Single molecule studies on the dynamics of the transcription initiation complex of yeast mitochondria

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Single molecule studies on the dynamics of the transcription initiation complex of yeast mitochondria

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Abstract

The transcription initiation complex in the yeast mitochondria of *Saccharomyces cerevisiae* comprises the RNA polymerase, Rpo41, the initiation factor, Mtf1, and the DNA including 6 base pair promoter sequence. The Mtf1 is known to recognize and help to open the promoter region during the initiation stage, but its exact role and mechanism still remains unclear. We designed a multi-color single molecule FRET assay to directly measure the dynamics of the complex during transcription initiation. The labels on the DNA report on its opening-closing dynamics, while the label on Mtf1 report on the recruitment, dynamics, and dissociation of the initiation factor. From these measurements, we can correlate the promoter opening dynamics, factor binding/dissociation, and the transition to the elongation phase. Mtf1 is also associated with controlling the production of abortive RNA transcripts. We observed the scrunching motion during transcription by stepping along the DNA template with various combinations of nucleotide substrates. The FRET distribution shifted toward the high FRET region as we stepped further. From these observations, we propose a mechanistic model of the transcription initiation in the yeast mitochondria.

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Ⅰ. Introduction

The transcription initiation step starts with several base pair of DNA promoter collapse. The RNA polymerase(RNAP) in yeast mitochondrial called Rpo41 which is known to recognize T7 promoter sequence is synchronized on base pairs adjacent to the transcription start site [1]-[2]. In yeast mitochondria, it is not possible to transcribe RNA by using only the Rpo41. Therefore, the pre-initiation complex is essential to transcribe RNA.

Initiation step is multistep process [8]. Previous research figured out transcription initiation factor called Mtf1 in yeast mitochondria is synchronized with Rpo41 and can open the DNA maintaining DNA melting state during transcription [1],[6]. However, it is not well understood when Mtf1 is recruited and dissociated from DNA. It can be an important information to characterize the Mtf1s motions. Studies of the multi-color foster resonance energy transfer(FRET) assay can be the clue to design the dynamics motion of Mtf1. If the Mtf1 has fluorescent dye, we can follow Mtf1's whereabouts by single molecule FRET assay.

In addition, it is interesting to watch the walking of Mtf1 the Mtf1 repeatedly binds and dissociates from the DNA again and again catching several base pair of DNA in its hand called "scrunching" [3]. It is known that 20 amino acid in C-terminal of Mtf1 is critical residue to make RNA abortive products. In this step, we used wild type of Mtf1 (non-labeled Mtf1), but the DNA has cy3 and Alexa647 dyes. The cy3 dye can transfer the energy to Alexa647 if we apply green laser which excites cy3 dye. Therefore, FRET efficiency increases when DNA crumples because distance between cy3 and Alexa647 are closed. After the "scrunching", Mtf1 releases DNA at one time. It means FRET efficiency goes down rapidly. This research is meaningful because scrunching phenomena is related with abortive RNA fragment producing by transcription initiation complex. we designed DNA to led Mtf1 stop at specific position of DNA when we added ATP, UTP, GTP, CTP one by one. Finally, we figured out scrunching mode of Mtf1 and walking mode from single molecule FRET assay by adding ATP, UTP, GTP, CTP step by step to make stop position to DNA.

Knowing the path way of transcription initiation complex is important for quantitative understanding of gene regulation [7]. From this research, we characterized the Mtf1's dynamics, which is recruitment, dissociation, and walking mode. It inspires researcher who work with Mtf1. In addition, the Mtf1 is initiation factor which is homologous with TFB2M in human mitochondrial in assisting the DNA promoter opening and maintaining these states. Accordingly, this paper will be helpful to design the single molecule studies about TFB2M's dynamics.

Ⅱ. Method and Materials

1. Labeling DNA

   DNA has amino modified C6 dT(iAmMC6T) for the labeling position. The NHS ester functional group can chemically react with iAmMc6T. Mixture of 14ul of dye (in DMSO), 7ul of dH_2O, 75ul of 0.1M Sodium Tetraborate buffer, 4ul of DNA stock (25ug/ul) produces labeled DNA. The incubation time is 6 hours at room temperature. To recover the labeled oligonucleotide by ethanol precipitation, 10ul of 3M sodium chloride and 250ul of cold Ethanol is added. After 30 minutes at -20℃, the labeled DNA can be recovered by centrifuging the solution during 30 minutes.

2. Labeling C315S Mtf1

   C315S Mtf1 has cysteine at 315 residue so the cy3 maleimide dye can attach to the C315S Mtf1 protein. The labeling buffer (50mM of sodium phosphate buffer pH 7.5, 200mM of sodium chloride, 0.1mM of EDTA) helps protein labeling reaction at room temperature. Mixing ratio of protein and dye is 1:4, and the labeling efficiency is 1:1 after purification.

3. Purification to get cy3-Mtf1

   Size exclusion chromatography can separate free dye from unlabeled C315S Mtf1 and labeled cy3-Mtf1 because free dye which is lighter than protein goes down slowly than labeled/unlabeled protein. The labeled/unlabeled Mtf1 goes down quickly by using P6 column from Bio-Rad.

   To purify labeled cy3-Mtf1 only, hydrophobic interaction chromatography is used. Cy3-Mtf1 is much more hydrophobic than unlabeled C315S Mtf1, so cy3-Mtf1 is washed away more slowly than the C315S Mtf1. The binding buffer is 1.8M $(NH_2)SO_4$, 0.1M sodium phosphate, and the elution buffer is 0.1M sodium phosphate [5].

4. Imaging buffer

   To prevent dye from bleaching, imaging buffer contains Tris acetate 50mM, potassium glutamate 100mM, magnesium acetate 10mM, and Glucose 0.6% w/v in saturated Trolox buffer [1]. Trolox can protect dyes from photobleaching and with 1% of gloxy, dye can survive longer because gloxy maintains pH condition in solution to protect dyes from photobleaching.

5. TIRF

A. 2-color experiment

2-color TIRF uses green and red dye. The green laser excites cy3 dye, and the cy3 energy can be transferred to Alexa 647. If these two dyes are closer enough, energy is transferred to Alexa647 so Alexa647 emits high FRET efficiency [4].

