MICRO TIMER
1. Circadian rhythm
2. Sensing the length of time
3. Behave accordingly

Tyrosine family recombinase: Cre/Flpe
Serine family recombinase: bxb1

Recombination target site (RTS)
Can a single recombinase unit define a time length accurately?

How can we construct a micro-timer based on these units?
Answer: invertase dynamics
Activated phase

Timing

Length

Product (inverted sequence)

Invertase

Mechanism: How invertase counts time

Quantity

Time

Timing length

Unactivated phase

RTS

RTS
Major Concerns

How to construct an available timing Module?
- Real-time invertase dynamics testing system

What factors determine the timing length?
System construction

Real-time invertase dynamics testing system

\[ P_{\text{IND}} \rightarrow \text{RBS} \rightarrow \text{Invertase-GFP} \rightarrow \text{Term} \]

\[ P_{\text{CONS}} \rightarrow \text{RBS} \rightarrow \text{RTS} \rightarrow \text{FPP} \]

\[ \text{plnv-gen} \rightarrow \text{Km}^r \]

\[ \text{plnv-rep} \rightarrow \text{Cm}^r \]
Our timing module exactly works!

- Relatively stable Cre generation
- A burst of Red signal
- A rough but obvious timing length
- Invertase can be a timer

Results

RFU/OD700

- Cre-EGFP(-ssra)
- mcherry(-ssra)

Burst

Time (h)

8hrs
Our timing module exactly works!

- 5 invertases
- 16 pairs of plnv-rep and plnv-gen.
- Similar pattern
What contributes to determination of timing length?

Major Elements

- Cre/Flpe/Dre/Vcre/Scre/Vika

- With ssra or not

- Ssra: a C-term tag leading to protein degradation

- 5 constitutive promoters

- 2 inducible promoters

- Promoter

- Invertase (and its RTS)

- RBS

- mCherry

- Term

- Rsra

- Term

- Rsra: a C-term tag leading to degradation
Results

Inversion efficiency is positively-correlated to promoter intensity.

Stronger promoter has:
- Higher mCherry expression at plateau phase.
- Shorter timing length.
Results

Fusing EGFP to invertase N-term deteriorates its activity.

- Cre-EGFP: stable growth, obvious mcherry burst.
- EGFP-Cre: Strong expression, deteriorated activity.
Results

Ssra might slightly reduce invertase expression, but not hinder inversion activity.

- Test it for later use;
- Reduce the leakage expression.
Results – Novel mechanisms

We introduced 4 new recombinases by de novo synthesis.

- Dre-Rox
- Scre-SloxM1
- Vika-Vox
- Vcre-VloxP

Rox, Scre, Vcre, and Flpe react at different rates.

Yat-sen Star Recombinases
Tool Kit:
- 7 recombinases,
- 7 RTS,
- 6 generators,
- 6 reporters,

Wide range of usage.
Conclusion:

1. We constructed invertase module that exactly works as a timer.

2. We introduced novel recombinases by de novo synthesis, and they work properly.

3. We understood how core elements determine timing length. We can create timers with flexible length of time.
We need a key!

The key is modeling to precisely measure timing length?
1. Precisely measure the timing length

2. Timing prediction
   - Expand “1 element” to “n elements”
   - Expand “1 period module” to “n period module”

Tool

Just design your own timer!
Time interval can be observed between invertase generation and a burst of product accumulation.

MODELING

Invertase-EGFP

mCherry by reporter

RFU

Timing length = T2-T1

Avoid random fluctuations
LOGISTIC EQUATION:

\[ N = \frac{K}{\frac{K-N_0}{N_0} e^{-rt}} \]  

Green light cre EGFP = leakage + Main production part

\[ = \frac{\text{od0} \cdot \text{Gre0}}{N(t)} + kt + b = G(t) \]
**MODELING**

**Experiment data**

- RFU/OD700
- Time (h)

**Fitting curve derived from model above**

General model:
\[
f(x) = \frac{K}{1 + ((K - N_0) \cdot \exp(-r \cdot x)) / N_0}
\]

**Coefficients (with 95% confidence b)**
- \(K = 0.7487 (0.727, 0.7703)
- \(N_0 = 0.2241 (0.1822, 0.266)
- \(r = 0.7123 (0.5612, 0.8634)

