SINGLE-MOLECULE STUDY
ON THE DNA HELICASE REGULATING GENOME STABILITY

BY

JEEHAE PARK

DISQUERATION
Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biophysics and Computational Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

Doctoral Committee:
Professor Taekjip Ha, Chair
Professor Paul R. Selvin
Professor Issac O. Cann
Assistant Professor Aleksei Aksimentiev
Abstract

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Jeehae Park, Ph.D.
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Taekjip Ha, Advisor

A DNA helicase, PcrA is an essential protein in gram-positive bacteria. PcrA utilizes its helicase activity and translocation activity to regulate various cellular functions including plasmid replication and counteracting deleterious recombination.

Using single molecule FRET technique and site-directed mutation, we discovered enzymes new activity: PcrA act as a powerful motor and remove RecA protein effectively from single strand DNA (ssDNA) as it pulls the RecA-coated lagging strand from the DNA junction. Our observation provides plausible mechanistic explanation for how a helicase can protect stalled replication fork from uncontrolled recombination in vivo.

PcrA is a prototypical translocating motor of which structure and kinetics are heavily studied for its kinetic mechanism. Utilizing the persistent reeling-in activity of PcrA, we conducted heterogeneity-free analysis and identified that the fundamental unit of translocation kinetics is a ‘one nucleotide uniform step’. Our result reconciles debates between the structural and biochemical studies on the motor mechanism, and moreover, clarifies a popular oversight regarding molecular heterogeneity.

Next, I developed new detection scheme to monitor translocation dynamics of unlabeled protein that carries metal containing domain. By utilizing protein’s ability to quench organic fluorophore in distance dependent manner, XPD helicase translocation on ssDNA was monitored
and, the differential effect of single-strand binding protein, RPAs on the XPD translocation kinetics were evaluated.

Lastly, I aim to observe translocating motor on a physiological length of ssDNA. FRET has limited working distance and therefore about 80 nucleotides (nt) or further distance change is undetectable. Custom made DNA nano-structure was devised and it is capable of providing up to 2000 nt stretched ssDNA tracks immobilized on the surface. I use fluorescence colocalization and FIONA technique to monitor hundreds of nanometer scale motion of translocases and activity of other single strand interacting proteins.
To my mother, Bokhyun Song (1957-2007)
Acknowledgements

My advisor, Taekjip Ha has provided a great guidance throughout my Ph.D. studies. He has been extremely supportive and patient with my development as a researcher. He has also given me a handful of research/academic opportunities and through those I learned to enjoy doing science and grew as a better scientist. I admire his brilliant insights and dedication for science and I am very thankful for many years of positive influence that I received from him.

Sua Myong has been an amazing teacher, mentor and colleague for me since the first day of my laboratory training. She never hesitated to spare her thoughtful advices as well as technical expertise. Moreover, my helicase study was initiated based on her pioneer work on Rep helicase.

Chirlmin Joo is an inspirational person in my Ph.D. He has been a great role model as a graduate student and as a researcher. He was not only excellent but also very generous and offered significant time and effort with younger graduate students including me.

Together with above mentioned people, Rahul Roy, Michelle K. Nahas and Burak Okumus taught me how to do single-molecule experiments. Ivan Rasnik and Sungchul Hohng gave me insightful advises and ideas. Sua Myong, Ben Stevens, Cathy McKinney taught me molecular biology techniques. Sean McKinney taught me optics and programming.

Ibrahim Cisse and Cheng Liu are my academic brothers. We joined the lab at the same year and shared so much on and beyond science and learned from each other. A lot of helpful discussion with Sinan Arslan had significant contributions to many helicase researches. Jaya Yodh and
Michael Schelif shared great ideas and technical expertise on mutagenesis studies. I had a great joy to be around Jiajie Diao, Reza Vafabakjsh, Ruobo Zhou, Kaushik Ragunathan, Prakrit Jena, Ankur Jain, Tae-young Yoon, Gwangrog Lee, Maria Sorokina, Jungmin Yoo, Michael Brenner, Seongjin Park, Sultan Doganay. I received many intellectual insights from Xinghua Shi, Peter Cornish, Yuji Ishitzuka, Hamza Balci, Eli Rothernberg, Hajin Kim, Ben Leslie and Hye Ran Ko. Sunghyun Kim, Victor Caldas and Jeongmin Yoon gave me positive influence even during their short visits. I thank my undergraduate student Emily Bozek, Jason Smith and Brian Zider for their enthusiastic contributions on the molecular biology work.

I thank my collaborators especially Timothy M. Lohman, Anita Niedziela-Majka and Eric Tomko for PcrA and UvrD studies; Maria Spies and Masayoshi Honda for XPD studies. Issac Cann and Yuyen Lin has been invaluable help in many aspects. It was a great experience to work with theorist Klaus Schulten, Jin Yu and Yang Liu. It has been exciting to build collaboration with Tim Liedl Lab and I thank his graduate student Stephanie Simmel for the DNA origamis.

Salman Syed has been great help with technical and secretarial aspects. He has also been a great friend and colleague. Julie Hall, Cindy Dodds, Wendy Wimmer, Karen Driscoll and Susan Flanegin were very helpful with secretarial support and their amazing kindness.

I would like to thank my prelim committee of Professors Bob Clegg, Ido Golding and Issac O. Cann as well as my defense committee Paul Selvin and Aleksei (Oleksii) Aksimentiev and Issac O. Cann for overseeing my graduate study. I thank Jae Youl Kim and Heeja Lim in Korea Foundation for Advanced Studies for their years of heartfelt support.
Lastly, I would like to thank my parents who raised and supported me with incredible love and sacrifice. I thank my husband Kyungsuk Lee with my heart for his encouragement and care in every aspect of my life.
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Chapter 1 Single-molecule fluorescence technique

1.1. Observing single biological complex using total internal microscopy

1.1.1. Advantage of single-molecule technique

Biomolecules regulating physiological functions in a cell are nano-machineries which change conformation and move physically in order to perform its function. In order to understand mechanism of how these nano-machineries work, it is crucial to observe their motion during its function. Classically, a function of a protein is being measured by measuring reactant or product concentration inside the cuvette during reaction. However, the actual motion of the protein is very difficult to detect in this way as there are millions of proteins inside the cuvette and their motion is not synchronized to be detected. There have been a few methods where researchers force reaction synchronization through fast mixing technique; however, growing evidence indicates that the individual molecules intrinsically have heterogeneous dynamics therefore imposing limitation to these approaches.

The simplest and most clear way of overcoming above mentioned problem is observing a single molecule at a time. Advancement of optics and detection devices now allows for tracking time trajectory of a single biomolecule. This method provides rich information that was not able to be obtained with ensemble techniques. Following are the advantage of Single molecule techniques.

1) Individual states are identifiable and the population distribution of each state can be measured.
2) Transition states which occur for a short time can be identified. 3) Structurally homogeneous molecules do show varying degree of dynamics and this heterogeneity in dynamics can be observed. 4) For a non-synchronized reaction, the reaction trajectory from individual molecule can be post-synchronized and for comparison and for further analysis. 5) At an equilibrium
condition, molecules may have dynamics of interconverting between different states. These
dynamics may play an important part of protein’s function however these equilibrium dynamics
are impossible to detect unless using single molecule approach.

1.1.2. Total internal reflection microscopy for single molecule detection

A powerful method of observing single molecule is use of fluorescence. In order to track
dynamics of a single molecule, fluorescently labeled biomolecules can be immobilized on the
microscopic slide surface. Individual molecule can be identified as an isolated fluorescent spot
when immobilized sparsely with average molecule-to-molecule-distance greater than diffraction
limit. However, signal that comes from a single fluorophore is finite and cannot be detected
clearly unless the background signals are eliminated. This becomes more problematic when
performing experiment with fluorescently labeled molecules present in solution. Effective
method of rejecting background fluorescence coming from solution is to illuminate only a finite
depth above the surface using Total Internal Reflection Microscopy.

Total Internal Reflection occurs when the light enters a medium of lower refractive index with an
incident angle larger than a critical angle. In this condition, entire incident light is reflected back
to the first medium. The critical angle can be calculated as the following.

\[
\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right)
\]

n1 is refractive index of original medium and n2 is refractive index of the second medium. For
our fused silica(n1=1.46)/water interface(n2=1.33), \( \theta_c \) is approximately xx. While all the light
is reflected back at the interface, light does penetrates in finite depth due to its continuous nature
of electromagnetic field. This finite depth of illumination is called evanescence field and its
intensity decays exponentially in relation to distance from the interface.
I₀ is the intensity at the interface, \( z \) is the distance from the interface and \( d \) is the penetration depth where the intensity becomes \( I₀/e \). Typically, the penetration depth \( d \) is referred as an illumination range of evanescence field. \( d \) falls \( \sim 100 \text{nm} \) and is dependent on wavelength of light, angle of incidence and diffraction index of the two medium in following relation.

\[
d = \frac{\lambda}{4\pi} \left( n_1^2 \sin^2 \theta - n_2^2 \right)^{-\frac{1}{2}}
\]

Typical depth of our experimental chamber is approximately 30 \( \mu \text{m} \) therefore \( \sim 99.5\% \) of the background noise can be effectively rejected while signal from single fluorophore illuminated by evanescence field can be detected effectively.

1.2. Measuring nm distance range dynamics using Föster resonance energy transfer

1.2.1. Föster resonance energy transfer (FRET)

Using Total internal reflection microscopy and surface immobilization, it is possible to detect the signal coming from a single molecule. Now the challenge is to detect a molecule’s motion accurately. One can either try to pinpoint molecule’s absolute position and track the distance change. However, this method is not trivial due to many technical difficulties such as stage drift and requirement for high photon counts for accurate position estimation. Average size of protein is \( \sim 5\text{nm} \) and many of the biologically relevant dynamics occur in nanometer range. In order to probe motions in such distance range with high sensitivity, Fluorescence Resonance Energy Transfer (FRET) can be used as an ideal tool which bypasses need for determination of absolute position.
Fluorescence Resonance Energy Transfer is a phenomenon that occurs between two fluorophores. Fluorophore is a molecule that absorbs a certain wavelength of light and emits another specific wavelength of light. The emitted light is called fluorescence. The energy absorbed through excitation wavelength is partially lost through the intermolecular vibration thus emission light becomes lower in energy making emission wavelength longer than excitation wavelength (Stokes shift). Energetic perspective of fluorescence can be described as Jablonski diagram below and the example of excitation and emission spectral profile of a fluorophore is as below.

Fluorophores have their own unique excitation/emission spectra. When there is a pair of fluorophores of one’s emission spectra overlaps with the other’s excitation spectra, Energy absorbed by the first fluorophore (donor) can be transferred to the second fluorophore (acceptor) so that the latter can emit fluorescence. This process is called Fluorescence resonance energy transfer (FRET) and the fraction of energy being transferred can be defined as FRET efficiency (EFRET). EFRET is increased with decreasing distance following the relationships as below.

\[
EFRET = \frac{IA}{ID + IA}
\]

\[
EFRET = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}
\]

ID and IA are fluorescence intensity from donor and acceptor each. R is the distance between donor and acceptor. R0 is a distance where EFRET becomes 0.5. Cy3 and Cy5 are the most popular fluorophore pair used as donor and acceptor each for FRET measurements due to many advantages: 1) Cy3 emission spectrum and Cy5 absorption spectrum overlaps significantly so that
energy transfer is efficient. 2) The peak of Cy5 emission spectrum is far (~100nm) from the peak of Cy3 emission spectrum so that fluorescence signals can be separated and detected. 3) Quantum yield of the two are comparable so that the anti-correlated signal of donor and accepter can be easily visible when FRET change occurs. 4) Cy3 and Cy5 are commercially available with various chemically functional groups so that they can be conjugated easily to various biological substrates. When $E_{\text{FRET}}$ is measured with Cy3 and Cy5 pair, its relation to the distance change follows as below.

**1.2.2. Advantage of single-molecule FRET technique and its limitations**

$E_{\text{FRET}}$ changes in distance range of 3-8nm with R0 around 5nm. This is a useful distance range to observe biomolecules: One helix turn of DNA is 3.4nm and the average size (diameter) of a protein is 3-6nm (Moran et al., 2010). When placing FRET pair on the system to probe the distance change, it is preferred to position them so that the distance change occurs around R0. Maximum sensitivity can be achieved by this strategy and especially for the short range dynamics, even a few Å difference can be distinguished.

Combined with Total Internal Microscopy, single-molecule FRET technique can provide detailed information of a molecular dynamics which was inaccessible in the past. Among other single-molecule techniques (such as AFM, optical/magnetic tweezer or FIONA), single molecule FRET is unarguably more stable and high throughput method. As the distance change is measured independent of absolute position detection, it is much less prone to stage drift. Also FRET is a ratio-metric unit therefore is independent of any fluctuation in excitation laser intensity. As FRET method is free from individual particle tracking, wide field TIR illumination and use of EMCCD camera is possible, allowing parallel detection of hundreds of molecules on
the surface at the same time. This high throughput capability is efficient way of obtaining data as well as ideal method to observe distribution of states in the population.

Single-molecule FRET is applicable in a limited distance range dynamics (up to around 8nm). Therefore, to observe longer range dynamics, single-molecule FRET is not suitable. Single particle tracking method such as FIONA can be more applicable for such occasions. Generally used time resolution for our single molecule FRET setup is 30ms. Therefore, if a certain dynamics occur faster than our detection frequency, the signals will be averaged out. Limitation on time resolution comes from the detection device. EMCCD camera that we use is suitable for imaging wide field illuminated area allowing parallel imaging of large populations. Faster time resolution (up to 8ms) can be achieved by reducing the area of imaging. If a faster time resolution is required, APD can replace EMCCD and push the resolution down to the system’s photophysical limits.

1.3. Other non-particle-tracking fluorescence methods to measure distance changes

1.3.1. Use of protein-induced fluorescence enhancement

Some of the fluorescent dyes are observed to show enhancement in fluorescence when a protein approaches nearby. This property has been employed in ensemble fluorescence techniques to study DNA translocating proteins. There are number of fluorophores that are found to show fluorescence enhancement upon protein binding. 2-aminopurine (2-AP), a fluorescence analogue of adenine can be substituted at the specific position of a nucleotide base and distance between the 2-AP incorporated position and the protein was measured by fluorescence enhancement (Dillingham et al., 2002). Cy3 is also used to measure distance change between the fluorophore labeled position and the protein. It was used to measure translocation activity of
UvrD and PcrA (Niedziela-Majka et al., 2007; Tomko et al., 2007) in ensemble experiment. In single molecule study, such property was reported in RecA study (Joo et al., 2006). It was used as a main technique to monitor polymerase activity in single molecule in 2008 (Luo et al., 2007). Recently in our lab, it was used to monitor RIG-I helicase translocation on the duplex DNA (Myong et al., 2009). It is used as a main tool to observe directionality and position of the PcrA at the beginning stage of my research (2006) when labeled protein was unavailable. Photophysics beyond PIFE is not fully understood. It is considered that the quantum yield of Cy3 is influenced by the torsional flexibility of the fluorophore along the absorption plane. The conjugate linker between the two resonance rings of the Cy3 can be twisted and the energy can be relaxed by such vibration. It is thought that the proteins which usually convey multi-charge can stabilize the conjugate plane therefore minimize energy loss thereby increase emission probability. Our unpublished result with TCSPC experiment has shown that the lifetime of the fluorophore indeed increases with increase in intensity induced by protein binding. Further characterization of the PIFE is conducted in Myong lab and shown that it can sensitively capture the dynamics of protein-Cy3 distance in 1-3nm range (communication with H. Hwang. Unpublished results).

