SINGLE-MOLECULE FRET STUDY
ON THE RECA-MEDIATED DNA REPAIR

BY

CHIRLMIN JOO

B.S., Seoul National University, 2002

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physics in the Graduate College of the University of Illinois at Urbana-Champaign, 2007

Urbana, Illinois
Abstract

Single-Molecule FRET Study
On the RecA-Mediated DNA Repair

Chirlmin Joo, Ph. D.
Department of Physics
University of Illinois at Urbana-Champaign, 2007
Taekjip Ha, Advisor

The RecA protein helps maintain genomic integrity through recombination. Using single-molecule assays and hidden Markov modeling, we show the most direct evidence that a RecA filament grows and shrinks primarily, one monomer at a time only from the extremities. Both ends grow and shrink, contrary to earlier predictions, and a higher binding rate at one end is responsible for directional filament growth. Quantitative rate determination also provides insights into how RecA might control DNA accessibility in vivo.

Next, we estimate that about five monomers are sufficient for filament nucleation. Using a vesicle encapsulation assay, we find that the nucleation is likely to occur through the binding of a pre-assembled oligomer rather than the simultaneous binding of several monomers. Although normally SSB prevents filament nucleation, single RecA monomers can easily be added to a pre-existing filament and displace SSB from DNA at the rate of filament extension. This supports the proposal for a passive role of RecA-loading machineries in SSB removal.

The RecA filament mediates homologous strand exchange resulting in the four-way DNA junction in vivo. We develop single-molecule assays to observe the strand exchange in real time to understand its molecular mechanism.

Finally, we explore the conformational repertoire of the four-way junction by characterizing the effects of metal ions. And we find that the four arms’ movements are synchronized to each other during conformational dynamics. We also seek to detect possible rare species such as intermediate and parallel conformations. While our study suggests that the intermediate form is achieved very frequently, we cannot detect even a transient existence of a parallel form.
To my parents
Acknowledgements

TJ (Taekjip) Ha has been a great advisor for his profound insights and continuous encouragement during my graduate study. It is needless to say that this work would not be possible without his outstanding guidance. Furthermore, he is an excellent example of how a new faculty can become a widely recognized big-shot scientist, through his sincere and never-ending curiosity in nature and science.

Sua Myong has taught me molecular biology techniques. She also enlightened me that a lab is not only where we learn how to carry out experiments but also how to communicate with people by getting to know ourselves.

Sean McKinney and Ivan Rasnik trained me to independently carry out single-molecule experiments. Sungchul Hohng taught me all the optical methods needed for single-molecule studies. Rahul Roy, Michelle Nahas and Keng (Chittanon) Buranachai have always been friendly coworkers. I would like to thank Ben Stevens and Cathy McKinney for their help with experiments.

Sean’s invaluable contribution made it possible for us to publish a RecA paper in Cell. Muneaki Nakamura joined the effort as an undergraduate. It was an exceptional experience to work with Burak Okumus and Ibrahim Cisse for the vesicle encapsulation of RecA. It was pleasing to work with Jeehae Park, Sunghyun Kim and Yongsun Kim for follow-up RecA studies. It has been the most memorable and gratifying experience to work with Kaushik Ragnunathan whose career is about to flourish.

I am also grateful to the new lab members of Peter Cornish, Masha (Maria) Sorokina, JJ (Jiajie) Diao, Bobo (Ruobo) Zhou, Reza Vafabakhsh and Yuji Ishitsuka. I have enjoyed being around Tae-Young Yoon, Cheng Liu, Gwangrog Lee, Mike Brenner, Kyung Suk
Lee, Prakrit Jena, Jaya Yodh, Sinan Arslan, and Hamza Balci. I must also mention Ziggy (Zigurt) Majundar, Jochen Fuchs, Stephanus Fengler and Nathan VanHoudnos who made their presence felt during their short stay in the lab.

It has been a great pleasure to collaborate with Peter (Yingxiao) Wang and Jihye Seong; Isaac Cann and Kent (Yuyen) Lin; Anita Niedziela-Majka and Tim Lohman; and David Lilley. I have also had great fun discussing biophysics with Jin Yu, Minsu Kim, Hyeongjun Kim, Hyokeun Park and Seung Joong Kim. It was also exciting to work with Heekyung Kim, Esther Jeng, and Paul Barone.

I appreciate the technical and the secretarial help of Salman Syed, Rick Renfrew, Julie Wright, Wendy Wimmer and Pat Collins. Salman’s generous help gave me the opportunity to write a RecA paper that was published in *Cell*.

I would like to thank the committee of Professors Bob Clegg, Yoshi Oono and Steve Granick for overseeing my graduate study.

I would like to thank Kaushik Ragunathan for carefully reading this dissertation.

Finally, Johnathan Milton has been my mentor in tutoring how to communicate not only in better English but also through the heart.

This work was supported by NIH (GM065367) and NSF (PHY-0134916 and DBI-0215869) grants.
# Table of Contents

List of Abbreviations .............................................................................................................. xi

## Chapter 1 Introduction ........................................................................................................ 1
1.1 Single-Molecule FRET Study .......................................................................................... 1
1.1.1 FRET ..................................................................................................................... 1
1.1.2 Single-Molecule Study ........................................................................................... 2
1.2 RecA Protein ................................................................................................................... 3
1.2.1 Recombination-Mediated DNA Repair .................................................................... 3
1.2.2 Structure, Formation and Dynamics of a RecA Filament ........................................... 4
1.3 Holliday Junction .......................................................................................................... 6
1.3.1 Open and Stacked Conformations ......................................................................... 6
1.3.2 Conformational Dynamics ....................................................................................... 7

## Chapter 2 Single-Molecule FRET Study ............................................................................ 9
2.1 Single-Molecule Microscopy .......................................................................................... 9
2.1.1 TIR Microscopy ...................................................................................................... 9
2.1.2 Objective-Type TIR Microscope ........................................................................... 10
2.1.3 Prism-Type TIR Microscope .................................................................................. 11
2.1.4 Confocal Microscopy ......................................................................................... 12
2.2 FRET ........................................................................................................................... 13
2.2.1 Fluorophore Selection ........................................................................................... 13
2.2.2 Construct Design .................................................................................................. 13
2.3 Surface Preparation ...................................................................................................... 15
2.3.1 BSA-coated Surface ............................................................................................. 15
2.3.2 Polymer-coated Surface ....................................................................................... 15
2.3.3 Immobilizing Single Molecules ......................................................................... 16
2.4 Single Molecule Data ................................................................................................... 17
2.4.1 Data Acquisition .................................................................................................... 17
2.4.2 Calculating FRET Efficiency ............................................................................. 18
2.4.3 Transition Trajectory Analysis ........................................................................... 18
2.4.4 FRET Histogram ................................................................................................. 20
2.5 Single-Molecule Three-Color FRET .......................................................................... 21
2.5.1 Need for More Colors ....................................................................................... 21
2.5.2 Fluorophore Selection ......................................................................................... 21
2.5.3 Design and Construction of Setup ...................................................................... 23
## Chapter 3 RecA Filament

3.1 Experimental Procedure

3.1.1 Detecting RecA Filament Formation via FRET
3.1.2 Reaction Condition
3.1.3 DNA Preparation

3.2 Nucleation of a RecA Filament

3.2.1 Observation on Nucleation Cluster Formation
3.2.2 Rapid Nucleation in a Confined Volume
3.2.3 Molecular Mechanism of the Filament Nucleation

3.3 Filament Dynamics at the 5'-Disassembly End

3.3.1 Construct Design
3.3.2 HaMMy and TDP Analysis
3.3.3 Transition Rates

3.4 Filament Formation with ATP\textsubscript{γS}

3.4.1 Property of <ssDNA|ATP\textsubscript{γS}>
3.4.2 Stability of <dsDNA|ATP\textsubscript{γS}>

3.5 Filament Dynamics at the 3’-Extending End

3.6 Molecular Mechanism of the Filament Dynamics

3.7 Future Study

## Chapter 4 Interaction of the RecA Filament with Other Proteins

4.1 SSB Displacement by RecA

4.1.1 Inhibitory Role of SSB
4.1.2 SSB Displacement During Filament Extension
4.1.3 Molecular Mechanism of Two Contrasting Roles of SSB

4.2 Interference with and by Helicases

4.2.1 RecA Filament Dynamics Around a Junction
4.2.2 Interference with Rep and UvrD Helicases
4.2.3 Interference by Repetitively Translocating Helicases

4.3 RecA Filament Dynamics \textit{in vivo}

## Chapter 5 RecA-Mediated Strand Exchange

5.1 Homologous Strand Exchange
5.2 Observation on Strand Exchange in Real Time

5.2.1 Strand Exchange in Single-Molecule Level
5.2.2 Pairing Assay

5.3 Regulation by Magnesium Ions

5.3.1 Role of Free Magnesium Ions
5.3.2 Separation Assay
5.3.3 Role of SSB

5.4 Observing the Interaction Between Three Strands Simultaneously

5.4.1 Three-Color FRET Assay
5.4.2 Preliminary Data
5.4.3 Observing the Intermediate Structure During Pairing
5.5 Ensemble Measurements ........................................................................................ 53
  5.5.1 RecA-Mediated Strand Exchange .................................................................... 53
  5.5.2 RadA-Mediated Strand Exchange .................................................................... 54

Chapter 6  Energy Landscape of Holliday Junction Dynamics ............................ 55
  6.1 Experimental Procedure .................................................................................... 55
    6.1.1 Sample Construct ....................................................................................... 56
    6.1.2 Reaction Condition ..................................................................................... 56
    6.1.3 DNA Preparation ....................................................................................... 56
    6.1.4 Data Acquisition and Analysis .................................................................. 57
  6.2 Conformational Exchange Between Stacking Conformers ............................ 58
    6.2.1 Dynamics of Junction 1 with Magnesium Ions .......................................... 58
    6.2.2 Dynamics of Junction 7 with Monovalent Ions Alone ................................ 60
    6.2.3 Dynamics of Junction 7 with Hexammine Cobalt (III) Ions ...................... 61
    6.2.4 Competition and Cooperation Between Mono- and Divalent Ions .......... 62
  6.3 Sequence and Ion Dependence ........................................................................ 63

Chapter 7  Exploring Rare Conformational Species in Holliday Junctions .......... 64
  7.1 Short-Lived Open Conformation .................................................................... 64
    7.1.1 Transitions Between Open and Stacked Conformations ............................ 65
    7.1.2 Frequently Visited Open Conformation ..................................................... 66
  7.2 Non-existence of Parallel Conformation .......................................................... 66
    7.2.1 No Observation on Parallel Conformation ................................................ 67
    7.2.2 No Observation of Parallel Conformation by a Three-Color FRET Assay 68
        7.2.3 Biological Irrelevance of Parallel Conformation ...................................... 69
  7.3 Synchronized Conformational Dynamics ....................................................... 69
    7.3.1 Movements of Four Arms ......................................................................... 69
    7.3.2 Correlated Movement Probed by a Special Two-Color FRET Assay ........ 70
    7.3.3 Correlated Movement Probed by a Three-Color FRET Assay ................. 70
    7.3.4 Complete Stacking and No Parallel Conformation ................................... 72
  7.4 Holliday Junction Dynamics and Branch Migration ....................................... 73

Appendix A  Supplements to Chapter 2: Single-Molecule FRET Study .................. 75
  A.1 Calculating $R_0$ ............................................................................................. 75
  A.2 Imaging Buffer ............................................................................................... 76
  A.3 Labeling DNA ................................................................................................. 77
  A.4 Change in Donor Quantum Yield .................................................................. 78
  A.5 Surface Preparation ....................................................................................... 79
    A.5.1 Cleaning .................................................................................................... 79
    A.5.2 Sample Chamber ...................................................................................... 80
    A.5.3 BSA-coated Surface ................................................................................ 80
    A.5.4 Solution Injection ..................................................................................... 81
    A.5.5 PEG-coated Surface ................................................................................ 81
Appendix B  Supplements to Chapter 3: RecA Filament

B.1 Interaction Between a Fluorophore and a RecA Filament ........................................... 83
  B.1.1 Effects of a RecA Filament on Fluorescence .................................................. 83
  B.1.2 Effect of a Dye on a RecA Filament ............................................................... 85
B.2 Filament Formation on a Short ssDNA ..................................................................... 85
  B.2.1 RecA Filament Formation with ATP .............................................................. 85
  B.2.2 RecA Filament Formation with Different NTP Factors .................................. 86
B.3 Filament Formation Around a dsDNA Mediated by ssDNA Tail ............................. 86
  B.3.1 Filament Formation on dsDNA is not Local to the ss-dsDNA Junction .......... 86
  B.3.2 <dsDNA|ATPγS> Protects dsDNA from Digestion by an Endonuclease ....... 87
  B.3.3 <dsDNA|ATPγS> Cannot Form Without ssDNA Tail ................................... 88
  B.3.4 Formation and Disassembly of <dsDNA|ATPγS> at High Temperature ....... 88
  B.3.5 Formation of <dsDNA|ATPγS> Mediated by 5’ ssDNA Tail ........................ 89

Appendix C  Optical Tweezers .......................................................................................... 90

C.1 Introduction ............................................................................................................ 90
  C.1.1 Principle .......................................................................................................... 90
  C.1.2 Force Calibration ............................................................................................. 90
  C.1.3 Experimental Scheme ...................................................................................... 92
C.2 Setup ....................................................................................................................... 92
  C.2.1 Components ..................................................................................................... 92
  C.2.2 Optically Conjugated System for Steering a Mirror ....................................... 93
  C.2.3 Fluorescence Microscope with Optical Trapping Capability ......................... 93
  C.2.3 Fine Adjustment .............................................................................................. 94
C.3 Program .................................................................................................................. 95
  C.3.1 How to Use ...................................................................................................... 95
  C.3.2 Computer Interface: Initialization ................................................................... 96
  C.3.3 Computer Interface: Mirror Control ............................................................. 97

Appendix D  Polymer-Mediated Protein Crosslinking ..................................................... 102

D.1 Protein Purification .............................................................................................. 102
  D.1.1 Over-Expression ............................................................................................ 102
  D.1.2 Purification .................................................................................................... 103
D.2 PEG-Mediated Dimer .......................................................................................... 106
  D.2.1 Dimerization .................................................................................................. 106
  D.2.2 Size Exclusion Purification ........................................................................... 107
D.3 Unwinding with PEG-Mediated Dimer ................................................................ 108

References ..................................................................................................................... 109

Figures ........................................................................................................................... 121

Author’s Biography ..................................................................................................... 181
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac</td>
<td>Acetate (CH$_3$COO$^-$)</td>
</tr>
<tr>
<td>APD</td>
<td>Avalanche photodiode</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATP$\gamma$S</td>
<td>Adenosine 5’-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>$\beta$ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>$C_c$</td>
<td>Critical concentration</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>cv</td>
<td>Column volume</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanine dye</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>$E$</td>
<td>FRET efficiency</td>
</tr>
<tr>
<td>$E_{app}$</td>
<td>Apparent FRET efficiency</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FIONA</td>
<td>Fluorescence imaging with one-nanometer accuracy</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>FRET</td>
<td>Föster (Fluorescence) resonance energy transfer</td>
</tr>
<tr>
<td>HaMMy</td>
<td>Hidden Markov modeling (HMM)</td>
</tr>
<tr>
<td>$I_A$</td>
<td>Emission intensity of an acceptor molecule</td>
</tr>
<tr>
<td>$I_D$</td>
<td>Emission intensity of a donor molecule</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide(s)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sm</td>
<td>Single-molecule</td>
</tr>
<tr>
<td>smFRET</td>
<td>Single-molecule FRET</td>
</tr>
<tr>
<td>SOS response</td>
<td>A DNA repairing mechanism initiated by RecA</td>
</tr>
<tr>
<td>SSB</td>
<td>ssDNA binding protein in <em>E. coli</em></td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TIR</td>
<td>Total internal reflection</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Tris</td>
<td>trishydroxymethylaminomethane</td>
</tr>
<tr>
<td>Trolox</td>
<td>6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid</td>
</tr>
<tr>
<td>T50</td>
<td>Solution containing 10mM Tris-HCl, pH 8.0 and 50mM NaCl</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

You can observe a lot by watching.

-Yogi Berra

1.1 Single-Molecule FRET Study

Single-molecule (sm) fluorescence detection is a powerful tool in probing biological events without time and population averaging (Cornish and Ha, 2007; Weiss, 1999). Single-molecule FRET (Föster/Fluorescence Resonance Energy Transfer), first introduced in 1996 (Ha et al., 1996), has been now widely adopted by many laboratories around the world to study a variety of biological systems including DNA, RNA (Zhuang, 2005), proteins and large macromolecular complexes (Ha, 2001a; Ha, 2004; Myong et al., 2006; Rasnik et al., 2006b). ¹

1.1.1 FRET

FRET is a spectroscopic technique for measuring distances in the 30-80 Å range. Excitation energy of a donor molecule is transferred to acceptor via interaction between two induced dipoles (Forster, 1948). As shown in Figure 1.1A, the efficiency of energy transfer, $E$, is given by

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

where $R$ is the distance between donor and acceptor and $R_0$ is the characteristic distance at which 50% of the energy is transferred (Clegg, 1992) (see Appendix A.1, Calculating

¹ Chapter 1.1 was excerpted from a published work:
C. Joo and T. Ha, ‘Single molecule FRET with total internal reflection microscopy’
$R_0$. $R_0$ is $\approx 60$ Å for a classical smFRET pair (Cy3 and Cy5) (Murphy et al., 2004). A sizeable change in $E$ is observed when the distance between donor and acceptor molecules changes over several angstroms and nanometers. Therefore, a structural change of a biological molecule or a relative motion between two interacting molecules can be detected via the change in FRET (Selvin, 2000).

1.1.2 Single-Molecule Study

In ensemble FRET measurements, it is often very difficult to synchronize the conformational changes of biological molecules and not feasible to detect the short-lived conformers (Figure 1.2A). smFRET opens up new opportunities by probing the structural changes of individual biological molecules in real time (Figure 1.2B) as follows.

1) smFRET readily determines the distribution of several conformations, not just the average of them (Lee et al., 2005; McKinney et al., 2003). This makes it possible to directly identify rarely-visited states and short-lived states (McKinney et al., 2005; Nahas et al., 2004).

2) Post-synchronization during data analysis eliminates the need of synchronization during a measurement (Blanchard et al., 2004) (Figure 1.2C).

3) It also opens a possibility of studying heterogeneity between molecules (Okumus et al., 2004; Zhuang et al., 2002).

4) Last, but not least, direct observation often reveals what people could not imagine or deduce based on ensemble measurements alone (Joo et al., 2006; Myong et al., 2005).

In comparison to other sm-techniques such as sm-tracking (FIONA), optical- and magnetic-tweezers, smFRET is less prone to environmental noise 1) because it is inherently a ratiometric technique where we measure the ratio between the two different
colors, and 2) because it reports on the internal movements of molecules in their center-of-mass frame. So, variations in the excitation and detection efficiencies between molecules are mostly tolerable. And relative drift of the molecule in the lab frame is much less of an issue. In addition, since it is relatively easy to acquire data from several hundred molecules compared to optical- and magnetic-tweezers, kinetic rates of biological events are determined with the highest accuracy screening out the intrinsic heterogeneity between single molecules. However, the photophysical properties of an organic dye limits its typical temporal resolution to several milliseconds (Ha, 2001b).

1.2 RecA Protein

1.2.1 Recombination-Mediated DNA Repair

DNA in a cell is often damaged by external factors such as UV light and toxic chemicals, resulting in the loss of the genomic integrity and ultimately leading to serious diseases such as cancer. RecA is essential in restoring the integrity of genetic information of the gut bacterium, *E. coli*, via homologous recombination, SOS response and mutagenic DNA repair (Cox, 1999; Kowalczykowski, 2000). A human homolog, Rad51, interacts with BRCA2 whose mutation increases susceptibility to breast and ovarian cancers (Venkitaraman, 2004). 

Several RecA- and RecA homolog-mediated recombination pathways have been found (Cox, 1999). In case a DNA polymerase complex encounters a damaged region, the replication fork collapses and fails to continue its function. The collapsed fork is restored through two RecA-mediated repair pathways in *E. coli* (Cox et al., 2000).

*RecBCD pathway:*

The replication fork collapses when it meets a ssDNA nick, resulting in a dsDNA break (Figure 1.3A). RecBCD processes the dsDNA into a partial dsDNA. (Spies

---

2 Chapter 1.2 was partially excerpted from a published work: C. Joo et al. ‘Real-time observation of RecA filament dynamics with single monomer resolution’ *Cell* 126: 515-27 (2006).
et al., 2003) At the same time, RecBCD loads RecA on the nascent ssDNA to form a RecA filament (Anderson and Kowalczykowski, 1997; Kowalczykowski, 2000).

**RecFOR pathway:**
When the fork is collapsed by a lesion, ssDNA gap appears (Figure 1.3B). RecFOR complex binds to the junction and loads RecA (Bork et al., 2001; Morimatsu and Kowalczykowski, 2003; Umezu et al., 1993).

The ssDNA within the filament is stretched along the longitudinal axis of the filament and has the capability of seeking out and recognizing the dsDNA of a homologous sequence (Figures 1.3Ad, Bd). Despite more than 20 years of effort on investigating the homology recognition, its molecular mechanism is still not understood.

After the homology recognition has occurred, other recombination proteins such as RuvAB facilitate branch migration, which results in the structure of a 4-way junction (Holliday junction) (Figures 1.3Ae, Be).

### 1.2.2 Structure, Formation and Dynamics of a RecA Filament

The copy number of RecA in *E. coli* is less than 10,000 in the basal level but increases by more than an order of magnitude during SOS response (Cox, 2003; Sommer et al., 1998), thus the physiologically relevant concentration of RecA is in the micromolar range. The protein binds to ssDNA to form a nucleoprotein filament that exhibits a helical structure (Story et al., 1992) with each pitch consisting of six RecA monomers and each monomer occupying approximately three nucleotides (nt) of the ssDNA (Figure 1.4A) (Egelman and Yu, 1989; Rajan and Bell, 2004; Zlotnick et al., 1993). The filament structure had been relatively well-known as above via X-ray crystallography (Figure 1.4Aa) and electron microscopy (Figure 1.4Ac); however, its dynamics were poorly understood until our sm-study has revealed the detailed molecular mechanism of the filament dynamics (Joo et al., 2006). The followings are the “old” pictures on the filament dynamics based on previous ensemble studies.
The formation of a RecA filament is highly cooperative and utilizes ATP as a cofactor (Menetski and Kowalczykowski, 1985). A number of monomers bind to ssDNA simultaneously during nucleation (Figure 1.4Ba), but this crucial event is poorly understood because it has been difficult to separate nucleation from the subsequent filament extension that immediately follows. After nucleation, additional RecA units bind and extend the filament in the 3’ direction of the ssDNA (Figure 1.4Bb). For this reason, we will refer to the 3’ end of ssDNA-RecA filament as the “3’-extending end”. The unit of filament extension, whether it is the RecA monomer or higher order complexes, is currently unknown for E. coli RecA. If the filament encounters a ss-dsDNA junction during extension, it continues to extend into the dsDNA (Register and Griffith, 1985). However, filament formation on dsDNA alone is extremely slow (Pugh and Cox, 1988) unless a 5’ ssDNA tail is present or the dsDNA is melted by applying strong force (Hegner et al., 1999) or torque (Fulconis et al., 2004). The rate of the filament extension on ‘dsDNA’ was determined by a sm-measurement (Shivashankar et al., 1999; van der Heijden et al., 2005), but the extension rate on ‘ssDNA’ has been difficult to measure because of complications arising from multiple nucleation events.

RecA is a DNA-dependent ATPase and hydrolyzes ATP at the rate of 0.5 sec\(^{-1}\) at 36 °C (Bedale and Cox, 1996), regardless of where it is located on the filament (Brenner et al., 1987). When a monomer at the 5’ end of the filament, termed here “5’-disassembly end”, hydrolyzes ATP, it dissociates from the filament (Figure 1.4Bc) (Lindsley and Cox, 1990; Register and Griffith, 1985) in a pH dependent manner (Arenson et al., 1999). If RecOR interacts with the 5’-disassembly end (Shan et al., 1997) or a slowly hydrolyzing ATP analogue, ATP\(_{\gamma}S\), is used, RecA dissociation becomes insignificant.

The difference between the two ends in terms of apparent stability may play a role during strand exchange via a treadmilling process in which monomers dissociating from the 5’-disassembly end can re-bind to the 3’-extending end so that a finite number of RecA monomers can be recycled efficiently and any discontinuity in the filament can be fixed (Menetski et al., 1990). A similar mechanism has been proposed for actin filaments, which show a large difference in critical concentrations between the two ends (Wegner, 1982). However, the rates with which RecA binds to the two ends and dissociates from
the 3’-extending end are not known yet. Thus, the molecular mechanism for the
directional growth of a RecA filament has not been clear.