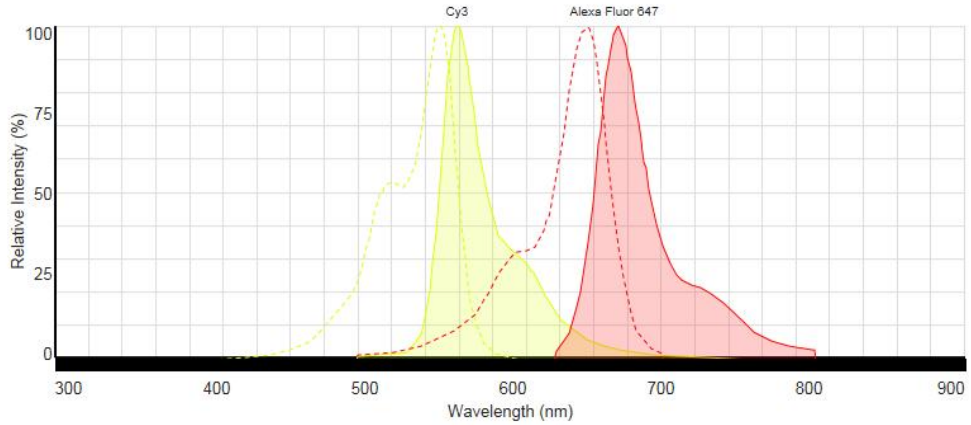
First, it is better to apply red laser just for 5 frames to see the number of Alexa647 dyes. Up to 1200 frames, the green laser excites cy3 dye to check the FRET efficiency. EMCCD catches the FRET signal. Movies which is recorded from smCamera program is needed for data analysis.

B. 3-color experiment

3-color FRET experiment is check the cy3, Alexa647 and ATTO740(or cy7). From the green laser, the FRET efficiency between cy3 and Alexa647 can be figured out. On the other hand, the FRET efficiency between Alexa647 and ATTO40 can be checked from red laser because the red laser excites Alexa647 and the energy of Alexa647 is transferred ATTO 740 dye.

The ALEX (alternative laser excitation) mode is used for measuring the distance per each dye. At first, cy3 dye excites from green laser and the energy is transferred to Alexa647[9]. From this FRET signal, the distance between cy3 and Alexa647 is figured out. And the red laser excited Alexa647 to know about the distance between Alexa647 and ATTO740 because the energy of Alexa647 is transferred to ATTO740.

(A)



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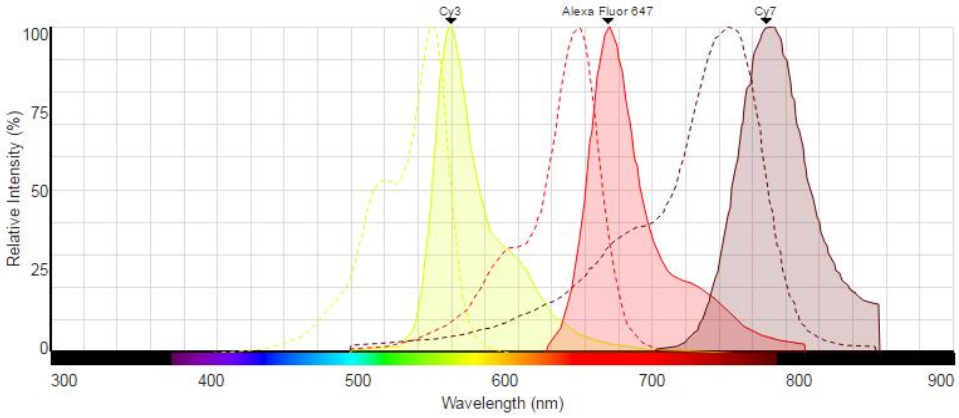

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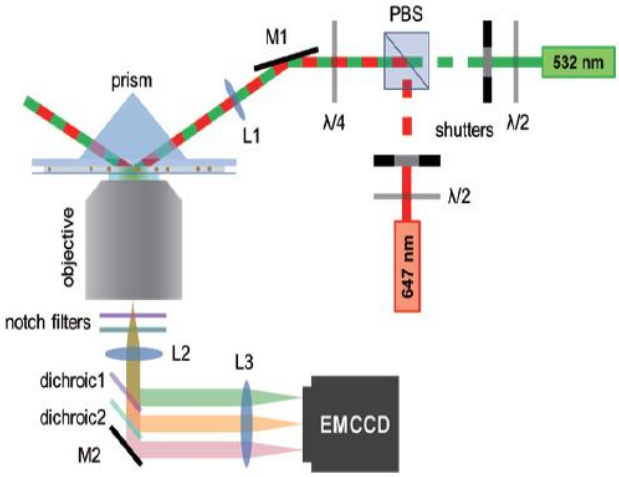


Figure1. wavelength of cy3, Alexa647 and ATTO740.

(A) There are two dyes for 2-color experiment, cy3 and Alexa647. emission wavelength

of cy3 dye (yellow area) is overlapped with excitation wavelength of Alexa647 (red dotted line). When green laser excites cy3 dye, emission energy of cy3 is transferred to Alexa647. Therefore, Alexa647 can emit its energy which captured by EMCCD.

(B) In 3-color experiment, we used cy3, Alexa647 and ATTO740 dyes. ATTO740 which is similar to cy7 dye is excited by Alexa647 and finally emits energy approximatively 800nm. Unlike 2-color experiment, two lasers, green laser and red laser, are used for 3-color experiment. From green laser, equally 2-color, we measure distance between cy3 and Alexa647. However, to measure distance between Alexa647 and ATTO740, red laser is used.

(C) ALEX (alternative laser excitation) is important for 3-color experiment. In ALEX, green and red laser are exchanged very quickly (5 times per 1 second) to measure distance between 3 dyes. If FRET signal is captured by EMCCD, researcher could see single molecule peaks from ‘smCamera’ program.