1.3.2. FeS induced Fluorescence Quenching

Distance change can be measured by the decrease of fluorescence affected by interaction between protein and a fluorophore. It is known that fluorescein can be quenched by the interaction with protein and was used to measure UvrD translocation (Tomko et al., 2007). Property of fluorescein as well as Cy3 can be used for proteins in general. But recently, it is found that certain classes of proteins that contain metal within the protein have ability to quench fluorophores. Cy5 was first used to measure distance change between XPD helicase binding in ensemble (Pugh et al., 2008a). Then I found that XPD can also quench Cy3 effectively and used
for the first time to measure XPD binding kinetics and translocation. Photophysics behind such quenching activity by the metal containing protein is unknown. Quenching of fluorescence by metal occurs in below nm range and it does not explain how it can read x-xx nt distance range. One explanation can be that it worked because it was attached to a flexible polymer rather than rigid body. All the measurements were done using single stranded DNA and the distance that we measure in our 30ms time resolution is average end to end distance within the time bin. It could as well be that distance controls the collision frequency of the fluorophore to the metal containing protein and results in gradual changes in fluorescence intensity that can report changes in several nanometer ranges.

1.4. Immobilization method

In order to track individual molecules for a desired duration of time, non-invasive way of immobilizing molecules on the surface is critical. Most conventionally, biotin-avidin linkage is most frequently used method. Because biotin is small (xkb) and easily conjugated with many commercial biomolecules such as DNA and PEG, biotin linked DNA was the first molecule to be studied using single molecule FRET. For protein DNA interaction studies, DNA was immobilized and proteins were added to solution for reaction. Use of lipid vesicle was developed as an alternating immobilization method mainly motivated by reducing surface effect. The method employs encapsulation of molecules of interest into lipid micells (vesicle) of desired size (x-xnm diameter) and immobilizing the vesicle on the surface by biotin conjugated on lipids. Additional benefit of this method are that molecules are confined in small volume (xfl) therefore

1) can observe reaction of given molecule over long time (while former method, proteins can dissociate and different protein can bind to the immobilized DNA under observation) and 2) can observe weak interaction that require high concentration by providing high local concentration
(xmM) (TIR method cannot detect signals from single molecule when concentration of fluorescently labeled species are at high concentration (>100nM) due to its background fluorescence).

Although vesicle method may be the most non-invasive method for protein studies, it is difficult to do vesicle experiment and analyze obtained data because 1) It is difficult to control number of molecules inside the vesicle 2) preparation procedures are time consuming and can be harsh for protein’s structure and function. Antibody method was used to pull down protein and is suitable immobilization method for studying static property such as concentration and complex stoichiometry but is not suitable for studying dynamics of the protein as antibody binding to the protein can hinder its motion. We have recently found a commercial protein that has both Ni-NTA and biotin conjugated on it (Ni-NTA Antibody biotin). I tested Hisx6 tagged PcrA over Ni-NTA-Antibody-biotin immobilized surface. Fluorescently tagged PcrA showed well distributed isolated single fluorophore spots on the surface and was stably bound for over 1hr. I performed functional assay and it showed its ssDNA pulling activity without noticeable perturbation (ref). Advantage of using Ni-NTA-Ab-biotin is that 1) procedure is simple, 2) fast and 3) not invasive. Additional benefit of the method is that 4) controlling oligomeric state of immobilized protein is possible as non-immobilized species can be washed away and only the isolated monomers can interact with later applied substrates.
Chapter 2 Discovery of reeling-in activity of PcrA

2.1. Introduction to PcrA helicase

DNA helicase, by definition, is a protein that unwinds duplex DNA. Helicase are known to be one of the first proteins to bind to origin of replication and initiate DNA replication by separating two single strand of DNA. However, other than this canonical role in replication, many other kinds of DNA helicases are found and shown to play role in non-canonical role such as DNA recombination and repair. Helicases can be divided into seven different families by their sequence and structural differences. Among the seven families, Superfamily 1 is the most heavily studied in structural perspective perhaps because of it is a minimal size of protein that carries conserved key features of motor proteins. Bacillus Staerootheromphilus PcrA is the first helicase ever crystalized providing insight to how the machinery of the motor protein looks like. Later, crystal structures of PcrA bound with and without ATP analogue were resolved which contributed important evidence for mechanism of how protein can move along the DNA. Physiological role of PcrA is relatively not studied well. PcrA is known to involve in rolling circle replication of plasmid. However, it does not explain why PcrA is essential in gram positive bacteria and recent studies point that its role is associated with DNA repair.

PcrA is known to be essential but so far the only reference I can find is knock out study on Bacillus subtilis (1998). I need to read three papers to figure out its role in rolling circle replication. 4 papers are directing to RepD. I also need to look at UvrD paper on repair again.

2.2. Experimental Design and the first observation

PcrA project started out as a follow up study of Rep helicase. Around year 2004, my colleague, Sua Myong in our lab discovered repetitive shuttling behavior of Rep helicase on the 3’tail of
partial duplex. As PcrA and Rep has ~40% sequence homology and have identical structure, it was suggested that PcrA may also act like a Rep helicase and show repetitive translocation on the 3’tail ssDNA. The first PcrA that I worked with is Staphylococcus aureus PcrA purified by Dr. Issac Cann, a professor in Animal Science department in our campus. There were only few published work on this protein and one of biochemical work that was done on this helicase is Dr. Khan’s work on unwinding assay (Anand and Khan, 2004). I was able to observe unwinding activity using an ensemble fluorescence assay (fluorometer) however, I could not see evidence of translocation activity using single molecule experiment. I tried various solution conditions (mono- and divalent ion concentration, PH, ATP), however, I could not observe any signal similar to repetitive shuttling nor any type of clear sign of translocation.

In 2006 fall, I attended Gibbs Conference on Biothermodynamics held in Carbondale, IL. At the meeting, one of my labmate Rahul Roy introduced me to Anita Neidziela – Majka, a postdoc of Timothy M. Lohman. She was working on PcrA helicase from Bacillus staero thermophilus, the one that is heavily studied for its structure as well as biochemical properties. She was about to finish with her project with it and was planning to move to her next job. She agreed that she will send me some PcrA sample so that I can test it on single-molecule.

I’ve tested several DNA construct with the new PcrA. The substrates I tested was 3’tailed and 5’ta ild partial duplex DNA (pdDNA) that have Cy3 labeled at the ssDNA tail end. With the 5’tailed DNA, I observed some gradual increase in fluorescence signal which could be an evidence of translocation. However, with the 3’tailed pdDNA, no distinctive signal was obtained. I discussed this with my lab mate Chirlmin Joo and he encouraged me to try testing DNA labeled with FRET pair. It would not give any changes in FRET if it is a simple translocation behavior,
but if there is more to this, then it may give extra information on the dynamics that PcrA is doing.

Figure 1 below shows the FRET pair labeled 5’pd DNA substrate that I used.

Figure 1 Experimental Design
With my amazement, FRET signal fluctuated with a beautiful pattern (see Figure 2.). It showed gradual increase in FRET followed by a fast drop and this signature of sawtooth pattern repeated continuously. This signal indicate that the two ends of ssDNA are somehow brought together in a gradual manner and then get pushed away from each other in an abrupt manner. And soon as the two ends fall apart, they are again brought together gradually so that the process gets repeated over and over.

![Figure 2](image)

**Figure 2** PcrA induces two ends of ssDNA to approach to each other gradually and move away rapidly in repetitive manner

### 2.3. Position of the binding site of PcrA

The initial sawtooth FRET signal I discovered was very fascinating but at the same time, very confusing. Nothing, until then, was known about any property of PcrA that can induce such ssDNA dynamics. Also, the FRET signal alone does not give any information on how PcrA plays a role in repetitive looping process. In order to identify the position of the protein, I went back to the single Cy3 dye labeled DNA experiment and examined the dynamics. As shown in Figure 3., only one end of ssDNA is labeled with Cy3 in order to monitor distance change between PcrA and the labeled position. With the 5’ tail end labeled DNA, PcrA showed sawtooth fluorescence intensity pattern indicating that PcrA is approaching toward 5’ tail end gradually.
This direction does match with the known directionality (3’→5’) of PcrA translocation. Rapid drop of fluorescence signal was followed indicating that PcrA moved away from the tail end rapidly, to perhaps where it began originally as the following pattern repeats the same pattern. On the other hand, with the junction labeled DNA substrate, PcrA did not show any repetitive dynamics. The signal fluctuated within a noise level, indicating that there are no noticeable distance changes between the junction and PcrA; ie, PcrA remains in a certain position relative to the junction and it should be far from the 5’ tail end (so that the protein can repetitively approach to the 5’ tail end). ssDNA is a one dimensional material so the only plausible explanation for this data is that PcrA binds at the junction and pulls ssDNA towards it so that 5’ tail end approaches gradually toward the junction. Moreover, it is likely that PcrA may run off the track at the end of the 5’ tail end and release the ssDNA inducing sharp FRET drop (Figure 2.) as well as fluorescence drop (Figure 3).

Figure 3 Verifying the position of the PcrA binding site
Although there can not be any other explanation than the model of ‘junction binding and pulling ssDNA’ for the given signals, I sought for more direct evidence using other techniques. In collaboration with Robert Pugh, a graduate student in Maria Spies’ lab, we performed exonuclease footprinting assay (Figure 4). ExoIII nuclease preferentially binds to the duplex and degrades nucleotides from 3’ end. If PcrA occupies some portion of DNA, it will be protected from further degradation. As shown in Figure 4, ExoIII can easily degrade 22nd-17th nucleotide resulting shorter fragments and PcrA bound DNA tend to show more prominent band on larger size of DNA (14-16nt) that are protected from degradation consistently with varying doses of Exonuclease. It is somewhat a corroborating result with a crystal structure bound with DNA which showed about 14-16 bp being occupied by the protein at the duplex junction (Lee and Yang, 2006; Velankar et al., 1999). However, I would like to note that this structure was made with 3’tailed partial duplex rather than 5’tailed duplex used in my study.

![Figure 4 Control Assay for verifying the binding position of PcrA](image)

Additional single-molecule experiment were conducted to verify that PcrA is actively inducing ssDNA looping at the buffer condition where exonuclease footprinting assay is being conducted (figure 5.). As a matter of fact, the signal appeared more robust and meticulous in this buffer
condition. Translocation rate appeared to be faster however no quantification was conducted on this data.

Figure 5 PcrA induces same type of ssDNA dynamics in Exonuclease experimental condition
Lastly, additional evidence of PcrA’s junction binding and ssDNA pulling was obtained by ensemble fluorescence measurement. Same type of DNA shown in figure 3. (except for the absence of biotin at the duplex end) was added to cuvette and fluorescence emission was measured (Figure 6). Upon PcrA addition, fluorescence signal increased due to PcrA binding. When ATP is added, further signal increase was observed only in the cuvette with the DNA labeled at the 5’tail end indicating that pulling activity brought PcrA and the tail end come closer.

![Figure 6 Fluorometer measurements that show evidence of ssDNA looping](image)

### 2.4. Monomeric state of PcrA during the reeling-in activity

One of the most debated topic in the field has been the functional state of the SF1 helicases. SF1 family helicases were crystalized as a monomer with and witout DNA or NTP substrates, however monomer of these helicase did not show any helicase activity in solution. Therefore verifying the oligomeric state of the protein is an important question to understand the mechanism of this machineries. Conventionally, though, it was assumed that if one adds protein to DNA in 1:1 ratio, protein interacts with DNA as a monomer(Dillingham et al., 1999; Dillingham et al., 2002; Tomko et al., 2007).However it is only assumption and protein binding can in principle should occur in statistical distribution with mean DNA:protein ratio being 1:1. In
previous ensemble assays, protein/DNA concentration of >10nM range were used and the system was still considered as a monomeric condition. Here in this PcrA study, we used 0.1nM protein/DNA concentration (Figure 1-3, 5) where chances of seeing oligomer should be much(<100 times) lower than the standard condition for monomer experiment. Although chances of protein being oligomer is small, I would like to more directly confirm its oligomeric states during the reeling-in activity. I’ve mutated one(C96A) of two native cysteins in the PcrA and conjugated Cy3 fluorophore using cystein-maleimide chemistry. This site-specifically labeled PcrA was added to 5’tailed DNA that is labeled at either 5’tail end or at the junction as below (Figure 7.). Upon the Cy3 labeled PcrA binding, fluorescence signal appeared and dissapeared abruptly as an indication for single molecule binding and dissociation (or photobleaching). No additional intensity increase was observed after the initial binding. These results indicate that the reeling-in activity occurs by a PcrA monomer and not in any higher oligomeric form. Additionaly, by this labeled protein experiment, I was able to verify the binding position of PcrA and the pulling activity in most direct manner.
Figure 7 ssDNA looping activity is induced by a PcrA monomer. Also the binding position of the PcrA is identified.
Monomeric state of PcrA can also be confirmed by protein immobilization method. It is known that PcrA is a monomer in solution (ref). Therefore, I prepared His-tag-antibody bound surface over PEG passivated slide in order to capture His-tagged PcrA protein added in solution. After several minutes of incubation, excess proteins are removed from the surface by buffer wash and fluorescently labeled DNA with ATP is flowed over the protein captured surface. Upon binding of the 5’pdDNA, PcrA showed the signature signal of repetitive reeling-in activity. As excess proteins are removed from the solution, any additional protein cannot be recruited to the PcrA/DNA pair. Therefore, this result supports that thereeling-in activity occurs with a monomeric form of PcrA.

