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Single-molecule FRET Studies on Factor-dependent Transcription Termination

인자 의존적 전사 종결 과정에 대한 단일분자 FRET 연구

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서울대학교 자연과학대학 대학원
협동과정 생물물리 및 화학생물학 전공

송 은 호

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지도 교수 홍 성 철

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송 은 호

송은호의 이학석사 학위논문을 인준함

2019 년 1 월

위 원 장 윤 태 영 (인)

부위원장 홍 성 철 (인)

위 원 이 남 기 (인)

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Graduate School of Nature Science
Seoul National University
Biophysics and Chemical Biology Major

Eunho Song

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Sungchul Hohng

Submitting a master's thesis of Public Administration

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Graduate School of Nature Science
Seoul National University
Biophysics and Chemical Biology Major

Eunho Song

Confirming the master's thesis written by Eunho Song

January 2019

Chair	Tae-Young Yoon	(Seal)
Vice Chair	Sungchul Hohng	(Seal)
Examiner	Nam Ki Lee	(Seal)

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Research Advisor **: Professor Sungchul Hohng**

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Graduate School of Nature Science
Seoul National University
Biophysics and Chemical Biology Major

Abstract

Single-molecule FRET Studies on Factor-dependent Transcription Termination

Eunho Song
Graduate School of Natural Science
Seoul National University
Interdisciplinary Program in Biophysics and Chemical Biology
Biophysics and Chemical Biology Major

Living organism has to survive under limited-resource condition with the consequent result that gene expressions in the organism have evolved to be more exquisitely controlled. Transcription termination is one of the mechanism involved in the gene expression. Compared to other part of transcription procedure, such as *initiation* and *elongation*, transcription termination is yet to be elucidated. In the case of microorganism, fortunately, many scientists have carried out studies on the mechanism of transcription termination. As a result, we have already known that there are two mechanisms in transcription termination, *intrinsic termination* and *factor-dependent termination*, by necessity of specific factor.

Intrinsic termination happens in specific sequence, so we have already known how it happen and what sequences it causes. On the other hand, factor-dependent termination, there are disputes concerning suggested mechanisms, even though we identified factor Rho as the factor causing this termination. One of them is contradiction between *pausing site* as essential component and torpedo model. If we accept the torpedo model, we thought factor Rho translocate faster than RNA polymerase, which means pausing site is not essential.

It's because of ignorance of the factor's role in Elongation complex during the transcription that there is dispute over the core mechanism of the factor despite of it being almost 40 years since identifying which the factors involved in factor-dependent transcription. In other words, knowing the factor-dependent transcription requires to understand whether there is only procedure for factor Rho's binding to *rut* site (Rho utilizing site) in RNA or there is another procedure for factor Rho's binding to RNA polymerase.

To solve the several problems of factor-dependent transcription, we designed *in vitro* transcription termination assay for the first time to image the transcription both temporally in real time and spatially in single molecule scale using single molecule FRET. By using this method, transcription procedure was observed in targeted situation.

We investigated several new factors by analyzing the data such as termination efficiency or several dynamics using this method. Firstly, we found there are two aspects in factor-dependent transcription, and these aspects are related to the RNA polymerase's remaining on DNA. Especially, whether RNA polymerase remains on DNA is effected by factor Rho's concentration, existence of freely diffusing factor Rho, and magnesium concentration for first time.

Moreover, we found that factor Rho binds to RNA polymerase with high affinity, and that factor Rho bound to the RNA polymerase cause the transcription termination. This termination is more sensitive to *rut* site or pausing site than the termination caused by factor Rho which is not bound to RNA polymerase. Based on these facts, we explained how the interaction between RNA polymerase and factor Rho takes role in transcription termination.

Finally, we observed transcription in the absence of factor Rho's binding to RNA polymerase using factor Rho mutant which lost function of termination and fluorescence dye labeled factor Rho which lost function of binding to RNA polymerase. Thus, we observed two different pathways proposed in previous studies. Furthermore, we found emergent properties of factor Rho that there is synergic effect when two different pathways of termination happen at once.

Keyword: Single-molecule fluorescence spectroscopy, Fluorescence Resonance Energy Transfer(FRET), Factor-dependent Transcription Termination, factor Rho, *mgt*A(Magnesium transport A) riboswitch, *rut*(Rho utilizing) site
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Chapter 1

Introduction

1.1. Transcription

1.1.1. Overview

Surviving in the limited resources, living organism with sophisticated gene regulation tends to be chosen in the long evolutionary process. After knowing some chemicals, like Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA), are the essential material of the heredity (Watson et al., 1953), many biological study have accumulated the knowledge of the way how this genetic information is delivered to the another generation and is utilized in each organism. These studies have filled the blank in the central dogma in the molecular biology, and sometimes added the unprecedented arrow in the diagram.

It's about seven decades that have passed since we discovered what store and deliver the genetic information. Although we have elegant model for explaining how the genetic code is passed down, there are still many question yet to be answered. This is because the accepted model suggested in the biological problem, like any other field of science, is the best interpretation for the given observation. Although the observation we got is still blur to exact understanding, it's more meaningful to make some effort to make the best interpretation than just to hesitate to do something.

Discovering some new technique which open the window of measurement for biological phenomena wide, we can make more clear interpretation. For instance, the X-ray diffraction data made by (Franklin et al., 1953; Wilkins et al., 1953) ends up the controversy in the structure of DNA. Like this, the work described in this thesis also the one of which try to make the existing knowledge more obvious. The method to open the window is the single-molecule FRET which have shed light on the biological phenomena happened in spatially single molecule scale and temporally about microsecond. Using this utile technique, we try to explain the transcription, the mechanism of synthesizing the RNA which is one of the chemical for delivering and storing genetic information. In detail, we try to explain how the external factor involved in termination of this procedure.

1.1.2. Transcription

Transcription is the mechanism in which a particular segment of DNA is copied into RNA (especially mRNA) by the RNA polymerase. In this reason, many thought that gene expression is start from transcription, as the replication can be considered as the step for storing the genomic information.

As the transcription is chemically synthesizing nucleic acid, it is sometimes compared with the replication which is also the procedure including synthesizing another type of nucleic acid. To understand the mechanism of transcription, it's useful to make comparison between transcription and replication. The difference between two procedure results from the objective of the material which is made from each procedure.

Conventionally, DNA the products from the replication is thought to store the genetic information, and RNA the product from transcription is considered as the material for the delivery of the genetic information. This convention had been accepted naturally because DNA is used for transcribing and stored in stable to be passed down to the next generation, but RNA is used for temporary translation only to be degraded, before the opposite case, like reverse transcription, and other functions of RNA are reported. (Watson et al., 2014)

To transcribe a gene, RNA polymerase proceeds through a series of well-defined steps categorized into three phases: initiation, elongation, and termination. (Watson et al., 2014) **Initiation** begin with RNA polymerase binding to the **promoter** where the DNA sequence that initially binds the RNA

polymerase, together with any initiation factors requires. The promoter-polymerase complex transformed into the structure which is required for initiation to proceed. The DNA around the point in which transcription will begins unwinds with the bae pairs disrupted only to produce a 'transcription bubble' of single-strand DNA. Transcription always occurs in a 5'-to-3' direction: the new ribonucleotide added to the 3' end of the growing chain. In the case of transcription, only one of the DNA strands as a template on which the RMA strand is built, and we called the DNA strands **template DNA** because of the role it has. Regarding the RNA polymerase only binding to the promoter, it is readily accepted always the same strand is transcribed. Moreover, the choice of promoter is thought to determine which stretch of DNA is transcribed and is the most common step which regulation is imposed at. (Watson et al., 2014)

The RNA polymerase has synthesized a short stretch of RNA (~10 bases), followed by shifting into the elongation phase. **Elongation** is the phase when the enzyme performs an impressive range of tasks in addition to the catalysis of RNA synthesis. RNA polymerase unwinds the DNA in front and reanneal it behind, and dissociates the growing RNA chain from the template as it moves along, performing proofreading functions. (Watson et al., 2014)

As all the mechanism done in the bio-system, transcription also is terminated after all the conduction is done. Once the RNA polymerase has transcribed the length of gene or genes, it must stop and is followed by releasing the transcript, RNA product, and dissociating itself from the template DNA.

This step is called **termination**. (Watson et al., 2014)

1.1.3. Termination

In the act, the actor not only should appear at appropriate time but also disappear in time. Like actor in act, chemical in the chemical reaction should be removed in suitable time as well as work in right time. To maintain gene stability, there are many mechanisms to get rid of the factors that exists when they are not expected to be. Transcription, like other steps of gene expression, has some mechanisms to remove needless factor.

RNA as the products of transcription become something to be removed when they are stalled at the template DNA, which happens when an elongation RNA polymerase becomes arrested and ceases transcribing or when it meets the pausing site in the template DNA. These remained elongation RNA polymerases can threat to gene stability, because they can hinder another RNA polymerase from using and so on.

There are some factors to deal with these remained products. One of them is TRCF (transcription-repair coupling factor), also called Mfd protein (mutation frequency decline-named for its activity to reduce mutagenesis through enhanced repair) (Alexandra et al., 2012), which removes polymerase and recruits repair enzyme. (Watson, 2014; Proshkin et al., 2016) This single protein, with ATPase activity, binds doub le-strand DNA upstream of the polymerase and translocate along the DNA using the ATPase motor until it encounters the stalled RNA polymerase. After touching the arrested RNA

polymerase, TRCF pushes polymerase forward, either allowing it to restart elongation or, more often, causing dissociation of the elongation complex, which is composed of template DNA, RNA transcript and RNA polymerase. This enzyme terminates transcription, which can make way for repair enzymes and for another RNA polymerase. (Watson, 2014)

The mechanism of TRCF is more like repair mechanism than ordinary termination, in the respect of its triggering by damaged DNA or by other unanticipated hindrances. Without any damage, termination should happen after transcription is complete, or termination is a normal and important function at the end of genes. There, sequences called **terminators** trigger the elongating polymerase to dissociate from the DNA and release the ENA chain it has made. In common, transcription come in two types: factor-dependent and intrinsic depends on the specific factor's involvement.

Factor-dependent transcription termination, as its name indicates, requires the specific factor to terminate the ongoing transcription. Historically, the concept of the factor-dependent transcription termination was suggested after the discovery of the factor releasing the RNA from template DNA in 1969. (Roberts, 1969). Several studies followed this discovery of first RNA-releasing factor.

(Roberts, 1969) named the first factor involved in the transcription termination ***factor Rho***, after the word 'release'. According to recent report (Peters et al., 2009), factor Rho is likely to take part in 20% of the total mRNA 3'-end formation in *Escherichia coli*, which used to be thought 50% estimate

that is often cited (Zhu et al., 1998), but it doesn't even found in the eukaryote. In recent, other transcription termination factor have been reported, and **Sen1** in *Saccharomyces cerevisiae* (Hannah et al., 2018).

Involvement of the specific factor is the key characteristic of the factor-dependent transcription termination compared with the **intrinsic transcription termination** which request not the specific factor but the specific sequence in template DNA, consists of two sequence elements: a short inverted repeat (of ~ 20 nucleotides) followed by a stretch of about eight A:T base pairs.

1.2. Factor Rho

1.2.1. Structure

Many studies during the 1980s and 1990s revealed that there are different functional domains of factor Rho. (Mitra et al., 2017) This factor compose oligomer (Finger et al., 1987) in various states, including dimer, tetramer, hexamer, and even higher depending on protein concentration, the ionic environment, and the presence of cofactors. (Geiselmann et al., 1992(1); Geiselmann et al., 1992(2)) Among the several states, the homo-hexameric form was found to be the principle state in the presence of the cofactor ATP. (Geiselmann et al., 1992(1))

```
MNLTELKNTP VSELITLGEN MGLENLARMR KQDIIFAILK QHAKSGEDIF
GDGVLEILQD GFGFLRSADS SYLAGPDDIY VSPSQIRRFN LRTGDTISGK 100
IRPPKEGERY FALLKVNEVN FDKPENARNK ILFENLTPLH ANSRLRMERG
NGSTEDLTAR VLDLASPIGR GQRGLIVAPP KAGKTMLLQN IAQSIAYNHP 200
DCVLMVLLID ERPEEVTEMQ RLVKGEVVAS TFDEPASRHV QVAEMVIEKA
KRLVEHKKDV IILLDSITRL ARAYNTVVPA SGKVLTGGVD ANALHRPKRF 300
FGAARNVEEG GSLTIIATAL IDTGSKMDEV IYEEFKGTGN MELHLSRKIA
EKRVFPAIDY NRSGTRKEEL LTTQEELQKM WILRKIIHPM GEIDAMEFLI 400
NKLAMTKTND DFFEMMKRS (419 a.a.)

22-116      Primary RNA binding site
179-183     ATP binding and hydrolysis (P-loop)
278-290     Secondary RNA binding site (Q-loop)
322-326     Secondary RNA binding site (R-loop)
```

Figure 1-2-1. Amino acid sequence of factor Rho

Monomer of factor Rho consists of 419 amino acids in known sequence. (**Figure 1-2-1**) The **primary RNA-binding site (PBS)** within the N-terminal domain (NTD) is encoded by amino acids 22-116). The ATP-binding and hydrolysis signature (Walker A and B) motifs within the C-terminal domain (CTD) are encoded by amino acids 179-183. (Dombroski et al., 1988) This region is called P-loop, which bears significant sequence homology to F1 ATPase and other nucleotide-binding proteins. (Dombroski et al., 1988; Dombroski et al., 1990) The **secondary RNA-binding site (SBS)** is formed by CTD and consists of a Q-loop (amino acids 278-290) and an R-loop (amino acids 322-326). (Mitra et al., 2017)

In recent, several crystal structure of factor Rho in complex with RNA and ATP analogs were solved. (Skordalakes et al., 2003; Skordalakes et al., 2006; Thomsen et al., 2009) The PBS of factor Rho, which touches to the RNA

first, consists of the oligonucleotide/oligosaccharide-binding (OB) fold, which can bind two pyrimidine bases (C, U), preferentially cytosine (C), but not purine (A, G). (Bogden, 1999) This is the structural basis of pyrimidine-rich *rut* (rho utilizing) site recognition by factor Rho (Galluppi et al., 1980; Richardson et al.1992). (Mitra et al., 2017) The crystal structure further proved that the P-loop is involved in ATP binding and Q-loop and R-loop are responsible for RNA binding.

The presence of RNA within the central channel containing the SBS induces factor Rho to adopt an asymmetric ring conformation (Boudvillain et al., 2013; Peters et al., 2011). RNA engagement at the SBS triggers ATP binding and hydrolysis, which culminate in 5' → 3' translocation of factor Rho along the RNA. The ATP-binding pockets are located at the C-terminal interface between adjacent subunits of factor Rho hexamer. In spite of all six pockets have the ability to bind nucleotide, studies indicated that factor Rho hexamer binds only three ATP or RNA molecules at the alternate sites owing to negative cooperativity (Geiselmann et al., 1992(3)); Stitt et al., (1998)). This implies that interaction between factor Rho submits may allow such alternate asymmetric functional states in each monomers (Geiselmann et al., 1992(3)); Modrak et al., 1994). (Mitra et al., 2017)

1.2.2. Rho terminator

To release the RNA from the template DNA, factor Rho needs interaction with the elongation complex spatially and some periods of the interacting temporally. To achieve the precise mechanism of Rho terminator, transcription terminator caused by factor Rho, there are some sequence components. As there being a variety, sequence components of the Rho terminator are different from those of the intrinsic terminator which has fewer variation in sequence composition.

Factor Rho's spatial and temporal requirement correspond to the existence of the factor Rho binding sites and pausing sites respectively. The former spatial requirement is ***rut* site**, which stands for Rho utilizing site. This site makes the factor Rho bind to the nascent RNA. Rho binding procedure starts from the primary binding which is the interaction between the factor Rho's **primary binding site (PBS)** and C-rich sites in the RNA. In recent, it is reported that anti-termination factor NusG triggers an allosteric relay in factor Rho to promote activiy on non-ideal substrates. (Lawson et al., 2018)

Interaction between the factor Rho and RNA can confirm the existence of the *rut* site. As there are several techniques for observing the interaction between bio-molecule, we can design several experiment to validate the *rut* site. For instance, Fluorescent Anisotropy, one of the technique for observing the interaction between bio-molecule, can provide us the position where the *rut* site is, by just measuring the fluorescence anisotropy between various fragment of RNA and factor Rho. We've already known that there can one or several *rut* site in one terminator, and there can be difference in the strength between *rut*

sites, which is thought to be decided by the sequence itself and its context.

The later temporal requirement is **pausing site** where the RNA polymerase stalls and takes time to reinitiate the elongation. Pausing site's hindrance to the elongation can bring factor Rho to catch up the elongation complex. Many scientists consider some pausing sites as **termination site** where the release of the nascent RNA happens.

The results of the gel electrophoresis experiment make it possible to assign pausing site and termination site by using the results of the gel electrophoresis experiment. After *in vitro* transcription termination assay, pulsed-field gel electrophoresis can show the length of the transcript as a result of the transcription. By measuring these length, scientists can assume the position where the termination occurred. Like *rut* site, the number of the pausing site or termination site is different depending on terminator. Besides the strength of the pausing site also different even in the same terminator.

Although there are some well-established experiments for assigning the sequence required for Rho terminators, there are arguments over the necessity of these sequence. Recent study, for instance, reported that the anti-termination NusG can make factor Rho bind to the RNA even in the case in the absence of *rut* sit in nascent RNA. (Lawson et al., 2018) Some alternative model for factor Rho binding suggest that the pausing site is optional. (Epshtein et al., 2010)

Besides, the sequences of the main components in the Rho terminator is too various to set rules. It is this absence of the understanding in the sequence that make it difficult to conduct the study of bioinformatics. (Peters et al., 2011)

Intrinsic terminators consist of an RNA hairpin followed by a U tract, which can be easily discovered in the full genome, compared to Rho terminator. Discovering Rho terminators is a slow process, with new examples largely being identified on a case-by-case basis only after isolation of suppressor mutations in factor Rho or when transcription regulation is observed without accompanying intrinsic termination signals (Stewart et al., 1985; Bossi et al., 2012). (Kriner et al., 2016)

It is reported that high-throughput techniques have led to a significantly improved picture of where in the genome factor Rho acts. (Kriner et al., 2016) For instance, measurement of change in mRNA abundance (Peters et al., 2012; Cardinale et al., 2008) and RNA polymerase occupancy (Peters et al., 2009) upon treatment with factor Rho-specific inhibitor BCM (bicyclomycin) identified transcripts and genomic locations, respectively, of BCM sensitivity with high resolution.

1.2.3. Phylogenic Traits

Factor Rho is a versatile protein, which is involved in transcription termination in bacteria (Robers, 1969) as well as suppression of antisense transcription (Peters et al., 2012), prevention of synthesis of aberrant and/or unnecessary transcripts when translation is impaired (Richardson, 1991), and resolving conflicts between transcription and replication machineries (Washburn et al., 2010). (Kriner et al., 2016) In this section, we will discuss the several functions of factor Rho.

To understand the function protein, it is useful to understand phylogenetic attribution which give us the correlation with other similar proteins. Phylogenetically, factor Rho belongs to the RecA like NTPase super family, which is characterized by NTP binding domain of F1 and V1H+ ATPase. (Sharmistha Banerjee et al., 2006) This family includes DnaB and related helicases, bacterial RecA and related eukaryotic and archeal recombinases.

Genes with sequences related to factor Rho are wide spread even amongst the most diverged organisms suggesting a common ancestor for the bacterial, archeal and eukaryotic factor Rho homologues. (Sharmistha Banerjee et al., 2006) N-terminal, the domain for RNA binding, among factor Rho and its homologs is more variable than its ATP binding domain. The RNA binding domain of the GC-rich gram-positive bacteria as compared to *Escherichia coli* has long insertions of up to 260 residues. Theses insertions are highly diverse in nature. (Nowatzke et al., 1996) It is reported that factor Rho in *Micrococcus luteus* could terminate transcription along λ-cro DNA with *E. coli* RNA polymerase at sites that are not accessible to factor Rho in *E. coli*, which means the possibility that these inclusions in RNA binding domain help factor Rho to bind to structured GC-rich sequence in these organisms. (Nowatzke et al., 1996)

The ATP binding domain is the most conserved segment. (Sharmistha Banerjee et al., 2006) The ATPaseA and ATPaseB motifs have blocks of conserved residues and are very similar to parts conserved in F1 ATPase β subunits. Within the ATP binding domain, though, there exist blocks specific to

factor Rho and not to any other members of RecA superfamily, which means theses residues are specific to factor Rho's function and therefore have been conserved exclusively among factor Rho molecules by strong selection during evolutionary process. (Opperman, T. et al., 1994)

Despite of belong to the RNA-DNA helicase, factor Rho is not that similar to other RNA helicase. (Sharmistha Banerjee et al., 2006) DEAD box that is the main characteristic of RNA helicase (Parick Linder et al., 1989) has no homology in factor Rho, which indicate that factor Rho is more diverse from RNA helicase than F1 ATPase. (Opperman, T. et al., 1994)

The factor Rho's closer resemblance to proton pumps proposes there should be another roles of factor Rho, such as building a protein gradient across membrane or acting like a proton driven rotatory molecule device like those of flagellated prokaryotes. (Sharmistha Banerjee et al., 2006) In spite of there being Rho homologues throughout bacterial world most of them have not been characterized for transcription terminator like factor Rho.

Although factor Rho is so conserved across the prokaryote, *rut* sites are highly deteriorated and unconserved. (Sharmistha Banerjee et al., 2006) Many assumed that only common feature of the *rut* sites among different Rho terminator is rich in cytosine residues. Random nucleotide substitutions in the *rut*A site of λtR1 showed that Rho is able to recognize a wide variety of similar *rut* site sequences. (Graham, J. E, 2004) These study show that an artificial terminator sequence of about 30 bp encoding (rUrC)n sequnce on the transcript

is sufficient to the formation of Rho terminator at a downstream non-terminator site. (Guerin, M et al., 1998)

1.3. Single-Molecule FRET

1.3.1. Fluorescence

Knowing the existence of something means we detect some signals come from the object. The physical meaning of flashing a light to see is almost same as that of throwing a ball to the wall, as in the both of the cases there is some aims of getting response from the object smashed by photon, as the part of light, or the ball. Without something come from object, it's impossible for us to see it. In other words, if we want to see something in the absence of the property that return the signal they received, we need to set up intentionally attribution of response to our input signal on what we are try to detect. Some of the molecules we tried to see don't have the characteristics we expected.

Using luminescence is one of the way to give property of giving back. **Luminescence** is the emission of light from any substance which is electronically exited states. Formally, luminescence can be divided into two categories, fluorescence and phosphorescence[1] in accordance with whether the nature of the excited state is singlet of triplet. (Lakowicz, 2006)

[1] The work described in the thesis used fluorescence, so we are going to focus on the fluorescence. We recommend (Lakowicz, 2006) as a good textbook to someone interested in the phosphorescence.)

Fluorescence happens when the excited state is singlet states, when the electron in the excited-state orbital is paired (by opposite spin) to the second electron in the ground-state orbital, which allows electron return to the ground state and occurs rapidly by emission of a photon. The emission rates of fluorescence are typically 10^8 sec^{-1}, so that a typical fluorescence lifetime is about 10 ns. The lifetime (τ) of a fluorophore is the average time between its excitation and return to the ground state. Considering the speed of light, about 300,000 km/sec, the light travels 30 cm in this 1-ns lifetime. (Lakowicz, 2006)

Even though the measurement of the time-resolved emission requires sophisticated optics and electronics to detect sub-nanosecond lifetimes of many fluorophores, we more widely use time-resolved fluorescence than the stationary or steady-state measurements due to much more information available from the data. In addition, technical requirement also has been easy to achieve because of the advances in technology that make time-resolved measurements easier, even when using microscope. (Lakowicz, 2006)

1.3.2. Single-molecule detection

Commonly, the detection of the fluorescence has been applied to the observing a large number of molecules prior to seeing single molecule, because of the amount of the signal. It's nature that the signal from a large number of molecules is much more intense than that from single molecule. Furthermore, it is of no use to say more intense signal is easy to detect. (Lakowicz, 2006)

Almost all experiments save the use of single-molecule detection (SMD) observe a large number of molecules, which reveal the ensemble average of the measured properties. The ensemble average can provide the overall property of the object, but the individual variety of molecule is impossible to approach because of ensemble averaging. (Lakowicz, 2006)

On the other hand, SMD provides a weak signal from a molecule. Instead, it can give us a more abundant information of the object, because the signals from SMD still keep the individuality of each molecule owing to avoiding the ensemble averaging. The fact derived from SMD measurement make us possible to understand various aspect of the molecule. (Lakowicz, 2006)

Although SMD requires the highest obtainable sensitivity, single-molecule experiments using SMD is now being performed in many laboratories, thanks to introducing the practical method (Roy et al., 2008) for SMD. At this time, most single-molecule experiments are performed on immobilized fluorophores, which are chosen for their high quantum yields and photo-stability. (The work in this thesis used Cy3, Cy5 and Alexa488, and so on.) A typical instrument for SMD is composed of laser excitation through microscope objective, a scanning stage to move the sample and confocal optics to reject unwanted signals.

Observations on single molecules represent the highest obtainable sensitivity. Single-molecule detection (SMD) is now being performed in many laboratories.33–34 At present most single-molecule experiments are performed on immobilized fluorophores, with fluorophores chosen for their high quantum yields and photo-stability. A typical instrument for SMD consists of laser excitation through microscope objective, a scanning stage to move the sample and confocal optics to reject unwanted signals. (Lakowicz, 2006)

1.3.3. FRET (Fluorescence Resonance Energy Transfer)

FRET (Fluorescence Resonance Energy Transfer) is one of the most widely used application of fluorescence including medical diagnostics, nucleic acid analysis, and optical imaging. (Lakowicz, 2006) To understand this phenomenon, we should understand resonance energy transfer, which occurs in the excited state.

To observe the FRET, donor with shorter excitation/emission wavelength is excited by a laser. Acceptor receive energy via FRET from a donor. By energy transfer, acceptor emits light instead of donor. Using both intensity of dyes, we can deduce distance between the dye in real time. The distant dependence of FRET is determined by the equation below.

$$\mathrm{E} = \frac{1}{1 + (R/R_0)^6}$$

Here, R is the distance between two dyes and R_0 is the distance when FRET efficiency becomes 0.5. The typical value of R_0 is about 5 nm. FRET changes in the range of 1-10 nm which is relevant to the size of typical biomolecules. The conformational change of biomolecule in real-time can be measured by FRET through labeled dyes on biomolecules. (Roy et al., 2008)

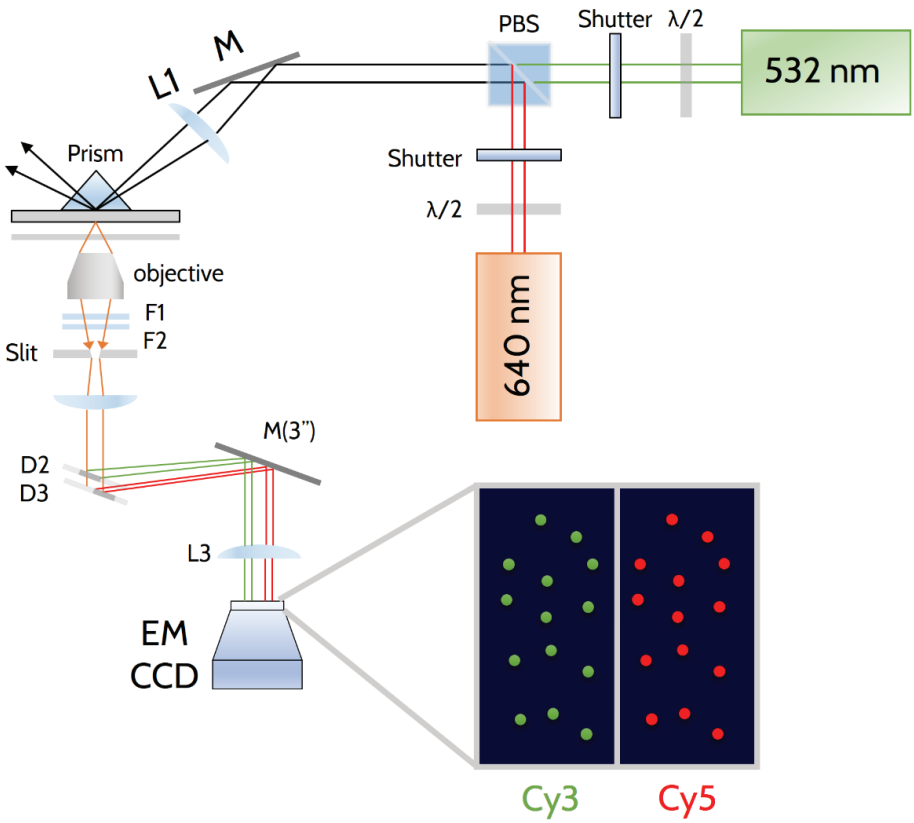

Figure 1-3-1. A schematic diagram of ALEX two-color FRET setup in TIRF microscopy

1.3.4. PIFE (Protein Induced Fluorescence Enhancement)

Protein's proximity to the fluorescent dye can cause fluorescence enhancement. This fluorescence change can be applicable to the single molecule fluorescence method, termed Protein induced fluorescence enhancement (PIFE). Compared to FRET which require two dyes, PIFE employs a single dye attached to DNA or RNA to which an unlabeled protein is applied. (Hwang et al., 2014)

PIFE is first discovered by (Aramendia et al., 1994) observing through steady-state fluorescence emission and fast photolysis that quantum yield of cyanine dyes was directly proportional to the viscosity of the local environment, but inversely proportional to temperature. (Hwang et al., 2014)

According to the review on PIFE (Hwang et al., 2014), this PIFE effect can arise from a protein that acts as an additional viscosity factor, increase of which of a fluorophore results in enhanced fluorescence intensity, (Sanborn et al., 2007; Levitus et al., 2010) as the cis-trans photo-isomerization reaction and the resulting fluorescence intensity of the cyanine dyes depends strongly on their local molecular environment. It further validates this elucidation (Hwang et al., 2014) that Cy3 dye resulted in distance dependent fluorescence increase and a corresponding increase in the lifetime of the dye, but Cy3B which does not undergo cis-trans isomerization exhibits no increase in quantum yield or fluorescent lifetime. (Hwang et al., 2011)

This PIFE effects can also report the distance like FRET. (Hwang et al., 2014) determined the distance sensitivity range of PIFE based on the restriction enzyme BamHI binding assay and RIG-I translocation data. (Hwang

et al., 2011) They dissected the single molecule traces obtained in RIG-I translocation into individual translocation events to analyze the PIFE sensitivity and the PIFE insensitivity portion. They observed the rapid intensity decrease corresponding to the PIFE sensitive distance range where RIG-I translocate away from the fluorophore, and interpreted the plateau as the PIFE insensitive portion where the intensity does not report on the protein movement. As they assumed a constant rate of translocation, they thought the data reflected the first 10-12 bp movement was detected by PIFE. (Hwang et al., 2011) They concluded that PIFE shows a sharp sensitivity within the 0-3 nm range, compared to FRET which reports on distance change in the 3-8 nm range, thereby adding sensitivity to the FRET insensitive short distance range.

Considering this calibration by (Hwang et al., 2014), PIFE can be used as a powerful alternative as well as a complement to FRET. In reality, this photo-physical phenomenon has been employed in stopped-flow measurements for translocase directionality (Tomko et al., 2010), ATP dependence (Fischer et al., 2004). (Fischer et al., 2012) It is in 2007 that, a method equivalent to single molecule PIFE to study T7 DNA polymerase binding to DNA showed a first demonstration that protein binding to fluorescently labeled DNA can be monitored by intensity change with single molecule resolution. (Luo et al., 2007) Following this study, (Myong et al., 2009) reported the ATP fueled translocation activity of a human antiviral protein, RIG-I. We also used PIFE to monitor the protein movement, or using PIFE we identified whether RNA polymerase arrived at the end of DNA, which will be given in **Chapter 3** in detail.

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Chapter 2

in vitro Transcription Termination Assay

2.1. Introduction

Many biologists already have studied the transcription by using several techniques. Traditional techniques used in biology, such as gel electrophoresis, can measure the transcription phenomena in a bulk scale, which means they provide only ensemble average data. These techniques give information about the dynamics of transcription and the position of transcription termination.

These traditional techniques, however, have limitation of resolution both temporally and spatially. As fate of the molecule ongoing transcription is decided by kinetics coupling between components, it's difficult to understand what happened in each molecule without real time temporal resolution (about second) and single molecule scale spatial resolution.

We designed ***in vitro*** **transcription termination assay** to monitor the procedure of transcription termination both temporally in real time and spatially in single molecule scale. This technique is more powerful tool to observe the transcription termination than existing tool. By using this technique, we expect to classify the molecule by the its fate whether termination happened and correlation between the kinetics information and the fate molecule as a result.