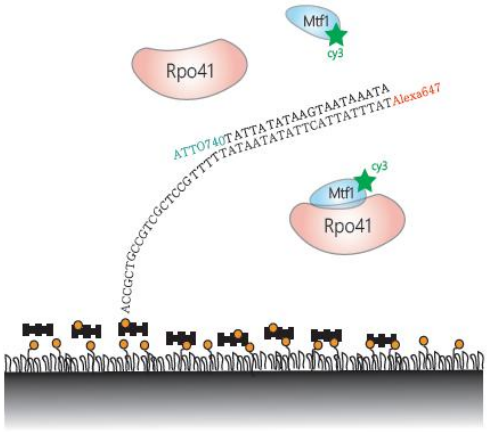
Ⅲ. Result

1. 3-color experiment with cy3-Mtf1 and ATTO740-DNA-Alexa647

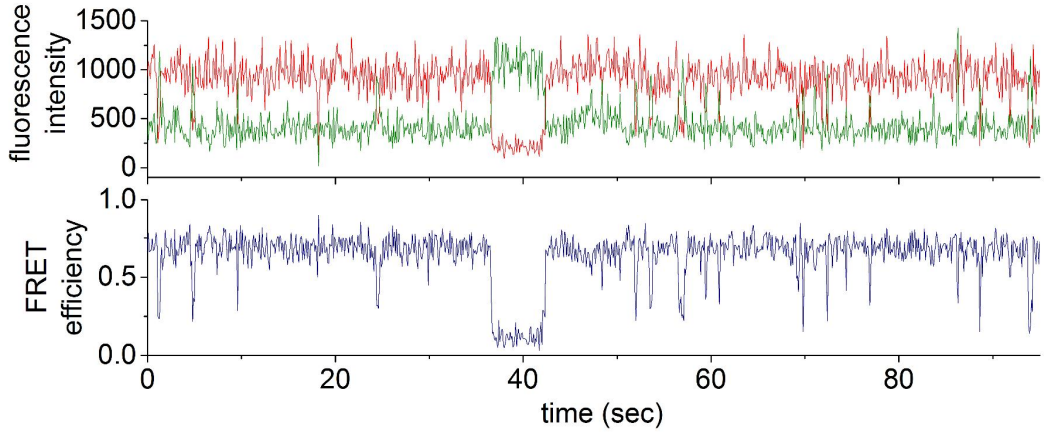
The 3-color experiment is to know about the Mtf1's recruitment and dissociation from DNA and Rpo41. The assumption is when the cy3-Mtf1 recruits to DNA, the DNA can be opened so the cy3 and Alexa647 can be closed. It means the high FRET efficiency can be shown between Alexa647 and ATTO740.

When the cy3-Mtf1 recruited to DNA, The DNA shows regular opening-closing dynamics.

(A)



(B)



(C)

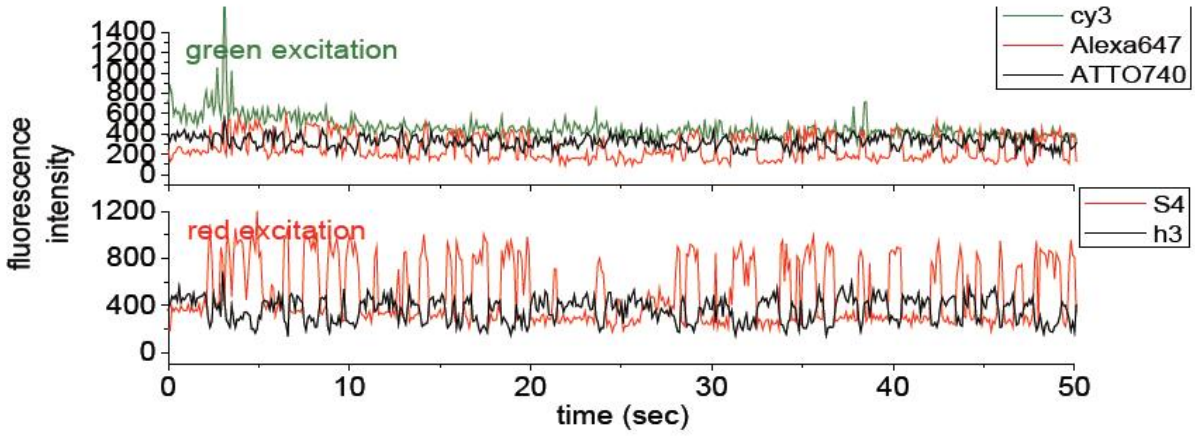


Figure2. 2-color experimental schematic model and example trace.

(A) 3-color experimental scheme. Cy3-Mtf1, ATTO740-DNA-Alexa647. cy3 energy is transferred to Alexa647, and Alexa647's energy is transferred to ATTO740. we check the distances of dyes per each.

(B) Example trace of 2-color experiment of cy3-Mtf1 + Non-template DNA + Template DNA-Alexa 647. When cy3-Mtf1 recruited to DNA, the energy of cy3 dye transferred to Alexa647. In this figure, we could know cy3-Mtf1 binds to the DNA.

(C) Example trace of 3-color experiment of cy3-Mtf1 + ATTO740- Non-template DNA + Alexa647-template DNA. When cy3-Mtf1 attached to DNA, the DNA opened and bent, So I could see the dynamics between Alexa647 and ATTO740. and I could also figure out the cy3-Mtf1's motion from the 3-color experiment.

2. 2-color experiment with wt-Mtf1 and cy3-DNA-Alexa647

Walking experiment

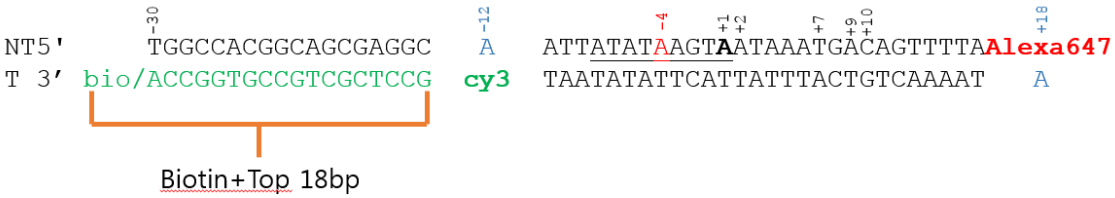
Wild type of Mtf1 is used for 2-color experiment with cy3-DNA-Alexa647 for the walking experiment.