![Figure 8 PcrA monomer immobilized on the surface can induce ssDNA looping](image)

Last control experiment to test the oligomeric state is a diffusion-dependent binding rate assay. This experiment was proposed by a reviewer of the journal when we did not yet present the labeled protein data. The principle behind this experiment is that the reaction rate can change depending on the requirement for number of components involved in the reaction complex. For instance

$$\text{DNA} + n\text{Protein} \rightarrow \text{DNAProtein}_n$$

Rate of complex forming reaction = \(k*[\text{DNA}][\text{protein}]^n\)
As the concentration of DNA is static as is immobilized on the surface, the complex forming rate will be dependent on protein concentration and the number of proteins required in a complex (n). The shape of the plot that shows rate of complex formation vs the concentration will follow an integered power law therefore number n can be estimated. Figure 9. Shows experimental setting and the a raw example trace. DNA is immobilized on the surface and is incubated in Mg++ free buffer. The recording starts and then PcrA together with ATP and Mg++ is flowed in to the chamber while recording the data. FRET gets elevated due to arrival of Mg++ (as ssDNA scrunches due to backbone charge screening). After several seconds, PcrA binds to the DNA and repetitive reeling-in begins. I measured time of solution arrival (Protein added time) and subtracted from PcrA binding time to obtain bind time (Δt). Then bind time was measured and average time was obtained from various PcrA concentrations. The binding rate (1/Δt) was plotted against PcrA concentration as shown in Figure 9. (top right). Five different color indicates experimental sets conducted in different days. Individual sets of data is shown in the following pannels. All five data showed that the relationship is linear and not quadratic (as shown in two fitting line). The fitness of linear vs 2nd power fit were summarized in the bottom right bar graph and in the Figure 10. Note that the plots with 4 data point showed much more difference in R² value between the linear and 2nd power fit. The absolute diffusion constant obtained from the slope of each linear fit varied giving about 30% variance. This may be due to the fact that the day to day illumination intensity varied and thus difference in photobleaching rate must have influenced the absolute diffusion constant. (Faster photobleaching cause lower number of lengthy bind time (Δt) therefor e shorten the apparent average bind time and increase binding rate (1/Δt)).
Figure 9 Evaluating diffusion kinetics to verify the monomeric state of PcrA
Figure 10 Evaluating possibility of monomer vs dimer
2.5. Model of PcrA reeling-in activity

In order to summarize the results shown above and visually describe the motion of PcrA, here I present the model for the reeling-in activity of PcrA (Figure 11). When PcrA monomer interact with 5’pd DNA, PcrA finds ss/dsDNA junction and anchors itself at this position (a). Then PcrA starts reeling-in ssDNA from the junction creating growing ssDNA loop (b). As the reeling-in continues, the loop continues to grow and the 5’tail end approaches gradually towards the ss/dsDNA junction (c). When PcrA pulls the ssDNA all the way until the end of the tail, then it runs off the strand and then ssDNA is released back to its original conformation (d). Then the process of reeling-in repeats itself (a-d) until protein dissociates from the DNA or until cannot be imaged due to fluorescence photobleaching.

Figure 11 A model showing that a PcrA monomer binds at the junction and pulls ssDNA in a repetitive manner
2.6. ATP concentration dependence of reeling-in activity of PcrA

Reeling-in direction of PcrA matches with the known directionality (3’→5’) of PcrA translocation. Therefore it is likely that the pulling activity is linked to the translocation activity of PcrA motor. Translocation activity is powered by ATP hydrolysis. Therefore, I tested dependence of ATP concentration to the reeling-in rate of PcrA. As predicted, the rate of reeling-in activity was strictly ATP concentration dependent. Lower concentration of ATP induced slowing down of the gradual phase of FRET increase. Time interval between each cycle (Δt) is measured and the rate (1/ Δt) was plotted against ATP concentration. It followed Michaelis-Menten relation, a characteristic for a one ATP binding to the protein per reaction. The fit gave Km value of 3.5μM, indicating the ATP binds relatively tightly to PcrA. The Vmax value of 1.44 indicates that repetition occurs about 1.44 times a second at maximum ATP concentration (The DNA substrate used here is a standard DNA shown in Figure 1, 5’pdDNA with dT40).

![Figure 12 ATP concentration dependence on PcrA reeling-in dynamics](image)

Figure 12 ATP concentration dependence on PcrA reeling-in dynamics
2.7. ssDNA length dependence on PcrA reeling-in activity

Although we can measure the time of ssDNA pulling for a given length of ssDNA (as shown in Figure 12), it is not sufficient information to deduce the translocation rate of PcrA. It is due to the fact that PcrA may occlude certain size of DNA and that one cannot measure exactly from where PcrA initiates pulling. This problem can be overcome by performing ssDNA length dependent assay. I prepared 5' pdDNA with various ssDNA length (40, 50, 60, 70, 80). As expected from the model shown in figure 11., the time interval that PcrA takes become longer with the longer length of the ssDNA (Figure 13).

Figure 13 ssDNA length dependence on PcrA reeling-in dynamics

The time duration for completing a single cycle of pulling was measured (Δt) and plotted against the length of ssDNA (Figure 14). It followed linear relationship supporting that PcrA translocates along the given length of ssDNA without dissociating from the middle (i.e. the processivity of translocation is larger than the maximum length of the DNA tested (80 nt)). From
the slope of the plot, translocation rate of 76nt/sec was obtained. This rate agrees well with the known translocation rate of PcrA measured from ensemble in similar but not identical solution condition (Dillingham et al., 1999; Dillingham et al., 2002; Niedziela-Majka et al., 2007).

Additional interesting number I obtained from this analysis is the value for y intercept. This value (0.06 ± 0.05 sec) indicates that PcrA may start pulling ssDNA from the almost the very first base near the junction. The number can be translated to <7nt depending on the error. PcrA ssDNA binding site can occlude 6-7nt shown from the crystal structure. However estimating the absolute number based on Figure 14 is not possible/meaningful considering the given error of fitting.

![Graph showing translocation speed and y intercept](image)

**Figure 14** Quantifying translocation rate from the repetition time interval

Length dependence could also be observed by using PIFE method. Here I prepared identical 5’pdDNA substrate except at this time, only Cy3 was labeled at the end of the 5’tail. Various lengths (40, 50, 60) of DNA was tested and the fluorescence time trajectory is shown below
As seen with the FRET data, time duration for each pulling cycle increased with increasing ssDNA length. Note that Cy3 intensity trajectories are more subject to noise compared with the FRET data where the effect of shot noise and/or laser fluctuation can be canceled out and give clearer signal.

Time interval analysis was conducted on the Cy3 intensity time trajectories. The time durations between each cycle (Δt) are measured and plotted against the length of ssDNA used for each experiment. The result again shows linear relationship corroborating with length dependent assay conducted with FRET. The slope gave translocation value of 82±23 nt/sec which is comparable with that from FRET assay above within error. These experimental sets were once used to demonstrate distance dependent relationship of Cy3 intensity against protein position from the dye.
Figure 16 quantifying translocation rate of PcrA from ssDNA length dependent assay using Cy3 PIFE.
2.8. DNA sequence dependence in PcrA reeling-in activity

Reeling-in activity of PcrA was tested with 5’ pdDNA that have non polyT sequence on the ssDNA part. ATGC Mixed sequence of 39mer was tested for reeling-in and PcrA faithfully pulled ssDNA repetitively as it did with polyT. The shape of repetitive sawtooth pattern appeared less uniform although the difference was not analyzed further. This difference may originate from different rate of translocation for different bases or it could be that the mixed sequence may form temporal secondary structure therefore inhibiting PcrA translocation at times.

![Figure 17 ssDNA looping is induced by PcrA regardless of DNA sequence](image)

2.9. Influence of solution condition in PcrA reeling-in activity

PcrA translocation rate is known to be dependent on the solution condition. In one recent study, this property was used to confirm that the underlying mechanism that they analyze is consistant regardless of the solution condition(Niedziela-Majka et al., 2007). Our collaborator Dr. Lohman asked if same effect of rate increase can be seen with ssDNA pulling activity as is powered by the same translocation mechanism. So I tested PcrA pulling activity in the solution condition (20 mm Tris (pH 7.5 at 25 °C), 10 mm NaCl, 3 mm MgCl₂, 10% (v/v) glycerol, 4 mm dithiothreitol) that is known to induce higher translocation rate(~2.6 fold). In the experiment
conducted on the same buffer condition (Figure 18. Bottom left), and is shown to move faster compared to the standard buffer condition used in my assays.

2.10. Lag time before the repetitive reeling-in initiation

At the beginning of the repetitive ssDNA pulling process, an elevated FRET plateau were observed. This period is relatively transient and often show some fluctuations that cannot be clearly distinguished within noise (Figure 19). It is difficult to interpret what PcrA is doing during this time but here are some potential guesses. As FRET between 5’tail end and junction is
changed, a part of ssDNA in between must have been influenced upon PcrA binding. From ensemble studies it has been assumed that PcrA can bind at a random position on the ssDNA (Niedziela-Majka et al., 2007). Also, a recent study more systematically that I did in collaboration with Dr. Lohman lab showed that PcrA has minimum junction specificity upon initial binding to the 5'pdDNA (Tomko et al., 2010). Therefore, it is highly likely that PcrA would bind at a random position in ssDNA and the FRET is influenced while it resides on ssDNA. Also as there are ATP in solution, PcrA may translocate towards 5' direction but not completely fall off until it eventually finds junction to anchor and start reeling-in ssDNA. It requires further investigation in order to verify if PcrA translocates multiple times on the ssDNA before junction recognition and/or if so, how PcrA can do so without complete dissociation. I measured the delay time before the initiation of looping (as shown in Δt on Figure 19. top) and build a histogram with that from many molecules. It showed exponential nature. The result may be interpreted as there is only a single step rate limiting process for potential confirmation change to the functional state of PcrA that engage a duplex junction. It might be an interesting future study to measure the delay time for different length of DNA. If PcrA indeed binds at a random position of ssDNA and translocates along the ssDNA until it finds duplex, then the delay time may increase with increase in length of the ssDNA.
Figure 19 A delay time was observed before the initiation of repetition as an elevated FRET plateau

t_1 = 1.04 ± 0.16 sec
Chapter 3 Mechanism of PcrA translocation

3.1. Introduction to translocation mechanism of PcrA and its stepsize

PcrA has been a model protein to study mechanism of a motor protein. Bacillus *staeothermophilus* PcrA is the first DNA helicase that is ever crystalized(Subramanya et al., 1996). In this paper it is found that PcrA has two RecA like domain which serve as DNA binding and translocation domain and that ATP binding domain sits in between the two. Unlike its homologue Rep or UvrD, PcrA is shown to be monomeric in solution. Two translocation models were proposed(Bird et al., 1998b). One is an active-rolling mechanism(Wong and Lohman, 1992) and the other is an inchworm model(Yarranton and Gefter, 1979). Active-rolling mechanism requires oligomerization of protein and may require larger step size (at least larger than the DNA binding site). On the other hand, inchworm mechanism doesn’t require oligomerization and can allow for smaller step size as to 1nt. Active rolling model was supported by researchers finding oligomeric form of protein and larger step size. Inchworm model was supported by researchers finding monomeric form of protein and small step size. UvrD is shown to exist as both monomer dimer and gets dimerized upon DNA binding(Runyon et al., 1993). Rep is shown to exist as a monomer and dimerizes upon ssDNA binding(Chao and Lohman, 1991). However, PcrA was shown to be a monomeric form and stays as a monomer upon ssDNA binding in solution(Bird et al., 1998a). SF2 helicase HCV NS3 was also shown as a monomer in crystal structure (Cho et al., 1998; Kim et al., 1998; Yao et al., 1997) as well as in solution(Porter et al., 1998). The debates were intense. Mutant study were followed that showed somewhat indirect support for inchworm mechanism. UvrD mutant that cannot dimerize were made and showed that dimerization is not required for duplex unwinding(Mechanic et al., 1999). Another interesting study showed that a PcrA mutatnt that can translocate but cannot unwind duplex can be made and show that the
translocation activity alone is insufficient for duplex unwinding, proposing an active mechanism (Soultanas et al., 2000).

There were many speculations on the step size of the protein also. For instance, 1bp step unwinding per 2ATP was proposed (Yarranton and Gefter, 1979) for Rep unwinding, 2bp step unwinding was proposed for HCV NS3 RNA helicase (Porter et al., 1998), and 4-5bp step unwinding was proposed for UvrD unwinding (Ali and Lohman, 1997).

Unwinding mechanism of SF1 helicase is still debated and functional state of the helicase such as monomer or dimer is still controversial. Even as late as 2006, Wrench and inchworm mechanism that assumes monomer helicase is proposed as an active unwinding model by the crystallographers (Lee and Yang, 2006). This paper and other crystal structure papers argue that only monomeric forms of helicase were found in crystal structures. However, based on other solution studies (Maluf et al., 2003b) and my own experience with single molecule observation, monomer itself is insufficient for unwinding duplex (as low protein concentration condition that allow only one protein binding cannot induce unwinding) although how the oligomeric form may act in concert is unknown.
In 1999, crystal structure of PcrA bound to DNA was resolved (Velankar et al., 1999). The DNA bound structure showed a single nucleotide shift in ssDNA/protein binding site when comparing to ATP analogue (AMPPNP) bound and phosphate analogue ($\text{SO}_4^{2-}$) bound state. These snapshots showed evidence that PcrA monomer may inchworm and may move 1nt as it hydrolyzes 1ATP.

However, translocation process of PcrA in solution was never observed in individual molecular detail. And it remained controversial what the minimum unit of motion that repeats to create stepping process is.

This minimum unit of motion is defined as “Kinetic stepsizse”. Formal definition of kinetic stepsizse is “number of nucleotide translocated between two successive rate limiting steps.” The importance of identifying kinetic step size lies that it is fundamental information to build any models for translocation process. If one can identify kinetic step size and then describe all the
process during this single step, then one can say that one understood translocation process. It is often confused with chemical step size which simply describes stoichiometry between numbers of nucleotide translocated per ATP hydrolysis. This stoichiometry can be unambiguously measured in ensemble solution study as it is an average property. However, to identify kinetic stepsize, one has to either 1) look at individual molecule directly to see pauses or 2) to estimate the hidden process by statistical analysis on the probability distribution.

The latter strategy has been applied to obtain kinetic stepsize of SF1 helicases translocation on ssDNA in solution. For UvrD translocation, various numbers were reported by the same group: 4nt(Fischer et al., 2004), 2nt(Lohman, 2003), 4nt(Tomko et al., 2007). PcrA translocation was measured by the same group and shown to take 4nt kinetic steps(Niedziela-Majka et al., 2007). Using the same ensemble stop-flow technique and the analysis method, unwinding kinetic stepsize were also measured and found that UvrD takes 4nt step size(Ali and Lohman, 1997) and later shown that PcrA also takes 4nt step size(Yang et al., 2008). Kinetic step size of 4 does not mean that PcrA cannot take 1nt steps. It can be explained that there are hierarchy on stepping process and that 1nt step occurs very fast to the major rate limiting step that appears to be 4nt. However, the possible explanation for such large kinetic step size estimated is lacking and consistently reported step number of 4nt cannot be explained from the crystal structures. There has been one report that claims that PcrA moves in 1nt steps(Dillingham et al., 1999) and some of the literature cites this article for 1nt stepping of PcrA. However in this paper, the authors only speculated in the discussion about the possibility of 1nt kinetic steps based on linearity (not step-like) of the DNA length vs time duration curve which is totally unsupported guess; Step-like relation can never be obtained with such assay because of the ensemble nature of the data.
To estimate kinetic step size in above ensemble measurements, a clever strategy was devised using stop-flow machine followed by careful mathematical model fitting. Experiments were devised by synchronizing translocation initiation of all the protein in solution and quench reaction as soon as complex dissociates by trapping proteins with excess heparin. It is called single-turnover experiment as individual protein will only translocate once on the bound DNA and generate signal. Such signal will have time dependent ensemble trajectory and such profile will reflect complexity of the hidden process. In order to draw information from such trajectories, length dependent titration as well as other assays needs to be conducted in order to provide reliable parameters. The global analysis of the various types of experimental data leads to reveal many of the hidden parameters that are valuable. However, the model explicitly assumes that the molecules would share same average translocation rate and it did not allow for the possibility of static variance in rate among the molecular population. Ensemble kinetic analysis essentially analyzes width of the peaked profile and if static variance is hidden in the population, it can only provide upper bound of step size.