1.3 Holliday Junction

Once homologous strand exchange has occurred mediated by RecA (Chapter 1.2), other
recombination proteins such as RuvAB join the effort of restoring the damaged DNA
(Figures 1.3Ae, Be). The central intermediate in this process is the four-way (Holliday)
junction (Holliday, 1964), which is described as a four-stranded DNA structure forming
four helices joined in the middle (Figure 1.5A) (Ho and Eichman, 2001; Lilley, 2000).
Although much is known about its static structure, there is only limited information on
the dynamic properties and their functional consequences. The dynamic characteristics of
the junction are likely to be important in understanding how different enzymes recognize
and process Holliday junction. sm-techniques offer a promising avenue into these aspects,
since they can reveal the structural changes of individual molecules in real time while
they are functioning, and do not require synchronization.³

1.3.1 Open and Stacked Conformations

In the absence of added metal ions, Holliday junction adopts an open conformation where
the four helices are directed to the corners of a square (Figure 1.5B) (Clegg et al., 1994;
Duckett et al., 1988). On addition of divalent metal ions such as magnesium, the structure
folds by the pairwise, coaxial stacking of helical arms to form the anti-parallel stacked X-
structure in which the continuous strands of the junction are oriented in opposite
directions (Duckett et al., 1988; Murchie et al., 1989). This structure was originally
proposed on the basis of electrophoretic (Cooper and Hagerman, 1987; Duckett et al.,
1988; Gough and Lilley, 1985), FRET (Clegg et al., 1992; Murchie et al., 1989) and
chemical probing experiments (Churchill et al., 1988), and has recently been confirmed

³ Chapter 1.3 was excerpted from a published work :
C. Joo et al. ‘Exploring rare conformational species and ionic effects in DNA Holliday junctions
by X-ray crystallography (Eichman et al., 2000; Nowakowski et al., 1999; Ortiz-Lombardia et al., 1999).

Formation of the stacked X-structure in the presence of Mg\(^{2+}\) ions lowers the symmetry of the junction, and there are two possible conformers of this structure that differ in the choice of stacking partners (Figures 1.5B). Thus a junction with arms named B, H, R and X (sequentially around the junction as depicted) may stack into alternative conformers based on the stacking of arms X on R (and consequently B on H), or X on B. These are termed the \textit{isoI} and \textit{isoII} conformers in this work (Grainger et al., 1998). In principle each of the stacking conformers could exist in predominantly parallel or antiparallel forms, although earlier studies have indicated that the antiparallel structure is the major form present in solution (Murchie et al., 1989) and in the crystal (Eichman et al., 2000; Ortiz-Lombardia et al., 1999). However, in the original proposals for the mechanism of recombination the structures were expected to be parallel (Holliday, 1964; Meselson and Radding, 1975), and have most frequently been drawn this way in textbooks (Figures 1.5C). Although not observed in bulk solution measurements (Duckett et al., 1988; Murchie et al., 1989), it is possible the parallel structure exists in a minor population rare enough to evade detection in ensemble experiments. sm-measurements should be able to detect even very rare conformations as long as they have distinct fluorescence signals and they are longer-lived than the time resolution of the experiment.

### 1.3.2 Conformational Dynamics

The four-way junction can undergo a number of important dynamic processes. First, a junction can exchange between the two alternative stacking conformers (Figures 1.5B). It is not possible to synchronize this process, and it was difficult to demonstrate in solution except by rather indirect experiments (Grainger et al., 1998; Miick et al., 1997; Murchie et al., 1991; Overmars and Altona). However, our lab has recently used smFRET analysis to demonstrate conformer exchange in a direct manner, and measured the rates of interconversion (McKinney et al., 2003). Four-way junctions can also undergo branch migration by virtue of the sequence homology, in which there is a step-wise exchange of basepairing partners that moves the point of strand exchange (Panyutin and Hsieh, 1994;
Thompson et al., 1976). Branch migration is an important element in the recombination mechanism (Holliday, 1964; Meselson and Radding, 1975; Orr-Weaver et al., 1981), and is accelerated in the cell by the action of specific proteins (Chen et al., 2002; Constantinou et al., 2001; Iwasaki et al., 1992; Muller et al., 1993; van Gool et al., 1998). Both conformer exchange and branch migration require the opening of the four-way junction to permit the required exchange of stacked helices or basepairing. In the case of branch migration this is consistent with the observed inhibition of the process by concentrations of metal ions known to stabilize the stacked X-structure (Panyutin et al., 1995).
Chapter 2

Single-Molecule FRET Study

In this chapter, we describe how we carry out sm-experiments. We show how to build sm-setups, how to design sm-assays, how to take sm-data and analyze them. We will also describe a three-color setup that we have recently developed. 4

2.1. Single-Molecule Microscopy

2.1.1 TIR Microscopy

Our setup is built around a commercial inverted microscope (Olympus IX71). Commercial solutions are available for TIR microscopy, but we prefer to build our own because of the lower cost and maximum flexibility. In brief, Cy3 molecules are excited by an Nd:YAG laser (532nm) via total internal reflection (TIR). The fluorescence signal from Cy3 and Cy5 that is collected by an oil-immersion (objective-type; Figure 2.1A) or by a water-immersion objective lens (prism-type; Figure 2.2A) goes through a long-pass filter that blocks out laser scattering. The donor and acceptor signals are separated by a dichroic mirror and are detected by a CCD camera with up to 16 ms time resolution (Figure 2.1B). The observation area is about 25 µm x 50 µm.

4 The work in Chapter 2 has been published as a book chapter and a paper:
C. Joo and T. Ha, ‘Single molecule FRET with total internal reflection microscopy’
In Single Molecule Techniques: A Laboratory Manual.(ed P. Selvin and T. Ha) (Cold Spring Harbor Laboratory Press, 2008) (in press) and
Signal amplification is necessary to suppress the readout noise in high-speed CCD imaging. The new generation of electron multiplying CCD cameras (iXon, DV887, Andor Technology) use on-chip multiplication that adds minimal noise (at worst, noise increases by about 50% compared to the shot noise limit) while eliminating readout noise through amplification. The back-thinned version achieves over 80-90% quantum yield over the entire visible light range, comparable to the best performing silicon avalanche photodiodes (APD). The time resolution is currently 30 ms for 512 x 512 pixels and 16 ms for 512 x 256. We set the temperature of the CCD chip at -75 °C and obtain sm-traces with a gain of 230.

2.1.2 Objective-Type TIR Microscope

The objective-type used to provide high background from the acceptor channel and was therefore deemed inadequate for FRET. However, the new generation of 1.4 NA oil immersion objectives (UPLSAPO100XO, 100x, Olympus) has largely solved this problem. There is still slightly higher background than in the prism-type as can be judged by a visual scan but the actual signal-to-noise ratio of sm-data is indistinguishable.

The schematic of the excitation optics is shown in Figure 2.1A. The expanded laser beam of 20-25mm width is focused by a 300 mm-focal length plano-convex lens (XY). If the focused beam (that is reflected by a dichroic mirror with 550nm cutoff wavelength) goes through the center of the objective lens, epi-fluorescence microscopy is achieved. By slightly shifting the beam pathway away from the center, it is converted into TIR microscopy. It is useful to have a red laser excitation optics installed to directly visualize acceptor molecules. For further details, see the following reference (Joo and Ha, 2008).

The schematic of the emission optics is shown in Figure 2.1B. The emission signal is filtered by a 550nm-longpass filter. A 2.5 mm-wide slit is placed in the imaging plane a few centimeters outside a side port. Then the beam collimated by an achromat (f, 100mm, L1) is split by a dichroic mirror (cutoff wavelength, 630nm, DM), focused by the other achromat (f, 150mm, L2), and finally imaged on the CCD.
Optimum FRET resolution requires minimal cross talk between the two detection channels, i.e., donor emission leakage to the acceptor detector and acceptor image leakage to the donor detector. The former is usually more significant because fluorescence emission spectrum is asymmetric with a long emission tail. In a typical experiment using Cy3 as the donor and Cy5 as the acceptor, approximately 10-15% of the donor signal is detected at the acceptor channel when a dichroic mirror with a cutoff at 645-630 nm is used to split the emission based on the wavelength.

2.1.3 Prism-Type TIR Microscope

Prism-type TIR has been a major imaging tool for smFRET since it was first introduced (Funatsu et al., 1995). The limitations of the setup are: 1) it is often difficult to find the excitation area on a sample surface, 2) the top side of the chamber is covered by a prism which is inconvenient, 3) relatively expensive and brittle quartz slides are needed. All these limitations are overcome by the objective-type. However, it is only the prism-type where a flow technique (Appendix A.5.4, Solution Injection) can be practiced reliably since the introduction of flow results in the bowing of the imaging surface, which is a coverslip in the objective-type, leading to defocusing during data acquisition.

The excitation beam is focused into a small pellin-broca prism which is placed on the top of a quartz slide with a thin layer of immersion oil in between to match the index of refraction (Figure 2.2). With a shallow incident angle of the excitation beam (<23°), the TIR at the interface between the quartz slide and aqueous imaging buffer is achieved. The location and the size of the excitation area are controlled by the position of the lens (f, 50mm). It is important that the prism holder be mounted and fixed relative to the body of the microscope so that the prism and the excitation beam do not move when the sample is laterally translated to image different sample areas. A water-immersion objective lens (UPLAPO60XW, 60x, 1.2NA, Olympus) is used.

The emission optics is virtually identical to that of the objective-type (Figure 2.1B). But to match the size of molecules on the CCD screen with that of the objective-type, 2.5-time magnification is required instead of 1.5 (since the water-immersion
objective lens is 60x, not 100x). Therefore, an achromat with $f = 250$ mm is used instead of that with $f = 150$ mm (L2), and 1.5 mm wide slit is used instead of 2.5 mm.

The temperature of the sample is regulated with a water-circulating bath flowing to copper tubing in contact with (i) a home-built brass collar on the objective, (ii) a plate that holds the sample cell, and (iii) metal pieces holding the prism (Figure 2.2B). The temperature is measured with a thermocouple sandwiched between a quartz slide and coverslip and placed on the microscope in place of the sample. The error in temperature measurement is estimated to be less than 1°C.

### 2.1.4 Confocal Microscopy

The recent improvements in the quantum yield and the time resolution of CCD cameras have outdated the confocal microscopy from carrying out sm-studies since the latter required immense amount of time in taking enough number of data. Therefore, we will discuss mainly one incarnation, TIR microscopy in Chapter 2. The remaining advantages of confocal detection using APD are (1) better instrumental time resolution (although ultimately the time resolution is limited by photophysics) and (2) possibility of doing lifetime measurements (Laurence et al., 2005).

In brief, donor fluorophores are excited using a solid-state 532 nm laser, with a power of 1-16 µW in a home-built confocal scanning microscope based on an inverted microscope with a 100x oil-immersion objective (NA 1.4) (Figure 2.3). A piezoelectric nano-positioner is used to locate a single molecule under the laser focal spot over 10 x 10 µm² area, and fluorescence signals are detected by a pair of detectors (Photon counting module, SPCM-AQR-14, Perkin Elmer). Data are collected at 20 (±2) °C and the temperature is monitored using a thermocouple.
2.2 FRET

2.2.1 Fluorophore Selection

Ideal dyes for sm-fluorescence studies have to possess as many as possible of the following characteristics. They have to be 1) photostable so that they emit millions of photons before photobleaching; 2) bright (high extinction coefficients and quantum yields); 3) showing little intensity fluctuation, at least, in the time scale of interesting biological events under study; 4) excitable and emitting in the visible wavelengths; 5) commercially available in a form that can be conjugated to biomolecules; and 6) relatively small so that they introduce minimum perturbation to the host molecules. Cy3 and Cy5 (Figure 2.4A) remain to be the dominant choices for smFRET measurements because of their superior photophysical properties. Cy3 is the most stable dye we have tested and Cy5’s stability is greatly enhanced if the oxygen scavenger system is used (Appendix A.2, Imaging Buffer).

An ideal pair of dyes for smFRET study would have 1) appreciable overlap between donor emission and acceptor absorption (Figure 2.4Ba), 2) large spectral separation in donor and acceptor emission to minimize the donor emission leakage into the spectral range of acceptor emission (Figure 2.4Bb) and to reduce the amount of direct excitation of the acceptor by the laser (Figure 2.4Ba) and 3) a comparable emission quantum yield for donor and acceptor. The latter is useful because it guarantees clearly anti-correlated intensity changes of donor and acceptor. Cy3 and Cy5 have been the most popular donor and acceptor pair for smFRET because 1) their spectral separation is large (~100 nm); 2) they are both photostable in oxygen-free environment; 3) the quantum yields (~0.25) are comparable; and 4) they are commercially available in amino-, thiol- and other reactive forms.

2.2.2 Construct Design

Attaching a fluorophore in an optimal location is crucial to successful smFRET experiments. FRET is the most sensitive when the donor and acceptor molecules are about $R_0$ apart (Figure 1.1B), therefore, it is common to place them on the DNA molecule
in such a way that the distance between the two dyes alternates around $R_0$ during the conformational change (Figure 2.4C). An internal labeling method (Appendix A.3, Labeling DNA) is used to move the fluorophore around to the optimal location (Figure 2.4Cb). In all cases, the presence of a dye and the physical constraint from immobilization may or may not interface with the activity of a protein; therefore, it needs to be cross-checked with biochemical ensemble data.

Some general rules for selecting the labeling site are: 1) if the protein recognizes a certain chemical feature of DNA such as the chemical group of its end, or if the protein quenches the fluorophore, the fluorophore should be placed elsewhere; 2) for the internal labeling, it is recommended to keep the backbone continuity of DNA (Appendix A.3, DNA Labeling); and 3) in the DNA the part that interacts with a protein may be placed away from the glass surface to avoid potential physical constraints. Finally, proteins may enhance the brightness of Cy3. Such an effect does not present much difficulty in data interpretation because of the cancellation of contribution to the commonly used approximation for FRET efficiency (Appendix A.4, Change in Donor Quantum Yield) (Joo et al., 2006).

In an experiment with an unlabeled protein, DNA with both donor and acceptor molecules is immobilized. Since it is impractical, in terms of yield and cost, to have all three modifications (biotin, donor and acceptor) in one single-stranded DNA, the modifications are often added to separate DNA strands that are annealed later. For example, if a ssDNA is required, it is common to use a partial dsDNA instead (e.g. Figure 3.1A) (Joo et al., 2006; Lee et al., 2005; Myong et al., 2005).

It is also useful to consider that smFRET is relatively insensitive to incomplete labeling of a host molecule. If it is the donor that is missing, the molecule is simply not observed; if the acceptor is missing, this donor-only species shows up as a zero-FRET population.
2.3 Surface Preparation

To study the conformational changes of individual molecules over extended time periods, molecules need to be localized in space. This is often achieved by surface immobilization (Rasnik et al., 2005). An ideal surface would allow specific immobilization of DNA, RNA or proteins while rejecting non-specific adsorption.

2.3.1 BSA-coated Surface

For nucleic acids studies, we prefer using a glass (or quartz) slide coated with biotinylated BSA and neutravidin (or streptavidin) because of this system’s simplicity (Figure 2.5A). We can immobilize DNA with high specificity and were able to reproduce their bulk solution activities faithfully (Hohng et al., 2004b; Lee et al., 2005; McKinney et al., 2003; McKinney et al., 2005; Murphy et al., 2004; Nahas et al., 2004; Tan et al., 2003). This is likely because all three surface constituents (BSA, neutravidin and glass) are negatively charged in neutral pH, repelling nucleic acids. A detailed protocol is in Appendix A.5 (Surface Preparation).

2.3.2 Polymer-coated Surface

For studies involving proteins, BSA-coated surface is too adhesive. Therefore, we use a PEG (polyethylene glycol)-coated surface that reduces the protein adsorption to an undetectable level (Figure 2.5B). Surface passivation using PEG, first introduced for sm-studies in 2002 (Ha et al., 2002), has now been adopted successfully by several other groups. If a dense layer of PEG is formed on a glass surface, it forms a polymer brush that prevents protein adsorption to the underlying surface. We incorporate a small fraction of PEG polymers that are end-modified by biotin to immobilize neutravidin, which further immobilizes biotinylated macromolecules. Proteins interact specifically with DNA immobilized to the PEG-coated surface and their bulk solution activities are well reproduced in all systems we have tested (Ha et al., 2002; Joo et al., 2006; Myong et al., 2005; Rasnik et al., 2004). A vesicle encapsulation technique that can measure
conformational dynamics of biomolecules free of surface tethering has been also developed recently (Figure 2.5C) (Chapter 3.2.2 Rapid Nucleation in a Confined Volume) (Cisse et al., in press; Okumus et al., 2004). We provide a detailed protocol in Appendix A.5 (Surface Preparation).

### 2.3.3 Immobilizing Single Molecules

Once a surface is prepared, we image the surface with buffer T50 (or with the imaging buffer) before immobilizing Cy3-labeled molecules (Figure 2.6A). We immobilize ~50 pM Cy3-labeled molecules via biotin-(neutr)avidin conjugation (see Appendix A.5.3 BSA-coated surface and A.5.5 PEG-coated surface for details) and compare the resulting density with that of the blank sample obtained with the same excitation intensity and same buffer (Figure 2.6B). Usually, the junk molecules are not as bright as Cy dyes. Unless both the density and intensity of the junk molecules are high, the surface is regarded clean enough for the sm-fluorescence experiment. If not, it is possible that 1) the surface was not cleaned properly; 2) the chamber assembled was kept for longer than a couple of weeks; 3) the double-sided tape was dirty; 4) the chemicals used for immobilization and imaging are dirty; or 5) the amino-silane is contaminated (if it was a PEG-coated surface).

sm-experiments with much lower surface density could give misleading results because contributions of the impurity can be significant. One can effectively lower the relative concentration of the impurities by adding a good number of single molecules. If a desired concentration on the surface (~200 molecules on 25 µm x 50 µm imaging area; Figure 2.6B) is not achieved even with >1nM, it is likely that a biotinylated sample or proteins used for immobilization are not properly prepared; for example, vortexing a protein such as neutravidin with a normal lab agitator easily inactivates the protein’s function.

Once a desired surface density is achieved, we wash out the unbound sample with buffer T50 (10mM Tris-HCl, pH 8.0, 50mM NaCl). If the sample is too dense, more than one molecule may be found under the same spot or nearby, making the interpretation
difficult. Even with a relatively low concentration sample, a small fraction of time traces can contain contribution from more than one molecule, thus giving unusual signatures.

2.4 Single Molecule Data

2.4.1 Data Acquisition

When acquiring sm-data with TIR microscopy through the CCD camera, fluorescence signal is recorded in real time using a home-written Visual C++ software (Microsoft) with the highest time resolution of 30 ms or shorter (see also Chapter 2.1.4 Confocal Microscopy). The software obtains each frame of the movie from the camera and writes it to the hard drive as a single large file that contains all the frames where each pixel is encoded as a single byte. Each byte is 8 bits (i.e. 0-255), which represents how bright a signal is. We use false colors to show the intensity in an effective manner (e.g. Figure 2.6). Typically, 1 min movie at 100 ms per frame occupies about 150 MBytes of hard drive space.

sm-traces are extracted from the recorded movie file using scripts written in IDL (Research Systems) (Figure 2.6Bb). Due to aberrations and imperfect alignments of the optical system, the donor and acceptor images can not be simply overlaid via a mere offset. By using IDL scripts and a calibration image obtained using fluorescence bead (see below), we can determine a polynomial map between the two channels, which includes rescaling, rotation and shear distortion.

The bead sample is made by diluting fluorescent beads (FluoSpheres carboxylate-modified microspheres, 0.2 µm, crimson fluorescent (625/645), Invitrogen) by a factor of 500 with 1M Tris-HCl (pH 8.0) solution, and injecting the solution into a sample chamber (Appendix A.5.2, Sample Chamber). The beads adhere to the imaging surface made of untreated glass surface. For long term use, we seal the holes to avoid solution drying.
2.4.2 Calculating FRET Efficiency

Once data are taken, data analyses on the sm-traces are carried out as described below with Origin and software written in Matlab. We calculate the apparent FRET efficiency by

\[ E_{\text{app}} = \frac{I_A}{(I_D + I_A)} \]

where \( I_D \) and \( I_A \) are the sensitized emission intensity of the donor and acceptor, respectively. What we actually measure are the raw intensities of the donor and acceptor channels, \( I_D^0 \) and \( I_A^0 \). We then need to correct for the leakage of donor signal to the acceptor channel, which is typically between 10-15% of the donor signal and can be determined from a donor-only molecule; we subtract a fixed fraction \( \alpha \) of \( I_D^0 \) from \( I_A^0 \) such that the corrected acceptor signal becomes zero (and \( E_{\text{app}} = 0 \)).

\[ E_{\text{app}} = \frac{(I_A^0 - \alpha \times I_D^0)}{(I_D^0 + I_A^0 - \alpha \times I_D^0)} \]

The absolute FRET value is estimated from the above corrected FRET by using a correction factor (Ha et al., 1999a). FRET efficiency is given by

\[ E = \frac{I_A}{(I_D + \gamma \times I_A)} \]

where \( \gamma \) is a parameter representing relative detection efficiencies and quantum yields of the two dyes, and is determined from photobleaching events. Our data using Cy3 and Cy5 show that \( \gamma \approx 1 \). Thus \( E \equiv I_A / (I_A + I_D) \) is an excellent approximation for FRET efficiency.

2.4.3 Transition Trajectory Analysis

If smFRET data display temporal fluctuations (Figure 2.7), the data are further analyzed by dwell-time, cross-correlation, or hidden Markov modeling, depending on the type of
fluctuation as described below. Note that a unique signature of a sm-trace is the single-step photobleaching (Figures 2.7Ca and D).

**Dwell-Time Analysis:**
If there are two FRET states, A and B, that are inter-converting (Figure 2.7A), we measure the dwell times of individual states from which we can estimate kinetic rates of the transitions (Figure 2.7B). After selecting the individual transitions either manually or via automated threshold analysis, we fit the dwell time histogram of each state to obtain the lifetimes ($\tau_A$ and $\tau_B$, Figures 2.7Bc, Bd) and the corresponding transition rates, $k_{A\rightarrow B}$ and $k_{B\rightarrow A}$.

**Cross Correlation Analysis:**
When the time scale of the fluctuation between the two states is too fast for a reliable dwell time analysis (Figure 2.7C), we resort to the correlation analysis. For example, cross-correlation tells us whether the donor and acceptor intensities are fluctuating in an anti-correlated manner, and, if so, what the time scale is

$$CC(\tau) = \int I_D(t)I_A(t-\tau)dt$$

Ideally, one would see a curve that starts from a negative value and decays to zero. Then, by fitting the curve using a single-exponential curve,

$$-Ae^{-(k_{A\rightarrow B} + k_{B\rightarrow A})\tau}.$$ 

we obtain the sum of the two rates (Figure 2.7Cc). If the FRET efficiency histogram is clean enough to yield a measure of the relative populations of the two species (Figure 2.7Cb), that is, $\frac{k_{A\rightarrow B}}{k_{B\rightarrow A}}$, in combination we can determine both $k_{A\rightarrow B}$ and $k_{B\rightarrow A}$ (Joo et al., 2004; Tan et al., 2003).
Hidden Markov Modeling:

If there are more than two states (Figure 2.7D), more complicated and time-consuming procedures are required when using dwell-time and correlation analysis. In such cases, unbiased analysis tools, such as hidden Markov modeling (HaMMy) are recommended. HaMMy is more reliable, reproducible and less susceptible to human bias than traditional thresholding algorithms (McKinney et al., 2006). Once idealized trajectories are generated using HaMMy, the existence of discrete FRET states and how they are interconnected can be evaluated by using a transition density plot, yet another layer of data reduction (Figure 3.3Da).

Not all the data are suitable for HaMMy. A good rule of thumb is: if you can almost distinguish individual states by eye but cannot carry out a quantitative analysis, then HaMMy is likely to be suitable. Additionally, there need to be multiple transitions within each trace.

2.4.4 FRET Histogram

FRET histograms are built by taking the average FRET efficiency of the first 10 frames for each molecule, typically out of more than 1,000 molecules from multiple imaging areas. When a single peak represents only one FRET state, it is typically less than 0.1 full-width-half-maximum wide (e.g. Figures 3.5, 4.2, and B.3). Statistical and instrumental noise inhibits further resolution of the peak.

Since the FRET histogram is built from a great number of molecules, it provides statistics in the quickest and most unbiased way. For example, as shown in Figure B.3, after a quick survey over several different solution conditions, we move on to a more rigorous and time-consuming step—to record smFRET traces and to carefully analyze them one by one.

The inactive acceptor molecules contributes to the peak at $E_{app} = 0$. While this FRET value cannot be smaller than 0 in principle, $I_A$ is very small for FRET approaching 0 and noise around the background level makes $I_A$ negative after background subtraction in practice, leading to calculated FRET values smaller than 0.
2.5 Single-Molecule Three-Color FRET

2.5.1 Need for More Colors

For complex molecular dynamics or multi-components binding interactions, it is beneficial to have more than one FRET pair to observe correlated changes. An example involving three points is shown in Figure 2.8A. Using a single pair of fluorophores attached to the point 1 and 2, we can determine how close the two points are but there is no information about where the third point is. If another dye is attached to the point 3 and three distances are obtained using FRET interactions between three dyes, we can determine the relative locations of all three points.

In spite of the obvious advantages of three-color FRET over the regular two-color FRET technique, its realization has been hindered due to contradictory requirements: a clear spectral separation of three fluorophores’ signals and an appreciable amount of FRET between them. For a clear signal separation, the spectral overlap should be small, but this leads to weaker FRET. Several recent studies demonstrated three-color FRET at the ensemble level (Horsey et al., 2000; Ramirez-Carrozzi and Kerppola, 2001; Liu and Lu, 2002; Watrob et al., 2003, Haustein et al., 2003). We have extended three-color FRET to the sm-level and measured FRET between one donor and two alternative acceptors to observe correlated movements of different helical arms of a DNA four-way (Holliday) junction undergoing conformational changes (Chapter 7.3, Synchronized Conformational Dynamics) (Hohng et al., 2004a).