When transcription complex transcribes RNA, the Mtf1 has DNA in its hand, after that, the Mtf1 releases DNA rapidly. This phenomenon is called "scrunching". In the scrunching mode, the FRET efficiency could increase because the transcription complex holds DNA out of shape so the distance between cy3 and Alexa647 closed. This experiment has novelty because scrunching motion is related with abortive RNA production. If transcription initiation step is failed, transcription complex releases DNA at one time not walks right direction of DNA. We figured 20 amino acids of C-terminal of Mtf1 is key position to make abortive RNA products. In in vitro transcription RNA products gel image, there are much amounts of RNA products which sizes are 2~7 mer than 8~12 mer. However, if we used mutated Mtf1 (D20) which is deleted 20 amino acids of C-terminal of Mtf1, 8~12 mer of RNA products are produced more than 2~7 mer. From FRET assay, which uses wild type of Mtf1, there are unstable FRET signal because transcription initiation is not perfect (dynamics mode and scrunching mode).

We wanted to stop transcription complex at specific position of DNA to watch walking motion of transcription complex. when we added ATP, the wt-Mtf1 could transcribe just 2 base pairs and stop that position. if ATP, UTP is added, the wt-Mtf1 could transcribe until 7 base pairs and stop walking. To stop the wt-Mtf1 after it transcribed 9 base pairs, it is enough to just add ATP, UTP, GTP. But the non-template DNA would be dissociated from the template DNA when the wt-Mtf1 transcribe 18 base pairs of RNA. because transcription complex scrunches DNA, if we add more kinds of NTP, it holds much more number of base pairs. For example, when in added just ATP, transcription complex crumples just 2 base pairs due to stop position. However, if we added ATP, UTP, transcription complex can hold up to 7 base pairs. Therefore, if we added more kinds of NTP, it causes FRET efficiency increasing because distance between cy3 and Alexa647 can be narrow. According this assumption, the FRET efficiency would be increasing from 0.2 to 0.8.

(A)

(B)



NTP mix	RNA length
A	2
A3'dU	3
AU	7
AU3'dG	8
AUG	9
AUG3'dC	10
AUGC	18

Figure3. schematic model of scrunching mode and designed DNA.

(A) schematic model of scrunching mode of Mtf1. Transcription complex crumples DNA and holds several base pair nucleotides for a while during transcription initiation step and releases DNA at one time.

(B) is an information of DNA and RNA length when we add NTP step by step.

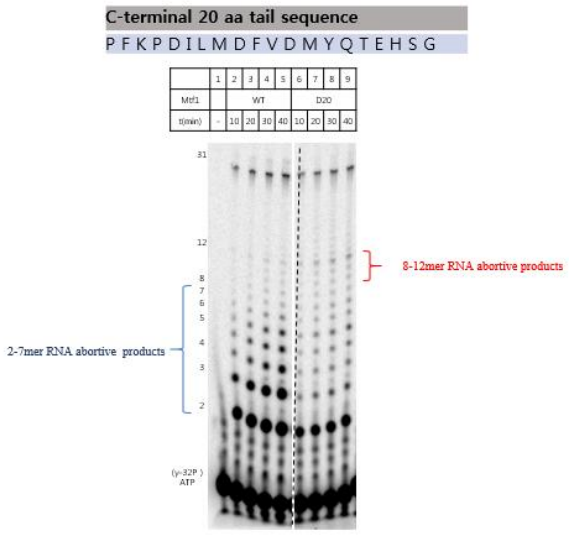


Figure4. in vitro transcribed RNA products.

With wild type of Mtf1, there are 2~7 mer RNA bands which is thicker than 8~12 mer RNA products. However, with D20 of Mtf1, there are 8~12 mer RNA products than before.

There are 3 types of FRET modes of example traces.

1. scrunching mode

The wt-Mtf1 has several base pairs of the DNA in its hand which means at this step the FRET efficiency is increasing step by step. After that when the RNA is transcribed or it is failed by transcription complex, the wt-Mtf1 release the DNA base pair which means the FRET efficiency decreases very rapidly. It is called “scrunching mode”.

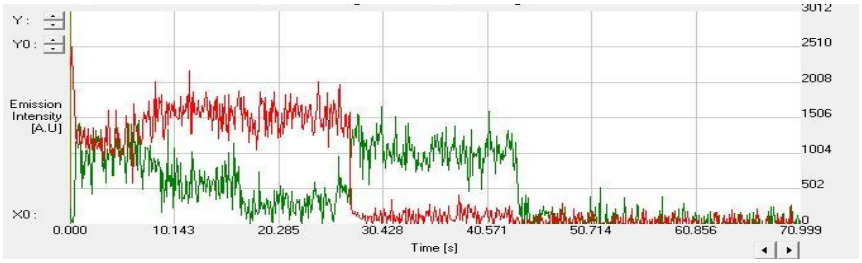
2. dynamics mode

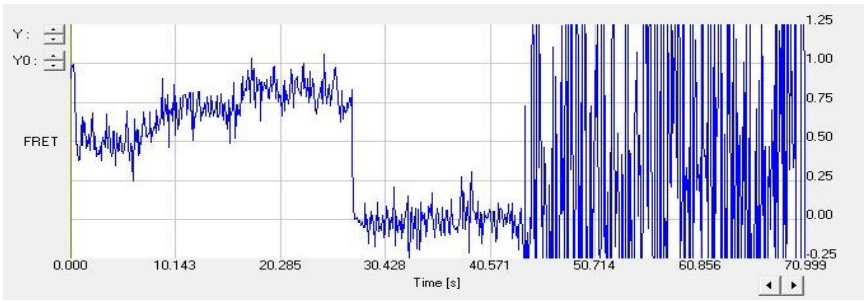
Sometimes the DNA is very unstable when the transcription complex recruits for transcription. The DNA is opening or closing very regularly. It means the transcription complex recruits or dissociates rapidly because transcription is failed. In this case, transcription complex could not proceed transcription and it is related with making abortive RNA products. It is not well understood why transcription complex makes abortive RNA products. However, single molecule FRET assay could offer transcription complex motion during making abortive RNA products for example when transcription complex recruits and dissociates.

3. Stable high FRET mode

When the transcription complex transcribes RNA, it attaches to the DNA for a while. At that moments, the wt-Mtf1 has the DNA in its hand. That is why the stable high FRET mode occurs.