Before embarking on the main experiment, we examine whether the technique we developed is well-established in observing the transcription mechanism in three aspects, initiation signal observation, photo bleaching control, and the pre-mature termination. In detail, our method has ability to observe the initiation signal which can be used correct the variation of the moment of transcription initiation. We examine whether the laser intensity in our experiment set-up can cause the phenomenon by photo-bleaching and that by real reaction. In this section, we will show the control experiment to examine if the technique is well-established.

2.2. Materials and Methods

2.2.1. DNA substrates preparation

Modified DNA strands and DNA splints (Table 2-2-1) were purchased from IDT, or Integrated DNA Technologies(USA), respectively.

Name	Sequence (5' → 3')
A1 (200 nt)	TATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCC ATACCGCCACAAAACTTATGGATTTATGCGTATAATCCGCGGCGCAAATT ATTTACTTACCGGAGGCGACATGGACCCTGAACCCACCCCTCTCCCGCGA TGGAGAATTTTCCTTTTCCGGTAAGCCTGCCTCTGCTGTCTTACCGGTGT
B1 (114 nt)	GTAAGACAGTGACACAATAACGTCCCTGTTTTTATTTAAACATTGCTCAT CGGGCAAGGCTTTGCCGTGCCTGAAGAATTTTCTGCGCCTGACTTCGGCG CGGAGGGATTACCT
A1B (40 nt)	TTATTGTGTCACTGTCTTACACACCGGTAAGACAGCAGAG
A2B (40 nt)	TTATTGTGTCACTGTCTTACACACCGGTAAGACAGCACAC

Table 2-2-1. Oligonucleotide sequences for DNA strands and splints

The following combinations were used to construct individual DNA substrates.

- ***mgt*A_WT** (Wild type): A1 + A1B + B1

[Annealing]

To prepare the full-length DNA substrate, DNA strands which compose the target DNA were annealed with a DNA splint by slowly cooling from 90 °C to 16 °C in annealing buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP (pH 7.5 at 25°C)) and ligated with T4 RNA ligase 2 (M0202T; New England Biolabs). (Lang and Micura, 2008).

[PCR – labeling]

We need to modify the DNA substrates both for immobilization and for florescence detection. To label the DNA substrate, we used the PCR (Polymerase Chain Reaction) with labeled primer. Primers were designed based on the melting temperature for reaction and purpose. To immobilize the DNA substrate on the quartz slide with streptavidin, we used **biotin** with high affinity to the streptavidin. To detect the DNA sample, we used **Cy5 fluorescent dye** for single-molecule detection. We used the GeneAll® Expin™ (GeneAll) following the PCR SV Protocol.

Name	Sequence (5' → 3')
Rho_*mgt*A_fw	Biotin - TATCAAAAAGAGTATTGACTTAAAGTCTAA
Rho_*mgt*A_bw	Cy5 - AGGTAATCCCTCCGC

Table 2-2-2. Oligonucleotide sequences for DNA primer

2.2.2. Elongation Complex assembly

Figure 2-1. Schematic diagram of elongation complex.

To reproduce the transcription in the test tube, we should assembly the elongation complex. **Elongation complex** is the RNA polymerase bound on the DNA. Elongation complex assembling require the transcription initiation and elongation, followed not by termination but by pausing. Pausing during the transcription happens when the appropriate rNTP (ribo-nucleoside triphosphate) is not provided, or when there is the specific sequence in DNA substrate, so-called pausing site.

Restriction of the rNTP is more efficient than introduction of the pausing site, as it occurs and lasts certainly before rNTP required is provided. To do this strategy, we should determine how long the transcript of elongation complex and which rNTP we are going to include of exclude. The longer length of the transcript, the more stable the elongation complex becomes. We, however, decide the length of the transcript is 5 nt, to observed initiation signal, mentioned in **Section 3-3-1**.

We also used the dinucleotide labeled by fluorescent dye to monitor the existence of RNA. We designed DNA substrate and selected proper combination of NTP to make all transcript begin from dinucleotide labeled by Cy3 fluorescent dye, Cy3-ApU.

As we took them into consider, we incubated DNA substrate (mentioned in **Section 2-2-1**), parts of rNTP (rATP, rCTP; 10 μM each; 27205601; GE Healthcare), Cy3-ApU (250 μM; Trilink), and RNA polymerase (~340 nM; New England Biolabs) for 20 minutes, in **Transcription buffer**, which contains 20 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 20 mM NaCl, 1 mM DTT.

2.2.3. Single-molecule experiment

Single molecule experiment required well-prepared slide to prevent the non-specific binding and to bind sample in adequate distance each other. So we prepared polymer-coated quartz slides prepared using a 1:40 mixture of biotin-PEG (biotin-PEG-5000; Laysan Bio, Inc.) and mPEG (m-PEG-5000; Laysan Bio Inc.). (Roy et al., 2008)

After streptavidin (0.2 mg/ml, Invitrogen) coating of the slides, elongation complex (~500 pM) were immobilized on the polymer-coated surface through biotin-streptavidin interactions. Molecules behaviors ongoing transcription is imaged using a home-made wild-field total-internal-reflection fluorescence(TIRF) microscope. An electron multiplying charge-coupled device camera (DU-897; Andor Technology) was used as a detector.

Experiments were generally performed with 50 ms exposure times in ALEX mode. (Kapanidis et al., 2004; Lee et al., 2010) Because the experiment was performed in an ALEX mode with alternating between a green laser (532 nm; Excelsior-532-50-CDRH, Spectra-Physics) and a red laser (640 nm; Excelsior-640c-35, Spectra-Physics), the actual time resolution of experiments was 1 sec (=1000 ms). For detecting factor Rho's existence, blue laser (473 nm; Excelsior-473-50-CDRH, Spectra-Physics) is also used.

A standard buffer (10 mM Tris-HCl (pH 8.0), 150 mM KCl, $MgCl_2$, 10 mM, 1 mM DTT and an oxygen scavenging system (5 mM PCA, 100 nM PCD, and saturated Trolox)) was used for all of the experiments. When we triggered transcription initiation again, we injected rNTP (rATP, rUTP, rGTP, rCTP; 200 μM (Standard in this thesis); 27205601; GE Healthcare). All experiments were performed at 37 °C. For data analysis, IDL (7.0, ITT), Matlab (R2018a, The MathWorks), and Origin (8.5, OriginLab) were used.

In this chapter we design two experiments to validate single-molecule *in vitro* transcription assay. Firstly, we confirmed that photo-bleaching didn't affect our assay. As we detect signal using fluorescence signal which always has a possibility to be photo-bleached, it's important to confirm that kind of photo-physical effect can be ignored. To confirm this, we just observed the population ratio of fluorescence bleaching depending on laser intensity. (**Figure 2-2-1**)

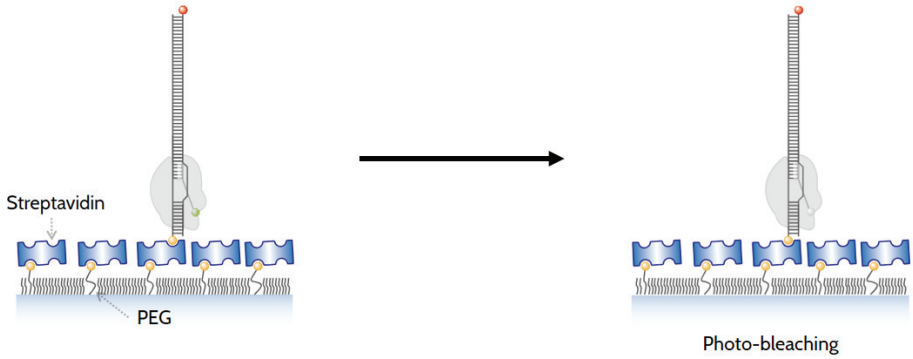

Figure 2-2-1. Schematic diagram of *in vitro* transcription assay without rNTP.

Secondly, as there can be difference between elongation complex just stalling on template DNA and going on the transcription elongation. To confirm this, we observed the RNA release signal depending on laser intensity. (**Figure 2-2-2**)

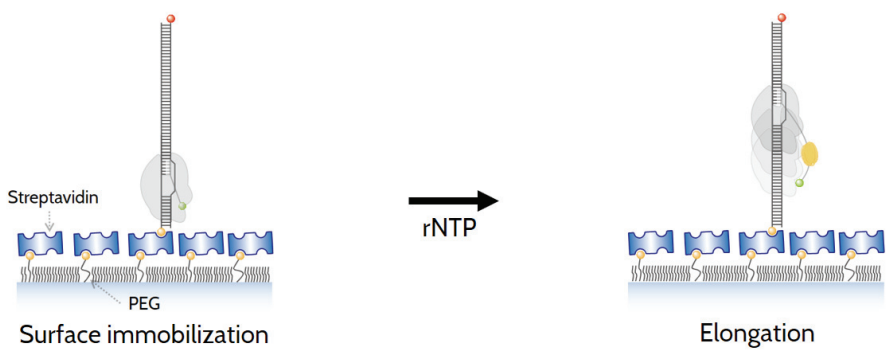

Figure 2-2-2. Schematic diagram of Elongation.

2.3. Results and Discussion

2.3.1. Photo-bleaching test

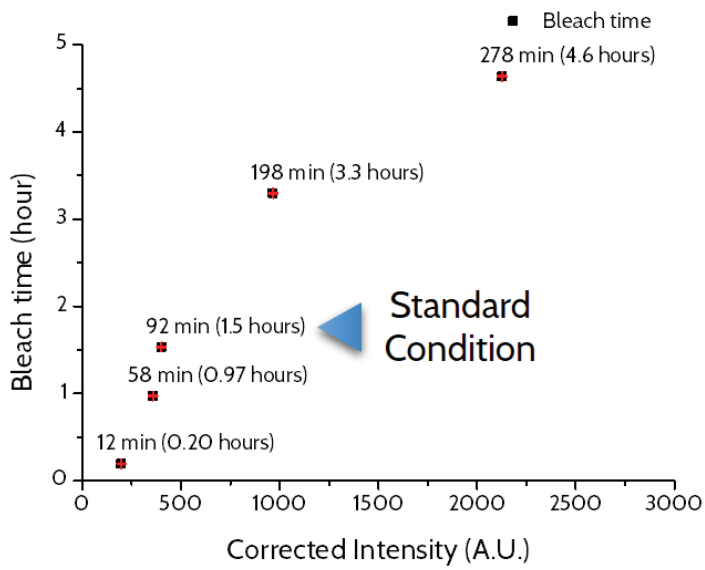

Figure 2-3-1. Photo-bleached times Bleach time at several corrected intensities, when the exposure time is 0.2 sec (12 min), 0.5 sec (58 min), 1.0 sec (92 min), 2.0 sec (198 min), 5.0 sec (278 min). Our experiment condition is 1.0 sec, indicated by blue arrow. (Time histograms for each data point is shown in **Figure S2-1**)

When designing the technique using fluorescence dye, we should already concern whether the photo-bleaching of dye. The number of the molecule whose dye is photo-bleached is decided by both the laser intensity and the period of measurement. The intense laser causes the faster photo-bleaching, but the long period of measurement requires long lifetime of the fluorescence dye. In other words, these two physical quantity are correlated in inverse proportion.

Both of the quantity is important in the measurement, because the laser intensity improves signal-to-noise ratio and the longer period of measurement provides much more information of object. To optimize our measurement, we should know the range of observing which did not cause the photo-bleaching problem.

2.3.2. Premature termination test

To consider certain terminator as Rho-dependent terminator, we need to confirm the effect of factor Rho, by comapring cases in the presence of factor Rho and cases in the absence of factor Rho. Before going on, we measure the termination efficiency in the absence of factor Rho, which can be considered as pre-mature termination.

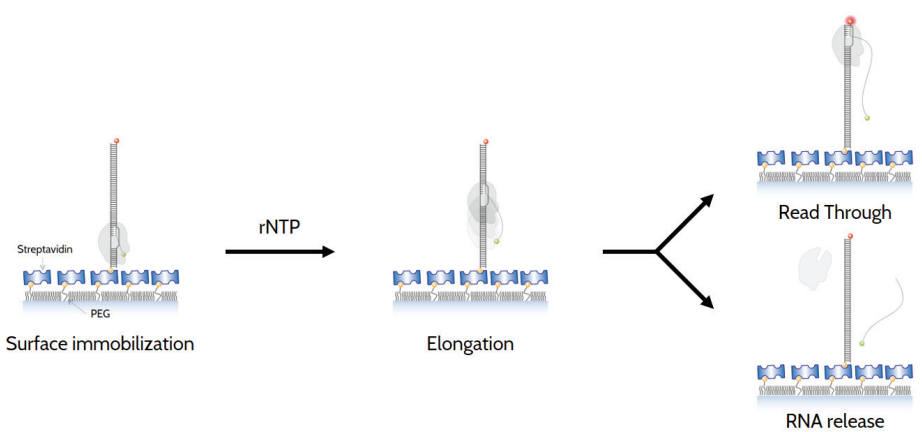

Figure 2-3-2. Schematic diagram of *in vitro* transcription assay without factor Rho.

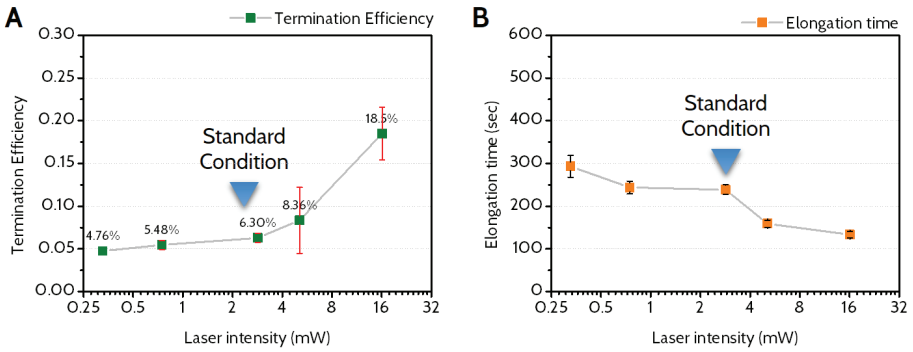

Figure 2-3-3. Termination efficiency and kinetics information in the experiment without factor Rho (A-B) Termination efficiency **(A)** and elongation time **(B)** at varying laser intensity. Standard condition is indicated using blue arrow.

We assumed that if RNA dissociates from elongation complex, then the Cy3 signal at the end of transcripts will disappear. (**Figure 2-3-2**) However, this Cy3 disappearance happens not only when transcript releases but also when Cy3 fluorescnce dye is photo-bleached. To confirm standard condition we decided can ignore the photo-bleaching problem, we measured termiantion efficiency at varying laser intensity. (**Figure 2-3-3 (A)**) We decided 1 sec as a standard condition, and in the standard condition is when the laser intensity is weak enough to ignore the fluorescence photo-bleaching. Elongation time, as well as termination efficiency, also show that the our standard condition is in interval where is credible. (**Figure 2-3-3 (B)**) (What is the elongation time is explained in **Section 3-3-3** in detail.) If laser intensity of our standard condition were too intense, elongation time would have been measured shorter because of fluorescence dye's photo-bleaching.

In spite of convincing that our standard condition is perpendicular to photo-bleaching problem, there still remain question, why the termination efficiency is about 6.3%, not zero. We assumed now that as photo-bleaching issue is excluded, termination efficiency even happen in the absence of factor Rho is premature termination, which happens regardless of factor Rho. Referring to data shown in the other chapter, this termination efficiency is almost similar to that in the experiment using other kinds of factor Rho (**Chapter 4**), other kinds of DNA substrates (**Chapter 5**), and so on. Moreover, by removing pausing site at the DNA substrate, we can control the speed of RNA polymerase. (**Figure 5-3-1 (E)**) Considering that the time distribution of when the Cy3 fluorescence signal disappeared changes depending on speed of RNA polymerase, RNA release observed in the absence of factor Rho happens as a result of something happend during the transcription elongation. (**Figure 5-3-1 (D)**)

2.4. Conclusion and Outlook

In this chapter, we tried to show that *in vitro* transcription assay using single-molecule FRET which we developed operates well. What we try to verify is whether photo-bleaching can be ignored in our experiment condition and how often pre-mature termination happen.

As we will deem disappearance of fluorescence signal the result from RNA release, it should be guaranteed that the photo-bleaching happens rarely. In addition, to validate the termination we observed is happened by factor Rho, the premature termination which occurs in the absence of factor Rho seldom, if ever, happens. Based on the vilification undergone in this chapter, we showed the factor Rho's effect in transcription termination in several cases in following chapter.

References

<Journal>

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Appendix

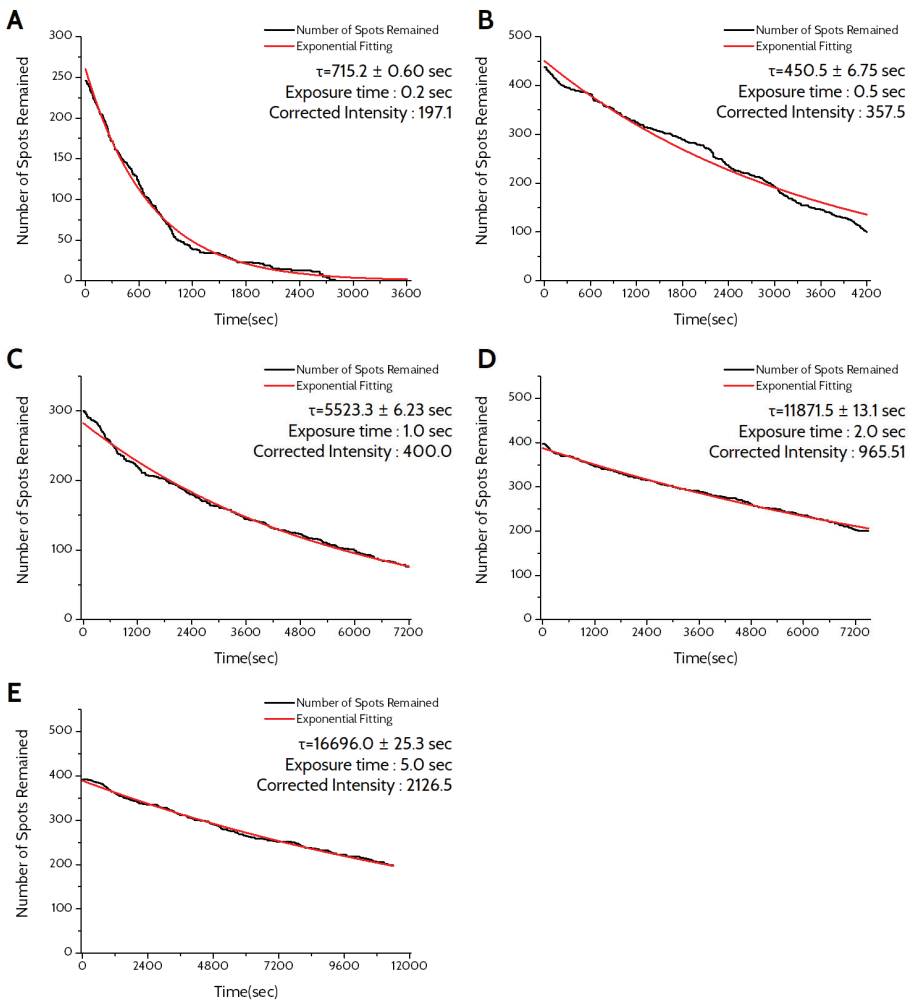


Figure S2-1. Photo-bleached times in the experiment without rNTP. (A-E) Number of spots remained at several exposure time, 0.2 sec **(A)**, 0.5 sec **(B)**, 1.0 sec **(C)**, 2.0 sec **(D)**, 5.0 sec **(E)**. Each corrected intensity corresponds to exposure time.

Chapter 3

Transcription termination by factor Rho

3.1. Introduction

3.1.1. Overview

In the previous chapter, we showed that *in vitro* transcription assay is well established. The aim of the work presented in this chapter was to observe the transcription termination caused by factor Rho. We show the way how we prepare the factor Rho, followed by single molecule method for observing transcription termination.

Single molecule FRET is powerful technique in the changing the environment because of its easy buffer changing, we can observe the transcription termination both when the factor Rho is pre-bound to the RNA polymerase by injecting factor Rho followed by wash out before rNTP injection

for transcription initiation (pre-binding factor Rho assay) and when the factor Rho and rNTP injected simultaneously only to remain factor Rho freely in buffer when transcription goes on (freely diffusing factor Rho assay).

The purpose of pre-binding factor Rho assay is to dissect the factor Rho transcription termination, which is thought to have two distinct pathways. This assay, which removes the factor Rho remained in buffer, exclude the transcription termination done by the freely diffusing factor Rho. In other words, transcription termination observed in pre-binding factor Rho assay is conducted by factor Rho pre-bound to the RNA polymerase.

On the other hand, freely diffusing factor Rho assay implies ambiguous meaning before conducting the experiment. We can expect to at least two plausible result, depending on how fast the factor Rho binds to the RNA polymerase. The longer the factor Rho takes time to bind to the RNA polymerase, the more the transcription done by factor Rho bound to the RNA polymerase will be excluded. Or, if the factor Rho binds to RNA polymerase relatively slow, freely diffusing factor Rho assay will show the transcription mediated only by RNA. If the factor Rho, though, binds to RNA polymerase relatively fast, the result come from this assay will be almost similar to the case when the factor Rho exists both before and after transcription. Namely, factor Rho's fast binding to RNA polymerase cause the transcription termination conducted in presence of factor Rho bound to RNA polymerase.

If there is association between factor Rho and RNA polymerase, it's be useful information how stable this association is. Besides, this obscurity in the interpretation of freely diffusing factor Rho suggested that the affinity between factor Rho and RNA polymerase should be measure to know the exact meaning of result that freely diffusing factor Rho assay will show.

We measured the termination efficiency and several kinetics parameters, such as elongation time and termination time, depending on the concentration of factor Rho. The assay described in the chapter also will be used to explain the experiments conducted in **Chapter 4**, which used various kinds of factor Rho, and **Chapter 5**, which used several kinds of DNA substrates.

3.1.2. *mgt*A terminator

A riboswitch is a regulatory segment of mRNA, usually found at the 5'-UTR (untranslated region), that regulates downstream gene expression upon binding to effector metabolite or metal ions (ligands). (Mitra et al. 2017). We thought that riboswitch can change the different conformations in response to different effector metabolites to switch on or off for the expression of downstream genes. (Proshkin et al., 2014, Serganov et al., 2012)

Some of Rho terminators have been found to regulate riboswitch action of the magnesium-sensing *mgt*A riboswitch from *Salmonella enterica*, and flavin mononucleotide-sensing riboswitch from *Escherichia coli*. (Mitra et al. 2017) The *mgt*A riboswitch from *Salmonella enterica* regulates transcription

elongation into coding region of the magnesium transport A (*mgt*A) by adopting two mutually exclusive conformations. High concentration of magnesium promotes a conformation of the *mgt*A leader RNA that favors factor Rho binding to this region only to induce transcription repression of the *mgt*A-coding region. On the other hand, low concentration of magnesium in which requires more expression of the magnesium transporter causes a conformation of *mgt*A leader RNA that disfavors factor Rho binding and represses the factor-dependent termination by factor, only to increase the *mgt*A-coding region. (Hollands, K et al., 2012; Hollands, K et al., 2012)

Factor Rho is also involved in the similar mechanism that regulate the flavin mononucleotide-sinsing *rib* riboswitch from *Escherichia coli* (Hollands, K et al., 2012) and the flavin mononucleotide riboswitch from the gram-positive *Corynebacterium glutamicum*. (Takemoto et al., 2014)

3.1.3. Kinetics-coupling Model

Kinetics-coupling model is proposed to explain how the factor Rho terminates transcription. The key concept of this model is factor Rho's chasing to the elongation complex. This phenomenon is similar to torpedo which travels under water, so someone called this model **Torpedo model**.

The procedure of kinetics-coupling model is well established and widely accepted. Factor Rho precedes through a series of steps categorized into five steps: (Sharmistha Banerjee et al., 2006) (a) Factor Rho binds to naked, not translated RNA at *rut* site, whose binding of factor Rho is achieved by the N-terminal or primary binding domain, (b) The bound RNA, passing through primary binding site reaches the secondary binding site that residues on C-terminal, (c) RNA-bound secondary sites sensitize factor Rho for ATP hydrolysis, (d) The energy derived from ATP hydrolysis powers both the translocase and helicase activity of factor Rho to unwind RNA/DNA duplex. (e) Finally, factor Rho release nascent transcript and disengage the transcribing RNA polymerase.

In detail, factor Rho generates sufficient force to displace streptavidin from biotinylated RNA, suggesting it can overcome RNA secondary structure and displace RNA-binding domain. (Schwartz et al., 2007) The termination zone usually lies about 60-90 nucleotides downstream of *rut* site, and elongation complex often pause inside this zone. Termination happens when factor Rho comes in contact with the RNA exit channel of the transcribing RNA polymerase. (Mitra et al. 2017)

3.1.4. Rho-RNAP interaction

According to the recent annual review on the Rho-depend transcription termination in 2017 (Mitra et al. 2017), the concept of kinetics coupling, which is described in **Section 3.1.3**, has been challenge by *in vitro* observation that factor Rho may be associated with the elongation complex from the beginning of the transcription cycle, even well before the nascent transcript containing *rut* site (Hart et al., 1994), leading to the transcription termination by the pre-bound factor Rho.

Moreover, termination defects of several mutants could not be suppressed by a slow moving RNA polymerase RpoB8 (Jin et al., 1988) or strong pause are necessarily to terminate transcription. (George A. Kassavetis et al., 1981) These facts means that Rho-dependent termination is not simple mechanism that just factor Rho's catching up with RNA polymerase but something more complicated. (Chalissery, Jisha et al., 2007)

To elucidate the mechanism of Rho-dependent termination in detail, we should found other pathway of this transcription termination. It has been reported that factor Rho interacts with RNA polymerase, supported by the result from SDS-PAGE data. (Darlix, J.l. et al., 1971) This association between the factor Rho and RNA polymerase is a prime candidate for another pathway for Rho-dependent transcription termination. Several mechanistic and structural aspects of this putative factor Rho-RNA polymerase association need to be resolved. (Mitra et al. 2017) To resolve association between factor Rho and RNAP, a stable association need to be observed. (Kalyani, B. Sudha et al., 2011)

3.2. Materials and Methods

3.2.1. Expression of factor Rho

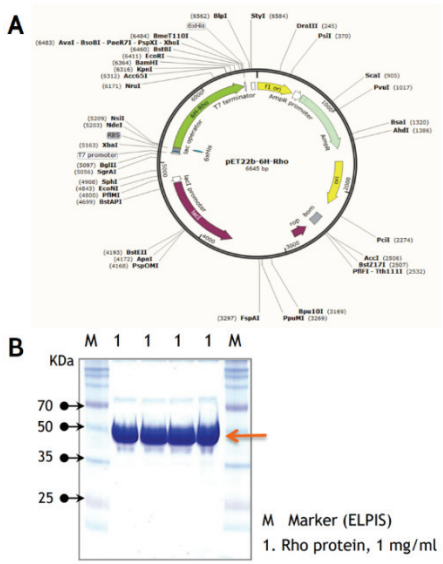


Figure 3-2-1. Vector map and quantitative result of factor Rho expression

(A) Vector map of pET22b-Rho-6H (6645 bp) **(B)** SDS PAGE gel results for factor Rho expression

Carboxy-terminal His-tagged factor Rho was purified after overexpression in *E. coli*, using commercial protein expression service (Bioprogen). The constructed plasmid pET22b-Rho-6H (**Figure 3-2-1**) was transformed into E. coli BL21-(DE3) strain for expression. The cells were incubated in LB media until they reached OD_{600} of 0.6 at 37 ℃. Protein expression was incubated with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 hours at 37 ℃. The cells were then harvested by spinning down (6,000 rpm) for 10 min, followed by 2 times washing with 50 mM Tris-HCl pH 8.0 and 1 mM EDTA. The cells were resuspended in 50 mM Tris-HCl pH 8.0 and 0.2 M NaCl. The

cells were fracted using sonication and spinning down (12,000 rpm) for 30 min. The supernatant was filtered and passed through a Ni-NTA column (Bio-read HR system) The collected fractions were dialzed against storage buffer (10 mM Tris-HCl pH 8.0, 0.1 mM DDT, 0.1 mM EDTA, 0.1 mM KCl and 50% (v/v) glycerol) (Nowatzke et al., 1996), flash frozen, and then stored at -80 ℃.

3.2.2. Single-molecule Experiment

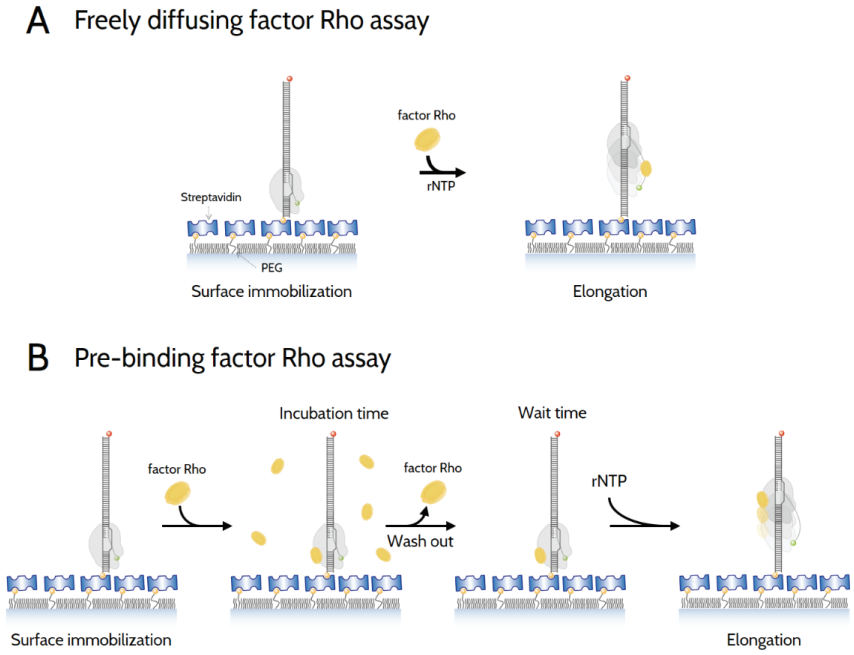


Figure 3-2-2. Schematic diagram of *in vitro* transcription assay in the presence of factor Rho (A) Freely diffusing factor Rho assay where we injected factor Rho and rNTP injected simultaneously. **(B)** Pre-binding factor Rho assay where we injected factor Rho followed by removing factor Rho freely diffusing in the buffer before rNPT injection.

We confirmed single-molecule *in vitro* transcription assay operates well in **Chapter 2**. In this chapter, we applied this assay to Rho-dependent termination.

As a result, single-molecule experiments conducted in this chapter is same as those described in Chapter 2 except the existance of factor Rho.

We designed two kinds of assay depending on when the factor Rho presents. **Freely diffusing factor Rho assay** is the assay that factor Rho exists from rNTP injection to the end of reaction, by injecting factor Rho when rNTP is injected. (**Figure 3-2-2 (A)**) On the other hand, **pre-binding factor Rho assay** is the assay which binds factor Rho to RNA polymerase before rNTP injection. This assay is consists of factor Rho injection before rNTP injection, followed by washing out carefully, only to remove all the factor Rho freely diffusing after transcription starts again. (**Figure 3-2-2 (B)**)

We conducted experients factor Rho concentration titration experiment in the range from the concentration whose termination efficiency is almost same as that in the absence of factor Rho to that whose termination efficiency is saturated, when the additional factor Rho didn't increase the termination efficiency.

To measure the affinity and stability of interaction between factor Rho and RNA polymerase, we used pre-binding factor Rho assay changing the incubation time and wait time. Incubation time ($\tau_{incubation}$) is the time we incubate the elongation complex with factor Rho. If affinity between factor Rho and RNA polymerase is high, then short incubation time is enough to exhibit termination. Wait time (τ_{wait}) is the time between the removal of factor Rho and rNTP injection. If the factor Rho-RNA polymerase is stable, then even tho long wait time will not affect termination acitivity.

3.3. Results and Discussion

3.3.1. Initiation Signal in Transcription

There is variation in the time when each molecule initiate again, so it's useful to monitor the exact moment of transcription initiation to measure the exact kinetics information. To monitor the moment of initiation, we assembly the elongation complex with short transcript for flourescent dye not to be exposed to outside of the RNA polymerase.

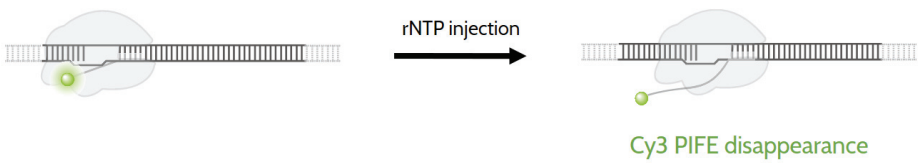


Figure 3-3-1. Schematic diagram of initiation signal.

