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Development of an in-vivo translation elongation reporter in yeast


A Thesis submitted in partial satisfaction of the Requirements
for the degree Master of Science


in


Chemistry


by


Wanfu Hou


Committee in charge:

    Professor Brian M. Zid, Chair
    Professor Thomas C. Hermann
    Professor Susan S. Taylor


2020

The Thesis of Wanfu Hou is approved, and it is acceptable in quality and form for publication

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<div align="right">Chair</div>

University of California San Diego

2020

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ABSTRACT OF THE THESIS

Development of an in-vivo translation elongation reporter in yeast

by

Wanfu Hou

Master of Science in Chemistry

University of California San Diego, 2020

Professor Brian M. Zid, Chair

Translational elongation plays crucial role in mediating the expression of proteins and finally affecting various biological reactions and processes in living organisms. Reversely, the efficiency and rate of translational elongation is commonly regulated by amounts of internal elements, such as special codon or sequences, and external conditions, such as different nutrient conditions. The relevant effects have been well investigated a lot based on transcriptome or proteome analysis and ribosome profiling. Specifically, previous researches commonly identified lessening of protein production and ribosomal loading, which suggest the variation of elongation efficiency or ribosome stalling derived from poor genetic elements

or worse nutrient conditions. We have found that, the existence of poly-proline sequence in nuclear-encoded mitochondrial proteins, such as ATP3 and Tim50, shows negative effects in the translation efficiency and the final products, while positive effects in mRNA localization on mitochondria. Since it is well-established that consecutive prolines will result in ribosomal stalling on mRNA, we assumed that the "stalling" may decrease the overall elongation rate, which can be measured and quantified in vivo. In addition, we also intend to monitor the translation variation of Yeast after switching from normal glucose condition to an unfamiliar condition, such different carbon source provide or even nutrient depletion. We finally realized that the translation of Yeasts is going to be impeded after alteration of nutrient supply, then great adaptation to the external "stress" is shown by organisms.

Introduction

Translation is a fundamental biological process in all living organisms, with proteins produced to carry out various cellular functions [1]. Translation is composed of several stages, including initiation, elongation, termination and ribosome recycling, which is a complicated circulating process, with message RNA carrying the genetic information, multiple factors to mediate every sub-processes and ribosome loading on mRNA as a "factory" to achieve the assembly of nascent peptides. The newly generated peptides can release after a nucleophilic attacking derived from eRF1 in eukaryotic and the left ribosomal complex will be recycled to prepare for the next circulation.

Initiation is typically thought to be the rate-limiting step of translational process, thus amounts of researches were done to investigate the mediations of translational initiation [2]. However, Elongation is also confirmed to be an important pathway to affect the translation efficiency and rate. In some cases, special codons or sequences are found to be less beneficial in peptide bond formation and result in stalling during translational elongation. For example, Proline is a typical poor amino acid both as donor or accepter during peptide formation [3]. Cryo-EM structure of ribosome processing in translational elongation with mRNA is found to be stalled when it goes to the PPP sits [4], where local resolution of peptidyltransferase center (PTC) of ribosome suggests that the existence of poly-proline destabilizes the peptidyl-tRNA loading and prevents the accommodation of new aminoacyl-tRNA; In addition, recent studies also reveal that the newly generated positive charged amino acid in nascent peptide also impedes the translational elongation because of its electrostatic interaction with the ribosomal exit tunnel [5]. With a poor codon or sequence as impediment, ribosome stalling may happen

a lot, and finally results in abortion or being rescued. Eukaryotic initiation factor 5A (eIF5A) is thought to be the key factor in alleviating ribosome stalling to avoid abortion for eukaryotes [6], similar to elongation factor P (EF-P) for bacteria. Besides, mRNA secondary structure, such as RNA pseudoknot or stem loop [7][8], and codon usage bias [9][10] are also crucial effects resulting in ribosomal pausing and mediating translational elongation.

Except for genetic elements, the metabolic condition is also an important role affecting translation efficiency and protein expression globally. Naturally, it is very common for microorganisms to encounter nutrient issues, such as, nutrient deprivation [11], less favored carbon sources provided [12][13] or switching from one type of carbon source to another, all of which should be adapted by microorganisms to maintain survival. It is well established that *Saccharomyces cerevisiae* alters expression pattern [14][15] and polysomal distribution [16] when switching between two carbon sources. Under poor nutrient conditions, such as glycerol, Ribosomal stalling is found to happen more frequently, because of the limiting of ternary complexes (TCs) [17]. Moreover, to achieve metabolic adaptation for cell survival, previous studies also identify some essential factors that mediates relevant cellular process. For example, eukaryotic elongation factor 2 kinase (eEF2K) is suggested to account for maintaining cell survival under acute nutrient deprivation by blocking translation elongation [18]. Thus, we assume that the intracellular reactions, such as the translational elongation, of microorganisms are capable of being affected by extracellular elements, such as nutrients alteration.

Mitochondrion is the cellular "factory" where carbon sources are consumed and energy is generated to drive amounts of biochemical reaction intracellularly. Thus, it is a common idea

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that the mitochondrial dynamics, such as morphology [19] or relevant proteasome [20], may alter under different nutrient supply. In 2019, a post-doctor in our lab quantified the Yeast mitochondrial morphology using mitochondrial volume fraction [21], by which studies the relationship between morphologic change and nutrients conditions. Two types of nuclear-encoded mitochondrial proteins – Tim50-type and ATP3-type, are identified according to their different behaviors in mRNA localization and protein translation. More in-depth investigations reveal that it is the existence of poly-proline that affects the translational elongation and finally makes the difference.

It is well established that translation efficiency and rate is highly influenced by various effects, including genetic elements and metabolic conditions, based on data of ribosomal profiling and transcriptome/proteasome analysis. In this thesis, we are going to measure the translational changes derived from multiple conditions, including the existence of poly-proline and metabolic switching. More importantly, we would like to develop an in-vivo reporter system to quantify translational elongation in Yeast in vivo, and this reporter system is believed to be useful in investigation in effects of more endogenous or exogenous factors on translation.

Chapter 1 Tetracycline (Tet) Inducible Expression

1.1 The basic mechanism of Tetracycline (Tet) Inducible Expression system

The Tet system, which was first developed by Manfred Gossen et al, is based on endogenous inducible promoters mediated by activation of extracellular chemical inducer. With the Tet system transformed into eukaryotic cells, scientists are able to control the initiation of specific gene expression easily by addition of tetracycline (or its analogs like doxycycline) extracellularly, which is a great favor for biological researches.
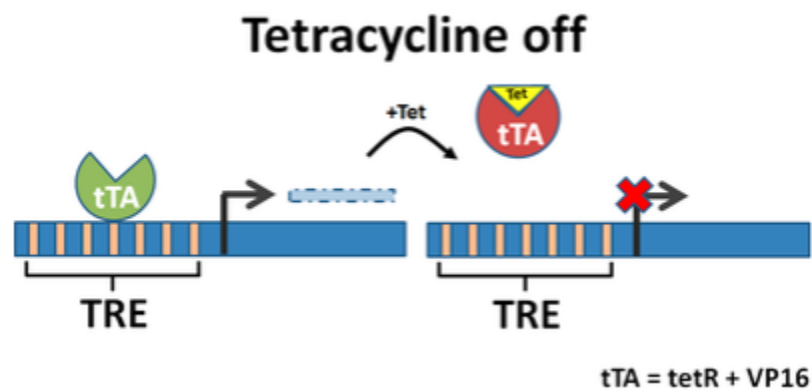


Figure 1.1.1 The mechanism of Tetracycline off (Tet off) system.
Cited from: https://www.addgene.org/collections/tetracycline/
    tTA binds with TRE sequence on the Tet off promoter, when the system is in "on" status for downstream gene expression. Tetracycline will preclude the binding and switch the system to "off" status and stops gene expression. (TRE: Tet Response Element; Tet: tetracycline; tTA: tetracycline-controlled transactivator; tetR: tetracycline repressor; VP16: virion protein 16)

The first Tet system was created in 1992 and was named as tetracycline off (Tet off) system. The inducible promoter is remained under "on" status, and is capable of being switched from "on" to "off" status after inducing of tetracycline or doxycycline. Initially, a tetracycline-controlled transactivator (tTA), which is created by fusing tetR with the C-terminal domain of VP16, recognizes and binds to Tet response element (TRE) of the promoter. Under this condition, the Tet off system is activated to get downstream gene

expression (or "on" status). The tetracycline (or doxycycline) works as regulator of gene expression, because its binding with tTA will precludes the binding between tTA and TRE, which finally inactivates the downstream gene expression (or switches to "off" status).



