 1.5ml tube at
room temperature



- reporters: eGFP, firefly luciferase
- detection: microscope, photo multiplier tube, platereader
- 1nM ~ 1 molecule per μm^3

One Gene:

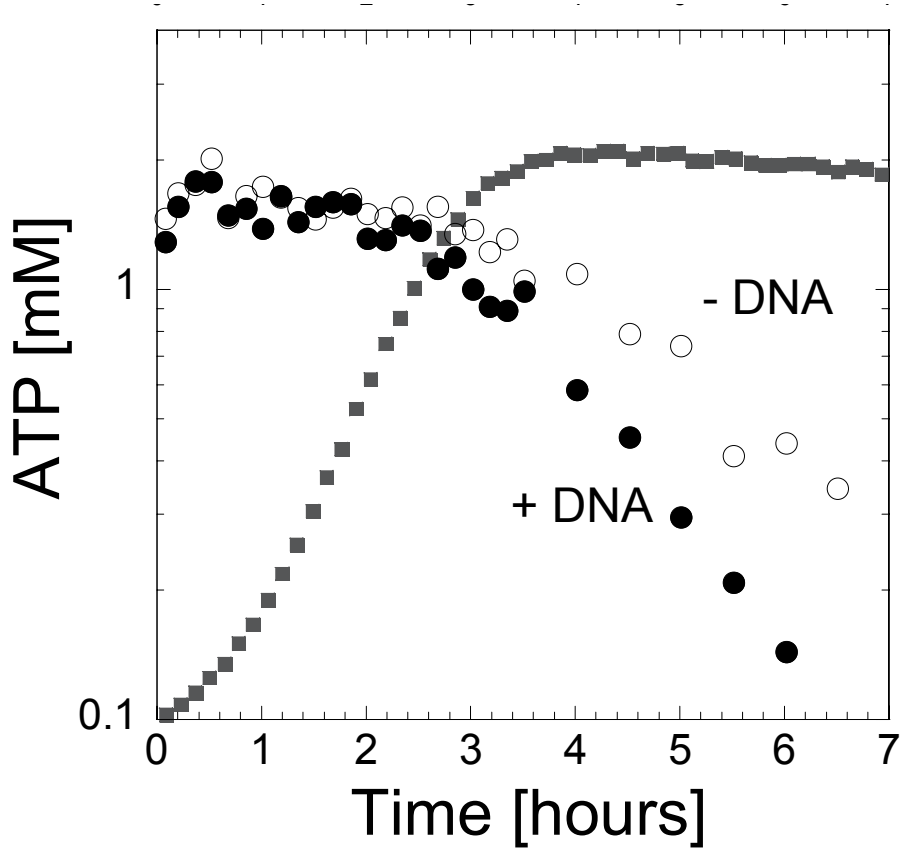




- 3-8 hours production
- 2μM proteins max.

20nM RNAP
1nM plasmid

Rate of protein synthesis



$$V(t) = V_0 \, e^{-b\frac{[ATP(t=0)]}{[ATP(t)]}}$$

The real parameter for energy is the energy charge
(Atkinson D.E. 60s)

Energy charge: $E_c = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$

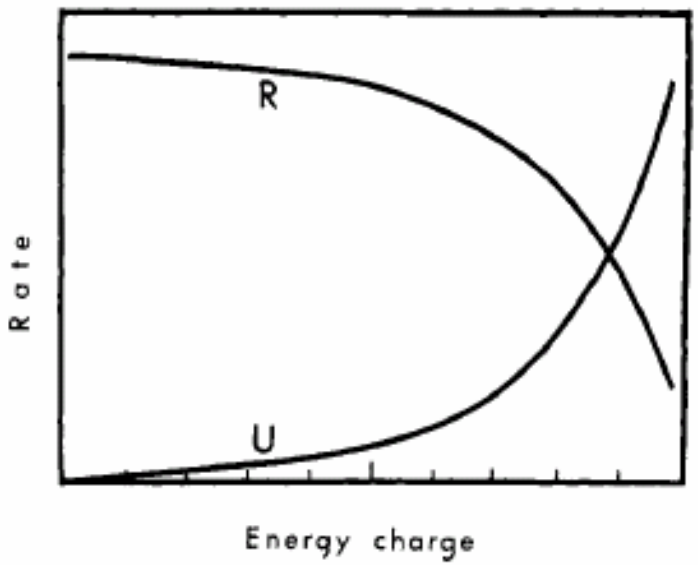


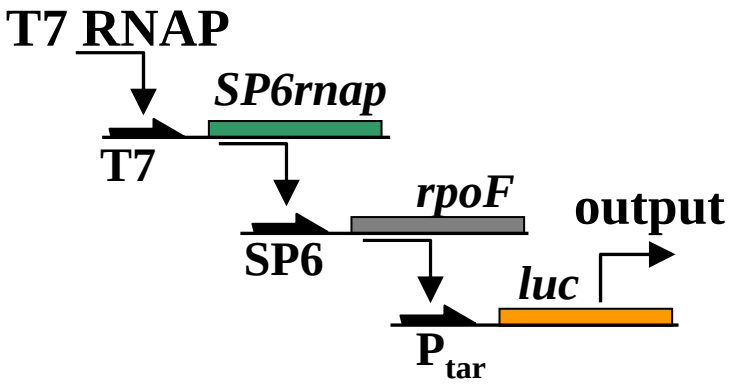
FIGURE 1: Generalized response to the energy charge of enzymes involved in regulation of ATP-regenerating (R) and ATP-utilizing (U) sequences.

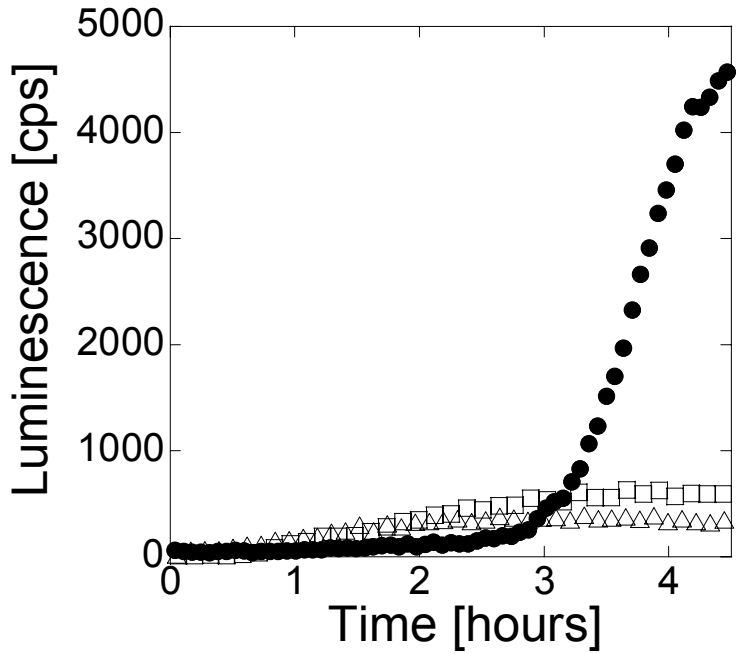
D.E. Atkinson, 1968.

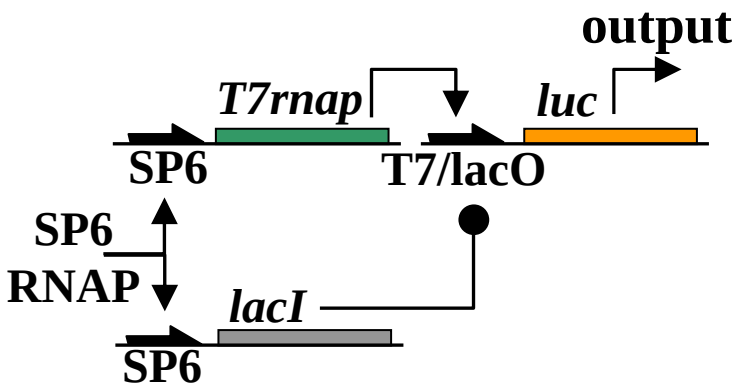
Batch mode limitations

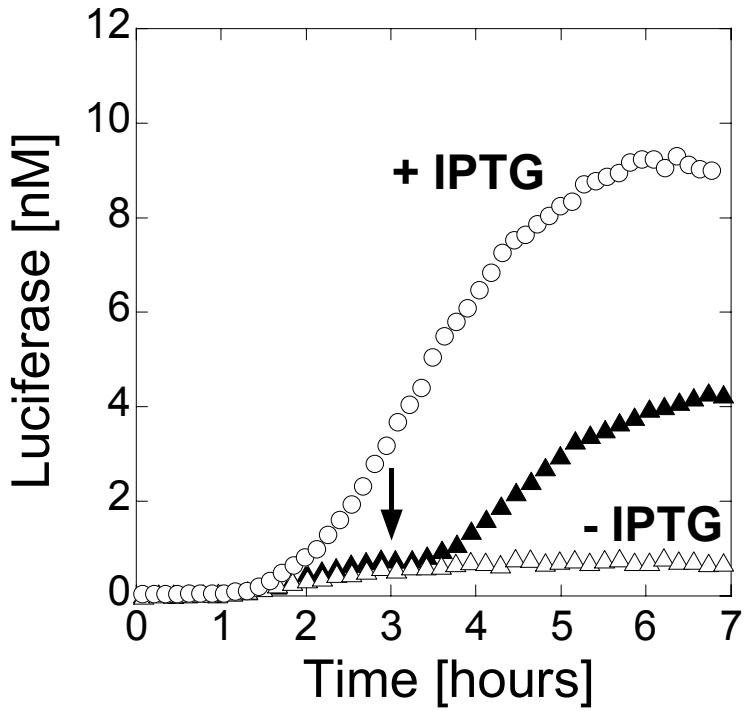
- energy charge

- finite resources (no feeding)
- pH variations
- amino acids degradations
- byproducts accumulation


T7 RNAP
SP6rnap
T7
rpoF
output
SP6
luc
P tar


5000
4000
3000
2000
1000
0
Luminescence [cps]
0
1
2
3
4
Time [hours]


output
T7rnap
luc
SP6
T7/lacO
SP6
RNAP
lacI
SP6


12
10
8
6
4
2
0
Luciferase [nM]
+ IPTG
- IPTG
0
1
2
3
4
5
6
7
Time [hours]

Conclusion:

● in batch mode, elementary genetic circuits of 3-4 well-known genes can be reconstituted.

limitation: transcription is too limited.

Noireaux, Bar-Ziv, Libchaber, PNAS, 2003.

Next steps:

● prepare a new type of extract: the complete transcription system from E. coli.

● add control mechanisms of mRNA degradation and protein degradation.

Part 3: cell-free gene expression Round2.

Jonghyeon Shin.

Optimization of a new CFE

Objectives:

- E. coli RNAP, sigma factors
  → choice of promoter/operator modules.
- protein production: at least a few micromolar.
- degradation of messengers.
- degradation of proteins.

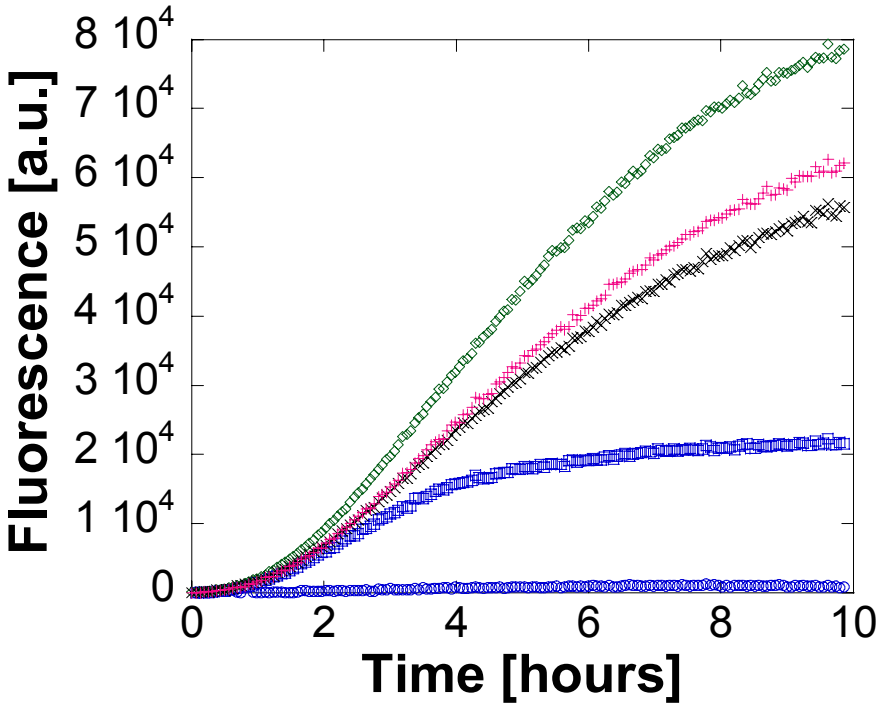
Optimization of a new CFE

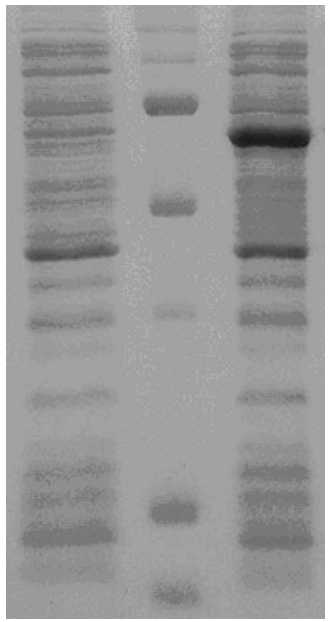
- cell-free extract prepared from E. coli, the endogenous E. coli RNAP and sigma factor70 are used to express all the other genes.
- the 6 other sigma factors are functional.
  S32, S28 > S38, S24 > S54, S19
- control of mRNA degradation rate is performed with MazF toxin. mRNA lifetime can be adjusted from 25 min to 0min.
- protein degradation is performed with ClpXP complex. Protein lifetime depends on the tag.