**Goodness of fit**
- SSE: 0.00516
- R-square: 0.9830
- Adjusted R-square: 0.9833
- RMSE: 0.02394

**Modeling results**

- \(od \sim N(t) = 0.7487 / (1 + ((0.7487 - 0.2241) \cdot \exp(-0.7123 \cdot t)) / 0.2241))
- \(leakage \sim 73 / (0.7487 / (1 + ((0.7487 - 0.2241) \cdot \exp(-0.7123 \cdot t)) / 0.2241))

**General model**
\[
f(x) = k \cdot x + b + (73 / (0.7487 / (1 + ((C))))
\]

**Coefficients (with 95% confidence b)**
- \(b = -406.8 (-615.9, -197.8)
- \(k = 145 (126.6, 163.5)

**Goodness of fit**
- SSE: 4.322e+05
- R-square: 0.9667
- Adjusted R-square: 0.9633
- RMSE: 207.9

**Modeling results**

\(G(t) = 145 \cdot t + 406.8 + (73 / (0.7487 / (1 + ((0.7487 - 0.2241) \cdot \exp(-0.7123 \cdot t)) / 0.2241))))
MODELING

Equations:

Michaelis-Menten equation

\[ v_0 = \frac{V_{\text{max}} [S]}{K_m + [S]} \]  \hspace{1cm} (3)

\[ \int v_0 \, dt = \int \frac{V_{d_m} \cdot G_0(t)}{K_{d_m} + G_0(t)} \, dt \quad (G_0(t) = kt) \]  \hspace{1cm} (4)

\[ S = S_p(t) + P_p(t) \]  \hspace{1cm} (5)

\[ V_{i0} = \frac{V_{im} \cdot S_p(t)}{K_{im} + S_p(t)} \]  \hspace{1cm} (6)

\[ V_i = G(t) \cdot V_{i0} \]  \hspace{1cm} (7)

\[ P_p = S - S_p(t) = \int_0^t V_i(t) \, dt \]  \hspace{1cm} (8)

\[ R(t) = \int (IP_p - V_0) \cdot \frac{k - N(t)}{N(t)} \, dt \]  \hspace{1cm} (10)

\[ \Rightarrow -S_p'(t) = G(t) \cdot \frac{V_{im} \cdot S_p(t)}{K_{im} + S_p(t)} \]  \hspace{1cm} (9)
The definition of precise timing length

T1: time of $\frac{1}{2}$ max growth rate of $G(t)$

T2: time of $\frac{1}{2}$ max growth rate of $R(t)$
The prediction of timing with any element

- Cre • Yes
- Dre • No
- Flpe
- Scre
- Vcka
- Vcre
- ...
- ...

- J23100
- J23101
- J23102
- J23103
- J23014
- ...
- ...
- ...
- ...

- Loxp
- Dox
- FRT
- SloxM1
- Vox
- Vloxp
- ...
- ...
- ...
- ...
The prediction of multiple-module timer

- Change mCherry to mCherry-Invertase
- Let $R(t) = G2(t)$, we got $R2(t)$, the second timer module
- Multiple module timer
Fusion: From single to multiple

PROKARYOTIC TIMER

Circuit 1

eGFP infused invertase

P_{soc} or P_{T7-loxC}

RBS

Term

P_{soc}

RTS

RBS

mCherry

RTS

P_{soc}

FRTr

RBS

flpe

ssrA

Term

FRTr

loxPf

RBS

Cre

ssrA

Term

loxPr

GFP

Term
PROKARYOTIC TIMER

Circuit 1

Circuit 2
PROKARYOTIC TIMER

2015.04

2015.09
PROKARYOTIC TIMER

RFU = \frac{F_{test} - F_{control}}{F_{M9broth}}

Relative Fluorescence Unit (a.u.)

Time (hour)

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Eukaryotic Timer

Integrase

attB

attP

attL

attR

Int

Int+

Xis

Xis1/2/3

STATE 0

PCDK

Integrase2

Integrase

RFP

BFP

suicide gene
Eukaryotic Timer

STATE 1
Eukaryotic Timer

Integrase

Integrate

Pck

Pck

 suicide gene

STATE 2

Xis 1/2/3

Integrate
Eukaryotic Timer

Integrase

Integrase

Integrase

suicide gene

Integrate

STATE 3
Eukaryotic Timer

Modified pAUR135

Bxb1 for the first step
• Fundamental system
• Assemble different units with different target genes