Figure 1.1.2 The mechanism of Tetracycline on (Tet on) system.
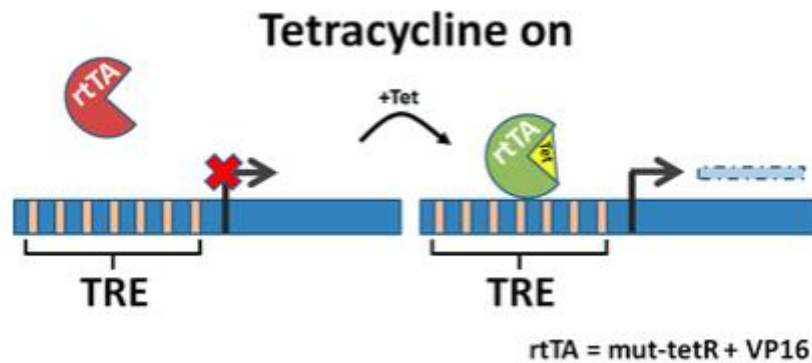Cited from: https://www.addgene.org/collections/tetracycline/
   rtTA is not going to bind with TRE sequence independently on the Tet on promoter, when the system is in "off" status for downstream gene expression. Reversely, Tetracycline will induce the binding of rtTA with TRE, which switches the system to "on" status and starts gene expression. (TRE: Tet Response Element; Tet: tetracycline; rtTA: reverse tetracycline-controlled transactivator; mut-tetR: mutated tetracycline repressor; VP16: virion protein 16)

In 1995, Manfred Gossen et al developed a second Tet system, where random mutagenesis was utilized to mutate tTA (or tetR). With mutations introduced, the tTA presents a totally reverse function, thus the new transactivator was named as reverse tetracycline-controlled transactivator (rtTA) and the new system is called Tetracycline on (Tet on) system. Without tetracycline addition, rtTA loses its binding affinity with TRE, which inactivates the initiation of gene expression (or "off" status). Reversely, the binding between tetracycline and rtTA drives rtTA to bind with TRE sequence and finally activates the Tet system and initiates gene expression (or "on" status).

The Tet on/off system is a very desirable technique in various areas of biological researches. For example, it shows amounts of advantages in gene therapy and gene transfer

experiment with combination of different types of genetic techniques, such as vectors [22][23][24] and genome editing tools [25][26]; In addition, Tet on/off system is also commonly utilized in investigation of the endogenous function of certain gene or proteins in basic researches [27][28][29]. As for my master research, the Tet on system (or rtTA system) is going to be used to control gene expression in *Saccharomyces cerevisiae*, specifically for quantification of eukaryotic translational elongation speed under various conditions.

1.2 Construction of Tet On system



## Constructed Tet on system:
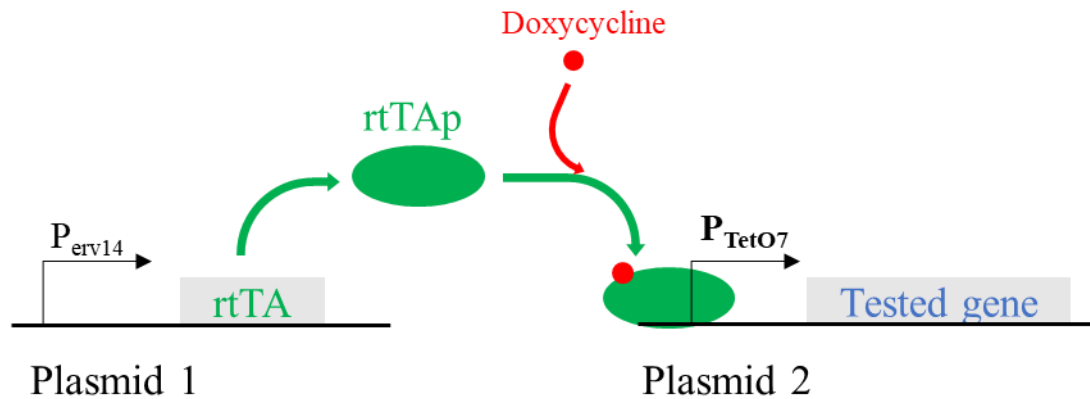
Figure 1.2 Construction of Tet On system
Two plasmids are transformed into *Saccharomyces cerevisiae*: one is responsible for expression of rtTA protein (green ellipse) and is controlled by erv14 promoter; The second plasmid is responsible for transcription of our tested genes and is controlled by TetO7 promoter.

Commonly, in an experimental design with Tet on system, two constitutive genes should be transformed into target cells (Figure 1.2). The first one is to introduce intracellular expression of rtTA protein and the expression is controlled by erv14 promoter, which is always under "activated" state. As for the second constructed plasmid, it is responsible for targeted gene expression and controlled by Tet on promoter. Before addition of doxycycline, TetO7 (or Tet On) promoter is inactivated because of the lack of rtTA binding. The addition of dox is going to drive the binding of rtTA protein to TRE sequence of TetO7 promoter, which finally activates the transcription of downstream "tested gene". With the Tet on system incorporated, we are able to control targeted gens expression easily by dox addition.

Chapter 2 Ribosomal "stalling" in poly-prolines sequence impedes translational elongation

2.1 Experimental design

In previous researches, we investigated the behavior of nuclear-encoding mitochondrial protein which we finally classified into two types (Tim50-type and ATP3-type). The Results suggest that even though the mitochondrial targeting sequence (MTS) of Tim50 and ATP3 is the reason of mRNA localization on mitochondria, it is the poly-proline in downstream coding sequence (CDS) that makes the difference. In addition, the ribosomal stalling on poly-proline sequence is well-studied, thus we assume that the existence of poly-proline is going to result in observable alteration of translational elongation in vivo.
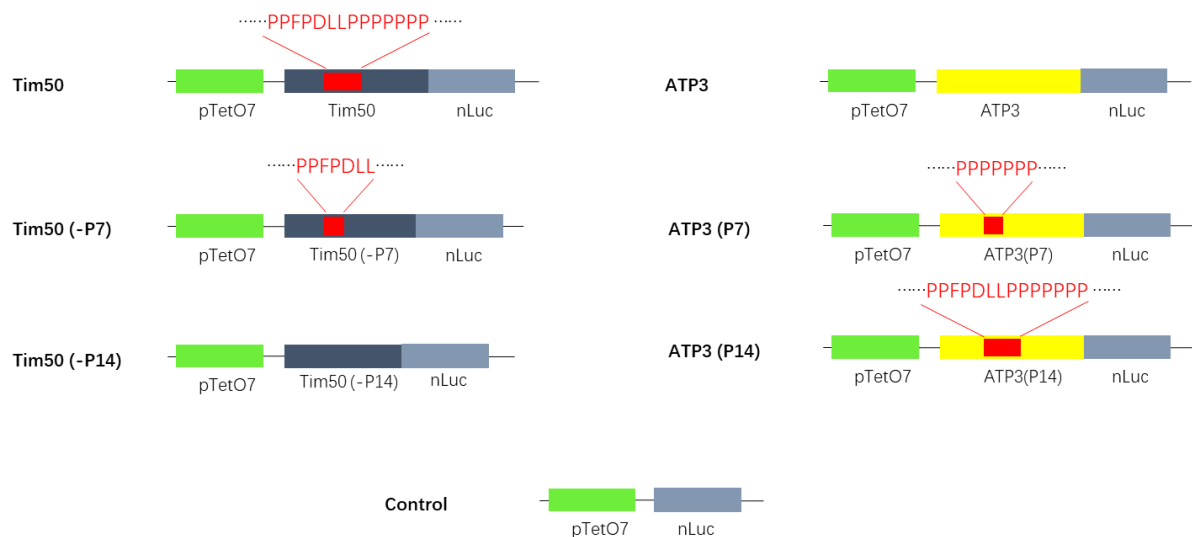


Figure 2.1: Plasmid design to investigate the effects of poly-proline.
pTetO7: truncated TetO7 promoter; nLuc: nanoLucPEST open reading frame (ORF); Tim50 5'UTR: the 5'UTR sequence of WT Tim50; Tim50: wild-type Tim50 ORF; ATP3: wild-type ATP3 ORF; (-P7) and (-P14): with 7 or 14 amino acids depletion in poly-proline sequence; (P7) and (P14): with 7 or 14 amino acids mutations to poly-proline sequence. (poly-proline in Tim50 is after 519bp from 5' terminal with length of Tim50 WT as 1428bp; poly-proline in ATP3 WT is after 300bp from 5' terminal with length of WT ATP3 as 933bp)(The consecutive poly-proline sequence is colored in red)