Fluorescent dye in the RNA polymerase cause Cy3 fluorescence enhancement induced by RNA polymerase as protein. This Cy3-PIFE disappears, after transcription initiates and fluorescent dye at the end of RNA gets out of the RNA polymerase. (**Figure 3-3-1**)

Cy3-PIFE disappearance can be observed in representative trace when green laser excitation (**Figure 3-3-2 (A)**). We named the time beween the rNTP injection and the beginning of Cy3-PIFE disappearance **initiation time**. In detail, the initiation time is followed by rNTP injection and doesn't depend on whether factor Rho exists, (**Figure 3-3-2 (D), (E)**) which effects only on the

after transcription initiation. (**Figure 3-3-3 (B-C)**) In this study we only collect trace showing the initiation signal, which is gurentted to transcription happens.

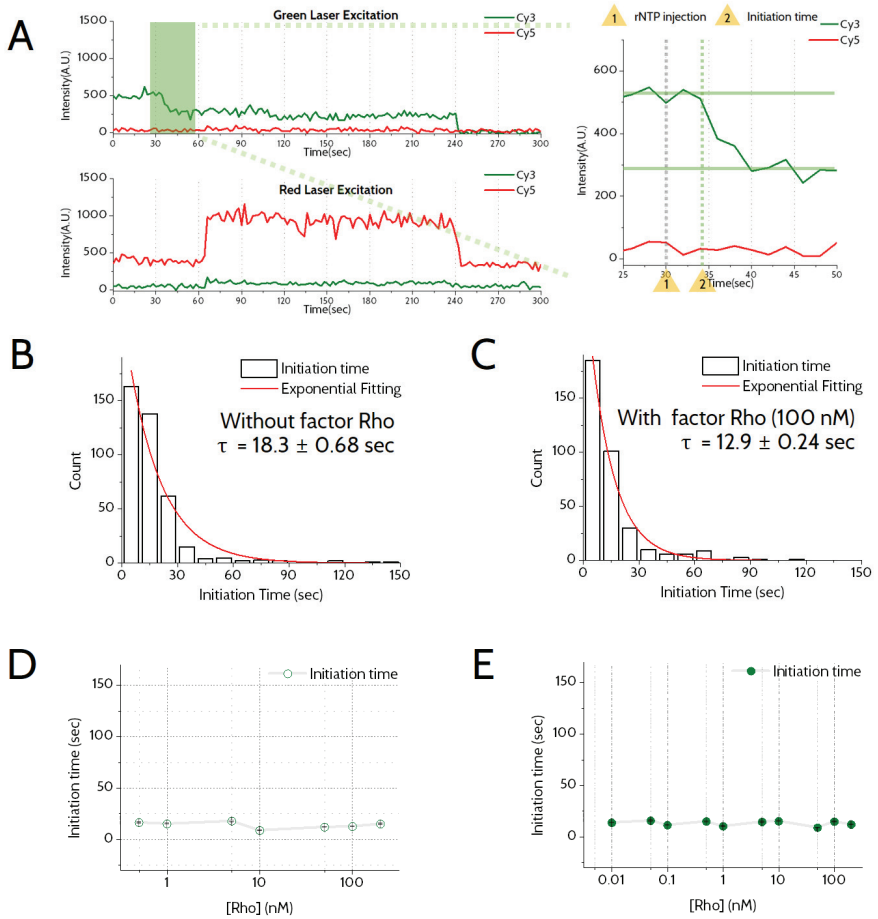


Figure 3-3-2. Representative time trace and distribution of initiation time. (A) Representative time trace of initiation time shows the Cy3-PIFE disappearance after rNTP injection. **(B-C)** Distribution of initiation time, its exponential fitting and fitting parameter when **(B)** without factor Rho and **(C)** with factor Rho (100 nM) both before and after rNTP (200 μM). **(D-E)** Exponential fitting parameter of initiation time at varying factor Rho concentration when factor Rho exists only **(D)** before rNTP injection and only **(E)** after rNTP injection.

(Time histograms for each data point is shown in **Figure S3-1** and **Figure S3-2**)

3.3.2. Representative Traces

Our *in vitro* transcription termination provides three kinds of time traces of the single-molecule experiments. We classify these traces by whether there is a Cy5-PIFE signal and the Cy3 signal disappearance. In our scheme, Cy5-PIFE appears when the RNA polymerase reach the Cy5 attached at the 5´-end of the template DNA. We can interpret the molecule showing Cy5-PIFE as the RNA polymerase arriving at the 5´-end of DNA. Cy3 disappearance means the RNA release, as the Cy3 dye is attached to the 3´-end. Combining these interpretation we can assign a kind of read through signal and the two kinds of termination singal.

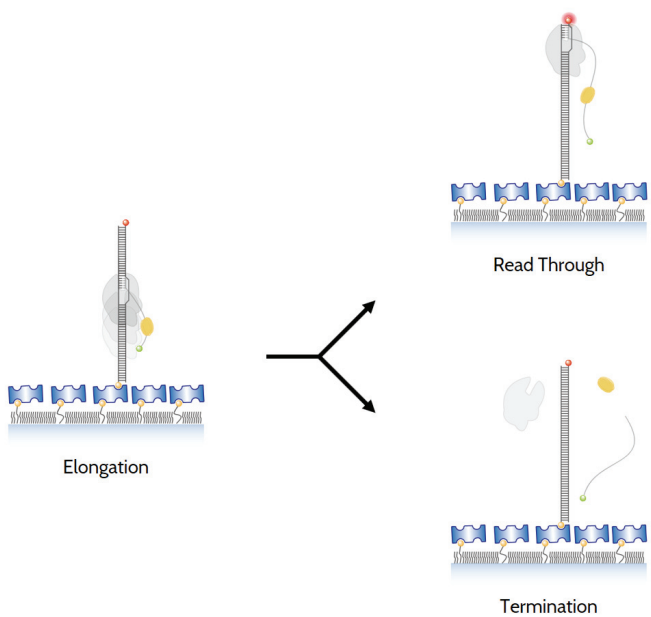


Figure 3-3-3. Schematic diagram of read through and termination

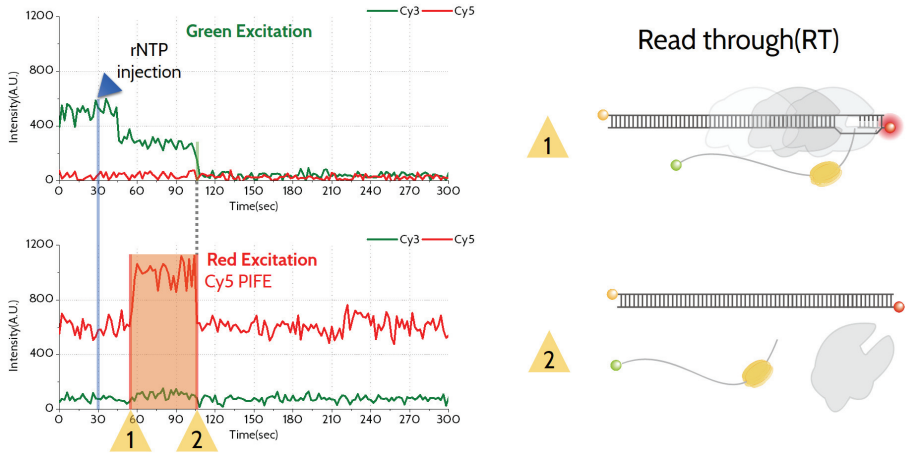


Figure 3-3-4 Representative time trace for Read Through (RT) cases

The representative time trace **(left)** and the schematic diagram **(right)** for read through cases. Yellow arrow denoted by number matches the moment in the time trace and the described phenomena.

Read through (RT) means RNA polymerase reaches end of the DNA template without RNA release. Time traces showing read through are expected to show the Cy5 enhancement with Cy3 signals kept as it is. Representative trace of read through is shown in (**Figure 3-3-4**). Arival at the end of the DNA is sometimes followed by RNA release of eloncation complex dissociation. As those kind of release happen after getting to the end point of DNA, they aren't considered as termination. In real situation, the end point of DNA is thought to be located further position from the initiation point.

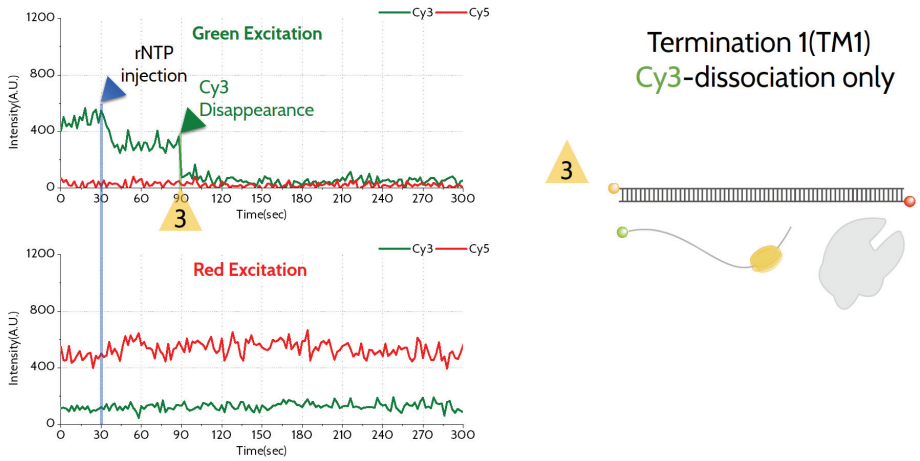


Figure 3-3-5. Representative time trace for type-1 Termination (TM1) cases

The representative time trace **(left)** and the schematic diagram **(right)** for type-1 termination cases. Yellow arrow denoted by number matches the moment in the time trace and the described phenomena.

Termination (TM) means the RNA is released from the template DNA. Even though the end point of DNA in our scheme is artifact component, we used this end point of DNA as the criterion for the termination. In other words, we consider some molecules with RNA release before RNA polymerase's arrival at the end point of DNA as molecules whose transcription was terminated. By definition, we assign the molecule with its Cy3 signal disappeared before RNA polymerase reach the end point of DNA as the termination signal.

There are two kinds of the termination depending on whether Cy5 PIFE following Cy3 disappearance. The first case is **type-1 termination (TM1)**, also called **Cy3-dissociation only**. Representative trace of TM1 is shown in (**Figure 3-3-5**) In this case, RNA polymerase dissociates from the template DNA after RNA release.

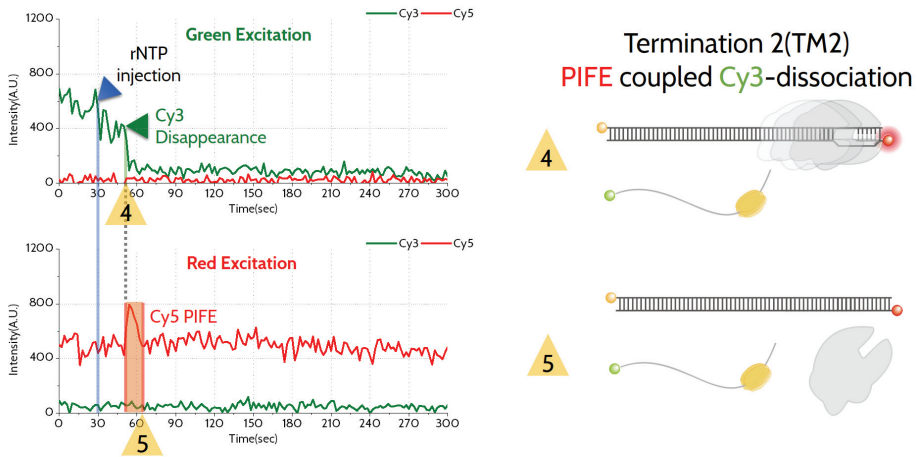


Figure 3-3-6. Representative time trace for type-2 Termination (TM2) cases
The representative time trace **(left)** and the schematic diagram **(right)** for type-2 termination cases. Yellow arrow denoted by number matches the moment in the time trace and the described phenomena.

The second case is **type-2 termination (TM2)**, also called **PIFE-coupled Cy3 dissociation**. Representative trace of TM2 is shown in (**Figure 3-5-6**). Referring to internal data from (Wooyoung Kang et al., unpublished), type-2 termination happens when the RNA polymerase remains in the teemplate DNA. Or, TM2 happens when the RNA polymerase remains in the template DNA only to arive the end of DNA causing the Cy5 PIFE.

3.3.3. Factor Rho's effect

We observed Rho-dependent transcription termination using single-molecule *in vitro* transcription assay. To validate transcription termination we observed is Rho-dependent transcription termination, we should observed difference in termination efficiency between in the presence of factor Rho (100 nM) and in the absence of factor Rho. We showed that termination efficiency observed in the absence of factor Rho is about 6.30%. (**Figure 2-3-3 (A)**) Termination efficiency increase when factor Rho is pre-bound to RNA polymerase, when factor Rho is injected with rNTP, and when both pre-bound factor Rho and freely diffusing factor Rho exist. (**Figure 3-3-7 (A)**)

Our assay can collect the kinetics information, such as termination time, and elongation time. (**Figure 3-3-7 (B), (C)**) **Termination time** represents periods from transcription initiation to RNA release if termination happens. In the absence of factor Rho, the average termination time is about 448.6 sec. The average termination time in the presence of freely diffusing factor Rho is more shorter, about 200 sec. Conversely, pre-bound factor Rho represents about 420.5 sec. Comparison between average termination time, termination caused by factor Rho is much faster thant that in the absence of factor Rho, which can be interpreted as the freely diffusing factor cause Rho not only quantative increase in the termination but also change in the termination time faster. Termination only caused by pre-bound factor Rho represnts less efficiency and not that difference in termiation, which suggest the difference between freely diffusing factor Rho and pre-bound factor Rho.

Elongation time represents periods from transcription initiation to arrival at the end of DNA causinf enhancement in Cy5 fluorescenec dye, if read through happens. In the absence factor Rho, average elongation time is about 266.9. This becomes shorter 229.8 sec (only pre-bound factor Rho), 210.3 sec (only freely diffuing factor Rho), and 174.9 sec (both pre-bound factor Rho and freely diffusing factor Rho). We understand this result by two aspects, reffering to kinetics-coupling model and involvement of factor Rho in translcription elongation.

Taking acount of kinetics-coupling model, the population of read through is different between cases with factor Rho and thouse without factor Rho, as the factor Rho's chasing to RNA polymerase cause RNA release, only to be classified to termination. As a result, the population of read through in the cases with factor Rho represents the faster average elongation time than that in the case without factor Rho. The concentration of factor Rho, 100 nM, can be deemed as saturated in the aspect of termination (**Figure 3-3-8**, **Figure 3-3-9**), which excludes the case elongation complex even not effected by factor Rho.

We can explain this difference in the elongation complex by involvement of foactor Rho in transcription elongation. Factor Rho's binding to factor Rho increases its elongation time. This fact is supported by the factor Rho mutants experiment, which will be showin in **Chapter 4**. We assumed that factor Rho's binding to RNA polymerase cause the changes in structure, which is needed to be supported other studies on structure of factor Rho bound to RNA polymerase.

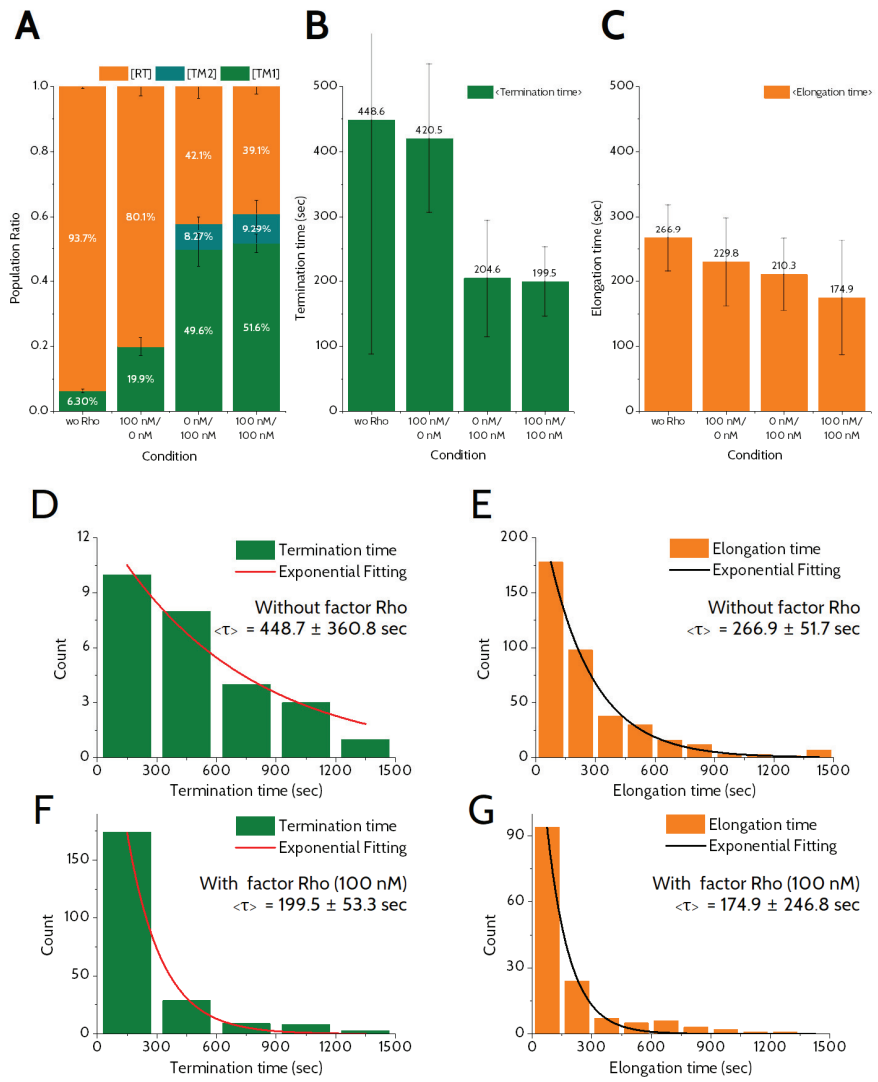


Figure 3-3-7. *in vitro* assay observed Rho-dependent transcription termination

(A-C) Population ratio **(A)**, termination time **(B)**, and elongation time **(C)** in the several of several cases. ('wo Rho' means we didn't inject factor Rho. 'A nM/B nM' means pre-bound factor Rho is A nM and freely diffusing factor Rho is B nM.) **(D-G)** Time histogram for termination time **(D, F)** and elongation time **(E, G)** when in the absence of factor Rho **(D, E)** and in the presence of both of pre-bound factor Rho (100 nM) and freely diffusing factor Rho (100 nM) **(F, G)**.

All the cases of cases in the present of factor Rho, *pre-bound factor Rho only*, freely diffisuing factor Rho only, and *both pre-bound factor Rho and freely diffusing factor Rho*, shows the changes in population ratio, termination time and elongation time. This implies that there is difference between pre-bound factor Rho and freely diffusing factor Rho. To clarify this implication, we conducted freely-diffusing factor Rho assay and pre-binding factor Rho assay in varying concentration of factor Rho. (**Figure 3-3-8**)

The result from factor Rho titaration experiment show that freely diffusion factor Rho is more effective in termination than pre-bound factor Rho. Saturate point of termination efficiency of freely diffusing factor Rho is almost 60%, but that of pre-bound factor Rho is about 20%. (**Figure 3-3-8 (A)**) [TM2] ratio as well as total termination is also different between two different states of factor Rho. Freely diffusing factor Rho always contatin some population of [TM2] and [TM2] take about 10~20% of total termination. Conversely, pre-binding factor Rho showed no [TM2] population over all Rho concetration. (**Figure 3-3-8 (B)**) To further analysis, we plotted [TM1] and [TM2] from freely diffusing factor Rho assay and total termination from pre-binding factor Rho assay at the same time. (**Figure 3-3-8 (C)**) It seemed that there are some correlation, but it's yet to explain the meaning of of this correlation.

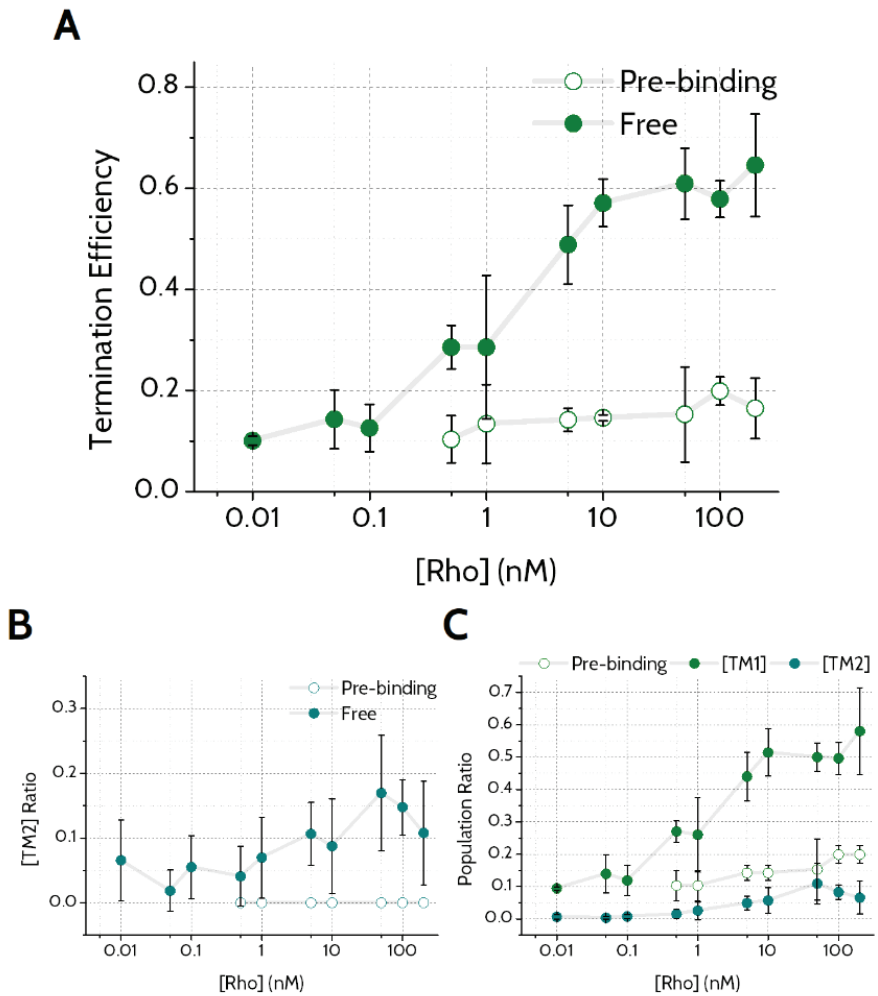


Figure 3-3-8. Termination ratio of two different pathway of termination

(A-B) Termination efficiency **(A)** and [TM2] ratio **(B)** of pre-binding factor Rho assay (denoted 'Pre-binding') and freely diffusing factor Rho assay (denoted 'Free') for varying [Rho]. **(C)** Population ratio of termination of pre-binding factor Rho assay (denoted 'Pre-binding'),

* [TM1] and [TM2] of freely diffusing factor Rho assay (denoted '[TM1]' and '[TM2]') In this condition, there is no [TM2] from pre-binding factor Rho assay, so all the termination observed in pre-binding factor Rho assay is [TM1].

(Time histograms for each data point is shown in **Figure S3-3**)

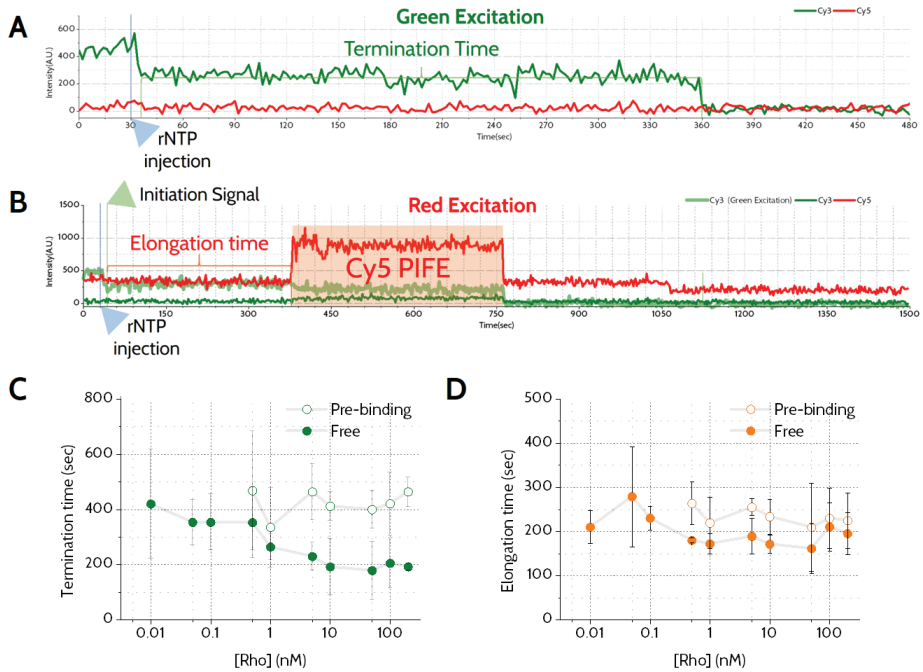


Figure 3-3-9. Kinetics information observed *in vitro* transcription assay

(A-B) Representative fluorescence time trace denoted by termination time **(A)** and elongation time **(B)**. The moment when we injected rNTP is indicated using blue arrow. Termination time is indicated using green line **(A)**, elongation time is indicated using red line **(B)**, and PIFE dwell time is shaped in red. **(B) (C-D)** The average termination time **(C)** and the average elongation time **(D)** at varying factor Rho concentration, measured pre-binding factor Rho assay (denoted 'Free') and freely diffusing factor Rho assay (denoted 'Pre-binding').

(Time histograms for each data point and summaries are shown in **Figure S3-4, Figure S3-5, Figure S3-6, Figure S3-7, Figure S3-8,** and **Figure 3-9**. Time histograms for PIFE dwell time and summaries are shown in **Figure 3-10**, **Figure 3-11**, and **Figure 3-12**)

As we mentioned, We collected kinetics information from fluorescence time trace obtaind from *in vitro* transcription assay. **(Figure 3-3-9)** We compared the kinetics traits obtained from freely diffusing factor Rho

assay and from pre-binding factor Rho assay. In the case of termination time, the average termination time from pre-binding factor Rho assay showed almost constant, or independent of factor Rho concentration. Considering all transcription termination happend in pre-binding factor Rho assay starts same state, due to factor Rho's already binding to RNA polymerase deemed as almost same each other, the time it takest to terminate transcription is regardless of the concentration of factor Rho. In other word, factor Rho concentration in pre-binding factor Rho decide not the time it takes to terminate transcription, but how much factor Rho binds to RNA polymerase, which decide the termination efficiency.

Conversely, the average time from freely diffusing factor Rho assay decreases when the concentration factor Rho increases. (Kim, Dong-Eun et al., 2001) showed that the binding kinetics of RNA to factor Rho consists of a several step before forming a stable active association. (Sharmistha Banerjee et al., 2006) Especially, a diffusion limited rate to randomly collide with factor Rho is inversely proportion to concentration of factor Rho. Referring to this result, the decreasing average termination time over concentration of factor Rho can be understood as a result of faster Rho binding, which increase the chance of collision between factor Rho and RNA.

In the case of elongation time, there is some irregular change, but all the change is within the standard deviation of each average value, which means that the change over varying concentration of factor Rho is not that meaningful, but just the perturbation between different observation. Only the tendency of

slight decreasement can be understood as the kinetics coupling and factor Rho's effect on elongation time, previously mentioned.

3.3.4. Two kinds of Termination

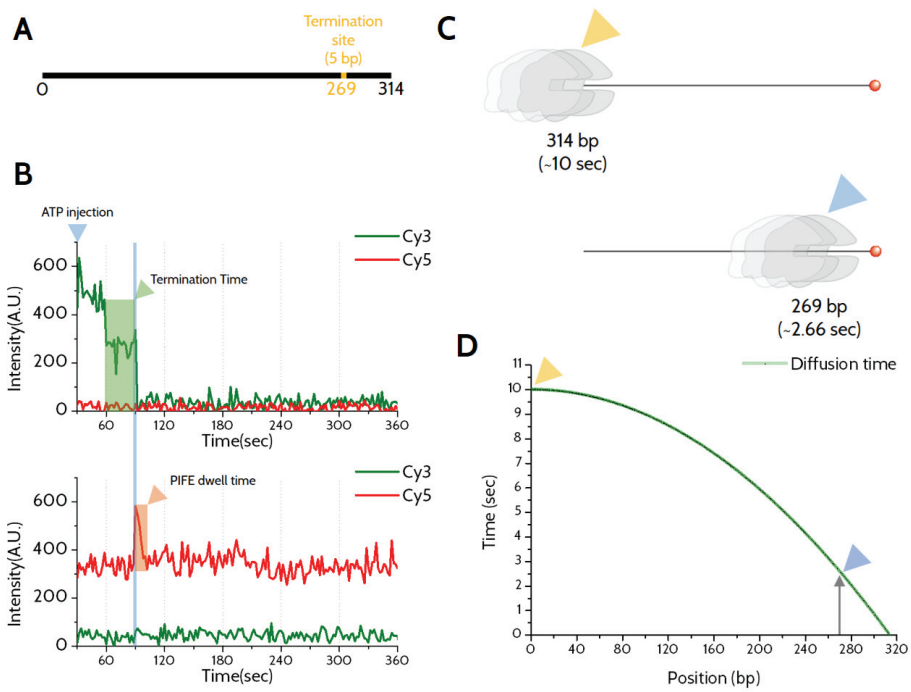


Figure 3-3-10. The diffusion time for type-2 Termination (TM2) cases

(A) The position of the termination site in the *mgtA*_WT. **(B)** Representative time trace of TM2 denoted by the termination time in green shade and by the PIFE dwell time in red shade. **(C)** Schematic diagram to show how the RNA polymerase diffuses. **(D)** The expected PIFE dwell time over the position of the termination site.

There are two kinds of the termination dependeing on whether Cy5 PIFE follows Cy3 disappearacne. Two different kinds of termination different in not only the shape of the time trace but also in the kinetics information. According to the our result showing the ratio between two kinds of termination (**Figure 3-**

3-7, **Figure 3-3-8**), type-1 termination (Cy3-dissociation only) is more common population in our condition. Moreover, the time representative traces of type-2 termination (PIFE-coupled Cy3-dissociation), which is relatively minor population, showed the more complicated shape than type-1 termination. (**Figure 3-3-9 (B)**)

The most specific characteristic of type-2 termination is that Cy3 disappearance and Cy5 PIFE happen at the same time. (**Figure 3-3-6**) We assumed that the RNA polymerase's rapid arrival at the end of Cy5 causes this simultaneous fluorescence disappearance and enhancement. (Wooyoung Kang et al., unpublished) suggested that the RNA polymerase doesn't dissociate from DNA substrate followed by the 1-dimensional diffusion at the DNA subtrate, by showing the result from the experiment that changes the length of DNA subtrates and DNA position, and by using the known model for 1D diffusion.

According to these results, it takes only one or two seconds, almost same or less than the exposure time of our experiments (**Figure 3-3-9 (A), (D)**), for RNA polymerase to arrive the end of DNA substrate. (**Figure 3-3-9 (C)**) As a result, the elongation time of the type-2 termination is too short to represent time difference between termination happens and PIFE appears, only to represent the coinstantanous fluorescence disappearance and enhancment induced by RNA polymerase.