E. Coli RNAP – sigma70

(endogenous activity)

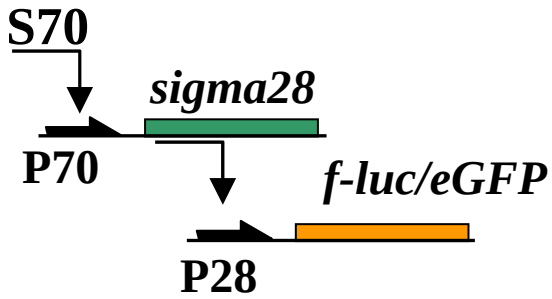
P70: sigma70 promoter

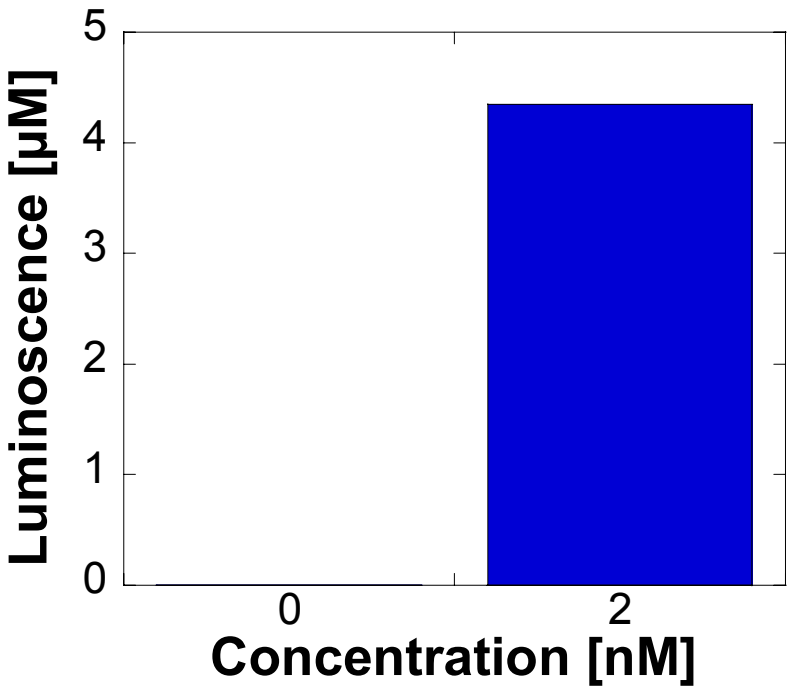


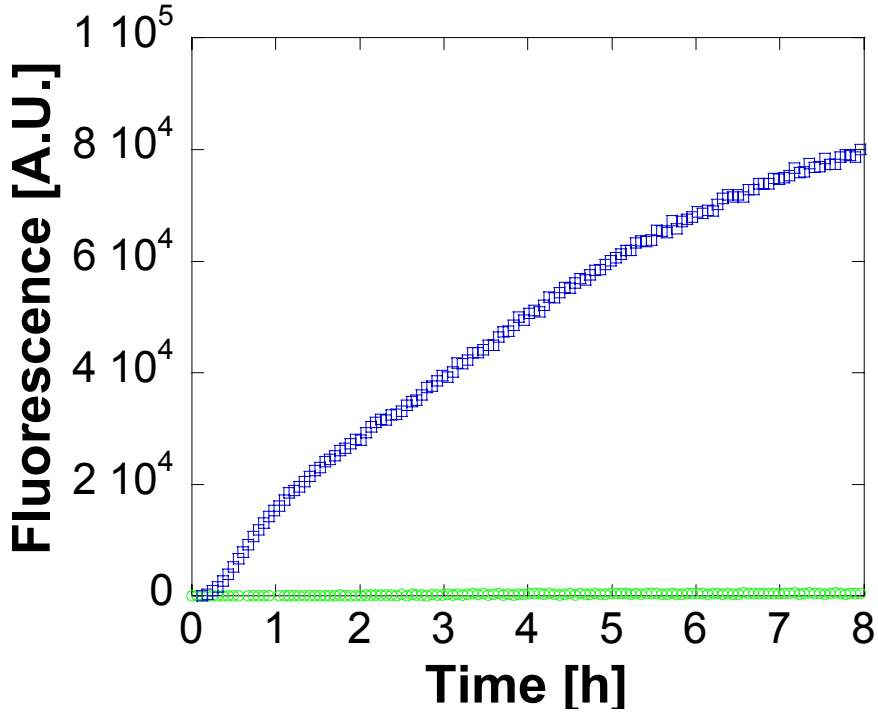


SDS PAGE 12%
0.6mg/ml of fLuc in test tube
(no feeding)