2.5.2 Fluorophore Selection

Three-color FRET may require additional criteria for the fluorophore selection depending on particular applications, and are classified into the following categories according to the spectral separation between three fluorophores (Figure 2.8B): (a) two-donor/one-acceptor scheme, (b) two-step FRET scheme, and (c) one-donor/two-acceptor scheme. These categories are neither exhaustive nor mutually exclusive, and may have pros or cons for specific applications. In the first scheme, the peak emission wavelengths of the two alternate donors are closer to each other than to that of the acceptor. Thus, a single
wavelength light source can excite both donors which will be selectively quenched in proportion to the proximity to the acceptor (Ramirez-Carrozzi and Kerppola, 2001). In the two-step FRET scheme, emission peaks of three fluorophores are evenly spaced so that the middle-energy fluorophore acts as a bridge to transfer the excitation from the high-energy to low-energy fluorophores (Haustein et al., 2003; Watrob et al., 2003). In the one-donor/two-acceptor scheme (Horsey et al., 2000; Watrob et al., 2003), emission peaks of the two acceptors are closer to each other than to the emission peak of the donor, so that the donor might be selectively quenched by either of the acceptor dyes. In this work, we focus on the third scheme.

Cy3 (donor), Cy5 (acceptor 1) and Cy5.5 (acceptor 2) are a good trio for the following reasons. As shown in Figure 2.8Ca, since Cy5 and Cy5.5 are separated by only ~30 nm in the excitation peak wavelengths and share similar extinction coefficients (250,000 M$^{-1}$cm$^{-1}$ for both of them according to GE Healthcare), the spectral overlap integral (Clegg, 1992) for Cy3-Cy5.5 pair is only slightly lower, a factor of 0.88, than that of Cy3-Cy5 pair. The spectral overlap integral for Cy5-Cy5.5 pair is substantially larger, being 3.31 times that of Cy3-Cy5 pair. Using measured values of quantum yields of DNA-conjugated Cy3 and Cy5 (Murphy et al., 2004) and assuming the orientational factor $\kappa^2$ is 2/3, Förster distances ($R_0$) are calculated (Appendix A.1 Calculating $R_0$) to be 6.0nm, 5.9nm, and 7.3nm for Cy3-Cy5, Cy3-Cy5.5 and Cy5-Cy5.5 pairs, respectively. This choice of fluorophores causes similar levels of FRET from the donor to either of the acceptors for a given distance, and is therefore ideal in determining which of the two alternative acceptors are closer to the donor.

Since FRET can occur between all three fluorophores, the data become too complicated to interpret if all three of them are brought close to each other. For example, if the two acceptors are in close proximity of each other, it will not be easy to tell whether Cy5.5 signal is due to FRET from Cy3 or due to sequential FRET from Cy3 to Cy5 and then to Cy5.5. It is recommended to design a construct in which the two acceptors are always far from each other in comparison to their $R_0$. The distance between Cy5 and Cy5.5 remains large for our model system of Holliday junction such that FRET between them is relatively small despite the large $R_0$ (Figure 2.8D).
2.5.3 Design and Construction of Setup

Because of the large spectral overlap between Cy5 and Cy5.5, a judicious selection of optimal dichroic mirrors and bandpass filters is crucial for clearly separating signals arising from three fluorophores.

2.5.3.1 Confocal Microscopy

The simplicity of a design made us realize the first generation of three-color smFRET with the confocal microscopy (Figure 2.3). Nominal cut-off wavelengths of dichroic mirrors were 650 nm (650drxr, extra reflection region, long pass) for separating Cy3 emission from others and 690 nm (690dclp, long pass) for further separating fluorescence signals from Cy5 and Cy5.5. To reduce bleed-through between detection channels, additional bandpass filters were used (HQ580/60m, 60nm transmission window centered at 580 nm, for Cy3 channel, HQ670/40m for Cy5 channel, and HQ715/30m for Cy5.5 channel). The cut-off wavelength of a dichroic mirror is a sensitive function of the incident angle. We intentionally made the incident angle to 690DCLP ~50° instead of 45° in order to minimize Cy5.5 signal bleed-through to Cy5 channel. Therefore, the true cut-off wavelength is ~ 685 nm. Bleed-through of Cy5 and Cy5.5 emission to Cy3 channel is negligible. However, there is significant amount of bleed-through of the Cy5 emission into the Cy5.5 channel and of the Cy5.5 emission into the Cy5 channel. Also a small but measurable amount of Cy3 emission appears in both the Cy5 and the Cy5.5 channels.

2.5.3.2 TIR Microscopy

In the need of increasing the data throughput, therefore, performing statistically significant analysis of even irreversible reactions, we have designed another three-color setup with TIR microscopy. Only after several trial-and-errors in designing, we have finally found an optimal configuration as shown in Figure 2.8E. The donor signal is first separated by a 645nm dichroic mirror, then Cy5 and Cy5.5 signals are split in the same way as the two-color FRET (but with a dichroic mirror of the 685nm cut-off wavelength).
Next, all the signals are put together by another 645nm dichroic mirror and sent to a CCD camera.
Chapter 3

RecA Filament

Here, we present smFRET assays which enable us to watch binding and dissociation of individual RecA monomers in real time. Combined with a novel analytic tool based on hidden Markov modeling, our work provides a new approach of determining not only the nature of the RecA binding and dissociation unit but also kinetic rates of the dynamics at both ends of the filament for the first time. In addition, we examine the filament nucleation process and determine its kinetic properties as well as the minimum number of monomers required for nucleating a RecA filament. 5

3.1 Experimental Procedure

3.1.1 Detecting RecA Filament Formation via FRET

The formation of a RecA filament is deduced from the change in the end-to-end length of ssDNA, which is reported by FRET. As illustrated in Figure 3.1A, the donor and acceptor fluorophores are attached at the end of the ssDNA tail and the ss-dsDNA junction, respectively. The ssDNA by itself is highly flexible (persistence length ~ 1.5-3 nm) such that its conformational fluctuation is averaged out on a much faster time scale than our time resolution (Murphy et al., 2004). Therefore, for (dT)21 tail, two dyes are in close proximity on average giving relatively high FRET efficiency, \( E \approx 0.55 \) (Figure 3.1Ca, white), in our standard RecA-reaction condition (Chapter 3.1.2, Reaction Condition).

5 This work in Chapter 3 has been published as papers:
C. Joo et al. ‘Real-time observation of RecA filament dynamics with single monomer resolution’ Cell 126: 515-27 (2006) and
Addition of 1 µM RecA and 1 mM ATPγS, our standard RecA and cofactor concentrations, leads to the filament formation and straightening of the ssDNA. This results in low FRET, $E \sim 0.1$ (Figure 3.1Ca, green), distinguishable from acceptor blinking ($E=0$) (Ha et al., 1999b). Likewise, the DNA in bulk solution shows high FRET without RecA, and low FRET when the filament forms with ATPγS (Figure 3.1B).

When shorter ssDNA tails (10-17nt) are used, we see that the longer the ssDNA, the lower the FRET efficiency when the filament is formed, which directly reflects the difference in their contour lengths (Figure 3.1D).

### 3.1.2 Reaction Condition

As shown in Figure 3.1A, DNA is immobilized on quartz surface, which is coated with poly-ethylene glycol (Appendix A.5.5 PEG-coated Surface) in order to eliminate non-specific surface adsorption of proteins (Ha et al., 2002). The immobilization was mediated by biotin-Neutravidin binding between biotinylated DNA (IDT DNA) (see also Chapter 3.1.3, DNA Preparation), Neutravidin (Pierce) and biotinylated polymer (PEG-MW 5,000, Nektar Therapeutics). About 100pM of DNA molecules are immobilized and observed in our standard RecA-reaction condition: 1 mM ATP (Sigma) or ATPγS (Calbiochem), 10 mM MgAc, 100 mM NaAc, 25 mM Tris-Ac at pH 7.5 in the presence of oxygen scavenging system of 1 mg/ml glucose oxidase (Sigma), 0.4 % (w/v) D-glucose (Sigma), 0.04 mg/ml catalase (Roche) and 1% v/v βME (2-mercaptoethanol, Acros) at the room temperature (22±1 °C) unless otherwise specified. The concentration of RecA (New England Biolabs) is 1 µM unless otherwise specified. SSB (10 nM, the standard concentration) (a gift from Dr. T. M. Lohman) was not added unless mentioned. The same condition was also used in Chapters 4 and 5 unless specified. A prepared solution ~100 µl was injected into an assembled chamber that holds ~20 µl. Therefore, the injection results in a very efficient change in chemical environments around immobilized DNA. When RecA and ATP are in the injected solution, the system inside the chamber reaches equilibrium in less than a minute.
3.1.3 DNA Preparation

Labeling of dye and biotin at 5’ or 3’ end of ssDNA was done during DNA synthesis (see also Appendix B.1 Interaction Between a Fluorophore and a RecA Filament). In case of internally-labeled ssDNA, Cy3 (NHS-ester from GE Healthcare) was attached to the base of a dT through a C6 amino linker in order to keep the DNA backbone intact (Appendix A.3 DNA Labeling). Annealing of dsDNA was performed by heating a sample that contained two complementary ssDNA molecules at micromolar concentrations to 90 °C and slowly cooling for 2 hours at the room temperature. The sequence of biotinylated ssDNA forming the dsDNA is: 5’-GCC TCG CTG CCG TCG CCA-biotin-3’ and that of ssDNA with 3’ tail is 5’- TGG CGA CGG CAG CGA GGC -(tail)- 3’. The sequences of (dT)$_{5+54}$, (dT)$_{10+49}$ and (dT)$_{13+46}$ are 5’ TGG CGA CGG CAG CGA GGC-T5-T*-T53-3’, 5’ TGG CGA CGG CAG CGA GGC-T10-T*-T48-3’ and 5’ TGG CGA CGG CAG CGA GGC-T13-T*-T46-3’ where T* stands for an amine-modified dT with Cy3 labeled.

3.2 Nucleation of a RecA Filament

3.2.1 Observation on Nucleation Cluster Formation

Whereas RecA forms a stable filament on (dT)$_{21}$ tail with ATP$_\gamma$S in our standard RecA-reaction condition, it does not form a filament stably when ATP is used as the cofactor; low FRET ($E$~0.1) shows up only a small fraction of the time (6%) (Figure 3.1Cb). Likewise, the fluorescence spectrum in bulk does not show any significant change when RecA is added with ATP (Figure 3.1B). This observation confirms previous measurements which found that RecA together with ATP does not form a stable filament on ssDNA lengths shorter than ~30 nt (Bianco and Weinstock, 1996; Brenner et al., 1987).

Examination of individual sm-traces reveals transient RecA binding, as evinced by occasional and brief transitions from high FRET ($E$~0.55) to low ($E$~0.1) (Figure 3.1E; more traces available in Figure B.2). Since the transient low-FRET state has
identical $E$ value to that observed with the stable ATPγS RecA filament (Figure 3.1Ca, green) we interpret it as representing brief formation of a RecA filament. While there must be several monomers in the filament formed on (dT)$_{21}$ tail, the transitions between the two FRET states display only a few data points at 100 ms time resolution, much faster than expected from binding and dissociation of a monomer at an established filament (see Figure 3.3C; binding and dissociation of monomers take several seconds under the same condition). This was also true of (dT)$_{17}$ and (dT)$_{19}$. Therefore, there must be either simultaneous binding of multiple monomers or binding of a pre-assembled oligomer in order for RecA to form a cluster of a filament on such short ssDNA.

We then determined the average dwell-time of the low-FRET state with different lengths of ssDNA tail and found that shorter ssDNA yields a shorter average dwell-time (Figure 3.1Fa). Meanwhile, the average dwell-time decreases as the temperature increases (Figure 3.1Ga), which is inversely-proportional to the increase of ATP hydrolysis rate (Bedale and Cox, 1996). These dependences suggest that the stability of the cluster is correlated to the number of monomers bound to ssDNA as well as with ATP hydrolysis. Next, we measured how frequently the cluster forms. The frequency sharply drops when shorter lengths of ssDNA than (dT)$_{21}$ are used (Figure 3.1Fb). Furthermore, there is no filament formation observed with (dT)$_{13}$ and (dT)$_{15}$. Finally, the frequency of cluster formation depends only weakly on the temperature (Figure 3.1Gb). These collective findings suggest that the transition observed here represents the formation and dissociation of a nucleation cluster that would likely lead to filament extension on a longer ssDNA. Other supporting sm-data taken with different NTP factors can be found in Appendix B.2.

### 3.2.2 Rapid Nucleation in a Confined Volume

The next question we ask is how a number of monomers bind together to ssDNA to nucleate. With the current assay we cannot tell whether it happens either by simultaneous binding of the monomers or by binding of a pre-assembled oligomer. We introduce a vesicle encapsulation assay, in which a spherical lipid vesicle provides a confined but inert environment for RecA-DNA interaction (Cisse et al., in press; Okumus et al., 2004).
We encapsulate a doubly-labeled ssDNA, (dT)_{20} (400 nM), together with several RecA monomers (3 µM) inside a lipid vesicle of 100nm radius (Figure 3.2A). If there is an oligomer present inside a vesicle, the effective concentration of it becomes several orders of magnitude higher than before encapsulation (Figure 3.2Ba). Therefore, if the nucleation occurs via the binding of a pre-assembled oligomer, the nucleation will become radically enhanced. In contrast, if it is the simultaneous binding of monomers that causes the nucleation, since the confinement will not lead to any change in the effective concentration of the monomers (Figure 3.2Bb), the nucleation rate will remain unchanged.

Shown in Figure 3.2C is the sm-trace obtained from the encapsulation assay. It shows rapid fluctuations between high and low FRET states. As previous (Chapter 3.2.1), the transition from the high- to the low-FRET state is abrupt, indicating that the nucleation occurs through binding of several monomers together. Also, abrupt change from the low- to the high-FRET state represents the complete dissociation of a filament from the ssDNA. The dwell time of the low-FRET state is 2.3 (±0.7) seconds on average (Figure 3.2Da), which is comparable with 6.0 (±2.0) seconds determined using partial dsDNA with a (dT)_{19} overhang, tethered to a surface.

What draws our attention is the extremely high frequency of the nucleation. The nucleation is observed on average ~1000 times per hour, which is two orders of magnitude higher than that measured in the previous surface-tethered assay (Figure 3.2E). This radical enhancement is explained if a filament remains assembled even after dissociation and if it readily rebinds to the ssDNA. Such rebinding will be rarely observed in the absence of a confinement since the dissociating oligomer will rapidly float away from a surface-tethered DNA.

### 3.2.3 Molecular Mechanism of the Filament Nucleation

RecA nucleation cluster forms briefly and infrequently on 17 to 21 nt-long ssDNA. The actual processes of formation and dissociation of the cluster were faster than we could resolve clearly with the time resolution of 100 ms. The dwell time of the cluster formed on 17 and 19 nt tails were 4.3 and 6.0 sec, respectively (Figure 3.1Fa), close to the
dissociation time of a single RecA monomer \( (k_{\text{off}}^*)^{-1}, 8.3 \text{ sec} \) (Chapter 3.3.3). This coincidence implies that dissociation of even a single monomer makes the cluster bound to 17-19 nt very unstable and induces rapid dissociation of the cluster. Furthermore, the nucleation frequency dropped precipitously when the ssDNA length was below 21 nt (Figure 3.1Fb), and no nucleation was observed with 13 and 15 nt. Therefore, ~5 monomers bound to 17 nt could be considered the minimum unit for nucleation.

We have studied the interaction between DNA and RecA in a confined volume. This unique environment has provided a chance to elucidate the molecular mechanism of the nucleation. We have found out that the nucleation through the binding of a pre-assembled filament is much more efficient than successive binding of individual monomers as well as have confirmed the requirement of the minimum number of RecA for nucleation (Figure 3.7).

3.3 Filament Dynamics at the 5’-Disassembly End

3.3.1 Construct Design

On a ssDNA longer than ~30 nt, a dynamic RecA filament stays stably even with ATP. In this case, FRET changes would reflect the assembly/disassembly occurring at the ends of the filament rather than the formation/dissociation of the nucleation cluster. Since FRET between the two ends of such a long naked ssDNA is relatively low, we sought to achieve high resolving power in FRET by placing the donor internally on the ssDNA tail without disrupting DNA backbone or perturbing RecA activity (Figure 3.3A). The ssDNA of \((dT)_{13+46}\) has the donor and acceptor separated by 13 nt, followed by 46 nt of 3’ tail so that FRET changes reflect the dynamics at the 5’-disassembly end. This DNA shows high FRET \((E\sim 0.65)\) by itself but exhibits low FRET \((E\sim 0.15)\) in the presence of RecA and ATPγS (Figure 3.3A), due to stable filament formation.

In contrast, a broad FRET histogram spanning from 0.15 to 0.7 is observed with RecA and ATP (Figure 3.3Bb). Examining the sm-traces we find that they exhibit stepping between well-defined FRET levels (Figure 3.3C). Since traces no longer exhibit
the simple two-state dynamics found in shorter tails, it suggests that different binding modes (i.e. different numbers of RecA monomers on the 13 nt region between donor and acceptor) are being observed. If these different FRET values correspond to different assembly states of the filament, we may expect to see up to five different FRET values (0, 1, 2, 3 and 4 monomers) because up to four monomers could bind to the 13 nt region. However, not only is it challenging to identify five states given the limited signal-to-noise ratio, but it could also bias the analysis to presume that there exist five states. Therefore, we employed a newly developed form of hidden Markov modeling (HaMMy) to analyze the data statistically without imposing a preconceived model (Chapter 2.4.3 Transition Trajectory Analysis) (McKinney et al., 2006). HaMMy analysis identifies distinct FRET states in each trace (Figure 3.3C, the fit in green) and determines the number of states, their FRET values, and the transition rates between them (Figure 3.3D).

3.3.2 HaMMy and TDP Analysis

Figure 3.3Da shows the result of HaMMy analysis as a transition density plot (TDP) of 17,203 transitions from 165 DNA molecules. A TDP is effectively a 2-dimensional histogram that shows how frequent transitions are between the different states. They are presented with the FRET levels prior to and after the transition on the horizontal and vertical axes respectively. It is evident that there are four distinct FRET values (0.15, 0.3, 0.5 and 0.7) along each axis, in addition to the no-FRET peaks due to acceptor blinking. The highest FRET value (E~0.7) agrees with the DNA-only value (Figure 3.3Ba green), therefore the state with E~0.7 is designated M0. The lowest FRET value (E~0.15) is close to the value observed with RecA and ATPγS (Figure 3.3Ba white). Thus the remaining FRET values (E~0.5, 0.3 and 0.15) are termed M1, M2, and M3 respectively, with each number representing the number of monomers bound contiguously, starting from the donor location, to the 13 nt segment (Figure 3.3A). Non-contiguous binding of RecA was not considered because we did not detect any binding of RecA to (dT)13 with ATP (Chapter 3.2.1) de novo nucleation of a filament in such a short ssDNA segment is extremely unlikely and the binding transitions here represent extension from the existing filament only.
It is not clear why only three monomers appear to bind instead of four. When we shortened the ssDNA segment between the donor and acceptor using (dT)$_{5+54}$ and (dT)$_{10+49}$, we observed that transitions occur primarily between two and three states, indicating that one and two monomers can access the 5 and 10 nt segments respectively (Figure 3.4). The TDP in Figures 3.3D and 3.4 show that the most frequent transitions are of the type $M_i \rightarrow M_{i\pm1}$, indicating that the majority, >78% for (dT)$_{13+46}$ and >93% for (dT)$_{10+49}$, of binding and dissociation events occurs one monomer at a time. Note that the mirror symmetry in the TDPs comes from the fact that the majority of transitions are of the type $M_i \rightarrow M_{i\pm1}$ and that the system under observation is in equilibrium, when the number of transitions of $M_i \rightarrow M_{i+1}$ is the same as that of $M_{i+1} \rightarrow M_i$.

### 3.3.3 Transition Rates

Once every transition in the plot is classified according to which $M_i \rightarrow M_j$ pair it belongs to, kinetic rates can be determined for each pair (Figure 3.3Db). For example, the binding rates ($k_{\text{on obs}}^{M_0 \rightarrow M_1}$, $k_{\text{on obs}}^{M_1 \rightarrow M_2}$ and $k_{\text{on obs}}^{M_2 \rightarrow M_3}$) can be determined as a function of RecA concentration (Figure 3.3Ea). As expected, all $k_{\text{on obs}}$’s increase with increasing RecA concentration. We also find that $k_{\text{on obs}}^{M_0 \rightarrow M_1} > k_{\text{on obs}}^{M_1 \rightarrow M_2} > k_{\text{on obs}}^{M_2 \rightarrow M_3}$ at two highest different RecA concentrations, 1 µM and 0.25 µM. This effect may be due to the reduction in the amount of naked ssDNA available for binding as the number of bound monomers increases.

The dissociation rates ($k_{\text{off}}^{M_1 \rightarrow M_0}$, $k_{\text{off}}^{M_2 \rightarrow M_1}$ and $k_{\text{off}}^{M_3 \rightarrow M_2}$) are largely independent of RecA concentration as one would expect (Figure 3.3Eb) but vary slightly with the position at the two highest RecA concentrations, 1 µM and 0.25 µM. We should point out that the average $k_{\text{off}}$ (averaged over all three dissociation rates) is not necessarily equal to or lower than the ATPase rate ($k_{\text{cat}}$). In fact, it was shown that, when a monomer leaves the 5'-disassembly end, $k_{\text{off}}$ of the adjacent monomer is twice $k_{\text{cat}}$ (Arenson et al., 1999). Thus, such an effect should be considered when $M_i$ has been arrived at via RecA dissociation. When the probability for $M_i$ to have come about via RecA dissociation is $p$ and the coupling efficiency of ATP hydrolysis to dissociation is $\varphi$,

$$k_{\text{off}} = \varphi \times (2k_{\text{cat}} \times p + k_{\text{cat}} \times (1 - p)).$$

32
Then, from \( p \) and \( k_{\text{off}} \) values determined by HaMMy analysis and TDP (Figure 3.3Db) we can calculate the “corrected” dissociation rate (\( k_{\text{off}}^* = \phi \times k_{\text{cat}} \)) (Figure 3.3Ec). This rate of 0.12 ± 0.02 (sec\(^{-1}\)), independent of the position of the dissociating monomer and RecA concentration, is comparable to the ATPase rate of 0.18 sec\(^{-1}\) at 22 °C, estimated from Bedale and Cox by extrapolation (Bedale and Cox, 1996). This comparison indicates that the coupling efficiency \( \phi \) is close to 1 in accord with the previous estimate at neutral pH (Arenson et al., 1999). Finally, the value of \( k_{\text{off}}^* \) is similar to \( k_{\text{on}} \text{(obs)} \), 0.11 ± 0.02 (sec\(^{-1}\)) in the presence of 100 nM RecA, so we estimate that the critical concentration (\( C_c \)) at which the filament neither grows nor shrinks is about 100 nM at the 5’-disassembly end.

### 3.4 Filament Formation with ATP\( \gamma \)S

Next, we attempted to observe the filament dynamics at the 3’-extending end using a ssDNA tail of (dT)
\(_{46+13}\), the same DNA as (dT)
\(_{13+46}\) but labeled differently so that FRET reports on the dynamics of the 13 nt segment at the 3’-extending end. At 1 µM RecA, this DNA gave stable low FRET indicating filament formation with no discernible dynamics at the 3’-extending end (data not shown). At 100 nM, we observed irregular FRET fluctuations likely as a result of both formation/dissociation of the nucleation cluster and the assembly/disassembly at the 3’-extending end (data not shown), precluding detailed analysis. We realized that what we needed was a method to separate the nucleation step from the filament extension. We could achieve just that, based on an unexpected finding that we will discuss in this section.

#### 3.4.1 Property of <ssDNA|ATP\( \gamma \)S>

As described earlier, stable filaments form on short ssDNA tails, from (dT)
\(_{13}\) to (dT)
\(_{21}\) (Figure 3.1Ca) if ATP\( \gamma \)S is used as a cofactor (Leahy and Radding, 1986). Here, we will refer to such filament as <ssDNA|ATP\( \gamma \)S>. With (dT)
\(_{13}\), <ssDNA|ATP\( \gamma \)S> results in low FRET (\( E \sim 0.2 \)) (Figure 3.5Ab white). If RecA is subsequently removed from solution via
flow while keeping the magnesium concentration constant (Figures 3.5Ac, d), the smFRET histogram is restored to that of DNA-only within 5 minutes (Figure 3.5Ab white), indicating the ssDNA tail becomes free of RecA.

sm-traces recorded during this disassembly reveal individual states (M_i) of (dT)_{13} and (dT)_{10}. We found that, if ATP\gamma S is kept in solution, sm-traces recorded during this disassembly visualize individual states (M_i) of (dT)_{13} and (dT)_{10} since monomers dissociate slowly one by one (Figure 3.5B). Among the molecules that exhibit a monotonic and stepwise change in FRET, we could count up to \~5 states as shown in an example time trace (Figure 3.5Ba), consistent with expectation from the ratio between RecA and nucleotides. Molecules with 4 states are also observed with a similar frequency (Figure 3.5Bb). The same experiment was carried out with (dT)_{10} tail that showed four different states in maximum (Figure 3.5Ca), while there were a similar number of molecules that show three states only (Figure 3.5Cb). Not all the molecules show a monotonic and/or stepwise change in FRET, therefore, it should be noted that this assay is only for counting the maximum number of FRET states but not for understanding the disassembly process of <ssDNA|ATP\gamma S>.