To explore the effect of poly-proline, we construct 7 plasmids (shown in Figure 1) which are transformed in Saccharomyces cerevisiae. These strains share the same nLucPEST gene

as a reporter, the same Tim50 5'UTR and TetOn promoter to control expression of constructed gene. The only difference among strains is the coding region, where Tim50 and ATP3 are incorporated into and fused with nLucPEST, respectively. To investigate the "stalling" effect of poly-proline, we construct three variants of Tim50 ORF, including "Tim50 wild-type", :Tim50 (-P7)" with 7 consecutive prolines deletion and "Tim50 (-P14)" with the whole poly-proline area deletion. Reversely, Three different variants of ATP3, including "wild-type ATP3", "ATP3 (P7)" and "ATP3 (P14)", are generated with sequence after 100aa(or 300bp) are mutated to poly-proline. Additionally, a control strain (named as "control") is also constructed, where there are not any Tim50 or ATP3 ORF included. Thus, by comparing the translational time/rate of strains, we are able to investigate the effects of ribosomal "stalling" on poly-proline sequence.
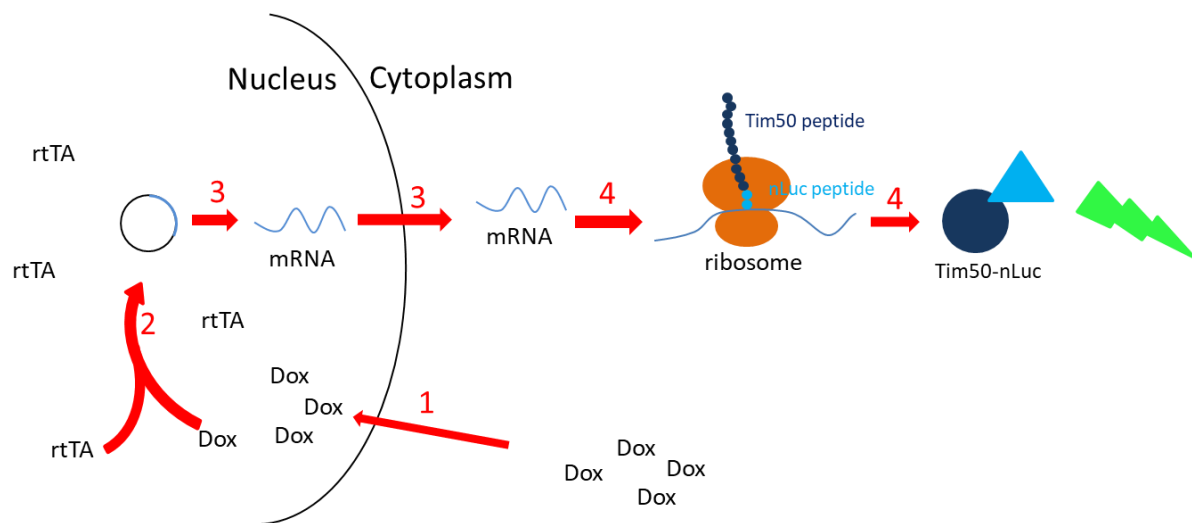


Figure 2.1.2 The intracellular reactions after dox addition to cultures.
1: doxycycline is absorbed Saccharomyces cerevisiae;
2: Tet On promoter is activated after the binding of rtTA and Dox;
3: mRNA is transcribed and transported to cytoplasm;
4: Protein is translated and secreted.

Figure 2.1.2 shows the intracellular reactions after addition of doxycycline into culture. The dox should be first absorbed by *Saccharomyces cerevisiae* into nucleus (Step1); then the

existence of doxycycline is able to drive the binding of rtTA to Tet On promoter and activate

the transcription of tested genes (Step 2); After mRNA is transcripted and transported

cytoplasm (Step 3), ribosomes in cytosol will load on mRNA and initiate translation; Only

after the tested proteins are produced and secreted, we are able to observe nLuc expression by

nLuc assay.
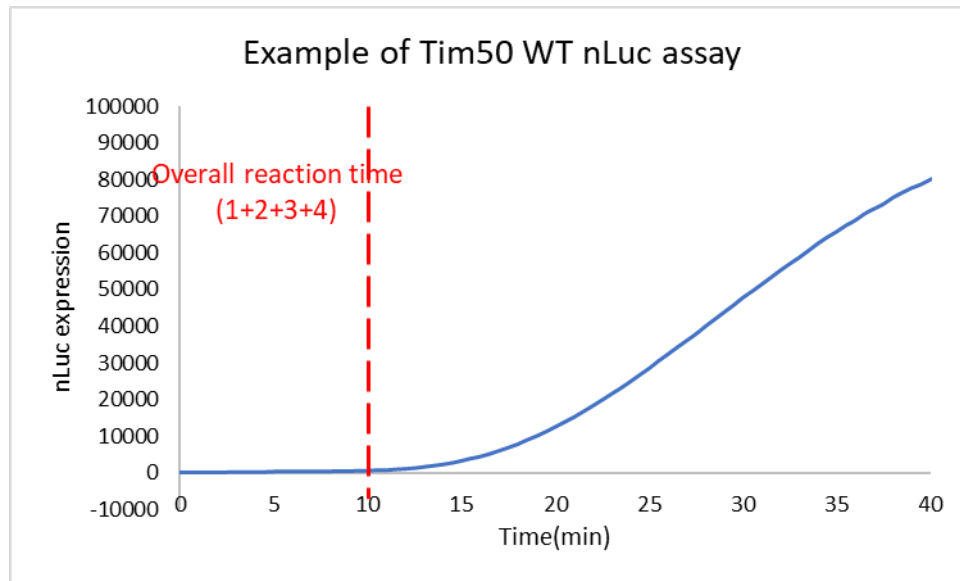


Figure 2.1.3 An example of nLuc assay.
This nLuc assay is done by Tim50 WT. The red dotted line shows the time when we observe nLuc expression,
and the time before is identified as "overall reaction time"

In the Figure 2.1.3, where I shows an example of nLuc assay, we find there is almost not

nLuc expression before 10 mins. Considering the intracellular reactions after dox addition

(Figure 2.1.2), we can easily identify that the 10 mins is when the step 1 to step 4 happens.

This time is what we are going to measure and quantify. To make thing easier, we identify it

as "overall reaction time"