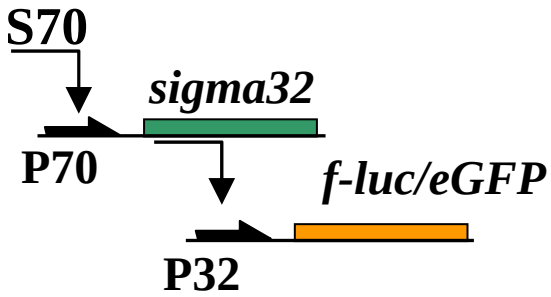
2-stages cascade for sigma28

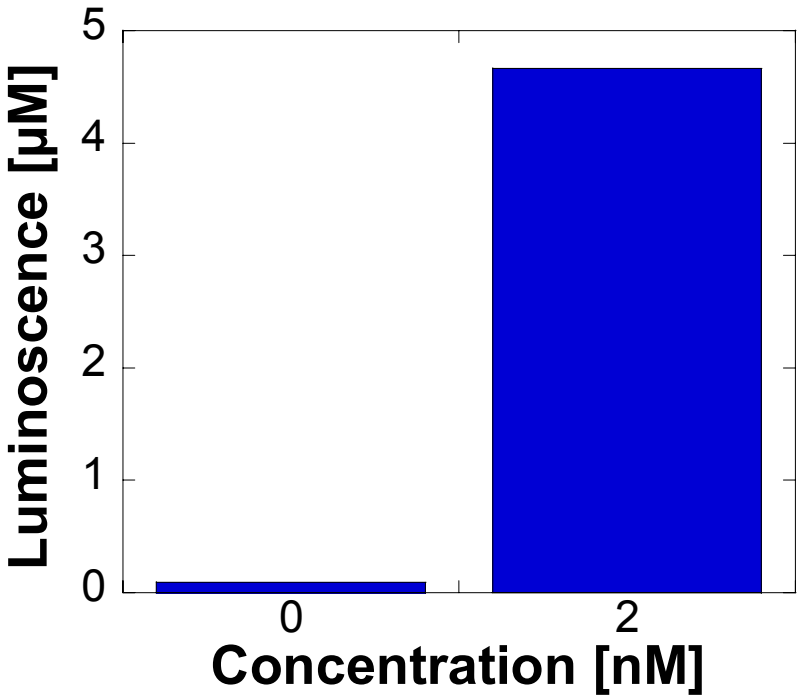


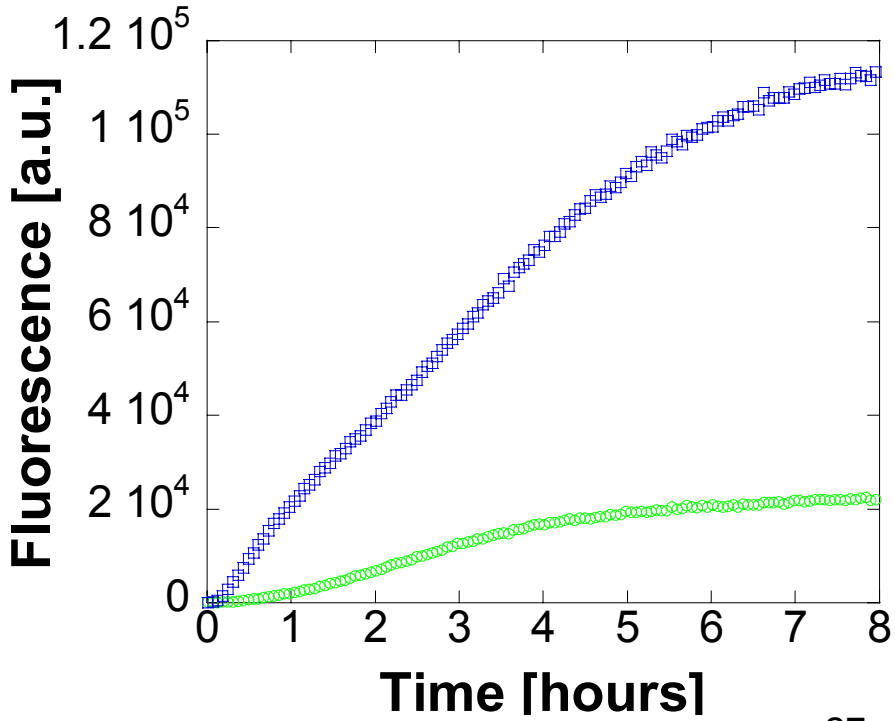




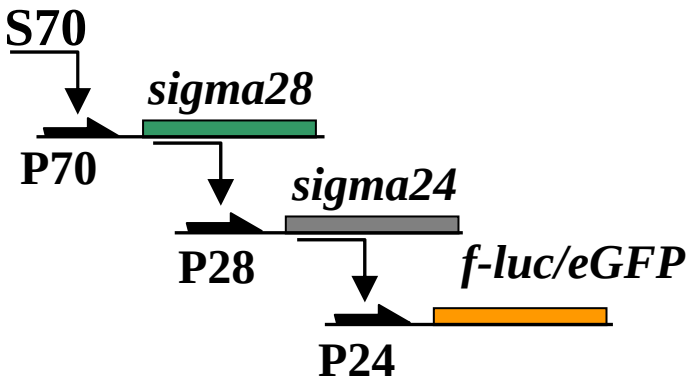
2-stages cascade for sigma32

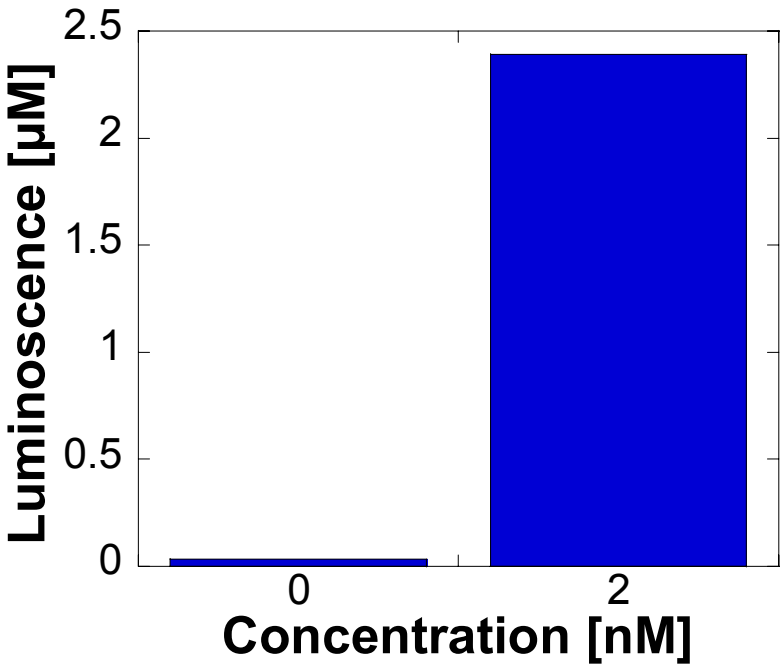


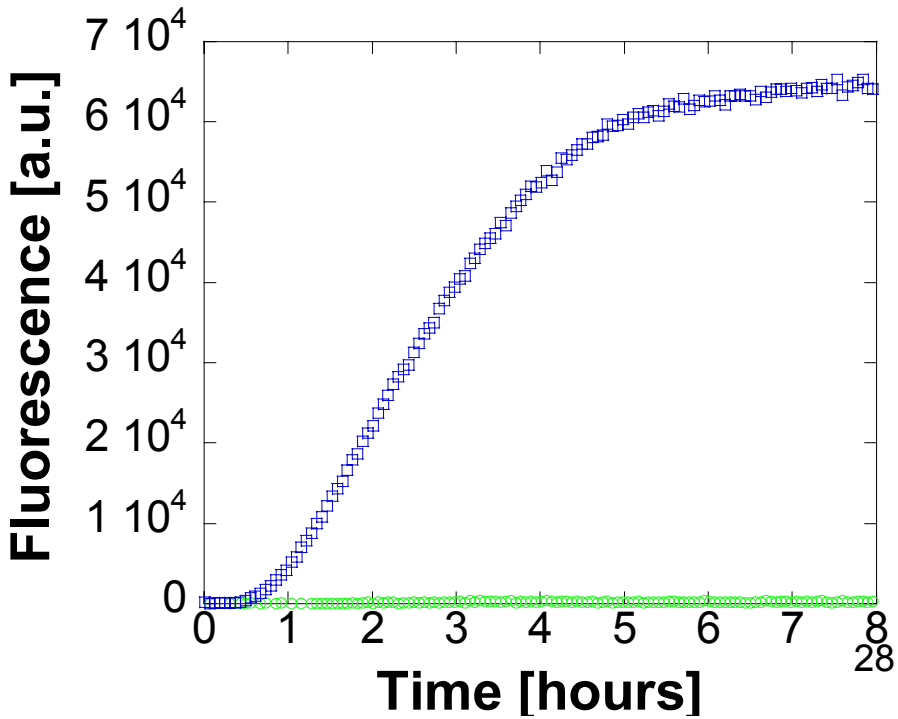




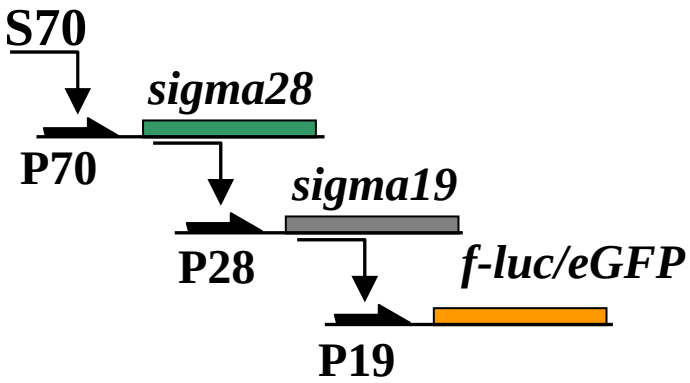
3-stages cascade for sigma24

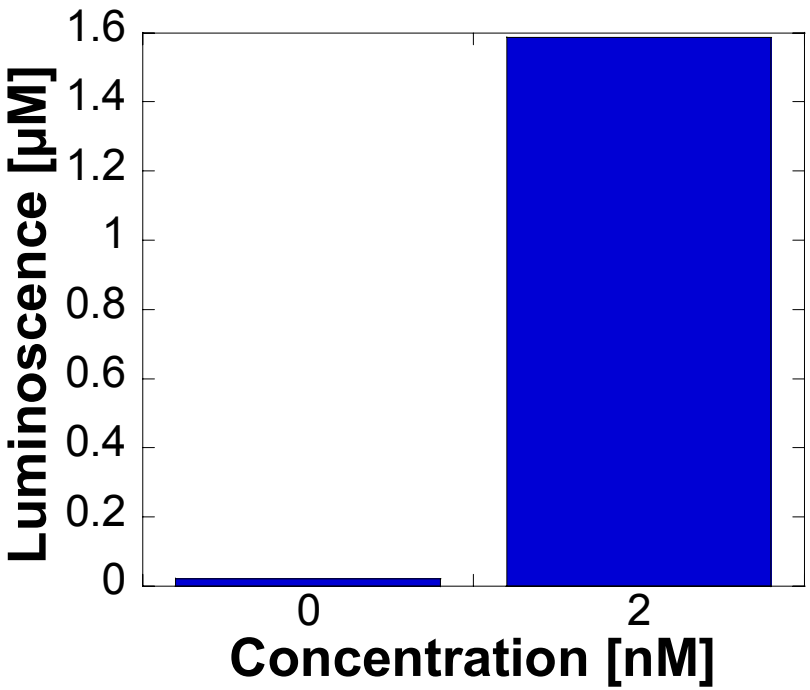


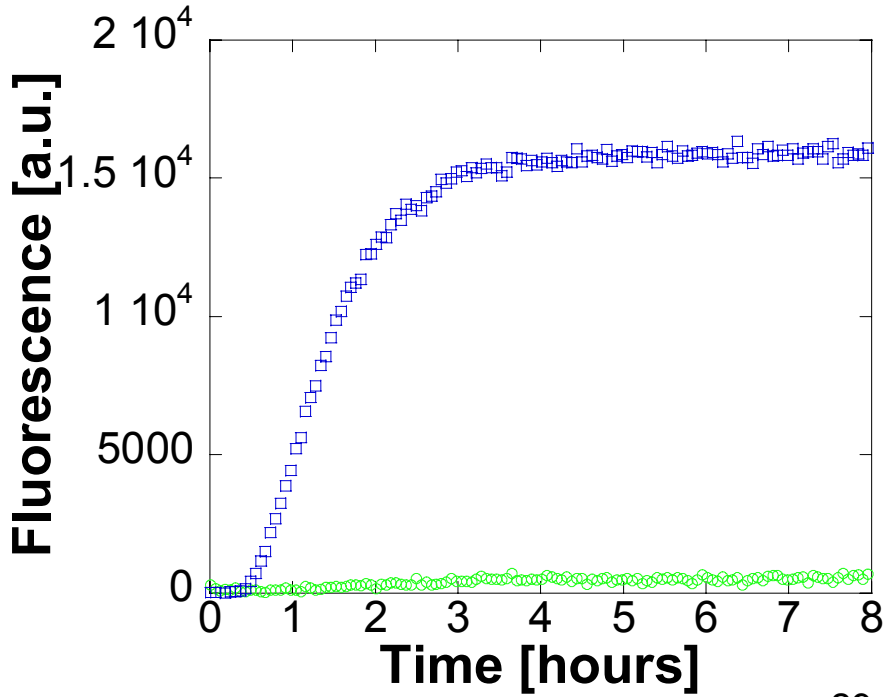




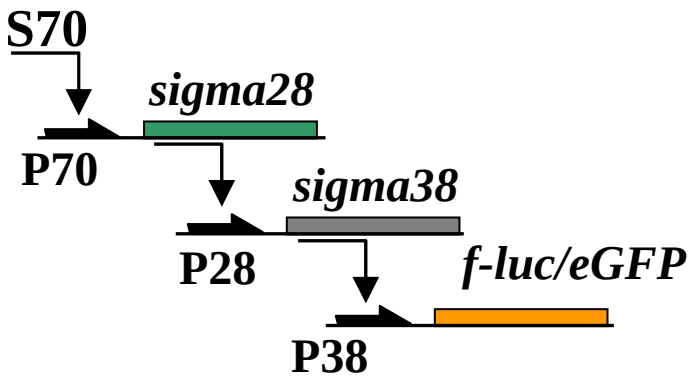
3-stages cascade for sigma19

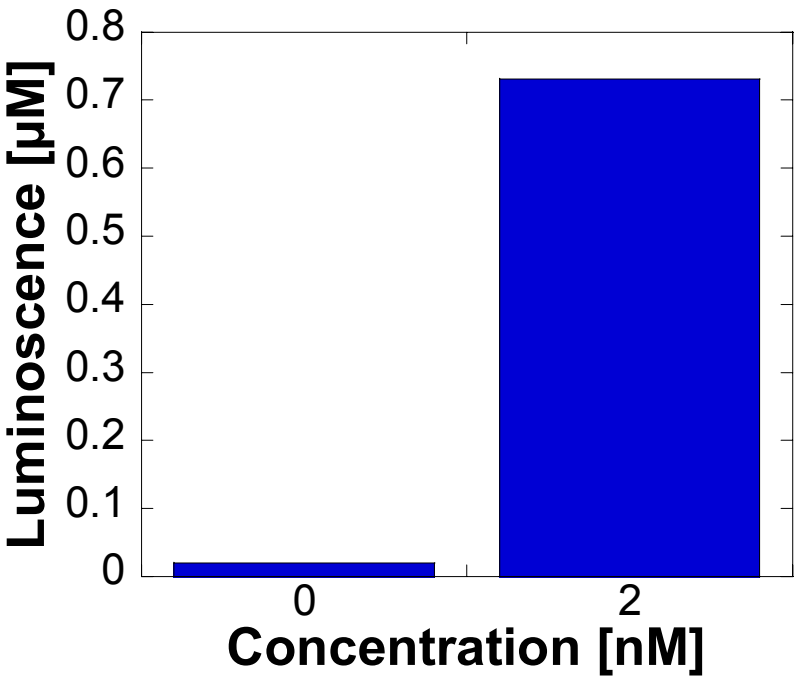


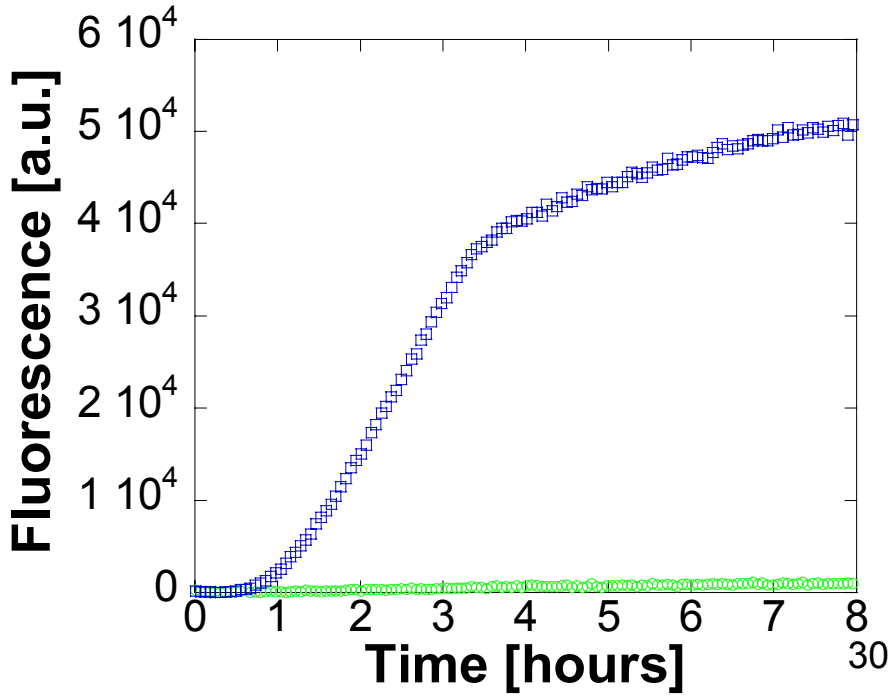




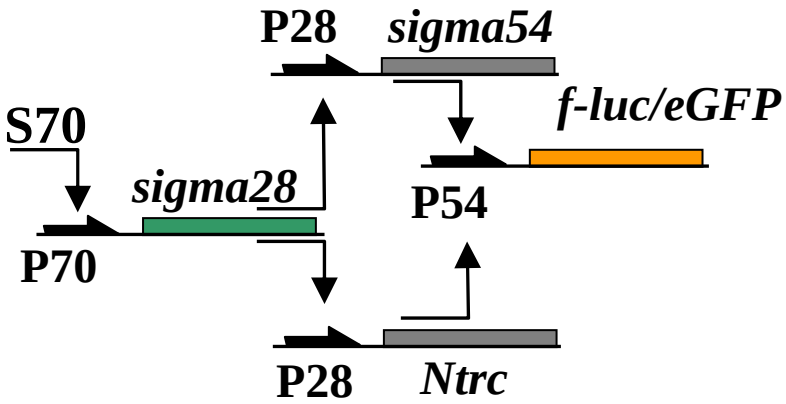
3-stages cascade for sigma38

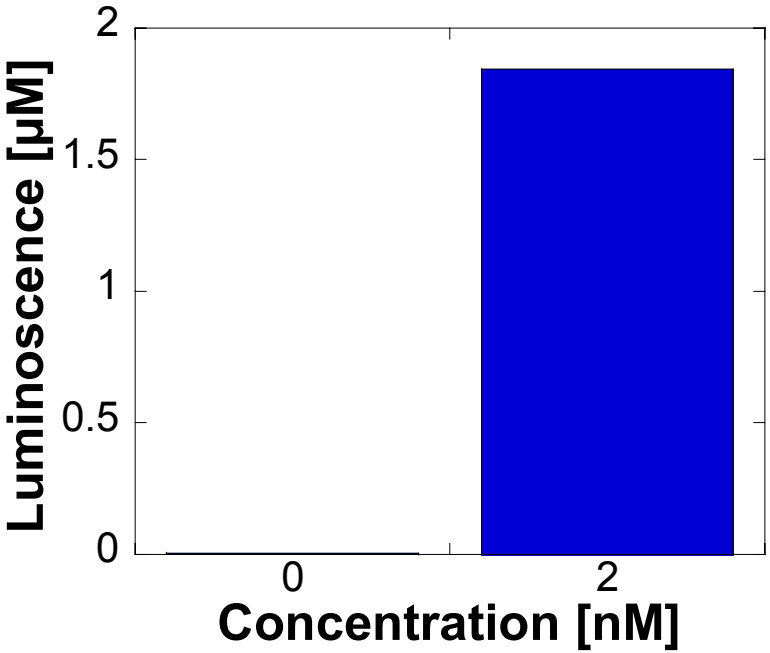


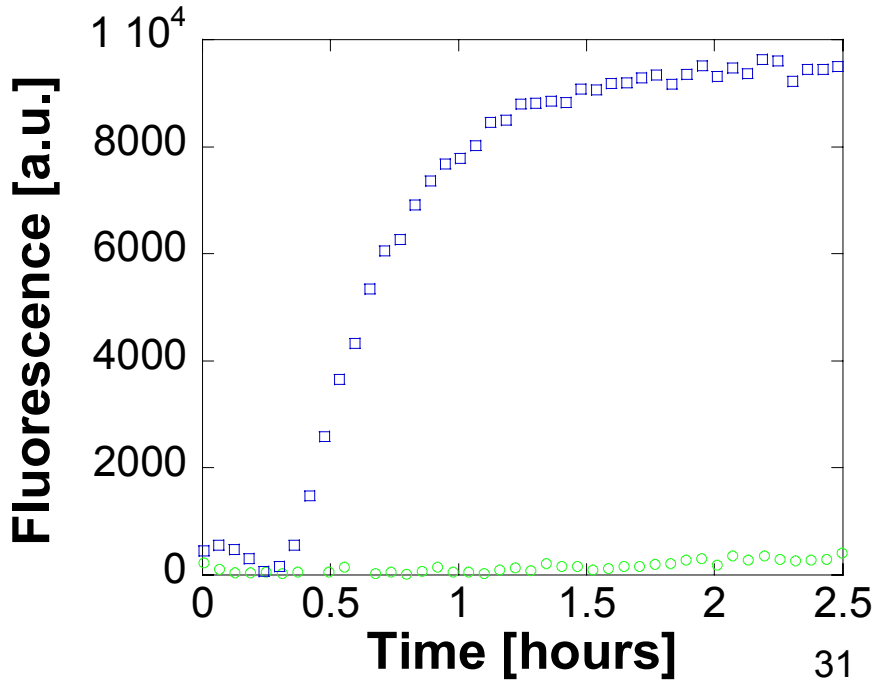




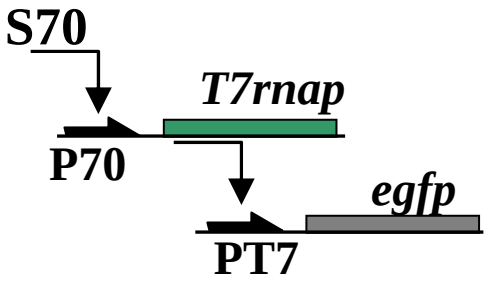
3-stage cascades for Ntrc-sigma54

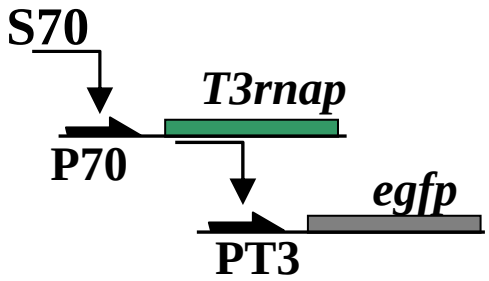