When I observed PcrA translocation using single molecule technique, individual molecules were shown to have various translocation rate that were static at least over the length of time of recording (<4min). Analysis of time trajectory that comes from single molecule allowed static variance free analysis and the results showed that PcrA moves in kinetic steps of 1nt. It resolves the long controversy between structure study and solution study. There is no hierarchy of steps for PcrA translocation and that it moves in uniform steps of 1nt. Hypothesis of stepping motor mechanism from static snapshots of crystal structure has now been demonstrated in solution study finally. This also emphasizes the fact that structurally identical molecules can show
heterogeneous dynamics therefore single molecule analysis is crucial to monitor movement of molecule with utmost accuracy.

3.2. Background of the analysis method

To obtain hidden number of steps during one cycle of reeling-in, I assumed a model as follows:

\[
\text{begin } \overset{k}{\rightarrow} \overset{k}{\rightarrow} \cdots \overset{k}{\rightarrow} \overset{k}{\rightarrow} \text{end}
\]

As the length of ssDNA is finite, there will be a limited number (N) of steps involved in the process between the beginning and the end. And each step would occur as a poissonian (random) process with a defined rate of k. This model assumes that each step is irreversible. If the system follows above simple model, then the total time it takes to move from beginning to the end follows gamma distribution, \((\Delta t) N - 1 \exp(-k\Delta t)\).

3.3. Evidence of 1nt step size

When PcrA binds to the 5’pdDNA and start reeling-in, the process was observed to repeat without any distinctive pause or disturbance. Also PcrA seems to bind tightly to the duplex junction and do not dissociate easily even in 100 fold lower concentration than the known Kd. Therefore, I was able to monitor repetitive signals for up to 120-180 seconds until fluorescence dyes photobleach and can no longer monitor the behavior. The number of time interval I can obtain from single molecule are around 100-250. These numbers are enough to conduct statistical analysis. Therefore, I build a histogram with cycle duration time (\(\Delta t\)) from a single time trajectory obtained by one PcrA reeling-in a 5’pdDNA (Figure. 21, top). When the histogram is fit to gamma function, it gave a step number (N) of 33.6, indicating that PcrA have taken at least 33 steps on 40nt ssDNA track that was provided. Therefore, if we assume that
PcrA takes and integer steps (that is 1nt, 2nt, 3nt, or 4nt, but not the numbers like 1.6, 2.1 or 4.9), then PcrA should be taking 1nt size steps (as it cannot be equal nor bigger than 2nt). More molecules were analyzed in a same manner and the gamma fit gave various step number (N) that falls in between 20 to 40, all strongly supporting 1nt step size (Figure 21, bottom).

Figure 21 Histogram of ΔT and the gamma analysis

As can be seen from the Δt distribution of various molecules, the mean translocation time varies from molecule to molecule. This is heterogeneity is also shown in gamma analysis result as well. From gamma fit, two parameters can be obtained which is step number (N) and rate of each step.
(k). If one assumes larger number of step, the rate of each step should be faster for a given data. Therefore, N and k are related as N/k = mean Δt. In the analysis result, both N and k are heterogenous and are not correlated (Figure 22). This result may indicate that heterogeneity is intrinsic to the molecules stepping rate.

![Figure 22 Heterogeneity in rate and step number](image)

If PcrA is indeed taking defined size of steps, the number of step should increase with increasing length of DNA. Therefore, I tested PcrA reeling-in activity with various length of ssDNA (as shown in figure 13) and analized Δt. Ganna analysis was conducted on Δt histogram obtained from various length of DNA and the result are shown below (Figure 23, top and middle panels). As expected, number of steps PcrA take increased with increase in length of ssDNA and all the N values support that PcrA take 1nt steps regardless of ssDNA length traveled (Figure 23, bottom left). The rate of steping (k) did not show any systematic change verifying that the fundamental kinetics are not influenced by the ssDNA length.
Figure 23 Gamma analysis on the various length of DNA
3.4. Molecular heterogeneity and stepsize estimation

Lastly, I become curious about the reason why previous ensemble experiments showed larger than 1nt step size so consistently. In order to mimic ensemble experimental condition/analysis, I combined all the Δt data that comes from different molecules in a same experimental chamber and build into a histogram. This histogram showed broadening of the width due to the contribution of various molecules having different mean values and as a result, gamma fit gave much lower step number (12) compared to that came from individual molecules (25-36). Therefore if one is to estimate a stepsize, the stepsize would become 3-4nt from this analysis. This result demonstrates the power of single molecule analysis and it teaches us that heterogeneity among molecules has to be considered and evaluated for the accurate kientic analysis. The source of this heterogeneity is unknown however, it is a general behavior found in various systems, even as small and simple system as a ribozyme (Nahas et al., 2004) or human telomeric DNA (Lee et al., 2005). For PcrA, the heterogeneity in rate was static over the observation time and the rate switching was not observed. However, it is still questionalble if the rate change might occur in extened observation time, perhaps in hours or days scale. It may still be able to conclude that in the energy landscape, there may be many local potential wells deep enough that the structure cannot escape esaily in RT condition. Further investigation may be required to resolve this seemingly static heterogeneity.
Figure 24 Gamma analysis on ensemble of data

Ensemble of the population:

N = 12.0
k = 19.9 s⁻¹
Chapter 4 PcrA 2B sub-domain’s role and its conformation

4.1. Introduction to the structure of PcrA

Structure of PcrA is composed of 4 sub-domains (1A, 2A, 1B, 2B). 1A and 2A domain has RecA-like structure and are responsible for ATP hydrolysis and ssDNA binding. 2B domain appears accessory domain and is linked to the two RecA-like domains via 2A-domain. Role of 2B domain has been most debated as its role doesn’t facilitate unwinding or translocation activity. Rather this highly conserved domain seems to inhibit protein’s potential for translocation and unwinding based on PcrA’s homolog Rep’s 2B domain deletion study (Cheng et al., 2002). In the crystal structure of PcrA (and UvrD) bound with 3’ pdDNA, 2B domain is shown to interact with duplex part of DNA. Therefore, it was once proposed that this domain may act as an active destabilizer for unwinding although 2B deletion mutant study eliminated the possibility of this hypothesis.

4.2. Perturbing the GIG box of the 2B domain

In order for PcrA to induce reeling-in ssDNA at the junction, it is crucial that a certain part of the protein should be able to recognize duplex DNA and stably maintain contact. There is no crystal structure for any SF1 helicase bound to 5’tail pdDNA until now. However, within such a simple and small structure containing only one accessory domain, it is not unreasonable to assume that 2B domain may be responsible for duplex recognition and binding. In fact, in crystal structure, 2B sub domain contains well preserved helix-turn-helix motif, that maintains good contact with a minor groove of dsDNA (figure 25).
Figure 25 GIG box of the 2B subdomain shown together with DNA that is bound to the protein. The amino acid positions for the mutation are indicated.

This motif is consist of Gly-Ile-Gly and is so called GIG box. Therefore, hypothesis was made that if this motif is perturbed, PcrA reeling-in activity would be influenced. I mutated the second Gly from GIG box to Thr and tested this mutant to the standard 5’pdDNA with dT40 overhang (Figure 26). Upon G423T mutant binding, FRET fluctuation was observed. However, robust sawtooth pattern as well as any high FRET signal was disappeared.

Figure 26 Identifying a role of 2B domain by perturbing GIG box
Compared with the wild type PcrA, any signal higher than FRET 0.8 was never observed and that most of the signals resided on FRET ~0.5 (Figure 27). This result indicates that PcrA may attempt to pull ssDNA but cannot pull all the way to the 5’ tail end. As the ssDNA binding is not perturbed, PcrA may still be able to translocate towards the 5’ tail end. Therefore, it is very likely that the other grip becomes loose and unable to hold the growing ssDNA loop. Equivalent mutation in UvrD was reported to severely reduce DNA binding activity (Lee and Yang, 2006). One of the reviewers of the Cell manuscript asked the reason why PcrA does not lose the grip completely and still be able to induce temporal looping. This may be that this first Gly may not be sufficient to destroy all the contact between protein and the duplex. In fact, initial residue that I tried to mutate was the first glycine to glutamic acid. In Rep, the GIG box is in a form of EIG and unlike PcrA and UvrD tested, Rep could not induce looping of ssDNA with 5’pdDNA. However, bacterial cells that harbor this type of mutant were never able to cultured therefore it maybe lethal to the organism. Further study of changing GIG box may be an interesting future study. Also one may think of using longer DNA to test the length of the shortened processivity.

![Figure 27 Quantifying the difference in pulling processivity between the G423T mutant and wildtype.](image)
As a control, I also mutated a residue nearby GIG box. T426A mutant did not show any distinctive difference with wildtype PcrA on the 5’pdDNA. Equivalent mutation in UvrD was reported to induce mild reduction in DNA binding. This data support the importance of the GIG contact in duplex recognition of PcrA for its pulling activity.

Figure 28 Perturbing a nearby residue of GIG box in 2B-sub domain.

4.3. Conformation of PcrA during the reeling-in process

Now I got curious how reeling-in activity can occur within the given structure. The only structure available for PcrA and other SF1 helicase bound to partial duplex DNA is with 3’tail DNA. As we know the two binding position of the protein (1A,2A on ssDNA and 2B on duplex DNA), I sought possible route for ssDNA to be reeled-in and the loop being formed.

In the PcrA (and UvrD) structure crystallized with a 3’ tailed partial duplex, the ssDNA binding site is shown as a small pocket enclosed by all four domains (1A, 2A, 1B and 2B)(Lee and Yang, 2006; Velankar et al., 1999). If the 5’ tail, instead of the 3’ tail, is extended and fed in to the ssDNA binding site with the correct orientation, the growing ssDNA loop that forms past ssDNA binding site on the 1A domain would not have enough room inside the small enclosed pocket of the UvrD structure. In addition, the 5’ ssDNA tail would need to traverse approximately 33 Å to
enter the 1A domain with the correct orientation whereas our tail length dependent data showed that translocation begins very close to the duplex part. Therefore, we conclude that the overall structure of PcrA during repetitive looping must be very different from what’s shown in the UvrD crystal structures.

Crystal structures of Rep bound to ssDNA (Korolev et al., 1997) showed that the 2B domain is flexible and can swivel between two conformations named open and closed conformations. The closed conformation of Rep is similar to what was observed with UvrD bound to a 3’ tailed duplex. Therefore, we made a hypothesis that PcrA may take an open conformation during looping and this conformation may allow 5’tail engagement more reasonably. I constructed a PcrA mutant with two cysteines, A533C and C247 that would be in close proximity, ~3.8 nm, in the closed conformation but far apart, ~5.5 nm, in the open conformation. The mutant was labeled stochastically with a mixture of Cy3 and Cy5 maleimides, and the labeled protein was observed on an unlabeled partial duplex DNA with a 40 nt 5’ tail (Figure 29). Fig. 5D shows the initial binding of PcrA to the DNA in the high FRET state, quickly followed by a transition to a low FRET state indicating that PcrA changes its conformation from closed to open at the initial stage of DNA binding and remains in an open conformation during translocation-coupled looping. Intermittent direct excitation of the acceptor showed that the low FRET state is not due to acceptor photobleaching or blinking but is rather due to a conformation with a large distance between the donor and the acceptor. Among the 189 molecules analyzed which also showed both donor and acceptor signals, about 80% exhibited primarily a low FRET state that we assign to the open state (Figure 29, bottom left). We conclude that PcrA most likely maintains an open conformation during ssDNA translocation coupled with looping.
Figure 29 Conformation of PcrA during the reeling-in activity.

4.4. Structural modeling of open conformation

We next used structural modeling to test if the open conformation would allow a proper engagement with the 5’ tail. We started with the closed form of the PcrA structure (PDB I.D. 3pjr), and rotated the 2B sub-domain together with the 3’-tailed DNA by 130°, to mimic the open conformation of Rep (PDB I.D. 1uaa) (Figure 30B). This resulted in the 3’ tail being positioned in an incorrect polarity relative to the ssDNA binding site of the protein (Figure 30D). Interestingly, however, the 5’ end of the duplex is in proximity to the ssDNA binding sites (8.5 Å
between F192 and the first base of the 5’ DNA end). A similar result was obtained from structural modeling using the UvrD structures (Figure 31). As a result, an extended 5’ tail could now interact with the ssDNA binding site of the translocase with the correct polarity starting from F192.

Figure 30 PcrA structural modeling and assessing possibility of the open conformation
4.5. PcrA dynamics at the forked DNA junction

As for the fork substrate, PcrA is also able to bind 3’ tail ssDNA and translocate toward the duplex. However, PcrA cannot unwind duplex as a monomer (Niedziela-Majka et al., 2007; Yang et al., 2008). When no more 3’ ssDNA tail is available for feeding as PcrA reaches the forked junction along the 3’ tail, PcrA may 1) get stuck at the junction, 2) dissociate from the DNA or 3) may switch its mode and interact with the 5’ tail duplex. In order to see what PcrA
would do with the forked duplex, I prepared forked DNA with two labels at the 5’ tail end and the junction. If 1) and 2) is occurring, no signal change is expected, but if 3) is occurring, PcrA will show repetitive sawtooth pattern of FRET signal changes. When ATP and Protein is added to such DNA substrate, I observed repetitive sawtooth pattern indicating 5’ tail engagement of PcrA. Existence of 5’ tail engaging mode of PcrA may explain why an addition of a 5’ tail to the 3’ tailed duplex inhibited DNA unwinding by Staphylococcus aureus PcrA ~10 fold (Anand and Khan, 2004).

**Figure 32 PcrA induces reeling-in with a forked DNA**

Although biochemical results have been seeing evidence that PcrA cannot unwind duplex as a monomer, even until nowadays, it is still suspected that SF1 helicase may be a monomeric helicase (mostly by the structural researchers). Therefore, I counted number of molecules that have FRET pair on the field of imaging area and compared before and after adding 1mM ATP and PcrA (100pM, standard translocation reaction condition used). If PcrA can unwind DNA in this condition, fluorescence spots should decrease due to strand separation. Figure 33 shows the
experimental result and is shown that unwinding activity is negligible (considering possible photobleaching effect or spontaneous dissociation over time and by buffer washes).

4.6. RecA-like domain orientation of PcrA on 5’tailed DNA

Our assumption behind interpreting the structural modeling is that PcrA maintains its ssDNA binding polarity within its ssDNA binding domains. However, because there is no crystal structure of PcrA (or any SF1 helicase) bound to 5’tail DNA, there is no direct proof that this assumption is true. Using single molecule FRET, it is possible to show binding orientation of protein relative to the substrate structure(Rothenberg et al., 2007). For PcrA, if the binding orientation of protein to ssDNA polarity is maintained, then 1A-domain should face the junction while 2A-domain should face the 5’tail. To test this distance difference, I have site specifically labeled Cy3 on each RecA-like domain of PcrA. Then I added these two types of labeled PcrA to either the 5’tail Cy5 labeled 5’pdDNA or the junction Cy5 labeled 5’pdDNA. The results are shown in Figure 34, 1A domain labeled PcrA showed low FRET with 5’ Cy5 labeled pdDNA.
(top, left) but showed high FRET with junction Cy5 labeled pdDNA, indicating that 1A domain stays closer to the junction than the 5’tail (bottom, left). Consistently, 2A domain labeled PcrA showed intermediate FRET with both types of DNA indicating that 2A domain is further away from 5’tail end compared to 1A domain (Figure 34, right top and bottom).