## 2.2 Optimization of experimental conditions
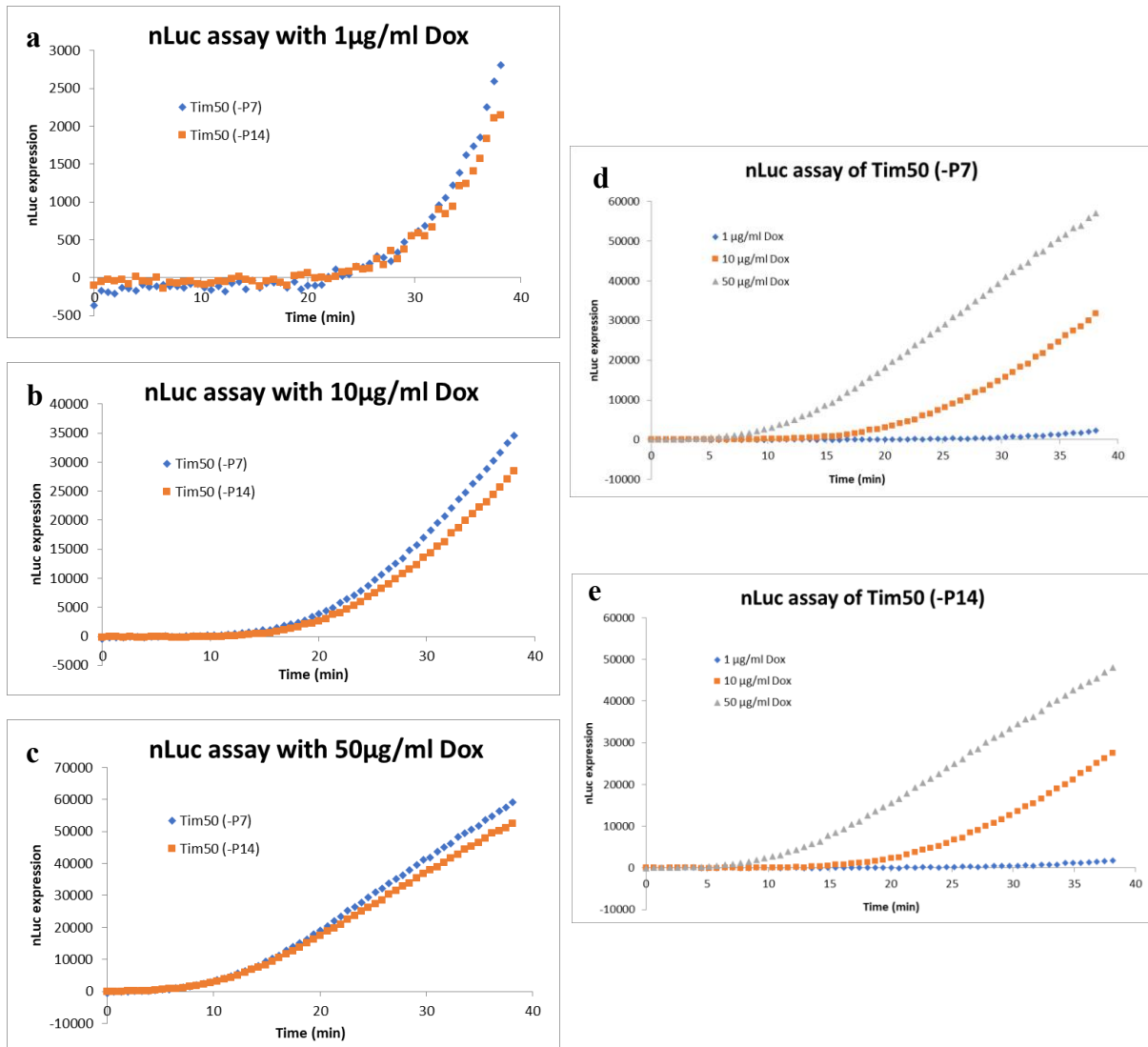


Figure 2.2.1 High concentration of doxycycline proceeds the nLuc expression.
a)   nLuc assay of Tim50 (-P7) and Tim50 (-P14) with 1 μg/ml doxycycline;
b)   nLuc assay of Tim50 (-P7) and Tim50 (-P14) with 10 μg/ml doxycycline;
c)   nLuc assay of Tim50 (-P7) and Tim50 (-P14) with 50 μg/ml doxycycline;
d)   nLuc assay of Tim50 (-P7) with 1, 10, 50 μg/ml doxycycline;
e)   nLuc assay of Tim50 (-P14) with 1, 10, 50 μg/ml doxycycline.

In the beginning of our research, we ought to identify the best experimental conditions.

What we want to optimize first is the concentration of Doxycycline, which is utilized to

activate initiation of Tet promoter with combination of rtTA. With addition of 1, 10 or 50

μg/ml Doxycycline, we run the nanoLuc assay with strains Tim50 (-P7) and Tim50 (-P14) at

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once and monitor the protein expression over time (Method). The results suggest that the concentration of doxycycline shows considerable effects on the overall reaction time (Figure 2.2 d and e), and we think the major reason of this effect is the molecular mechanism of rtTA system, where doxycycline may accumulate more and faster intracellularly under higher extracellular Dox concentration, which finally initiates the transcription earlier. We also conclude that 10μg/ml of dox is an ideal condition. When it is a very low dox concentration (e.g. 1μg/ml in Figure 2.2 a), we are able to observe a little difference between Tim50 (-P7) and Tim50 (-P14), but the nLuc expression is very small, and the deviation affects a lot for our future quantification; As for high dox concentration, such as 50 μg/ml (Figure 2.2 c). the concentration is 5 times of 10 μg/ml dox shown in Figure 2.2 b, while the final nLuc expression in Figure 2.2 c is only two-fold of the nLuc expression in Figure 2.2 b. This incompatibility means 50 μg/ml is an excess concentration and is potential to be toxic.
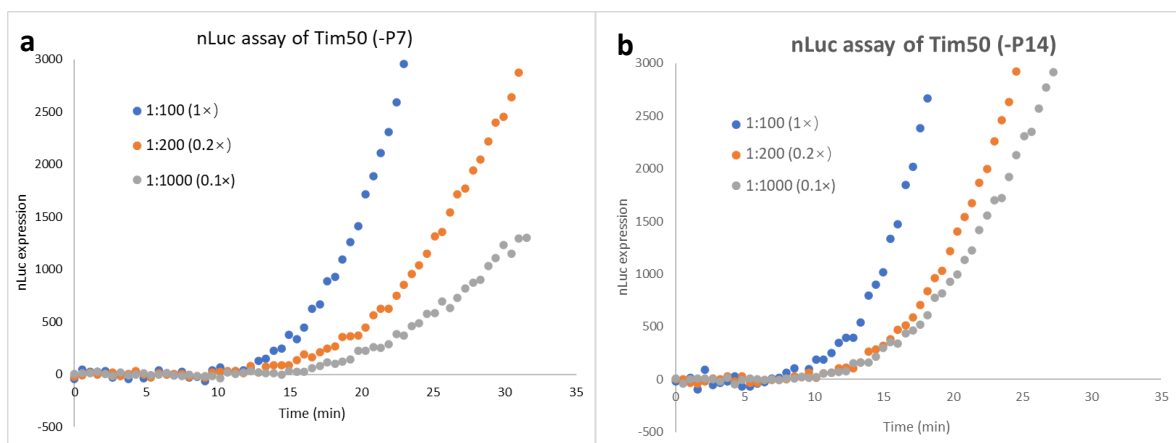


Figure 2.2.2 concentration of nLuc substrate shows little effects to translation rate. (The y-axis is enlarged to get better observation of the nLuc expression increasing)
a) nLuc assay of Tim50 (-P7) with 1:100 (1×), 1:200(0.5×), 1:1000(0.1×) nLuc substrate;
b) nLuc assay of Tim50 (-P14) with 1:100 (1×), 1:200(0.5×), 1:1000(0.1×) nLuc substrate.

The next experimental condition we want to test is the concentration of nLuc substrate. Three nLuc substrate concentrations are also tested, including 1:100 (1×), 1:200(0.5×),

1:1000(0.1×), and this experiment is also done with Tim50 (-P70) (Figure 2.2.2 a) and Tim50 (-P14) (Figure 2.2.2 b), and both of them reveal similar results. First of all, under different concentration of nLuc substrate, we are capable of observing different levels of nLuc expression, which is what we expect in the beginning. In addition, compared with dox, the overall translational rate is almost not affected by nLuc substrate, even though there is a 10-fold difference in concentration. To avoid large deviations during experimental operations, higher concentration of nLuc substrate is a better choice, thus we finally identify the 1:100 (1×) nLuc substrate as the best condition.
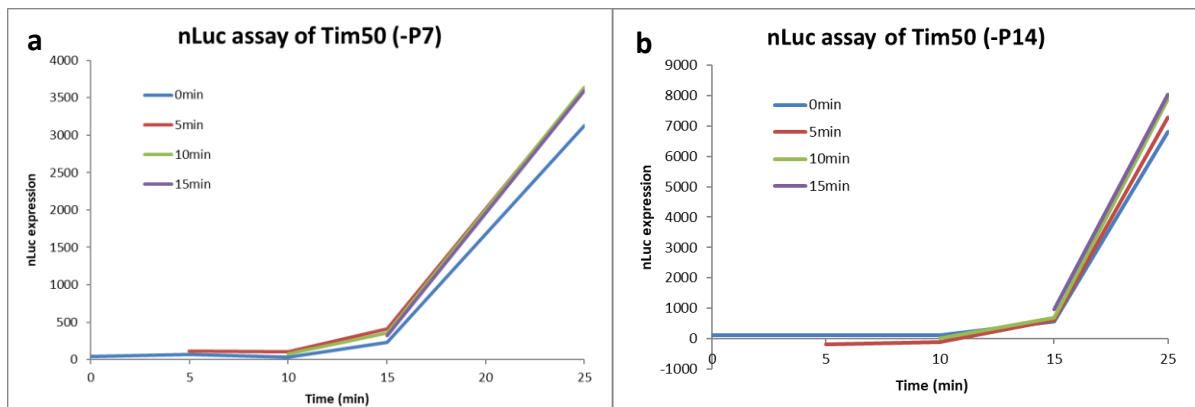


Figure 2.2.3 Complete mixing of reagents has a little effect on final protein expression.
a)   nLuc assay of Tim50 (-P7) with 0, 5, 10, 15 min re-incubation;
b)   nLuc assay of Tim50 (-P14) with 0, 5, 10, 15 min re-incubation

Another important factor that may influence the credibility of nLuc assay is the mixing of reagents, especially the doxycycline, which was confirmed to affect translation a lot. We may acquire less accurate data, once the doxycycline doesn't mix well before nLuc measurement. To solve the problem, we decide to incubate our cultures again (or a re-incubation step) for minutes after the addition of dox to activate Tet system. In Figure 2.2.3, we re-incubate our yeast samples for 0, 5, 10, 15 mins after dox addition, then take out the activated cell sample and run nLuc measurements every 5 mins. This experiment is also

done with Tim50 (-P70) (Figure 2.2.3 a) and Tim50 (-P14) (Figure 2.2.3 b) with 10 μg/ml of

dox and 1 × nLuc substrate. We finally realize that for both Tim50 (-P70) and Tim50 (-P14),

taking a second incubation for more than 5 mins shows slightly positive effect on final nLuc

expression level. In this experiment, we are not able to identify the exact "overall reaction

time" directly, while the "re-incubation" is valuable to avoid inconsistency in "overall

reaction time" Thus, we decide to take a 5 mins re-incubation after dox activation.