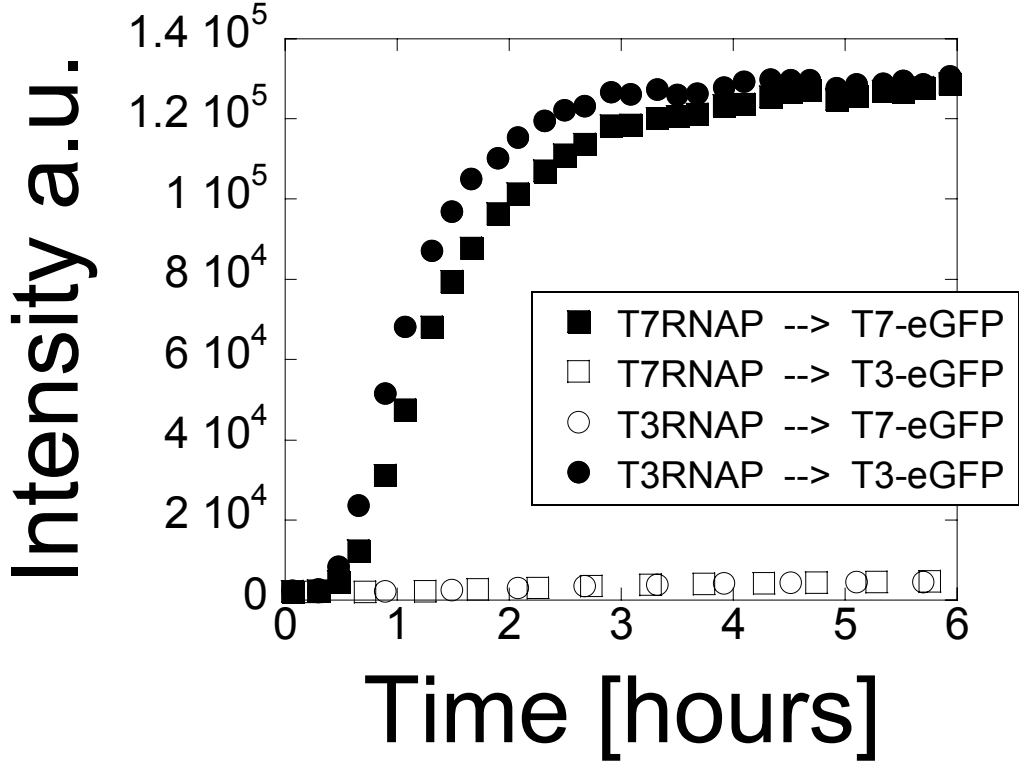




T7 and T3 RNAP

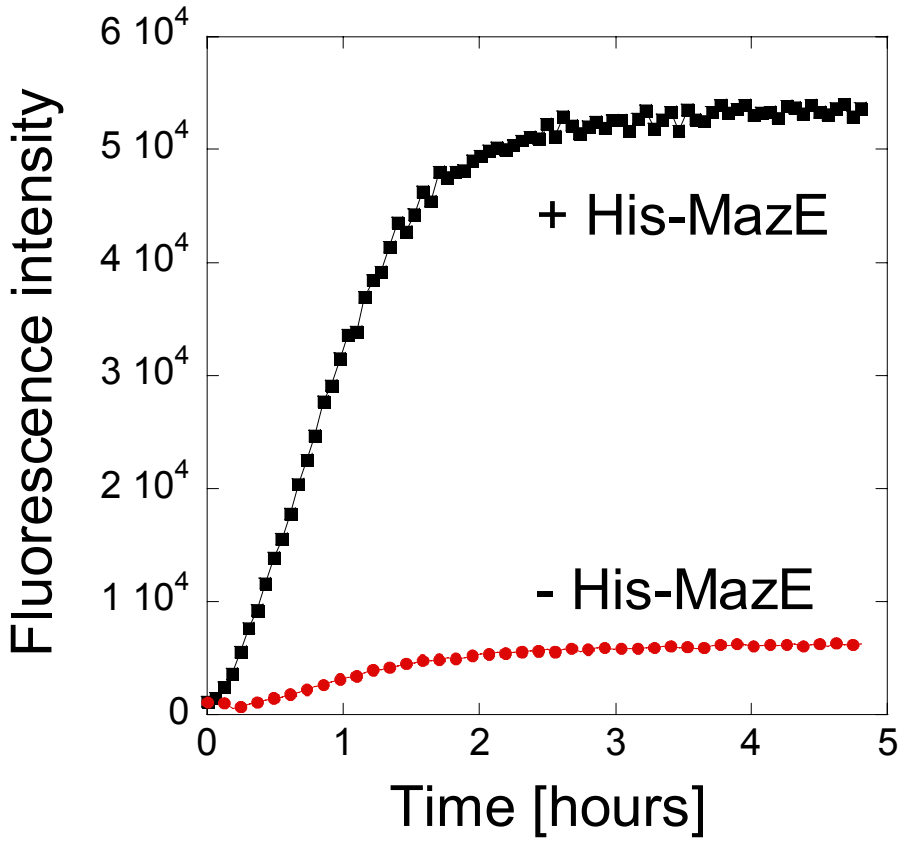






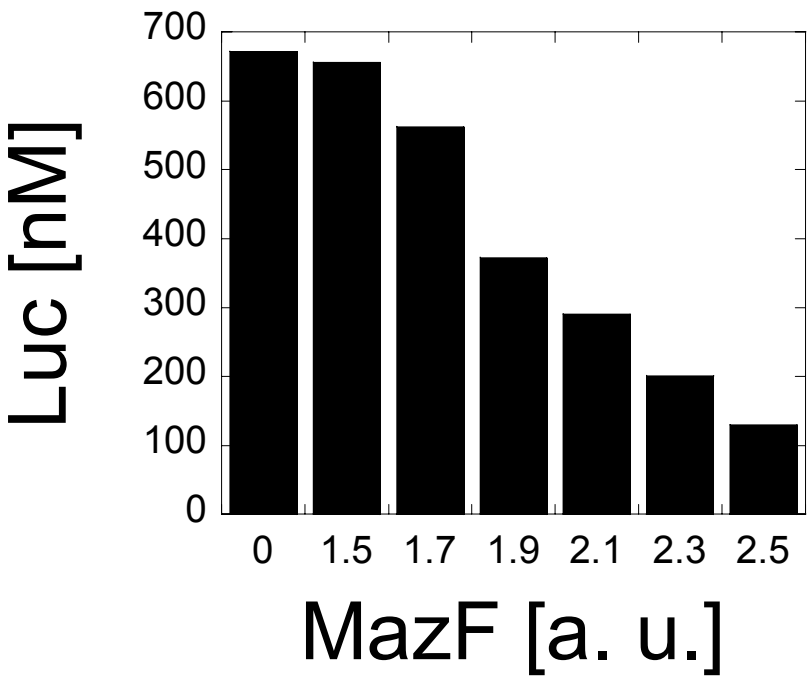
mRNA degradation

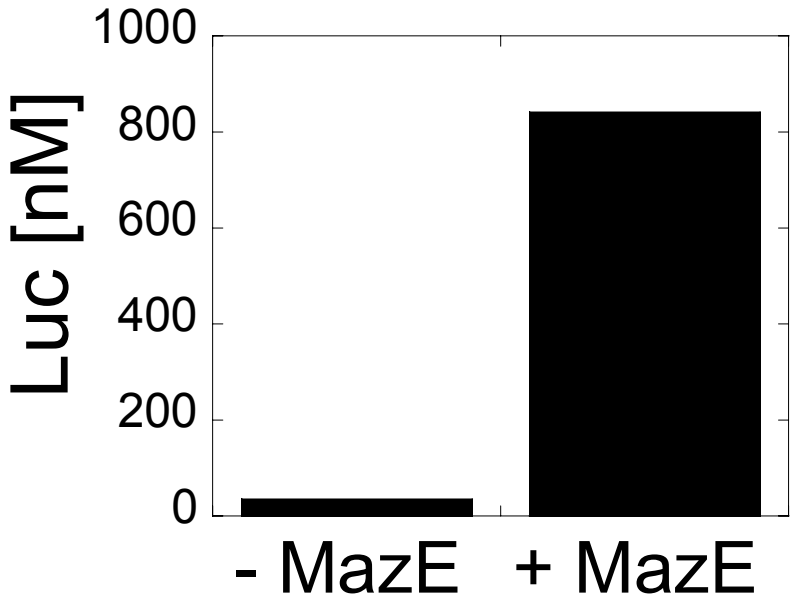
- MazF: toxin, specific mRNA cleavage.
- MazE: antitoxin, binds to MazF.



mRNA degradation rate tuning

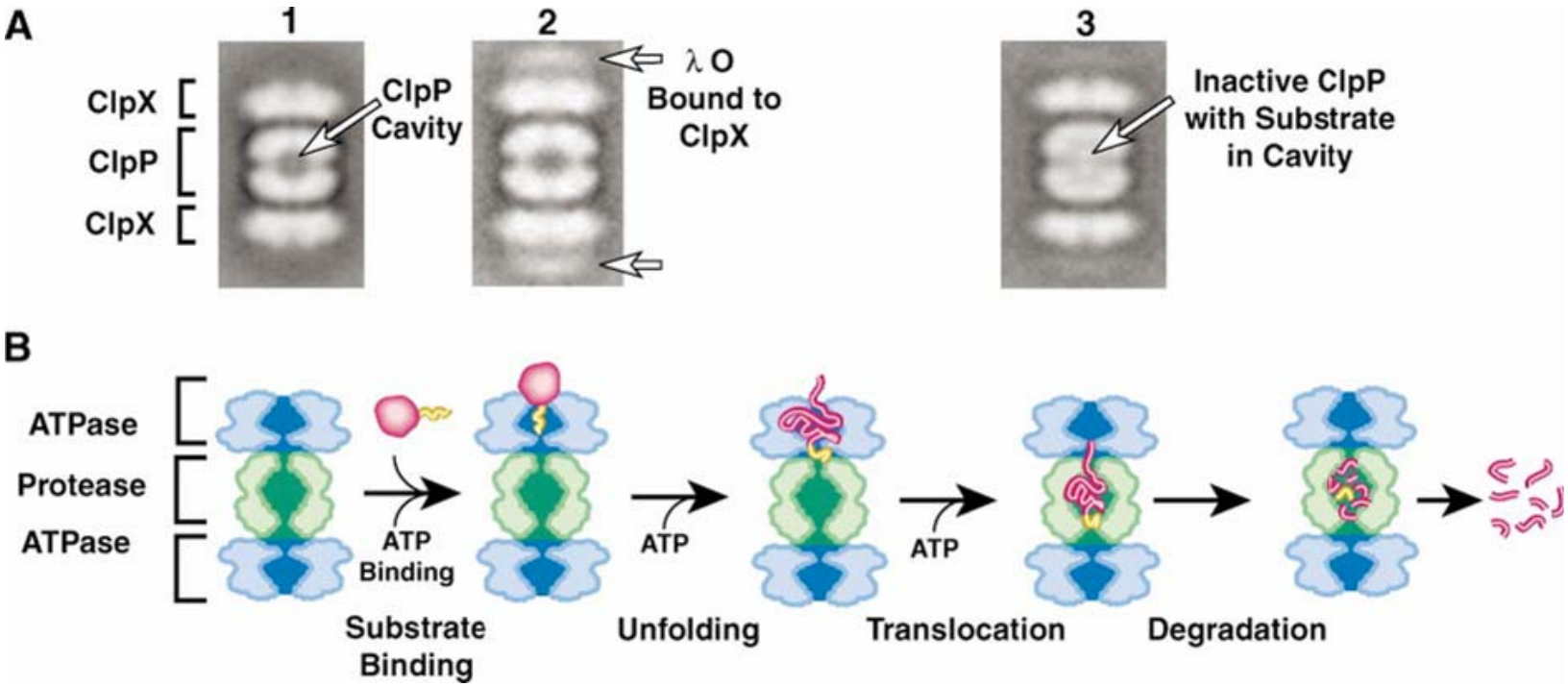
MazEF specific messengers degradation





Specific protein degradation

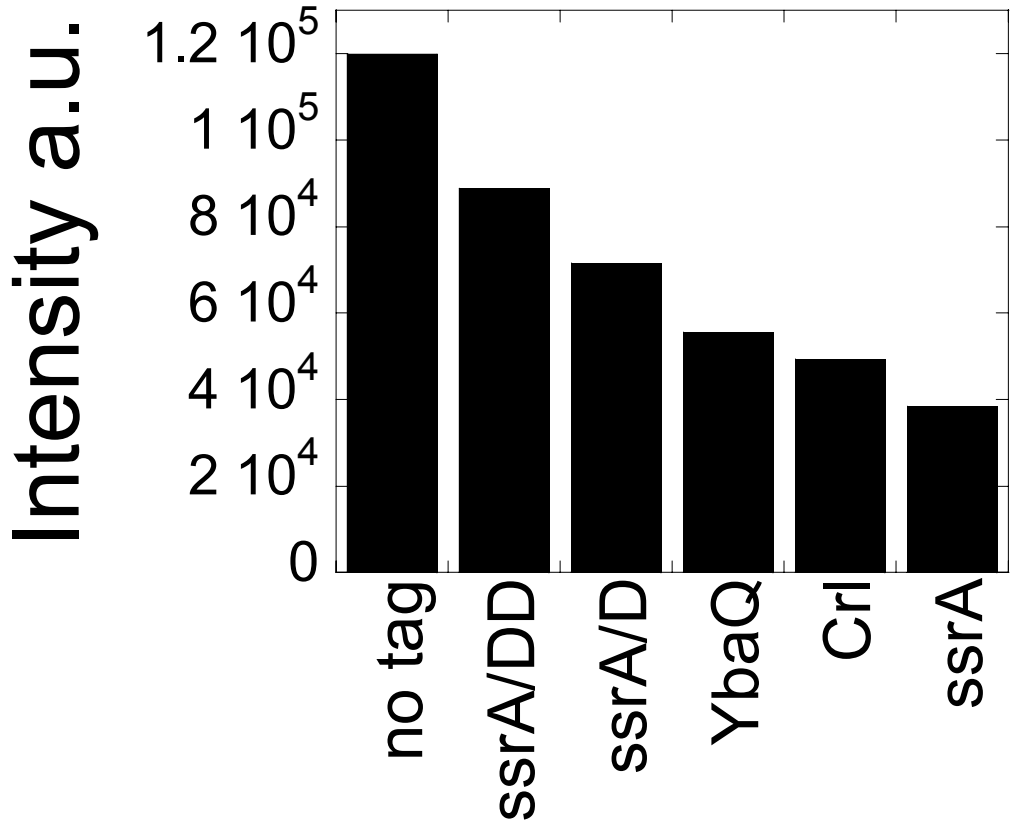
ClpXP degradation complex E.coli



(Gottesman, 2003)

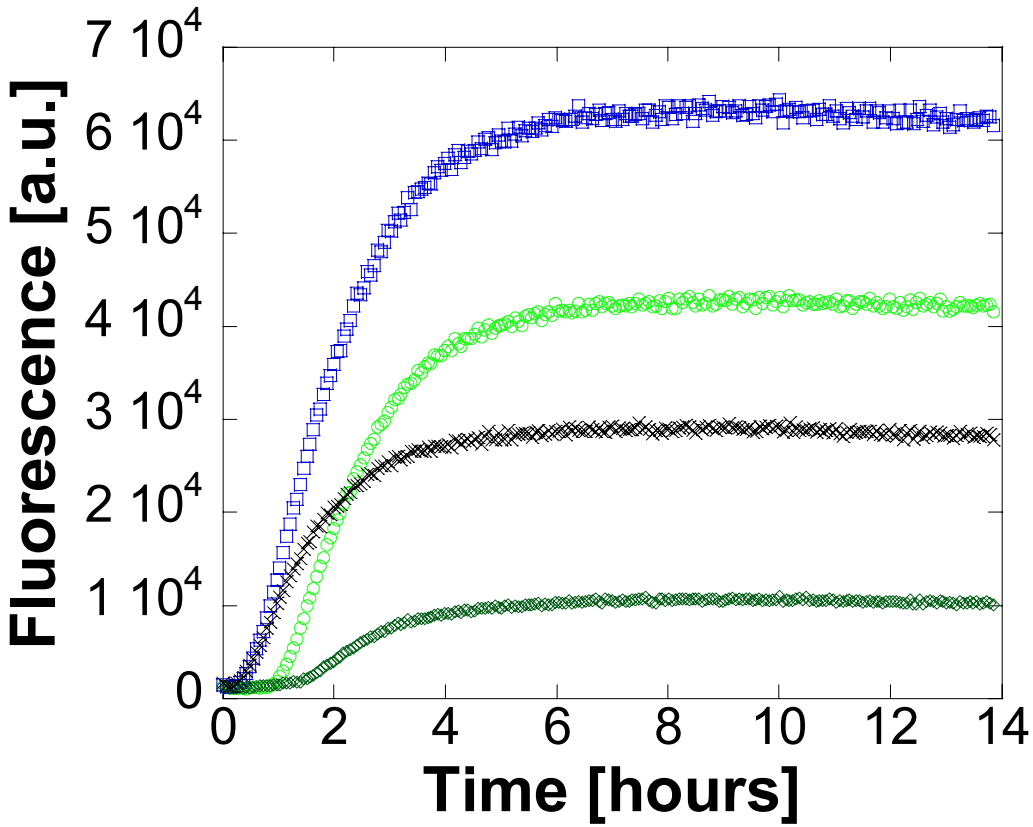
ClpXP tag

ClpXP degradation complex E.coli



(Flynn et al. – 2003)

Coupled mRNA/protein degradation



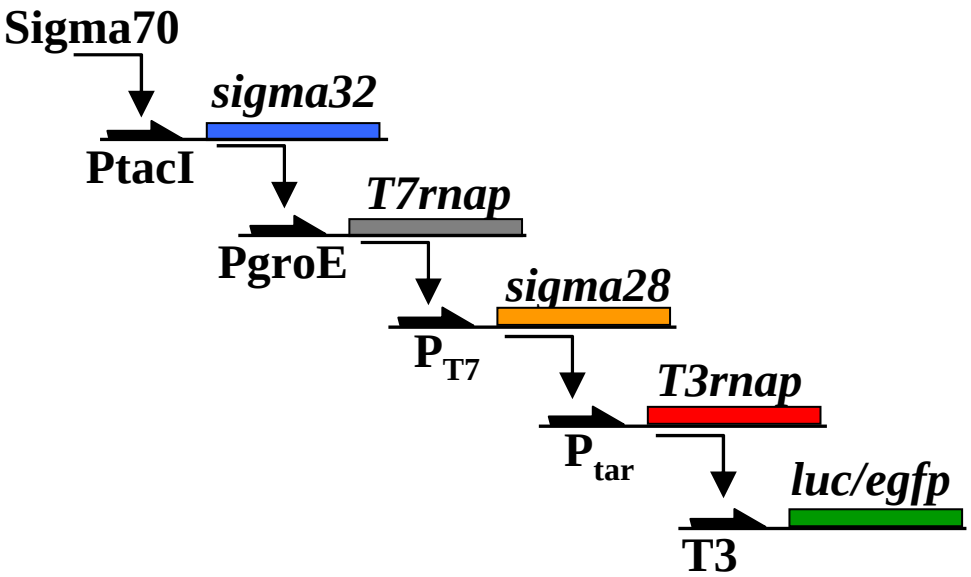
Conclusion:

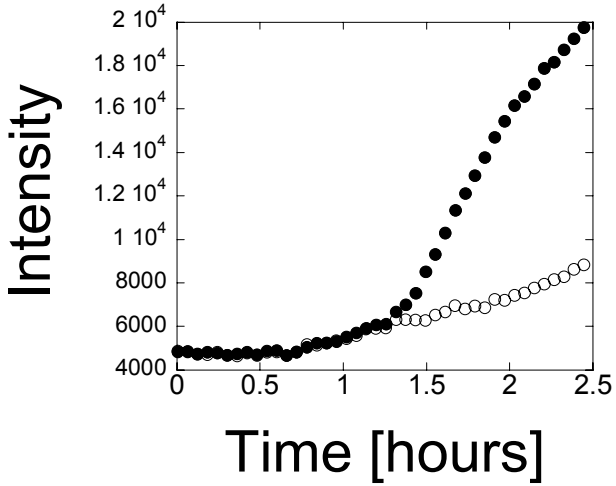
- in batch mode, elementary genetic circuits of …….
- E. coli cell-free system with 9 different types of RNA polymerases, degradation of mRNA and proteins can be adjusted.