While the disassembly was also observed with (dT)_{15}-(dT)_{21} tails (data not shown), longer tails, (dT)_{40} and (dT)_{70} however supported stable <ssDNA|ATP\gamma S> even after removing free RecA and ATP\gamma S (data not shown) and required SSB for RecA removal from ssDNA (Chapter 4.1 SSB Displacement by RecA).

### 3.4.2 Stability of <dsDNA|ATP\gamma S>

To study a possible filament formation of <dsDNA|ATP\gamma S>, we attached the donor and acceptor fluorophores at the ends of dsDNA, which has a 3’ tail of (dT)_{20} (Figure 3.5Da). E decreases from 0.25 to 0.1 when RecA and ATP\gamma S are added, indicating the formation of <dsDNA|ATP\gamma S>. Since it cannot form in the absence of ssDNA tail (Appendix B.3.3), its formation here must be through extension of <ssDNA|ATP\gamma S> into dsDNA (Shaner et al., 1987; Shaner and Radding, 1987). Furthermore, by utilizing a different configuration of labeling (Appendix B.3.1) and by a footprinting assay (Appendix B.3.2), we found that the filament forms over the entire length of dsDNA.
Next, when RecA and ATPγS are removed from solution, no significant change in FRET is observed for an hour, while it slowly starts to disassemble in two hours (Figure 3.5Db) suggesting unexpectedly high stability of \( <\text{dsDNA}|\text{ATPγS}>\) (> 1 hour) compared to \( <\text{ssDNA}|\text{ATPγS}>\) formed on 13-21 nt ssDNA (< 5 min). A similar property was observed at high temperature (35°C) (Appendix B.3.4) and also with dsDNA that has 5’ ssDNA tail (Appendix B.3.5).

### 3.5 Filament Dynamics at the 3’-Extending End

The difference in stability between \( <\text{ssDNA}|\text{ATPγS}>\) and \( <\text{dsDNA}|\text{ATPγS}>\) in the absence of free RecA results in a unique configuration in which a filament is present only around the dsDNA portion of a partial duplex DNA (Figure 3.5Ac). We suspected the \( <\text{dsDNA}|\text{ATPγS}>\) in such a construct might act as a nucleation cluster, thus facilitating the formation of \( <\text{ssDNA}|\text{ATP}>\) when RecA is added with ATP. Indeed, we observe stable filament formation on (dT)$_{13}$ within a second of adding RecA and ATP (Figures 3.5Ae, f), whereas there was no or rare filament formation observed with 13-21 nt ssDNA without \( <\text{dsDNA}|\text{ATPγS}>\) (Figure 3.1Fb). The similar observation was made also at a higher temperature (35°C) (Appendix B.3.4). This unexpected finding allows us to decouple filament extension from nucleation and enables the observation of filament dynamics at very low RecA concentrations.

Figure 3.6B shows FRET fluctuations after the addition of 8 nM RecA with ATP, indicative of filament dynamics at the 3’-extending end (Figure 3.6A). Transitions between multiple FRET states detected in sm-time traces are presented in the TDP (Figure 3.6Ca; 82 molecules and 4,635 transitions) where five different states are clearly discerned (\( E \approx 0.2, 0.3, 0.55, 0.75 \) and 0.85; \( M_4, M_3, M_2, M_1 \) and \( M_0 \), respectively), consistent with the maximum number of states counted in Figure 3.5B. Almost all transitions occur between the nearest neighbors, \( M_i \leftrightarrow M_{i+1} \), again suggesting that the unit of binding and dissociation is a monomer also for the 3’-extending end. It also provides the most direct evidence thus far that RecA cannot dissociate from the middle of the filament.
3.6 Molecular Mechanism of the Filament Dynamics

Since free *E. coli* RecA exists in solution in diverse assembly states including a monomer, trimer, hexamer and so on (Brenner et al., 1990), there has been no definitive way to determine the size of a binding unit in the filament assembly. Because the filament binding and dissociation transitions occurred predominantly between nearest neighbor states as revealed in the TDPs (Figures 3.3, 3.4 and 3.6), we conclude that this unit of binding as well as of dissociation is primarily a single monomer. The data further indicate that dissociation occur from the extremities of the filament because extensive dissociation from the internal sites on the filament would have yielded much more significant amount of non-nearest neighbor transitions.

The individual monomer binding rates $k_{\text{on}}^{\text{(obs)}}$ have been directly measured at both 3’-extending and 5’-disassembly ends. In previous works, binding of RecA to the 5’-disassembly end had been largely ignored in describing the dynamics of a filament, but we found that the $k_{\text{on}}^{\text{(obs)}}$ was even higher than $k_{\text{off}}$ at the 5’-disassembly end at 1 µM RecA. The RecA filament was thought to exhibit a monotonic disassembly followed by a rapid recovering assembly in a previous analysis which neglected RecA binding at the 5’-disassembly end (Tlusty et al., 2004), but we observed continual binding and dissociation
of monomers (Figure 3.3C). Thus, RecA binding at the 5′-disassembly end should be included in the description of filament dynamics.

We also determined the heretofore immeasurable $k_{\text{on}}^{(\text{obs})}$ at the 3′-extending end (0.18 sec$^{-1}$ at 8 nM RecA). As a rudimentary comparison, if we extrapolate to 1 µM, $k_{\text{on}}^{(\text{obs})}$ becomes 23 sec$^{-1}$, which coincides with the rate determined with dsDNA (20 sec$^{-1}$ in the same RecA concentration (van der Heijden et al., 2005)).

The dissociation rates $k_{\text{off}}$ were also measured at both ends. $k_{\text{off}}$ at the 5′-disassembly end was consistent with the previous report (Arenson et al., 1999) and $k_{\text{off}}$ at the 3′-extending end was determined for the first time. $k_{\text{off}}$ at the 5′-disassembly end and at the 3′-extending end were essentially the same. Meanwhile, there is about an order of magnitude difference in critical concentration, ~100 nM at the 5′-disassembly end and ~8 nM at the 3′-extending end. Therefore, the directionality in the filament growth, is mainly due to the difference in the binding rates (Figure 3.7).

The dissociation rate after correction ($k_{\text{off}}^*$) was close to the ATPase rate showing that disassembly of a filament is tightly coupled with ATPase activity. $k_{\text{off}}^*$ was independent of the position of the dissociating monomer, indicating that there is no significant coupling between neighbor monomers in terms of ATPase activity (Shan et al., 1996).

### 3.7 Future Study

This new assay developed is more sensitive and accurate than any other existing methods in studying the filament dynamics. With that, we will further characterize the detailed properties of the RecA filament. We have demonstrated that we can count the number of RecA monomers bound to a short, defined stretch of ssDNA. We will do the same measurement but with different lengths and sequences (poly-dT, poly-dA, poly-dC, etc) so that we can determine the precise number of nucleotides covered by a single monomer and the sequence dependence of it. Since a DNA strand has never been resolved in RecA crystal structures, this study will provide the first glimpse on how differently a RecA interacts with different bases of ssDNA. In addition, there is a compelling need for
carrying out the same approach at different chemical conditions. By measuring the RecA filament dynamics as a function of concentrations of ATP, magnesium, and monovalent ions and as a function of pH, we hope to answer numerous questions that have been raised from previous biochemical studies.

Rad51 is a eukaryotic homolog of RecA. Despite its importance in understanding human disease, relatively few kinetic studies on the Rad51 filament have been carried out so far (Baumann and West, 1998) (Ogawa et al., 1993; Shinohara et al., 1992). We will apply the new assay in studying the Rad51 filament. We will first investigate the nucleation by trying different lengths of short ssDNA as we have done with RecA. Once we determine the minimum length of ssDNA required for the nucleation, we will observe the binding and the dissociation of Rad51 on a longer DNA and determine their rates. A similar experiment is planned for an archaeal homolog, RadA. We have already carried out the RadA-catalyzed strand exchange reaction in ensemble (Chapter 5.5.2) and found that the temperature is needed to be above 30°C for the optimal activity which can be achieved by a temperature control system integrated with our sm-setup.
Chapter 4

Interaction of the RecA Filament with Other Proteins

In a cell, RecA is not the only protein that interacts with ssDNA. Those include SSB (ssDNA binding protein) that universally binds to any ssDNA and helicases that translocate along the ssDNA. Based on an assay in which a cluster of the filament is pre-nucleated near the ss-dsDNA junction, we find that RecA, once nucleated, is able to replace SSB almost as quickly as filament extension. Furthermore, by visualizing the dynamics of the filament near the ss-dsDNA junction, we provide insight into the role of RecA and its interactions with other proteins.  

4.1 SSB Displacement by RecA

4.1.1 Inhibitory Role of SSB

In *E. coli*, most of a nascent ssDNA becomes occupied by SSB preventing other proteins from accessing the ssDNA. Because of the highly cooperative nature of RecA binding, absence of a naked ssDNA precludes RecA filament nucleation on SSB-coated ssDNA.

---

6 This work in Chapter 4 has been published as papers:
C. Joo et al. ‘Real-time observation of RecA filament dynamics with single monomer resolution’ *Cell* **126**: 515-27 (2006) and
Therefore, for RecA-mediated DNA repair to initiate on SSB-coated ssDNA, accessory proteins are necessary to help load RecA (Amundsen and Smith, 2003). Once RecA is loaded, SSB should no longer present a barrier to the 3’-extending end RecA filament (Kowalczykowski and Krupp, 1987; Thresher et al., 1988). To comprehend these two different modes of SSB function in relation to RecA, we need techniques that can observe the dynamic interactions between RecA and SSB in real time.

In the double-strand break repair (Cox et al., 2000; Kowalczykowski, 2000; Smith, 2001), a blunt ended dsDNA is processed into a partial dsDNA with a 3’ tail which becomes coated with SSB (Figure 4.1). After RecFOR occupies the ss-dsDNA junction (Morimatsu and Kowalczykowski, 2003), RecA displaces SSB assisted by RecOR (Bork et al., 2001; Umezu et al., 1993). How RecFOR helps load RecA on SSB-coated ssDNA is not yet clear. Likewise, RecA displaces SSB assisted by χ-modified RecBCD (Anderson and Kowalczykowski, 1997) and, in eukaryotes, Rad51 removes RPA supported by Brh2, a BRCA2 homolog (Yang et al., 2005).

### 4.1.2 SSB Displacement During Filament Extension

The binding of a SSB tetramer to 3’ (dT)70 ssDNA tail of a partial dsDNA, a structure that would be produced in the double-strand break repair, results in high FRET ($E \sim 0.7$) in our solution condition (Figure 4.2A). This is likely caused by the wrapping of ssDNA around SSB observed both in crystallography and ensemble FRET studies (Kozlov and Lohman, 2002; Raghunathan et al., 2000). In comparison, FRET is very low in the absence of any protein ($E \sim 0.1$) and in the presence of RecA ($E \sim 0$) (Figures 4.2Bb, Bc). Once SSB binds to the ssDNA, it does not come off appreciably even after 1 hour in the absence of free SSB in solution (data not shown). Upon addition of RecA and ATP, SSB remains even after 1 hour (Figure 4.2Ad), indicating that SSB is extremely difficult to be displaced by RecA.

We wondered whether RecA might form a filament by replacing SSB if $\langle$dsDNA$|$ATPγS$>$, the pre-assembled nucleation cluster around the junction, is present. As illustrated in Figure 4.2B, adding RecA and ATPγS to the partial dsDNA results in very low FRET (Figure 4.2Bc). If we then add 10 nM SSB but remove RecA and ATPγS
from solution, SSB slowly binds to ssDNA by disassembly of $<\text{ssDNA}|ATP\gamma S>$ (Figure 4.2Bd), eventually returning to the sm-FRET histogram identical to what was obtained with SSB alone (Figure 4.2Ac) with a time scale of ~5 min (Figure 4.4; see its legend for details), suggesting SSB has replaced the RecA filament on the ssDNA. Strikingly, upon subsequent addition of RecA and ATP, the FRET peak shifts to a very low FRET within seconds (Figure 4.2Be), indicating efficient formation of a stable filament. Such rapid filament formation is in sharp contrast to the data obtained without the pre-assembled nucleation cluster (Figure 4.2Ad), where RecA and ATP could not displace SSB during a one-hour observation window. The detailed process by which RecA replaces SSB is shown in sm-time traces (Figures 4.2Ca-c). After the solution containing RecA and ATP is flowed in to replace the SSB solution at $t = 0$, FRET efficiency drops largely monotonically via diverse pathways, with a rate of 0.55 sec$^{-1}$ (Figure 4.2Cd).

We carried out a similar experiment but removed the DNA-bound SSB before adding RecA and ATP in order to find out how much SSB influences the formation/extension of a RecA filament. DNA-bound SSB was efficiently removed by adding 100 nM of 80-mer ssDNA (Figure 4.3Ae), which restored the FRET histogram into that of DNA-only ($E \approx 0.1$) (Figure 4.3Ab). When RecA and ATP were added, FRET became even lower (Figure 4.3Af). The detailed transitions are shown in Figure 4.3B. After RecA was added at $t = 0$, FRET dropped from $E \approx 0.1$ to 0 through two different ways; 1) FRET becomes high briefly, right before $E$ becomes 0 (Figure 4.3Ba), or 2) it changes monotonically (Figure 4.3Bb). The origin of the brief high-FRET state is not known at this point. The time taken for the transition was measured from 55 molecules, which gave the transition rate of ~0.8 (sec$^{-1}$) (Figure 4.3Bc), which is close to the value obtained in the presence of SSB, 0.55 (sec$^{-1}$) (Figure 4.2Cb). Note that the former rate is over-estimated since it reflects not only the filament extension but also the filament formation de novo, which makes the comparison even more plausible. It implies that SSB is displaced with little resistance by the extending end of the filament, therefore, the binding of SSB must be very weak compared to the force exerted during extension of a RecA filament.
**4.1.3 Molecular Mechanism of Two Contrasting Roles of SSB**

It has been long known that RecA can displace SSB from ssDNA (Kowalczykowski and Krupp, 1987). Using a pre-assembled nucleation cluster, here we provided the most comprehensive evidence that SSB is removed by an extending RecA filament. The rates of SSB removal and the unhindered extension rate of the RecA filament were found to be almost identical suggesting that the hindrance provided by SSB to a growing filament is minimal. Even though an unraveled section of ssDNA between the nucleation cluster and SSB must be very small, RecA could still bind and extend the filament with ease. Therefore, how SSB can function in two contrasting roles in relation to RecA is clearly explained; the ssDNA binding energy of SSB is smaller than that of the RecA filament so that SSB can be easily removed by an extending filament but the exposed length in SSB-saturated ssDNA is shorter than ~17 nt such that a *de novo* nucleation of a filament is inhibited (Figure 4.5).

Our result supports the proposal that a sufficient role for the RecA-loading machineries in helping RecA replace SSB is to provide a nucleation cluster-like structure to initiate RecA filament extension (Anderson and Kowalczykowski, 1997). If so, no active SSB-clearing mechanism is required for the RecA-loading machineries (Kowalczykowski, 2005). Crystallography revealed that the interface between BRCA2 and Rad51 bears a clear resemblance to the interface between neighbor Rad51 monomers in Rad51 filaments (Conway et al., 2004; Pellegrini et al., 2002). It is possible that the RecA-loading machineries, RecBCD (Spies and Kowalczykowski, 2006) and RecFOR, might have adopted the same strategy by emulating the surface of the filament that free RecA monomers in solution easily access and bind to.

**4.2 Interference with and by Helicases**

**4.2.1 RecA Filament Dynamics Around a Junction**

There were no dissociation events observed from <ssDNA|ATP> when its 5’-disassembly end was stabilized by <dsDNA|ATPγS> at 1 µM RecA (Figure 3.5Af), analogous to the
stabilization of the 5’-disassembly end by RecOR (Shan et al., 1997). We therefore expect that once a RecA filament forms assisted by RecA loading proteins, other proteins would not be able to access the ssDNA or the junction (Figure 4.6Aa).

In the absence of such stabilizing factors, for example, after the RecA-loading proteins have left the junction, a stretch of ssDNA next to the junction would become exposed due to the intrinsic instability of the 5’-disassembly end (Figures 4.6Ab, c). Since the FRET histogram of (dT)_{13+46} was broadly biased towards low FRET at 1 µM RecA (Figure 3.3Bb), the exposed ssDNA is shorter than ~13 nt on average. Stronger bias toward low FRET was observed with a longer segment between donor and acceptor as expected (Figures 4.6B, C). When RecA concentration was lowered to 0.1 µM, the histogram of (dT)_{13+46} shifted toward high FRET (data not shown), indicating that more than ~13 nt is routinely exposed.

Our data therefore suggest that ssDNA accessibility at the junction of a 3’ tailed dsDNA is modulated by RecA concentration. When the RecA concentration is above ~1 µM, the filament would not be immediately destroyed but would go through constant fluctuations of limited range (Figure 4.6Ab). Therefore, RecA would still retain to a large extent the capacity to perform its role, the degree of which would be higher than previously thought since the binding at the 5’-disassembly end is significant. On the contrary, at sub-micromolar concentrations the filament would disassemble swiftly (Figure 4.6Ac).

### 4.2.2 Interference with Rep and UvrD Helicases

Indeed, we find that the activity of Rep and UvrD helicases that unwind 3’-tailed dsDNA is affected by the presence of a RecA filament, the degree of which varies with different RecA concentrations. We have carried out ensemble FRET measurements as follow. We incubated 10 nM partial dsDNA with 18bp stem (5’-TGG CGA CGG CAG CGA GGC with the tail and its complementary strand 5’-Cy5-GCC TCG CTG CCG TCG CCA-3’) and (dT)_{13+46} tail at different RecA concentrations with ATP for 3 minutes (Figure 4.7A). Then we added either 500nM Rep or 100nM UvrD helicase with ATP and unlabeled 0.1-1µM 18mer ssDNA (5’-GCC TCG CTG CCG TCG CCA-3’) and observed how much
the unwinding activity is affected by the filament formed on ssDNA tail. As shown in Figure 4.7B, 1 µM RecA delays the Rep unwinding by ~100 sec. Similar, it slows down the UvrD unwinding; the higher the concentration of RecA, the slower the unwinding, as proposed (Figure 4.7C).

We have observed a similar behavior in sm-experiments as follows. We immobilized the same construct of DNA as above. After incubating it with 1 µM RecA and ATP for longer than 5 min, we introduced 100nM UvrD together with 1 µM RecA in solution. The unwinding occurred with the time scale of 15 sec (Figure 4.7D, navy), while it occurred much faster without a RecA filament formed (shorter than our measurement dead-time, ~5 sec) (data not shown).

Next, we wondered whether the inhibition by a RecA filament would be greater if the 5’-disassembly end is held by \(<\text{dsDNA}|\text{ATP}_{\gamma}\text{S}>\) which emulates the role of a RecA-loading protein (Figure 4.6Aa) and the configuration of which cannot be achieved in ensemble assays. After the same procedure as Figure 4.2B, we introduced 100nM UvrD together with 1 µM RecA in solution. Indeed the unwinding speed further decreased (Figure 4.7D, black). As a control, we measured unwinding speed with only \(<\text{dsDNA}|\text{ATP}_{\gamma}\text{S}>\) formed on DNA by practicing the same procedure as Figure 4.3Ab-Ae. In this case, the unwinding occurred faster than our measurement dead-time (<5sec), which indicates that UvrD easily removes the filament formed on dsDNA and the enhanced inhibition by RecA in Figure 4.7D (black) is mainly due to the connection between \(<\text{dsDNA}|\text{ATP}_{\gamma}\text{S}>\) and \(<\text{ssDNA}|\text{ATP}>\) (data not shown).

4.2.3 Interference by Repetitively Translocating Helicases

As for the opposite case, we consider how the activity of RecA is affected by other DNA-interacting proteins. *E. coli* Rep helicase is now known to repetitively translocate along a 3’ overhang ssDNA (Myong et al., 2005). Suspecting that such repetitive shuttling along the ssDNA may interfere with the filament formation, we monitored the RecA filament formation with Rep present and observed that the formation with 1 µM RecA was delayed substantially even at Rep concentrations as low as 1 nM (Figure 4.8).
What might be the biological role of the inhibition by the repetitive shuttling? *E. coli* remains viable even with the deletion of both Rep and UvrD if RecFOR machinery is defective. RecFOR is crucial in forming a RecA filament *in vivo*, so the lethality of the double deletion of Rep and UvrD may indeed arise from uncontrolled recombination via the RecA filament. On the basis of the present results, we propose that repetitive shuttling of Rep may be an effective means of keeping the ssDNA clear of unwanted proteins such as RecA.

### 4.3 RecA Filament Dynamics *in vivo*

Based on our findings in this chapter, we propose the following scenarios of protein interactions during the first steps of the double-strand break repair (see Figures 4.1, 4.5 and 4.6). When a blunt ended dsDNA is processed into a partial dsDNA, SSB binds to the 3’ ssDNA tail generated. Then, the RecA-loading machinery binds to the junction, recruits and holds RecA (Anderson and Kowalczykowski, 1997; Bork et al., 2001; Kowalczykowski, 2005; Morimatsu and Kowalczykowski, 2003; Pellegrini et al., 2002; Spies and Kowalczykowski, 2006; Umezu et al., 1993; Yang et al., 2005), nucleating a RecA filament. Additional RecA monomers bind successively to extend the filament in the 3’ direction, displacing SSB (Kowalczykowski and Krupp, 1987; Thresher et al., 1988). The filament is very stable not only because the 5’-disassembly end is held by the RecA-loading machinery (Shan et al., 1997) but also because the *C_c* of the 3’-extending end is much lower than the concentration of RecA in the basal level. Therefore, neither the ssDNA tail nor the junction is easily accessible for binding by other proteins. The 5’-disassembly end becomes unstable if the RecA-loading machinery leaves the junction, but because the basal level of RecA is still higher than the *C_c* of the 5’-disassembly end, only a small region of ssDNA near the junction becomes accessible to other proteins. Future experiments including the RecA-loading machineries may test these ideas directly.
Chapter 5

RecA-Mediated Strand Exchange

When a car breaks down in the middle of a road, a truck tows the car to a body shop.

RecA is an excellent truck driver for its speed but a so-so guide for its low fidelity in finding the best body shop.

5.1 Homologous Strand Exchange

The ssDNA within a RecA filament is stretched along the longitudinal axis of the filament (Egelman and Yu, 1989) (Figure 5.1Aa) and has the capability of seeking out and recognizing a dsDNA of a homologous sequence to initiate the strand exchange reaction (Kowalczykowski, 2000). The process of the homologous strand exchange is often divided into four steps: 7

i) **Pairing** between the ssDNA-filament and a homologous incoming dsDNA (Figure 5.1Ab)

ii) **Strand transfer** over a local region in which the embedded ssDNA in the filament replaces its equivalent in the incoming dsDNA (Figure 5.1Ac)

iii) **Branch migration** by the extension of the partial strand transfer (Figure 5.1Ad)

iv) The completion of the strand exchange by the disassembly of the RecA filament (Figure 5.1Ae)

Note that the distinction between the first two steps are not clear; the pairing (first step) might happen by the strand transfer over a local region (second step) (Figure 5.1B).

---

7 This chapter is from unpublished work of:
K. Ragunathan, C. Joo, and T. Ha (in preparation)
The intermediate ternary structure (i.e. involving three strands) that forms during the pairing has not been directly observed with any biochemical and structural methods due to the transient nature of this weakly interacting complex. It is likely that there are not intertwined interactions between all three strands but rather that there is only Watson-Crick base-pairing between the embedded ssDNA and its complementary ssDNA within the incoming dsDNA and that RecA merely lowers the activation energy barrier (Folta-Stogniew et al., 2004; Mazin and Kowalczykowski, 1998) (Figure 5.1B). Presumably, RecA achieves this by providing a binding site to the incoming dsDNA or by holding partially unzipped, incoming dsDNA (Mazin and Kowalczykowski, 1998). A similar idea but based on the entangled interactions between all three DNA strands has also been suggested (Bertucat et al., 1999; Zhurkin et al., 1994). Other researchers argue that the stretched structure of ssDNA inside the filament is crucial (Klapstein et al., 2004); however, it lacks of experimental evidence. FRET-approaches (Bazemore et al., 1997a; Folta-Stogniew et al., 2004; Xiao and Singleton, 2002) combined with sm-methods are ideally suited for detecting these transient intermediates during the reaction, without having to stall it.

After the pairing and/or the strand transfer over a local region, the part where the strand transfer has occurred propagates, which is called branch migration. The branch migration is known to occur with a certain directionality (5’ to 3’ end of the ssDNA filament), which behavior vanishes when non-hydrolysable ATP analog (ATPγS) is used (Jain et al., 1994). The origin of the directionality is still not known.

After the strand transfer, the displaced strand from the incoming dsDNA leaves the complex. (Figures 5.1Ac, Ad). It is likely that the displaced strand is weakly bound before it dissociates; however, it was impossible to observe it directly in real time due to its transient nature. In addition, it was proposed that SSB might capture this displaced strand, which will facilitate the strand exchange by preventing the reverse strand-exchange (Mazin and Kowalczykowski, 1998). It still remains to be tested.