2.3 The poly-Proline sequence results in a "stalling" effects in translation
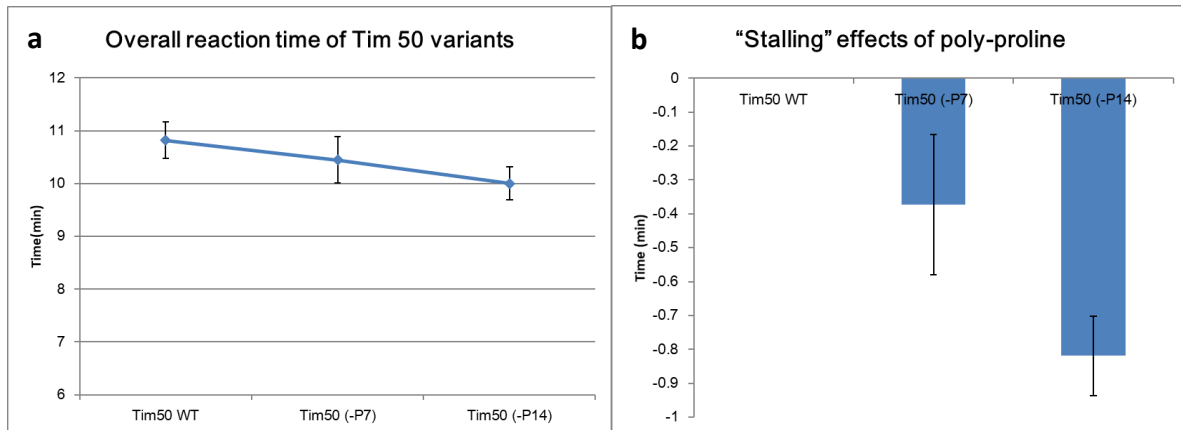


Figure 2.3.1 The existence of poly-proline has considerable effects on the translation elongation.
a)  The overall reaction time of Tim50 variants, including Tim50 WT, Tim50 (-P7) and Tim50 (-P14); (3 parallel experiments)
b)  Poly-proline stalls the translation elongation for tens of seconds. (Tim50 WT as control and subtract out its overall reaction time)

With the best nLuc assay conditions identified, we then try to investigate the effects of poly-proline sequence in translational elongation of Tim50 ORF, by comparison of Tim50 WT, Tim50 (-P7) and Tim50 (-P14). What's more, based on the Schleif Plot [30], we develop a standard protocol (Chapter 5.3) to analyze nLuc assay data and quantify the overall reaction time. Considering that there is always a deviation in our experimental protocols by hand, in Figure 2.3.1 b, we subtract the overall reaction time of Tim50 WT and study the "stalling" time derived from poly-proline.

Several conclusions can be suggested in Figure 2.3.1: first, it takes about 15mins for yeasts to express Tim50-nLuc (5mins incubation is considered); second, after depletion of poly-proline sequence, Tim50 is translated faster, which confirms our assumption that the existence of consecutive prolines causes ribosomal stalling and impedes overall translational elongation. In Figure 2.3.1 b, we can easily calculate the stalling caused by poly-proline, which is about 0.8 min for the whole sequence, but about 0.4 min for half of it, even though

there is a very large deviation. In summary, based on in-vivo measurement, we confirm that the existence of poly-proline in Tim50 coding region will cause a stalling (tens of seconds) in translational elongation process.
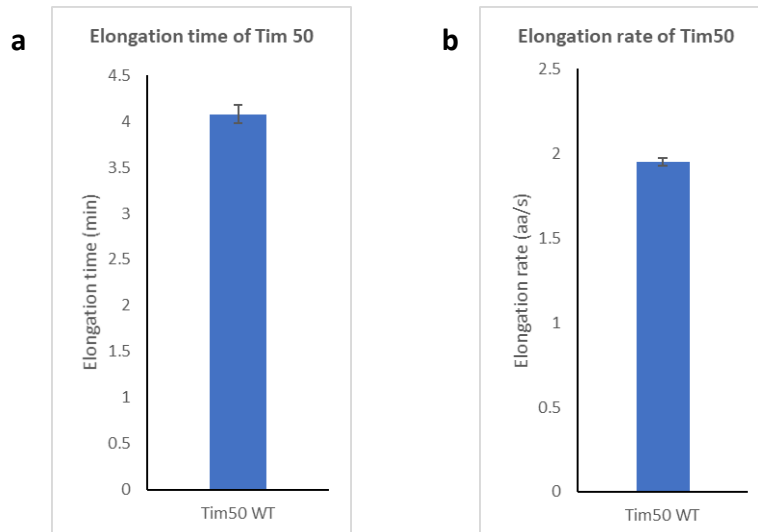


Figure 2.3.2 Translation elongation time and rate of Tim50 WT ORF. (3 parallel experiments)
a)  Translation elongation time of Tim50 WT ORF; (transcriptional elongation is subtracted.)
b)  Translation elongation rate of Tim50 WT ORF. (transcriptional elongation is subtracted.)

With the "stalling" time being quantified, we still don't know how much does translation be affected by the stalling, even though tens of second is observable in nLuc measurements. To solve the problem, we construct a control strain (Figure 2.1), with the same genetic elements except for the Tim50 ORF. Following the same experimental protocols and data analysis, we are able to quantify the elongation time of Tim50 WT ORF itself (Figure 2.3.2 a) using the formulas:

Overall reaction time of Tim50 WT – Overall reaction time of control = Transcriptional elongation time of Tim50 WT ORF + Translational elongation time of Tim50 WT peptide ·························· Formula 2.3.1

Elongation rate of Tim50 = The length of Tim50 WT ORF/Elongation time of Tim50 ORF····· Formula 2.3.2

The final translation elongation time of Tim50 WT can be calculated as about 4 mins (transcriptional elongation rate in yeast is about 2 kp/min, and length of Tim50 WT is 1428bp). Considering that the "stalling" of poly-proline in Tim50 WT is about 0.820 min,

which is 20% of the overall Tim50 WT elongation. This difference, obviously, can't be explained by the depletion of 14 amino acids (Tim50 ORF has 476 aa, the poly-proline takes up about 3 %). In addition, with translational elongation time calculated, we can also know about the overall translational elongation rate of Tim50 WT, and the final result is 2 amino acid/second.

2.4 Summary and Future prospection

Until now, we have confirmed that the existence of consecutive prolines in Tim50 ORF is going to cause extra impediment in translational elongation of Tim50, which is observable in in-vivo measurements. We assume this impediment in elongation process is caused by ribosomal stalling on consecutive prolines. The effect of "stalling" is quantified in this thesis, while the data calculated shows large deviations (Figure 2.3.1), which, in our opinions, can be alleviated with more repeats in the future.

What's more, to acquire more credible conclusion, we are planning to test the effects of poly-proline in ATP3 (plasmids construction is shown in Figure 2.1). Different from Tim50, for ATP3 where no consecutive prolines existed, we plan to introduce mutations into ATP3 WT to the same poly-proline sequence. We assume that the introduction of poly-proline is going to impede translation elongation of ATP3.

Chapter 3 Translational elongation process is affected by nutrient condition

3.1 Experimental design
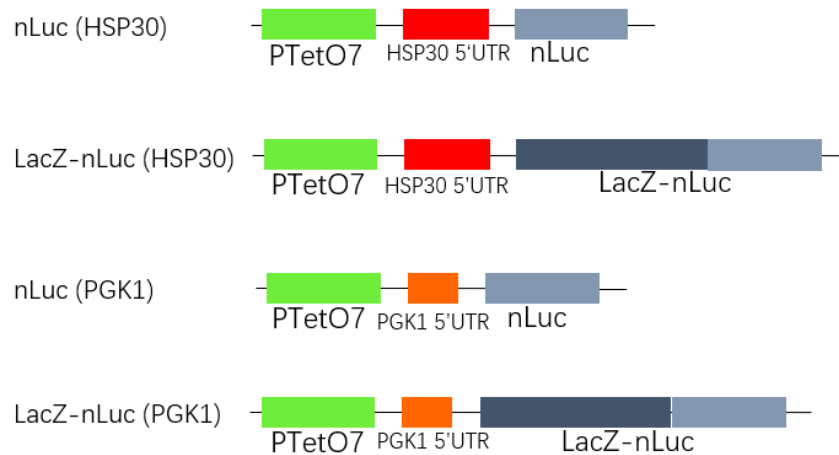


Figure 3.1: Plasmid design to investigate the effects of nutrient conditions.
pTetO7: truncated TetO7 promoter; nLuc: nanoLucPEST ORF; HSP30 5'UTR: the 5'UTR sequence of WT HSP30; PGK1 5'UTR: the 5'UTR sequence of WT PGK1; nLuc (HSP3): plasmid with HSP30 5'UTR and nLuc; LacZ-nLuc (HSP30): plasmid with HSP30 5'UTR and LacZ-nLuc; nLuc (PGK1): plasmid with PGK1 5'UTR and nLuc; LacZ-nLuc (PGK1): plasmid with PGK1 5'UTR and LacZ-nLuc;

Except for genetic elements, it is also well-established that translation process of microorganisms is also highly affected by external factor, such as switching carbon sources, and nutrient starvation. More generally, microorganisms will behave differently intracellularly under different nutrient conditions, which is also suggested in researches done by Tatsu et al, where mRNAs of Tim50 and ATP3 behave differently in localization on mitochondrial after switching between respiratory and fermentative stages with different nutrient supply. Here, instead of mitochondria protein, we would like to investigate the translation alteration of a commonly studied gene LacZ, which encodes beta-galactosidase with function in catalyzing the cleavage of lactose to form galactose and glucose.