Part 4:
application to gene networks

Jonghyeon Shin.

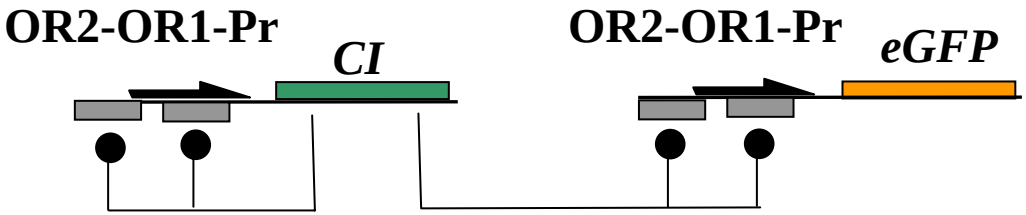
5-gene cascade

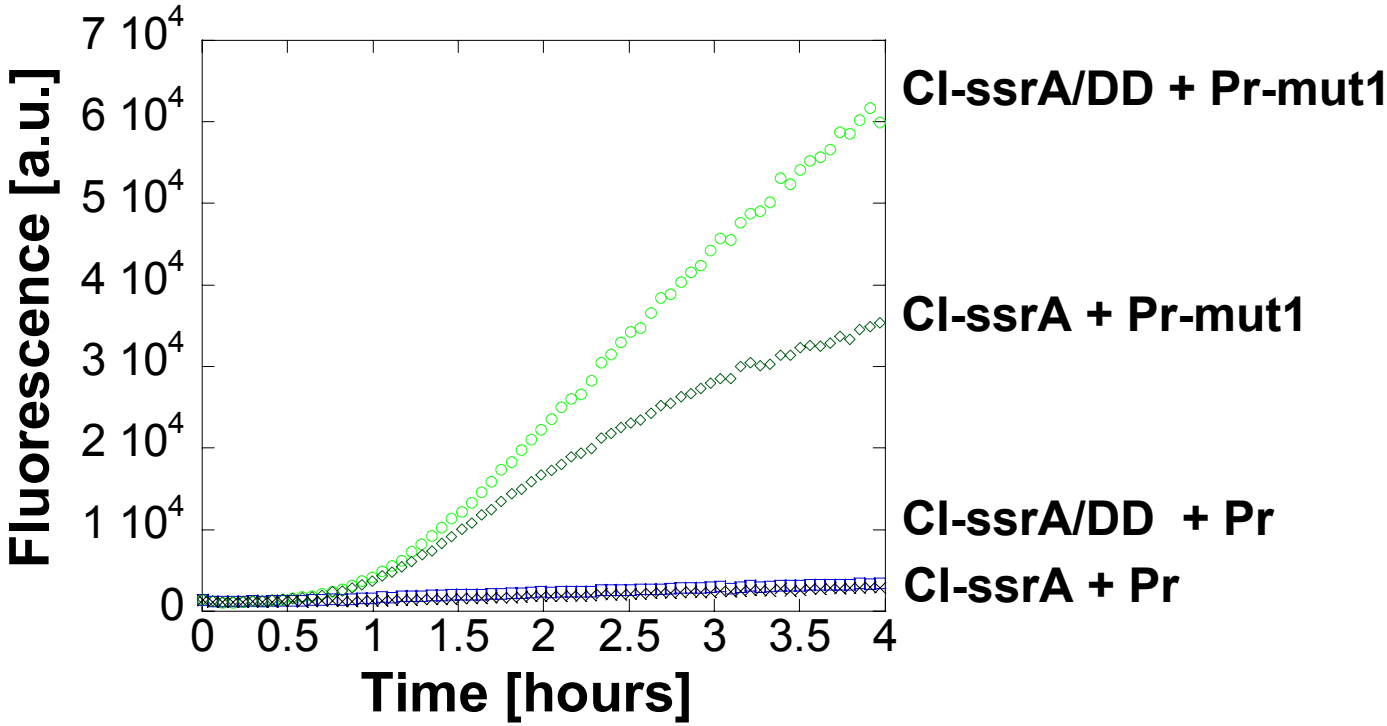




Parameters: gene concentration, mRNA degradation, protein degradation, ….

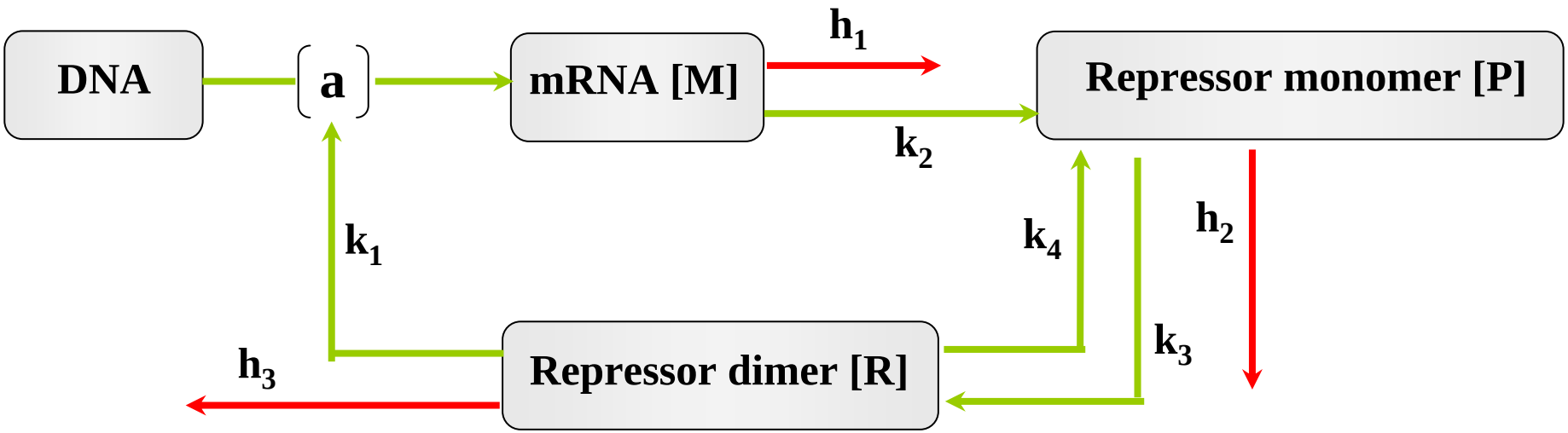
Negative feedback loop (1/2)





Auto-regulation system

(Transcriptional negative feedback loop)



Model I: high cooperativity

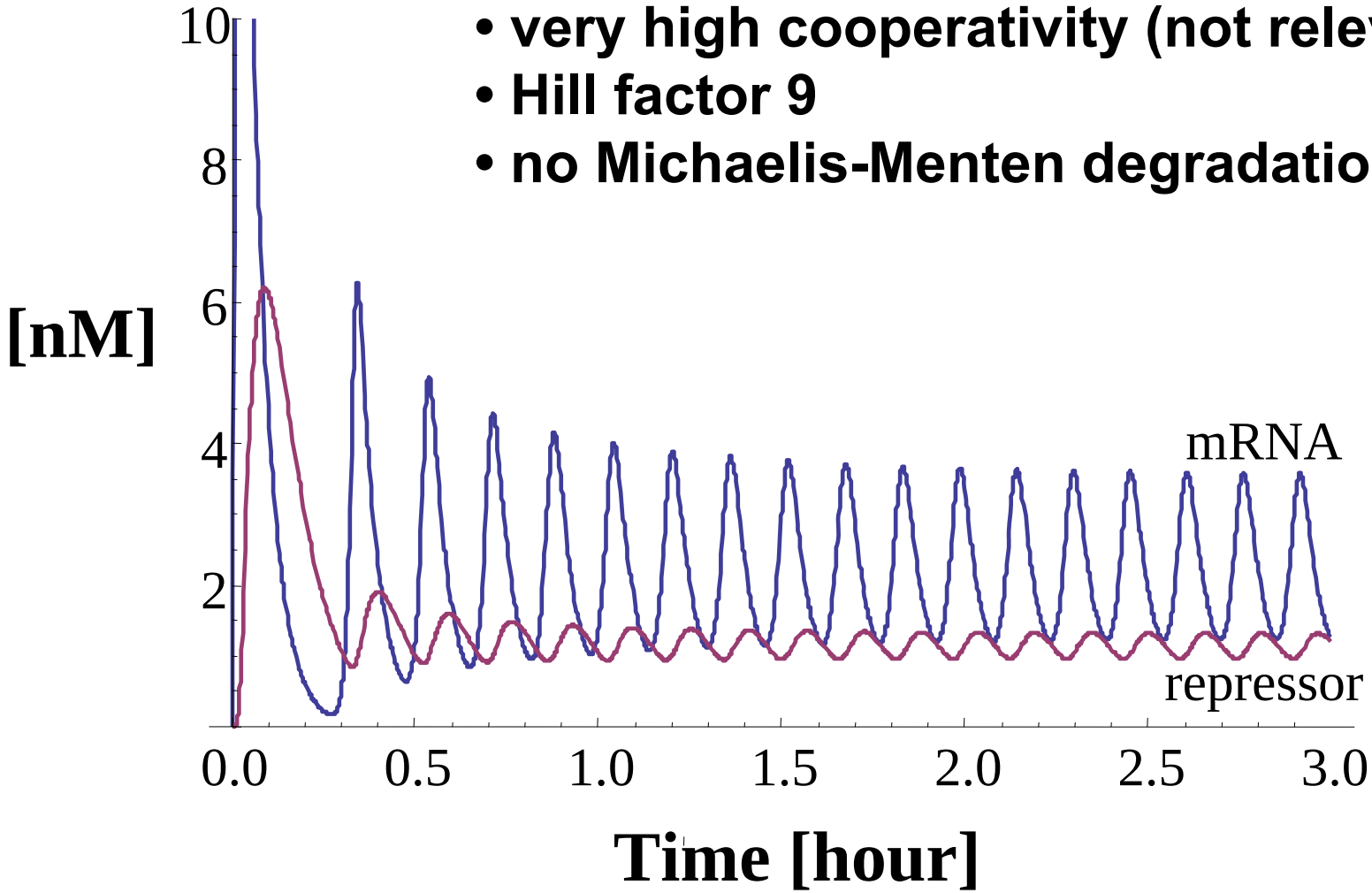
$$\frac{d[M]}{dt} = \frac{a}{1 + k_1[R]^m} - h_1[M]$$

$$\frac{d[P]}{dt} = k_2[M] + k_4[R] - k_3[P]^2 - h_2[P]$$

$$\frac{d[R]}{dt} = k_3[P]^2 - k_4[R] - h_3[R]$$

- Ref) B.C.Goodwin. Adv. Enzyme Regul. 3, 425-439 (1965)

- very high cooperativity (not relevant)
- Hill factor 9
- no Michaelis-Menten degradation



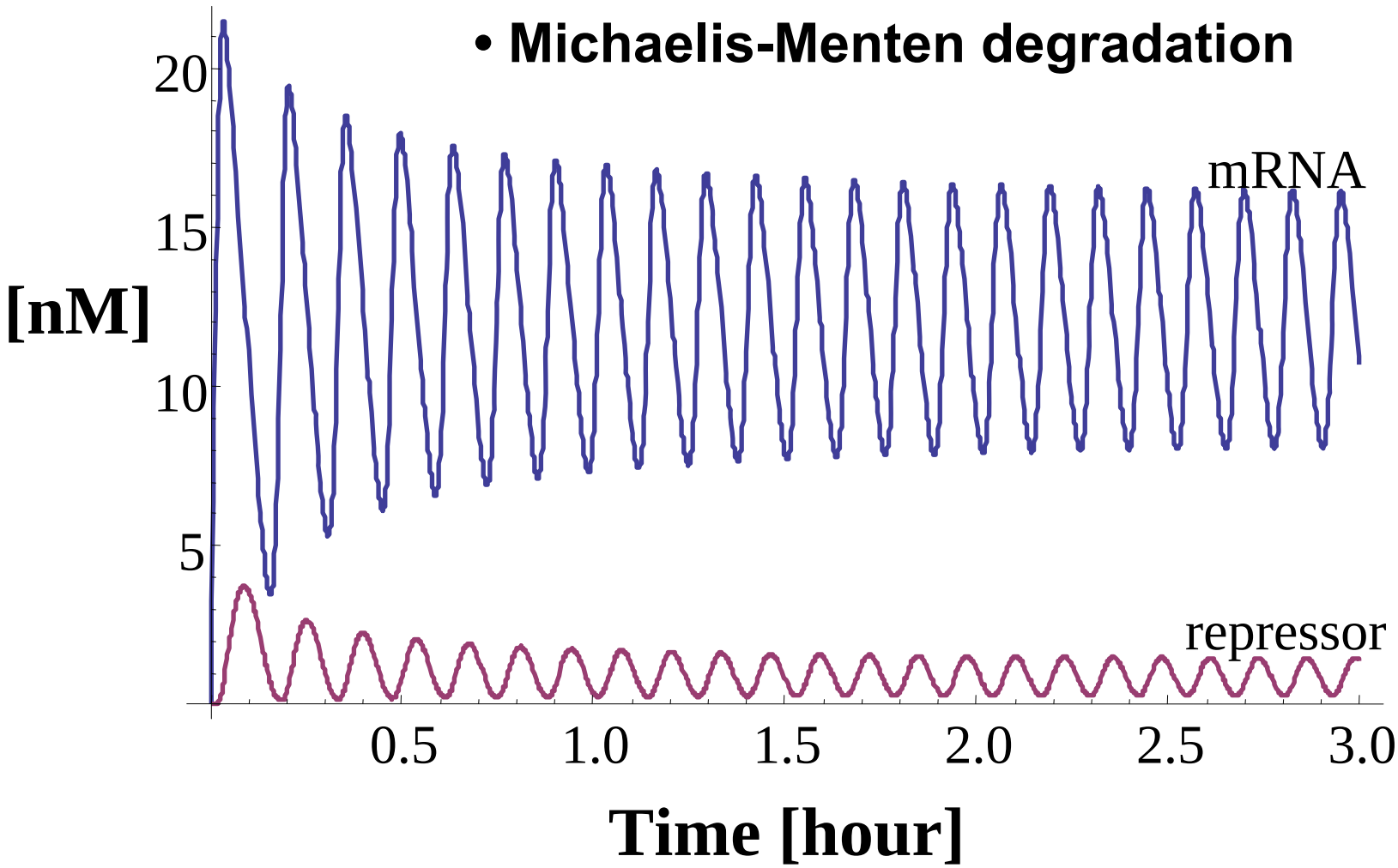
Model II: low cooperativity

$$\frac{d[M]}{dt} = \frac{a}{1 + k_1[R]^m} - \frac{h_1[MF][M]}{b + [M]}$$

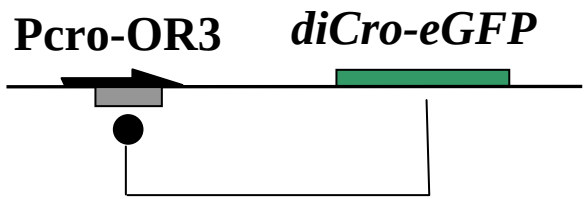
$$\frac{d[P]}{dt} = k_2[M] + k_4[R] - k_3[P]^2 - \frac{h_2[CX][P]}{c + [P]}$$