Figure 34 Domain orientation of PcrA upon 5’tailed DNA binding

4.7. Model for PcrA dynamics at the fork DNA

Based on all the experiments shown in this chapter as well as on the information obtained from the structural modeling, here I present a model for PcrA’s dynamics on the DNA mimicking stalled replication fork (Figure 34). PcrA is ssDNA binding protein therefore it may bind at either 3’tail or 5’tail. When it binds to 5’tail, it may translocate away from the junction and dwell on the ssDNA perhaps by multiple round of translocation until it finally engages in the junction. On the other hand, if PcrA binds to the 3’tail, it will translocate toward the junction. When it hits
the junction, 2B domain may interact with duplex DNA and may induce conformation change to an open conformation. This change in conformation will cause duplex junction position shift from 2A domain side to 1A domain side. This conformation will allow ssDNA binding domain of PcrA to contact the available 5’ ssDNA close by and start to feed in the 5’ ssDNA region near the junction then step toward the 5’ end. As it is now in open conformation, 5’ tail will be able to freely access the ssDNA binding domain, making it easier to form a single stranded bulge or loop form behind F192 and grow without steric constraints as translocation continues.

Figure 35 The structural model of PcrA reeling-in
Chapter 5 Physiological role of PcrA’s reeling-in activity

5.1. Introduction to the physiological role of DNA translocases

In 1989, Iordanescu and Bargonetti at department of plasmid Biology in Public Health Research Institute in New York found that if they knock out one gene in host chromosome of staphylococcus aureus, only one kind of plasmid (pT181) were replicated very slowly while other kinds of plasmids are not affected (Iordanescu, 1989). Same team in 1993 verified that PcrA is an essential helicase for S. aureus survival (Iordanescu, 1993).

In 1998, Petit et al, a French team identified PcrA from Bacillus Subtilis by homology search with S. aureus PcrA and E.coli Rep and UvrD. They found that B. subtilis PcrA is 1) essential for the survival, 2) conditional mutant can decrease chromosomal DNA replication slightly and inhibit plasmid pT181 replication, 3) can replace uvrD deletion mutant with UV sensitivity but 4) cannot replace rep deletion mutant with bacteriophage M13 replication. Additionally, the expression level of PcrA was shown to be low (about 30 molecules/cell) (Petit et al., 1998).

The lethality of PcrA family was brought into attention. Double knockout of PcrA’s cousin Rep and UvrD in E. coli were shown to induce lethality (Taucher-Scholtz, 1983).

In all three proteins, when deleted or suppressed, it is found that the recombination gets increased. Recombination between tandem repeats show 10 fold increase in uvrD mutant (Bierne et al., 1997a; Zieg et al., 1978), 20 fold in rep mutant (Bierne et al., 1997b). In 2002, Petit et Ehrich established PcrA inducible mutant in B.subtilis and found that 30 fold suppression of PcrA in B. subtilis caused 15 fold increase in recombination between tandem repeats (Petit and Ehrlich, 2002).
Recombination can occur by two pathways: RecFOR dependent pathway and RecBCD (in E.coli)/AddAB (in B.subtilis). Petit and Ehrlich investigated relationship between PcrA lethality and RecFOR and AddAB and found that it is linked to RecFOR but not AddAB. Mutation in RecF, RecO, RecR suppressed lethality of pcrA mutation which indicates that toxicity comes from RecFOR dependent recombination pathway and PcrA is involved in regulation of it (Petit and Ehrlich, 2002). In the same paper, it is reported that mutation in RecF, RecO, RecR suppressed lethality of uvrD and rep double knockout in E.coli suggesting that the essential role of these helicase family comes from their common role in regulating RecFOR dependent recombination.

Toxicity of RecFOR dependent recombination is suggested to be linked to replication. There have been evidences that recombination process may occur at the stalled replication fork (Michel et al., 2001). This link may explain why the chromosomal fork progression rate is decreased in rep mutant in E.coli (Colasanti and Denhardt, 1987; Lane and Denhardt, 1975) and replication is slowed down in PcrA suppression mutant (Petit et al., 1998). UvrD is shown to co-purify with DNA polymerase III holoenzyme (Lahue et al., 1989).

Later studies that focus on anti-recombinase activity of such helicases on the replication fork were done on E.coli as the system is most well characterized and several strategy of stalling replication fork were available. In 2004, Replication fork reversal assay was devised and is used to verify UvrD’s role in resetting blocked replication fork (Flores et al., 2004). Further study has identified that UvrD’s repair function on stalled replication fork is by counteracting RecQJFORA as mutation on those protein did not require UvrD for replication fork reversal (Flores et al., 2005). In 2007, Lestini and Michel showed that ATPase activity and unwinding activity is not required for one type (created by mutation on DnaE, polymerase subunit of Polymerase III
holoenzyme) of stalled replication fork, while those activities were required for the other type (created by mutation on DnaN, beta-clamp of the PolIIIh) of the stalled fork (Lestini and Michel, 2007). In this work B. subtilis PcrA was expressed in uvrD- E.coli and was found that PcrA can reverse the replication fork as UvrD does. Additionally, relation of UvrD and PcrA on replication fork restart was studied on E.coli chromosome replication where replication is blocked by Tus protein binding on Ter site of DNA. In this study, UvrD was shown to be essential for Tus removal and the role can be substituted by B.subtilis PcrA (Bidnenko et al., 2006).

Molecular mechanism of PcrA’s anti-recombinase activity is not fully understood. In vitro assay to test anti-recombinase activity were first conducted by Yeast homologue Srs2 (Krejci et al., 2003; Veaute et al., 2003). In these two studies Rad51 (RecA counterpart in yeast) filament is formed on circular ssDNA and Srs2 is added to remove Rad51 it resulting loss of filamentous structure. UvrD was tested using same strategy and was found that it can remove RecA filament in 10fold lower concentration(Veaute et al., 2005). Rather unique study was conducted by Anand et al in 2007 where they report RecA removal by PcrA(Anand et al., 2007). Contrary to formerly mentioned studies, they used 10,000 fold lower concentration of DNA which results in same order of concentration of RecA bound to it. As a result, effectively 10,000 fold higher concentration of helicase (to that of RecA) is added to the nucleoprotein filament in their assay. It is very unusual way because in other studies, 10 to 100 fold LOWER concentration of helicase were used over RecA (or Rad51) to test helicase activity. In the same paper, RecA removal from dsDNA is also conducted. Pitfall of their assay is that they removed RecA from solution when adding PcrA. It is known that a spontaneous dissociation of RecA can occur in the absence of RecA in solution therefore removal can occur regardless of helicase. No control experiments were presented to show that the data is valid.
Regardless of validity of some of the former studies, kinetic information on anti-recombinase activity or the mechanistic details of how helicase machinery can process such function was not studied before. I used single-molecule FRET technique to obtain dynamic information of individual PcrA in action. More over unexpected finding of reeling-in activity was shown to be a highly efficient mode of PcrA in dismantling RecA filaments: it showed 10-fold higher effective removal with 10-fold higher reaction rate compared to former studies that tested removal of RecA (or Rad51) on ssDNA that lack duplex element. Detailed time trajectory of individual RecA filament removal was also revealed showing tug-of-war between RecA and PcrA.

5.2. RecA filament formation on ssDNA and its kinetics

It is known that when RecA binds to DNA, it stretches DNA by 50% compared to its length as a duplex. Single strand is a flexible polymer and therefore its average end-to-end distance is even shorter than the duplex DNA. This property is useful to monitor RecA filament formation as formation of RecA stretches ssDNA and induce increase in end to end distance. Single-molecule FRET method were devised as to add FRET fluorophores flanking the ssDNA of interest to monitor RecA filament dynamics (Joo et al., 2006). Here I used same principles and used standard 5’pdDNA and added RecA and ATP to form a filament. As shown in Figure 36, FRET histogram changed from mid FRET to low FRET up on addition of RecA.
The time course of RecA filament formation could be monitored by taking movies of molecules at different time point and compare the population changes. As shown in Figure 37 top, high FRET shifts gradually toward lower FRET over the course of 2-3 minutes and reached equilibrium ~5 min. Filament formation can also be observed by tracking individual DNA molecules. Figure 37 bottom shows a representative trace where FRET gradually increases over 2 minutes. Extensive fluctuation in Cy3 signal was observed which might be caused by multiple RecA interacting with fluorophore as the ssDNA polymer can adapt ~13 RecA molecules.
5.3. PcrA removes preformed RecA-filament

On a pre-formed RecA filament prepared from above (Chapter 5.2.), PcrA was added in various concentration while keeping the RecA and ATP concentration constant. When 1nM of PcrA was added, significant amount of low FRET population were shifted to higher FRET, indicating that
some of the RecA filaments were dismantled. Higher concentration of PcrA further shifted the lower FRET population toward higher FRET and the trend was PcrA concentration dependent (Figure 38). For all the measurement, RecA concentration was constant (250nM). Therefore, the result showed that even hundreds fold less amount of PcrA can counteract RecA in equilibrium.

Figure 38 PcrA dismantles RecA filament in PcrA concentration dependent manner
5.4. RecA removal kinetics

RecA filament dissociation kinetics can be monitored by taking snapshots of movies at different time points after adding PcrA (Figure 39). RecA dissociation was observed as early as 10 seconds after PcrA addition as indicated by shift of low FRET population towards higher FRET population. The trend continued until about 2 minutes and reached equilibrium sometime between 2-5min.

Figure 39 Time course of population change during RecA removal by PcrA

The kinetic data is analyzed further by fitting the time dependent histograms using two gaussians. The areas under each curve were quantified and the fraction of dissociated population
were obtained. These numbers are summarized in Figure 40. Figure 40 top right shows clear pattern of PcrA concentration dependent RecA dissociation that occurs rapidly at the initial phase and reaches equilibrium between 2-5 minutes. The percents RecA dissociated at the equilibrium are plotted on Figure 40 top right. It shows that even a few nanomolar concentration of PcrA can dismantle RecA filament formed in 100 fold excess. Few nanomolar concentration corresponds to a few number of molecules inside a bacterial cell therefore PcrA must be able to act as a highly effective anti-recombiase. The plot shown in Figure 40 top left were fit to exponential fit and the decay time was obtained as in Figure 40 bottom. It shows that the rate itself is not PcrA concentration dependent. This may be interpreted as that PcrA replacement of RecA is determined by spontaneous RecA dissociation and unable to rebind due to the action of PcrA. But further experimental supports are required to verify if this is truly the mechanism.
**Figure 40 Analysis result of time course population change during RecA removal**

Most exciting part of single-molecule measurement is that one can follow the dynamics of individual molecule and see the individual process during the reaction. Individual molecules’ FRET signals were monitored upon PcrA addition (Figure 41). Over a course of time, FRET signal gradually increased from low FRET to mid FRET. Eventually, repetitive sawtooth pattern appeared as an indication of PcrA taking over entire ssDNA. Before repetitive pattern begin to appear, intermittent FRET fluctuation as well as some gradual FRET increase was observed which may indicate a period where PcrA is gradually taking getting RecA removed. At the
equilibrium, repetitive sawtooth pattern was often interrupted and this may be due to competition with RecA that are still present in high concentration in solution.

Figure 41 Single-molecule time trajectory of RecA-preincubated DNA upon PcrA addition

It is difficult to interpret in detail what is happening from the FRET time trajectory, because there will be at least one PcrA and up to 13 RecA may be involved for a given DNA. Additional analysis that can be done is repetition duration time ($\Delta t$) analysis. When I collected $\Delta t$ of the repetitive part of the FRET signal on the RecA removal time trajectory, following histogram was obtained (Figure 42). The result indicates that the average or FWHM is comparable to the result from the experiment without RecA. Therefore, I can conclude that at least during the period
where repetitive sawtooth were seen, RecA must be fully removed from the DNA and thus do not hinder with reeling-in activity.

Figure 42 time duration (t) analysis of PcrA repetitive reeling in at the equilibrium with RecA filament
Chapter 6 UvrD helicase dynamics

6.1. Introduction to UvrD helicase

*E. coli* UvrD, like PcrA is an SFI family helicase. It has 3’→5’ translocation activity and shows unwinding activity possibly as a dimer *in vitro* (Heller and Marians, 2007; Maluf et al., 2003a; Maluf et al., 2003b). Its *in vivo* function is known to be related to methyl-directed mismatch repair (Iyer et al., 2006), DNA excision repair (Sancar, 1996) replication restart (Flores et al., 2004; Flores et al., 2005; Lestini and Michel, 2008; Michel et al., 2007) and plasmid replication (Bruand and Ehrlich, 2000). Recently, it was suggested to be involved in anti-recombinase activity by counteracting ReA filament (Flores et al., 2005; Long et al., 2009; Veaute et al., 2005). It was shown that UvrD can be closely related to PcrA for its *in vivo* function. Several studies have shown that PcrA replacement on the UvrD deleted strain can restore UvrD’s function such as replication fork restart or tus removal from ter site (Bidnenko et al., 2006; Lestini and Michel, 2007). These activities however cannot be restored by Rep helicase.

6.2. UvrD can reel-in ssDNA of 5’overhang

*E. coli* Rep and UvrD are homolog of PcrA and have over 40% sequence similarity and identical structure. After the discovery of PcrA reeling-in activity, I tested Rep as well as UvrD on the same DNA substrate and tested if they may also have the same activity. On the 5’tail substrate, Rep did not show any evidence of reeling in. Rather showed repetitive shuttling behavior as it showed with 3’tailed pdDNA (communication with S. Arslan). However with UvrD, distinctive FRET fluctuations were observed indicating that the ssDNA is being pulled by UvrD (Figure 43). Compared with PcrA reeling-in, number of difference was observed. 1) UvrD reeling-in occurs with faster frequency. This is possibly due to higher translocation rate that is known to be ~3
times higher than of PcrA. 2) The shape of sawtooth pattern was not as regular to that of PcrA. This may be due to possible slippage or dissociation from one or both end of ssDNA. 3) The duration of repetition was shorter. This may indicate that the UvrD’s ability to stay localized at the junction may be weaker than that of PcrA.

Figure 43 UvrD reeling-in ssDNA of the 5’pdDNA in comparison with that of PcrA

6.3. ATP dependence of UvrD-reeling in

Although UvrD reeling in pattern was less ordered compared to that of PcrA, UvrD also showed a clear dependence on the ATP concentration (figure 44). With low concentration of ATP, gradual increase phase of FRET time trajectory become slower while the sharp drop period did not show distinctive change. The time duration of each cycle (Δt) was measured and the repetition rate (1/Δt) was plotted against the concentration of ATP used (Figure 44 Top right).
Because of the more disordered repetition, wide distribution of repetition rates was observed in an identical ATP condition. However, the mean value did increase with increasing ATP concentration and when this mean value is plotted, it followed Mechalis-Menten relation. The fitting gave $K_m = 23.2 \pm 4.1 \mu M$ indicating that UvrD may have lower affinity to ATP compared to PcrA.