To achieve the goal, in Figure 3.1, a TetO7 promoter is utilized to control the expression of LacZ, and the nLuc works as a reporter protein. To understand the effects of LacZ on

translation, we fuse the LacZ gene with nLuc. Considering the long sequence of LacZ (~3kb), strains with LacZ fused inside should present nLuc expression much later, which means that the difference of elongation time should be very observable. In addition, two different 5'UTR are tested at the same time to investigate the effects of 5'UTR.

## 3.2 nLuc assays of Yeast strains under switching from glucose to glycerol condition
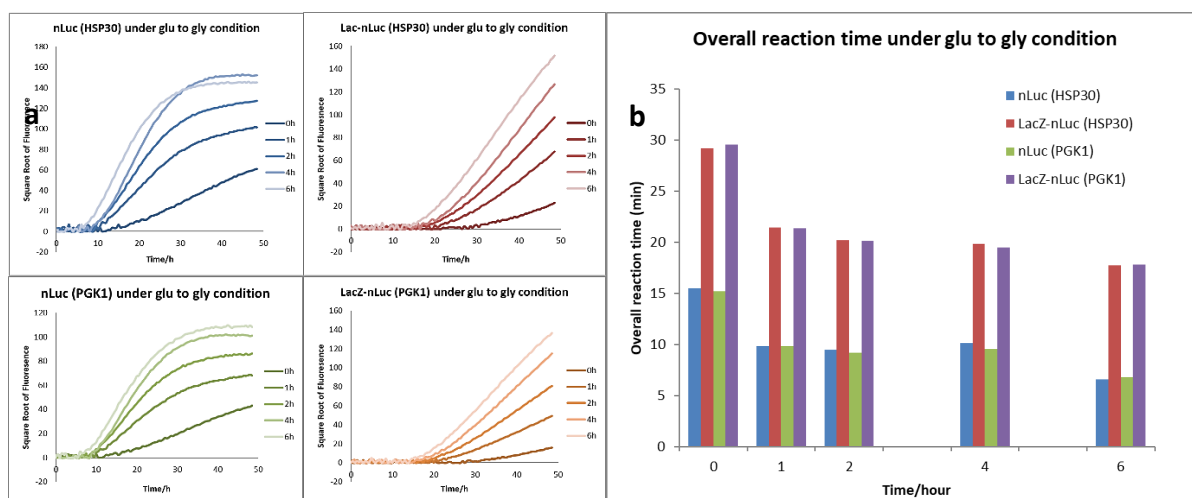


Figure 3.2 nLuc measurement after switching Yeast strains from glu to gly condition.

a) nLuc assay of Yeast strains measured at 0, 1, 2, 4, 6 hours after switching from glu to gly conditions. (Top left: nLuc (HSP30); Top right: LacZ-nLuc (HSP30); Bottom left: nLuc (PGK1); Bottom right: LacZ-nLuc (PGK1));

b) Overall reaction time of Yeast strains after switching Yeast strains from glu to gly condition.

To investigate the effects of switching nutrient supply on translation elongation, the 4 yeast strains are incubated overnight under glucose condition and kept under log phase. We then harvest the cells and switch them from glucose to glycerol condition. Then we do nLuc assays every 1h (Figure 3.2) to see if Yeast behaves differently after switching to new nutrient condition. There are several important conclusions suggested: first of all, after being switched to a new condition, all four strains present very low nLuc expression level in the beginning, and then start recovering to a higher level, which looks like an adapting process to new condition; what's more, the phenomenon of "adapting" is also reflected by the shorting of overall reaction time (Figure 3.2 b), which suggests that switching nutrient supply acts as a stress to microorganisms, whose intracellular reactions, such as translation, will be influenced a lot. After intracellular mediations, microorganisms, such as Yeast, will acquire adaptations to new extracellular environments, such as new carbon source.

## 3.3 Translational Elongation of LacZ



Figure 3.3 Quantification of translation elongation of LacZ under different nutrient conditions.
a)  Translational elongation time of LacZ under glu to gly condition;
b)  Translational elongation rate of LacZ with HSP30 5'UTR under three nutrient conditions, with 3 parallel experiments. (glu to gly condition: red columns; glu condition: red solid line; gly condition: red dotted line)
c)  Translational elongation rate of LacZ with PGK1 5'UTR under three nutrient conditions, with 3 parallel experiments. (glu to gly condition: green columns; glu condition: green solid line; gly condition: green dotted line)

To evaluate the effects of switching carbon source on translational elongation, we subtract the overall reaction time of LacZ-nLuc strain with nLuc strain. Even though other transcriptional and translational processes are also affected, we think that the same level of effects should be imposed on the same biological reaction, such as the initiation when it comes to the same promoter and 5' UTR. Based on this assumption, a series of simplified formulas can be generated:

Overall reaction time of LacZ-nLuc (HSP30) - Overall reaction time of nLuc (HSP30) = Transcriptional elongation time of LacZ-PKT sequence + Translational elongation time of LacZ-PKT peptide ⋯ Formula 3.3.1

Overall reaction time of LacZ-nLuc (PGK1) - Overall reaction time of nLuc (PGK1) = Transcriptional elongation time of LacZ-PKT sequence + Translational elongation time of LacZ-PKT peptide ⋯ Formula 3.3.2

Transcriptional elongation rate of LacZ-PKT sequence = length of LacZ-PKT sequence/Transcriptional elongation time of LacZ-PKT sequence ⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯Formula 3.3.3

Translational elongation rate of LacZ-PKT peptide = length of LacZ-PKT peptide/Translational elongation time of LacZ-PKT peptide ⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯ Formula 3.3.4

It was established that the common translational elongation rate in Yeast is about 2kb/min, and the length of LacZ-PKT sequence is 3096 bp, thus the peptide of LacZ-PKT is composed of 1032 amino acids. Based on these known data, we are able to calculate the translational elongation time/rate of LacZ and the variation over time after switching to new condition (Figure 3.3). In addition, to get better evaluation on the effects of nutrients alteration, we repeat this experiment under glucose and glycerol conditions, then compare the elongation rate of LacZ under three different nutrient conditions (Figure 3.3 b and c).

From the variation of transcriptional elongation rate of LacZ, we are able to realize some interesting behaviors of Yeast elongation under different nutrient condition. In the first place, similar to the variation of overall reaction time, the elongation time of LacZ is largest in the very beginning (0h in Figure 3.3 a), then goes down as time goes, which suggests an increasing elongation rate of LacZ (Figure 3.3 b and c); What's more, in Figure 3.3 b and c, the elongation rate of LacZ under glucose (solid lines) and glycerol (dotted lines) are shown, and we find Yeast translates slower in glycerol, which suggests to be a worse carbon source for yeast; If we compare the elongation rates under glucose to glycerol condition (columns) with glucose (solid line) and glycerol (dotted line), the Yeast is found to be stressed by alteration of carbon source, but get adaptation to new glycerol condition gradually. Finally, 5' UTR doesn't show much effects in this "adapting" process, by comparison between LacZ with HSP30 5'UTR (Figure 3.3 b) and LacZ with PGK1 5'UTR (Figure 3.3 c).