Intriguingly, type-2 termination has distinctive attribution in kinetics which is different from type-1 termination, and read through. Firstly, the average termiantion time of type-2 termination is much shorter than that of

type-1 termination. For example, in standard condition, the average termination time of type-1 termination is about 192.0 sec, but that of type-2 termiantion is about 48.66, almost the quarter of it. (**Figure 3-3-11 (A), (B)**)

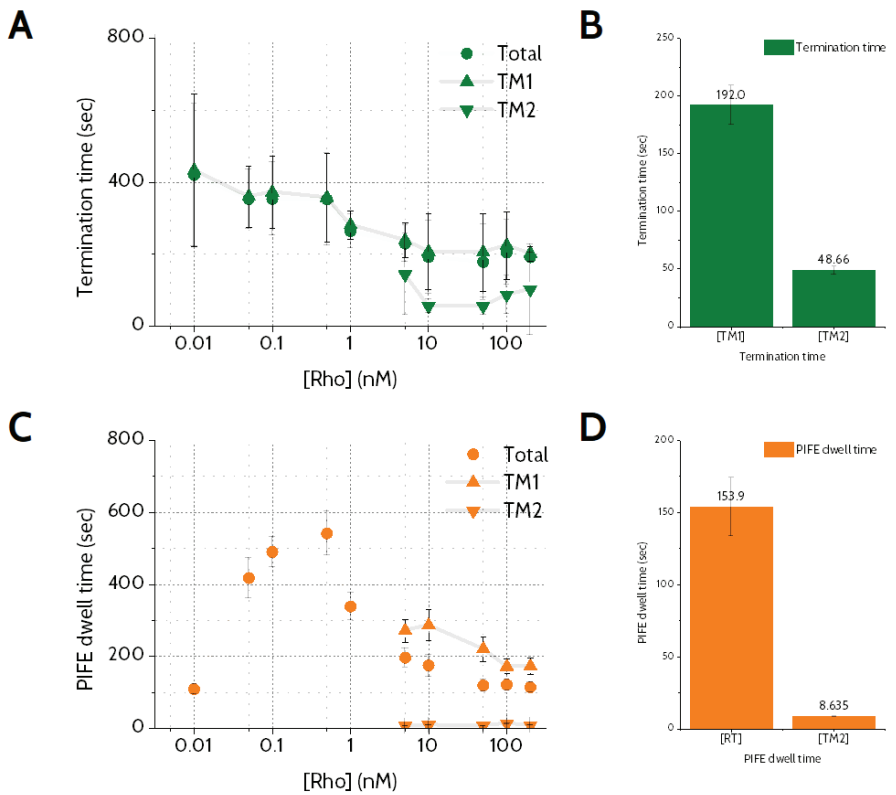


Figure 3-3-11. Termination time and PIFE dwell time in [TM1] and [TM2]

(A) Termination time of the [TM1] population and [TM2] population observed using freely diffusing factor Rho assay at varying factor Rho. (Total include both [TM1] and [TM2]. **(B)** Comparison between the average termination time of [TM1] and [TM2] when [factor Rho] is 100 nM. **(C)** PIFE dwell time of the [TM1] population and [TM2] population observed using freely diffusing factor Rho assay at varying factor Rho. ('Total' include both [TM1] and [TM2]) **(D)** Comparison between the average PIFE dwell time of [TM1] and [TM2] when [factor Rho] is 100 nM.

(Time histograms for each data point and summaries are shown in **Figure S3-13,** and **Figure 3-14**)

Taking account of the two facts that type-2 terminatian takes shorter termiantion time and that high concentration factor Rho increases the population ratio of type-2 termination, we assumed that the correlation between the concentration of factor Rho and the average time is attribute not only to the high chance of collision between factor Rho and RNA, but also to the contribution of another termination pathway.

The average PIFE dwell time, how long the Cy5-PIFE dwells, was also different between type-2 termination and read through. The average PIFE dwell time when read through happened is about 153.9 sec, but that of type-2 termination was about 8.64 sec less than a tenth in the standard condition. This difference was not that odd, because originally the arrival of RNA polymerase at the end of DNA is different from read through. The later happened as a result of completion of trnascription, but the former occured during the 1-dimensional diffusion. The difference in the context of arrival at the end DNA, which is correlated to the how RNA polymerase tightly binds to DNA template, supports the difference in PIFE dwell time.

3.3.5. factor Rho-RNA polymerase interaction

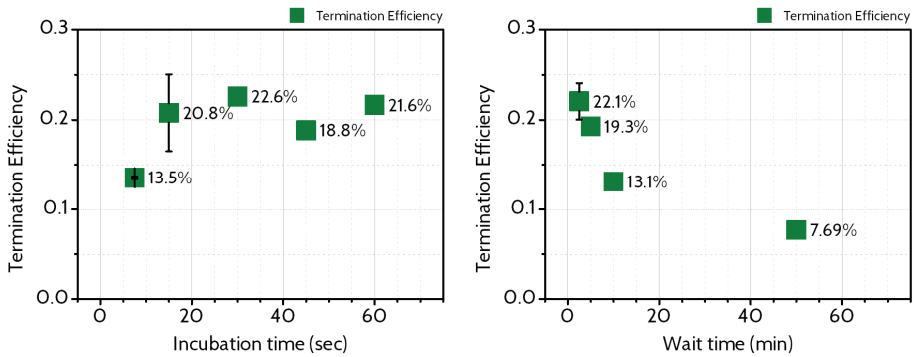


Figure 3-3-12. Affinity and stability of pre-bound factor Rho

(A-B) The result from pre-binding factor Rho assay at varying incubation time **(A)** and wait time **(B)**. Termination efficiency over incubation time showed the affinity between factor Rho and RNA polymerase. **(A)** When incubation time is 7.5 sec, the termination became median value between the background value (without Rho) and saturated value. Termination efficiency over wait time showed the stability between factor Rho and RNA polymerase. It became almost same as background value at 50 min.

To observe stable interaction between factor Rho and RNA polymerase, we conducted pre-binding factor Rho assay at varying incubation time and wait time. (**Figure 3-3-12**) Our *in vitro* transcription assay detect signal not from the interaction itself, but from the RNA release, so strickly speaking what we observed is association between factor Rho and RNA polymerase with potential for terminating transcription. In spite of chance to exclude factor Rho-RNA polymerase interaction without function of termiantion, that cance can be ignored as our main purpose of detecting this interaction is wheter termination happen.

The termination efficiency at varying incubation time shows how

strong the affinity between factor Rho and RNA polymerase. The stronger affinity the association between factor Rho and RNA polymerase has, the shorter time it takes for factor Rho to bind to RNA polymerase. Our *in vitro* assay showed that it takes less than 15 sec to achieve saturate termination efficiency, and only 7.5 sec to represent half of the saturated termination efficiency. Considering that Rho-dependent termination takes at least 200 sec (**Figure 3-3-9**), binding time of factor Rho to RNA polymerase short to enough to assumed that other steps will be the time-limiting step in termination.

Moreover, this result can give a clarity to freely diffuisng factor Rho assay with ambiguity of pre-bound factor Rho's existance. Taking acount of plausible binding time of factor Rho to RNA polymerase, factor Rho injected with rNTP binds to RNA polmyerase before transcription termination happens. This means that transcription termination observed in freely diffusing factor Rho assay included caused not only by a freely diffusing factor Rho but also by a RNA polymerase-bound factor Rho and by both a freely diffusing factor Rho and a RNA polymerase-bound factor Rho. Furthermore, this means pre-binding factor Rho assay is almost similar to the assay including both pre-binding factor Rho and injecting factor Rho with rNTP. Only difference is time interval of factor Rho's binding to RNA polymerase assumed least than 15 sec.

Experiments with wait time, which is between washing out freely diffusing factor Rho and rNTP injection, shows how stable the association between factor Rho and RNA polymerase. Once factor Rho dissociats from RNA polymerase in the absence of freely diffusing factor Rho, it's almost

difficult to binds RNA polymerase, because the effect concentration of factor Rho is almost zero. The result that factor-dependent termination happens even though the long-lasting wait after removal of freely diffusion factor Rho, which keep RNA polymerase from newly being bound, means the factor Rho stably bound to RNA polymerase. Considering most of termination happens in least than about 7 min (~420 sec), interaction between factor Rho and RNA polymerase, which lasts more than 15 min, can be considered stable condition. Stability we observed is not just factor Rho's binding, rather than bound factor Rho that can cause termination.

3.4. Conclusion and Outlook

In this chapter, we used single-molecule in vitro transcription assay to observed Rho-dependent termination. In **Chapter 2**, we have already confirmed our assay, and even in the presence of factor Rho, our assay well worked, distinguishing the molecules that termination happend (**Figure 3-3-5**, **Figure 3-3-6**) and those that read through happend (**Figure 3-3-4**). (**Figure 3-3-3**) To confirm the termination events we observed is caused by factor Rho, we should compare the termination eifficiency. In our thesis termination efficiency is the portion of the number of molecules which happens termination in total molecules with initiation signal (**Figure 3-3-1**, **Figure 3-3-2**) Factor Rho caused termiantion, or the termiantion efficiency increased when we injected factor Rho. (**Figure 3-3-7**)

We observed how fast the factor Rho binds to RNA polymerase and

how stable the association between factor Rho and RNA polymerase by using pre-binding factor Rho assay. Our results show than it takes less than 7 sec for factor Rho to bind, and it takes over about 50 min for all the association and RNA polymerase to dissociate. (**Figure 3-3-12**) Reffering to these data, the results from freely diffusing factor Rho assay were included both termination involving factor Rho-RNA polymerase association and that not involving.

We conducted both pre-binding factor Rho assay and freely diffusing factor Rho assay at the varying concentraiton of factor Rho. We observed that the termination efficeincy increased with the increase in concentration of factor Rho. (**Figure 3-3-8**) We understood that in the case of pre-binding factor Rho assay, more the concetration of factor Rho, the higher the probaility for factor Rho to bind to RNA polymerase, which is candidate of occuring termiantion. Freely diffusing factor Rho assay, where molecules are exposed to the freely diffusing factor Rho during transcription, the faster Rho's binding to RNA polymerase can be thought as a candidate of factor that make the average termiantion time faster. We asumed that this accelerating the termination reaction enhance the probability of termination.

When we conducted freely diffusing factor Rho assay, we found two different kinds of trace assigned as transcription termination, which means there were two distinct fate for transcription termination. To understand the different between two different classes of termination, we should pay attention to the different characteristic, whether threre is Cy5-PIFE just after the Cy3 signal's disappearance. (**Figure 3-3-6**) As Cy5-PIFE means the RNA

polymerase's proximity to the end of Cy5 and Cy3 signal's disappearance can be interpreted as RNA release, we can assume that the differences come from where the RNA polymerase is after the RNA release.

We thought type-1 termination which is not followed by Cy5-PIFE (**Figure 3-3-5**) means not only RNA but also RNA polymerase dissociates from template DNA after termination happens. While type-2 termination followed by Cy5-PIFE can be interpreted by RNA polymerase remains on the template DNA even after the termination. (**Figure 3-3-10**) Type-2 termination are also observed in the our internal data of the experiment studying the intrinsic termination. (Wooyoung Kang et al., unpublished) In the study of intrinsic termination, type-2 termination is main portion of transcription termination.

There are interesting traits in kinetics of type-2 termination. (**Figure 3-3-11**) Compared to the type-1 termination, the average time of type-2 termination is faster. This results means that not only faster factor Rho's binding to RNA polymerase, but also the increase in the population ratio of faster type-2 termination also contributed to the faster the average termination time only to increase the termination efficiency. Besies, the difference in PIFE dwell time represents that there is different RNA polymerase's binding to template DNA when read through happens and when type-2 termiation happens.

Our resutls showed that type-2 termination is only observed in freely diffusing factor Rho assay, not in pre-binding factor Rho assay. The main difference between two assay is whether there is freely diffusing factor Rho in buffer. In other words, single factor Rho participated in transcription

mechanism in pre-binding factor Rho assay, so we assumed that multiple factor Rho is required to occure type-2 termination. However, to confirm this we need to validate several factor Rho is involved in transcription termination in real.

It is useful to find what other factor causes type-2 termination. As type-2 termination take a major part in the intrinsic termination, finding a factor which cause type-2 termination can give a correlation between Rho-dependent terminator and intrinsinc terminator. Moreover, Rho-dependent terminator's similarity to intrinsic terminator implies two different terminataor may share structure or mechanism in trascription termination. This implication, though, need to be supported by structure evidence.

The difference between the results from pre-binding factor Rho assay and from freely diffuisng factor Rho assay was also interesting. As we mentioned, data from pre-binding factor Rho assay only show the termination mediated by assciation between factor Rho and RNA polymerase, but that from freely diffusing factor Rho assay show the termination not depending on whether association between factor Rho and RNA polymerase mediates the reaction. Therefore, these several assay can elucidate what is going on in the transcription termination.

Comparing results from pre-binding factor Rho to those from freely diffusing factor Rho assay, when the termination happens only by pre-bound factor Rho has lower efficiency and slower. Although it's early to say wheter there is a causal relation between efficiency and speed, only a pre-bound factor Rho showed a relatively inefficient performance in the respct of termination.

Considering that factor Rho also binds to the factor Rho in freely diffusing factor Rho assay, it is still difficult to say what the association between factor Rho and RNA polymerase takes a role in Rho-dependnet termination.

In spite of ambiguity in interpreting the data, we can assume some model for explaining these two kinds of pathway. One of the plausible description is there is two different pathway in trasncription termination, whether there is additional factor Rho to the RNA polymerase-bound factor Rho. When there is no other save RNA polymerase-bound factor Rho, RNA-polymerase-bound factor Rho solely cause transcription termination. While there is another factor Rho can involve in the transcription termination, the transcription termination happen more often and fasten than transcription termination caused by sole RNA polymerase bound factor Rho.

This model, though, need to shore more evidences to clarify the detail of elucisation. For instance, it's need to explain how RNA polymerase-bound factor Rho cause transcription, because so-called RNA polymerase-mediated pathway still yet to be explained. One possible idea is even RNA polymerase-bound factor Rho also need to bind *rut* site in RNA transcript to terminate transcription. This can be confirmed by using DNA substrate removed *rut* site, and results are shown in **Chpater 5**. Another question is that is there any pathway that happens exclusing association between factor Rho and RNA polymerase. This also cam be examined by blocking factor Rho's binding to RNA polymerase, but this is something more difficult than excluding factor Rho's binding to some position of RNA, like temporally exclusing by injecting

factor Rho before transcrip is formed or using DNA substrate with mutation in *rut* site. In spite of this difficulty, we found and designed some method to observe the transcription termination without association between factor Rho and RNA polymerase in **Chapter 4**.

Ultimately, we assumed that our model can change the paradigm of understanding Rho-dependent termination. Traditionally, the debate on the mecahnism of Rho-dependent transcription termination has focused on which mutually exclusive model is right or dominant in real, RNA-mediated pathway or RNA polymerase-mediated pathway. Howevr, our model in this thesis will suggested that existance of each pathway can be validated by using our *in vitro* transcription assay, and that these two pathway is not mutually exclusive but synergic each other. Our results from freely diffusing factor Rho assay in this chapter showed that transcription termination happend more efficient and more fast than that happens in pre-binding factor Rho assay. This emergent property of factor Rho is novel concept in Rho-dependent termiation.

Appendix

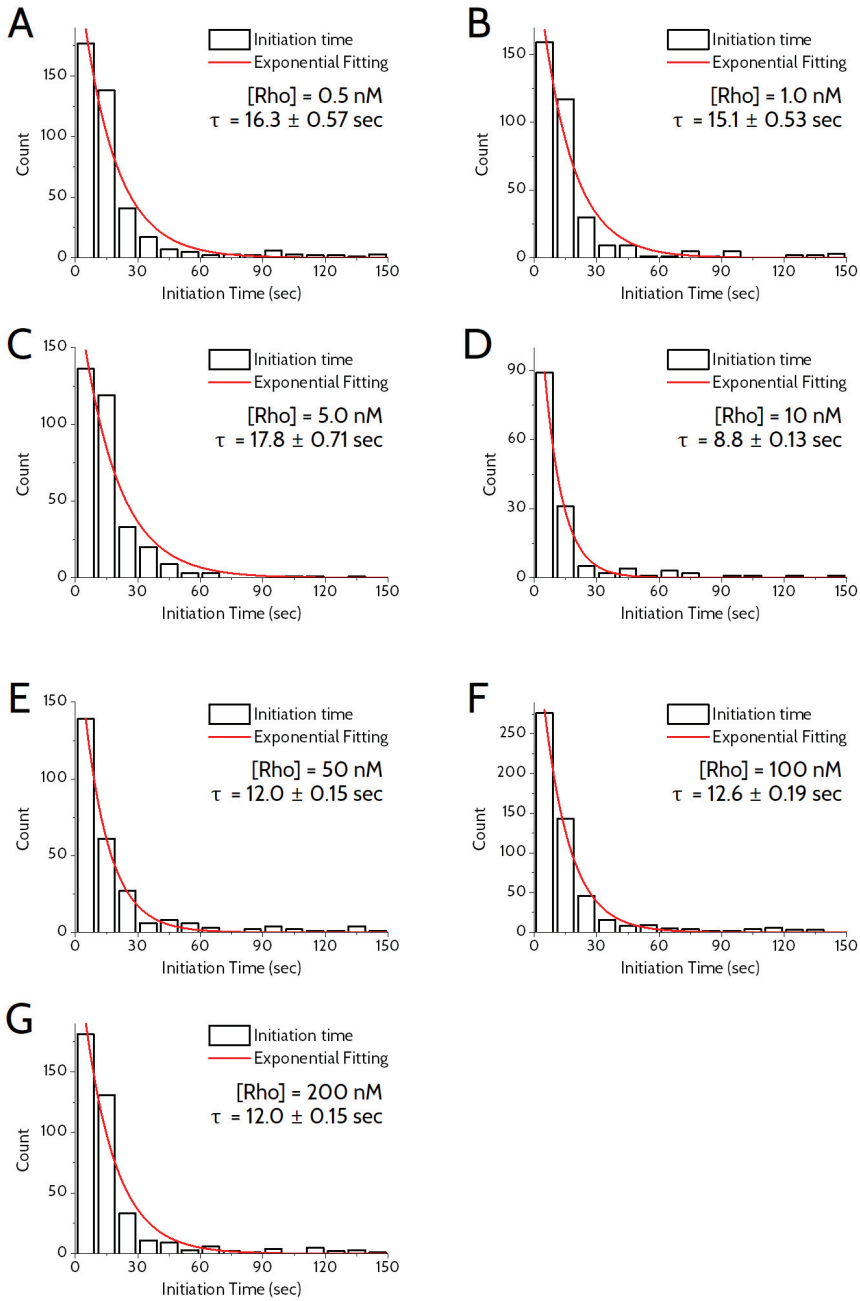
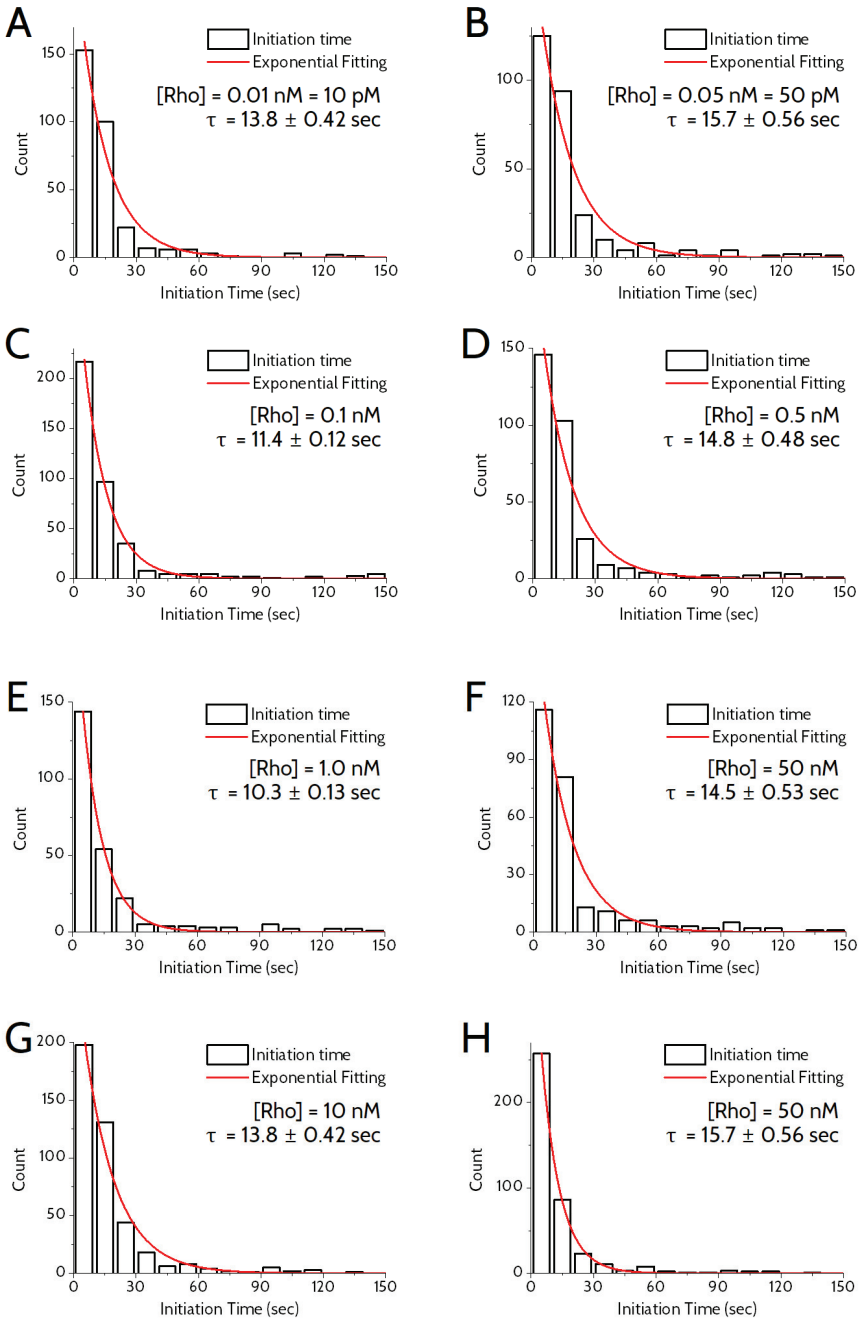


Figure S3-1. Distribution of initiation time (τ) for varying [Rho] (pre-binding)


A
Initiation time
Exponential Fitting
[Rho] = 0.01 nM = 10 pM
τ = 13.8 ± 0.42 sec
B
Initiation time
Exponential Fitting
[Rho] = 0.05 nM = 50 pM
τ = 15.7 ± 0.56 sec
C
Initiation time
Exponential Fitting
[Rho] = 0.1 nM
τ = 11.4 ± 0.12 sec
D
Initiation time
Exponential Fitting
[Rho] = 0.5 nM
τ = 14.8 ± 0.48 sec
E
Initiation time
Exponential Fitting
[Rho] = 1.0 nM
τ = 10.3 ± 0.13 sec
F
Initiation time
Exponential Fitting
[Rho] = 50 nM
τ = 14.5 ± 0.53 sec
G
Initiation time
Exponential Fitting
[Rho] = 10 nM
τ = 13.8 ± 0.42 sec
H
Initiation time
Exponential Fitting
[Rho] = 50 nM
τ = 15.7 ± 0.56 sec
Count
Initiation Time (sec)

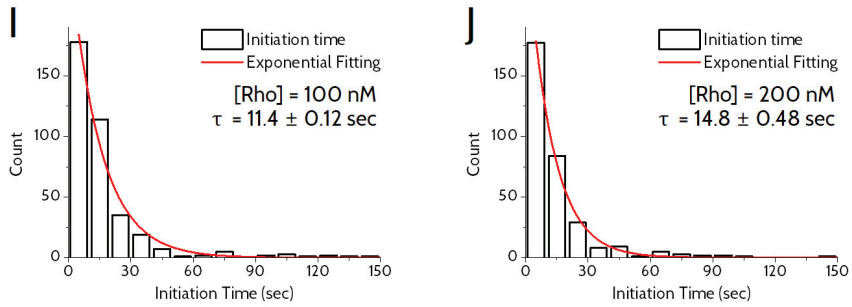


Figure S3-2. Distribution of initiation time (τ) for varying [Rho] (free)

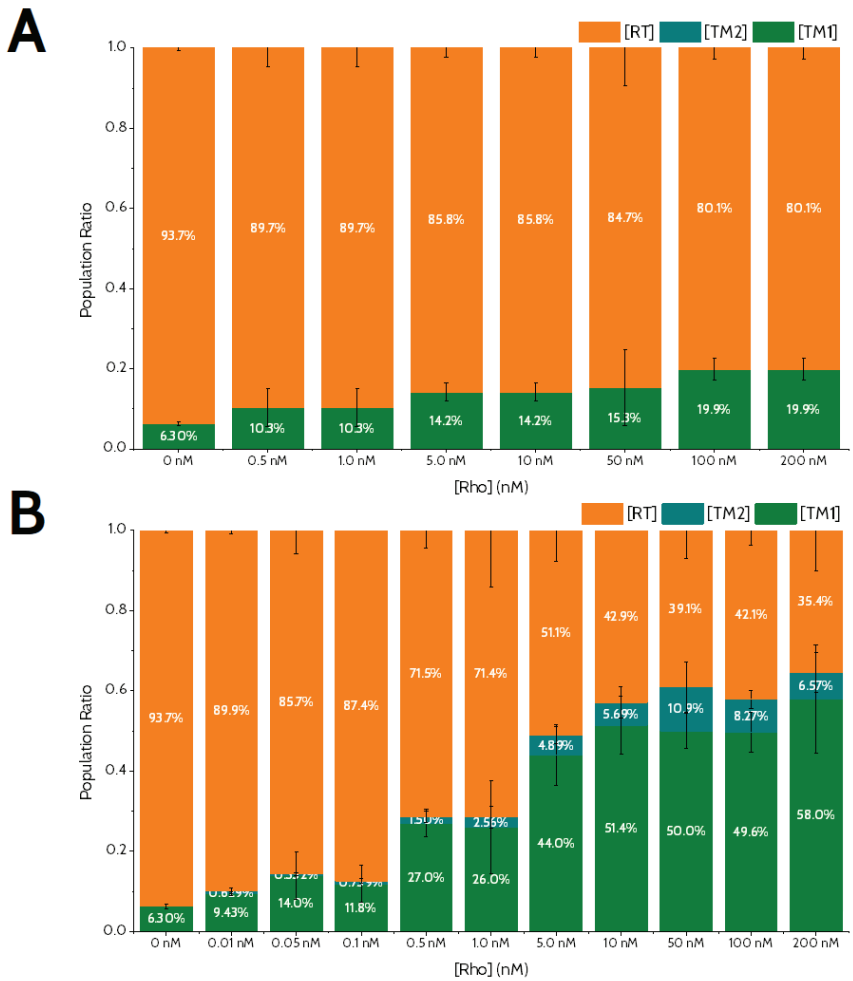


Figure S3-3. Population Ratio for varying [Rho]

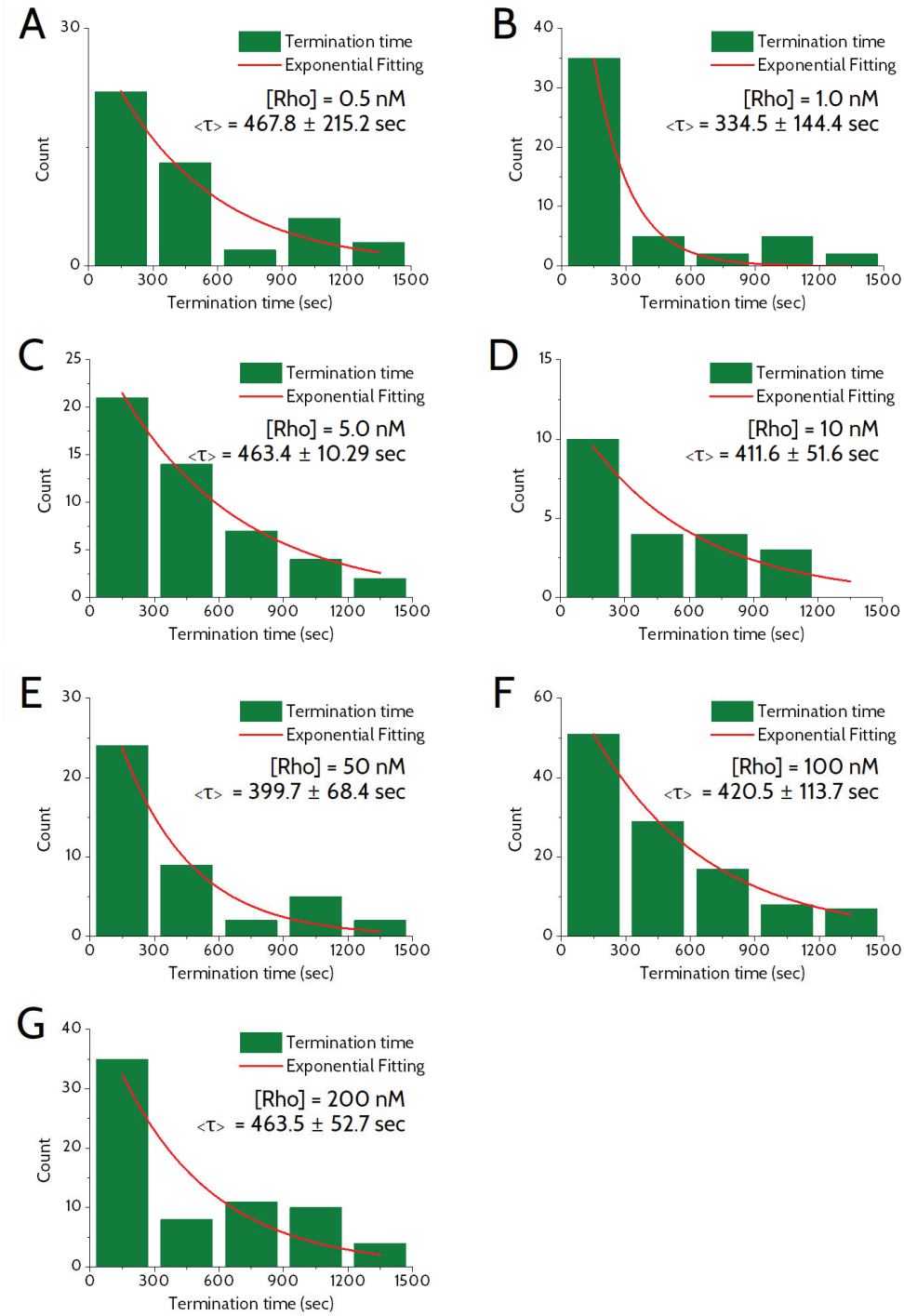
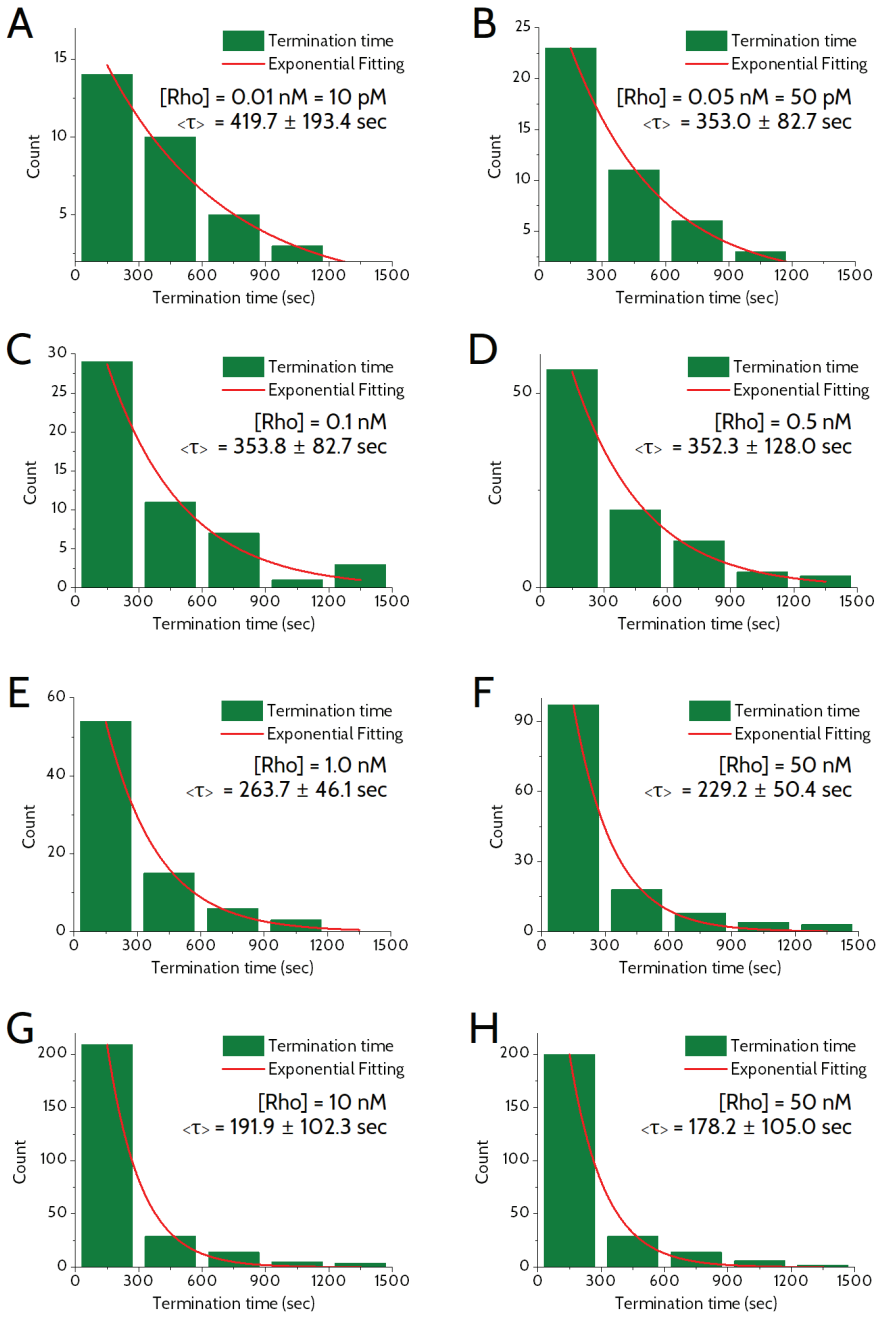


Figure S3-4. Distribution of termination time (τ) for varying [Rho] (pre-binding)