We have developed smFRET assays to directly observe how a ssDNA-filament and its homologous dsDNA interact during the strand exchange. We attach donor and acceptor molecules at several different places of DNA (Figures 5.2-5.7) so that FRET...
reports on the different stages of the *strand exchange* reaction. We discover that the free magnesium ions regulate the *strand exchange* reaction by preventing the *displaced strand* from being dissociated in its absence. Such inhibition effect is completely lifted away when SSB is introduced. We plan to investigate the detailed structure of the intermediate ternary complex utilizing the three-color FRET assays.

### 5.2 Observation on Strand Exchange in Real Time

#### 5.2.1 Strand Exchange in Single-Molecule Level

As illustrated in Figure 5.2Aa, a RecA filament forms along ssDNA that is labeled with acceptor. dsDNA brings donor, and the completion of the strand exchange leads to a high-FRET state. We call it the “pairing assay” and carry it out by immobilizing ~100 pM of partial dsDNA which has 39-nt or longer ssDNA tail then by introducing donor-labeled homologous dsDNA into the solution. Though TIR is effective in reducing the background signal, we cannot introduce donor-labeled molecules of a concentration higher than ~1nM in solution since its contribution to the background becomes too serious, deterring proper sm-measurements. We initially doubted that the *strand exchange* reaction would occur when sub-nanomolar concentrations were used (100pM on surface, <1nM in solution); however, surprisingly there were a lot of reactions observed within a few minutes when we introduced 100-500pM dsDNA (39mer-I, TTT ACT TGT ACT TCA TTC ATT CAC ATT CCT ATC ATG TTT and its complementary strand) together with 1 µM RecA and 1 mM ATP (Chapter 3.1.2, Reaction Condition), therefore, we confirmed that a RecA filament is good at finding the homologous sequence for its speed.

#### 5.2.2 Pairing Assay

As shown in the sm-trace in Figure 5.2B, there is no fluorescence signal detected in the beginning since there is only acceptor in the immobilized complex (Figure 5.2Aa, state I).
Once a donor-labeled dsDNA arrives (*pairing*), there is sudden increase in total signal (Figure 5.2Ab, state II), which is followed by high FRET, which indicates the completion of the *strand transfer* (Figure 5.2Ac, state III).

The dwell time of ‘state II’ might represent how long it takes for a paired complex to complete the *strand transfer* (or at least around where the dyes are placed at). Figure 5.2C shows the distribution of the dwell time, where a group of molecules show ~110 msec of a lag and the other do not show any delay. A similar distribution was seen when the reaction at the other end of the filament was watched (Figure 5.2Da). To accurately understand the nature of the state II, we have carried out several different control experiments as follows.

First, we varied the length of the *embedded ssDNA* tail (39, 39+dT30, 39+dT60) while keeping the length of the *incoming dsDNA* fixed. With that, we did not see any change of the dwell time within error (Figure 5.2Db). If the “lag-phase” were due to lateral diffusion of the *incoming dsDNA* along the ssDNA-filament after *pairing*, we would have seen the dwell time become longer with a longer tail. Therefore, the independence of the tail length rules out the possibility of lateral diffusion in our system. Concerned that the lag-phase might be unique only to a certain sequence, we designed another sequence of DNA (39mer-II, ATG AAC GTC GCG GGT GAT CTG AAT ATC AAT CTC TAA GCT; Figure 5.2Dc); however, we did not observe any significant difference. Third, because the stability of the filament is known to be affected by sodium ions, we tried different sodium concentrations only not to see any change (data not shown). Finally, when ATPγS was used instead of ATP, we did not see any change either (Figure 5.2Dd).

We did observe increase when we introduced mismatch in the sequence between the *embedded ssDNA* and the *incoming dsDNA*. The mismatch of 9nt was placed at either end of the ssDNA, then, the dwell time increased 3-4 times (Figure 5.2De). We also observed increase of the dwell time (5-fold, data not shown) when we used 1mM Mg\(^{2+}\) instead of 10mM Mg\(^{2+}\) together with 1mM ATP (i.e. no magnesium ions free from the ATP-Mg complex).

To investigate the molecular mechanism of the lag-phase, we have designed another type of a *pairing assay*, in which it is a ssDNA-filament that approaches the
immobilized complex, here dsDNA (Figure 5.2Ea). As expected from the slow diffusion of the ssDNA-filament (the ssDNA-filament is 5 times thicker and 1.5 times longer than dsDNA), the reaction yield dropped dramatically (data not shown). Intriguing was the increase in the dwell time (5-10 times; Figure 5.2Eb). It may imply that the lag-phase represents a transition which requires the relative rotational motion between the ssDNA-filament and the incoming dsDNA, which is directly affected by the diffusion constant. Note that the lateral diffusion is not considered since it was not detected in Figure 5.2Db.

5.3 Regulation by Magnesium Ions

5.3.1 Role of Free Magnesium Ions

*Strand exchange* occurs only in the presence of free magnesium ions. Recently, it was found that the regulation is due to the conformational change of the C-terminal domain induced by the free magnesium (Lusetti et al., 2003). What still remains unclear is its molecular mechanism: in which step of strand exchange the regulation kicks in. Here, we show that the regulation is involved with *post-strand transfer* and also it is lifted away by a SSB protein.

5.3.2 Separation Assay

We have designed an assay in which FRET reports on how incoming dsDNA gets separated during strand exchange (Figure 5.3). An unlabeled ssDNA-filament is immobilized on a surface (Figure 5.3Aa, state I) and a high-FRET signal is observed when a doubly-labeled incoming dsDNA pairs up with it (Figure 5.3Ab, state II). At the end, the acceptor-labeled displaced strand leaves the complex, therefore, there is no FRET observed (Figure 5.3Ad, state IV). What we can uniquely observe via a sm-study is the transient, state III (Figure 5.3Ac), where fluctuation in FRET is seen. We interpret it as a ternary complex, in which the displaced strand is weakly bound before leaving.
From this assay we can tell how long it takes for the incoming dsDNA to be unwound after the pairing. For instance, if the entangled interaction between all three strands is the key in the strand exchange, the unwinding speed (inverse of the dwell time of state II) would not be the same with the strand transfer rate (inverse of the dwell time of state II in pairing assay, Figure 5.2B) (Bertucat et al., 1999; Zhurkin et al., 1994). Further data acquisition is required for reliable data analysis yet.

### 5.3.3 Role of SSB

When we measured the combined dwell time of states II and III, we found that there was more than an order of magnitude difference in it with and without free magnesium ions (Figure 5.3C). That is, the displaced strand cannot leave the complex after the strand transfer without the assistance of the free magnesium ions. Therefore, the regulation by the free magnesium ions must be involved with the post-strand exchange. This is further supported by the normal high yield which the strand transfer-sensitive pairing assay shows even in the absence of free magnesium ions (data not shown).

We wondered whether SSB might relieve this suppression because SSB might be able to grab the displaced strand stuck in the complex. As shown in Figure 5.3C, the presence of SSB lowered the dwell time back to normal, therefore, indicating that the displaced strand has been captured and removed away by SSB very effectively. With that, we conclude that the role of the free magnesium is to finalize the homologous strand exchange, which can be substituted by SSB.

### 5.4 Observing the Interaction Between Three Strands Simultaneously

#### 5.4.1 Three-Color FRET Assay

Since there are three strands involved in the strand exchange reaction, a conventional two-color FRET leaves some ambiguities even when several different combinations of
labeling positions are used sequentially. By increasing the information content using the three-color FRET method (Chapter 2.5, Single-Molecule Three-Color FRET), we anticipate that we will be able to resolve ambiguities and propose a better model for the transient interactions among the three strands.

5.4.2 Preliminary Data

Before we go into a three-color FRET assay, we present another two-color assay. In this configuration, donor and acceptor molecules are attached to the opposite ends of the ternary complex thus there is no significant amount of FRET after the strand exchange has completed (Figure 5.4Ac). However, about 10% of molecules show a very brief period of a high-FRET state between the pairing and the completion of strand transfer (Figure 5.4Ab, state II).

While further investigation is required to understand the origin of this unexpected, transient high-FRET state, we have designed a three-color FRET assay (Figure 5.5) combining this and the pairing assays. The signal from Cy5.5 reports as the pairing assay did and that from Cy5 does as the assay above did. An example trace in Figure 5.5B show that Cy5 and Cy5.5 signals are exclusively anti-correlated, indicating that the state II in Figure 5.4 represents a certain reaction occurring before the strand transfer.

5.4.3 Observing the Intermediate Structure During Pairing

With another three-color FRET assay (Figure 5.5C), we propose to test whether the pairing occurs via triplex-DNA formation (Bertucat et al., 1999) or Watson-Crick base-pairing (Folta-Stogniew et al., 2004; Mazin and Kowalczykowski, 1998). If there is only Watson-Crick base-pairing involved, only two (not three) strands are base-paired locally, therefore, there will be distinct high-FRET states of Cy5 and Cy5.5 showing up in different times. If not, only Cy5.5 signal will be observed all the time since all three dyes are close to each other before Cy5 leaves.
5.5 Ensemble Measurements

Insight frequently comes when data measured with different techniques are compared such as those from ensemble and sm-FRET measurements. We have carried out the ensemble measurements with RecA and with the similar set of the DNA strands of the pairing assay (Figure 5.6A). We incubate 5 nM ssDNA (78mer with accepter at T*; 5’-TGG CGA CGG CAG CGA GGC AAA CAT GAT AGG AAT GTG AAT GAA TGA AGT ACA AGT AAA TT* T TTTTTT TTT TTT TTT TTT-3’) in a solution that contains 1 µM RecA or 4 µM RadA, 1 mM ATP, 0.1 mg/ml BSA, 1% v/v βME, 100 mM NaAc and 1-10 mM MgAc in a buffer that contains 25 mM Tris-Ac (pH 7.5) for about three minutes. Then we initiate the reaction by mixing it with 15 nM of 39mer dsDNA (5’-Cy3-TTT ACT TGT ACT TCA TTC ATT CAC ATT CCT ATC ATG TTT-3’, and its complementary sequence).

5.5.1 RecA-Mediated Strand Exchange

When we measure the FRET change by watching the change in donor and acceptor signal in bulk, we observe that FRET reaches ~0.5 in 2 min (Figure 5.6Ba, green) in a standard reaction condition (Chapter 3.1.2 Reaction Condition). As mentioned earlier (Chapter 5.3 Regulation by Magnesium Ions), the deprivation of free magnesium ions inhibits the strand exchange reaction, which is observable in Figure 5.6Ba (blue). Not only the reaction becomes slow (τ~5min), but also the reaction yield drops significantly (final FRET ~ 0.1).

Since such inhibition is removed when the C-terminal domain of a RecA protein is truncated (Lusetti et al., 2003), we have tried a mutant of RecAΔC17 (a gift from M. Cox in University of Wisconsin at Madison), which lacks of 17 amino acids at its C-terminus. As shown in Figure 5.6Ba (red and black), the mutant carries the reaction very well both with and without free magnesium ions (τ~0.6 and 1.6min respectively). In addition, RecAΔC17 makes the reaction occur faster.
RecA not only mediates the strand exchange but also facilitates annealing. The traces in Figure 5.6Bb show that the annealing occurs very rapidly and does not depend on the free magnesium ions at least in our time resolution.

5.5.2 RadA-Mediated Strand Exchange

We have carried out the same experiment with *Methanosarcina acetivorans* RadA (RecA archaeal homolog) with *Methanosarcina acetivorans* RPA1 (SSB archaeal homolog) at higher temperature, 50°C. The concentrations of ssDNA and dsDNA are 60 and 30nM, respectively. When RPA1 was included in the assay, it was added 2 min before RadA was introduced. ⁸

Figure 5.7A (No RPA1 WT) shows the reaction with RadA alone, which reaches FRET ~0.3. Upon adding different amount of RPA1, the reaction yield drops, though the reaction rate (k = 0.29 min⁻¹) does not (Figure 5.7A), which implies that RPA1 competes the ssDNA binding site with RadA.

To further investigate the binding property of RPA1 on ssDNA, we have tried different truncated versions. RPA1 has four predicted OB-folds. As shown in Figure 5.7Ba, we tested mutants with three different degrees of truncation from the C-terminus (RPA1ΔC1, RPA1ΔC2 and RPA1ΔC3) and observed that the inhibition by RPA1 becomes weaker with longer truncation. In contrast, mutants truncated from the N-terminus did not show either the strong inhibition or clear dependence on the truncation lengths (Figure 5.7Bb, RPA1ΔN1, RPA1ΔN2 and RPA1ΔN3), which suggests the importance of the first OB-fold. Neither did the mutant that was truncated from both sides (Figure 5.6Bb, RPA1ΔC1ΔN1).

---

⁸ This section is from unpublished work of:
Chapter 6

Energy Landscape of Holliday Junction Dynamics

After the RecA-mediated homologous strand exchange, Holliday junction structure emerges (Figure 1.3). Holliday junction is described as a four-stranded DNA structure, forming four helices joined in the middle (Figure 1.5). We use smFRET method to demonstrate conformer exchange in a direct manner and measure the rates of interconversion.

6.1 Experimental Procedure

We have studied 4H DNA junctions with four perfectly base-paired arms each of 11 bp. We have measured FRET between donor and acceptor fluorophores attached to the ends of different helical arms to distinguish different junction conformations as shown schematically in Figure 6.1A. The conformational interconversion of these vectors has been analyzed using smFRET spectroscopy, whereby the junction molecules were immobilized onto a glass surface via biotin covalently attached to the end of one arm that is free from fluorophore conjugation.⁹

⁹ The work in Chapter 6 has been published as a paper:
6.1.1 Sample Construct

Fluorescent vectors are named according to the arms carrying the donor and acceptor in that order; for example the XB vector carries Cy3 on the end of the X arm, and Cy5 on the end of the B arm. We have used a number of vectors in this study to report on the various possible conformations of the junction.

**XB vector** (Figure 6.1A)

The donor-acceptor pair should be physically close (and therefore result in elevated energy transfer) only for the antiparallel conformer of the X on R stacked form. Conversion to the alternative (X on B) stacking conformer leads to separation of the fluorophores, and thus lowered energy transfer. The vector BX (Figure 6.1B) has the fluorophores reversed with respect to XB, and should behave equivalently.

**XR vector** (Figure 6.1C)

The fluorophores will only be close in the antiparallel form of the alternative stacking conformer with X on B stacking.

6.1.2 Reaction Condition

Unless otherwise specified, all measurements were made in a 10 mM Tris-HCl, pH 8.0, oxygen scavenger system (0.4% (w/v) glucose, 1% (v/v) βME, 1 mg/ml glucose oxidase and 0.04 mg/ml catalase) with specified amounts of MgCl₂, NaCl, Co(NH₃)₆Cl₃ and EDTA. Unless specified, the same condition was also used in Chapter 7.

6.1.3 DNA Preparation

Vectors of junction 1 were prepared as following. Oligonucleotides of the following sequences were purchased from IDT DNA. All the strands were purified by PAGE.

- J1b is 5'-Cy5-CCC TAG CAA GCC GCT GCT ACG G
- J1Lb, 5'-CCC TAG CAA GCC GCT GCT ACG G
- J1Rb, 5'-Cy3-CCC TAG CAA GCC GCT GCT ACG G
- J1h, 5'-CCG TAG CAG CGA GAG CGG TGG G
J1Th, 5'-Biotin-CCG TAG CAG CGA GAG CGG TGG G
J1Qh, 5'-Cy5-CCG TAG CAG CGA GAG CGG TGG G
J1r, 5'-Biotin-CCC ACC GCT CTT CTC AAC TGG G
J1Tr, 5'-Cy5-CCC ACC GCT CTT CTC AAC TGG G
J1x, 5'-Cy3-CCC AGT TGA GAG CTT GCT AGG G
J1Rx, 5'-Cy5-CCC AGT TGA GAG CTT GCT AGG G
J1Ax, 5'-CCC AGT TGA GAG CTT GCT AGG G

Each junction is constructed by the hybridization of four strands — b, h, r and x.

- Junction 1-XB comprises strands J1b, J1h, J1r and J1x,
- Junction 1-XR comprises J1Lb, J1Th, J1Tr and J1x,
- Junction 1-BX comprises J1Rb, J1h, J1r and J1Rx, and
- Junction 1-BR comprises J1Rb, J1Th, J1Tr and J1Ax.

The four oligonucleotides were mixed with the ratio of 1:2:2:2 for Cy3-, Cy5-, biotin-labeled and unlabeled strands, respectively, in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, with a Cy3-strand concentration of approximately 2 µM. The mixture was cooled from 90 to 24 °C over 3-4 h. Annealed junctions were stored at -20 °C. Junction 7 was prepared analogously (McKinney et al., 2003).

### 6.1.4 Data Acquisition and Analysis

The data were acquired with the confocal microscopy (Chapter 2.1.4 Confocal Microscopy). The locations of junctions were manually identified in images scanned. The fluorescence signals of the chosen molecules were recorded for 15-30 sec. Junctions with four perfectly base-paired arms should exhibit an anti-correlated behavior with respect to signals of Cy3 and Cy5 during conformational change or when Cy5 photoblinking / photobleached, and the photobleaching of Cy3 or Cy5 should be one step process. These criteria select 10-50 % out of all data recorded. For each chemical condition, about 5-20 molecules were analyzed. The set of characteristic time (τ) for a given condition gave the average and the standard deviation as represented on figures. The standard deviation of τ reflects the statistical, local and possibly intrinsic heterogeneity of molecules in a given condition.
6.2 Conformational Exchange Between Stacking Conformers

6.2.1 Dynamics of Junction 1 with Magnesium Ions

Ensemble experiments indicated that junction 1 adopts predominantly the antiparallel isoI stacked X-structure (Duckett et al., 1988). We chose the XB vector for initial study which should exhibit high FRET efficiency for isoI (Figure 6.1A). Figure 6.2A shows a time record of donor and acceptor fluorescence signals of a single junction 1 molecule in the presence of 10 mM Tris-HCl, pH 8.0, 30 mM Mg²⁺, and 50 mM Na⁺. I_D and I_A exhibit two-state fluctuations that are clearly anti-correlated. Figure 6.2B shows the histogram of the apparent FRET efficiency E_app. The E_app histogram has two peaks corresponding to the two stacking conformers with a strong bias toward the higher FRET state. We assign the E_app = 0.69 state as isoI and the E_app = 0.15 state as isoII, as indicated in Figure 6.1. The forward and backward stacking conformer transition rates (k_I→II and k_II→I) were obtained through a combination of cross-correlation analysis and the ratio between numbers of data points corresponding to each FRET state (Chapter 2.4.3 Transition Trajectory Analysis). We obtained k_I→II = 9.3 s⁻¹ and k_II→I = 28 s⁻¹, averaged over 41 molecules. Thus junction 1 significantly favors the isoI conformer as expected (Duckett et al., 1988).

In our previous study of junction 7 we discovered that the conformer transitions became faster as the Mg²⁺ concentration was reduced. The conformer transition of junction 1 exhibits the same dependence on the concentration of Mg²⁺ ions. Comparison of the FRET efficiency histograms in Figures 6.2B and D shows that the ratio between isoI and isoII remained constant regardless of Mg²⁺ ion concentration. This behavior was true for all concentrations of Mg²⁺ ions, as long as two FRET states were distinguishable in the histogram. Thus Mg²⁺ ions must stabilize both stacking conformers equally by screening of electrostatic repulsion between phosphate groups around the point of strand exchange. Stacking conformer transitions require the disruption of base stacking at the
point of strand exchange, and should become slower if the stacked structure is stabilized by elevated Mg\(^{2+}\) ion concentration.

In order to study the fast dynamics under more physiologically relevant conditions, cross-correlation analysis was used to provide accurate values of the conformer transitions rate \(k_{CT}\), where \(k_{CT} \equiv k_{I\rightarrow II} + k_{II\rightarrow I}\). Figure 6.2G shows how the correlation time \((\tau = k_{CT}^{-1})\) changes with different Mg\(^{2+}\) ion concentrations. The resulting \(k_{CT}\) rates (each an average of ~ 10 molecules) are plotted as a function of ionic conditions in Figure 6.2H. Error bars represent the heterogeneity in \(k_{CT}\) values between molecules (Chapter 6.1.4 Data Acquisition and Analysis).

As illustrated in Figure 6.1A, while only \(iso I\) has high FRET, low FRET states might result from any of the other conformations including the \(iso II\), open and parallel forms (Chapter 7). In contrast to the XB vector, high FRET efficiency is expected for the \(iso II\) conformer of the XR vector (Figure 6.1C). If the low FRET states of the XB vectors arise only from \(iso II\), XR vectors should show the same transition rates of junction dynamics regardless of their different labeling. In Figure 6.2H, transition rates measured for XB (triangles) and XR (squares) vectors fall on a single line with deviations that are small compared to the error bars, indicating the low FRET state of XB vectors mainly consists of \(iso II\) in the presence of high Mg\(^{2+}\) ion concentration. In addition, this indicates that there is no significant effect of fluorophores or attachment position on junction dynamics.

Taken together with our previous studies of junctions 3 and 7 (McKinney et al., 2003), the new data on junction 1 show that the two-state FRET fluctuations observed represent stacking conformer transitions, and provide further evidence of the sensitivity of the conformation of the four-way junction to the sequence immediately at the point of strand exchange (Table 1).
6.2.2 Dynamics of Junction 7 with Monovalent Ions Alone

If the role of Mg\textsuperscript{2+} is primarily in the screening of electrostatic interactions, high concentrations of monovalent ions may achieve the same effect as Mg\textsuperscript{2+}. Indeed, in previous studies the stacked-X structure has been observed in the presence of monovalent ions only (e.g., 1 M Na\textsuperscript{+}) (Duckett et al., 1990). We have therefore used smFRET spectroscopy to examine whether the stacking conformer transitions can be observed in Na\textsuperscript{+} ions alone, how Na\textsuperscript{+} ion concentration affects the transitions rates, and whether the conformer bias seen in Mg\textsuperscript{2+} is maintained. The transition rate of junction 7 is lower than that of junction 1 (Table 1) thus measurements on junction 7 are more accessible. So, junction 7 was used to examine the ionic effects of monovalent and trivalent ions.

We studied the HB vector of junction 7 at high Na\textsuperscript{+} concentrations (400 mM to 2 M) in the absence of Mg\textsuperscript{2+} ions. The buffer included 2.5 or 5 mM EDTA to chelate any residual divalent ions. Figure 6.3A shows the time record of a single junction 7 molecule in the presence of 1.5 M Na\textsuperscript{+}. The corresponding $E_{\text{app}}$ histogram (Figure 6.3B) comprises two peaks, although there is broad overlap that probably arises from time-averaging due to fast exchange between states. $iso\text{I}$ is favored over $iso\text{II}$ by 3 to 2, in contrast to the 1 to

<table>
<thead>
<tr>
<th>Ion</th>
<th>Junction 1 50 mM</th>
<th>Junction 1 50 mM</th>
<th>Junction 3 50 mM</th>
<th>Junction 7 1 M Na\textsuperscript{+}</th>
<th>Junction 7 2 M [Co(NH\textsubscript{3})\textsubscript{6}]\textsuperscript{3+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso I (%)</td>
<td>77.9</td>
<td>22.6</td>
<td>48.3</td>
<td>64.3</td>
<td>61.3</td>
</tr>
<tr>
<td>iso II (%)</td>
<td>22.1</td>
<td>77.4</td>
<td>51.7</td>
<td>35.7</td>
<td>38.7</td>
</tr>
<tr>
<td>$k_{\text{CT}}$ (s\textsuperscript{-1})</td>
<td>20.1</td>
<td>15.5</td>
<td>11.8</td>
<td>60.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Sequence</td>
<td>CC GA</td>
<td>GG CT</td>
<td>CC GC GG GC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Junction 7 50 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion</td>
<td>Mg\textsuperscript{2+}</td>
</tr>
<tr>
<td>iso I (%)</td>
<td>77.9</td>
</tr>
<tr>
<td>iso II (%)</td>
<td>22.1</td>
</tr>
<tr>
<td>$k_{\text{CT}}$ (s\textsuperscript{-1})</td>
<td>20.1</td>
</tr>
<tr>
<td>Sequence</td>
<td>CC GA</td>
</tr>
</tbody>
</table>

\textsuperscript{a} from our previous study
\textsuperscript{b} in the presence of 50 mM Na\textsuperscript{+}
\textsuperscript{c} central sequence at the point of strand exchange
1 distribution observed in the presence of Mg$^{2+}$ ions (Table 1). The transition rates averaged over 13 molecules were $k_{I\rightarrow II} = 24$ s$^{-1}$ and $k_{II\rightarrow I} = 36$ s$^{-1}$.

Figure 6.3E shows transition rates for junction 7 measured as a function of Na$^+$ ion concentration from 400 mM to 2 M. As in the case of Mg$^{2+}$, increased Na$^+$ ion concentration in this range reduced the rate of the conformer transitions, while leaving the ratio between $iso_I$ and $iso_{II}$ unchanged (data not shown). Therefore, the effect of Na$^+$ ions appears to be very similar to that of Mg$^{2+}$, except that much higher concentrations are needed to stabilize the stacked structures.