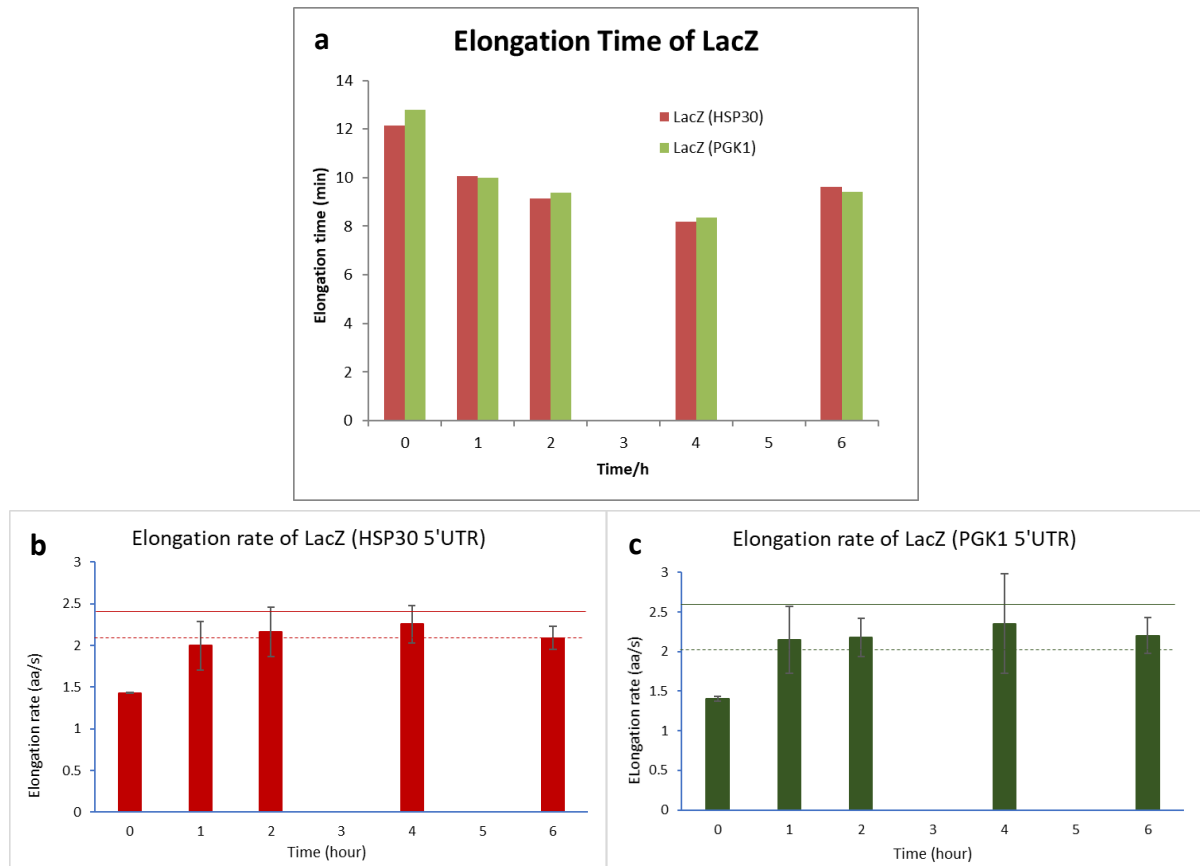## 3.4 Translational elongation time and rate of LacZ under glucose depletion condition
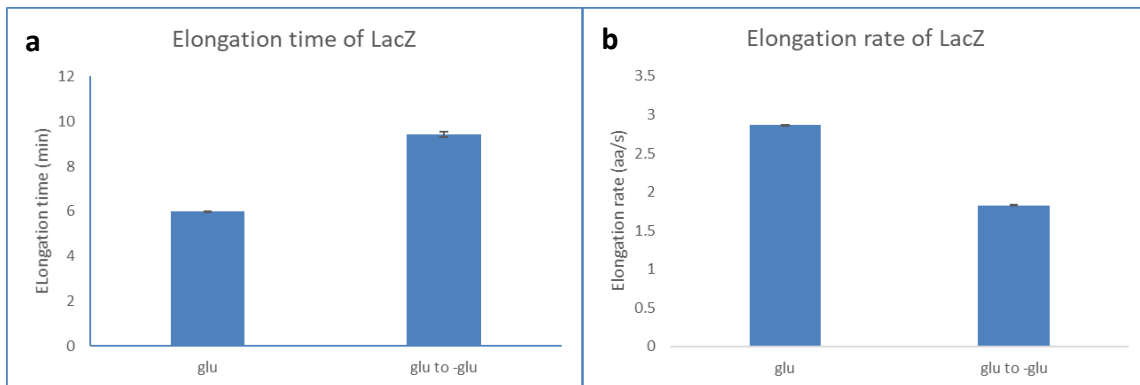


Figure 3.4 Quantification of the Translation elongation of LacZ under nutrient depletion.

a) Translation elongation time of LacZ under glucose(glu) condition and +glucose to -glucose (glu to -glu) condition;

b) Translation elongation rate of LacZ under glucose(glu) condition and +glucose to -glucose (glu to -glu) condition.

In addition to glycerol, we also study how *Saccharomyces cerevisiae* behaves under glucose deprevation (Figure 3.4). Following the protocols in chapter 3.3, we do nLuc assay with *Saccharomyces cerevisiae* under switching from glucose incubation (+glu) to glucose depletion (-glu) condition, which is compared with *Saccharomyces cerevisiae* with only glucose incubation. The final results suggest that, after switching to -glu condition, the elongation time of LacZ increases and the elongation rate decrease, which also suggests a "stress" to *Saccharomyces cerevisiae*.

3.5 Summary and future prospection

In this portion, we investigate how is the translational elongation of Yeast been affected by the alteration of nutrient condition extracellularly. We hypothesize that when *Saccharomyces cerevisiae* gets carbon source switching, such as from glu to gly (chapter 3.2), the cells get "stress" in the beginning, but they will try to get adaptation to new nutrient condition thought unclear intracellular mediations, such as the change of translation process (shown in Figure 3.3).

We are trying to examine this hypothesize under various nutrient conditions, and one condition we are working on the glucose depletion (Chapter 3.4), which shows some basic conclusions (Figure 3.4), which more relevant experiments should be completed in the future.

Chapter 4 Conclusion

In this thesis, we investigate various factors that show great impactions to the translational elongation process of *Saccharomyces cerevisiae*. In chapter 2, we do investigations in the effects of consecutive prolines in Tim50 coding region. We hypothesis that it is the poly-proline sequence that stalls the ribosomes during translation elongation process, which impedes the overall elongation rate. In addition, the decreasing of translational rate is going to affects other biological process, such as the mitochondrial localization of Tim50 mRNA and the final Tim50 expression level. Through in-vivo measurement, we are capable of observing the "stalling" effects derived from poly-proline in Tim50, even getting quantification. The quantified results suggest a considerable effect (~20%) of poly-proline on the overall translation elongation, which confirms our hypothesis. We are planning to test more nuclear-encoded mitochondrial protein, such as ATP3, to acquire more credible confirmation.

Except for poly-proline, which is an endogenous factor, we are also interested in study the alteration of Yeast translation by extracellular elements, such as the nutrient condition. In Chapter 3, we run similar in-vivo measurements to study the translation elongation change of Yeast, when it is transferred from one nutrient condition to another, such as from glucose to glycerol. We finally realize that this operation introduces a stress to Yeast, whose intracellular reactions are influenced right now, such as the decreasing translational elongation rate. While *Saccharomyces cerevisiae* is able to get adaptation to new nutrient condition in hours through some mediations intracellularly. To get more integrated comprehension of their behaviors under nutrient alteration, we ought to test other types of nutrient condition, such as glucose

depletion, which is ongoing in out lab.

Chapter 5 Materials and Methods

5.1 Yeast strains and Plasmids

The *Saccharomyces cerevisiae* strain I use in this project is called "W303-1a MATa ade2-1 can1-100 his3-11 leu2-3 trp1-1 ura3". The Plasmids I use in this research are listed on the following table:

Table 5.1 Plasmids required in thesis

| Name of Plasmids | Functions | Source |
|---|---|---|
| ZP42 pRS306-Perv14-rtTA(S2) | rtTA expression | Lab Stock |
| ZP15 pRS305-12xMS2 v4 | Backbone for plasmids construction | Lab Stock |
| ZP35 TetO7-lacZ (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/P30314.html) | PCR for TetO7 and LacZ | Lab Stock |
| ZP256 305-HSP30prUTR-nLuc-pest-(v6) 12XMS2 | PCR for nLucPEST and HSP30 5'UTR | Lab Stock |
| TTP155_TIM50p-TIM50orf-flagyoGF | PCR for Tim50 5'UTR and ORF (Tim50 variants are all from Tatsu.) | Lab Stock |
| pRS305-pTetO7-Tim50 5'UTR-Tim50-nLucPEST-12xMS2 v4 | Investigation of poly-proline | Constructed |
| pRS305-pTetO7-Tim50 5'UTR-Tim50(-P7)-nLucPEST-12xMS2 v4 | Investigation of poly-proline | Constructed |
| pRS305-pTetO7-Tim50 5'UTR-Tim50(-P14)-nLucPEST-12xMS2 v4 | Investigation of poly-proline | Constructed |
| pRS305-pTetO7-HSP30 5'UTR-nLucPEST-12xMS2 v4 | Investigation of nutrients conditions | Constructed |
| pRS305-pTetO7-HSP30 5'UTR-LacZ-PKT-nLucPEST-12xMS2 v4 | Investigation of nutrients conditions | Constructed |
| pRS305-pTetO7-PGK1 5'UTR-nLucPEST-12xMS2 v4 | Investigation of nutrients conditions | Constructed |
| pRS305-pTetO7-PGK1 5'UTR-LacZ-PKT-nLucPEST-12xMS2 v4 | Investigation of nutrients conditions | Constructed |