(A)





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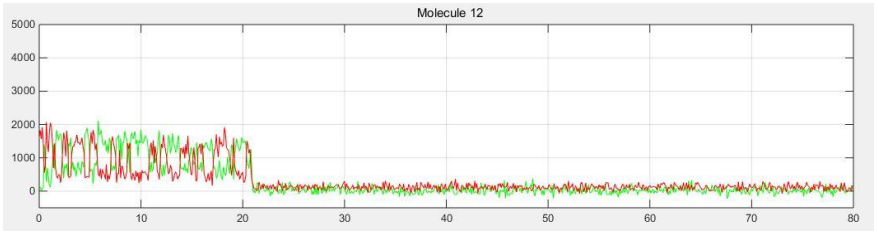
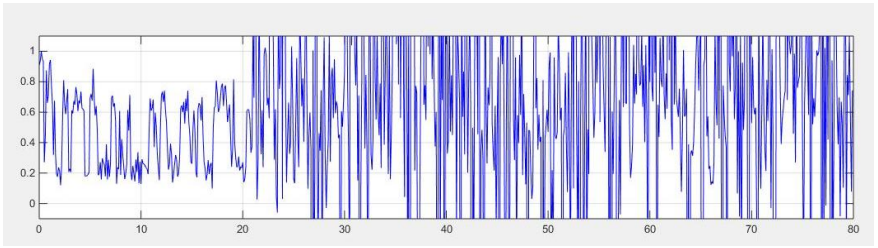




(C)

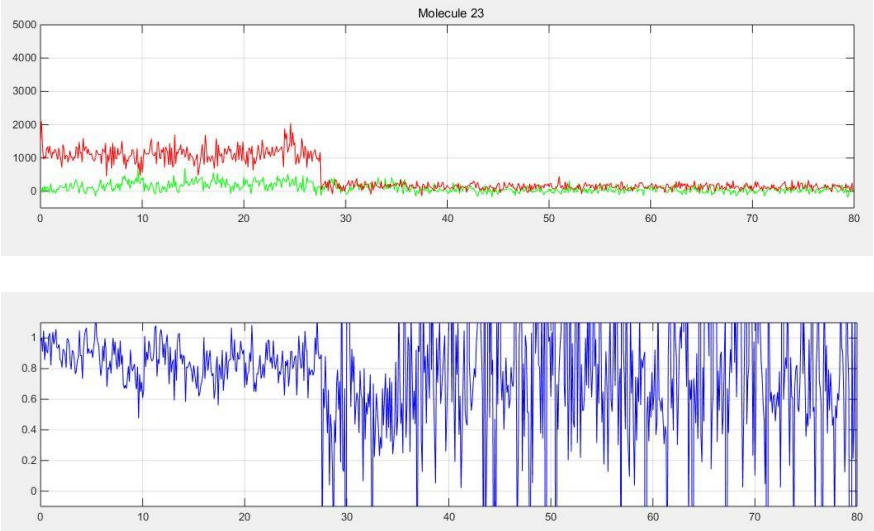


Figure5. example traces of 2-color experiment.

(A) scrunching mode of transcription complex. transcription complex crumples several base pair of DNA which could be explained by FRET efficiency increasing and releases DNA at one time.

(B). dynamics mode of transcription complex. if transcription complex could not proceed transcription, it is not able to make transcription complex go right direction of DNA and it recruits and dissociated from DNA repeatedly which means FRET efficiency could not increase anymore.

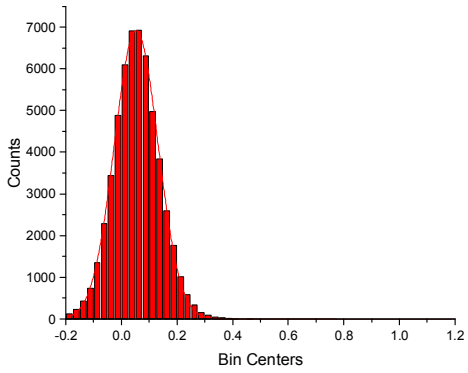
(C). stable high FRET mode. When transcription complex stops perfectly at specific position which we make, FRET trace shows stable high FRET mode.

FRET histogram

At the DNA only condition, the DNA is not opened which means the FRET efficiency is low around 0.2. However, when the Rpo41 and Mtf1 is added, the DNA can be opened. Histogram (figure6(B)) shows the peak which increased a little bit. when adding the NTP, not only the DNA is opened, but also the distance of two dyes is closed because of the scrunching mode. However, adding ATP condition (figure6(C)), there is no significant difference than before. because the Mtf1 walks just 2 base pair, the histogram does not show big differences. but when the Mtf1 walks 7 base pair of DNA which condition is adding ATP and UTP, there is mid-FRET peaks around 0.5. there are 3 peaks at the adding ATP, UTP, GTP condition. First one means closed DNA, second one is opened DNA, third one is affected from the NTP, which is mixed several phenomena, for example the transcription complex stop at specific position before transcribes 9 base pair of RNA, or after transcribing the 9 bases pair of RNA. even though the Mtf1 walks longer than others condition at adding ATP, UTP, GTP, CTP, because the non-template DNA is dissociated from template DNA, the FRET efficiency goes down again such like DNA only condition.

Comparing to without washing and washout condition (figure6 and figure7), there are different shapes about multi-peaks. For example, at adding ATP, UTP, GTP, 3'dCTP condition, the FRET peaks are broad without wash condition. in this solution, protein, Rpo41 and Mtf1, nucleotides are remained. On the other hand, there are much clear three peaks in washout condition. It means the Rpo41 and Mtf1 complexes recruits to DNA again and again. Therefore, wash out condition is better for experiment because we could reduce multiple peaks from multiple protein complexes.

(A)



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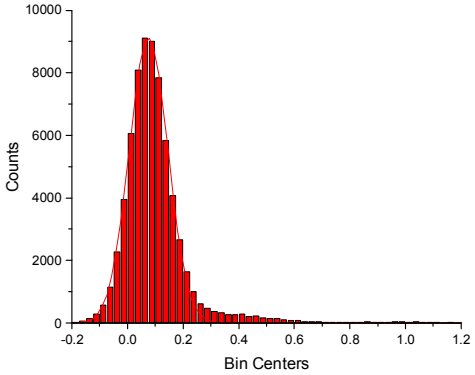