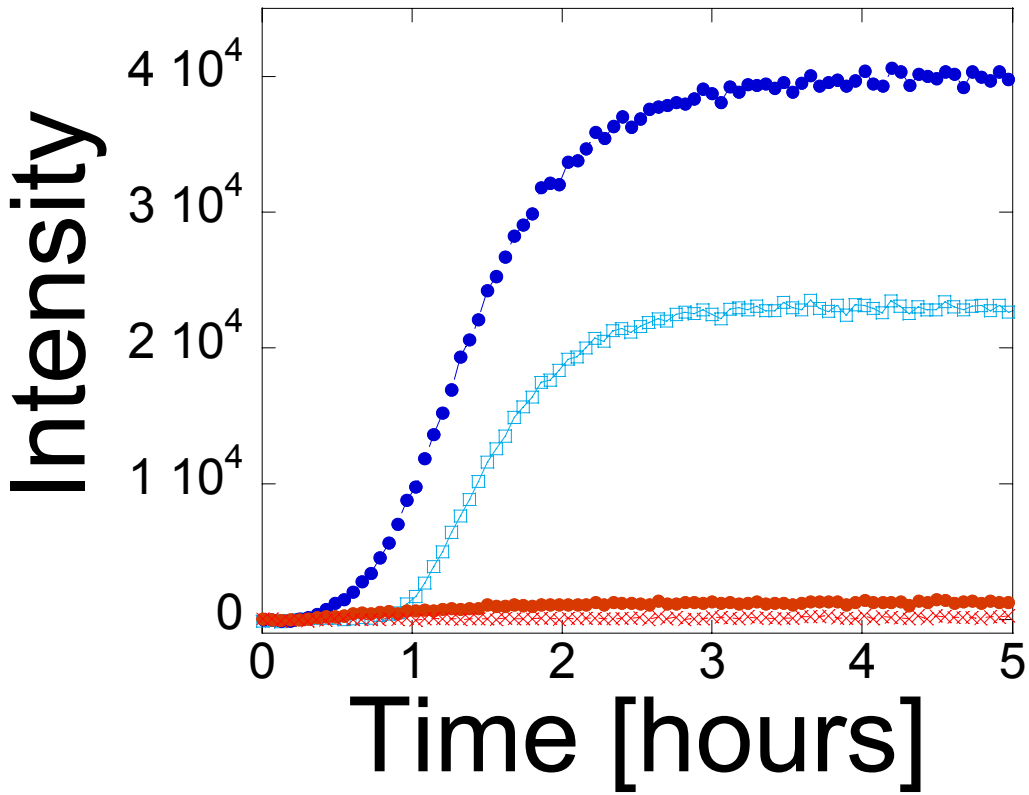
$$\frac{d[R]}{dt} = k_3[P]^2 - k_4[R] - \frac{h_3[CX][R]}{d + [R]}$$

- **low cooperativity (relevant)**
- **Hill factor 1.8**
- **Michaelis-Menten degradation**



Negative feedback loop (2/2)





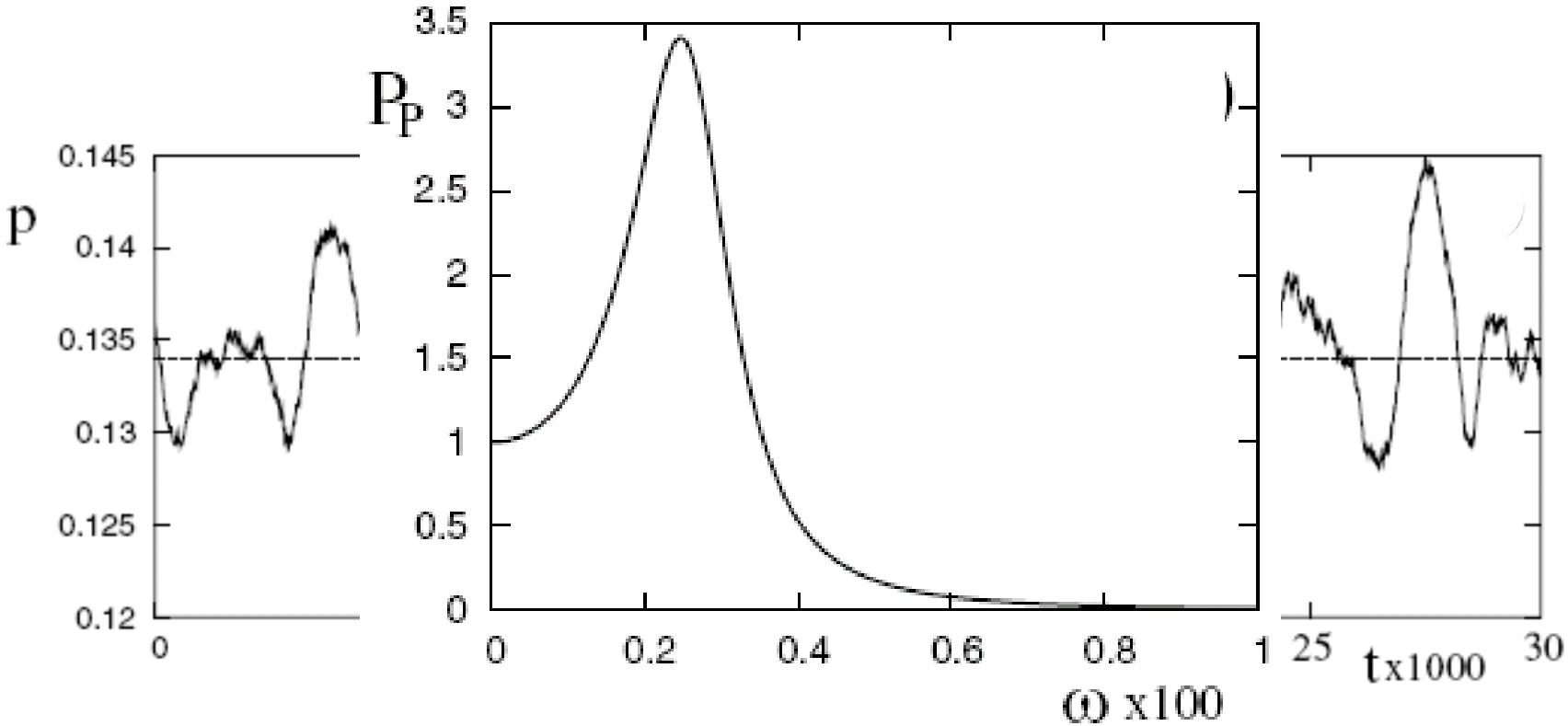
diCro-eGFP-ssrA/DD + Pcro-mut1

diCro-eGFP-ssrA + Pcro-mut1

diCro-eGFP-ssrA + Pcro

diCro-eGFP-ssrA/DD + Pcro

Model III: stochastic model



- Ref) A.J.Mckane and co-workers.
- Journal of Statistical Physics **128**, 165-191 (2007)

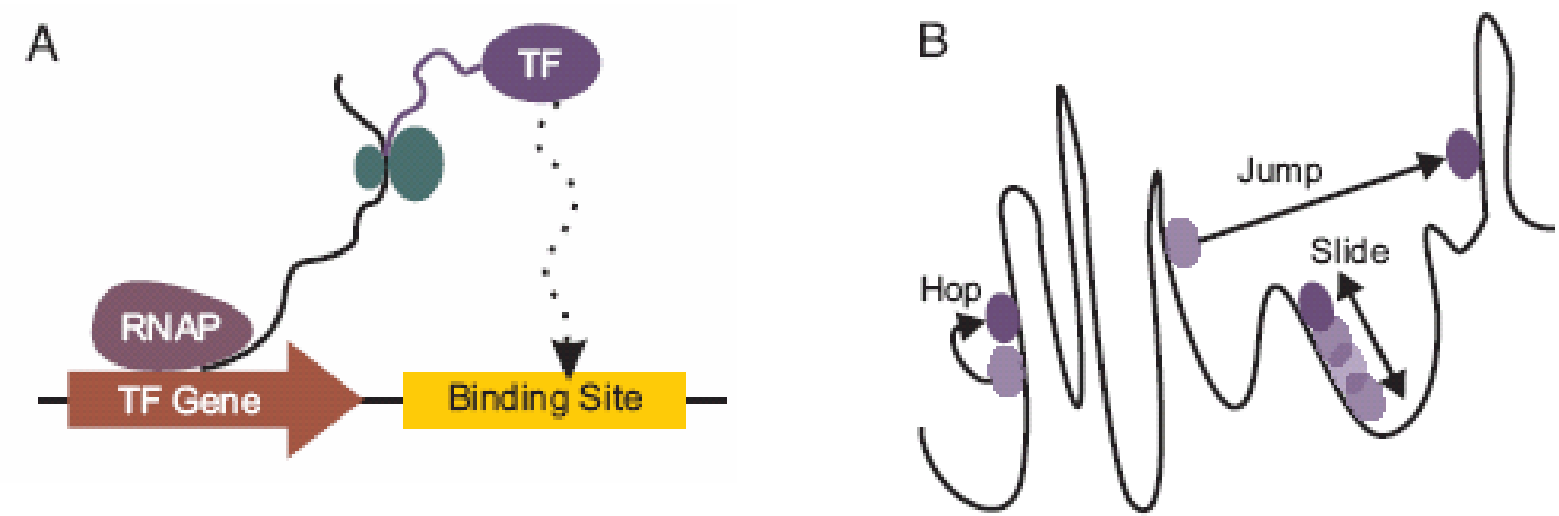
Part 5:
application to the DNA-binding protein search problem.

Jonghyeon Shin and Nadezda Monina.

The problem:

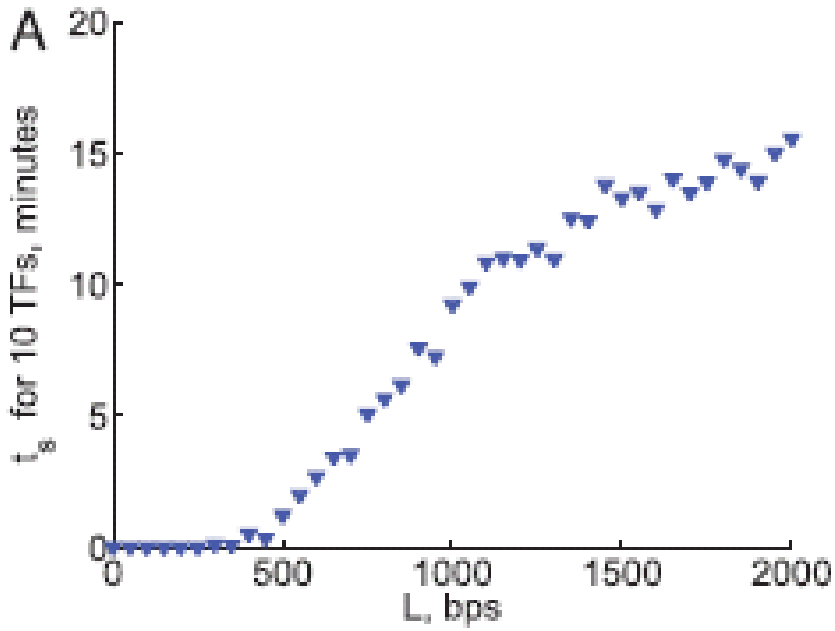
how a TF finds its target sequence (30bp) among 510^6 bp in a few seconds?

→ 1D and 3D diffusion problem.



Kolesov et al - 2007

Model prediction:

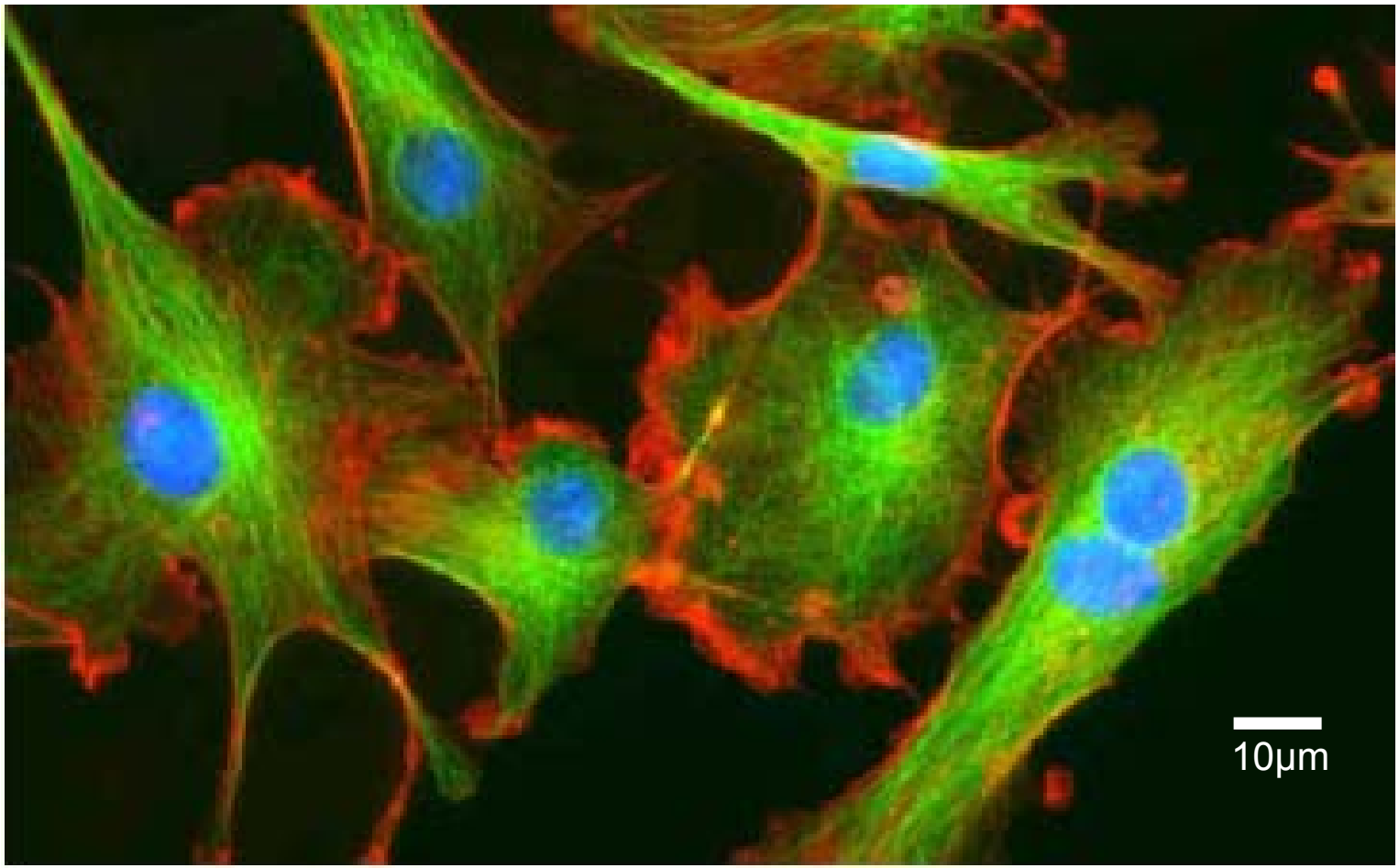


genetics under construction

Part 6:
application to diffusion from a source point.

Jonghyeon Shin
Nadezda Monina
Jonathan Gapp.