Significance

- Lifetime limitation
- Periodical administration
- Safer ferment engineering
Significance

Precursors → Intermediate Protein → Final Products

- **Enzyme 1**
- **Enzyme 2**
Significance

INPUT TIME LENGTH

Promoter1 → Recombinase1 → RTS1 → Degradation tag1

OUTPUT COMBINATION
- Summer School
- 2015 iGEM Conference
- Newsletter
- SKLBC Meet Up
- Team Selection
- Biocamp
- High School Science Camp
- Freshmen Work
Safety

• Use well-developed and relatively safe strains.
• Mainly use biobricks from iGEM kit plate.
• Strictly follow the lab treatment disciplines of disposable biological equipment.
• Strictly observed lab regulations.
• Provide a idea to deal with implanting strains safety concerns.
THANKS FOR YOUR ATTENTION
Appendix
Oscillator vs Our timer

- **Repressor**
- **Stimulator**
- **Stage A**
- **Stage B**

**Damping**

- Flipping Threshold (Constant)
- Recombinase Fast Accumulates
- Recombinase Fast Degrades
4. Ligation
Use T4 ligase to ligate two fragments. Now, you have high quality and high quantity ligation products!

3. Clean-up
Use a clean-up kit to wash away the small fragments produced by digestion.

2. Digest
Use SpeI and PstI (or EcoRI and XbaI) to digest the plasmid with a shorter fragment; use XbaI, PstI and EcoRI (or EcoRI, SpeI and PstI) to digest the plasmid with a longer fragment (one more restriction enzyme is used to destroy the backbone of insertion fragment).

1. Insertion and miniPrep
Fragments are already inserted into backbones.
Excision Won’t Cause Chaos
Excision Won’t Cause Chaos
EcoRV

Simulated Agarose Gel of Different Stages of Circuit 2 Digest with EcoRV
Project Safety

How will your project work?

The purpose of our project is to build an internal clock in microbes, in order to let them perform complex self-regulating activities. We mainly used E.coli and yeast as experimental subjects. In E.coli, we produced proteins we need by plasmid transformation, while in yeasts, we integrate genes needed into the genome by gene recombination. There were different fluorescent proteins to report each gene expression. By testing the starting point and variation of their concentrations, we can infer how this system works from the fluorescent protein accumulation pattern. When the fluorescent proteins are expressed accordingly and regularly, the system was built successfully. With further development, this system may be applied to other fields.
What risks might your project pose, if it were fully developed into a real product that real people could use? What future work might you do to reduce those risks?

Our project aims at providing existing micro organism products a more self regulating control and a safer method, which can be used to deal with those safety problems lots of iGEM teams failed to answer. For example, for a currently impossible project about implanting redesigned microbes into human bodies, our system would provide it a very attractive alternative solution to its safety concerns. Certainly, our project itself has its own risks, if the self-regulating suicide genes lost control, precious strains might die. Also, this system cannot avoid the problem of genetic shift.
With those concerns, we added a reset system in the yeast cycle system, in order to prevent the system from accidentally turning on by human error or other factors. This system could save the strains, though we have not done experiments of this area. As for genetic drift, we consider that there has not been any efficient technology to solve this problem, while our system can reduce the possibility of genetic drift to a certain extent by artificially controlling bacteria’s life.

Of course, risks need good managements, so these kind of products should be properly preserved to avoid the cost of inappropriate turning on.

Laboratory Safety
What risks does your project pose at the laboratory stage? What actions are you taking to reduce those risks?

We have experiments on E.coli and yeasts at the same time, though the E.coli strain DH5a and Top10 are well-developed and relatively safe strains, treating them unscrupulously might still cause contamination to the environment, or even harm to humans. Yeasts cause less damage to human, but as fungi, they might contaminate prokaryotic culture and nearby cell culture rooms. For safety concern, we strictly acquired team members to wear properly during the experiments, which means they had to wear trousers, long sleeve tops, shoes that cover the whole feet, the lab gown and latex gloves. When using harmful reagents, team members must wear masks and operate in the fume hood.
In order to prevent E.coli and yeasts from polluting the environment, we strictly follow the lab treatment disciplines of disposable biological equipments. All used petri dishes and culture will be sent to the fixed collecting locations and go through uniform disposal. Besides, the experiment region and resting area are strictly separated. Also, we have a spare rocking bed used on yeast culturing only and placed in another room, in order to prevent yeasts contamination.