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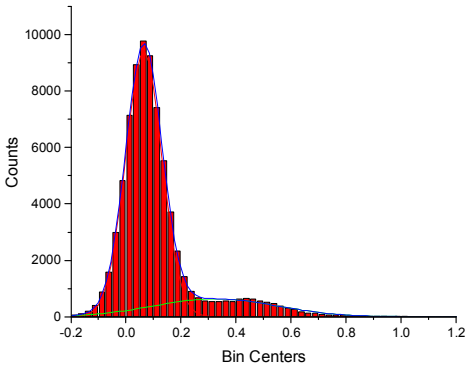
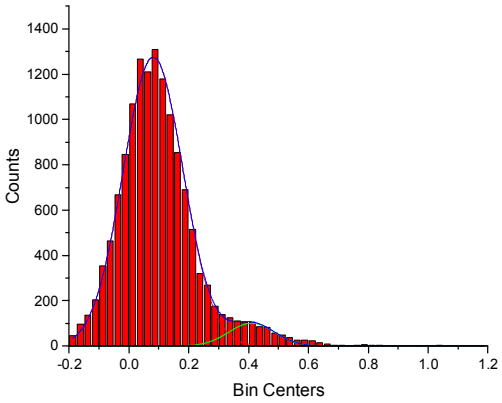
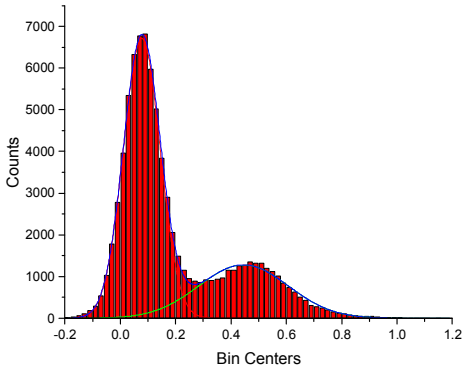


(D)



(E)



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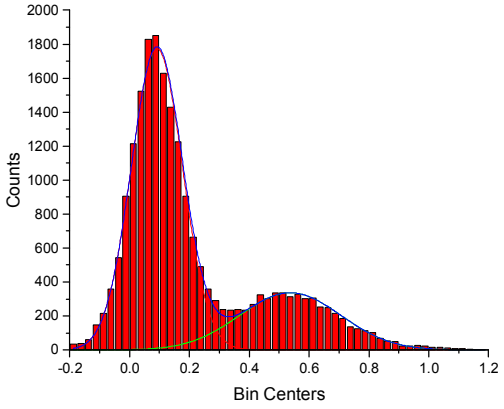

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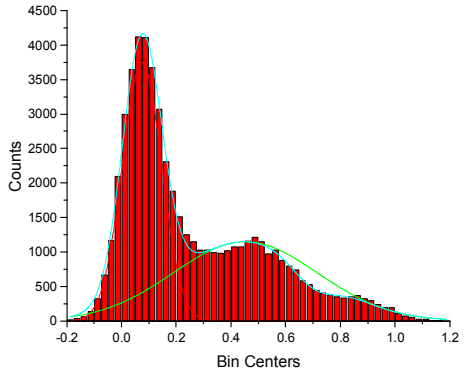
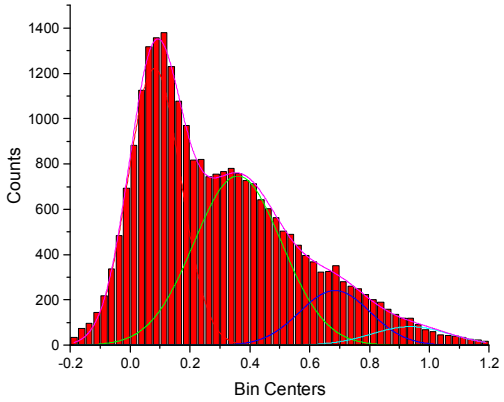


(H)



(I)

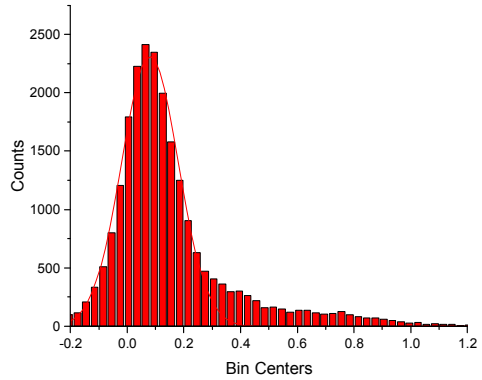


Figure6. FRET histogram in each condition without washing.

(A) DNA only condition. FRET efficiency is low about 0.2 because DNA is not opened and very stable without any effect from the outside.

(B) DNA + Rpo41 + Mtf1 condition. Although we did not add NTP, several DNA molecules are opened by effect of transcription complex.

(C) DNA + Rpo41 + Mtf1 + ATP condition. FRET peak around 0.5 is increasing by effect of ATP.

(D) DNA + Rpo41 + Mtf1 + ATP, 3'dUTP condition. it is similar to figure6(C)

(E) DNA + Rpo41 + Mtf1 + ATP, UTP condition. There are tree peaks in histogram. FRET peak

around 0.5 is due to ATP and UTP.

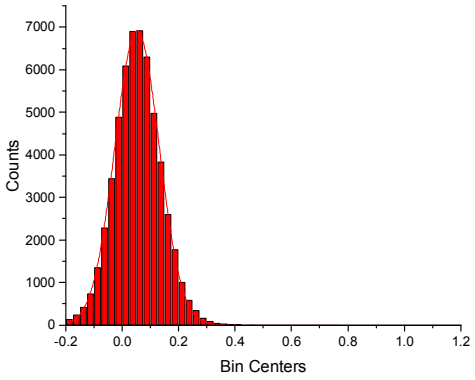
(F) DNA + Rpo41 + Mtf1 + ATP, UTP, 3'dGTP condition.

(G) DNA + Rpo41 + Mtf1 + ATP, UTP, GTP condition. There is another peak around 0.8 which is not shown previous condition. it is due to GTP.

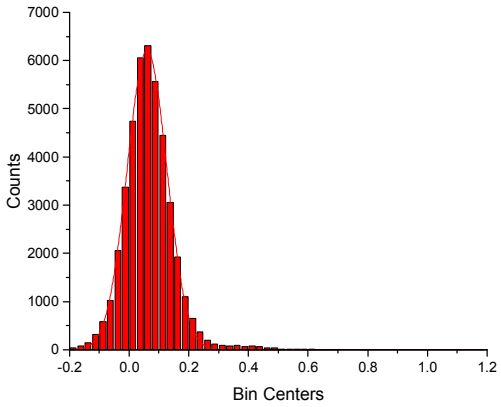
(H) DNA + Rpo41 + Mtf1 + ATP, UTP, GTP, 3'dCTP condition. there are four peaks. One is around 0.2 with DNA closing, second is DNA opening peaks. And then third one and fourth one appeared because of scrunching mode and multiple transcription complex.