Anomalous diffusion



Diffusion from a local source

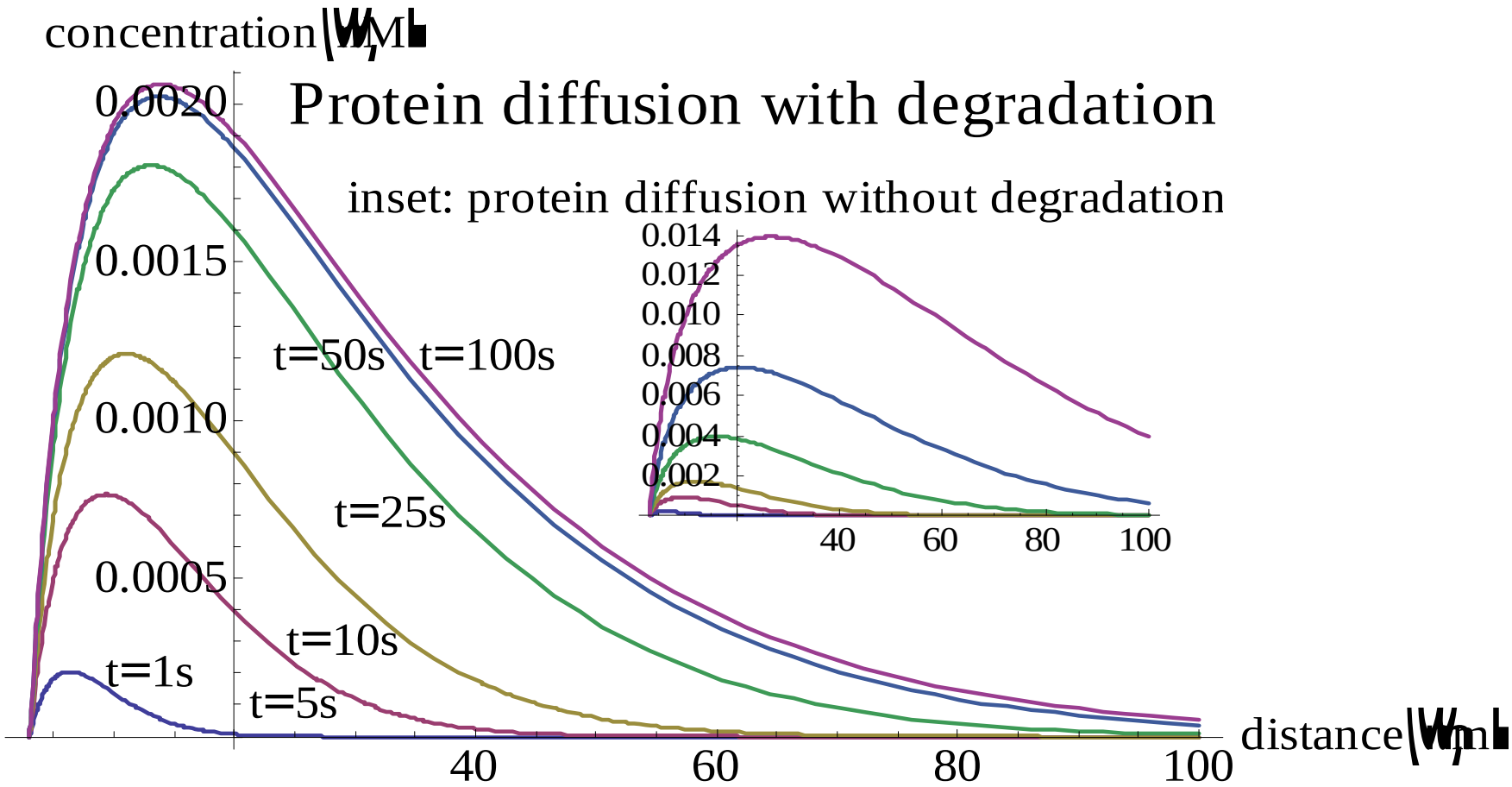
$$\frac{d[M]}{dt} = \frac{D_1}{r}\partial_r(r\partial_r[M]) + S_1\delta(r - r_0)$$

$$\frac{d[P]}{dt} = \frac{D_2}{r}\partial_r(r\partial_r[P]) + k[M]$$

$$\frac{d[M]}{dt} = \frac{D_1}{r}\partial_r(r\partial_r[M]) + S_1\delta(r - r_0) - \frac{h_1[MF][M]}{1 + [M]}$$

$$\frac{d[P]}{dt} = \frac{D_2}{r}\partial_r(r\partial_r[P]) + k[M] - \frac{h_2[CX][P]}{1 + [P]}$$

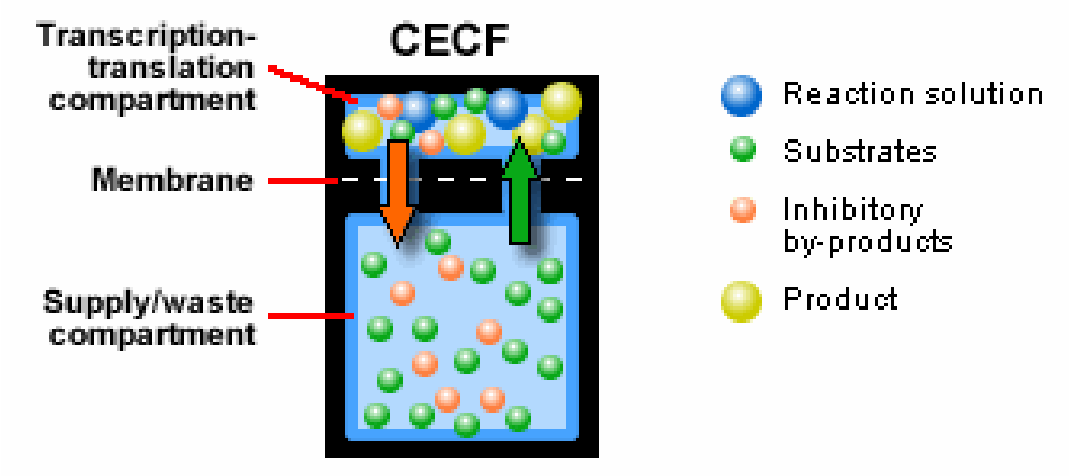
mRNA and protein gradient profile of diffusion motion



Part 7:
application a protocell system.

Jonghyeon Shin
Nadezda Monina.

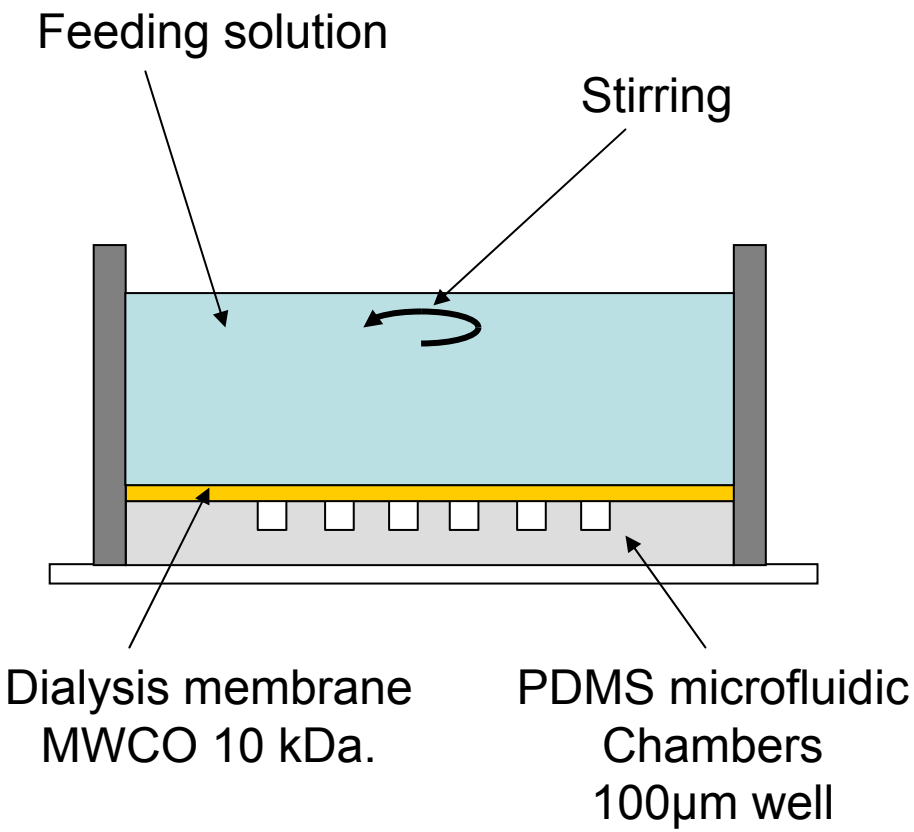
Continuous cell-free expression (in vitro coupled transcription-translation)

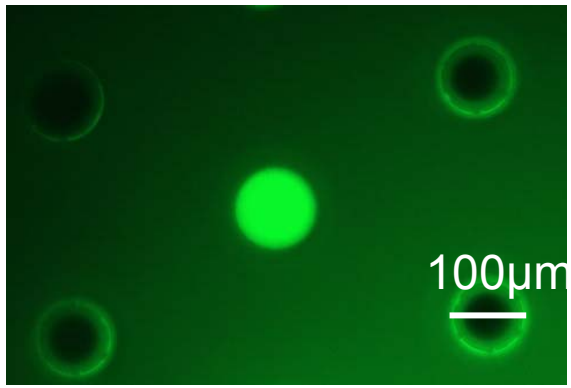


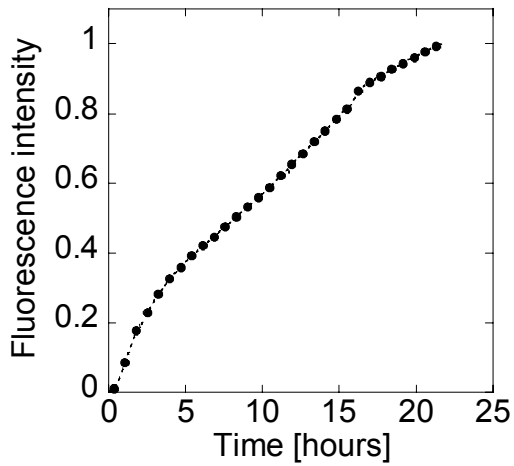
(RTS: Rapid Translation System, E. coli)

Spirin A. S. et al, 1988.

Continuous expression





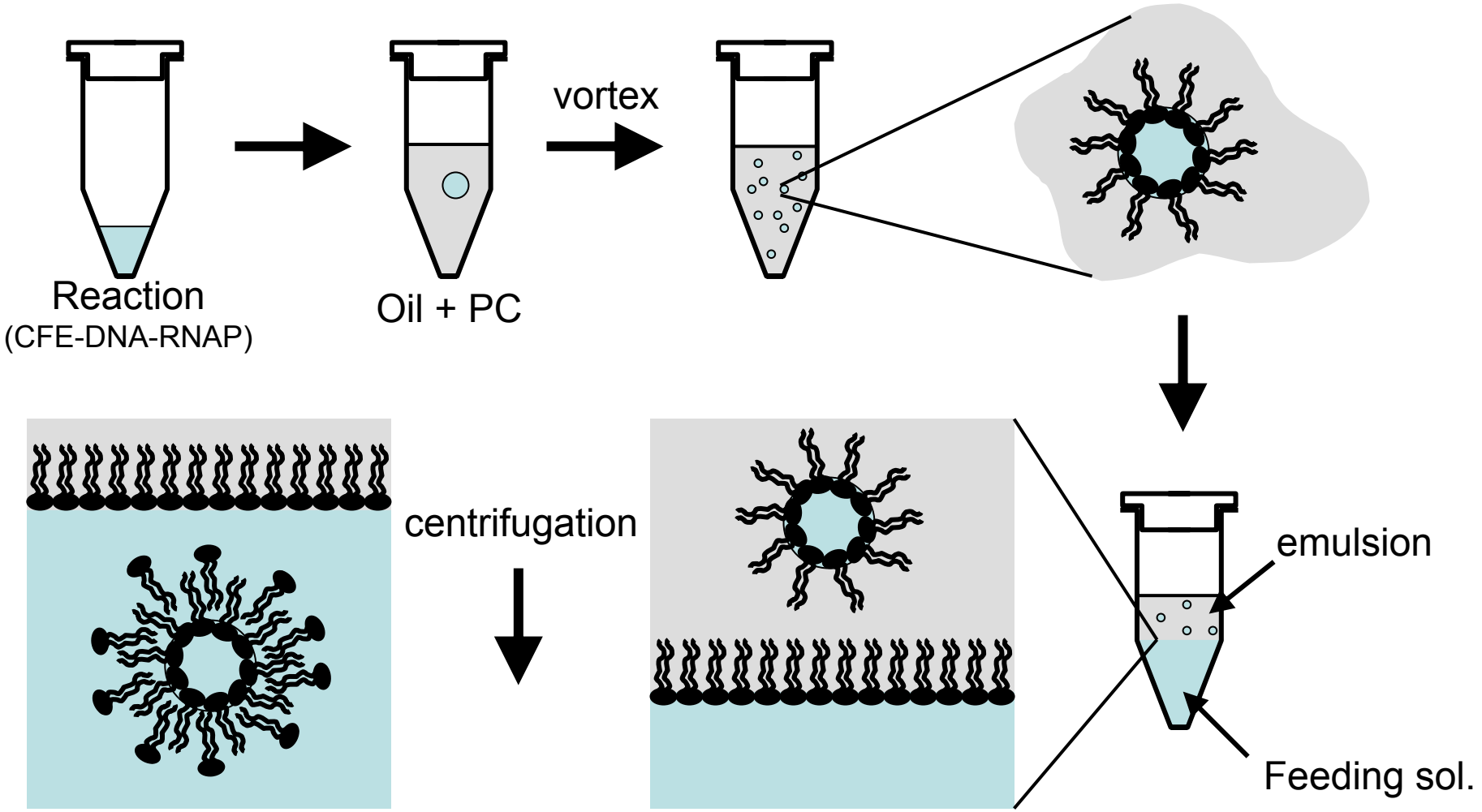


Encapsulation in synthetic vesicles

Conditions/limitations to encapsulate a cell-free extract in phospholipids vesicles:

- CFE is a very dense solution.
- temperature range < 40ºC.
- fragile system (vortex/freeze-thaw)
- phospholipids solvents are toxic.
- osmotic pressure balance.

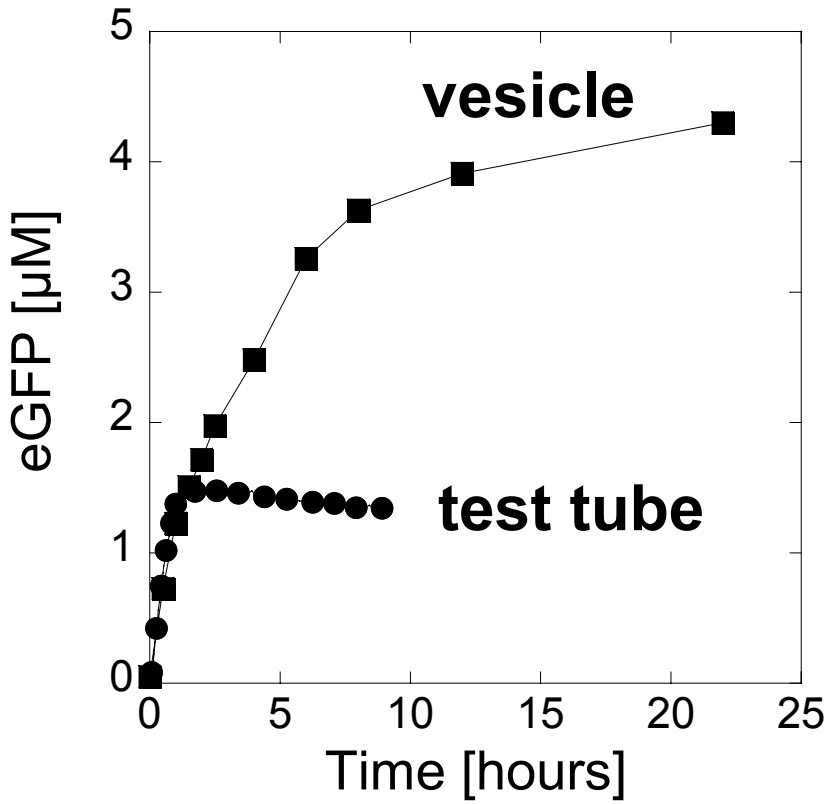
Encapsulation in synthetic vesicles

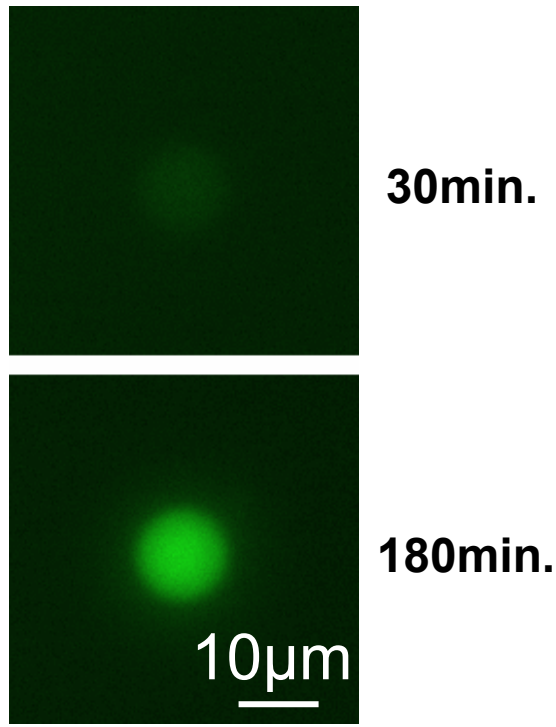


Noireaux - libchaber, 2004.