A
Termination time
Exponential Fitting
[Rho] = 0.01 nM = 10 pM
<τ> = 419.7 ± 193.4 sec
Count
Termination time (sec)
B
Termination time
Exponential Fitting
[Rho] = 0.05 nM = 50 pM
<τ> = 353.0 ± 82.7 sec
Count
Termination time (sec)
C
Termination time
Exponential Fitting
[Rho] = 0.1 nM
<τ> = 353.8 ± 82.7 sec
Count
Termination time (sec)
D
Termination time
Exponential Fitting
[Rho] = 0.5 nM
<τ> = 352.3 ± 128.0 sec
Count
Termination time (sec)
E
Termination time
Exponential Fitting
[Rho] = 1.0 nM
<τ> = 263.7 ± 46.1 sec
Count
Termination time (sec)
F
Termination time
Exponential Fitting
[Rho] = 50 nM
<τ> = 229.2 ± 50.4 sec
Count
Termination time (sec)
G
Termination time
Exponential Fitting
[Rho] = 10 nM
<τ> = 191.9 ± 102.3 sec
Count
Termination time (sec)
H
Termination time
Exponential Fitting
[Rho] = 50 nM
<τ> = 178.2 ± 105.0 sec
Count
Termination time (sec)

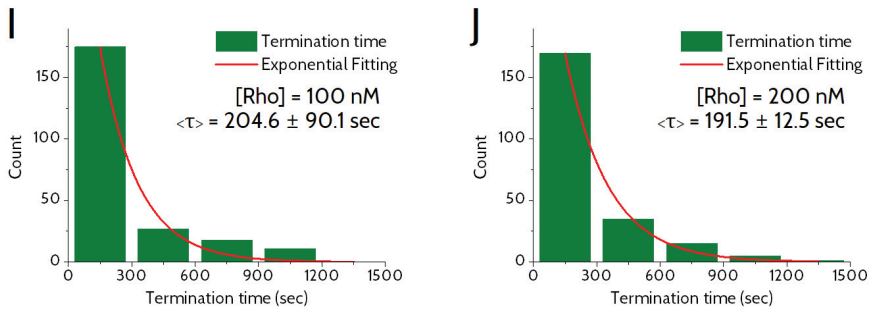


Figure S3-5. Distribution of termination time (τ) for varying [Rho] (free)

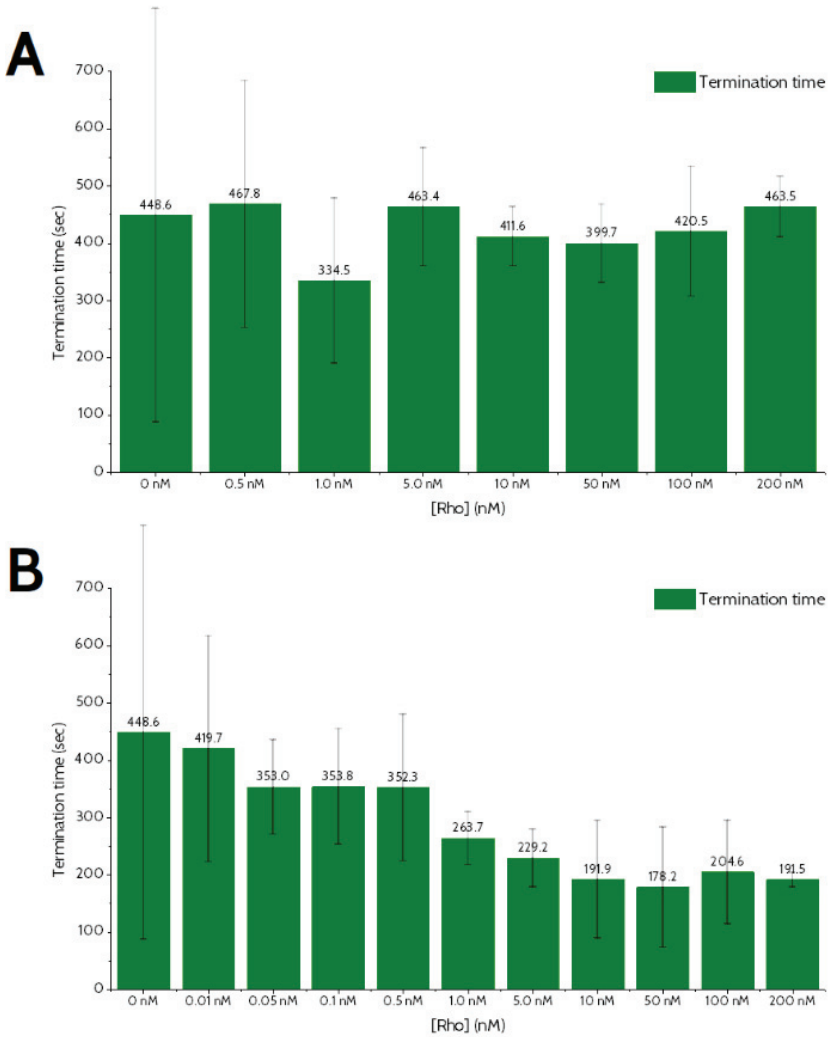


Figure S3-6. Termination time (τ) for varying [Rho]

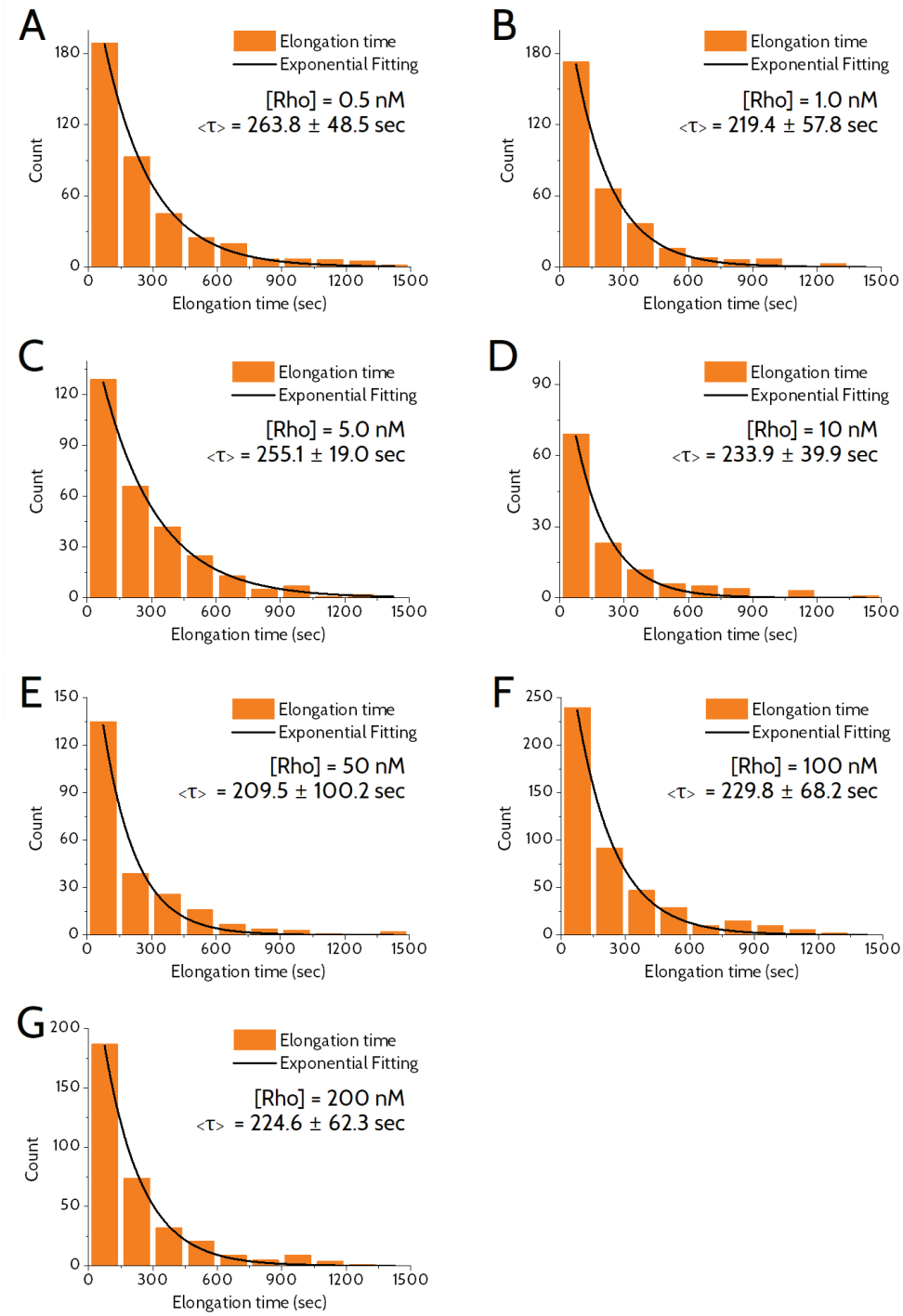
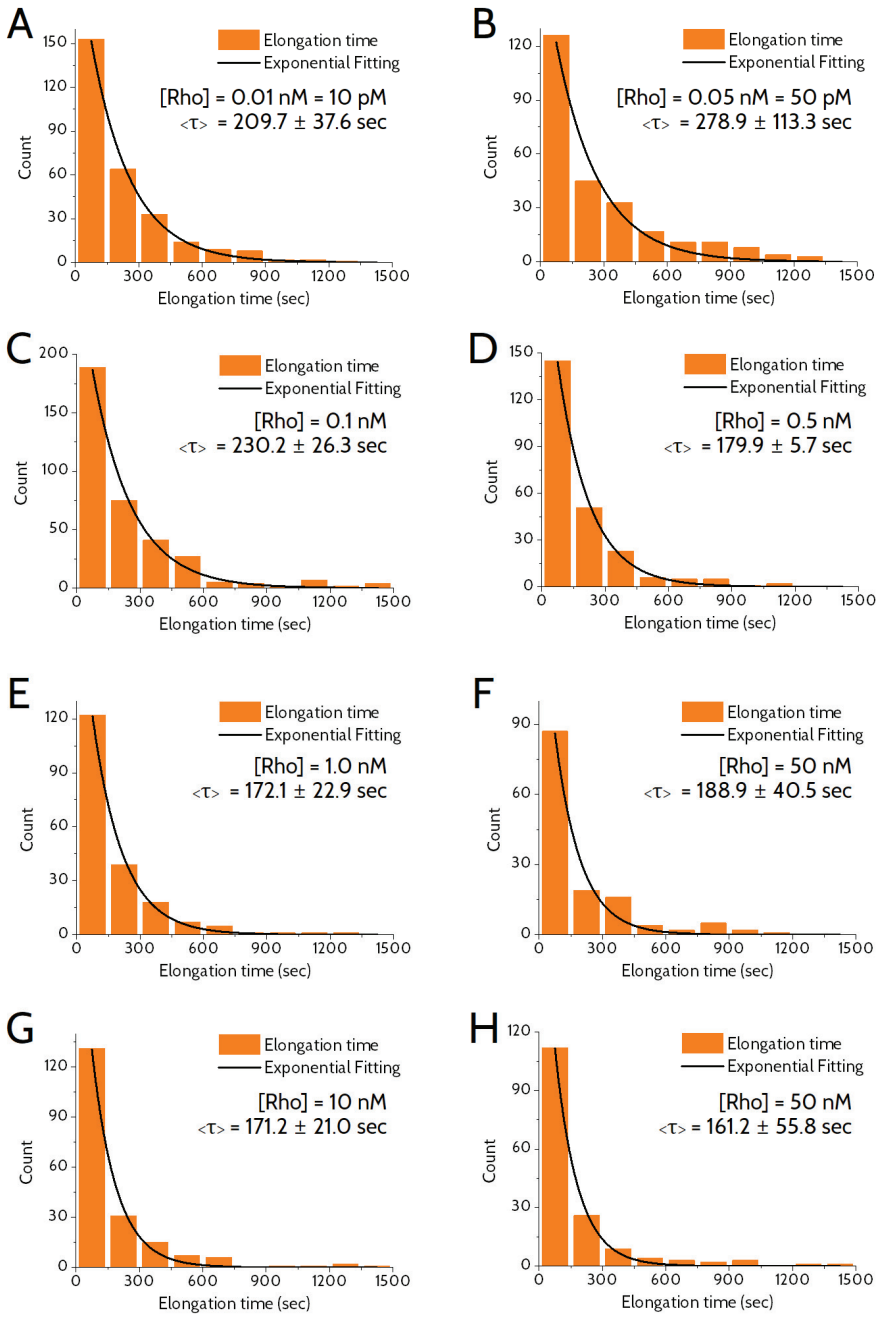


Figure S3-7. Distribution of elongation time (τ) for varying [Rho] (pre-binding)


A
Elongation time
Exponential Fitting
[Rho] = 0.01 nM = 10 pM
<τ> = 209.7 ± 37.6 sec
Count
Elongation time (sec)
B
Elongation time
Exponential Fitting
[Rho] = 0.05 nM = 50 pM
<τ> = 278.9 ± 113.3 sec
Count
Elongation time (sec)
C
Elongation time
Exponential Fitting
[Rho] = 0.1 nM
<τ> = 230.2 ± 26.3 sec
Count
Elongation time (sec)
D
Elongation time
Exponential Fitting
[Rho] = 0.5 nM
<τ> = 179.9 ± 5.7 sec
Count
Elongation time (sec)
E
Elongation time
Exponential Fitting
[Rho] = 1.0 nM
<τ> = 172.1 ± 22.9 sec
Count
Elongation time (sec)
F
Elongation time
Exponential Fitting
[Rho] = 50 nM
<τ> = 188.9 ± 40.5 sec
Count
Elongation time (sec)
G
Elongation time
Exponential Fitting
[Rho] = 10 nM
<τ> = 171.2 ± 21.0 sec
Count
Elongation time (sec)
H
Elongation time
Exponential Fitting
[Rho] = 50 nM
<τ> = 161.2 ± 55.8 sec
Count
Elongation time (sec)

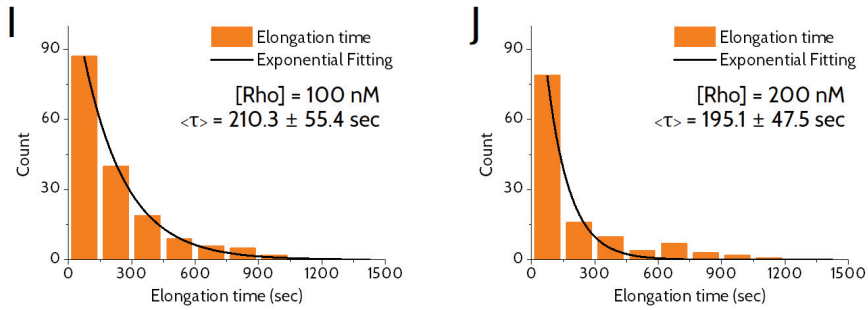


Figure S3-8. Distribution of elongation time (τ) for varying [Rho] (free)

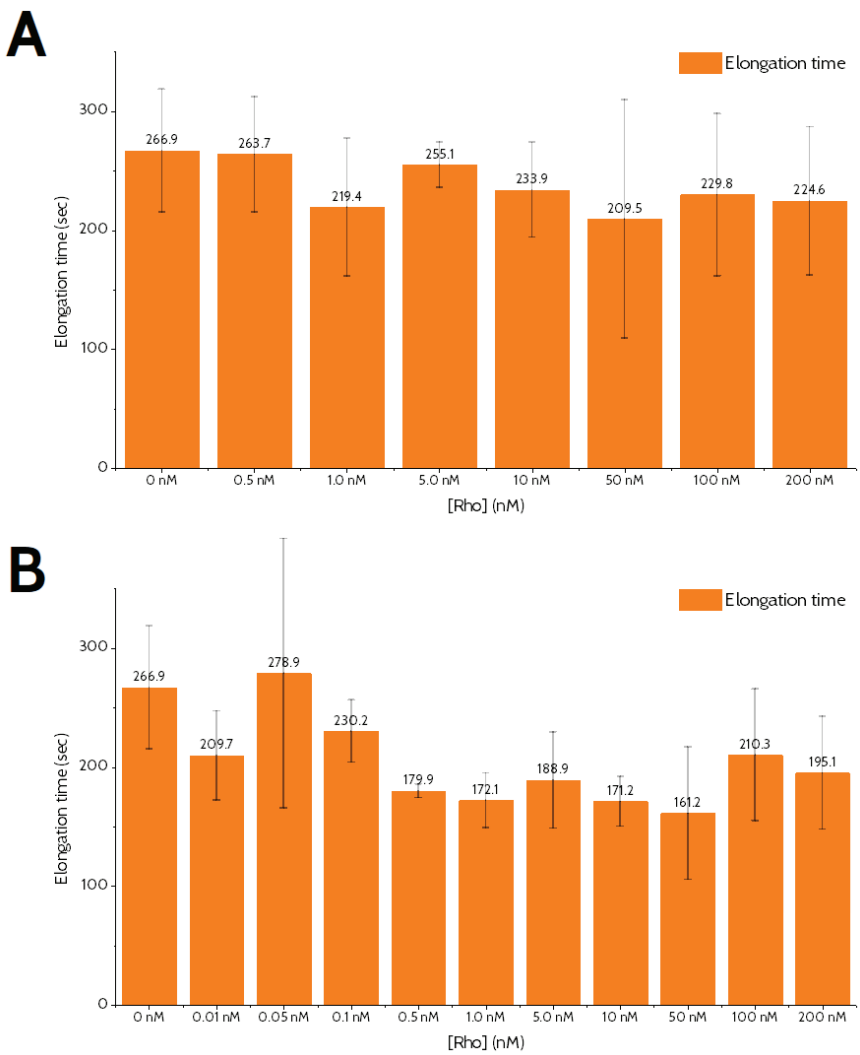


Figure S3-9. Elongation time (τ) for varying [Rho]

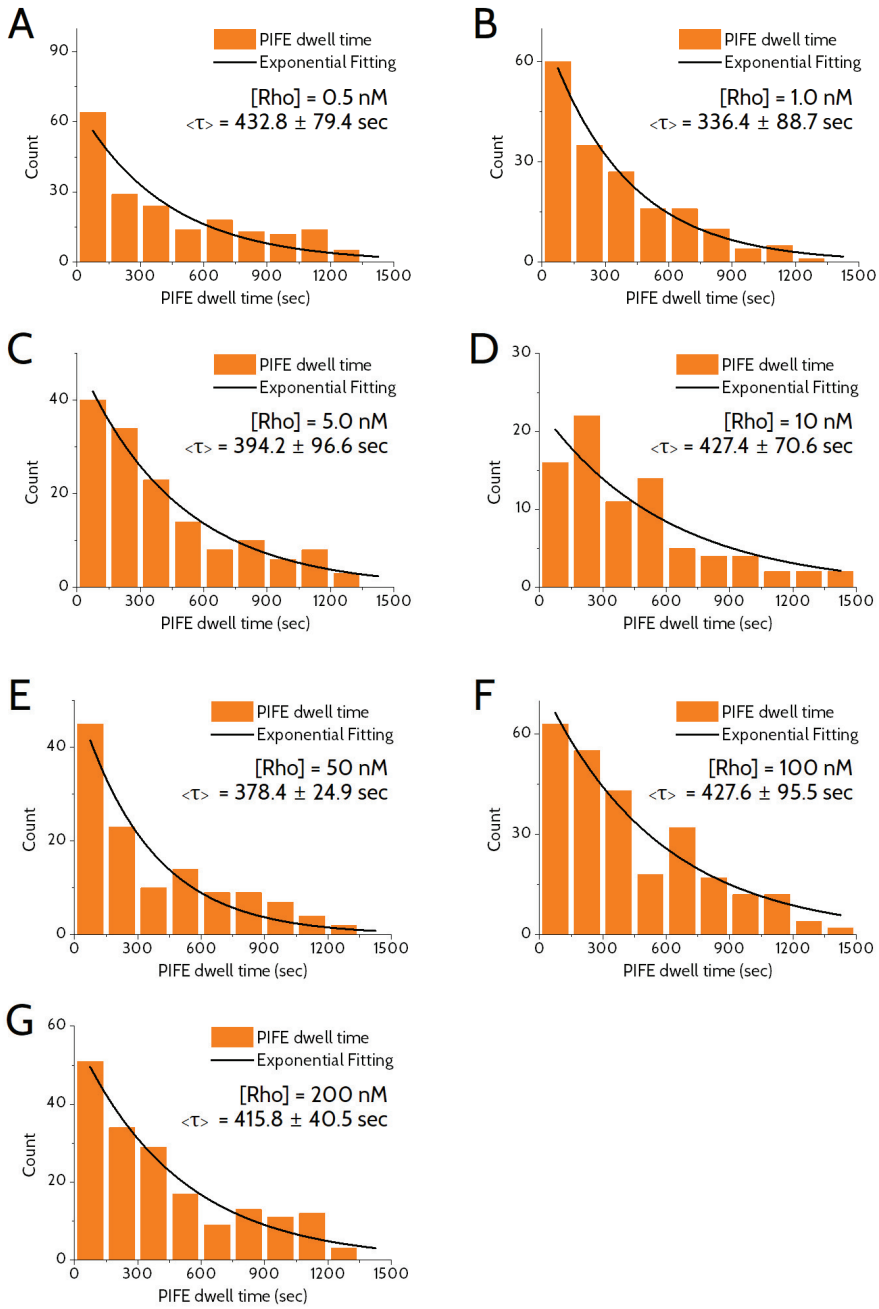
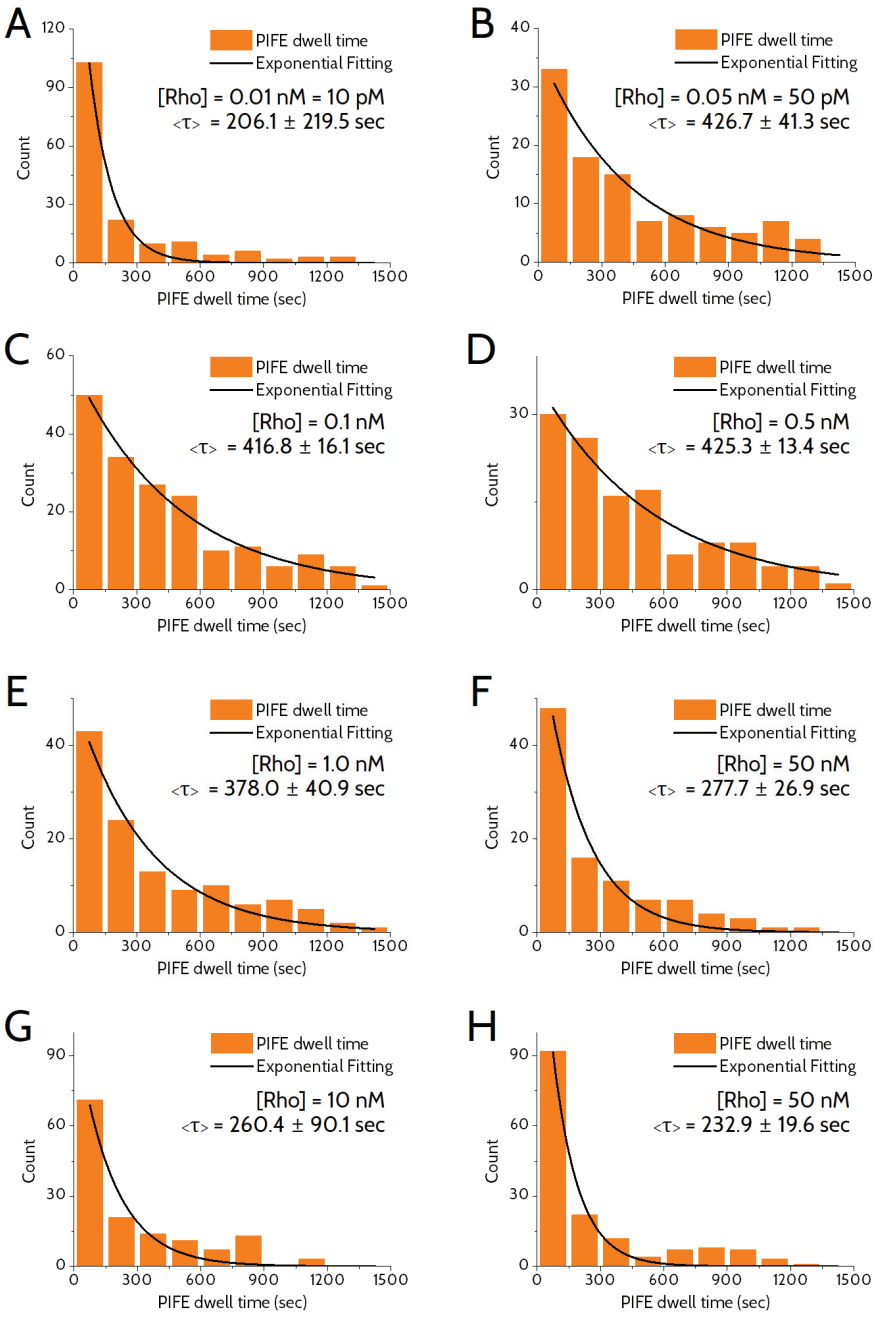


Figure S3-10. Distribution of PIFE dwell time (τ) for varying [Rho] (pre-binding)


A
PIFE dwell time
Exponential Fitting
[Rho] = 0.01 nM = 10 pM
<τ> = 206.1 ± 219.5 sec
Count
PIFE dwell time (sec)
B
PIFE dwell time
Exponential Fitting
[Rho] = 0.05 nM = 50 pM
<τ> = 426.7 ± 41.3 sec
Count
PIFE dwell time (sec)
C
PIFE dwell time
Exponential Fitting
[Rho] = 0.1 nM
<τ> = 416.8 ± 16.1 sec
Count
PIFE dwell time (sec)
D
PIFE dwell time
Exponential Fitting
[Rho] = 0.5 nM
<τ> = 425.3 ± 13.4 sec
Count
PIFE dwell time (sec)
E
PIFE dwell time
Exponential Fitting
[Rho] = 1.0 nM
<τ> = 378.0 ± 40.9 sec
Count
PIFE dwell time (sec)
F
PIFE dwell time
Exponential Fitting
[Rho] = 50 nM
<τ> = 277.7 ± 26.9 sec
Count
PIFE dwell time (sec)
G
PIFE dwell time
Exponential Fitting
[Rho] = 10 nM
<τ> = 260.4 ± 90.1 sec
Count
PIFE dwell time (sec)
H
PIFE dwell time
Exponential Fitting
[Rho] = 50 nM
<τ> = 232.9 ± 19.6 sec
Count
PIFE dwell time (sec)

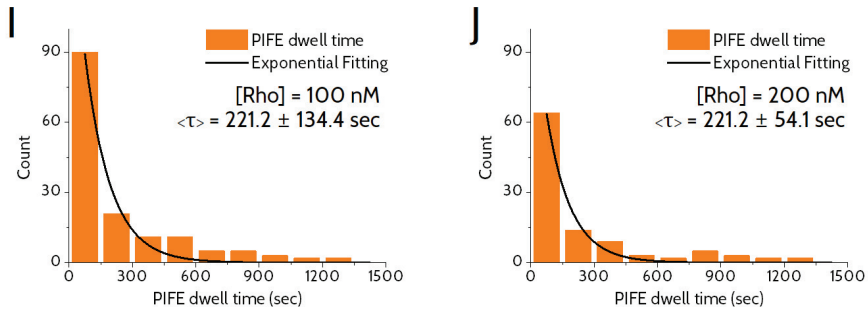


Figure S3-11. Distribution of PIFE dwell time (τ) for varying [Rho] (free)

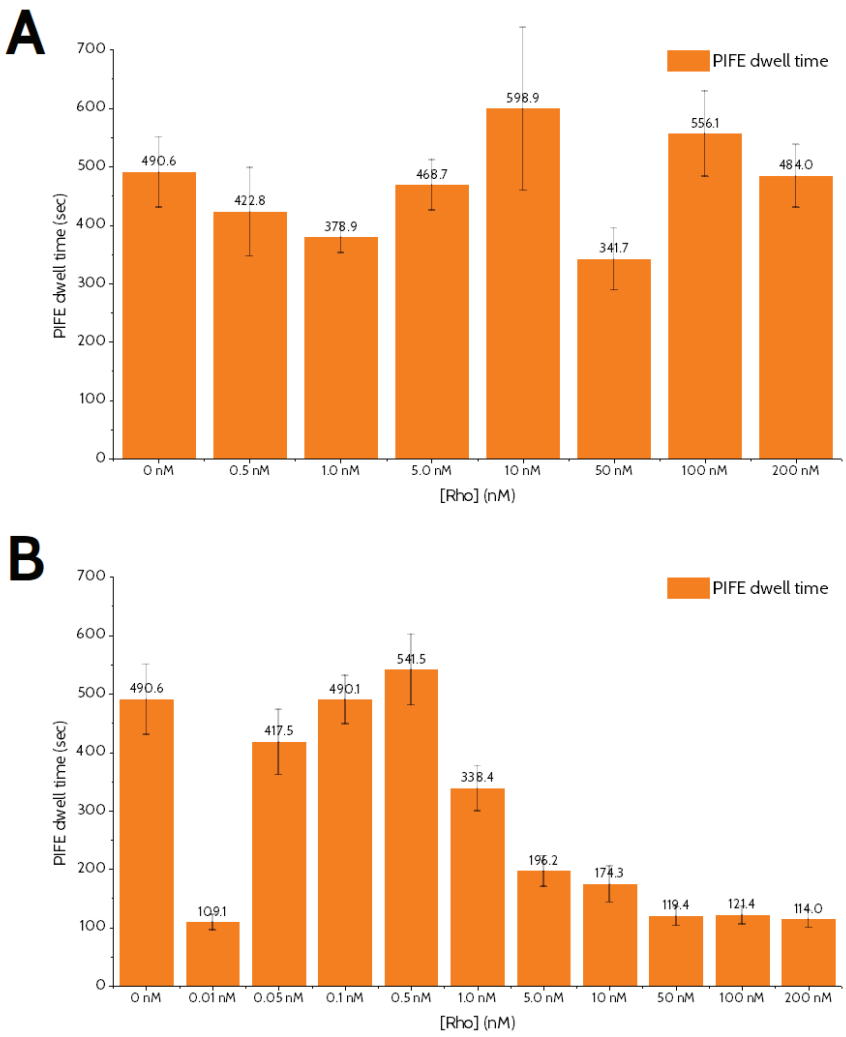


Figure S3-12. PIFE dwell time (τ) for varying [Rho]

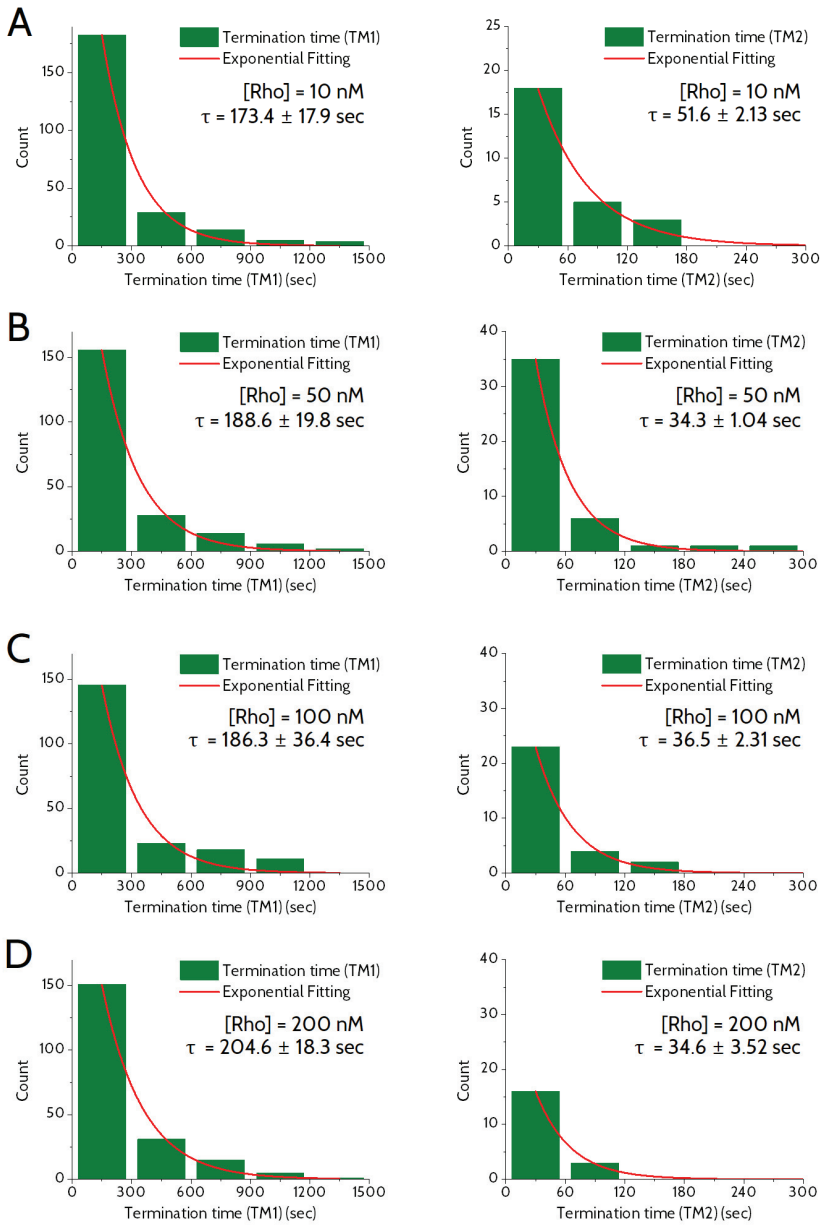


Figure S3-13. Distribution of Termination time (τ) for varying [Rho] (free)

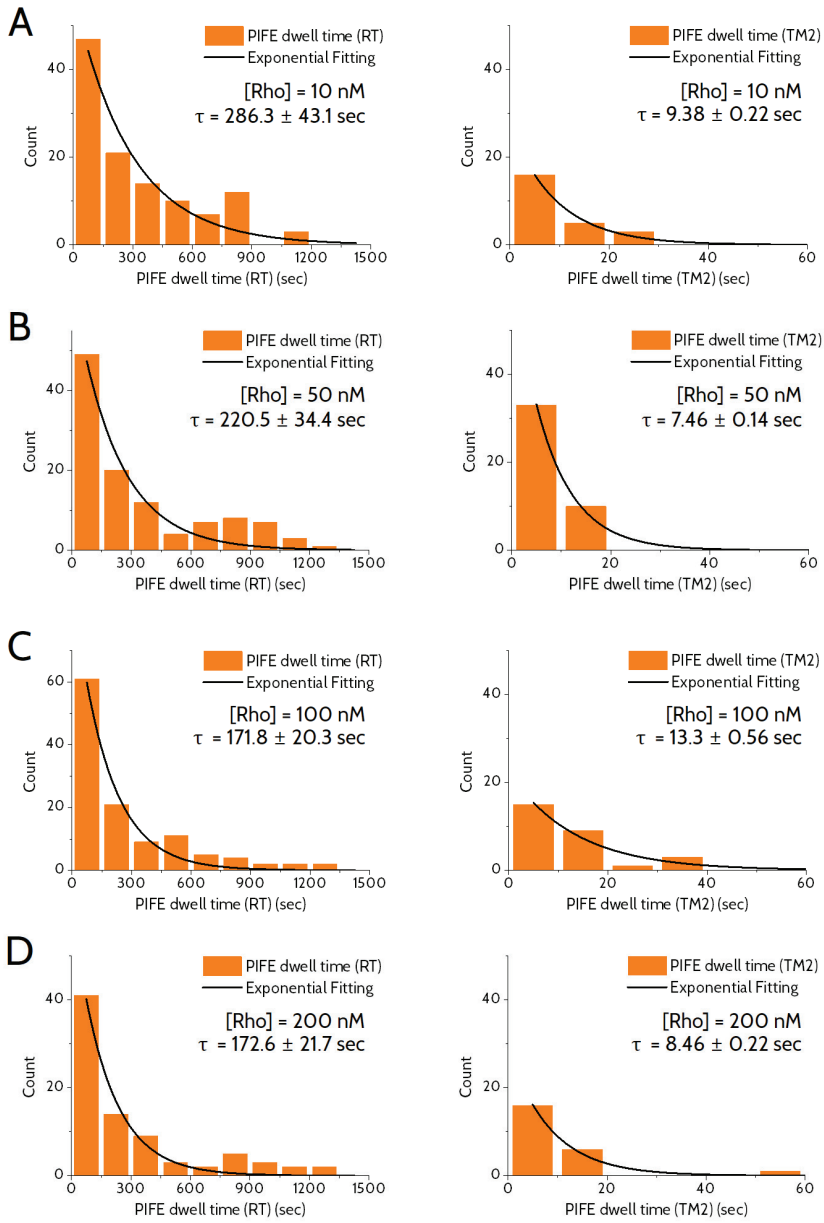


Figure S3-14. Distribution of PIFE dwell time (τ) for varying [Rho] (free)

<Detailed description for each additional data>

Figure S3-1. Distribution of initiation time (τ) for varying [Rho] (pre-binding).
Elongation complex is incubated with factor Rho in **(A)** 0.5 nM, **(B)** 1.0 nM, **(C)** 5.0 nM, **(D)** 10 nM, **(E)** 50 nM, **(F)** 100 nM, and **(G)** 200 nM, followed by washing out unbounded factor Rho before rNTP injection (200 μM).