### 6.2.3 Dynamics of Junction 7 with Hexammine Cobalt (III)

Ions

Hexammine cobalt (III) ions have a similar octahedral geometry to Mg$^{2+}$ ions, but differ in two important respects. The charge on the ion is +3, and the NH$_3$ ligands of the cobalt ion are very good H-bond donors. These ions are highly efficient in promoting structural transitions in DNA, including stabilizing left-handed Z-DNA (Gessner et al., 1985) for example. Previous studies have demonstrated the formation of the stacked X-structure in micromolar concentrations of (Co(NH$_3$)$_6$)$_3^+$ ions (Duckett et al., 1990). We tested whether the stacking conformer transitions can be observed in (Co(NH$_3$)$_6$)$_3^+$ ions, how the transition rates are affected by the concentration of the trivalent ion, and whether the conformer bias observed in the presence of Mg$^{2+}$ and Na$^+$ ions is maintained.

Figure 6.3C shows a time record of FRET efficiency of junction 7 in the presence of 2 mM (Co(NH$_3$)$_6$)$_3^+$, 50 mM Na$^+$, 0.1 mM EDTA. The corresponding $E_{app}$ histogram (Fig. 6.3D) contains two peaks. The $iso_I$ conformation (lower FRET state) is favored over $iso_{II}$ by 3 to 2 in contrast to the 1:1 partitioning observed in Mg$^{2+}$ solution, (Table 1) consistent with previous studies using gel electrophoresis (Grainger et al., 1998). The transition rates averaged over 16 molecules were $k_{I\rightarrow II} = 28$ s$^{-1}$ and $k_{II\rightarrow I} = 42$ s$^{-1}$.

Figure 6.3F shows transition rates measured from junction 7 as a function of (Co(NH$_3$)$_6$)$_3^+$ ion concentration in the range from 0.1 to 2 mM in the presence of 50 mM Na$^+$ and 0.1 or 0.25 mM EDTA. Increased (Co(NH$_3$)$_6$)$_3^+$ concentration decreased the
conformational transition rates, similar to the effects of Mg$^{2+}$ and Na$^+$ ions. In addition, the ratio between $isoI$ and $isoII$ was constant over this range of (Co(NH$_3$)$_6$)$_3^+$ concentration, as it was with other ions (data not shown). Therefore, while the (Co(NH$_3$)$_6$)$_3^+$ ion promotes the formation of the stacked X-structure of the DNA junction at much lower concentrations, the effects on the dynamics of the interconversion are closely similar to those of other metal ions.

6.2.4 Competition and Cooperation Between Mono- and Divalent Ions

In an earlier study (McKinney et al., 2003), we found that the rate of stacking conformer transitions occurring in Mg$^{2+}$ ion-containing solution was accelerated by the addition of 50 mM Na$^+$ ions. However, we have seen that high concentrations of Na$^+$ as the only cation slows the rate of transitions (Figure 6.3E). Na$^+$ ions thus play dual roles in determining conformational dynamics, and it is therefore expected that the interconversion rate in the presence of Mg$^{2+}$ ions will exhibit a non-monotonic dependence on Na$^+$ ion concentration, with a maximum rate at some concentration. Figure 6.4 shows the dependence of transition rates on Na$^+$ ion concentration in the presence of three different background Mg$^{2+}$ ion concentrations of 2, 5 and 20 mM. Clearly, the overall trend is non-monotonic, with a maximum rate in each case. For example, at 5 mM Mg$^{2+}$, addition of Na$^+$ ions to a concentration of 300 mM led to a marked increase in the transition rate. Beyond 300 mM Na$^+$, however, further increase in Na$^+$ ion concentration led to a reduction in rate. This non-monotonic trend was observed for all Mg$^{2+}$ concentrations examined, and indicates that the stability of the stacked structures is not a simple function of ionic strength. Evidently, at low concentrations, Na$^+$ ions screen the interaction between Mg$^{2+}$ ions and the junction, reducing the conformer stability and hence accelerating the dynamics. At high concentrations, Na$^+$ ions play a similar role as Mg$^{2+}$ ions, and the two cooperate to stabilize the stacked structures.
6.3 Sequence and Ion Dependence

The conformational dynamics of four-way DNA junctions are affected by the base sequence at the point of strand exchange, and the nature and concentration of metal ions present (Table 1). Although junctions 1, 3 and 7 differ only in the identities of the nucleotides located immediately at the point of strand exchange, they exhibit markedly different conformational properties. In the presence of Mg$^{2+}$ ions, junction 1 exists predominantly in the $isoI$ stacking conformer; junction 3 favors $isoII$, while junction 7 favors neither significantly. The type of cation present may also affect the bias; while junction 7 has equal populations of $isoI$ and $isoII$ conformations in the presence of Mg$^{2+}$ ions, $isoI$ is favored in Na$^+$ or hexammine cobalt (III) ions. However, the concentration of a given cation does not affect the conformer bias.
Chapter 7
Exploring Rare Conformational Species in Holliday Junctions

In this work, we have used smFRET to probe for the existence of minor species, the anticipated open intermediate of conformer transitions, and the parallel conformations of the stacked form of the junction. We find that the population of parallel species is exceedingly low, and the open species is achieved very frequently, hundreds of times per second under physiologically relevant conditions even though it is too short-lived to be detected directly. And we find that the movements of the four arms are synchronized to each other during exchange between the two stacking conformers. 10

7.1 Short-Lived Open Conformation

In order for the conformer transitions to occur, base stacking at the exchange point has to be disrupted, suggesting that the transition trajectory should pass through the open structure that is stable in the absence of divalent ions (Figure 7.1). The same open species should lie on the trajectory for spontaneous branch migration; however, in that case the base pairing must also be disrupted. In the branch migration process the open structure is not therefore likely to be the transition state, but instead must be a high energy intermediate. It is therefore likely that the open structure is a common intermediate in

10 The work in Chapter 7 has been published as papers:
C. Joo et al. ‘Exploring rare conformational species and ionic effects in DNA Holliday junctions using single-molecule spectroscopy’ J Mol Biol 341: 739-51 (2004) and
both stacking conformer transitions and branch migration. This then raises the question of whether the open structure might be detected as a stable structure of measurable lifetime.

7.1.1 Transitions Between Open and Stacked Conformations

We studied the XB vector of junction 1 as the Mg\(^{2+}\) ion concentration was reduced, seeking the observation of a new peak emerging between the two peaks in the \(E_{\text{app}}\) histogram shown in Figures 6.2B and D. However, instead of revealing a new peak, the two peaks merged into a single peak as the Mg\(^{2+}\) concentration was reduced (Figure 6.2F). Such behavior can be fully explained if the lifetime of the states is less than the time resolution, leading to averaging of FRET values.

As an alternative way of seeking the open structure, we performed experiments at low Mg\(^{2+}\) concentrations, where conformer transitions are not fully resolved. Figure 7.1C shows the time-averaged \(E_{\text{app}}\) values measured from single molecules of the XB vector of junction 1 at Mg\(^{2+}\) ion concentrations between 1 \(\mu\)M and 1 mM, in the absence of Na\(^{+}\) ions (circles). Since junction 1 is biased strongly toward to \textit{isol} (high FRET state), \(E_{\text{app}}\) averaged over multiple transitions is expected to be high, close to the \(E_{\text{app}}\) of \textit{isol}, as long as junction 1 is folded into stacked conformations. This appears to be the case at Mg\(^{2+}\) ion concentrations above 100 \(\mu\)M Mg\(^{2+}\). By contrast, at sufficiently low Mg\(^{2+}\) concentrations, the junction would exist primarily in the open structure with \(E_{\text{app}}\) value significantly below that of \textit{isol}, which is the case below 10 \(\mu\)M Mg\(^{2+}\). In between these extremes, in the range 10 - 100 \(\mu\)M Mg\(^{2+}\), a clear and gradual transition in the average \(E_{\text{app}}\) is observed, which we interpret as the transition between open and stacked structures. This interpretation is consistent with earlier ensemble studies (Duckett et al., 1990) that showed that stacked X-structures become stable at \(\sim\) 80 \(\mu\)M Mg\(^{2+}\). A control experiment with the XR vector did not show significant changes in \(E_{\text{app}}\) (squares in Figure 7.1C; see also Figure 7.1B) ruling out possible Mg\(^{2+}\)-dependent effects on fluorophore properties as the source for the observed transition.
7.1.2 Frequently Visited Open Conformation

The fit in Figure 7.1C is made to the Hill binding model with the Hill coefficient of 1. Although the fit can not be interpreted as the site binding of one magnesium ion, the excellent agreement with data suggest that the transition from open to stacked species is relatively gradual, instead of being sharp and highly cooperative. Therefore it is likely that the open state is significantly populated and frequently visited even in higher magnesium concentrations and is probably a stable intermediate during conformer transitions. However, we cannot estimate the lifetime of the open intermediate directly because of limited time resolution and signal level. As a note: recently, our lab found out that the structure of the open conformation is asymmetric tetrahedral, via molecular dynamics simulation (Yu et al., 2004) and via the combination for force and FRET spectroscopy (Hohng et al, unpublished work), which might explain why the FRET values in the open form are slightly different between the vectors XB and XR in Figure 7.1C.

7.2 Non-existence of Parallel Conformation

Original models of Holliday junction were almost exclusively in a parallel conformation, and it therefore came as a surprise when the first structural data indicated that the four-way DNA junction in free solution preferentially adopted an antiparallel conformation (Duckett et al., 1988; Murchie et al., 1989). This bias was later shown to be true in the crystal too (Eichman et al., 2000; Ortiz-Lombardia et al., 1999). Nevertheless, it is possible that in solution the junction might exist in a parallel conformation during some small fraction of the time, such that it would be undetectable in ensemble measurements. We therefore set out to try to detect this conformation by sm-spectroscopy, directly exploring the possibility that parallel conformations might exist in some small proportion.
7.2.1 No Observation of Parallel Conformation

We studied the BR vector of junction 1.

**BR vector** (Figure 7.2A)

The fluorophores will be close in either of the parallel forms. It will thus identify parallel conformers as a group, but not distinguish between stacking conformers. The fluorophores are attached to the alternative pair of helices in the XH vector, which would therefore be expected to behave equivalently to BR.

Time traces for this vector (Figures 7.2B and D) exhibited a constant level of $E_{\text{app}}$ (~0.3) without any transition to high $E_{\text{app}}$ values, and lacking any anticorrelation between donor and acceptor intensities (Figures 7.2C and E) at a time resolution of 5 ms in several different solution conditions, i.e. 1, 10 and 50 mM Mg$^{2+}$ with 50 mM Na$^+$, 30 and 50 mM Mg$^{2+}$ without Na$^+$, 50 and 1000 mM Na$^+$ without Mg$^{2+}$.

A quantitative limit on the existence of parallel conformations was established by examining the BR vector in the presence of 30 mM Mg$^{2+}$ with no Na$^+$ ions, at 5 ms time resolution. No transition to a higher FRET state was observed within a total observation time of 50 s measured from 23 molecules. Therefore, a stable parallel conformation with a lifetime greater than 5 ms must be exceedingly rare, with a probability lower than 0.01%. We have performed the same measurements on the XH vector, which should also be sensitive to the presence of parallel conformations. In addition, no evidence of parallel conformations was obtained for this vector in the presence of 50 mM Mg$^{2+}$ at 6 ms time resolution (Figures 7.2F and G).

It is still possible that parallel conformations exist with lifetimes shorter than 5 ms. With time resolution less than 5 ms, we did see short-lived high FRET states, which could correspond to short-lived parallel forms. But, as shown in Figures 7.2C, E and G, cross-correlation analysis with 1-2 ms time resolution shows no anti-correlation. And, at a low temperature (2 °C), the lifetimes of short-lived high FRET states of junction 1 did not increase (data not shown). From these observations, the short-lived high FRET states of less than 5 ms are unlikely to arise from a population of parallel forms, but are rather due to shot noise or some other artifact.
In contrast to these properties of DNA junctions, our lab has observed a significant population (~25%) of the parallel form of a four-way RNA junction, using equivalent labeling strategy in sm-experiments (Hohng et al., 2004b). The failure to detect the parallel conformation of the DNA junction is therefore unlikely to be an artifact of the experimentation, and we conclude that its population is vanishingly small for four-way DNA junctions.

7.2.2 No Observation of Parallel Conformation by a Three-Color FRET Assay

This study with the BR and XH vectors strongly indicate the lack of parallel states longer than 5-6 ms. However, one of the most time-consuming and subjective steps in this study was to discern a “real” molecule, a complete four-way junction with all intended fluorophores, from other contaminants such as incomplete molecules or impurities. This can be a common problem when one tries to detect a very rare event since there is no easy way to tell whether an interesting time record is from a “real” molecule or other artifacts. In this respect, having an additional acceptor, for example Cy5.5 attached to the helix B of the XH vector, is very helpful because FRET between Cy3 and Cy5.5 shows whether the molecule is “real” while FRET between Cy3 and Cy5 can be used to seek parallel states. Starting here, each vector is named by writing the helical arms superscripted by a number denoting the fluorophore attached to them. The unmentioned arm is labeled with biotin. X^3H^5B^5.5 depicted in Figure 7.3A has Cy3 (green) on arm X, Cy5 (red) on arm H, Cy5.5 (navy) on arm B, and biotin (black) on arm R.

Figure 7.3B shows fluorescence time records obtained from a X^3H^5B^5.5 vector obtained in 10mM Tris:HCl, pH. 7.4, 50 mM Mg^{2+} at 20 °C with 6 ms time resolution. While the anti-correlated fluctuation between Cy3 and Cy5.5 shows that the molecule displays the expected Holliday junction conformational dynamics, there is no high FRET state observed between Cy3 and Cy5 indicating that the parallel state, even if it exists, must be short-lived. We did not observe any parallel state for a total observation time of 147 s from 17 molecules which showed both FRET fluctuation between Cy3 and Cy5.5 and between Cy3 and Cy5. No parallel states were observed at lower temperature (2 °C).
achieved via Peltier cooling in contact with the sample slide; data not shown), and regardless of the presence of 50 mM NaCl. We conclude that parallel states longer than 6 ms are exceedingly rare.

7.2.3 Biological Irrelevance of Parallel Conformation

In summary, despite the dynamic character of these branched DNA species, our studies have shown that parallel forms of the DNA junction are not observed. This contrasts with the corresponding RNA four-way junction, where a significant population of parallel forms exist (Hohng et al., 2004b). It is possible that the antiparallel form of the DNA junction is stabilized by favorable backbone-groove interactions (Timsit and Moras, 1991; von Kitzing et al., 1990), which are not possible in RNA due to the different geometry of the A-form helix. The lack of parallel states does not appear to be a peculiarity of non-migratable junctions because our preliminary measurements on migratable junctions did not detect any evidence of parallel forms (McKinney et al., 2005). Thus, even if the parallel structure has a transient existence, it is unlikely to be important for the mechanism of genetic recombination unless stabilized by the interaction with proteins.

7.3 Synchronized Conformational Dynamics

7.3.1 Movements of Four Arms

So far a continuous exchange between the two stacking conformers was observed. These studies, however, cannot tell if the movements of the four arms are simultaneous or some segments can move independently of others. For instance, does helix B approach helix X as helix R simultaneously moves away from helix X? These types of questions cannot be addressed using only two fluorophores. Below, we show data obtained using one donor and two identical acceptors (Cy3-Cy5-Cy5); and one donor and two different acceptors (Cy3-Cy5-Cy5.5). Each vector is named as mentioned earlier (Chapter 2.5, Single-Molecule Three Color FRET).
7.3.2 Correlated Movement Probed by a Special Two-Color FRET Assay

First, we measured the $X^3R^5B^5$ vector using a two-color setup for Cy3 and Cy5 signals (Figure 7.4A). In one stacking conformer, the helix R is close to the helix X that carries the donor (Cy3) while in the other conformer, it is the helix B that is close to the helix X. Since both helix R and helix B carry one acceptor (Cy5) each, we expect to see a constant level of FRET provided that (i) there is no other conformation populated and that (ii) the distance between the donor and the acceptor on helix R in the X-on-B conformer is identical to the distance between the donor and the acceptor on helix B in the X-on-R conformer.

As Figure 7.4B shows, FRET signals remain constant when both acceptors are active except for brief excursions to lower FRET values. These apparent FRET values were calculated as the ratio between the signal in acceptor channel and the total signal detected by both channels. Experiments were performed with 30 mM Mg$^{2+}$ and 50 mM Na$^+$. After the photobleaching of one of the acceptors, marked by a dotted vertical line, fluorescence signals are due solely to $X^3B^5$ or $X^3R^5$ and favor high or low FRET states respectively depending on which acceptor bleaches first. This result suggests that the junction structures are symmetric enough between the two conformers so that the two alternative acceptors share similar distances to the donor (~8 nm) when each is brought close to the donor. However, the observation of short-lived low FRET states here with 8 ms time resolution cannot be interpreted unambiguously. They can be due to photophysical properties of the fluorophores (for example, blinking of Cy5) or due to other minority species of Holliday junction such as parallel or partially stacked structures.

7.3.3 Correlated Movement Probed by a Three-Color FRET Assay

To observe correlated conformational changes of Holliday junction directly, we made the $X^3R^5B^{5.5}$ vector which is labeled with Cy3 on helix X, Cy5 on helix R, and Cy5.5 on
helix B (Figure 7.5A). Figures 7.5B and C are typical three-color time traces of $I_3$, $I_5$, and $I_{5.5}$ (intensities of Cy3, Cy5 and Cy5.5, respectively) after bleed-through has been corrected. All data are collected with 20 ms bin time. About 5% of molecules in our preparation had all three fluorophores active. This low yield could be due to inactive acceptor molecules (typically less than half of Cy5 or Cy5.5 molecules are active for two-color FRET experiments) as well as molecules that are not complete since we did not purify four-stranded molecules after junction assembly. For an easier inspection of the data, intensity time traces of the three dyes (Cy3 in green, Cy5 in red, and Cy5.5 in navy) are plotted in pairs in three different graphs.

In Figure 7.5B, Cy5 bleaches first at ~4 seconds (marked by a dotted red line) and the behavior of the $X^3B^{5.5}$ vector appears afterward. When Cy5.5 bleaches first as in Figure 7.5C, a bias to the low FRET state is observed as in $X^3R^5$ vector. When all three dyes are active, Cy5 and Cy5.5 signals fluctuate in an anti-correlated manner, which is clear evidence for an unambiguous separation of Cy5 and Cy5.5 signals despite significant bleed-through between the two channels. Two-dimensional histogram of FRET efficiencies to Cy5 and Cy5.5 in Figure 7.5D shows two separated peaks corresponding to two conformers. The anti-correlated fluctuation of Cy5 and Cy5.5 signals is consistent with the presently accepted model that helix X alternatively comes close to helices R and B. The donor signal fluctuates by a relatively small amount but is slightly more quenched when the Cy5 signal is strong compared to when the Cy5.5 signal is strong. Therefore, the donor is continuously quenched by either of the acceptors suggesting that movements of helices B and R to and from helix X are well-synchronized. Slightly better quenching of Cy3 by Cy5 than by Cy5.5 may be due to a larger spectral overlap between Cy3 and Cy5 rather than to geometric differences such as distance and orientation because we observed no time-dependent changes of the donor signal when two identical acceptors were used instead (Figure 7.4B). It is noticeable that the Cy5.5 signal intensity of the high FRET state increases after Cy5 bleaches in Figure 7.5B and vice versa in Figure 7.5C. Competition between FRET from Cy3 to Cy5 and FRET from Cy3 to Cy5.5 partly explains the difference but there definitely exist effects of two-step FRET from Cy3 to Cy5 to Cy5.5; Cy5.5 intensity in its low FRET state is higher when Cy5 is active than after Cy5 bleaching (Figure 7.5B).
7.3.4 Complete Stacking and No Parallel Conformation

In a parallel state as illustrated in Figure 7.2 (see also Chapter 7.2, Non-Existence of Parallel Conformation), the $X^3R^5B^5.5$ vector would give low FRET for both Cy5 and Cy5.5 acceptors. Or, if stacking is incomplete, for instance if one pair of helices unstacks significantly earlier than the other pair of helices, the transitions in Cy5 and Cy5.5 signals may not be simultaneous. In these respects, the time traces in Figure 7.5 support the current model of Holliday junction conformations; complete stacking and no parallel state. To elucidate these matters more quantitatively, we performed additional data analyses as shown in Figures 7.5B and E.

The two-dimensional histogram of apparent FRET efficiencies of Cy5 vs. those of Cy5.5 shows two well-separated regions (Figure 7.5D, obtained from ~60 molecules). Negligible number of data point in the left-bottom region indicates the absence of parallel states. Highly-populated regions are elongated with a negative slope due to bleed-through correction. Therefore, a noise-induced increase of signal in one channel brings about a decrease in the other channel leading to an anti-correlated behavior between Cy5 and Cy5.5 signal within each region.

We also tested for the coincidence of Cy5 and Cy5.5 transitions. Transition times were determined using a computer algorithm. In the algorithm used, the apparent FRET efficiencies were first normalized so that the low FRET state is set to zero and the high FRET state is set to 1. Molecules were considered to be in the low FRET state if the normalized value is less than 0.3 and in the high FRET state if the normalized value is larger than 0.5. When values are between 0.3 and 0.5, molecules were assigned to the previous state to avoid false identification of fast transitions due to noise. The difference between a Cy5.5 transition event and Cy5 transition event, $\Delta t$, was determined by matching recorded conformational transitions in both channels. The result from 20 molecules is summarized in Figure 7.7E as a histogram of $\Delta t$. 84 % of data points fall within the integration time used (20 ms). This, therefore, supports the conclusion that the stacking and unstacking of the two pairs of helices are very well synchronized within 20 ms although noise in the data prevents us from placing a precise limit on the degree of synchrony.
7.4 Holliday Junction Dynamics and Branch Migration

Branch migration is a key step in genetic recombination, involving the step-wise sequential exchange of basepairing between the complementary strands of homologous duplexes. Two broad models have been suggested for the mechanism. Initially, Holliday junction was believed to exist as a parallel structure, and it was proposed that simple rotational motion around the helical axes might induce branch migration without the need for unstacking the helices (Meselson, 1972; Sigal and Alberts, 1972). However, it is now well established that four-way DNA junctions adopt an antiparallel conformation, and that conclusion has now been reinforced and extended in this study (Chapters 6 and 7). It therefore seems inescapable that branch migration must require an open conformation as an obligate intermediate, consistent with earlier observations (Panyutin and Hsieh, 1994) that the rate is 1000-fold greater in the absence of Mg$^{2+}$ ions. A recent AFM study of surface-immobilized four-way DNA junctions in the absence of Mg$^{2+}$ ions also showed that the open form must be achieved for branch migration to occur (Lushnikov et al., 2003).

Conformational interconversion between $isoI$ and $isoII$ conformers becomes slower as the concentration of Mg$^{2+}$ ions increases. This is qualitatively similar to the behavior of spontaneous branch migration, the rate of which also decreases with increased Mg$^{2+}$ concentration (Panyutin et al., 1995). Therefore, it is likely that the open structure is a common intermediate for branch migration and stacking conformer transitions (McKinney et al., 2003). Because the frequency with which the open intermediate is accessed cannot be greater than the measured interconversion rate, this sets the upper limit on the stepping rate of branch migration, $k_{BM}$. At high Mg$^{2+}$ concentration, $k_{CT}$ is low, so the open intermediate is visited less frequently, and consequently $k_{BM}$ is reduced. For example, in the presence of 10 mM Mg$^{2+}$, $k_{CT}$ for junctions 1 and 7 is 47 and 17 s$^{-1}$ respectively. Thus, the frequency with which the open intermediate is visited, $4 / (k_{I\rightarrow II}^{-1} + k_{II\rightarrow I}^{-1})$ sets the upper limit as 30 and 17 s$^{-1}$ on the $k_{BM}$, with the caveat that this limit can be highly dependent on the local sequence. Here
the factor of four stems from the fact that the open state is likely to be visited four times on average during one cycle of conformer transition, twice during the actual transitions back and force and twice during failed attempt that returns the molecule to the previous conformer.

In the branch migration experiments of Panyutin and Hsieh (Panyutin and Hsieh, 1994) $k_{BM}$ was measured as 3.9 s$^{-1}$ in the presence of 10 mM Mg$^{2+}$, which is much lower than the upper limits calculated from the data of junction 1 and 7 even though branch migration was measured at a higher temperature than conformer transitions (37°C vs. 20°C). This indicates that during extended branch migration, multiple conformer transitions may occur before the branch point migrates a given step. Moreover, this does not appear to be a unique property of non-migratable junctions (i.e. those incapable of branch migration), because our measurements on migratable junctions showed the expected behavior (McKinney et al., 2005). It will be of great interest if we directly test this hypothesis by seeing the branch migration in real time and by measuring its speed at different magnesium concentrations via single-molecule spectroscopy.