Pautot et al., 2004.

Expression of eGFP





Conclusion:

- in batch mode, elementary genetic circuits of …….
- E. coli cell-free system with 9 different types of ……
- cell-free extracts can be encapsulated in synthetic phospholipid vesicles.
  The method is reproducible with fluctuations.
  Osmotic pressure is a serious limitation.

Similar work:

FEBS 28879 FEBS Letters 576 (2004) 387–390

Expression of a cascading genetic network within liposomes

Keitaro Ishikawa[a,1], Kanetomo Sato[a,1], Yasufumi Shima[a], Itaru Urabe[a], Tetsuya Yomo[a,b,c,d,e,*]

Continuous CFE in phospholipids vesicles

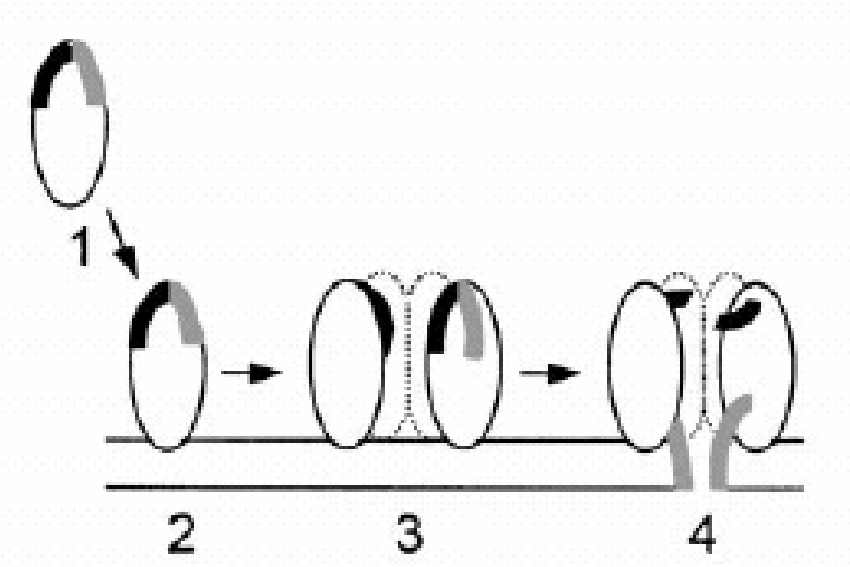
Conditions:

- CFE has no integral membrane protein insertion mechanisms.
- selective permeability, molecular mass cutoff below 10 kDa, above 1 kDa.
- better if established by expression inside the vesicle.

Membrane selective permeability

Alpha Hemolysin

- toxin Staph. Aureus
- soluble monomer
- membrane heptamer
- channel of 1.4nm: 2-3kD



Feeding Solution

Extract

BSA-rhodamine

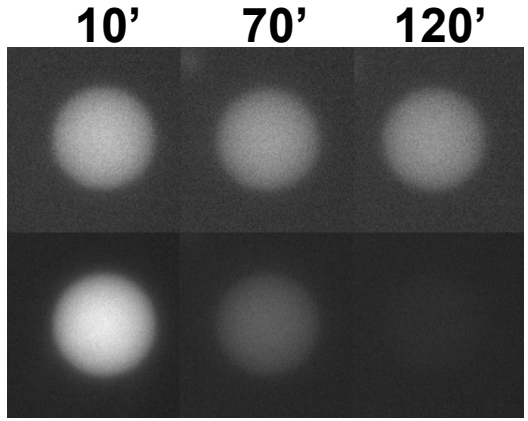
Fluorescein-UTP

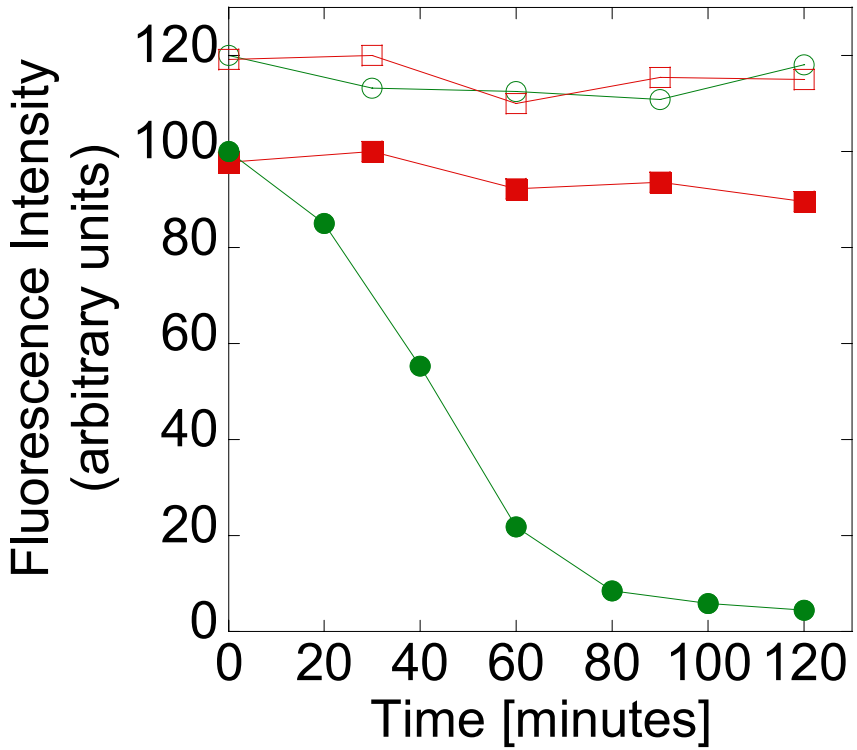
T7- αHemolysin

BSA-rhodamine: 60 kDa

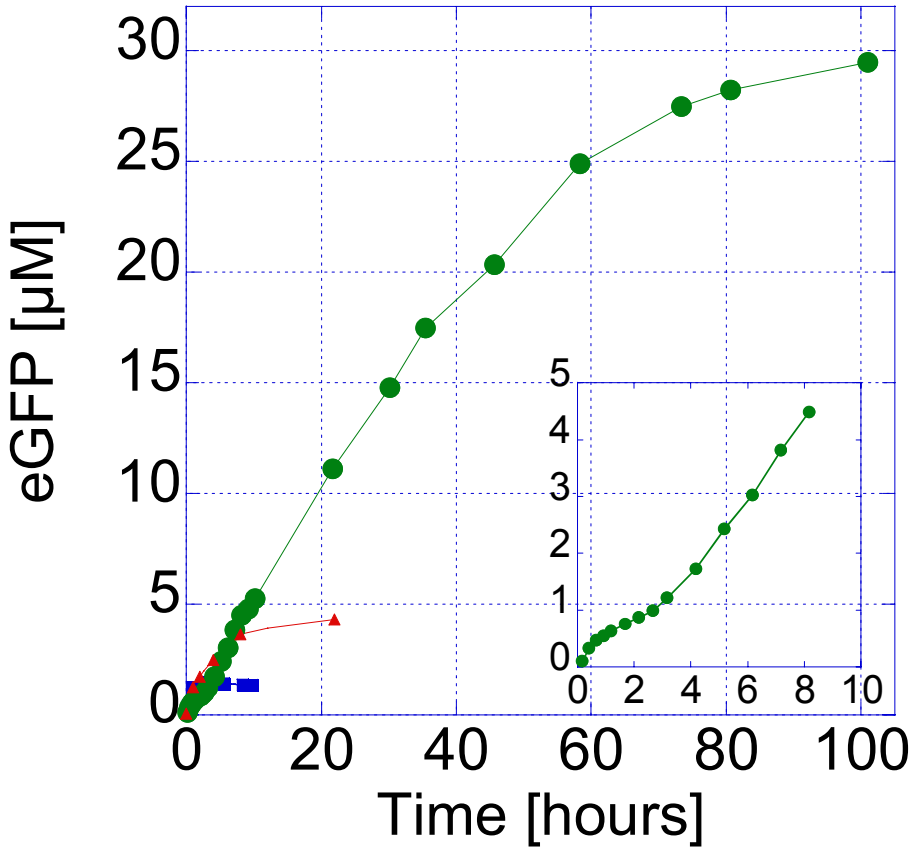
Fluorescein-UTP: 1 kDa

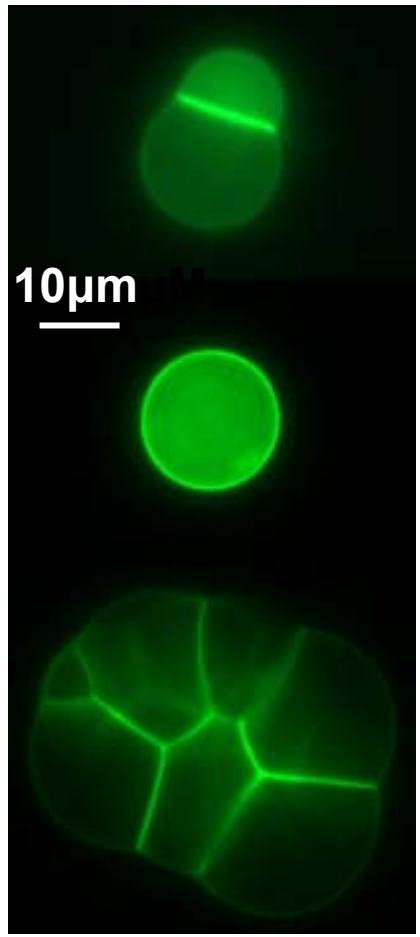
(Song L et al., Science, 1996)


10’
70’
120’
BSA RITC
Fluorescein UTP


Fluorescence Intensity
(arbitrary units)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Time [minutes]

Long-lived bioreactor





Conclusion:

- in batch mode, elementary genetic circuits of …….
- E. coli cell-free system with 9 different types of ……
- cell-free extracts can be encapsulated in synthetic ……
- continuous cell-free expression in the vesicles can be carried out with the internal expression of the toxin alpha-hemolysin.

Noireaux and Libchaber, PNAS, 2004.

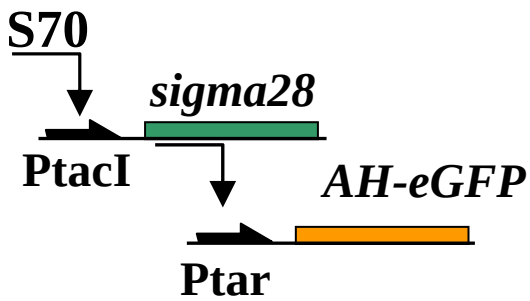
Block copolymer vesicles

(work of Nadezda Monina)

- Vesicles formed with:

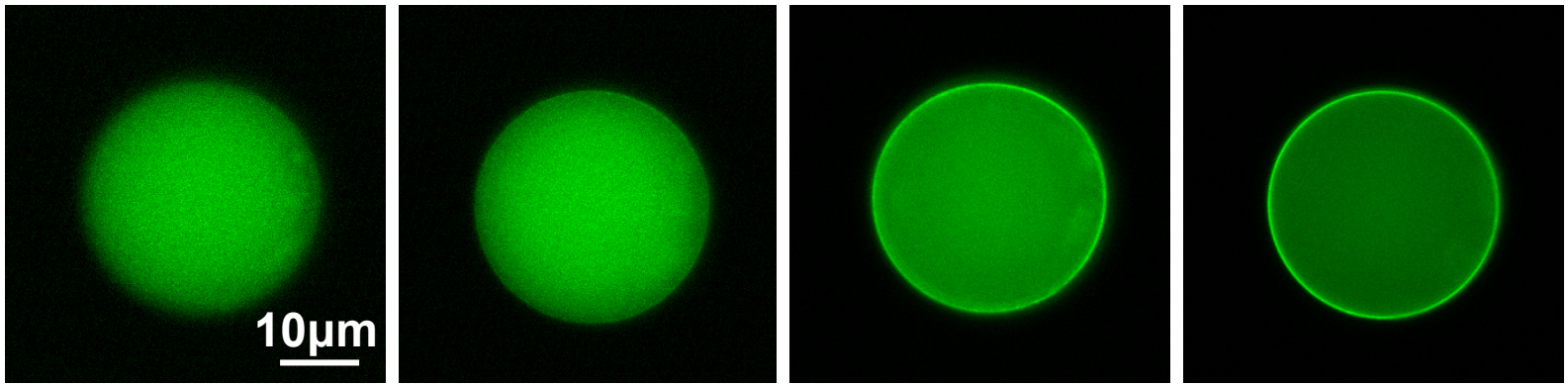
- phospholipids only (2-3nm thickness)
- block copolymer only (20nm thickness)
- mixture of phospholipids and block copolymer

- inside vesicles, expression of AH-eGFP

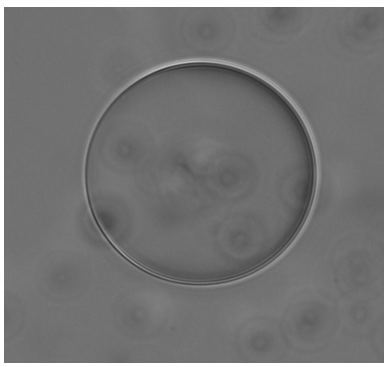


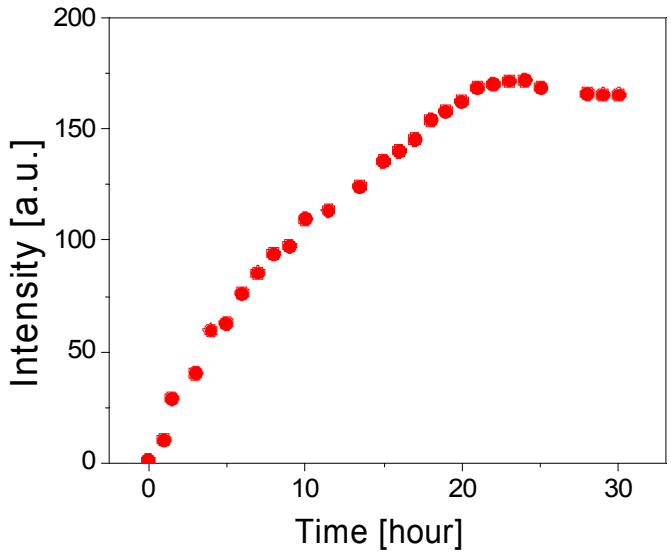
Phospholipids/copolymer vesicles

(work of Nadezda Monina)



3 hours **5 hours** **6 hours** **21 hours**





Lab

Jonghyeon Shin.
Nadezda Monina.
Jonathan Gapp.

Collaborators (block copolymer)

Frank Bates, University of Minnesota.
Kevin Davis, University of Minnesota.