Figure S3-2. Distribution of initiation time (τ) for varying [Rho] (free).
Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with factor Rho in **(A)** 0.01 nM (=10 pM), **(B)** 0.05 nM (= 50 pM), **(C)** 0.1 nM, **(D)** 0.5 nM, **(E)** 1.0 nM, **(F)** 5.0 nM, **(G)** 10 nM, **(H)** 50 nM, **(I)** 100 nM and **(J)** 200 nM.

Figure S3-3. Population Ratio for varying [Rho]
Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with varying concentration of factor Rho from pre-binding factor Rho assay **(A)** and freely diffusing factor Rho assay **(B)**.

Figure S3-4. Distribution of termination time (τ) for varying [Rho] (pre-binding)
Elongation complex is incubated with factor Rho in **(A)** 0.5 nM, **(B)** 1.0 nM, **(C)** 5.0 nM, **(D)** 10 nM, **(E)** 50 nM, **(F)** 100 nM, and **(G)** 200 nM, followed by washing out unbounded factor Rho before rNTP injection (200 μM).

Figure S3-5. Distribution of termination time (τ) for varying [Rho] (free)
Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with factor Rho in **(A)** 0.01 nM (=10 pM), **(B)** 0.05 nM (= 50 pM), **(C)** 0.1 nM, **(D)** 0.5 nM, **(E)** 1.0 nM, **(F)** 5.0 nM, **(G)** 10 nM, **(H)** 50 nM, **(I)** 100 nM and **(J)** 200 nM.

Figure S3-6. Termination time (τ) for varying [Rho]
Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with varying concentration of factor Rho from pre-binding factor Rho assay **(A)** and freely diffusing factor Rho assay **(B)**.

Figure S3-7. Distribution of elongation time (τ) for varying [Rho] (pre-binding)
Elongation complex is incubated with factor Rho in **(A)** 0.5 nM, **(B)** 1.0 nM, **(C)** 5.0 nM, **(D)** 10 nM, **(E)** 50 nM, **(F)** 100 nM, and **(G)** 200 nM, followed by washing out unbounded factor Rho before rNTP injection (200 μM).

Figure S3-8. Distribution of elongation time (τ) for varying [Rho] (free)
Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with factor Rho in **(A)** 0.01 nM (=10 pM), **(B)** 0.05 nM (= 50 pM), **(C)** 0.1 nM, **(D)** 0.5 nM, **(E)** 1.0 nM, **(F)** 5.0 nM, **(G)** 10 nM, **(H)** 50 nM, **(I)** 100 nM and **(J)** 200 nM.

Figure S3-9. Elongation time (τ) for varying [Rho]
Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with varying concentration of factor Rho from pre-binding factor Rho assay **(A)** and freely diffusing factor Rho assay **(B)**.

Figure S3-10. Distribution of PIFE dwell time (τ) for varying [Rho] (pre-binding)
Elongation complex is incubated with factor Rho in **(A)** 0.5 nM, **(B)** 1.0 nM, **(C)** 5.0 nM, **(D)** 10 nM, **(E)** 50 nM, **(F)** 100 nM, and **(G)** 200 nM, followed by washing out unbounded factor Rho before rNTP injection (200 μM).

Figure S3-11. Distribution of PIFE dwell time (τ) for varying [Rho] (free)
Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with factor Rho in **(A)** 0.01 nM (=10 pM), **(B)** 0.05 nM (= 50 pM), **(C)** 0.1 nM, **(D)** 0.5 nM, **(E)** 1.0 nM, **(F)** 5.0 nM, **(G)** 10 nM, **(H)** 50 nM, **(I)** 100 nM and **(J)** 200 nM.

Figure S3-12. PIFE dwell time (τ) for varying [Rho]
Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with varying concentration of factor Rho from pre-binding factor Rho assay **(A)** and freely diffusing factor Rho assay **(B)**.

Figure S3-13. Distribution of termination time (τ) for varying [Rho] (free)

Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with factor Rho in **(A)** 10 nM, **(B)** 50 nM, **(C)** 100 nM, **(D)** 200 nM. Each panel contained two cases, one collected from type-1 termination (TM1) cases, **(left)** and another collected from type2-termination (TM2) cases. **(right)**

Figure S3-14. Distribution of PIFE dwell time (τ) for varying [Rho] (free)

Elongation complex is re-initiate the transcription by injecting rNTP (200 μM) with factor Rho in **(A)** 10 nM, **(B)** 50 nM, **(C)** 100 nM, **(D)** 200 nM. Each panel contained two cases, one collected from read through (RT) cases, **(left)** and another collected from type2-termination (TM2) cases. **(right)**

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<Journal>

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Chapter 4

Factor Rho mutants Experiment

4.1. Introduction

There have been several studies on understanding the function of each domain in the factor Rho. One of the way to shed light on these study is using factor Rho mutants which has a point mutation in specific domain. By comparing between the result from wild-type factor Rho and factor Rho mutants, we can understand what function each domain of factor Rho has. (Chalissery et al., 2007) developed several kinds of factor Rho which has point mutation in each specific domain and studied the function of each domain of the factor Rho.

These kinds of mutation are not only applied to studying the function of each domain, but also to masking the association between factor Rho and RNA polymerase. For instance, (Epshtein et al., 2010) showed that pre-

incubation of RNA polymerase with a catalytically inactive factor Rho mutant resulted in defective Rho termination, in spite of followed by the addition of wild-type factor Rho. They concluded that the initial binding of factor Rho to RNA polymerase is strong enough to preclude an exchange of factor Rho molecules.

(Epshtein et al., 2010) used factor Rho mutant to prove the association between factor Rho and RNA polymerase take an important role in Rho-dependent termination. Referring to the (Epshtein et al., 2010), transcription termination, which bypassed binding to RNA polymerase, doesn't even exist. However, several follow-up studies have been disputed this result. For instance, (Kalyani, B. Sudha et al., 2011) insisted that the model proposed by (Epshtein et al., 2010) undermine the importance of the specific *rut* site interaction at the binding step. They also argued that factor Rho does not bind to an elongation complex in the absence of *rut* site and found no evidence for a direct interaction between factor Rho and RNA polymerase prior to termination. Moreover, (Koslover, Daniel J. et al, 2012) asserted that the result come from assay they developed didn't support (Epshtein et al., 2010).

We have already shown that the interaction between factor Rho and RNA polymerase has both high affinity and stability in the previous chapter, which support the (Epshtein et al., 2010)'s opinion. We, though, need to examine whether there is transcription termination without going through factor Rho's binding to RNA polymerase. To examine the existence of transcription termination which detour binding to RNA polymerase, we used factor Rho

mutant like (Epshtein et al., 2010).

Considering the highly controversial topic of whether the association between factor Rho and RNA polymerase involves in the transcription termination, it's meaningful to answer this question by suggesting the existence of transcription termination inhibited by preoccupancy of factor Rho mutant. In this section, we will propose the experiments or resolving this question, followed by the results from them and interpretation to them.

4.2. Materials and Methods

4.2.1. Factor Rho mutant with point mutation

Factor Rho mutants were purified by Palinda Ruvan Munasingha who worked as a post-doctoral researcher at Professor Yeon-Soo Seo [2] 's laboratory according to (Chalissery et al., 2007).

(Chalissery et al., 2007) mapped the positions of mutations on the hexameric closed ring structure of factor Rho, which has both the primary and secondary RNA binding sites occupied with nucleic acid. G51V and Y80C are in the primary binding site (PBS), and P279S are in the Q-loop. As we mentioned in **Section 1.2.1**, primary binding site is required when factor Rho binds to the RNA, and Q-loop is proposed to be involved in transferring RNA between the subunits during translocation.

[2] Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST)

(Chalissery et al., 2007) conducted biochemical experiment to understand how the point mutation in each factor Rho mutant alter the properties of factor Rho. The termination defect of all the factor Rho mutants we used could not be overcome under the most relaxed conditions that have been tested by (Chalissery et al., 2007). In spite of G51V's and P279S's higher affinity to the primary RNA binding site than wild-type, both of them has lower affinity to secondary RNA binding site[3]. All the factor Rho mutants has defect in ATP binding. (Table 4-1-1)

Mutants	1ST RNA	2nd RNA	ATP
WT	+	++	+++
G51V	++	+/-	++
Y80C	-	-	+
P279S	++	-	+

Table 4-1-1. Summary of different properties of the factor Rho mutants

4.2.2. Factor Rho mutant by fluorescence labeling

Welabeled the factor Rho the fluorescence dye. The maleimide-mediated methologies are widely used in putting probes such as fluorescent labels at protein, becasue of exceptionally fast reaction rates and significantly highly selectivity towards cysteine residues in proteins. (Francis, Matthew B. et al., 2008)

[3] Binding activies are for rC10 and rC25 templates. +/- indicates ~100 fold reduced binding on rC25 template.

```
MNLTELKNTP VSELITLGEN MGLENLARMR KQDIIFAILK QHAKSGEDIF
GDGVLEILQD GFGFLRSADS SYLAGPDDIY VSPSQIRRFN LRTGDTISGK 100
IRPPKEGERY FALLKVNEVN FDKPENARNK ILFENLTPLH ANSRLRMERG
NGSTEDLTAR VLDLASPIGR GQRGLIVAPP KAGKTMLLQN IAQSIAYNHP 200
DCVLMVLLID ERPEEVTEMQ RLVKGEVVAS TFDEPASRHV QVAEMVIEKA
KRLVEHKKDV IILLDSITRL ARAYNTVVPA SGKVLTGGVD ANALHRPKRF 300
FGAARNVEEG GSLTIIATAL IDTGSKMDEV IYEEFKGTGN MELHLSRKIA
EKRVFPAIDY NRSGTRKEEL LTTQEELQKM WILRKIIHPM GEIDAMEFLI 400
NKLAMTKTND DFFEMMKRS (419 a.a.)

22-116      Primary RNA binding site
179-183     ATP binding and hydrolysis (P-loop)
278-290     Secondary RNA binding site (Q-loop)
322-326     Secondary RNA binding site (R-loop)
202         Cysteine (for Labeling site)
```

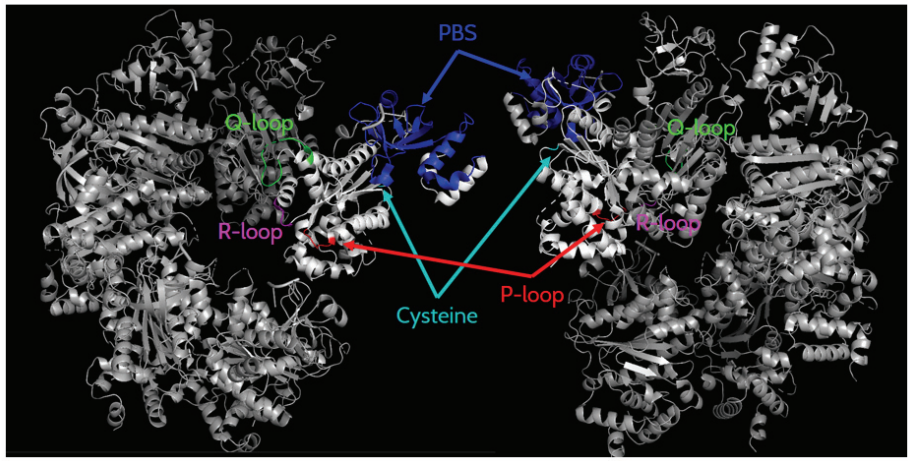

Figure 4-2-1. Protein sequence and structure of factor Rho.

Protein sequence of monomer factor Rho denoted by the position of cysteine. **(above)** The structure of the factor Rho showing the position of the cysteine and other known major domain. **(below)**

Before labeling, we examined the number and the position of the cysteine in the factor Rho. According to the study in advance (Mitra et al. 2017), the monomer of factor Rho contain a cysteine, which means factor Rho in hexamer has the six cysteine. (The structure is plotted in **Figure 3-3-3**, based on the 1PVO in Protein Data Bank (PDB), X-ray crystal structure of Rho transcription termination factor in complex with ssRNA substrate and ANPPNP[4], using Pymol (Schrödinger) which is the program for molecular visualization.)

We incubated overnight factor Rho with fluorescence dye with maleimide group, such as Alexa488 (A10254; Alexa Fluor™ 488 C_5 Maleimide; Invitrogen), Cy5 (45-001-251/GE Healthcare PA15636; Cy5 Maleimide; GE Healthcare), Alexa750 (A10254; Alexa Fluor™ 750 C_5 Maleimide; Invitrogen). After incubation is over, we removed free dye by using centrifuge and changing buffer until the concentration of free dye is less enough than that of factor Rho.

4.2.3. Single-molecule Experiment

Single-molecule experiments conducted in this cahpter is alsmost same as those described in Chapter 3. The only difference is that the factor Rho injected is various, not only wild-type but also mutant with point mutation or fluorescenc dye-labeled.

[4] DOI: 10.2210/pdb1PVO/pdb

4.3. Results and Discussion

4.3.1. Factor Rho mutant experiment

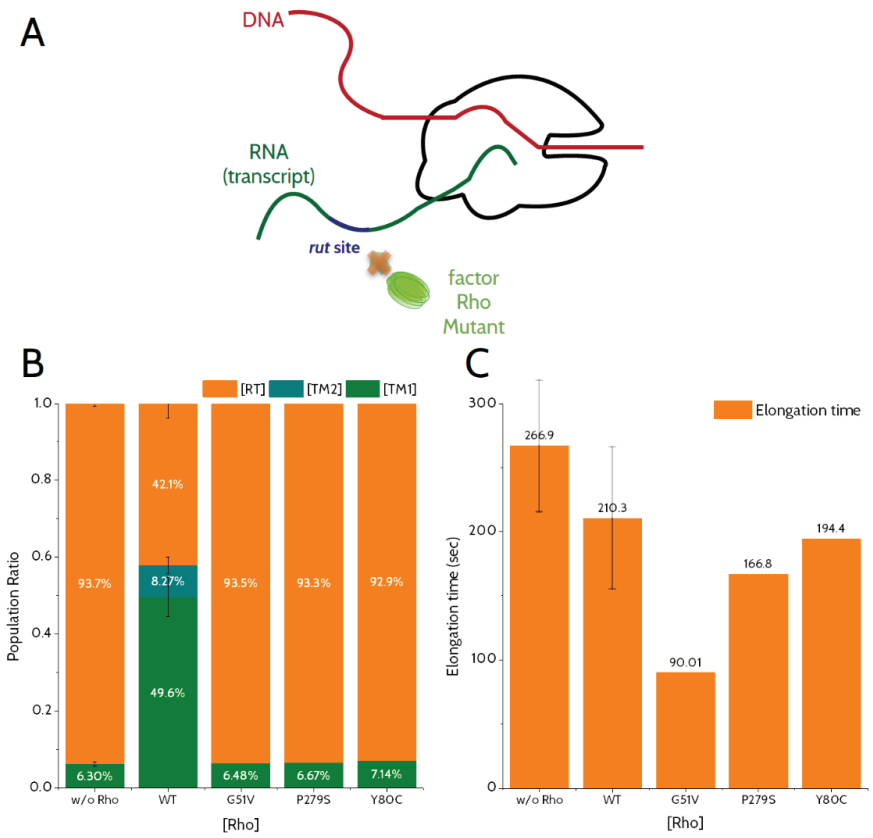

Figure 4-3-1. Termination efficiency and elongation time of mutants of factor Rho
(A) Termination efficiency of mutants of factor Rho. **(B)** Elongation time of mutants of factor Rho in single-exponential fitting.

(Time histograms for each data point are shown in **Figure S4-1** and **Figure 4-2**)

The result showed that all kinds of mutants have no transcription termiantion activity. (**Figure 4-3-1 (A), (B)**) Intriguingly, elongation time of the each mutant was less than the wild type. Besides, elongation time between mutants of factor Rho was also different each other. (**Figure 4-3-1 (C)**) There were some reports that factor Rho is involved not only in termination but also in elongation. Moreover, it was also reported that factor Rho enhance the elongation rate of RNA polymerase. In this point, the results come from freely difusing factor Rho assay using Rho mutants were make sene.

As we confirmed factor Rho mutant lost the function of transcription termination, we coducted the masking experiment using these factor Rho mutant. First of all, we confirmed the possibility that factor Rho mutant can binds to RNA polymerase and keeps other factor Rho from binding to RNA polymerase. To condifrm this we injected factor Rho mutants, followed by wild-type factor Rho before rNTP injection. We removed all the free protein sufficiently, between changing buffer in the reaction chamber. (**Figure 4-3-2 (A)**) Population ratio of these experiments show that termination efficiency was almost same as that of experiments in the absence of factor Rho. (**Figure 4-3-2 (B)**) Elongation time was slightly increased. (**Figure 4-3-2 (C)**) Thus, we confirmend that factor Rho mutant can be used for maksing factor Rho's binding to RNA polymerase.

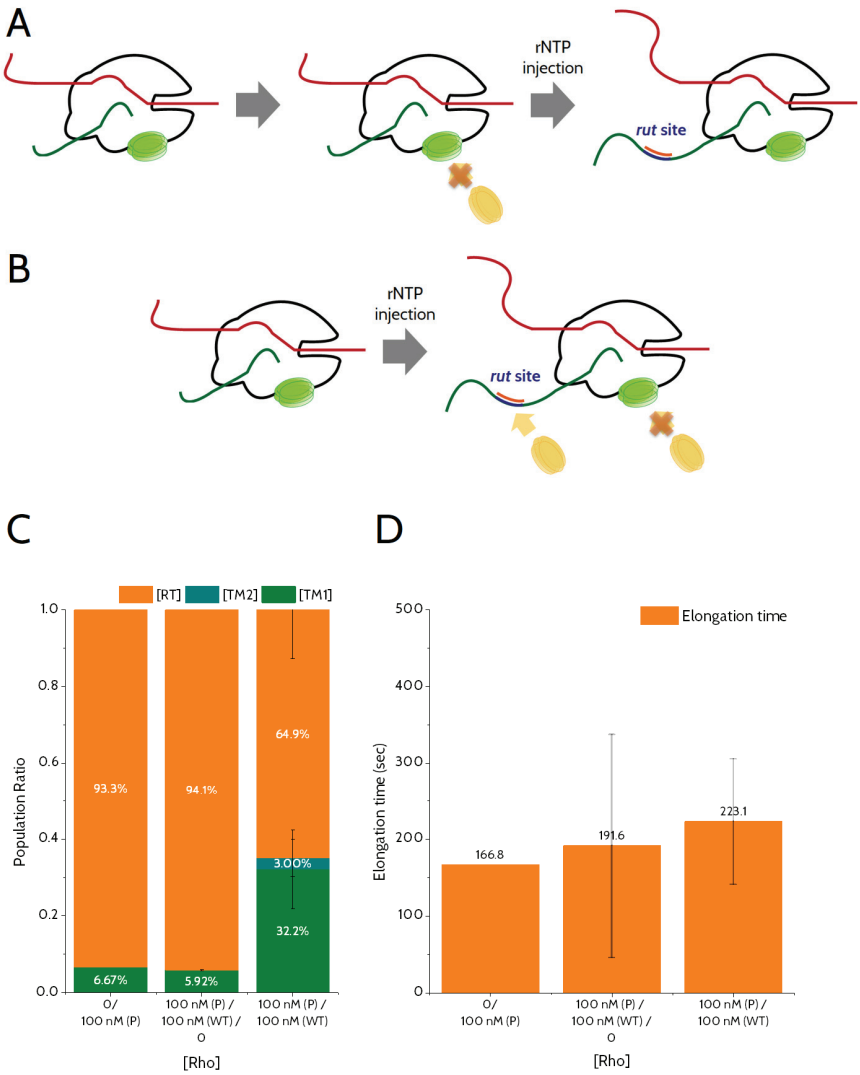

Figure 4-3-2. The results from masking experiment using Rho-mutants.

(A-B) Schematic diagram of fluorescence dye labeled factor Rho. **(C-D)** Population ratio **(C)**, termination time **(D)**, and elongation time **(D)** in the several of several cases. ('0 / 100 nM (P)' means injecting rNTP with P279S factor Rho (P). '100 nM (P) / 100 nM (WT) / 0' means injecting P279S factor Rho, followed by injecting wild-type factor Rho (WT), before we injected rNTP. '100 nM (P) / 100 nM (WT)' means injecting P279S factor Rho followed by injecting wild-type factor Rho and rNTP. Between different type factor Rho injection, all free protein was removed from reaction chamber.) (Time histograms for each data point are shown in **Figure S4-3**)

Based on these confirmation, we conducted masking experiment to observe transcription termination which is not mediated by association between factor Rho and RNA polymerase. We injected factor Rho mutants, followed by injection of rNTP and wild-tpye factor Rho. (**Figure 4-3-2 (B)**)

The results from masking experiment show that termination happend. The termination efficiency is about 35.2%, which is lower than that of freely diffusing factor Rho assay using wild-tpye Rho, but higher thant that of pre-binding factor Rho assay. (**Figure 3-3-7 (A)**) The average termination time is about 296.5 sec (**Figure S4-3 (E)**), which is also slower than that from freely diffusing factor Rho assay but faster than pre-binding factor Rho assay.

The results from masking experiments, though, are need to be optimized, because the variation between dataset is too large to accept the result. It seemd that the masking experiment is still need to be more precise.

4.3.2. Fluorescence dye labeled factor Rho experiment

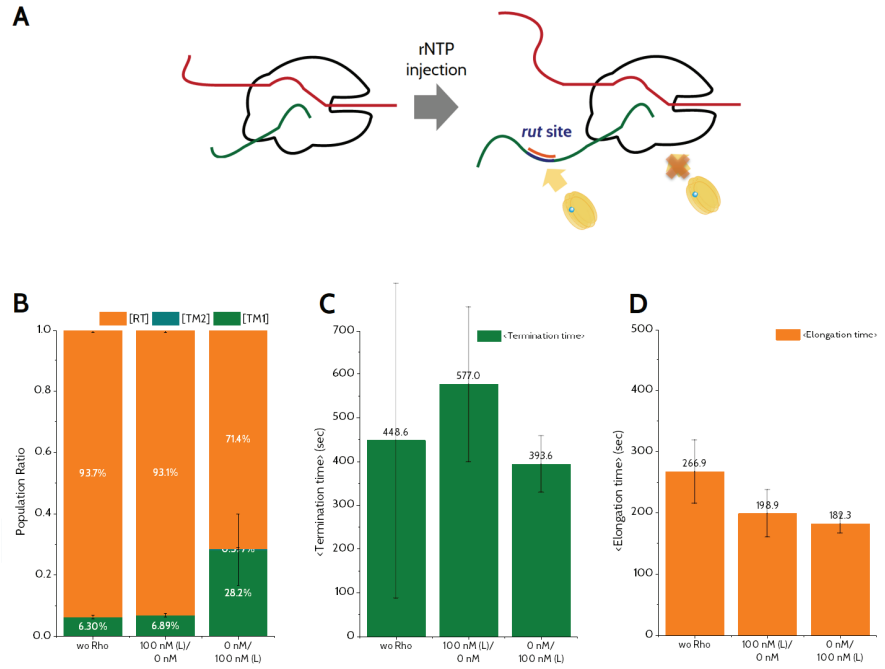

Figure 4-3-3. Results from fluorescence dye labeled factor Rho

(A) Schematic diagram of fluorescence dye labeled factor Rho. **(B-D)** Population ratio **(B)**, termination time **(C)**, and elongation time **(D)** in the several of several cases.

('wo Rho' means we didn't inject factor Rho. 'A nM / B nM' means pre-bound factor Rho is A nM and freely diffusing factor Rho is B nM. '(L)' means fluorescence dye labeled factor Rho.)

(Time histograms for each data point are shown in **Figure S4-4**)

We labeled the factor Rho the fluorescence dye (Alexa488). Fluorescence labeled factor Rho is prepared to monitor the binding of factor Rho in real time, at first. We, though, confirmed by accdident that fluorescence-labeld factor Rho cannot bind to the RNA polymerase only to interact with RNA transcript. (**Figure 4-3-1 (A)**) Pre-bindnig factor Rho assay using Alexa488-labeled shows the termination efficciency almost same as that in the absence of any factor Rho. (**Figure 4-3-1 (B)**).

The average termination time measured only in freely diffusing factor Rho assay was credible due to enough number of traces to have assurance. (**Figure 4-3-1 (C)**)The average termination time obtained from freely diffusing factor Rho assay was about 393.6 sec. Which was much larger than that from freely diffusing factor Rho assay using wild-type factor Rho (~204.6 sec) but almost same as that from pre-binding factor Rho assay also using wild-type factor Rho (~420.5 sec). The average elongaiton time was slightly decreased when we injected factor Rho. The difference of results from pre-binding factor Rho assay is within a standard deviation, which means it was not meaningful difference. On the other hand, freely diffusing factor Rho assay showed difference larger than a satard deviation, which means it is more probable the average termiantion time is different. We assumed that this probable difference is caused by exclusion of slow population of molecule which is terminated by factor Rho.

4.4. Conclusion and Outlook

In this chpaer, we tried to observe Rho-dependent transcription termination when factor Rho's binding to RNA polymerase is blocked. We suggested two methods to achieve this purpose, factor Rho mutants, referring to (Epshtein et al., 2010), and fluorescence dye-labeled factor Rho.

To reproduced masking experiment using factor Rho mutants, we conducted freely-diffusing factor Rho assay using factor Rho mutants. (**Figure 4-3-1**) We confirmed that factor Rho mutants lost the function of termination. Besides, we examined whether RNA polymerase-bound factor Rho mutant can hinder other factor Rho from binding to RNA polymerase. (**Figure 4-3-2**) According to result, RNA polymerase-bound factor Rho mutants shows blocking performance. We conducted freely diffusing factor Rho assay with masking by factor Rho mutant. (**Figure 4-3-2**) Results show that masking decreases the termination efficiency (~35.2%) and slow down the termination time (~296.5 sec). This results mean that Rho-dependent termination can occur in spite of situation when RNA polymerase is blocked from factor Rho.

We found that fluorescence dye labeled factor Rho lost the function of binding to RNA polymerase. (**Figure 4-3-3**) We conducted freely diffusing factor Rho assay using Alexa488-labled factor Rho. Results from those experiments show that transcription termination happened in spite of factor Rho without binding ability. Like the results from masking experiment using factor Rho mutants, termination efficiency became lower (~28.5%) and termination time became slower (~393.6 sec).

Even though we designed two methods to achieve same purpose, the results from masking experiment using factor Rho mutants and those from fluorescence dye labeled factor Rho are different quantitatively. We assumed that this difference resulted from whether the factor Rho-binding to RNA polymerase. If association between factor Rho and RNA polymerase causes the change in structure of RNA polymerase, we can explain these difference. Freely diffusing factor Rho assay using factor Rho mutants show that factor Rho's binding to RNA polymerase accelerates the speed of RNA polymerase. Although those results support for difference between results from factor Rho mutants and fluorescence dye labeled factor Rho, direct observation of structure seems to be required.