(I) DNA + Rpo41 + Mtf1 + ATP, UTP, GTP, CTP condition. because non-template DNA is dissociated from template DNA, FRET efficiency decreased rapidly.

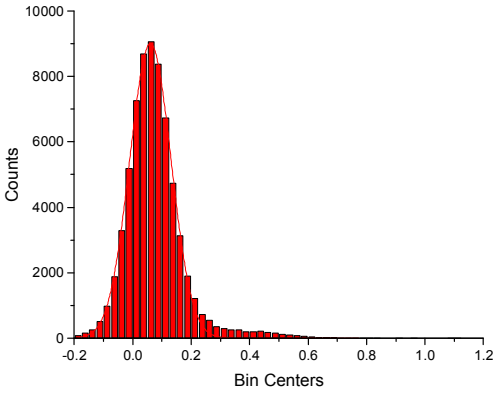
(A)



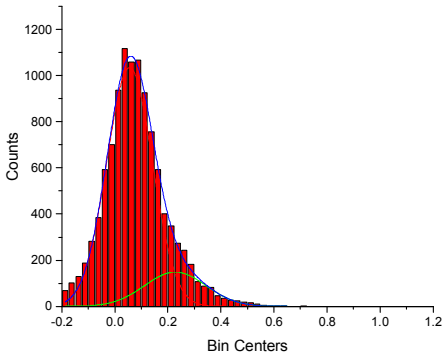
(B)



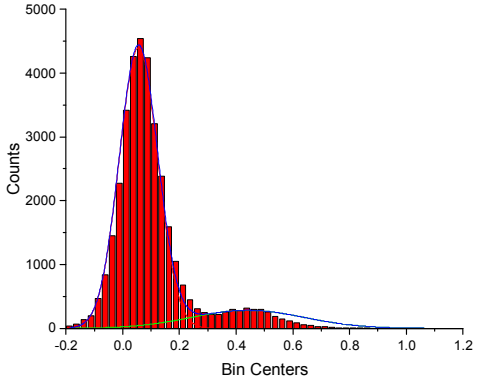
(C)



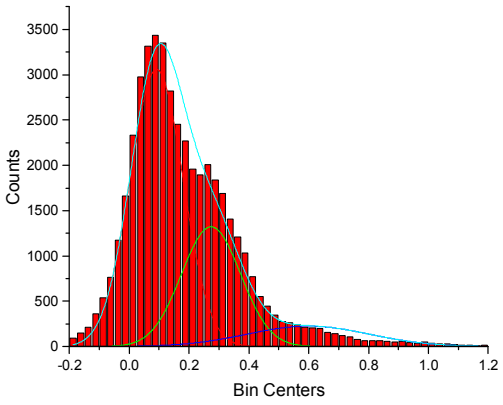
(D)



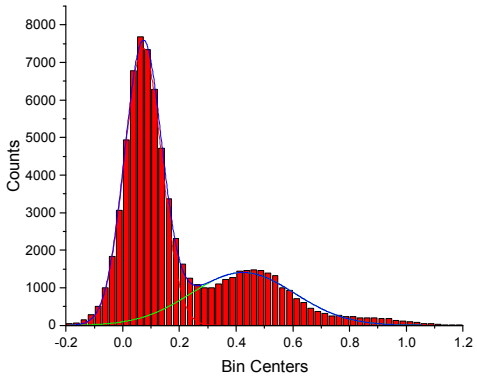
(E)



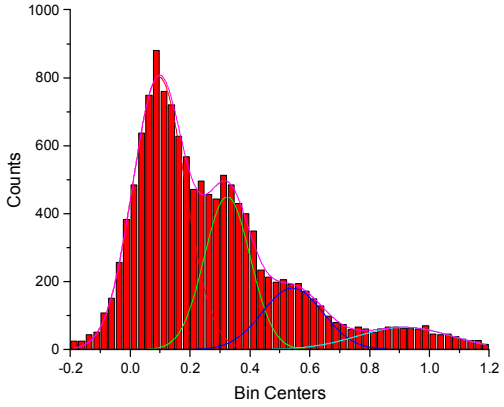
(F)



(G)



(H)



(I)

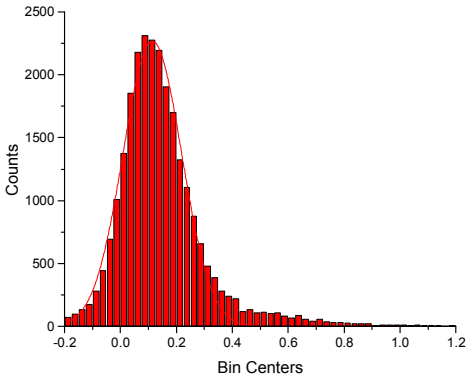


Figure7. FRET histogram in each condition with washing.

(A) DNA only condition. FRET efficiency is low about 0.2 because DNA is not opened and very stable without any effect from the outside.

(B) Washout Rpo41 and Mtf1 condition. Although we did not add NTP, several DNA molecules are opened by effect of transcription complex.

(C) Washout with ATP condition.

(D) Washout with ATP, 3'dUTP condition.

(E) Washout with ATP, UTP condition.

(F) Washout with ATP, UTP, 3'dGTP condition.