To conclude, the four-way DNA junction intrinsically adopts an antiparallel stacked X-conformation that is in constant exchange between stacking conformers via a short-lived open intermediate. Any other behavior that might be required by the exigencies of recombination mechanisms must be conferred by protein binding. Indeed, it is striking that many proteins targeted to four-way DNA junctions (junction-resolving enzymes for example (Bennett and West, 1995; Déclais and Lilley, 2000; Duckett et al., 1995; Pöhler et al., 1996; White and Lilley, 1997)), do indeed distort the local and global structure (Lilley and White, 2001). A clear example is provided in branch migration, accelerated by the RuvAB proteins in \textit{E. coli} (West, 1997). The RuvA protein stabilizes the structure of the junction in the unstacked square form (Parsons et al., 1995), effectively driving the formation of a conformation with the properties of the open intermediate. Manipulation of DNA structure may therefore be one of the most important functions of recombination proteins, working to overcome the intrinsic conformational properties of the DNA junction.
Appendix A

Supplements to Chapter 2:

Single-Molecule FRET Study

A.1 Calculating $R_0$

$R_0$ is calculated as below:

$$R_0^6 = \frac{9000(\ln 10)\Phi_D \kappa^2 J(\nu)}{128\pi^6 N_A n^4}$$

where $\Phi_D$ is the quantum yield of the donor molecule, $N_A$ is Avogadro’s number and $n$ is the index of refraction of the medium. $\kappa^2$ is an orientation factor, whose average is 2/3. $J(\nu)$ is the normalized spectral overlap of the donor emission ($f_D(\nu)$) and the acceptor absorption ($\epsilon_A(\nu)$) as below.

$$J(\nu) = \frac{\int \epsilon_A(\nu) f_D(\nu) \lambda^4 d\lambda}{\int f_D(\nu) \lambda^4 d\lambda}$$

where ($\epsilon_A(\nu)$) is in units of $[M^{-1} \text{cm}^{-1}]$ and $[M^{-1}]$ is 1000 cm$^3$/mole (Clegg, 1992).
A.2 Imaging Buffer

Triplet-state quenchers or reducing agents greatly improve the photostability of Cy dyes—we use 140mM (i.e. 1% v/v) βME (Zhuang et al., 2000). Recently our lab discovered that Trolox is a better substrate than βME (Rasnik et al., 2006a). The photostability of Cy dyes (especially Cy5 and Cy5.5) is further improved by the oxygen scavenger system, which consists of the following components:

- 0.4% w/v β-D-glucose or 0.8% w/v d-glucose
- 1 mg/ml glucose oxidase
- 0.04 mg/ml catalase

The coupled reactions are:

\[
\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2
\]

\[
H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2} O_2 \quad \text{(Benesch and Benesch, 1953)}
\]

Glucose may be included in a buffer stock solution without any degradation for a long time. We prepare the 100x “gloxy” solution (a mixture of glucose oxidase and catalase) as follows:

1) Add 20 µl of catalase into 80 µl of buffer T50
2) Add 10 mg of glucose oxidase to the mixture
3) Mix the solution by tapping (no aggressive vortexing)
4) Centrifuge for 1 min
5) Take the supernatant and keep it at 4°C until use (it can be stored for several months).

The reaction by the oxygen scavenger system generates the gluconic acid; therefore, the solution becomes very acidic over a long time period, if the sample is allowed to equilibrate with air. This problem is greatly reduced by adding the gloxy to a buffer just before a measurement and also by minimizing exposure of the solution to air (Section A.5.2, Sample Chamber).
A.3 Labeling DNA

Several companies offer fluorescently labeled DNA oligonucleotides. For example, IDT DNA (Integrated DNA Technologies, Inc) provides 5’-end labeling for a 5-150 nt long DNA, and 3’-end labeling for 5-50 nt long as of now. Labeling in the middle of the strand (internal labeling) is also offered commercially. While a dye can be inserted using phosphoramidite chemistry during the oligonucleotide synthesis, this interrupts the backbone of the oligonucleotide which may perturb biological function. We, therefore, recommend an alternative method for the internal labeling as described below.

An oligonucleotide is synthesized with an amine-modified thymine added in a desired location (Figure 2.4Cb). Reaction of the amine group with an NHS-ester form of a dye followed by a simple purification procedure results in a purely labeled DNA in a day. The protocol shown below is optimized for 5 nmol of DNA that has an amine-modified thymine in its sequence. The original protocol is available from Invitrogen Inc. and this is its simplified and revised version.

1. Dissolve the DNA in water (MilliQ, 18.5MΩ) to a final concentration of 1mM. Tris or compounds carrying amine groups interfere with the labeling reaction and hence should be removed by ethanol precipitation prior to labeling.

2. Prepare a 0.1 M fresh labeling buffer by dissolving 380 mg of sodium tetraborate in 10 ml of water (MilliQ). By adding 65 µl of 12.1M HCl or equivalent, get the pH 8.5. At higher pH, the aminolysis of dyes occurs faster; however, hydrolysis rates parallel aminolysis rates, lowering the total yield.

3. Dissolve 1mg of dye in 56 µl of DMSO to a final concentration of 20mM. Excess of dye solution can be stored at -20°C, however, it is not highly stable in solution.

4. Mix the listed components in an eppendorf tube. This composition results in 20 dye molecules for each reactive amine group. If there is not enough DNA available, linearly re-scale the amount of the dye and the labeling buffer. Then
incubate the mixture six hours at room temperature or overnight at 4°C with gentle mixing and in darkness.

5 µl of dye in DMSO
25 µl of 0.1 M labeling buffer
5 µl of 1 mM DNA or equivalent (5 nmol in total)

5. Ethanol precipitation
Add 87.5 µl of cold ethanol and 3.5 µl of 3 M NaCl into the mixture and keep it at -20°C for 30 min. Centrifuge at 12,000g for 30 min at 4°C. Remove the supernatant carefully and rinse the pellet with cold ethanol several times very gently. Dry the DNA pellet to evaporate ethanol away. Dissolve the pellet in a solution relevant.

6. Check the labeling efficiency by comparing the absorption spectra of the DNA (260nm) and of the conjugated dye. Typically, it is close to 100%. If not, run an additional purification such as PAGE (if <=100%) or repeat Step 5 (if >100%).

A.4 Change in Donor Quantum Yield

While the donor quantum yield change does affect the absolute FRET efficiency, it does not affect the apparent FRET efficiency we and others typically use for smFRET analysis as shown below.

The donor quantum yield $\phi_D$ is given by

$$\phi_D = \frac{k_r}{k_r + k_{nr}},$$

where $k_r$ is the radiative decay rate and $k_{nr}$ is the non-radiative decay rate. The apparent FRET efficiency $E_{app}$ is given by

$$E_{app} \equiv \frac{I_A}{I_A + I_D} = \frac{\eta_A I^0_A}{\eta_A I^0_A + \eta_D I^0_D},$$
where \( I_D \) and \( I_A \) are the measured intensities of donor and acceptor molecules, \( I_D^0 \) and \( I_A^0 \) are the true intensities of donor and acceptor emission, \( \eta_D \) and \( \eta_A \) are the detection efficiencies of donor and acceptor signals determined by instrumentation. Since

\[
I_D^0 \propto \frac{k_r}{k_{ET} + k_r + k_{nr}} \quad \text{and} \quad I_A^0 \propto \frac{k_{ET}}{k_{ET} + k_r + k_{nr}}
\]

where \( k_{ET} \) is the energy transfer rate and \( \phi_A \) is the acceptor quantum yield, we obtain

\[
\frac{I_D^0}{I_A^0} = \frac{k_r}{\phi_A k_{ET}}.
\]

Finally, inserting this into the formula for \( E \), we obtain

\[
E_{app} = \frac{1}{1 + \left( \frac{\eta_D}{\eta_A} \right) \left( \frac{k_r}{\phi_A k_{ET}} \right)}
\]

which is independent of changes in the non-radiative decay rate of the donor, \( k_{nr} \).

Note that the apparent FRET efficiency \( (E_{app}) \) is not equivalent to the real FRET efficiency; therefore, the exact distance between a FRET pair cannot be directly obtained from \( E_{app} \), without further corrections.

### A.5 Surface Preparation

#### A.5.1 Cleaning

Two 0.75 mm-diameter holes are drilled into a glass slide to form the inlet and outlet. For a flow technique (Appendix A.5.4, Solution Injection), one of the holes is further bored (~0.8mm) to have a tube inserted. The slides and coverslips are cleaned as follows. The slides are sonicated in a glass staining dish for 20 min in 10% alconox, 5 min in tap water, 15 min in acetone and 20 min in 1 M KOH. The coverslips are sonicated in another glass staining dish for 20 min in 1 M KOH. Next, the slides and coverslips are rinsed with deionized water (MilliQ, 18.5MΩ). Finally, the surface to be imaged is burned using a propane torch in order to remove any fluorescent organic molecules (a quartz slide for the prism-type TIR, half a minute; a coverslip for the objective-type TIR, limiting the process to a few seconds to prevent glass deformation). We have tried plasma cleaning (200W,
20-400 mTorr O₂, 3-10 min) and a piranha solution (3:1 mixture of sulfuric acid and 30% hydrogen peroxide). The quality of the surface was improved but not significantly.

To prepare a BSA-coated surface, assemble a chamber as described below (Appendix A.5.2). In order to coat a surface with PEG, place the coverslips and slides back into the previous glass staining dishes in methanol for the next steps (Appendix A.5.5, PEG-coated Surface).

### A.5.2 Sample Chamber

We attach two pieces of a double-sided tape (~100 µm thick, 3M) to a cleaned slide such that there is about 5 mm gap between the tapes to be used as spacer. Then, we form a 10-20 µl volume sample chamber by putting a cleaned coverslip over the slide (Figure A.1A). When assembling a PEG-coated chamber, care must be used to place the PEG-coated sides facing inside the chamber. Remaining boundaries between the sample chamber and outside are sealed using 5-min epoxy. This system has the advantage that many solution conditions can be explored by flowing different solutions through the same sample chamber. Small holes minimize evaporation during prolonged measurements and reduce oxygen uptake by the solution thereby reducing photobleaching effects and solution acidification. After use, put the chambers in tap water overnight, which helps remove the double-sided tape and epoxy for slide recycling.

### A.5.3 BSA-coated Surface

BSA-coated surface is prepared right before each experiment (Figure 2.5A). First, flow through the chamber 30 µl of 1mg/ml biotinylated BSA solution in buffer T50 (10mM Tris-HCl, pH 8.0, 50mM NaCl). BSA nonspecifically adsorbs to the chamber surfaces. After 5 min incubation, wash biotinylated BSA solution away by flowing through 100 µl of buffer T50. Then, introduce 0.2mg/ml neutravidin or streptavidin solution (30 µl) in buffer T50, incubate for 1 min and wash out in the same manner. Finally, add 30 µl of biotinylated DNA of 50 pM concentration in an appropriate buffer (usually buffer T50).
This protocol allows the stepwise deposition of reagents without sample drying and typically results in the surface concentration of DNA suitable for sm-imaging.

A.5.4 Solution Injection

Through the holes drilled, a solution can be easily injected by using a 200 µl pipette tip. When the solution needs to be changed during data acquisition, we utilize a syringe system as follows (Figure A.1B). On a chamber assembled, a flow system is created by attaching a 28 GA tube to the 0.8mm-wide hole with epoxy. The tube is linked to a 26 G 3/8 needle, which is connected to a 1 ml syringe. The solution is injected into the chamber by snuggly plugging the 200 µl pipette tip with solution to the inlet and then pulling the syringe either manually or via an automated pump.

A.5.5 PEG-coated Surface

To coat the surface with PEG, we clean slides and coverslips as above (Appendix A.5.1, Cleaning), then use amino-silane coating followed by conjugation with the NHS-ester form of PEG as described below (Figure 2.5B).

The slides and coverslips are amino-modified as follows. The cleaned coverslips and slides are in the glass staining dishes in methanol (from Appendix A.5.1, Cleaning). In a flask which is cleaned with methanol via 5 min sonication, 100 ml of methanol, 5 ml of acetic acid and 1 ml of amino-silane are mixed. Immediately, the methanol in the glass staining dishes is replaced by this mixture, incubated for 20 min and sonicated for 1 min. Then, the solution is replaced by methanol and the slides and the coverslips are stored under this condition until the next step.

The amine-modified surfaces are coated with PEG as follows. The slides and coverslips are rinsed with water (MilliQ) and placed well-leveled. 70 µl of the following solution is applied on each slide and a coverslip is laid over the slide to spread the solution evenly and to prevent drying. The reaction solution is made by dissolving 0.2 mg of biotin-PEG and 16 mg of mPEG in 64 µl of freshly made 0.1 M sodium bicarbonate (pH 8.5) and by centrifuging it (7,200 g) for a minute to remove bubbles. Then the
slide/coverslip sandwich is incubated in a dark and humid environment overnight in a box, which is kept humid by adding water to the bottom. Finally, they are disassembled, rinsed with water (MilliQ) and stored in -20 °C until use.

The chamber is assembled immediately before each experiment (Appendix A.5.2, Sample Chamber). After assembly, the quality of the slide is tested. In order to check the non-specific binding of DNA, we inject 1 nM Cy3-labeled DNA in buffer T50. Next, we check the non-specific binding of a protein by adding 1 nM solution of Cy3-labeled protein (we use Rep helicase but any sticky protein should work fine). In both cases, on a good PEG-coated surface, fewer than 10-20 molecules should be visible in each 25 µm x 50 µm imaging area. Once the chamber passes the quality test, we practice the same procedure as Appendix A.5.3 (BSA-coated Surface) starting with adding neutravidin (i.e. skip adding biotinylated BSA). We found that DNA sticks to the PEG surface nonspecifically if the pH is lower than 7.4.
Appendix B

Supplements to Chapter 3:

RecA Filament

B.1 Interaction Between a Fluorophore and a RecA Filament

The spectra of 50 nM partial dsDNA molecules with (dT)$_{21}$ and (dT)$_{13}$ tail labeled with Cy3 and/or Cy5; and 10 nM 77mer ssDNA (5’ TGG CGA CGG CAG CGA GGC-T13-T*-T45 3’, T* is amine-modified dT with Cy3 labeled) were measured by Cary Eclipse (Varian) in the identical chemical condition as sm-measurements after 10 min incubation at the room temperature. The fluorescence signal was recorded by exciting with 540 nm (640 nm in case of directly exciting Cy5) light and by collecting signal from 550 to 750nm (650 to 750 in case of directly exciting Cy5). The recorded fluorescence signal was corrected for the wavelength-dependent sensitivity of the detector. The absorption spectrum was measured by Cary Bio 100 (Varian) under the identical condition.

B.1.1 Effects of a RecA Filament on Fluorescence

A fluorescence spectrum of a partial dsDNA with (dT)$_{13}$ ssDNA tail that was labeled with Cy3 and Cy5 (Figure B.1A) was measured in ensemble as above. While DNA alone shows high FRET (red in Figure B.1Ba), introduction of RecA with ATP$_\gamma$S leads to 60% decrease in FRET (green in Figure B.1Ba) due to the filament formation.
To study an effect of a RecA filament on Cy3 (donor), the measurement was repeated with Cy3-only sample (Figure B.1Bb). Cy3 attached at the end of ssDNA tail became 70% brighter when the RecA filament formed with ATPγS, which has been also observed in sm-measurements (Figure B.2B). The increase in fluorescence signal is likely due to conformational constraint imposed by a protein nearby which decreases the non-radiative decay rate and consequently increasing the fluorescence quantum yield of Cy3. While the donor quantum yield change does affect the absolute FRET efficiency, it does not affect the apparent FRET efficiency we and other typically use for smFRET analysis (Appendix A.4 Change in Donor Quantum Yield).

In addition, the RecA filament formation did not cause any change in the fluorescence emission spectrum of Cy3. The same measurement was carried out with a ssDNA internally-labeled with Cy3 (that was used in Figure 3.3A), with which we again observed the increase in fluorescence signal (50%) but no change in the emission spectrum (Figure B.1Eb).

A similar measurement was carried out by directly exciting Cy5 (acceptor) that is positioned at the junction of ss-dsDNA (Figure B.1Bc). We observed a decrease of the signal (30%), smaller than that in FRET (60%), when RecA formed a filament with ATPγS. One possible reason for the fluorescence decrease is photoblinking of Cy5 which may be enhanced by the filament formation on the ssDNA as well as dsDNA. The photoblinking frequently showed up in sm-traces in the same condition (Figure B.1C). We can easily distinguish such photoblinking (E=0) from a normal active state of Cy5 (E>0) in sm-traces based on a FRET value. Regardless of the source of Cy5 signal reduction, our data analysis on FRET trajectories do not rely on the absolute values of FRET but rather on its relative changes. Therefore, the kinetic rates measured 1) from all-or-none events of a nucleation cluster (Figures 3.1 and 3.2), 2) from stepwise change in FRET (Figures 3.3-3.6) would not be influenced by the decrease in Cy5 signal.

As shown in sm-traces taken with (dT)$_{13}$ and (dT)$_{21}$ tail (Figures B.1C, D), the low-FRET state which results from a RecA filament formed with ATPγS is distinguished from photoblinking or photobleaching of Cy5 by the difference in FRET (E>0 vs. E=0). We further checked the activity of Cy5 by exciting Cy5 directly using a 633 nm laser (Figure B.1D).
When absorption spectra were measured, there was only slight change observed (Figures B.1Bd, B.1Ec), indicating insignificant influence of a RecA filament on the absorption properties.

**B.1.2 Effect of a Dye on a RecA Filament**

We have measured ATPase activity with 77mer ssDNA as following. 50 nM of labeled 77mer ssDNA molecules (5’ TGG CGA CGG CAG CGA GGC-T13-T*-T45 3’, T* is amine-modified dT with Cy3 labeled) and unlabeled DNA (5’ TGG CGA CGG CAG CGA GGC -T59 3’) were, respectively, mixed with 1.3 µM RecA and 2 mM ATP in the identical chemical condition as sm-measurements at room temperature. ATPase activity was measured by EnzChek kit (Invitrogen) based on the increase in absorption at 360nm, which was detected by Cary Bio 100 (Varian). The rates were determined by fitting the change of absorption in the first minute. The measurement was repeated three times for each sample.

ATP hydrolysis rates were 0.14 ± 0.00 and 0.14 ± 0.01 (sec⁻¹ RecA⁻¹) with labeled and unlabeled ssDNA, respectively, indicating that the dye (Cy3) positioned in the middle of DNA does not affect ATPase activity of a RecA filament. The minimal influence of organic dyes on a RecA filament was reported previously (Bazemore et al., 1997b; Gourves et al., 2001; Xiao and Singleton, 2002). We could also carry out RecA-mediated homologous strand exchange with a ssDNA that was internally-labeled in a sm-assay (unpublished observation).

**B.2 Filament Formation on a Short ssDNA**

**B.2.1 RecA Filament Formation with ATP**

Figure B.2C shows traces taken with (dT)₂₁ tail in the same condition as Figure 3.1E. A fraction of filament assembly/disassembly events are slow enough to display intermediate FRET values during transition (Figures B.2Cc, d).
The change in FRET is well-correlated with the change in total fluorescence intensity as has been discussed above (Figure B.1Bb). When a 2-dimensional density plot is made for $E$ vs. ‘fluorescence intensity’, the anti-correlation between FRET and intensity is clearly visualized (Figure B.2B). This anti-correlated property was carefully considered in analyzing traces, especially if FRET is too low ($0 < E << 1$) to distinguish from an inactivated state of acceptor molecules ($E = 0$).

B.2.2 RecA Filament Formation with Different NTP Factors

$(dT)_{21}$ ssDNA tail free from a RecA filament shows high FRET ($E \sim 0.55$) (Figures B.3Ba), while it shows low FRET ($E \sim 0.1$) with the filament (Figures B.3Bb). When dATP is used as a NTP factor, a stable filament forms (Figure B.3Bc; arrows) in contrast to the brief filament formation with ATP (Figure B.3Ba) (Menetski and Kowalczykowski, 1989). The sm-trace also shows more stable low-FRET states (Figure B.3Ca). A similar property was observed with $(dT)_{17}$ tail (Figure B.3Cb) while the filament formation was rarely observed with ATP (Figure 3.1Fb).

In the absence of NTP factors, RecA still forms a filament but in a collapsed form with a lower pitch than usual (Bell, 2005; Story et al., 1992). The FRET efficiency ($E \sim 0.3$) is higher than that with ATP$_\gamma$S ($E \sim 0.05$) possibly reflecting the lower pitch (Figure B.3Bd). The broad distribution in a FRET histogram is due to two-state fluctuation between $E \sim 0.3$ (filament) and $\sim 0.55$ (no filament) (Figure B.3Cc).

B.3 Filament Formation Around a dsDNA

Mediated by ssDNA Tail

B.3.1 Filament Formation on dsDNA is not Local to the ss-dsDNA Junction

In order to test whether $\langle$dsDNA$|$ATP$_\gamma$S$\rangle$ forms all over the dsDNA or only a part of it (e.g. only near the junction of ss-dsDNA), we relocated Cy5 at the junction (Figure 3.5D).
into the middle of the dsDNA as shown in Figure B.4A. (The sequence of the ssDNA that is labeled with biotin and Cy5 is 5’-GCCTCGC T* GCCGTCGCCA-biotin-3’, where T* stands for an amine-modified dT with Cy5 labeled.) While $E$ is ~0.6 without a RecA filament, $E$ becomes as low as ~0.4 when RecA and ATPγS are added (Figure B.4Ab). Since the change in FRET reflects a filament formation over a portion of dsDNA that is far from the junction and since $<\text{dsDNA}|\text{ATPγS}>$ cannot form with a blunt end of the dsDNA (Figure B.4C), $<\text{dsDNA}|\text{ATPγS}>$ is not likely to form only near the junction, but rather to form over the entire length of 18 bp duplex DNA.

**B.3.2 $<\text{dsDNA}|\text{ATPγS}>$ Protects dsDNA from Digestion by an Endonuclease**

DNase I footprinting was carried out to check the presence of $<\text{dsDNA}|\text{ATPγS}>$ using an independent, non-FRET based method (see the next paragraph for a detailed procedure). A dsDNA with 3’ (dT)$_{13}$ tail was labeled with a dye at the junction (Figure B.4B). The DNA was incubated with RecA and ATPγS, which was followed by cleavage by DNase I. When the DNA was incubated with RecA and ATPγS for a different amount of time (lanes 1-5 in Figure B.4Bb) before cleavage reaction, a different degree of cleavage activity was observed—the longer incubation, the less cleavage. Similarly, when the DNA was incubated with RecA and ATPγS for 10 minutes but with different RecA-concentrations, the less cleavage reaction was observed with the larger amount of RecA (lanes 8-11). Such protection of dsDNA by RecA was not observed when ATP was used (lane 7). Therefore, this footprinting assay further supports the formation of a filament around dsDNA with ATPγS mediated by 3’ ssDNA tail, which has not been reported previously.

DNase I footprinting was carried out as following. 600 nM of a partial dsDNA molecules with (dT)$_{13}$ tail was incubated with RecA and 1 mM cofactor (ATPγS or ATP) for a given time, in a buffer that contains 2.5 mM MgCl$_2$, 0.5 mM CaCl$_2$, 1% v/v βME, and 10 mM Tris-HCl (pH 7.6). Next, 10 µl of the solution was cleaved by 0.05 U/µl of DNase I (New England Biolabs) for 20 seconds, which was stopped by adding SDS, EDTA and tRNA (0.3%, 70 mM and 1 µg/ml in final). The solution was ethanol-
precipitated, then, the DNA-pellet was dissolved in 10µl formamide. It was run in 12% denaturing PAGE for 2 hours (20W) and imaged in a fluorescence imager (FLA-3000, Fujifilm) with 633 nm light exciting Alexa647 that is attached to the dsDNA.

**B.3.3 <dsDNA|ATPγS> Cannot Form Without ssDNA Tail**

When the same DNA as that in Figure 3.5D but without ssDNA tail (Figure B.4C) was incubated with RecA and ATPγS, we could not observe any filament formation around dsDNA for up to 2 hours (Figures B.4Cc). This rules out the possibility that <dsDNA|ATPγS> which formed in a 3’ tailed DNA was due to the dyes acting as nucleation sites, as the blunt dsDNA used for the control here also contained the dyes in the same location but did not show evidence of filament formation.

**B.3.4 Formation and Disassembly of <dsDNA|ATPγS> at High Temperature**

The same experiment as Figure 3.5 was carried out at 35°C instead at the room temperature (Figure B.5). The formation of <ssDNA|ATPγS> was observed when RecA and ATPγS were added in the same way as at the room temperature, resulting in a transition from high to low FRET (Figures B.5Aa, b). When RecA and ATPγS were removed from solution, the disassembly of <ssDNA|ATPγS> was observed as at the room temperature but at a faster rate (<1min) (Figures B.5Ac, d). Finally, when RecA and ATP were added after 10 minute-incubation in the previous condition (no RecA and ATPγS in solution at 35°C), only ~50% of the molecules in the high-FRET state shifted into the low-FRET state (Figures B.5 Ae, f). Therefore, <dsDNA|ATPγS> is less stable at 35°C than at the room temperature. Note that <dsDNA|ATPγS> is still more stable than <ssDNA|ATPγS> at 35°C for a short 3’ tail (13 nt).

Similarly, the formation of <dsDNA|ATPγS> was directly observed at 35°C (Figure B.5Ba). A half of the molecules lost the filament 10 minutes after RecA and ATPγS were removed from solution at 35°C (Figure B.5Bb) consistent with the result above (Figure B.5Af).
B.3.5 Formation of $\text{<dsDNA|ATP}_\gamma\text{S>}$ Mediated by 5’ ssDNA Tail

The same experiment as shown in Figure 3.5 was carried out with a dsDNA with 5’ (dT)_{20} ssDNA tail, instead of 3’ tail (Figure B.6). When RecA and ATP\gamma S were added, a filament formed (Figures B.6Aa, b) in less than half a minute. When RecA and ATP\gamma S were removed from solution, the disassembly of $\text{<ssDNA|ATP}_\gamma\text{S>}$ was observed in a similar time scale as that with 3’ tail (~5 min) (Figures B.6Ac, d). Finally, when RecA was added with ATP, most of the population in the high-FRET state was shifted into low FRET (Figure B.6Ae, f). Such transition was not observed in the absence of $\text{<dsDNA|ATP}_\gamma\text{S>}$ (Figure B.6B).