5.2 Culture preparations and nLuc assay

nLuc measurements should be run when *Saccharomyces cerevisiae* is under exponential stage. To avoid the effects of cell concentration, Strains that are going to study together should have similar concentrations. The exact preparation protocols are shown blow:

For stains used for poly-proline proline projects (Chaper 2.3):

1. Cell samples are cultured with glucose media until exponential stage;
2. When cultures are ready, each culture will be divided in two portions (One is named "sample"; another one is named as "blank");
3. In the "sample" culture, doxycycline (10 μg/ml) should be add in to activate the Tet on system and initiate transcription, while the "blank" is lack of dox activation;
4. Both "sample" and "blank" cultures are incubated again for 5mins (Re-incubation) to acquire better reagents mixing;
5. nLuc assay is ready to run now, with 90 μl culture 10 μl nLc substrate (1:100)

For stains used for investigation of nutrients conditions (glu to gly or +glu to -glu)

(Chaper 3):

1. Cell samples are cultured with glucose media until exponential stage;
2. Cells are harvested and switched from glucose media to new media (glycerol or -glu media);
3. When cultures are ready, each culture will be divided in two portions (One is named "sample"; another one is named as "blank");
4. In the "sample" culture, doxycycline (10 μg/ml) should be add in to activate the Tet on system and initiate transcription, while the "blank" is lack of dox activation;
5. Both "sample" and "blank" cultures are incubated again for 5mins (Re-incubation) to acquire better reagents mixing;
6. nLuc assay is ready to run now, with 90 μl culture 10 μl nLc substrate (1:100)

For stains used for investigation of nutrients conditions (glu and gly) (Chaper 3):

1. Cell samples are cultured with glucose or glycerol media until exponential stage;
2. When cultures are ready, each culture will be divided in two portions (One is named "sample"; another one is named as "blank");
3. In the "sample" culture, doxycycline (10 μg/ml) should be add in to activate the Tet on system and initiate transcription, while the "blank" is lack of dox activation;
4. Both "sample" and "blank" cultures are incubated again for 5mins (Re-incubation) to acquire better reagents mixing;
5. nLuc assay is ready to run now, with 90 μl culture 10 μl nLc substrate (1:100)

5.3 Quantification of "Overall Reaction Time"

An important direction in this research is to get quantification of translation elongation. To do that, the "overall reaction time" should be quantified first, then the translation elongation of target genes can be easily calculated.
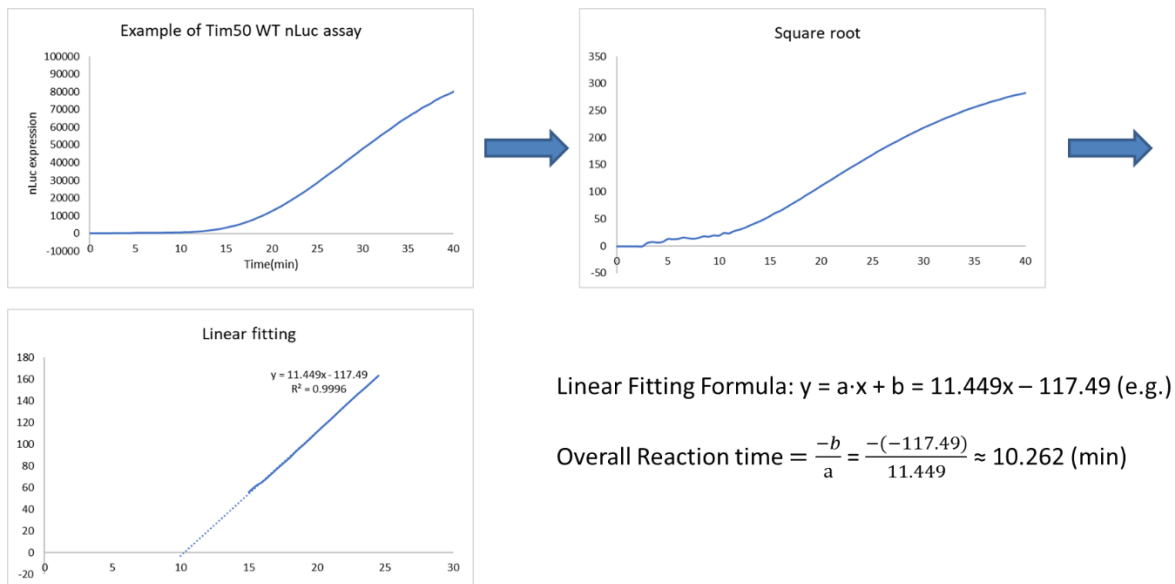


Figure 5.3.1 An example to do data analysis based on Schleif plot [30].

Figure 5.3.1 is an example to show how we do data analysis to quantify the "overall reaction time". The idea is that: nLuc expression (or protein production) is proportional to both mRNA amounts and time (t). What's more, the mRNA amount is proportional to both DNA amounts and time (t). Since DNA amounts is a constant, the nLuc expression is finally proportional to $t^2$. If we make "square root" to the raw data of nLuc assay, theoretically, the data points after "overall reaction time" are localized in a line. In Figure 5.3.1, we find that after "square root", data from 15 mins to 25 mins has wonderful linear fitting, while data after 25 mins diverges. We assume that the diverging comes from degradation of mRNA or nLuc, thus we decide to take data from 15 mins to 25 mins and do linear fitting. The x-intercept is the exact "overall reaction time" we want.
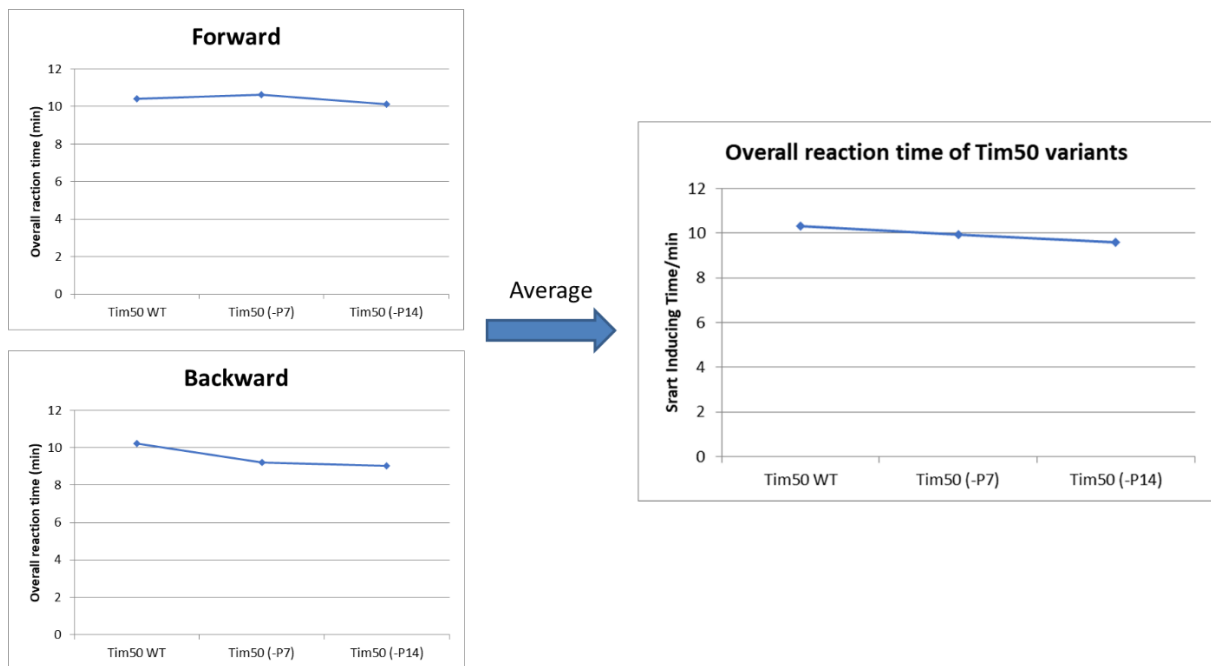
Figure 5.3.2 An example to show how much does the sequence affects

.

During nLuc measurements, since we are going to test multiple strains at the same time, there is an important factor to influence the accuracy of final quantification. That is the "sequence" to deal with strains, including the sequence we add reagents (doxycycline and nLuc substrate) and the sequence to measure the luciferase. In Figure 5.3.2, we run nLuc assays with Tim50 WT, Tim50 (-P7) and Tim50 (-P14) together and we run both "forward" (from Tim50 WT to Tim50 (-P14)) and "backward" (from Tim50 (-P14) to Tim50 WT) direction. It looks like the deviation derived from "sequence" is less than 1 min, while affects a lot in investigation of elongation. To solve the problem, for every trial, we run in both "forward" and "backward" directions and finally average them (Figure 5.3.2).

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