Appendix

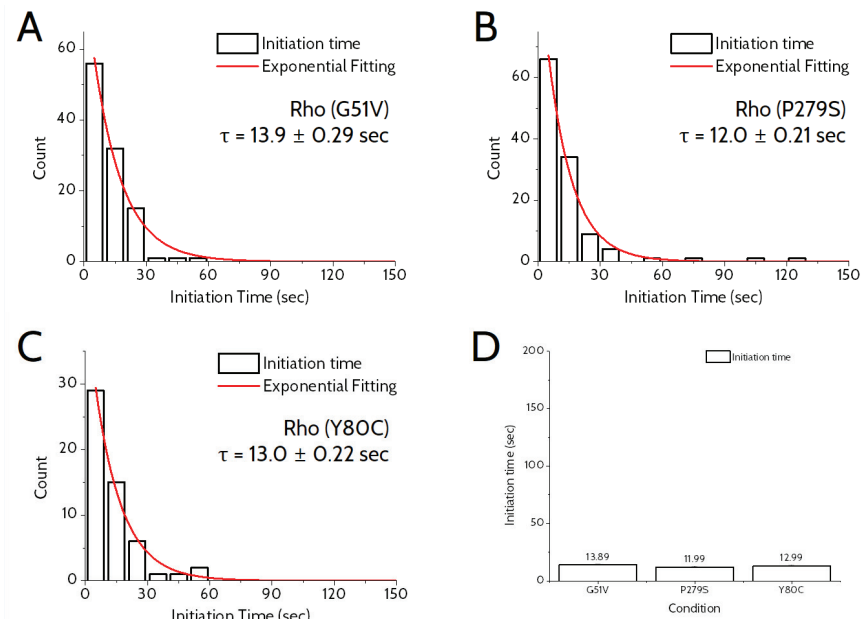

Figure S4-1. Distribution of initiation time (τ) for varying mutant factor Rho

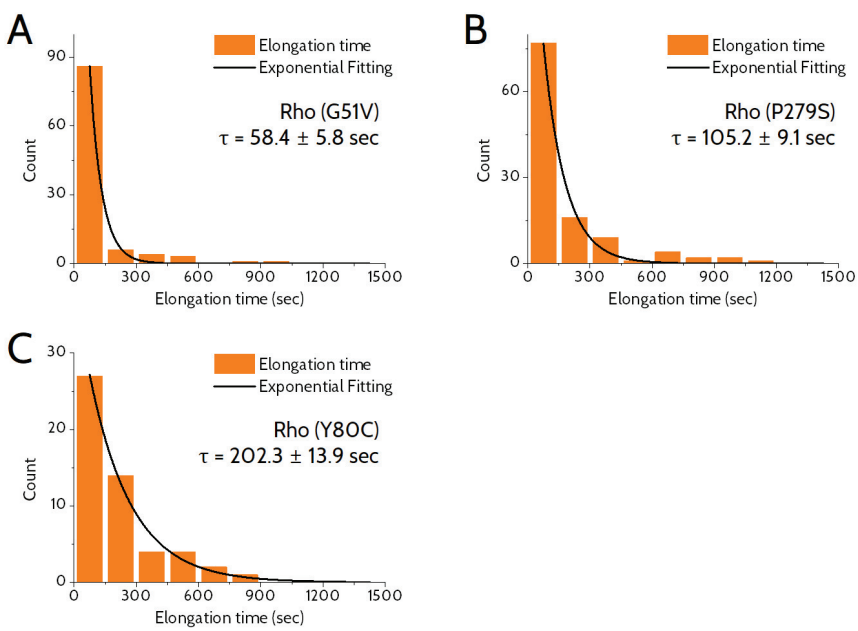

Figure S4-2. Distribution of elongation time (τ) for varying mutant factor Rho

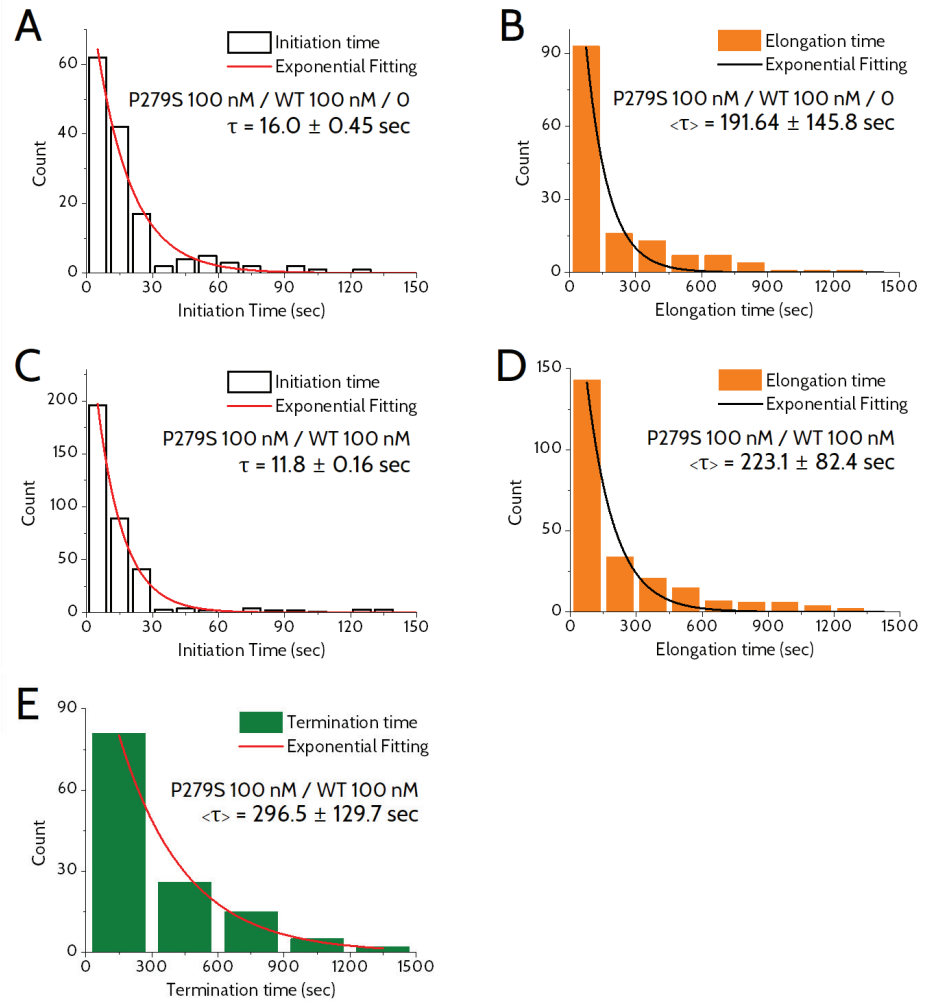

Figure S4-3. Kinetics information from factor Rho mutant's masking experiment

(A-B) Initiation time **(A)** and elongation time **(B)** when we injected P279S factor Rho, followed by injecting wild-type factor Rho (WT), before we injected rNTP.

(C-E) Initiation time **(C)**, elongation time **(D)** and termination time **(E)** when we injected P279S factor Rho followed by injecting wild-type factor Rho and rNTP.

(Between different type factor Rho injection, all free protein was removed from reaction chamber.)

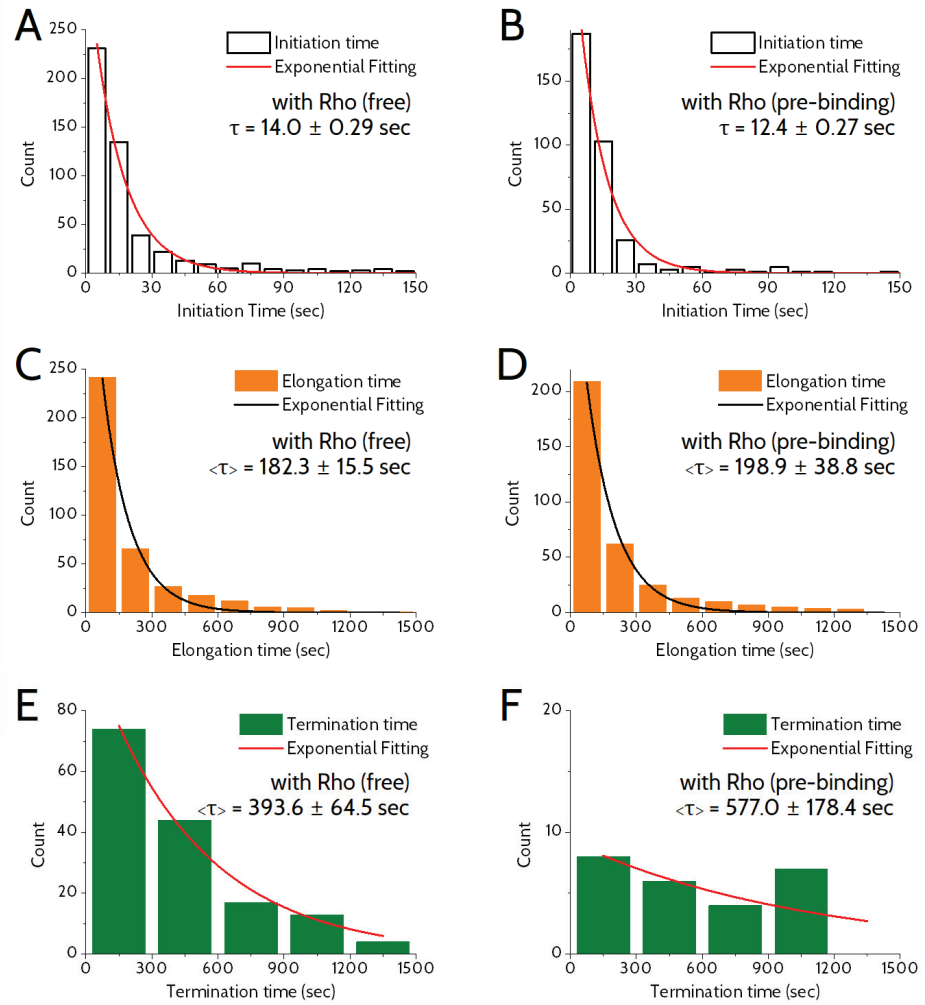

Figure S4-4. Kinetics information from Alexa488-labeled factor Rho experiment

(A-B) Initiation time of freely diffusing factor Rho assay (A) and pre-binding factor Rho assay (B), using Alexa488-labeled factor Rho.

(C-D) Elongation time of freely diffusing factor Rho assay (D) and pre-binding factor Rho assay (C), using Alexa488-labeled factor Rho.

(E-F) Termination time of freely diffusing factor Rho assay (E) and pre-binding factor Rho assay (F), using Alexa488-labeled factor Rho.

<Detailed description for each additional data>

Figure S4-1. Distribution of initiation time (τ) for varying mutant factor Rho

(A-C) Initiation time when the elongation complex was incubated with factor Rho mutant (100 nM), G51V **(A)**, P279S **(B)**, Y80C **(C)**. **(D)** Summary of initiation time for varying mutant factor Rho.

Figure S4-2. Distribution of elongation time (τ) for varying mutant factor Rho

(A-C) Elongation time when the elongation complex was incubated with factor Rho mutant (100 nM), G51V **(A)**, P279S **(B)**, Y80C **(C)**.

Figure S4-3. Kinetics information from factor Rho mutant's masking experiment

(Written in below the figure)

Figure S4-4. Kinetics information from Alexa488-labeled factor Rho experiment

(Written in below the figure)

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Chapter 5

Nucleic Acid Sequence Mutation Experiment

5.1. Introduction

Transcription termination happens remarkably in the specific positon, which means the specific sequence take a role in transcription termination. Like intrinsic terminators whose sequence component is well studied and accepted, factor-dependent terminators also have the specific sequence involved in transcription termination. The difference between those terminators is whether transcription termination is triggered by specific factor, which is factor Rho in the case of Rho-dependent terminators. Transcription termination triggered by factor Rho was already shown in Chapter 3.

In this chapter, we examined the properties two components, pausing site and *rut* site, consisting Rho-terminator. These two components have been considered essential, but sometimes this fact is also challenged. In the case of

pausing site, the refutation to the components is same as that to the kinetics coupling, which explains the core of factor Rho's role is the chasing the elongation complex. The necessity of pausing site is challenged because the translocation rate of factor Rho, 23±11 bp/s (Larson, Matthew H. et al., 2011), is faster than elongation rate of RNA polymerase, 56±3 bp/s (Gocheva, Veronika., 2015), which means factor Rho can catch up RNA polymerase without pausing site. As we mentioned in **Section 1.2.1**, factor Rho binds to RNA in the presence of NusG.

To resolve these problem, we conducted both freely diffusing factor Rho assay and pre-binding factor Rho assay to observe how two different pathway of termination is effected by deletion of pausing site of *rut* site.

5.2. Materials and Methods

In the case of *mgt*A, it is already studied that the position of the pausing site and rut site. (Hollands, K et al., 2014) According to this study, point mutation on these sites effect on the termination efficiency both *in vitro* and *in vivo*. We designed DNA substrates based on these previous studies.

Using these DNA substrates, we measure the elongation time when the transcription is done in the absence of factor Rho to confirm the mutation at the pausing site. After that we measure the termination efficiency conducted by factor Rho by using *in vitro* transcription assay, and compared the result with the wild type DNA substrate.

5.2.1. DNA substrates preparation

Modified DNA strands and DNA splints (Table S5-2-1) were purchased from IDT, or Integrated DNA Technologies(USA), respectively.

Name	Sequence (5' → 3')
A1 (200 nt)	(Same as described in **Chapter 2**)
B2 (114 nt)	GTAAGACAGTGACACAATAACGTCCCTGTTTTTATTTAAACATTGCTCAT CGGGCAAGGCTTTGCCGTCCCTGAAGAATTTTCTGCGCCTGACTTCGGCG CGGAGGGATTACCT
B3 (114 nt)	GTAAGACAGTGACACAATAACGTCCCTGTTTTTATTTAAACATTGCTCAT CGGGCAAGGCTTTGCCGTCGGACAAGAATTTTCTGCGCCTGACTTCGGCG CGGAGGGATTACCT

A1B (40 nt)	TTATTGTGTCACTGTCTTACACACCGGTAAGACAGCAGAG
A2 (200 nt)	TATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCC ATACCGCCACAAAACTTATGGATTTATGCGTATAATCCGCGGCGCAAATT ATTTACTTACCGGAGGCGACATGGACCCTGAACCCACCCCTCTCCCGCGA TGGAGAATTTTCCTTTTCCGGTAAGGGTGGGTGTGCTGTCTTACCGGTGT
A2B (40 nt)	(Same as described in **Chapter 2**)

Table S5-2-1. Oligonucleotide sequences for DNA strands and splints

The following combinations were used to construct individual DNA substrates.

- ***mgt*A_G269C** (pausing site mutant): A1 + A1B + B2
- ***mgt*A_pausing** (pausing site mutant): A1 + A1B + B3
- ***mgt*A_*rut*** (*rut* site mutant): A2 + A2B + B1

The preparation is done same as described in **Chapter 2**.

5.2.2. Single-molecule Experiment

Single-molecule experiments conducted in this cahpter is alsmost same as those described in **Chapter 3**. The only difference is that the DNA substrate used is various, such as pausing site mutation and *rut* site mutation.

5.3. Results and Discussion

5.3.1. Pausing site mutation Experiment

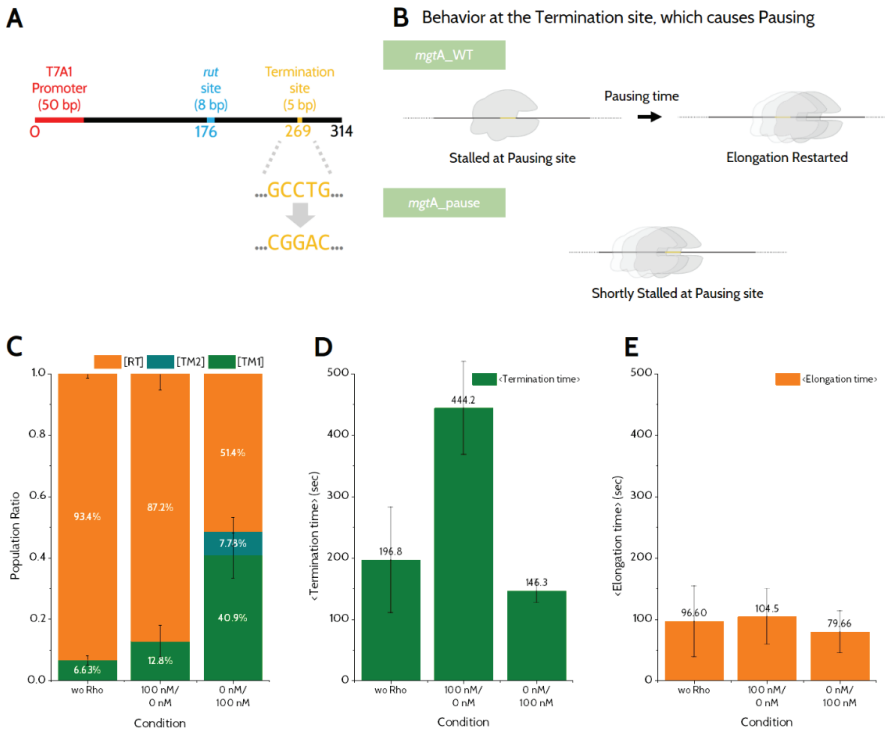

Figure 5-3-1. Results from pausing site mutation sample

(A) DNA design of *rut* site mutation. (*mgt*A_pause) **(B)** Schematic diagram of effect of pausing site mutation, compared to wild-type DNA substrate. **(C-E)** Population ratio **(C)**, termination time **(D)**, and elongation time **(E)** in the several of several cases. ('wo Rho' means we didn't inject factor Rho. 'A nM/B nM' means pre-bound factor Rho is A nM and freely diffusing factor Rho is B nM.)

(Time histograms for each data point are shown in **Figure S5-2**)

We observed how Rho-dependent transcription termination changes in the absence of pausing site, using *mgt*A_pause DNA substrate. We designed DNA

substate by removing unique pausing site in *mgt*A_WT referring to (Hollands, K et al., 2014). (**Figure 5-3-1 (A)**) We expected that elongation complex on this DNA substrate will stall shortly at the pasuing site, compared to that on wild-type DNA substrate. (**Figure 5-3-1 (B)**) We confirmend the removal of pausing time by measuring the average elongation time in the absence of factor Rho, which presents 96.6 sec, much shorter thant that of wild-type showing about 266.9 sec. (**Figure 5-3-1 (E)**)

Removal of pausing site reduces the termination efficiency of freely diffusing factor Rho and pre-bound factor Rho. Reduction of the termination efficiency of pre-bound factor Rho is more larger than that of freely diffunsing factor Rho, which measn the termination done by pre-bound factor Rho is morse sensitive to the delition of pausing site. (**Figure 5-3-1 (C)**) Intriguingly, change in the termination time is different between pre-binding factor Rho assay and freely diffusing factor Rho assay. Pre-bound factor Rho assay show that the average termination time is about 444.2 sec, which is almost same as 420. 5 sec resulted from wild-type DNA substrate. On the other hand, freely diffusing factor Rho assay show that the termination time is 146.3 sec, which is shorter than that of wild-type DNA substrate. (**Figure 5-3-1 (D)**)

This difference implies that termination pathway which is mediated by association between factor Rho and RNA polymerase requires time windows for pre-bound factor Rho to move from RNA polymerase to RNA. Moreover, this longer termination time means that pre-bound factor Rho has more probability to fail in transcription termination.

5.3.2. *rut* site mutation Experiment

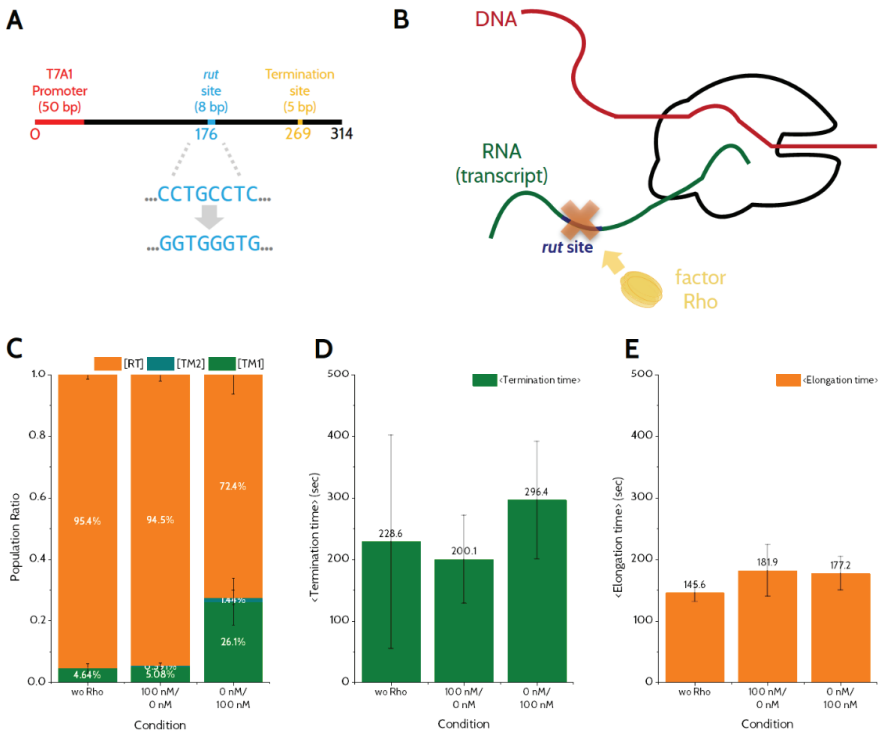

Figure 5-3-2. Results from *rut* site mutation sample

(A) DNA design of *rut* site mutation. (*mgt*A_*rut*) **(B)** Schematic diagram of effect of *rut* site mutation. **(C-E)** Population ratio **(C)**, termination time **(D)**, and elongation time **(E)** in the several of several cases.

('wo Rho' means we didn't inject factor Rho. 'A nM/B nM' means pre-bound factor Rho is A nM and freely diffusing factor Rho is B nM.)

(Time histograms for each data point are shown in **Figure S5-3**)

We also conducted single-molecule in vitro transcription assay using *mgt*A_*rut* DNA substrate. The DNA substrate is designed to observe what will happen when *rut* site is removed, referring to (Hollands, K et al., 2014). (**Figure 5-3-2 (A)**). We expected that it can be difficult for factor Rho to bind to RNA, only to decrease in its termination activity. (**Figure 5-3-2 (B)**)

Removing the *rut* site suppress transcription termination observed in the pre-binding factor Rho assay, which means pre-bound factor Rho cannot terminate transcription in the DNA substrate without *rut* site. This is different from the result come from DNA substrate with defective pausing site, whose termination efficiency of pre-bound factor Rho became almost half, but not zero. The termination efficiency seen in freely diffusing factor Rho assay also become half, compared to the wild-type DNA substrates. This means defective in *rut* site is more fatal to Rho-dependent termination. (**Figure 5-3-2 (C)**)

The average termination times are different from those from wild-type DNA substrate. Average termination time of cases in the absence of factor Rho and in the only existence of pre-bound factor Rho became about 200 sec. (**Figure 5-3-2 (D)**) Faster elongation speed of RNA polymerase and almost absence of termination caused by pre-bound factor Rho seemed to attribute this result. Elongation speed of RNA polymerase became faster in the absence of *rut* site, which can accelerate overall reaction. (**Figure 5-3-2 (E)**) Moreover, the Termination efficiency of cases without factor Rho and that of cases with only pre-bound factor Rho is almost same, which means the population of termination is almost same each other.

The acceleration of elongation time was due to the change in the structure of riboswitch. (**Figure 5-3-2 (E)**) The modeled of (Hollands, K et al., 2014) asserted that riboswitch structure in *mgt*A_WT substrate cause unusually long pause. In other word, change in the sequence of riboswitch can change the pausing time of RNA polymerase. We assumed that change in the sequence of

rut site accelerated transcription termination. But this points should be elucidated by further study.

We should take note the result from freely diffusing factor Rho assay. Although the termination efficiency become half, transcription termination still happens, but slowly. This relatively low efficiency and slow termination are attributed to difficulty in factor Rho's binding to RNA. Moreover, considering only change in *rut* site effect decrease pre-bound factor Rho, RNA-binding is essential to all the case of termination caused by factor Rho.

5.4. Conclusion and Outlook

In this chpaer, we tried to observe Rho-dependent transcription termination when one of essential components of Rho-dependent terminator, such as pausing site, or *rut* site, is removed. The importance of components accepted as essence in termination has been controversial. Many studies to elucidate how important or not these components is.

The resuts from DNA substrate removed pausing site, *mgt*A_pause, firstly showed the effect of removal of pausing site. The average elongation time is dramatically decrease in the experiment in the absence of factor Rho. **(Figure 5-3-1)** The poulation ratio showed the temrination efficiency both from pre-bindin factor Rho assay and from freely diffusing factor Rho assay decreased. However termination efficiency in the pre-binding factor Rho assay more decreased than that in the freely diffusing factor Rho assay. Besides, the average termination time both from experiments in the absence of factor Rho and from freely diffusing factor Rho decreased, but that from pre-binding factor Rho assay didn't change. We assumed that termination occured by sole pre-bound factor Rho takes time to terminae transcription.

The results from DNA substrate removed *rut* site, *mgt*A_rut, we conducted in the same way. In this case, transcription termination is rarely observed in pre-binding factor Rho assay, which has similar termination efficiency to the experiments without factor Rho. This means the *rut* site in this case did a essnce role, which implied that even RNA polymerase-bound factor Rho need to bind *rut* site in the RNA. Conversely, freely diffusion factor Rho

assay still show transcription termination, with the average terminaiton time increased. Thus, transcription terminaiton can happened in DNA substrate removed *rut* site, but there is something disturbance because of deletion of *rut* site.

The results from this chapter suggest that the components are not essential but necessary in transcription termination. Some cases, pre-binding factor Rho assay using DNA substrate without *rut* site, seemed that components is essential. However, it's still yet to elucidate whether components are essential or promoting, because the small time window in some cases excluded the possibility to occur the slow transcription termination. We should also consider that there are still other weak pausing site or *rut* site.

Even though there are still ambiguity in interpreting data, our results are enough to construct the model for elucidating Rho-dependent termination. In spite of removing the major pausing site, there is no change in the average termination time in pre-binding factor Rho assay, which implies that it is time-limiting procedure that RNA-polymerase bound factor Rho was activated and moved to RNA. This can be evidence for low efficiceny and slow termination time of transcription termination of pre-binding factor Rho assay. Besides, removing of *rut* site decrease termination efficiency dramaically in pre-binding factor Rho assay means the RNA-binding is required for pre-bound factor Rho to terminate transcription.

Appendix

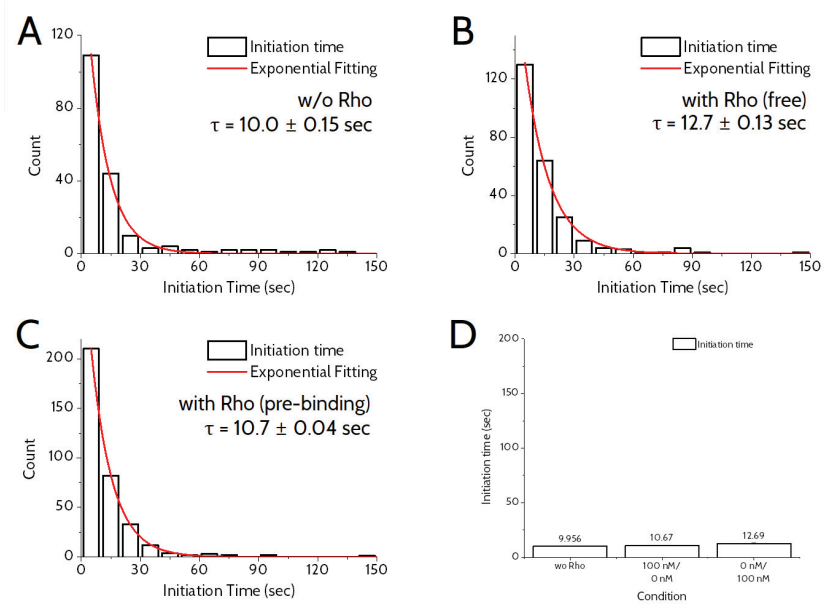

Figure S5-1. Distribution of initiation time (τ) for *mgt*A_pause

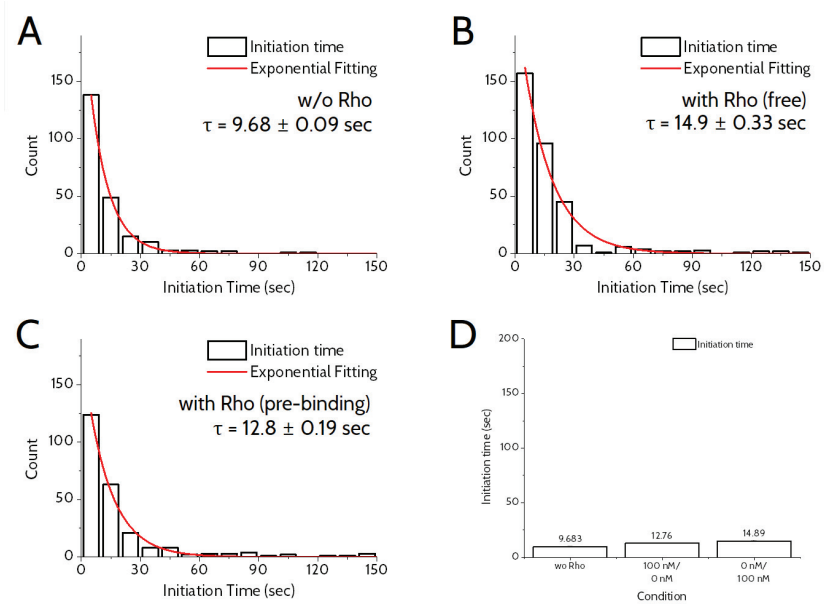

Figure S5-2. Distribution of initiation time (τ) for *mgt*A_rut

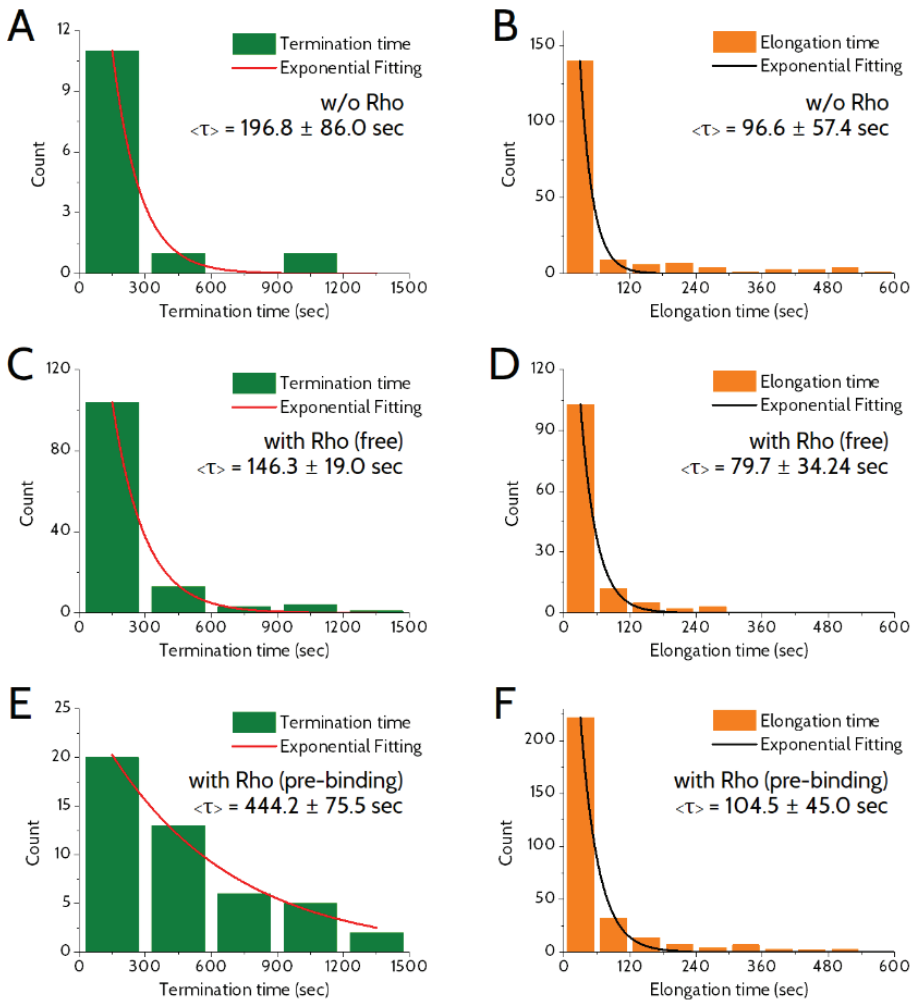

Figure S5-3. Kinetics information from *mgt*A_pause

(A-B) Initiation time **(A)** and elongation time **(B)** in the absence of factor Rho.

(C-D) Initiation time **(C)** and elongation time **(D)** in freely diffusing factor Rho assay.

(E-F) Initiation time **(E)** and elongation time **(F)** in pre-binding factor Rho assay.