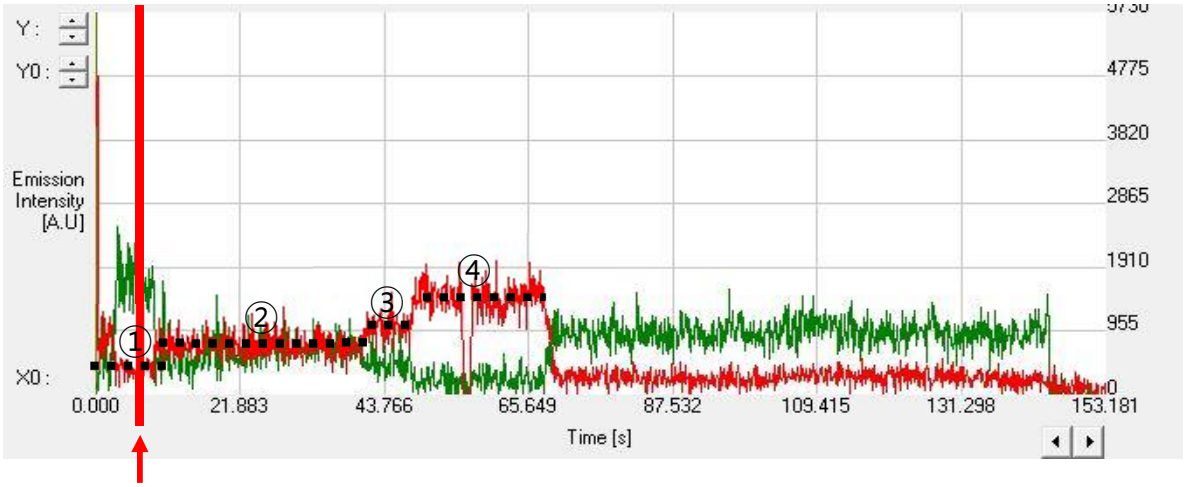
(G) Washout with ATP, UTP, GTP condition. because the transcription complex walked 9 base pair from starting point, FRET efficiency increased which is around 0.5.

(H) Washout with ATP, UTP, GTP, 3'dCTP condition. there are four peaks. One is around 0.2 with DNA closing, second is DNA opening peaks. And then third one and fourth one appeared because of scrunching mode and multiple transcription complex.

(I) Washout with ATP, UTP, GTP, CTP condition. FRET efficiency decreased because non-template DNA (without biotin) is dissociated from template DNA (with biotin).

Flow in experiment

The transcription complex walks toward right direction (from +1 to +18 of DNA) when the NTP is offered. We flowed all kinds of NTP (ATP, UTP, GTP, CTP) at 5 seconds after recording movie to watch step wise motion. By flow in experiment, the stepwise FRET trace can be shown. The figure shows the 4 steps of FRET efficiency increasing. Not only the stepwise phenomenon but also the protein complexes of wt-Mtf1 and Rpo41 are recruited to the DNA randomly. As conclusion, the transcription is not occurred just one of transcription complex. That is why the histogram peaks are overlapped.



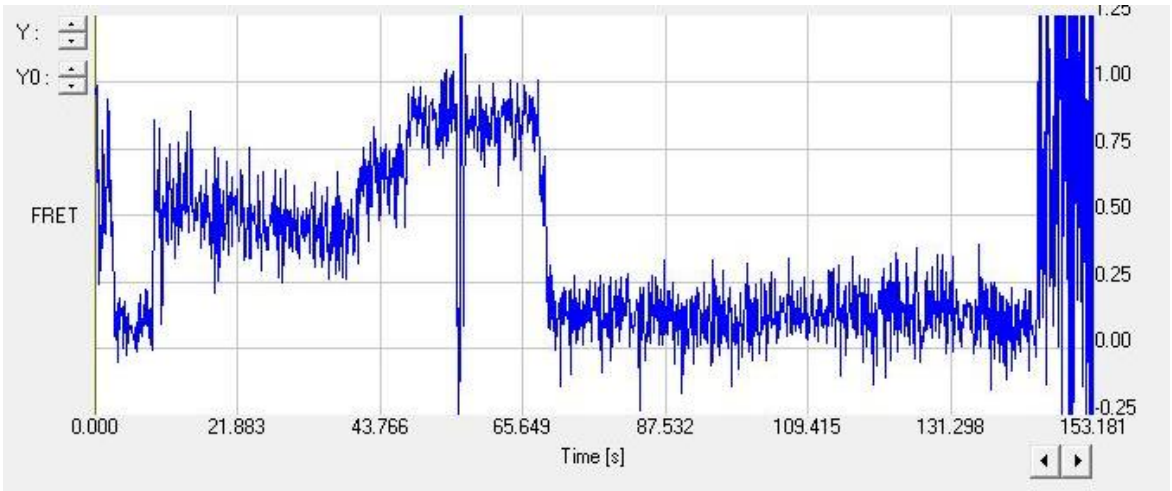


Figure 8. Flow in experiment FRET trace.

Red line is position of flowing NTP at one time. after transcription complex is affected from NTP, it walks right direction of DNA transcribing RNA. there are 4 steps of transcription complex walking motion. As a result, FRET efficiency increases from 0.2 to 0.8.

Ⅳ. Conclusion and Discussion

3-color experiment

From 3-color experiment, we figured out when Mtf1 recruits, DNA's opening-closing dynamics appears. Although Mtf1 attaches to DNA over 50 seconds, it is just one movie not normalized one. it is due to traces which looks good are very few. we doubt binding efficiency of Mtf1 to DNA through colocalization efficiency. If Mtf1 bind DNA perfectly, cy3 peaks and Alexa647 peaks should be overlapped at least 10%. However, in our case, the colocalization efficiency is just 3~5%. Because cy3-Mtf1's binding quality is quite low (low quality of cy3-Mtf1), we need to make fresh cy3-Mtf1 to get meaningful result.

2-color experiment

There are 3 types of Mtf1 walking mode. When transcription proceeds well, transcription complex stops at specific position as we expected. However, some of traces appear unstable dynamics mode and scrunching mode. Transcription complex recruits and dissociates very rapidly or it crumples DNA for a while and after release DNA at one time. these mechanisms are related with making abortive RNA products. Abortive RNA products are incomplete RNA fragments because of unstable transcription complex.

The gel image shows 20 amino acid in C-terminal of Mtf1 is fundamental residue to make the abortive RNA products. The reason why transcription complex makes abortive RNA products is not well known. Therefore, comparing wild type of Mtf1 and mutated Mtf1 which has 20 amino acid deletion in c-terminal (D20 Mtf1) by single molecule FRET assay is crucial. We expect that there are few of traces which shows scrunching mechanisms but much more dynamics mode because of RNA transcription failing when we use mutated Mtf1 (D20 Mtf1).

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무엇보다도 지금까지 저를 사랑으로 키워 주신 부모님께 저의 모든 공을 돌리고 싶습니다.

앞으로도 개인의 성장을 넘어 사회에 보탬이 되는 구성원으로서 성장하겠습니다.