Figure 44 ATP dependence of UvrD reeling-in

6.4. ssDNA length dependence

UvrD’s processivity is known to be longer than 100nt. Therefore, at least when UvrD maintains contact with the duplex junction, reeling in activity can occur for multiple cycles on longer than
40nt ssDNA overhang that is initially tested (as in Figure 43). Various length of ssDNA was tested for UvrD reeling-in activity (Figure 45). And indeed, UvrD did show ssDNA length dependence. The interval between the two peaks becomes longer with use of longer DNA substrate. Time duration between each cycle (Δt) was measured and built into a histogram as shown in Figure 45 top right. Again, due to the irregular nature of signal, wide distributions of dwell time were shown for a given condition. However, the peak value did shift toward longer duration with increasing ssDNA length. When the peak value were extracted and plotted against the ssDNA length, very distinctive linear relationship was observed (Figure 45 bottom right). The slope of the linear fit gave 184nt/sec as a translocation rate which is comparable to the known translocation rate from previous ensemble study (Tomko et al., 2007).
6.5. Difference between PcrA and UvrD at the initiation of reaction

An interesting observation was made by Eric Tomko at Dr. Lohmans lab when he was testing UvrD and PcrA reeling-in activity using ensemble single-turnover method with stop-flow machine. He has found that when UvrD is preincubated for binding, UvrD tend to find ss/dsDNA junction and favorably bind to it compared to other sites of ssDNA. However, he did not see such strong junction preference with PcrA. As a result, less amount of ssDNA looping was observed with PcrA at his experimental setting. With the single-turnover stop-flow technique, only an initial few seconds of reaction can be captured and it cannot monitor dynamics at equilibrium. I suspect this difference may have caused difference in probability of seeing the
pulling activity of these two helicases. To test this hypothesis, I prepared a flow chamber and
pre-incubated both UvrD and PcrA. Then I flowed in ATP without any protein. In this condition,
only the bound protein may react to ATP and induce signal changes and additional binding of
protein is minimized. The result showed two major differences between UvrD and PcrA (Figure
47). 1) After the flow of ATP, FRET increase was immediately observed in UvrD. However, for
PcrA, FRET signal showed a certain delay time until it rise to high FRET peaks. 2) UvrD FRET
signal showed a few number of spikes then is disappeared while PcrA FRET peaks continued
spiking and continued for minutes. I measured the delay time between the buffer flow initiation
and the beginning of the spikes. Figure 46 B and D shows the results. It is clearly shown that
PcrA require time to start reeling-in while with UvrD reaction occurred without delay. This
result explains why less pulling signal was observed with PcrA compared to that with UvrD.
Also new information that is revealed is that UvrD can be a fast acting molecule as it can
recognize junction immediately and starts reeling in. However, it is not persistent molecule and
can easily dissociate after few seconds. On the other hand, PcrA may be relatively slow acting
molecule that takes time to find junction. However when junction is found, PcrA become a
powerful motor and persistently reels-in ssDNA without dissociating from it.
Figure 46 Comparison of delay time between PcrA and UvrD

6.6. Stepsize of UvrD may be 1nt

The method used to verify the kinetic stepsize of PcrA requires following condition. 1) A single protein should give translocation signal and it should be guaranteed that the signal is not contaminated by potential replacement of other proteins. 2) It should be guaranteed that the protein translocates a defined length of DNA and should not bind or dissociate at random position. 3) Translocation trajectories should have enough statistics to allow reliable analysis. For UvrD, repetitive translocation is often terminated without giving enough numbers of repetitions. Also it is not fully certain if UvrD completes full length translocation as individual time duration statistics show broad histogram. Therefore, it may not be suitable to conduct same
analysis on UvrD to find out its step size. For UvrD, individual molecules show extreme variation in both repetition number and the disorderedness among each repetition. However, at times, some molecules are shown to present lengthy repetition trajectory as shown in Figure 47. When this trajectory is analyzed for time duration of each repetition (Δt), and the histogram was analyzed with gamma distribution, it was found that UvrD at least took 26 steps for translocating on a 40nt ssDNA. This indicates that UvrD may also translocate on steps on 1nt. Here I would like to note that the more irregularity seen in UvrD reeling-in trajectory is most likely due to its less active ability to stay anchored at the duplex junction. Therefore, it is most likely irrelevant to the function of motor domain therefore its stepping mechanism. I strongly believe that if one can devise an experiment that allows UvrD to translocate a full length of given ssDNA and can obtain substantial statistics on translocation time, it will show 1nt steps as I have seen in my preliminary data as below.

![Figure 47 UvrD may translocate with 1nt step size](image)

We found that repetitive looping is also observed with UvrD, but not with Rep (data not shown).
6.7. Comparison of UvrD and PcrA and their common role

Although UvrD and Rep double mutants are lethal in vivo suggesting an overlapping function, several non-overlapping roles of UvrD have also been reported. Unlike Rep, UvrD can help restart replication by Tus removal (Bidnenko et al., 2006) and dismantle recombination intermediates from the lagging strand (i.e. the 5’ ssDNA tail) of the replication fork in (Lestini and Michel, 2007). Interestingly, Bacillus subtilis PcrA was capable of substituting for UvrD in both studies while Rep was not able to compensate for the absence of UvrD. Our finding that both PcrA and UvrD stably anchors on the duplex while actively translocating on the 5’ tail provides a plausible mechanism of stripping deleterious RecA filaments from the lagging strand (Flores et al., 2004; Morel et al., 1993; Veaute et al., 2005; Zieg et al., 1978). Although in vivo data implied RecA removal by UvrD at the stalled replication fork, it was not clear if and how UvrD can target RecA on the replication fork. Previous in vitro study showed UvrD induced removal of RecA filament on circular ssDNA that lack dsDNA (Veaute et al., 2005). In that experiment, over 100nM of UvrD was required to observe effective RecA filament removal (RecA : UvrD =20 :1). In our assay using partial duplex DNA molecules, we observed effective RecA removal at PcrA concentration as low as 1nM (RecA : PcrA = 1000 :1). Our results show that that anti-RecA activity conserved in PcrA and UvrD is highly effective in the context of ss/ds junction and that the robust looping dynamics shown with 5’ tail is likely to be a mechanism for negative regulation of RecA function at the stalled replication fork. A similar mechanism may in play for the function of eukaryotic superfamiily 1 helicases such as Srs2 in removing potentially deleterious recombination intermediates (Krejci et al., 2003; Veaute et al., 2003)
Chapter 7 XPD helicase

7.1. Background of XPD helicase

XPD helicase belongs to Superfamily 2(SF2) of which members are related many disease such as premature aging, susceptibility to cancers, immunodeficiency and mental retardation (van Brabant et al., 2000). XPD helicase are known to be involved in DNA repair as hyperphotosensitivity and defect in nucleotide excision repair as well as skin cancer susceptibility can be resulted when XPD is mutated(van Brabant et al., 2000). XPD belongs to a Rad3 sub-family within SF2 helicase which has two unique features. One is the iron-sulfur cluster(Pugh et al., 2008a; Rudolf et al., 2006) and the other an arch domain(Fan et al., 2008; Liu et al., 2008; Wolski et al., 2008). FeS domain is known to involve in coupling ATP hydrolysis to translocation and also was proposed to provide secondary binding site for DNA (Gupta et al., 2005; Pugh et al., 2008a; Voloshin and Camerini-Otero, 2007). Also, for the unwinding and G-quadruplex remodeling function of XPD as well as its homolog FancJ, ssDNA binding protein, RPA are shown to be required for facilitation(Gupta et al., 2007; Pugh et al., 2008b; Wu et al., 2008).
7.2. Monitoring XPD binding kinetics using FRET

XPD helicase is 5’→3’ translocase like other SF2 helicases (Cantor et al., 2004; Farina et al., 2008; Hirota and Lahti, 2000; Pugh et al., 2008a; Rudolf et al., 2006; Sung et al., 1993; Voloshin et al., 2003), therefore 5’tailed partial duplex is a suitable substrate for testing DNA unwinding. Although many biochemical studies were done on XPD, it is first time for XPD to be tested in single molecule. To start out with testing some basic properties, I conducted binding/dissociation kinetics using single-molecule FRET. The DNA used is shown in Figure 48 left. Short ssDNA overhang is used in order to prevent multiple XPD binding. The FRET pair separated by ssDNA of 12dT gave 0.8 FRET. Upon addition of XPD, this high FRET population shifted to low FRET population. Note that there is no distinctive intermediate population appearing. This trend was XPD concentration dependent and can distinctively probe protein binding.
Figure 49 XPD helicase binding to FRET pair labeled DNA. Cy3 is bound at the 5's end and Cy5 is bound at the dupelex junction to monitor any changes in distance between two labeled sites upon protein binding. Various concentrations of XPD helicase are added.

XPD binding kinetics can be observed by following individual fluorescence time trajectories (Figure 50). XPD binding was shown to be a dynamic process where binding and unbinding constantly occurs for a given DNA. As observed from the histogram data, binding and dissociation occurred without any distinctive intermediate step. As higher concentration of XPD is in solution, higher frequency of binding appeared shown as an increasing frequency of lower FRET state visited.

It is unclear why FRET decrease was observed with XPD binding. In fact, at the moment that is expected for PcrA binding, FRET was shown to increase (Ch. xFigurex). One possibility is that...
the PcrA binding scrunched ssDNA to certain extent while XPD binding mode may be different from PcrA and stretches ssDNA. Another more probable explanation is that XPD’s iron-sulfur cluster quenched Cy5 when it binds to the junction and is placed closer to the junction than to the 5’ tail end. In fact, the latter explanation is plausible explanation as the FRET change comes more from Cy5 signal decrease and not much from Cy3 signal increase.

Figure 50 (continued)
It was known that iron-sulfur cluster of XPD can quench Cy5 fluorescence (Pugh et al., 2008a). Therefore, the project was originally proposed by my collaborator to study binding/dissociation kinetics of XPD using Cy5. However, Cy5 is a poor fluorophore for molecule tracking experiments due to its severe photo-instability. Therefore, I asked if Cy3 can be used instead. DNA construct was prepared as Figure 50 with only Cy3 bound at the junction. On this DNA
substrate, XPD was added in various concentrations. XPD indeed quenched Cy3 very effectively and decrease in fluorescence signal appeared in XPD concentration dependent manner. It is a first demonstration of monitoring any iron-sulfur containing protein’s activity in single molecule level, using its quenching ability. It is a very convenient method as no specific labeling is required and the natural fluorescence intensity modulator is built in naturally inside a protein.
Unlike FRET measurement, it is difficult to construct histogram of binding profiles because absolute intensity of individual molecules in an imaging area is different intrinsically due to uneven illumination. Although details of population analysis cannot be done, general trend of
ensemble quenching behavior was observed using fluorometer (Figure 52). The result showed XPD concentration dependent quenching with varying $k_d$ depending on the concentration of DNA substrate used. When tested with low concentration of DNA (1nM), $k_d$ of about 1nM was observed.

![Figure 52 Quenching of Cy3 fluorescence measured by fluorometer (by M. Honda)](image)

**7.4. Characterizing Cy3 quenching effect**

It is not known why and how FeS cluster of XPD can quench fluorescence. Only close example of fluorescence quenching that is reported is GFP quenching by iron of hemin and is explained as intermolecular long-range-dipole-dipole coupling (Takeda et al., 2003). Although details of quenching mechanism may require further research, one simple hypothesis that can be tested was if presence of FeS cluster creates another non-radiative energy transfer pathway therefore decreases fluorophore’s overall excited-state lifetime. We used a time-correlated-single-photon-
counting (TCSPC) method to verify this. For Cy3, most of the protein binding induces fluorescence enhancement and so did PcrA. I prepared PcrA and XPD and observed fluorescence lifetime change due to protein binding. As shown in Figure 53, PcrA binding indeed increased Cy3 fluorescence lifetime by about 30% and on the other hand, XPD decreased Cy3 fluorescence lifetime by about 50%.

Figure 53 Cy3 fluorescence lifetime change upon protein binding is monitored using time-correlated single photon counting method. X axis is time (fs) and Y-axis is count.

7.5. Rate analysis on FRET data using Hidden-Markov analysis

XPD binding kinetics was monitored by FRET and Cy3 quenching and both data yield a consistent result. Most powerful perspective of single molecule lies on that it can measure kinetic rate rather than the simple equilibrium binding constant. To obtain the Kon and Koff, further analysis was conducted on FRET time trajectories. Great thing about FRET signal is that it is ratio-meteric measure and is not subjected to the absolute fluorescence intensity. Therefore,
effect of Cy5 quenching extent is pre-normalized when using FRET. Hidden Markov analysis using HaMMy software (McKinney et al., 2006) was conducted for individual trajectories and the transition frequency was calculated. An efficient way of visualizing the transition frequency is by 2-dimensional transition density plot (TDP). Figure 54 shows TDP of data at various XPD concentrations. At the low concentration regime, no significant size of cluster was observed indicating that there was no defined transition state. This may be due to the fact that very few molecules show binding signal and that rather the noise fluctuation were captured by the analysis program as false positives. As the XPD concentration increases, defined cluster was observed which shows transition from one defined FRET state to the other. Extreme symmetry observed in TDP indicates that the reaction is at equilibrium.

![Image of Transition Density Plot showing HaMMy analysis of XPD binding kinetics measured by FRET. Initial FRET value is shown in x-axis and the y-axis indicates the FRET that is changed to.](image-url)
From TDP plot, FRET states were identified and grouped for analysis. And from this grouped data, transition rate were calculated. As a result, Kon and Koff were obtained. Figure 55 shows resulting Kon and Koff for various XPD concentrations.

Figure 55 Kon and Koff of XPD binding analyzed form TDP
As observed from TDP, the analysis on the data obtained from low concentration range is susceptible to noise and the false positive transition can be picked up by the HaMMy analysis. Based on the shape of the TDP, I represent below an analysis result on the reliable range. The result shows that the binding rate increases linearly with increasing XPD concentration while dissociation rate remains constant.

![Graph showing Kon and Koff of XPD binding on the reliable concentration range](image)

**Figure 56 Kon and Koff of XPD binding on the reliable concentration range**

### 7.6. Rate analysis on Cy3 quenching data using Hidden-Markov analysis

As the binding kinetics are fully characterized and it was confirmed that there are no other states than two states (XPD binding and dissociation), now Cy3 fluorescence quenching signals can be normalized and analyzed with Hidden Markov method. This normalization process was done by the help of Yang Liu, a postdoc of Dahmen lab. Resulting normalized time trajectories were fed to HaMMy and the TDP was built with the output data. As shown in Figure 57, tightly isolated FRET transition states were found. Transition rate calculated from each cluster gave Kon and
Koff of XPD. Kon and Koff are each plotted against XPD concentration and the same trend was also observed as seen with FRET data. The binding rate increased with XPD concentration while dissociation rate remained constant independent of XPD concentration. From both FRET and Cy3 quenching data, one intrinsic value were obtained which is XPD dissociation rate of ~0.7s⁻¹. This indicates that XPD binding is not very stable and does not dwell on the DNA only about 1.5 second. It will be an interesting study to vary the length of ssDNA of the pdDNA and see if the Koff changes with substrate that can potentially provide more (or less) stable contact.

Figure 57 Transition Density Plot obtained from HaMMy analysis on Cy3 quenching data
7.7. XPD unwinding fork DNA substrate

Here I present some preliminary data on XPD unwinding experiment I conducted. XPD is 5’→3’ translocase and helicase. I used forked DNA substrate as illustrated in Figure 58 and added XPD toether with ATP. At low (200nM) concentration of XPD, no significant evidence of unwinding was observed although severe quenching of both fluorophore is observed. Extent of unwinding can be confirmed by imaging after high salt wash as removal of XPD allows verification of remaining FRET paired DNA that resisted unwinding. However, after applying high (500nM) concentration of XPD with ATP, significant amount of unwinding is observed as a disappearance of fluorescence spots on the surface due to loss of donor labeled ssDNA by XPD unwinding.