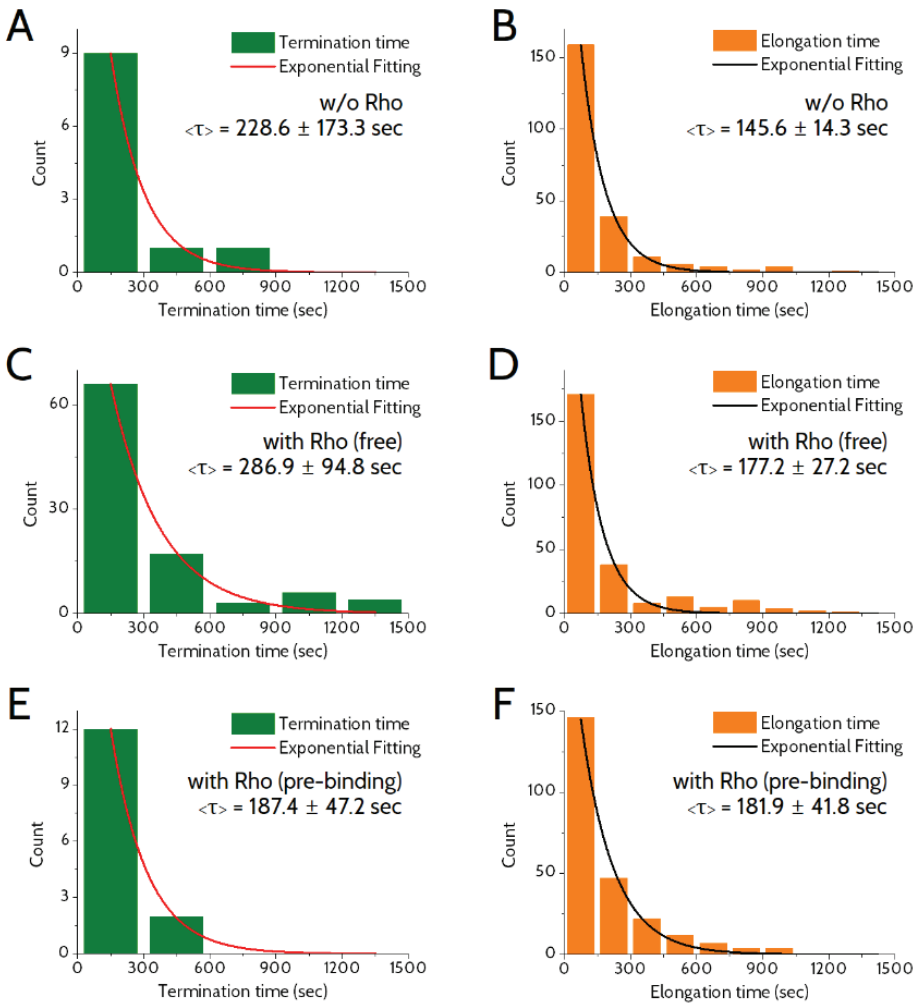

Figure S5-4. Kinetics information from *mgt*A_rut

(A-B) Initiation time **(A)** and elongation time **(B)** in the absence of factor Rho.

(C-D) Initiation time **(C)** and elongation time **(D)** in freely diffusing factor Rho assay.

(E-F) Initiation time **(E)** and elongation time **(F)** in pre-binding factor Rho assay.

<Detailed description for each additional data>

Figure S5-1. Distribution of initiation time (τ) for *mgt*A_pause

(A-C) Initiation time from experiments without factor Rho **(A)**, from freely diffusing factor Rho assay **(B)**, and from pre-binding factor Rho assay **(C)**. **(D)** Summary of initiation time for varying experiments conditions.

Figure S5-2. Distribution of initiation time (τ) for *mgt*A_rut

(A-C) Initiation time from experiments without factor Rho **(A)**, from freely diffusing factor Rho assay **(B)**, and from pre-binding factor Rho assay **(C)**. **(D)** Summary of initiation time for varying experiments conditions.

Figure S5-3. Kinetics information from *mgt*A_pause

(Written in below the figure)

Figure S5-4. Kinetics information from *mgt*A_rut

(Written in below the figure)

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Chapter 6

Summary and Future Directions

6.1. Summary of Findings

6.1.1. Overview

This thesis has developed and optimized *in vitro* transcription assay based on single-molecule FRET for observing factor dependent transcription especially by factor Rho. We dissected each pathway of Rho-dependent termination by using single-molecule *in vitro* transcription termination assay. We confirmed there are two kinds of pathways which has already been reported and emergent property when both pathways are activated. This results shed light on the mechanism of factor dependent transcription termination. Moreover, Transcription termination is still intriguing field and below are described possible future developments from the work presented in this thesis that

incorporate development from other research groups.

6.1.2. Development of *in vitro* transcription assay

We developed the single-molecule *in vitro* transcription assay which can observe the transcription temporally in real time and spatially in single molecule. Before confirming the effect of factor Rho, we validated the fluorescence assay well reports the fate of each molecule's transcription in two aspects, photo-bleaching problem and premature transcription termination problem. The results come from experiment controlling laser intensity and whether rNTP is injected or not showed that *in vitro* transcription termination we developed reports appropriately.

6.1.3. Two different fate of RNA polymerase

Using single-molecule *in vitro* transcription assay, we observed there are two kinds of termination fate, type-1 termination (TM1; Cy3-dissoncation only) and type-2 termination (TM2; PIFE-coupled Cy3-dissociation). These two kinds of termination can be distinguished by whether RNA polymerase remains on the template DNA even after termination happens, by referring to the other internal data accounting for re-initiation in the intrinsic termination (Wooyoung Kang et al., unpublished)

Our experiments results report the process as well as the result of two different terminations is different. Comparing the termination time of two population of termination, TM2 takes shorter time than TM1 to terminate

transcription. Moreover, the binding property to the DNA of RNA polymerase remained on template DNA after termination happens seems to be different from that of RNA polymerase under gone read through. The population ratio changes depending on the concentration of factor Rho, and on the presence of freely diffusing factor Rho.

6.1.4. Two different termination pathway

Single-molecule *in vitro* transcription assay also showed that there are two different pathways in Rho-dependent termination, which is already reported. Widely accepted model is kinetics coupling which explain the Rho-dependent termination happens by factor Rho's binding to specific site, called *rut* site, followed by translocation, only to catch up RNA polymerase and release RNA from template RNA.

In addition to this widely accepted model, we observed transcription termination mediated by binding between factor Rho and RNA polymerase. We observed stable factor Rho-RNA polymerase complex and transcription termination solely by RNA polymerase-bound factor Rho, using pre-binding factor Rho assay at varying incubation times and wait times. High affinity of factor Rho to RNA polymerase means that freely diffusing factor Rho assay included transcription termination both mediated by RNA polymerase-bound factor Rho and not mediated by that. Considering the results from DNA substrate removed pausing site or *rut* site, RNA polymerase-bound factor Rho also need to binds to *rut* site and it takes time to move on.

To confirm whether there is termination pathway not mediated by factor Rho's RNA polymerase binding, we conducted masking experiment using factor Rho mutant, which lost function of termination, and experiment using fluorescent dye labeled factor Rho, which lost function of factor Rho binding. Both of the experiments showed that low termination efficiency and slow termination efficiency. These results means that there is pathway not only mediated by factor Rho's binding to factor Rho, but also not mediated. However, both of them showed relatively low efficiency and slow termination time than both of them happened at once, which suggests something can be called emergent property.

6.1.5. Emergent properties

As we mentioned, the debated on the mechanism of Rho-dependent transcription termination has concentrated on which model is right or dominant in real, RNA-mediated pathway or RNA polymerase-mediated pathway. Our results show that both of pathway are validated. Moreover, we observed that two pathways are not mutually exclusive, rather those are synergic in transcription termination.

Single-molecule *in vitro* transcription termination assay could dissect Rho-dependent transcription termination in largely three aspects: RNA polymerase mediating pathway only, RNA polymerase mediating only, and both RNA mediating and RNA polymerase mediating. When both RNA mediating and RNA polymerase mediating are activated, termination efficiency

became higher and the average termination time became faster than only one of them is activated. We concluded that there is synergic effect between RNA-mediated pathway and RNA polymerase-mediated pathway. Our studies are not enough to elucidate why the synergic effect happen. Considering our results, we assumed that synergic effect attribute to increase in type-2 termination, which happens faster than type-1 termination. We expected that the structure of factor Rho-bounded RNA polymerase can explain the reason why the population ratio of efficient type-2 termination increased.

It is yet to say why the synergic effect

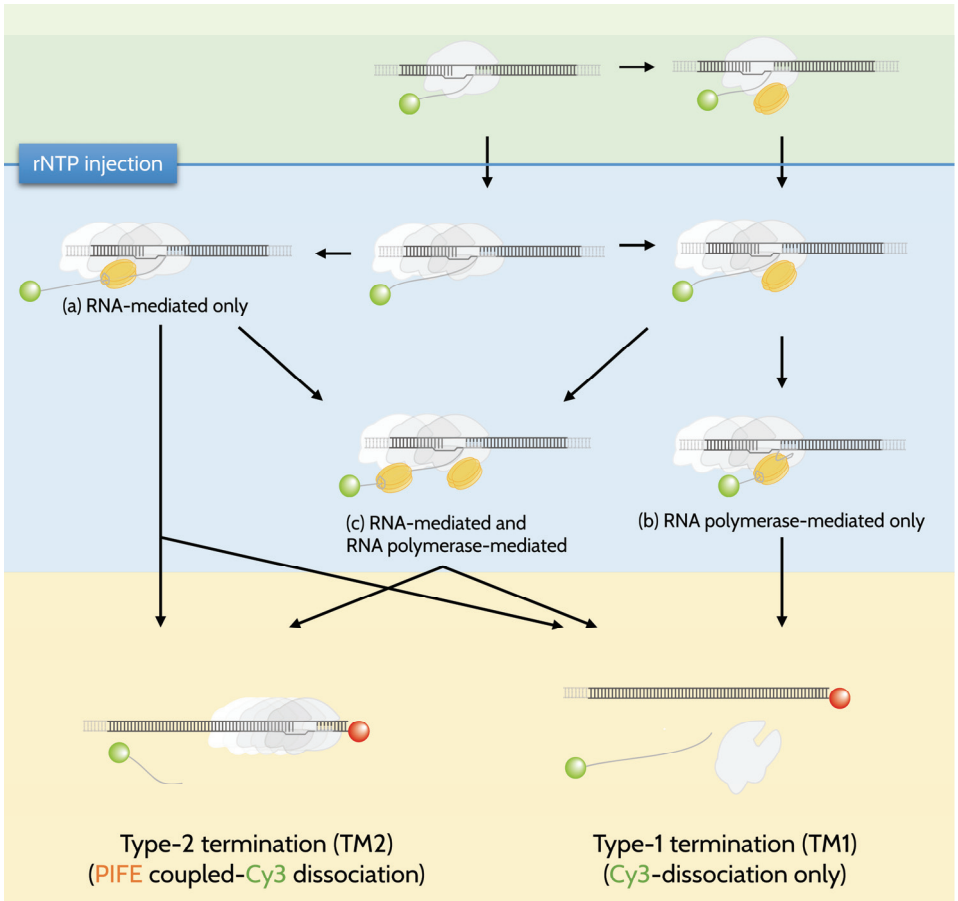

Figure 6-1-1. Mechanism of Rho-dependent transcription termination

We summarized Rho-dependent transcription in three phases: before elongation (green shaded), elongation (blue shaded), and termination (orange shaded). **(a)** 'RNA-mediated only' is almost same as classical kinetics-coupling model. **(b)** 'RNA polymerase-mediated only' described RNA polymerase-bound factor Rho solely terminates transcription. **(c)** 'RNA-mediated and RNA polymerase-mediated' described two different factor Rho involved in transcription termination. In termination phase, we described type-2 termination and type-1 termination.

6.2. Future Directions

6.2.1. Further studies of the factor-dependent transcription

We have showed several results about factor-dependent transcription termination using single-molecule *in vitro* transcription assay. We suggested novel model to explain the factor-dependent transcription termination. There are many topics yet to be elucidated.

These topics are classified into three groups. First is generalizing our findings. This thesis focused on the *mgt*A, but our internal data of another terminator λTR1 showed something different from *mgt*A. It seemed that the components of Rho-dependent terminator, such as *rut* site and pausing site, are different in number and intensity each other. We need to examine how that kind of difference attributes to transcription termination.

The comparison between different terminator can give a new insight into the Rho-dependent termination. For example, it seems that the number of the pausing site of Rho-terminator depends on the where the Rho terminator is. Rho terminator exists both in the leader part of coding RNA region and in the following the coding RNA region. There seems tendency that the Rho terminator followed by coding region, such as *mgt*A, has single intense pausing site, but that following coding region, like λtR1 and *trp* t', has multiple relatively weak sites. We guess that Rho terminator in the leader region takes a role as one/off switch, but that in the following coding region takes a role as block only to terminate transcription. Finding the sequences involved in the

Rho-dependent terminator followed by studying correlation between termination efficiency and kinetic traits of terminator is helpful to validate this guess.

Another approach is the study more about magnesium-sensing riboswitch in *mgt*A, which are in the leader site of coding region for magnesium transporter. (Hollands, K et al., 2014) To control the expression of magnesium transporter gene *mgt*A from *Salmonella enterica serovar Typhimurim*, magnesium sensing riboswitch modulates the activity of factor Rho, by responding to intracellular level of magnesium adopting one of two mutually exclusive conformations. (Hollands, K. et al., 2012; Cromie, Michael J., 2006) High magnesium concentration makes an RNA conformation permitting factor Rho to interact with the nascent RNA followed by terminating transcription within the *mgt*A leader. Conversely, in the conformation fostered by low magnesium conditions, rut site is sequestered, only to hinder factor Rho from terminating transcription and permit to continue in to transcribe *mgt*A coding region. (Hollands, K et al., 2012)

We have also tested some other conditions with other magnesium condition. In low magnesium, such as 2 mM (considering four kinds of rNTP were injected 200 μM each, effective magnesium concentration is 1.2 mM), we observed how the Rho-dependent transcription changes. As we expected, termination efficiency become lower than our standard condition. (10 mM) Intriguingly, the degree of effect is different between results from pre-binding factor Rho assay and from freely diffusing factor Rho assay. Besides, the ratio

of [TM2] increased in low magnesium, and [TM2] is observed in the absence of factor Rho when the effective magnesium concentration is almost zero, because of concentration of magnesium being same as that of total rNTP.

Last thing to suggest is to observe the effect of the other factors, such as anti-termination factor and factor Rho inhibitor. Anti-termination factor, like NusA (Qayyum, M. Zuhaib et al., 2016), NusG (Chalissery, Jisha et al., 2011; Li, J., S. W. Mason et al., 1993; Pasman, Zvi et al., 2000), are reported to involve in transcription termination. These reports are usually based on bulk-scale experiment, so there are many reported effects, yet to be elucidated in detail. BCM is reported as factor Rho inhibitor by disrupting ATP hydrolysis. (Kohn, Harold et al., 2005) Our transcription assay can give a more detail information how BCM changes the transcription termination mechanism in detail.

6.2.2. Evolutionary study of transcription termination

After finding key structural and functional similarities between Rho-dependent termination and intrinsic termination pathways, (Epshtein et al., 2010) argued that allosteric mechanism of termination is general and likely to be preserved for all cellular RNA polymerase throughout evolution.

Our single-molecule *in vitro* transcription assay showed some results that supports assertion of (Epshtein et al., 2010). [TM2], one of the termination pathway, is observed both of Rho-dependent termination and intrinsic termination. However, this results can be challenging to their opinion, because

it proposes difference between two different terminators. We assumed that further study can shed a light on what is common and what is different between two different terminators.

Another report also asked that there be some correlation between two different terminators. For instance, (Dar, Daniel et al., 2018) insist the fact that these structures are energetically indistinguishable from those found in intrinsic terminators implies that terminators could easily switch between Rho-dependent terminator and intrinsic terminator during evolution, simply by altering the uridine content downstream the stem-loop. They gave an indicative example of transition state between termination mechanism, the reduced and confined uridine enrichment downstream of stem-loop structures. This case defines the steady-state termini of Rho-dependent transcripts where the gene is terminated by inefficient intrinsic mechanisms due to lower uridine content but is also terminated by factor Rho to achieve efficient control of gene expression.

The result from (Dar, Daniel et al., 2018) showed that terminator's factor Rho-dependence is anti-correlated with gene expression level, because Rho-dependent termination would be averagely less efficient mechanism than intrinsic termination, which naturally would be less robust to perturbations that influence factor Rho abundance or activity, because of not being effected by external factor. They concluded that Rho-dependent genes have significantly lower expression means higher expression of Rho-dependent genes may result in unwanted read-through into neighboring genes, which might disrupt tightly

regulated expression programs or even generate interference through antisense transcription. (Georg, J. et al., 2011)

We tend to believe Rho-dependent terminator and intrinsic terminator behaves exclusively each other, even though we don't know how different the sequence components of two different terminators or how high the specificity of factor Rho's reaction. This tendency, though, is not that odd because previous study focus on the most typical case of Rho-dependent terminator and that of intrinsic terminator. We, however, need to imagine the existence ambiguous to classify, which can be considered intermediate state in the context of evolution, or just the moderate mixed-up terminator in the context of function.

Thus, imagination about evolutionary flow that transcription termination underwent is required to design the further experiments about something between Rho-dependent terminator and intrinsic terminator. We think that these kinds of studies can give not only insights on the evolution of terminator more information about terminator which is difficult to be detected because of not completing the strict condition.

6.2.3. Study on translational polarity

The most representative attribution of eukaryotic cell is compartmentalization, which means the formation of cellular compartment that are often, but not always, defined as membrane closed regions. Compartmentalization is considered one of ways to enhance the cellular metabolism, because it can satisfy the different cellular environments that different cellular compartment needs. The unique trait of eukaryotic cell means it was widely accepted that compartmentalization is not found if prokaryotic cells, but there are some reports that some of the prokaryotic cells can make compartmentalized structure, which is not surrounded by a lipid bilayer like eukaryotic cell, but by pure proteinceous. In spite of some case of prokaryotic cells with compartmentalization, most of prokaryotic cells don't have compartmentalized structure.

As there is no spatial separation in prokaryotic cells, all the cell metabolism happens in the shared space. As gene expression also is no exception, transcription and translation takes place temporally and spatially at once. This is different from eukaryotic cells whose transcription happens in nucleus but translation happens in cytoplasm only to naturally satisfy temporally separation. The characteristic of prokaryotic gene expression that transcription and translation simultaneously happen called **transcription-translation coupling**. The main issue of transcription-translation coupling is that how they regulate each other.

This transcription-translation coupling cause translational polarity (Mitra et al., 2017). **Translational polarity** means translational termination within the first of gene of an operon leads to Rho-dependent transcription termination of the downstream genes of the same operon, as transcription and translation are simultaneous in prokaryotes. In detail, under optimal translation conditions, the translating ribosomes physically block the *rut* sites on nascent RNA, only to make in inaccessible to factor Rho. When translation cases under nutrient starvation or upon encountering nonsense mutations, factor Rho binds to the available *rut* sites on the nascent transcript and proceeds to terminate transcription, thereby hindering excess mRNA accumulation. Thus, the translation in the upstream gene can effect on the gene expression of downstream.

We assumed that developing single-molecule assay to monitor the transcription and translation simultaneous to understand translational polarity. This information can be useful to understand that the gene expression is regulated by the completion of upstream gene. Precise kinetics information obtained from single-molecule assay can contribute to the understanding translational polarity.

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Abstract in Korean(국문 초록)

인자 의존적 전사 종결 과정에 대한 단일분자 FRET 연구

서울대학교 자연과학대학

대학원 협동과정 생물물리 및 화학생물학 전공

송 은 호

생명체는 한정된 자원을 바탕으로 생존해야 하기 때문에, 유전자 발현 과정은 다양한 과정을 통해서 정교하게 조절이 되도록 진화되어 왔다. 전사 종결 과정 역시 그런 유전자 발현 과정 중에 한 가지이다. 전사 종결 과정은 전사 과정의 다른 과정인 개시 과정이나 신장 과정과 달리 그 동안 상대적으로 연구가 많이 진행되지 않았다. 그나마 미생물에 있어서의 전사 종결 과정은 많이 알려져 있는데, 그 과정은 크게 내재적 종결 과정(intrinsic termination)과 인자 의존적 종결 과정(factor-dependent termination)으로 나누어진다.

내재적 종결 과정은 특정 서열에서 전사 종결 과정이 일어나는 것으로, 그 작용 원리와 작동하는 서열에 대해서 잘 알려져 있다. 반면, 인자 의존적 종결 과정은 그 인자가 Rho 인자라는 것은 알려져 있지만, 제안된 작동 원리에 대해서

는 다양한 논란이 존재한다. 가장 대표적인 지점은 멈춤 지점(pausing site)가 Rho 인자의 작용에 필수적이라고 알려져 있지만, 이 사실은 Rho 인자의 작동 원리를 설명하는 대표적인 원리인 어뢰 모형(torpedo model)에 전면으로 배치된다. 어뢰 모형은 Rho 인자가 RNA 중합 효소보다 빠르게 이동을 한다는 것이 핵심 전제인데, 그 전제를 받아들인다면 굳이 멈춤 지점이 필요하지 않기 때문이다.

미생물의 인자 의존적 종결 과정의 원인 인자가 특정이 된 지 40년이 넘었음에도 불구하고, 핵심 메커니즘에 대한 논란이 이어지고 있는 것은 인자가 전사 과정이 진행되는 신장 복합체(Elongation Complex)에 어떻게 작용하는지가 정확하게 규명되지 않았기 때문이다. 즉, 인자가 작용하지 않는 경우와 달리, 인자가 RNA에서 Rho가 붙는 *rut* 위치(*rut* site, Rho utilizing site)에 붙는 과정만 존재하는지, 인자가 RNA 중합 효소에 직접 붙는지에 대해서 정확하게 규명을 해야 인자 의존적 종결 과정에 대한 정확하게 이해할 수 있게 된다.

본 연구에서는 인자 의존적 전사 종결 과정과 관련된 이런 다양한 문제를 해결하기 위해서, 처음으로 단일분자 FRET 연구방법을 이용해서 전사 종결 과정을 단일분자 수준에서 실시간으로 영상화하는 '슬라이드 안[*in vitro*] 전사 종결 방법'을 고안하였다. 이 방법을 이용하면 원하는 상황에서 전사 과정을 진행해서 전사 종결 과정을 확인할 수 있다.

이 연구 방법을 이용해서 종결 효율 종결 효율(termination efficiency)이나 각종 동역학(dynamics)적 지표를 집계한 결과 다양한 새로운 사실을 알 수 있었다. 먼저, 전사 종결 과정이 일어나면 크게 두 가지 다른 양상이 나타난다는 것을 처음 발견하였으며, 이 양상은 RNA 중합 효소가 DNA에 남는 것과 연관

이 있음을 확인하였다. 특히, RNA 중합 효소가 DNA에 남는 경우는 크게 Rho 인자의 농도, 전사 과정에서 자유롭게 떠다니는 Rho 인자의 유무, 마그네슘 농도 등에 영향을 받는 다는 것도 최초로 발견하였다.

한편, Rho 인자가 RNA 중합 효소에 높은 친화도를 가지면서 비교적 안정적으로 부착하며, 나아가 RNA 중합 효소에 붙은 Rho 인자가 전사 종결 과정을 일으키는 것을 발견하였다. 이 과정은 *rut* 위치나 멈춤 위치에 더 민감하게 영향을 받는다는 것을 본 연구에서 처음으로 확인하였다. 이러한 사실을 바탕으로 본 연구는 RNA 중합 효소와 Rho 인자 사이의 상호작용이 전사 종결 과정에 어떤 역할을 하는지에 대해서 설명하였다.

뿐만 아니라, 전사 종결 기능을 상실한 Rho 인자의 돌연변이나 형광 분자를 달아서 RNA 중합 효소에 붙을 수 없는 Rho 인자를 통해서, RNA 중합 효소에 붙지 못하더라도 전사 종결 과정이 일어나는 것을 확인하였다. 이를 바탕으로, 본 연구는 기존에 제기된 두 가지 전사 종결 경로를 확인하였고, 나아가 이들 두 전사 종결이 동시에 일어날 때 전사 종결 과정의 효율이 높아지는 시너지 효과가 나타나는 것을 통해 Rho 전사 종결 과정의 창발적 속성을 확인하였다.

핵심어: 단일분자 형광 분광학, 형광 공명 에너지 전달(FRET), 인자 의존적 전사 종결 과정(factor-dependent transcription termination), Rho 인자(factor Rho), *mgt*A 리보 스위치(*mgt*A riboswitch), *rut* 위치(*rut* site)

학번: 2017-24825

감사의 글

이 학위논문은 석사과정을 마치는 동시에 박사과정을 다시 시작하는 과정에서 작성되었습니다. 자연스럽게 석사과정 그 자체와 석사과정을 시작할 수 있게 한 많은 일들이 주마등처럼 지나갔고, 제가 이렇게 석사과정을 마치는 동안 도움을 주셨던 많은 분들이 떠올랐습니다.

누구보다 저희 부모님에게 감사의 말씀을 드리고 싶습니다. 부모님은 부족한 저에게 항상 믿음을 주시고, 제가 학위과정 동안에 여러 도움을 주셨습니다. 만약 부모님의 믿음이나 도움이 없었더라면 제가 학위과정을 시작하는 데에 더 많은 용기가 필요했을 것입니다. 새롭게 시작하는 학위과정 동안에도 부모님의 믿음과 도움에 항상 감사하고, 그 기대에 부응하는 아들이 되고자 합니다.

한편, 지도 교수님으로서 학문적으로 저에게 항상 연구 기회를 주신 홍성철 교수님에게 감사의 말씀을 드립니다. 부족함 많은 저에게 항상 진심 어린 조언을 주시고, 저의 부족에 대해 채울 기회를 항상 주셔서 많은 발전이 있었다고 생각합니다. 특히, 생물물리학 특강이나 물리생물학 등의 교과목을 통해서 생물물리학 전반에 대한 안목을 키워 주셨습니다. 앞으로 저의 연구자로서의 면모나 제가 연구했던 부분에 보였던 부족한 부분을 채워나가는 박사과정이 되었으면 좋겠습니다.

석사과정 동안에 학문적으로나 연구실 생활에 큰 도움을 주었던 엄희수 박사님께 감사드립니다. 제가 석사과정 동안에 진행하였고, 이번 학위 논문의 주요 주제인 Rho 의존적 전사 종결 과정은 엄희수 박사님의 가르침에서 시작되었고, 엄희수 박사님과의 토론을 통해서 무르익었습니다. 특히, 실험 결과를 해석하는 과정에서 방향을 잡아주는 조언은 연구 과정에서 큰 도움이 되었습니다.

본고에는 게재가 되지 않았지만 석사 과정을 진행하는 과정에서 Werner, HLTF, SHPRH가 fork reversal activity를 통해서 손상된 DNA 단백질을 어떻게 복구하는지에 대한 연구를 진행하였습니다. 신수철 박사님의 꼼꼼하고 세심한 프로젝트 인수 인계 과정이 있었기에 프로젝트를 시작할 수 있었다는 점에서 감사드립니다. 한편, 기초과학연구원 (IBS)의 명경재 유전체 항상성 연구단 단장님과 연구단의 강석현 박사님과 박지은 박사님께도 해당 프로젝트 진행과정에서 여러 조언을 주신 점이 큰 도움이 되었습니다.

연구실을 생활하는 동안, 곽재원 박사님, 박상준 박사님, 신수철 박사님, 이종진 박사님 등 네 분은 선배가 박사학위를 취득하였습니다. 제가 연구실 생활을 할 수 있었던 것은 박사님이 되신 네 분의 선배님이 계셨기 때문이라고 생각합니다. 사회의 여러 위치에서 뜻하는 바를 이루시기를 바랍니다. 또, 박사 후 연구원으로 저희 연구실에 계셨던 이주연 박사님, 그리고 영국에서 새로운 연구 생활을 시작하는 엄희수 박사님도 새로운 위치에서 좋은 연구를 하시기를 기대합니다. 이와 더불어, 저의 새로운 박사 과정 동안에 함께 계속 연구할 강우영 연구원, 강찬신 연구원과 임건형 연구원 모두 좋은 연구를 하기를 바랍니다.

아울러, 학위과정 동안에 학회활동이나 수업 등을 통해서 도움을 주셨고, 이번 졸업 과정에서 논문 심사를 맡아주신 서울대학교 자연과학대학 생명과학부의 윤태영 교수님과 화학부의 이남기 교수님께도 감사드립니다.

제가 지금도 연구를 할 수 있는 것은 아마 연구에 대한 열정이 넘치는 학부 동기와 후배, 그리고 당시에 여러 가르침을 주셨던 은사님이 계셨기 때문이라고 생각합니다. 서울대학교 물리천문학부에서 응집물리 분야의 연구를 하고 있는 김광탁 연구원은 서강대학교 물리학과 학부시절부터 지금까지 여러 도움을 주었던 동기입니다. 김광탁

연구원의 물리학에 대한 탁월함과 열정은 항상 저에게 연구에 대한 열정을 가지는 데에 도움을 주었습니다. 김광탁 연구원 외에도 서강대학교 물리학과 대학원에서 X선 물리학 분야의 연구에 전념하는 윤규석 연구원, 최성욱 연구원 등 전국 곳곳에서 연구하는 동기가 있었기에 제가 연구 생활을 결심하는 데에 큰 도움이 되지 않았나 생각합니다.

대학원 진학을 고려하고 있는 이재혁 학생, 생물물리학 분야에 관심을 가지고 저와 깊은 토론을 나눈 유정헌 학생, 이제 학부를 마치고 새롭게 학위 과정을 시작하는 천예륜 학생 모두 좋은 결과가 있기를 바랍니다. 서강대학교 생명과학과에서 불철주야 연구에 전념하는 최주영 학생은 생명과학 분야의 탁월한 지식과 안목으로 저의 생명과학 분야에 부족한 부분을 항상 채워준 부분에 대해 감사하며, 본인의 연구에도 좋은 결과가 있었으면 좋겠습니다.

서강대학교 자연과학부 물리학과 연성물리분광학 연구실의 김도석 교수님은 학부 시절 '막연'만 가득하던 저에게 많은 가르침을 주셨습니다. 가끔 아침에 교수님 연구실 칠판에서 토론을 하던 것이 떠오를 때마다 다시 연구에 대한 열정이 사라집니다. 당시 연구실에 있던 이종관 선생님, 이재진 선생님, Zaure 선생님, 차선철 박사님, 성웅모 박사님, Adam 박사님 등에게도 감사드립니다.

제가 현재 생물물리 및 화학생물학을 전공할 수 있었던 것은 서강대학교 학부 시절 물리학뿐만 아니라 화학과 생명과학 분야의 훌륭한 가르침 덕분이라고 생각합니다. 서강대학교 물리학과의 박정혁 교수님 (통계물리학, 일반상대론), 김원태 교수님 (수리물리학), 김도석 교수님 (고체물리학, 현대광학)은 연구 과정에서 큰 도움이 되었습니다. 서강대학교 생명과학과의 이규호 교수님 (미생물생리학, 환경과학론), 정광환 교수님 (생물물리학), 이호석 교수님 (신경생리학) 교수님의 수업

내용은 생물물리학 연구에 큰 도움이 되었습니다. 서강대학교 화학과와 바이오융합기술 협동과정의 조규봉 교수님의 분자생물공학, 생물물리화학과 바이오융합프로젝트 수업 등은 저의 연구에 큰 뒷받침이 되고 있습니다.

한편, 연구 외적으로 힘들 때마다 힘이 되어준 많은 분들이 있습니다. 서강대학교 수학과에서 위상수학을 전공하고 있는 이준엽 연구원이나 서울대학교 약학대학에서 연구하고 있는 박주찬 연구원과 같이 연구에 전념하는 경우도 있지만, 국가 공무(公務)에 관심을 갖는 동기나 후배도 많습니다. 이미 올해 좋은 소식을 얻은 수학과의 배재권 학생은 물론, 시험을 치르게 되는 물리학과의 오랜 동기인 이동희 학생, 수학과의 후배인 이새결 학생과 물리학과의 후배인 김동욱 학생에게 모두 좋은 결과가 있기를 바랍니다. 한편, 물리학을 전공을 하고 반도체 산업 발전에 이바지하는 후배인 강은혜 선생님과 위영서 선생님도 직장에서 좋은 일만 있기를 바랍니다.

또, 새로운 캠퍼스에서 만난 서울대학교 공과대학 재료공학부의 홍기명 선생님에게도 감사드립니다. 공학자로서의 소양과 다양한 분야에 대한 관심을 가진 덕분에, 저의 정신을 맑게 환기시켜주는 다양한 토론은 연구생활을 이어가는 데에 큰 도움이 되었습니다. 반면, 용산고등학교 시절부터 저보다 한 발 앞서서 좋은 연구자의 길을 걷고 있는 한국과학기술원의 장진형 연구원도 연구가 잘 진행되기를 바랍니다.

학부를 다닐 때, 먼저 졸업을 하시던 어느 선배님이 '예비 과학자'로 불러주신 것이 엊그제 일인 것 같은데 벌써 석사학위를 마칩니다. 어쩌면 긴 연구 생활의 여정 속에서 지금까지의 2년은 아주 일부분에 불과할지 모르지만, 이렇게 한 발을 내딛는 것으로도 저에게 큰 의미가 있는 일이라고 생각합니다.

마지막으로 제가 좋아하는 시를 통해서 마무리를 짓고자 합니다.

농담

유하

그대 내 농담에 까르르 웃다
그만 차를 엎질렀군요
미안해 하지 말아요
지나온 내 인생은 거의 농담에 가까웠지만
여태껏 아무것도 엎지르지 못한 인생이지만
이 순간, 그대 재스민 향기 같은 웃음에
내 마음 온통 그대쪽으로 엎질러졌으니까요
고백하건데 이건 진실이에요

석사과정에 그만 **차**를 엎질러 버렸을 때, 그 순간만큼은 그 실험 결과로 말미암아 **재스민 향기 같은 웃음**에 **제 마음이 엎질러진** 순간이 있었습니다. 힘들 때마다 그 순간을 되새기면서 석사과정 동안의 **농담**과 같은 연구가 **진실**이 되도록 하는 박사과정이 되기를 스스로 기대해 봅니다.