Figure 58 Forked duplex unwinding by XPD helicase
Individual DNA substrate can be tracked during XPD is unwinding. Representative FRET time trajectories are shown in Figure 59. FRET trajectory shows that FRET decrease in gradual manner indicating that the duplex is separated. Cy5 signal seems to be more influenced by XPD movement perhaps due to the fact XPD follows 5′ tail for unwinding. Due to quenching effect, anti-correlation between Cy3 and Cy5 is not as perfect as conventional FRET experiments. But, it is clear that Cy3 signal increase when Cy5 decreases. This experiment includes three parties of each signal influence one another. In order to clarify detailed dynamics of unwinding, further experiments will be required.

![Figure 59 Time trajectory of XPD during unwinding of forked duplex](image)

**7.8. XPD unwinding activity on RPA pre-incubated DNA**

From previous ensemble studies, it was observed that RPA facilitate XPD unwinding. Therefore I tried to see if I can observe the same RPA effect in XPD unwinding (Figure 60). First I incubated RPA on immobilized forked duplex. Then I added XPD together with same RPA
concentration and see if unwinding occurs. As an effect of FeS cluster, significant quenching of both fluorophore were observed indicating that XPD can bind to RPA coated DNA. After the reaction, high salt wash was applied to surface to remove XPD and verify amount of unwinding occurred. Interestingly, no significant amount of unwinding was observed indicating that XPD could not effectively unwind RPA coated DNA substrate. This is somewhat contradictory to the literature. However I would like to note that this is a preliminary result and there are several possible reasons for this difference. Some of the reason could be less accessibility of the tracking strand due to surface immobilization.

Figure 60 XPD unwinding activity on RPA coated DNA

7.9. XPD translocation on ssDNA

As monitoring XPD signal is complex with two FRET pair, I conducted unwinding experiment with Cy3 only labeled DNA substrate. From this experiment, I discovered that gradual increase
(or decrease) in signal in Cy3 fluorescence. The fact that the signal is not step-like but is gradual reveals the fact that the quenching is occurring with reasonable distance dependence to work with. This result reveals the possibility that XPD translocation or unwinding can be monitored by Cy3 quenching signal. I used 5’end Cy3 labeled ssDNA and immobilized on the surface and tested if translocation can be monitored in this scheme. First, I added XPD without ATP (Figure 61). The fluorescence time trajectory showed a step-like transitions of signal indicating XPD binding and dissociation.

![Figure 61 XPD binding on ssDNA](image)

Now, on the same DNA substrate, ATP was introduced together with XPD. Fluorescence time trajectories clearly showed that gradual changes in signal (Figure 62) that is dramatically
different from protein alone. This was a preliminary experiment with a short (dT18) length of ssDNA. Using longer ssDNA and less protein concentration would elucidate translocation activity with better detail.

Figure 62 XPD translocation on ssDNA
Chapter 8 RecA filament dynamics

8.1. Introduction to RecA

RecA is an essential protein that involves in homologous recombination, SOS response, and DNA repair (Cox, 1999; Kowalczykowski, 2000). RecA binds to ssDNA and forms a filament and then can search for the homology for pairing with the dsDNA and therefore induce strand-exchange. When RecA binds to ssDNA, it occupies about three nucleotides (Egelman and Yu, 1989; Zlotnick et al., 1993) and stretches ssDNA. RecA filament has helical structure with repeats of six RecA monomers (Story et al., 1992).

RecA filament dynamics is dependent on ATP. ATP acts as a cofactor for cooperative formation of RecA filament (Menetski and Kowalczykowski, 1985). RecA hydrolyzes ATP in DNA dependent manner with rate of 0.5 s⁻¹ at 36°C (Bedale and Cox, 1996). ATP hydrolysis induces RecA to dissociate from the end of the filament (Lindsley and Cox, 1990; Register and Griffith, 1985). Therefore, when non-hydrolysable ATP analog ATPγS is replaced with ATP, RecA dissociation is minimized.

RecA filament formation requires at least 5 monomers for nucleation (Joo et al., 2006). When nucleation is achieved RecA monomers can bind to both end of DNA and grow. However the binding rate towards 3’ direction is much faster than towards 5’ direction whereas dissociation rate remains the same (Joo et al., 2006). Therefore 3’ end is termed “3’-extending end” while 5’ end is termed 5’ disassembly end.

At the initiation of double-strand break repair by homologous recombination, ssDNA generated by RecBCD is coated with SSB. RecA displaces SSB mediated by RecOR (Bork et al., 2001; Umezu et al., 1993) which may help RecA filament nucleation at the ss/ds DNA junction. When
nucleation complex is formed RecA was shown to displace SSB as it grows by monomer binding at the end (Joo et al., 2006).

8.2. Experimental settings

From C. Joo’s pioneer work on RecA, an optimal platform was provided in order to test binding kinetics of RecA in single monomer level. Effect of any factors that influence RecA filament dynamics can be observed in detail. RecA monomer binding and dissociation is monitored using single-molecule FRET with the following experimental scheme (Figure 63). 3’tailed partial duplex with FRET labels is immobilized on the surface. The fluorophores are separated by 10 nt between each other and it was shown as an effective condition to monitor up to two RecA binding and dissociation giving three state FRET transition (0 monomer bound, 1 monomer bound, 2 monomer bound)(Joo et al., 2006).

Figure 63 Experimental scheme. 3'tail partial duplex with fluorescence labels indicated as above is immobilized on the surface and RecA and ATP are added to see RecA filament dynamics
8.3. pH dependence on RecA filament dynamics

The stability of RecA filament was reported to be dependent on pH (Arenson et al., 1999). However, it was not clear if pH is influencing binding affinity of the protein or the stability of RecA/ssDNA complex after binding. I have prepared three buffers with different pH and tested its effect on filament dynamics. For low pH and high pH conditions, I used Hepes as a buffering reagent instead of Tris as Tris is not suitable for buffering in such range. As shown in Figure 64, high pH induced DNA population shift towards high FRET state while low pH induced DNA population shift towards low FRET states. This result indicates a trend that Higher the pH cause unstable RecA filaments.

Figure 64 FRET histograms representing population distribution of filaments at each pH condition.
On individual FRET time traces (not shown), HaMMy analysis was conducted to find out the FRET states visited and TDP is constructed as shown in Figure 65. Data shows two sets of data obtained from different movie taken on a same day. Filament stability observed in the histogram (Figure 64) is also reflected on the TDP as fewer numbers of transitions between high-mid FRET were observed in low pH data. Along the same line, TDP of High pH data also show less frequency of low-mid FRET transitions.

![Figure 65](image)

**Figure 65 Transition Density Plot showing frequency of transition between 3 different RecA bound states at various pH conditions**

From the TDP, isolated islands were identified to extract transition rates of the each. In the figure 66, transition frequencies are indicated for the TDP data shown in Figure 65. Reference transition rates are from the literature and placed for comparison (pH condition of the referenced
work is at 7.5). Average value between two sets of data taken with identical condition is shown in bold.

![Table showing transition rates](image)

**Figure 66 Transition rates calculated from TDP shown in Figure 65**

If the mechanism for binding (or dissociation) is influenced distinctively, it should influence both of the first and the second monomer binding (or dissociation). Therefore, I classified binding and dissociation rates and plotted as Figure 67. It is shown that both dissociation and binding seems to be influenced by the pH. Dissociation occurs slower with decrease in pH and the binding occurs faster with decrease in pH. Note that the influence of dissociation was not fully clear while binding rates showed clear trend.
To identify if the dissociation rate decrease is noteworthy or negligible, it was compared with the preliminary data taken about a week before this experiment. The experimental scheme and buffer conditions used were identical. As shown in Figure 68, similar results were obtained.

Dissociation rate did not show distinctive trend but the binding rates are clearly influenced. Therefore, I conclude that the pH may not change RecA’s ATP hydrolysis process which governs dissociation. However the binding of RecA to ssDNA process is highly affected by pH.

Perhaps RecA have ssDNA binding site with residues that vary its protonation state and cause pH sensitivity in binding affinity.
8.4. ATP concentration dependence on RecA filament dynamics

ATP hydrolysis is involved in RecA dissociation. If ATP hydrolysis is suppressed, RecA dissociation was shown to be minimal based on ATPγS study. To test the effect of ATP, 10 fold variation of ATP conditions are prepared and tested for the filament dynamics. DNA construct used is same as shown in Figure 63. With individual time trajectories, HaMMMy analysis was conducted and the results are presented as TDP shown in Figure 69. Lower ATP concentration tend to show broader range of FRET for each FRET states but overall frequency appeared similar for all transition states.
Figure 69 Transition Density Plot showing frequency of transition between 3 different RecA bound states at various ATP concentration

From the TDP, transition states are identified and each rate is calculated. The result is shown in Figure 70. Reference transition rates are from the literature and placed for comparison (ATP condition of the referenced work is at 1mM). Average value between two sets of data taken with identical condition is shown in bold.
Figure 70 Transition rates calculated from TDP shown in Figure 69

If the mechanism for binding (or dissociation) is influenced, it should influence both of the first and the second monomer binding (or dissociation). Therefore, I classified binding and dissociation rates and plotted as Figure 71. It is shown that both dissociation and binding seems to be influenced but only very subtle. Dissociation occurs slightly faster with decrease in ATP and the binding appears to occur somewhat faster with decrease in ATP but is less conclusive.
In order to identify if the dissociation rate increase is noteworthy effect or negligible, this experiment is repeated but with lower concentration range because it is likely that >1mM concentration can be saturating condition. The experimental scheme and buffer conditions used were identical. As shown in Figure 72, similar but not identical results were obtained. Dissociation rate showed only minor (negligible) increase and the binding rates were also did not definitive trend.
As the result was puzzling, repeat experiment were conducted to verify the reliability of the experiment and analysis method. For this test, 1mM and 100 μM concentration of ATP is used. The experimental procedure, buffer conditions and analysis methods are all identical. The results are shown in Figure 73. The dissociation rate again showed increase with lower ATP although the difference was minor consistent with other two measurements. However, this time binding rate were somewhat appeared lower than previous measurement and rather showed decrease in rate with lower ATP concentration. Therefore, I conclude that higher ATP concentration slightly decreases rate of dissociation but negligible changes in binding rate. However, there is a chance that the range of ATP tested was all around the saturating condition as ATP hydrolysis rate of RecA is very slow (0.5/sec). Therefore, further experiment may be required to elucidate the effect of ATP on RecA filament kinetics.

**Figure 72 Repeat rexperiment conducted on different day**
8.5. Size of RecA occupancy on ssDNA

RecA monomer is shown to occupy 3nt of ssDNA when forming filament (Chen et al., 2008). However, in single-molecule experiment, 5nt, 10nt, 13nt of ssDNA were probed by Cy3 and Cy5 and optimally probed 1, 2 and 3 monomer binding and dissociation (Joo et al., 2006). When considering 10 nt ssDNA length, it is enough size to accommodate at least 3 RecA monomer however only 2 RecA dynamics were clearly observed without ambiguity. This is somewhat puzzling results and appeared worthy to look into for further characterization. To verify if 10 nt indeed is an optimum condition for probing dynamics of 2 RecA monomer, ssDNA length probed by FRET pair is varied slightly. The results are shown in Figure 74. TDP clearly shows distorted FRET transition states for the shorter (9nt) distance used and also for the longer (11nt).
is used. 9nt flanking substrate (short) showed higher FRET as a major population while 11nt flanking substrate showed lower FRET as a major population. Possible interpretation for this result can be with the shorter probing distance, RecA become difficult to bind in between therefore lower FRET barely appeared. Whereas with the longer probing distance, binding between two probes maybe easier for 2 RecA to accommodate and thus lower FRET became dominant. When transition rates were calculated, slight trend of faster binding was observed with longer flanking length. This result also supports my preliminary hypothesis. On the other hand, presence of fluorophore seems only to interfere with binding perhaps by steric hindrance but does not appear to influence dissociation kinetics maybe the interaction breakage may occur between ssDNA backbone and the protein.
Figure 74 Transition Density Plot showing frequency of transition between 3 different RecA bound states probed by fluorescence probe positioned at various distances (top), and the transition rate (binding/dissociation rate) calculated from TDP (bottom).
Chapter 9 DNA Origami

9.1. Introduction to DNA origami: custom shaped DNA nano-structure

DNA is a genetic molecule and is important for cellular function. But also strand of DNA itself can be a very interesting and useful material. Utilizing its unique base-pairing property, people can now design DNA sequences such that the DNA can self-assemble to form a desired structure. First demonstrations of such structure were bundles of branched helix of 1-100nm size structure (Fu and Seeman, 1993; Li et al., 1996; Seeman, 1982, 2003). Various dimensions were tried from two-dimensional crystal(Winfree et al., 1998), nanotubes (Douglas et al., 2007; Liu et al., 2004; Mathieu et al., 2005; Rothemund et al., 2004; Yan et al., 2003; Yin et al., 2008) and 3D polyhedral (Chen and Seeman, 1991; Goodman et al., 2005; He et al., 2008; Shih et al., 2004; Zhang et al., 2008; Zhang and Seeman, 1994). Then “scaffold-DNA-origami” method was devised which utilizes short “scaffold strands” that staples regions of the structure together by specifically designed base-pairing (Rothemund, 2006) thereby guide and fix the individual helical structure as desired. By stalking conjugated sheets of antiparallel helices, 3D structure could be designed more effectively (Douglas et al., 2009a) and graphical software is developed for efficient design (Douglas et al., 2009b). Further progress was made by targeted insertion or deletion of base-pair which can be used to create curvature effectively therefore enabling creation of more intricate 2D and 3D structures (Dietz et al., 2009). Using DNA origami method, tension bearing structure can also be made holding pre-stressed force of up to 14pN (about twice the stall force of kinesin or myosin)(Liedl et al., 2010). These structures are so called tensegrity (tensional integrity) DNA origami.
9.2. The Bull Horn structure, a tensegrity DNA origami

In collaboration with Dr. Shih and his post-doc T.Liedl, a project of applying DNA origami for testing bio-molecule’s property has initiated. The very first design made is shown in Figure 75. It has a “Bull Horn”-like shape of helix bundle with two ends of the horn being connected by ssDNA. The length of the ssDNA can be adjustable and can be modulated in order to change the tension applied on the ssDNA. This force can be calculated from simulation and is known to show 7-2 pN when 600-900 nt ssDNA is used. The structure has four site at the bottom that has ssDNA exposed, which can be annealed to biotinylated ssDNA and used to fix the whole structure specifically on the PEG-biotin-avidin conjugated surface.

![Figure 75 Structure of the Bull Horn DNA origami](image)

My original goal for this project is to see translocation of ssDNA motor proteins. In order to get a working condition, preliminary tests were done to test if Bull horn is indeed on the surface and does provide platform for protein binding. As shown in Figure 76, Cy3-labeled PcrA were added to two different chambers on the same microscope slide. One chamber has ssDNA immobilized
on the surface and the other with biotin-ssDNA annealed Bull Horn. The absolute number of fluorescence spots appeared is not directly comparable as the Bull Horn was immobilized in 10 fold lower concentration due to limited amount that I had. A distinctive difference that is noteworthy is that the Cy3 spots on the Bull horn appeared brighter than that on the short ssDNA. As the Bull Horn that I used has about 1000 nt ssDNA, more than on PcrA can in principle bind to one structure

+ 250pM cy3-PcrA

Figure 76 Binding of Cy3 labeled PcrA on the Bull horn and a short ssDNA

Number of proteins bound to each spot can be quantified by counting photobleaching steps in the individual time trajectories (figure 77). Due to stochastic nature of fluorophore photobleaching
event, number of fluorophores can be counted by following sharp drop of signals until the signal gets fully disappeared.

Figure 77 Representative time trajectory of Cy3-PcrA bound to Bull horn

Number of photobleaching steps was plotted for both Bull horn and short ssDNA (figure 78). For the short ssDNA, less than 10% of molecules showed two step photobleaching indicating that chance for potential aggregation or two molecule bind in proximity is small (but in fact 40nt ssDNA is also long enough to adapt 2 or more PcrA binding). Less number of statistics were obtained with Bull horn however, distinctively many fractions of molecules showed two or more photobleaching steps. The result indicates that the Bull horn is indeed bound to the surface and the structure is able to capture many PcrA proteins.
An excess amount of Cy3-labeled PcrA was added to both structures to test if this trend is PcrA concentration dependent. The result is shown in Figure 79, more number of fluorescence spots are observed with about 4 fold increase in protein concentration. Also more distinctive high intensity spots are observed with the Bull horn immobilized surfaces. This trend was clearly shown in intensity distribution (Figure 79 right) that sharp narrow distribution near low intensity was shown with short ssDNA while higher intensity tails are developed with the Bull horn.
Figure 79 binding of excess amount of Cy3-PcrA on the Bull horn and a short ssDNA

9.3. The Bow structure, a rigid DNA origami

PcrA translocation on a long ssDNA was never tested before. Therefore, it is not known if PcrA binding and translocation endure tension applied on the ssDNA of the Bull horn structure. In order to minimize tension that can potentially interfere with PcrA translocation, newer design of origami was made. The new structure is named as The Bow and the structure is as shown in Figure 80. Instead of having a curved helix bundle, Bow structure has stalks of sheets of rigid helixses and ssDNA is connected by two ends with no tension except the ones that come from its own length used. Also, higher number (25) of biotin sites is prepared at the bottom for stable immobilization with desired orientation. This short biotinlyated strand is added at the time of
construct assembly to increase chance of incorporation. Additionally, Cy5 fluorophore is added at the 5’end of the ssDNA track so that the presence of the structure can be identified as well as possible FRET can be monitored at the end of the translocation when Cy3-PcrA is used.

Figure 80 Structure of the Bow DNA origami

One problem of the experiment is that DNA origami is custom made and the concentration (~1nM) and the amount is very low.
Appendix

Protocol for PcrA Protein Purification

Updated June 17th 2009

Growth and Induction of BL21 cells

Grow 500ml cell culture

1. Prepare 500ml LB medium:
   Heat the outside of the LB medium before adding the antibiotics.
   Add 2.5mL of ampicillin and 175uL of chloramphenicol to the LB medium
2. Remove 5ml overnight culture from shaker.
3. Add remainder of cells (4 mL) to a 500 mL LB+ampicillin+chloramphenicol in a 2L flask.
4. Place in shaker at 250 rpm, 37° C for 1 hour.
5. Prepare glycerol stocks: Cut edge of pipette tip to add glycerol to a microcentrifuge tube.
6. Add 200 uL of glycerol to microfuge tube. Make sure to label the tubes before adding cells.
7. Add 500 uL of cells, mix well and store at -80° C (this is an important stock).

Glycerol stock detail

1. Obtain two microfuge tubes and place on a rack.
2. Cut the end off of a 1000ul pipette tip. Collect 200uL of glycerol using blunt cut tip. Put at the bottom of microfuge tube. Do the same for the second tube.
3. Add 500uL of BL21 cells to the microfuge tubes (each).
4. Mix the glycerol and cells using a precut 200uL pipette tip and pipettor.
5. Label as BL21, hisPCRA, date and name. Store these in the -80 °C freezer.

Measure OD<sub>600</sub> until OD<sub>600</sub> = 0.6~0.8

8. After 1 hour, remove 1 mL of solution using a 10 mL surgical pipette.
9. Place in a clean cuvette(disposable).
10. Using a spectrophotometer, measure the OD<sub>600</sub>.
11. Remove 1 mL from cuvette and place in a microfuge tube and store at -20°C.
12. Repeat process of checking the OD<sub>600</sub> every hour until it reaches 0.8-1.
13. Once OD<sub>600</sub> is in the range of .4-.5, check the sample every 20 minutes.
14. After reaches 0.6-0.8, add 500 uL IPTG (500mM stock) to 2L flask. Label the sample taken out as IPTG 0hr. (final concentration of IPTG= 0.5mM, ref. REP= 0.3mM, Anita PcrA= 1mM)

Spectrophotometer detail
1. To measure the OD, make sure the spectrophotometer is on, and the computer program Cary WinUV is set to the “Scan” setting. Set the wavelength to be between 600 and 200 nm.

2. For the first reading, obtain 1 mL of the broth sample. Transfer to a cuvette. Make sure cuvette is clean and does not have fingerprints on it.

3. Measure the OD by placing the cuvette with the sample in the spectrophotometer.

4. The reading should be low. After finishing the reading, transfer the sample from the cuvette to a microfuge tube (label the tube), place in a rack, and put in the -20 °C freezer. Record the OD value in your manual.

5. After 1 hour, repeat steps 3 and 4. When the reading is near .4 - .5, take a sample every 20 minutes. Everytime you take a sample and measure the OD, transfer the sample to a new microfuge tube and place in the rack in the freezer.

Protein Induction

15. Continue to grow at 37°C and 250 rpm for 4 hours.

16. Continue to take 1 mL samples every hour and record OD600 and store samples at -20°C freezer (label as “IPTG 2hr, 3hr, 4hr”).

17. Weight empty 250 mL centrifuge bottle and write the number on the bottle.

Pellet Down

18. Transfer the cell broth sample that is on the shaker to a 250 mL centrifuge bottle. Centrifuge half of the sample (because there should be 500 mL of the sample) using a JA-14 rotor, at 5,000 rpm, at 4°C for 15 minutes. Weigh the bottle for balance before placing them to the centrifuge.

19. Remove supernatant and pour reminder of solution into centrifuge bottle. Make sure the centrifuge is balanced.

20. Place in centrifuge again for 15 minutes and remove supernatant.

21. Weight bottle and calculate g of cell pellet obtained. Record it on your labnote.

22. Store the pellet in -80°C freezer.

Purification of Proteins from BL21 cells

Do every step in the cold room

Thaw/Resuspension of Cell Pellet

1. Thaw the cell pellet on ice for 1 hour (in the cold room).
(Read the protocol ahead and prepare things needed up to step 11.)

2. Add 12.5 mL of Lysis Buffer in the cold room. The Lysis Buffer should have the following inside: 1X Tris HCl, .2M NaCl, 20% sucrose (no DTT or EDTA)
3. Add 150uL of PMSF. The PMSF should have a concentration of 17.4mg/10mL (of isopropanol).

4. Mix the cells with a 10mL pipettor. Use the end to separate the cells from the bottom of the bottle. Blend the cells by taking up about 12 mLs of the cell solution each time, and leaving approx. 1mL of the sample at the bottom to avoid bubbles. Repeat 30-40 times.

5. Transfer sample to a round-bottomed 50mL test tube

**Sonication**

6. Sonicate the cell sample. Do so by placing the opened test tube in a beaker with ice and water. Place the sample on the side of the beaker. Raise the beaker on the platform so the sonication needle is one-third of the way from the bottom of the test tube. Make sure the ice in the beaker is propping up the test tube so it does not move around.

7. The sonicator should be set for 2 minutes, have an amplitude of 20%, have a “pulse on” of 0.5, and a “pulse off” of 0.5. Keep an eye on it so that the tube does not move around and touch the tip.

8. When finished, check the viscosity by pipetting about 1 mL of the lysed cell solution. If it has been sonicated correctly, it should be very fluid, not thick.

9. Add another 150uL of PMSF to the round-bottomed tube with the cell sample in it.

10. Wrap the top of the test tube with parafilm because the blue cap will not be able to fit in the centrifuge. Cut off excess parafilm from the sides.

11. Have the centrifuge set with these parameters: Rotor, JA-20; Rpm, 16,000; time, 2:00 (hours); temperature, 4°C.

*(Read the protocol ahead and prepare things needed upto step 8 in following section.)*

12. do Ni-column pre-equilibration(next step) before centrifugation is over

13. Check if there’s enough buffer A and make more if it is not enough

   200ml of 250mM Tris pH7.5

   30ml of 5M NaCl

   770ml of MilliQ water

   1000ml buffer A

**Procedure for Column Chromatography**

**Ni-NTA column pre-equilibration**

1. Fill a 15ml round-bottom Corning tube with 2ml of Ni-NTA agarose solution.

2. Fill the tube with approximately 10ml (or enough to increase the volume to 12ml total) of Buffer A.

3. Place the tube from the previous step into the **balanced** centrifuge in the cold room and allow the centrifuge to spin for approximately 10 seconds.
4. Remove the tube and carefully dispose of the supernatant in a waste container (non-biohazard, use beaker). Fill the tube with buffer A until the volume reaches 12ml and centrifuge again for approximately 10 seconds.

5. Repeat step 4 (pour off supernatant, refill, centrifuge, pour off supernatant). Repeat 3 times in total.

Batch Mixing

6. Prepare clean 50ml round bottom tube and transfer the supernatant fraction of the cell lysate.

7. Pipette out as much column solution as possible and transfer into the soluble fraction of cell lysate. Add 0.5-1.0ml of buffer A to the Corning tube. Pipette 2ml of this solution into the soluble fraction of the cell lysate using a P1000 pipette. Be sure that the precipitate has not settled before pipetting.

8. Secure this mixture onto a tube rotator using tape and/or the clamps on the machine (if they fit correctly). The machine should turn the tube over and back upright in a circular motion. Allow the machine to turn the tube for 2 hours in the cold room to incubate. Check to see it is secure every 20 minutes.

9. Meanwhile, prepare 50ml of elution buffer to use in the elution stage. (Read the protocol ahead and prepare things needed up to step 16.)

Column Pre-wash

10. Once the incubation is complete, remove the tube and place it on ice. Transport the tube to the centrifuge with the correct size rotor for that specific Corning tube. Cut a piece of Parafilm, remove the lid from the tube, and cover the top with the Parafilm. Use a razor to cut the film around the threads on the tube so that it fits properly in the centrifuge.

11. Centrifuge mixture at 2000rpm, JA-20, 4°C for 15 minutes and remove supernatant. (balance the tubes before start centrifuge)

12. When centrifuge is complete, immediately remove and place on ice. Transport the tube to the cold room.

13. Very carefully pour out the supernatant. Stop pouring when a white stream of fluid is visualized down the length of the tube.

14. Fill the tube with buffer A until the volume reaches 40ml. Cover the tube with another piece of Parafilm and place on ice. Transport the tube back to the centrifuge.

15. Centrifuge the tube using the same parameters as in step 11 (if the centrifuge has not been used since it was used in step 10 then just press start).

16. Repeat steps 13 through 15 two additional times. When this is completed, keep the tubes on ice in the cold room for the next stage of the procedure. [You should have completed a total of 3 cycles of centrifugation and removal of the supernatant]

(During the 15min wait period, read the protocol ahead and prepare things needed up to step 20.)

Check if there’s enough buffer A and make more if it is not enough

200ml of 250mM Tris pH7.5
30 ml of 5M NaCl

770 ml of MilliQ water

680.8 mg of Imidazol (FW 68.08) (10mM final)

1000 ml buffer A (10mM imidazol)

* imidazol may increase pH.

Column Wash (+ TCEP, labeling)

17. Get a column from the lower center-lower cabinet at the lab table and take it to the cold room. Get a column rack that contains a collection box from the right hand table in the cold room. Place the column in the top of the rack.

18. Fill the column with the entire solution from the Corning tube using a P1000 pipette. Allow any liquid to drip out the bottom of the column and into the container below it. This is waste and will eventually be discarded.

19. Measure the volume of the column in column units using the scale on the side of the column tube. Take that number and multiply by 15. This is the volume of buffer A you will add. Add the buffer A and allow it to flow through the column completely.

20. Prepare 2 ml of TCEP (0.1 mM) for each sample. Dilute 10 mM TCEP stock (-20°C) to buffer A (100 fold dilution)

21. Add 2 ml of 0.1 mM TCEP solution to each column, wait until ~1 ml goes through. Cap the bottom and incubate for 10 min.

22. Open the cap and let the TCEP solution goes through completely. Add buffer A for wash. Use 20x cv of Buffer A

(Read the protocol ahead and prepare things needed upto step 23.)

23. Divide the columns into 3 by the following protocol: Prepare 2 microfuge tubes for each sample, label them ahead of time. Cap the bottom of the column. Add 1/2 cv of buffer A and mix the column beads well by pipetting up and down (now volume increased to 3/2 cv). Take 1/2 cv of mixed column beads and transfer to a microfuge tube. You'll have two tubes for labeling and the rest of beads will stay in the column. Add enough buffer A to prevent the remaining samples from drying. Make sure that the cap at the bottom is closed tightly.

24. Bring the samples in microfuge tubes out from the cold room. (in ice bucket). Open the microfuge tube cap and blow the nitrogen gently over the surface for de-gasing. (1 min) Close the cap and leave it for 1 min. Add the 6 ul cy3-maleimide dye (or cy5-maleimide dye) to the microfuge tube. Wrap it with foil to avoid light exposure. Bring the samples to the cold room. Place it in the rotor and let it incubate overnight (12 hrs)
(next day)
Elution

25. Prepare the 2 columns (for cy3 and cy5) for each sample on the rack and label them. Load the samples to the column (the overnight labeled beads).
26. Add Excess amount of buffer A until no dyes come out from it.
27. Elute out proteins using elution buffer (as below)

28. Get another column rack (this time without a collection box) and place it over two stable objects of the same size so that it bridges a gap between them. Place the column in the upper part of the rack somewhere in the middle, and place a clean microfuge tube directly below the column. The microfuge tube must be able to collect any liquid that passes through the column.
29. Prepare to use the elution buffer by opening the container and bringing it close to the column. Also prepare a P1000 pipette and set it to 370 µl. Also note the amount of precipitate in the column.
30. Pipette the elution buffer into the column and allow the liquid to pass through. When the column is no longer dripping carefully remove the microfuge tube, close the lid, and label with name and the number of the tube in order.
31. Repeat step 21 until the column becomes colorless (expect 5-10 tubes).
32. When this process is complete, pipette 300 µl of glycerol into each tube (do not mix yet). Be sure to cut the end off of the pipette tip.
33. Use a P200 pipette and set it to 200 µl. Pipette up and down in each microfuge tube to mix thoroughly.
34. When complete place the microfuge tubes in a rack and store at -20 C in the proper freezer.
References


Heller, R.C., and Marians, K.J. (2007). Non-replicative helicases at the replication fork. DNA Repair (Amst) 6, 945-952.