However, a small population were in high and intermediate FRET (Figure B.6f, arrows), the origin of which is revealed when sm-traces are analyzed. As shown in Figure B.6Ag, $E$ fluctuates between high and low FRET dynamically. This large fluctuation is in clear contrast with a trace recorded with 3’ (dT)_{19} tail (Figure B.6C), where $E$ always stays low. This difference between 3’ and 5’ tails, observed in FRET histograms and sm-traces, is anticipated since $\text{<ssDNA|ATP>}$ on 5’ tail corresponds to the unstable 5’-disassembly end of a filament ($C_c \sim 100\text{nM}$), while $\text{<ssDNA|ATP>}$ on 3’ tail corresponds to the stable 3’-extending end ($C_c \sim 8\text{nM}$). Conversely, it implies that the polarity of $\text{<dsDNA|ATP}_\gamma\text{S>}$, which appears to act as a nucleation cluster, is not random but rather follows that of $\text{<ssDNA|ATP}_\gamma\text{S>}$.

Formation of $\text{<dsDNA|ATP}_\gamma\text{S>}$ was directly observed by attaching the dye pair at two ends of the dsDNA (Figure B.6Da) and $\text{<dsDNA|ATP}_\gamma\text{S>}$ remained stable in the absence of RecA and ATP\gamma S for ~2 hours until it started to disassemble after ~3 hours (Figure B.6Db).
Appendix C

Optical Tweezers

C.1 Introduction

C.1.1 Principle

When a transparent spherical bead is placed where a laser beam is tightly focused, the bead gets trapped by force induced by the laser beam (Figure C.1A) (Ashkin and Dziedzic, 1987; Ashkin et al., 1987). The generated force originates from the momentum conservation of the bead and the photons that go through the bead, as explained in the following. A beam that enters the left side of the sphere becomes bent and heads towards the right when it exits (Figure C.1B). The change of the photon’s direction (i.e. its momentum change, $\vec{k}_2 - \vec{k}_1$) results in a force against the sphere, due to the momentum conservation. When the laser beam enters a bead symmetrically, the generated forces cancel with each other thus the bead stays captured in equilibrium (Figure C.1A). But, if the bead is placed aside, the net force is generated toward the center of the focus (Figure C.1Ca). It is also true when the bead is placed aside center along the vertical axis (Figure C.1Cb).

C.1.2 Force Calibration

It is crucial to accurately measure the force applied to a bead. One of the estimating methods is theoretical calculation based on the laser’s intensity, its profile and the size of the bead (Ashkin, 1992). It is also possible to estimate it based on the thermal fluctuation of the bead and the distance of the bead from the focal point (Neuman and Block, 2004).
Here, we estimate it based on a drag force as following (Figure C.1D) (Felgner et al., 1995).

A bead experiences a viscous drag force when it moves in a solution, which is measured as:

\[ F = 6\pi \eta \times a \times V \]

where \( \eta \) is the viscosity of a solution, \( a \) is the radius of the sphere, and \( V \) is the speed of the movement. When a bead of 5 \( \mu \text{m} \)-radius moves with a speed of \( V \) (\( \mu \text{m/sec} \)) in water (\( \eta = 0.001 \text{ N-sec/m}^2 \)), it experiences a force of \( \sim 0.1V \) (pN). First, we move the trapped bead with a certain speed \( (V) \). By recording the image of the bead and the position of the optical trap during the translation, we measure the distance \( (d) \) between the trap and the bead centers at the given speed \( (V) \) (Figure C.1D). Since the magnitude of the drag force is the same as that of the optical trapping force during the translation \( (F_{\text{trap}} = F_{\text{viscous}} = 6\pi \eta \times a \times V) \), we can estimate how strong force will be exerted by a trap when the trap center is offset from the bead center by a distance \( d \). We repeat the procedure to obtain a calibration curve of the force \( (F_{\text{trap}}) \) dependence on the distance \( (d) \). Once the curve is obtained, in order to apply a certain magnitude of force, we put the trap a corresponding distance away from the bead center (Figure C.2).

Another calibration method is to carry out the same drag force measurement but only to measure the largest trap force. If the highest speed that can be achieved is \( V_m \), the maximum trapping force is \( F_{\text{viscous}} = 6\pi \eta \times a \times V_m \). Then based on the maximum trapping force and Ashkin model, we can estimate the trapping force at different positions. With the setup described in Section C.2, we estimate the maximum force to be \( \sim 300\text{pN} \) with a 5 \( \mu \text{m} \)-radius bead.

Note that when we measure the drag force, we have a bead trapped slightly above the glass surface so that the interaction between the surface and the bead is minimized for the reliable force measurement. This is carried out by lifting up the focal plane of the objective lens.
C.1.3 Experimental Scheme

There are numerous applications of the optical tweezers in studying biological molecules and their interactions (Bustamante et al., 2003). Here, we will utilize the optical tweezers in applying several hundred-pN force to a single cell in a precise and well-controlled way to study the response of a cell to the external force (Figure C.2) (Wang et al., 2005).

To carry out an experiment, we first immobilize cells on a glass surface (Figure C.2A). Then we introduce polystyrene beads that are coated with fibronectin or polylysine that specifically interact with the cell surface. A bead is captured by the optical trap, is brought to the cell surface and is attached to a desired spot (Figure C.2B). The other beads in solution are flushed away to prevent further binding. We turn off the optical trap and then move the position of the optical trap; the traveling distance depends on the degree of force that we want to apply later (see Section C.1.2). Finally, we turn on the trap while we are imaging the cell via fluorescence microscopy in order to see the response of the cell to the external force in real time (Figure C.2C).

C.2 Setup

C.2.1 Components

As follows are the basic instruments and optics required for building the optical tweezers. We use a fiber-coupled IR (infra-red) laser (1064nm, 5W, 5mm diameter, YLD-5-1064-LP, IPG Photonics). We use a piezo-electric system for a steering mirror (see also Section C.3.2). The piezo-mirror system is designed with a closed loop so that it does not experience hysteresis during operation. We use an automated shutter (LS6ZM2, Uniblitz) with a shutter controller (VCM-D1, Uniblitz). Mirrors (designed for IR), lenses (BK7, plano-convex) and other basic optics are purchased from Thorlabs. A hot mirror (FM01, wide band, Thorlabs) is installed inside a microscope to block the IR scattering. For crude imaging, an inexpensive CCD camera (Genwac / Watec WAT-902H3SUPREME, near IR) and a commercial TV are used.
C.2.2 Optically Conjugated System for Steering a Mirror

To manipulate the position of a trapped bead, we either move the microscope stage or move the trapping beam itself. We choose the second to have a full degree of freedom in using the microscope stage area. As shown in Figure C.3B, the trapping position moves when the incident angle of a beam through the objective lens changes, which is achieved by tilting the steering piezo-mirror.

The distance between the piezo-mirror and the lens-L1 should be the focal length of the L1; and the distance between the lens-L2 and the objective lens should be the focal length of the L2 so that the change of the angle ($\theta_1$) induces the change of the incident angle of $\theta_2$ only (Figure C.3B). Otherwise, the beam hits off the center of the objective lens which introduces severe geometrical aberration deterring proper optical trapping. The distance between the two lenses (L1 and L2) should be the sum of the focal lengths of L1 and L2 for the proper beam collimation (Figure C.3A).

C.2.3 Fluorescence Microscope with Optical Trapping Capability

To achieve the optically conjugated system described above, we need to put two coupled lenses before the objective lens. However, the fluorescence microscope (Axivert 200, Zeiss) has already four lenses installed inside the microscope, which are necessary in fluorescence imaging using a filament lamp (Figure C.4). To circumvent the presence of the lenses or to compensate it, we have analyzed the beam pathway. It has turned out that the four lenses are coupled in such a way that, when a parallel beam enters the microscope, it reaches the objective lens as if it is focused by the first lens (L1$_{int}$) (Figure C.4A); and when a focused beam enters, it reaches the objective lens as a parallel beam (Figure C.4B). That is, the three other lenses (L2$_{int}$, L3$_{int}$ and L4$_{int}$) are present without affecting the beam pathway except they elongate it. Therefore, we have realized that we only need to put one lens to build the optically conjugated lens system with the fluorescence microscope.
As shown in Figure C.4C, we couple a lens, $L_{1\text{ext}}$, $(f, 150\text{mm})$ with $L_{1\text{int}}$, by placing it a distance of $f_{1,\text{ext}} + f_{1,\text{int}}$ away from $L_{1\text{int}}$. The distance between the lens ($L_{1\text{ext}}$) and the piezo-mirror is set as the focal length of the lens ($L_{1\text{ext}}$). Since this combination reduces the beam waist $\sim 3$ times smaller, it becomes unreliable to get good trapping. So, we expand the beam by introducing a beam expander which is composed of two lenses ($L_{2\text{ext}}, f, 125\text{mm}$ and $L_{3\text{ext}}, f, 40\text{mm}$). It expands the beam by $\sim 3$ times back, therefore, the beam enters the objective lens with a waist of about $10\text{mm}$, which is slightly larger than the back aperture of the exit pupil, $8\text{mm}$. Note that the beam is magnified twice by the combination of $L_{2\text{int}}, L_{3\text{int}}$ and $L_{4\text{int}}$ lenses (not described).

**C.2.4 Fine Adjustment**

The position of the trap on the coverslip surface is fine-adjusted by moving the position of the $L_{1\text{ext}}$ on the plane perpendicular to the excitation beam pathway. If the position of the trap is severely off from the center, both $L_{1\text{ext}}$ and $L_{2\text{ext}}$ need to be moved through beam walking to avoid introducing geometrical aberration. For that, the $L_{1\text{ext}}$ and the $L_{2\text{ext}}$ are mounted on micrometer stages.

The height of the trapping position from the coverslip surface is carefully adjusted. In the beginning, we put the trap as far as possible from the surface. Then, while comparing on CCD screen the image of the trapped bead (5 $\mu\text{m}$ in radius) with those of other identical beads stuck on surface, we lift the trap position down by gradually moving the lens $L_{1\text{ext}}$ away from the microscope. We continue to lift it down until we see the image of the trapped bead becomes the same as those of the others stuck on surface, which indicates that the trap is 5 $\mu\text{m}$ above the surface. Then by moving back the $L_{1\text{ext}}$ toward the microscope a few millimeters we can have the bead trapped just above the surface (a few micrometers above) but does not touch the surface.
C.3 Program

C.3.1 How to Use

To start controlling the position of the optical trap on the microscope stage, click START (Figure C.5A). Once START is clicked, three parameters at the left are locked; so, they need to be properly modified before the clicking. To move the trap, drag the scroll bars (horizontally and/or vertically), which can be done either by a computer mouse (crude but fast movement) or by a keyboard (fine but slow). When ORIGIN is clicked, the trap goes back to the first original position. The current positions are visualized by the angles of the piezo-mirror, displayed in real time (bottom left). In addition, the current forces exerted (calculated based on the parameters entered) in the horizontal and vertical directions are also displayed in real time (bottom left).

When the parameters need to be modified, click RESET. The trap will go back to the origin and all the parameters will become editable. With the new parameters entered, click START to make the new parameters recognized and to re-start controlling. To end the program, click EXIT.

Parameters:

The top two parameters need to be measured empirically (top left in Figure C.5A). The first parameter (mrad/µm) determines how large angle of the piezo-mirror (mrad) needs to be tilted to have the trap 1 µm moved on the microscope stage, which depends upon the excitation optics including the objective lens. When the other parameters are set as default (10µm/100pN and 100pN), moving one of the scroll bars all the way up to down (or left to right) should result in the movement of ±10 µm. It is obvious that, when a random value is entered for the first parameter (1 mrad/µm by default), it would not lead to the movement over a correct distance when looking at it on a CCD screen. With a 100x objective lens and the optics scheme shown in Figure C.4C, the value of the parameter is estimated to be around 0.5. The second parameter (µm/100pN) is determined by the calibration carried out in Section C.1.2. The third parameter (pN) is to set the maximum
range of the traveling distance, that is, the maximum range of the force that can be applied when moving the scroll bars.

*Speed:*
When controlled by the scroll bars or when ORIGIN is clicked, the piezo-mirror responds immediately therefore relocates the position of the trap right away. In some cases, the speed of the trap movement needs to be regulated. For example, the trap might need to move slowly with a certain upper limit, or very fast with a specific speed (Section C.1.2 calibration). The speed is set by entering a desired value (µm/sec) (right bottom) and by changing OFF into ON. With the state ON, even if the position of the trap is changed quickly by moving a scroll bar, the trap moves within the limited speed. In the same way, when ORIGIN is clicked, the trap goes to the origin with the specified speed. It is the same when RESET is clicked. Note that RESET does not change the status of the speed mode.

The absolute value of the speed is valid only for one axis due to a limited capability of the piezo-mirror system. For example, when ORIGIN is clicked, if the position of a trap is offset from the origin only along the horizontal direction, the speed will be accurate. However, if it is also offset from the origin in the vertical direction, the trap will follow a strange trajectory. It is programmed such that the magnitude of the speed is correct only for the axis that has been accessed recently. That is, if the trap is offset from the origin in both vertical and horizontal directions and if it is the vertical axis that has been controlled the last, the absolute value of the speed is correct for the vertical direction only.

**C.3.2 Computer Interface: Initialization**

The piezo-mirror (S-334.2, (PI) Physik Instrumente) is installed together with the computer interface module of (E-516.I3, PI). The interface module set up with the other drivers (E-503.00 and E509.S3, PI) makes it possible to control the piezo-mirror system by a computer. For the communication between the interface module and the computer, we need another interface card installed in the computer such as a GPIB-interface card.
(NI PCI-GPIB, National Instruments) or a parallel port. We prefer GPIB for its convenience. For the first start (and for good), the communication options of the E-516.I3 need to be reset as follows. The communication method is set IEEE instead of RS-232, for the GPIB control. The address of the E-516.I3 module is set a certain number, say, 8.

When writing a C program, the following files are incorporated. GPIB-32.OBJ is included as one of the library files. In the start of the program, Decl-32.h is included as shown below.

```c
#include "Decl-32.h" // for GPIG
```

The system is initialized as:

```c
int iPZTMount;
iPZTMount = ibdev(0, 8, 0, T100ms, 0, 0); // Open and initialize a device descriptor  
    // 8 indicates the address assigned above in text
ibclr(iPZTMount);  // Clear a specific device
ibeos(iPZTMount, 0x040A); // Configure the end-of-string (EOS) termination mode or character
```

The piezo-driver becomes activated by:

```c
char command[100] = {0}; // a string that will be sent
short no_of_byte;
sprintf(command, "ONL 1"); // To make the controller ONLINE
no_of_byte = length(command);
ibwrt(iPZTMount, command, no_of_byte);

sprintf(command, "SVO A1 B1"); // To make the Servo ON
no_of_byte = length(command);
ibwrt(iPZTMount, command, no_of_byte);
```

C.3.3 Computer Interface: Mirror Control

We control the orientation of the piezo-mirror by specifying absolute values of the angles. Below is an example of controlling the angles for x and y axes.
void MovePZTMount(double dA, double dB){
    char command[100] = {0};
    short no_of_byte;
    double tempA, tempB;

    tempA = dist_to_voltA(dA); // see below for the definition of this function recalled
    tempB = dist_to_voltB(dB);

    sprintf(command, "MOV A%5.3f  B%5.3f\n", tempA, tempB); // to control the angle
    no_of_byte = length(command);
    ibwrt(iPZTMount, command, no_of_byte);
}

double dist_to_voltA (double dA) {
    double tempA;
    dA = dA * distance_to_volt; // Physical distance (on the coverslip) into a voltage
    tempA = dA + dMountAngleAOffset;
    return tempA;
}

The forces (and the physical distances) on the stage are converted into the angles of the piezo-mirror with the following formula.

\[
angle(\text{mrad}) = \text{offset}(\text{mrad}) + \text{force}(\text{pN})
\]
\[
= \text{offset}(\text{mrad}) + a \times \text{distance}(\mu\text{m})
\]
\[
= \text{offset}(\text{mrad}) + a \times b \times \text{voltage}(\text{V})
\]
\[
= 25(\text{V}) + a \times b \times \text{voltage}(\text{V})
\]

where, \( a \) is a conversion factor of force(pN)-to-distance(µm), which is the second parameter in the controlling panel; and \( b \) is a conversion factor of distance(µm)-to-voltage(V) or distance(µm)-to-angle(mrad), which is the first parameter in the panel. Note that the magnitude of voltage (V) is the same as that of the angle (mrad) in the current piezo-system. Also, the piezo-mirror move either 25 ± 25 mrad or 0 ± 25 mrad; the first is the case with the current setting, therefore, the offset of 25mrad (i.e. 25V) is added. The formula is written as below.
double distance_to_volt = 1.; // The conversion factor between the voltage and the distance on the objective lens. This should be empirically measured. For example, 1 means 1V / 1um (i.e. 1mrad / 1um)

double dMountAngleAOffset=25;
double dMountAngleBOffset=25;
double stage_x=0, stage_y=0;

stage_x = (m_calibration / 100.) * force_x; // Converting the force into distance. For example, if m_calibration is 10um/100pN, the requested 100pN will result in 10um.

stage_y = (m_calibration / 100.) * force_y;
MovePZTMount(stage_x, stage_y); //see the previous paragraph for this function recalled

When the piezo-mirror is tilted by the angle of $\theta$ horizontally, the angle of the reflected beam changes by $2\theta$. However, when it is tilted vertically, the angle of the reflected beam changes by $\sqrt{2} \theta$, which is not intuitively apparent (Figure C.5B). To compensate this difference, a magnification of $\sqrt{2}$ is required for the vertical rotation. Furthermore, the rotational plane of the piezo-mirror is 45° (or 135°) rotated when manufactured. Therefore, the following transformation matrix is used:

\[
\begin{pmatrix}
V_x \\
V_y
\end{pmatrix} = \begin{pmatrix}
\cos 135^\circ & -\sin 135^\circ \\
\sin 135^\circ & \cos 135^\circ
\end{pmatrix}\begin{pmatrix}
1 & 0 \\
0 & \sqrt{2}
\end{pmatrix}\begin{pmatrix}
\theta_x \\
\theta_y
\end{pmatrix}
\]

which is incorporated into a program as:

double m=1.414; // this value is \sqrt{2}, which compensates the difference between the effects of two types of angle rotation

double theta=135; // rotation for 45+90 degree

temp_x = stage_x;
temp_y = stage_y;
stage_x = m*cos(theta*3.14159/180)*temp_x - 1*sin(theta*3.14159/180)*temp_y;
stage_y = 1*cos(theta*3.14159/180)*temp_y + m*sin(theta*3.14159/180)*temp_x;

With all the source codes written above, the positions of the trap in x-y plane are controlled in real time when we drag the scroll bars as written below:
int PZT_resolution=10; // 100 gives 1/100 pN resolution when adjusting a scroll bar

void CMirrorDlg::OnHScroll(UINT nSBCode, UINT nPos, CScrollBar* pScrollBar)
{
    double force_x, force_y;
    double temp_x, temp_y;

    if (velocity_on==1) speed_x(m_speed); //see the next paragraph for this function

    force_x= (double)m_x_control.GetPos()/PZT_resolution;
    force_y= (double)m_y_control.GetPos()/PZT_resolution;

    stage_x = (m_calibration / 100.) * force_x;
    stage_y = (m_calibration / 100.) * force_y;

    temp_x = stage_x;
    temp_y = stage_y;
    stage_x = m*cos(theta*3.14159/180)*temp_x - 1*sin(theta*3.14159/180)*temp_y;
    stage_y = 1*cos(theta*3.14159/180)*temp_y+ m*sin(theta*3.14159/180)*temp_x;

    MovePZTMount(stage_x, stage_y);
    UpdateData(FALSE);
    CDialog::OnHScroll(nSBCode, nPos, pScrollBar);
}

void CMirrorDlg::OnVScroll(UINT nSBCode, UINT nPos, CScrollBar* pScrollBar) {
    // the same as above
    CDialog::OnVScroll(nSBCode, nPos, pScrollBar);
}

Finally, the speed of the movement is controlled as described below. Note that the speeds in the x- and y-directions are calculated differently for the reason mentioned earlier.

void VelocityControlOn() {
    char command[100] = {0};
    short no_of_byte;
    sprintf(command, "VCO A1 B1\n");
    no_of_byte = length(command);
    ibwrt(iPZTMount, command, no_of_byte);
    velocity_on=1;
}
void speed_x(double mspeed) { // speed when moving along the x-axis.
    char command[100] = {0};
    short no_of_byte;
    double speed;

    // Setting up the speed
    speed = mspeed * distance_to_volt; // um/sec is converted into V/sec
    speed *= m/1.414213562; // m is sqrt(2) for the speed_x(); m is 1 for speed_y().

    sprintf(command, "VEL A%5.3f B%5.3f\n", speed, speed);
    no_of_byte = length(command);
    ibwrt(iPZTMount, command, no_of_byte);
}
Appendix D

Polymer-Mediated Protein Crosslinking

*E. coli* Rep is a helicase that unwinds a partial dsDNA with 3’-overhang. The functional unit required for unwinding is thought to be a dimer or a higher oligomeric complex (Ha et al., 2002; Lohman and Bjornson, 1996). To investigate this nature, we developed an assay in which we link two Rep monomers by polymer (PEG). We hypothesize that the unwinding from this PEG-mediated dimer form of Rep would show enhanced activity.

D.1 Protein Purification

D.1.1 Over-Expression

*Autoclave;*

LB broth was prepared by dissolving 12.5g of Miller Base (Fisher) into 500ml of deionized water (Milli-Q) in a clean 2L flask. The flask was covered with a piece of aluminum foil on top. The medium was autoclaved with a liquid cycle at 121°C for 20-30min. Proper autoclaving was confirmed by the color change of the autoclave tape. The autoclaved medium was slowly cooled down to room temperature before use.

*Start-up culture; (wear gloves at all times)*

500μl of kanamycin stock (50mg/ml in water) was added to the 500ml of sterile medium to achieve the final concentration of 50μg/ml. 5ml of LB with kanamycin (LB<sub>kan</sub>) was added to a 14ml falcon polypropylene tube (kept sterile). The inoculation was performed
by carefully scraping the top of -80°C cell stock (desired BL21-Rep) with a yellow pipette tip and adding the whole tip into the 14ml tube containing LB<sub>kan</sub>. The culture was overnight grown by shaking at 37°C with 260-300rpm for 12-16 hours (overnight is recommended).

**Cell Growth and Protein overexpression;**
5ml of overnight inoculum was applied to 500ml LB<sub>kan</sub> in the 2L flask. The culture was grown for 2-3 hours at 37°C with 260-300rpm until OD<sub>600</sub> reached 0.8-1. At this stage, cells were induced by adding 500µl of IPTG (isopropyl β-D-1-thiogalactopyranoside; stock = 300mM; final=0.3mM) and shaken for 3-4 hours (275rpm). For some mutants with less solubility we induced at lower temperatures (15°C-30°C) for longer period of time (5 to 15 hrs). 1ml aliquot is taken at each hour after induction, centrifuged and the pellet is stored at -20°C for SDS-PAGE analysis (Figure D.1, lane 1).

**Storage;**
Induced cell culture was poured into 250ml centrifuge bottle and centrifuged at 5000 rpm for 10-15 min at 4°C (10,000g with JA-14, Avanti J-E, Beckman Coulter). The supernatant was discarded and the cell pellet was stored at -20°C or -80°C until purification.

**D.1.2 Purification**

**Lysis;**
Frozen cells were thawed slowly on ice (this takes >1hr). 12.5ml of lysis buffer (50mM Tris pH 7.64 at room temperature, 0.2M NaCl, 15% v/v glycerol and 20% sucrose) was added to resuspend the pellet (~2g). The cell mix was transferred to 50ml centrifuge tube. 275 µl of lysozyme (stock 20mg/ml in H<sub>2</sub>O) and 15µl of PMSF (phenylmethylsulphonyl fluoride, 17.5mg/ml in isopropanol, prepared fresh every time) were added to the cell mix. The lysis mix was stirred at 4°C for 1 hr. 20µl of lysed cell was taken and frozen for SDS-PAGE analysis (Figure D.1, lane 2).
Next, the cells were further lysed with sonication as following. While keeping the centrifuge tube cool by putting wet ice around it, we sonicated the lysis mix for 5 minutes at 20% power (400W in full power) with 50% duty cycle (0.5 sec ON; 0.5 sec OFF) (20 kHz, Sonic Dismembrator Model 500, Fisher Scientific). We checked the solution fluidity by gently pipetting with a P-1000 pipette. 20µl of lysed cell was taken and frozen for SDS-PAGE analysis (Figure D.1, lane 3).

**Centrifugation:**
The lysed cell was centrifuged at 14,000 rpm (35,000g with JA-20, Avanti J-E, Beckman Coulter) at 4°C for 30-60 min. After centrifugation, the supernatant was poured into a clean 50ml centrifuge tube. 20µl of the supernatant and a small amount of pellet were stored as soluble and insoluble fractions respectively, for later SDS-PAGE analysis.

**Ni-NTA column (batch mixing):**
2 ml of pre-equilibrated Ni-NTA agarose column was added to the 50ml tube containing the supernatant. (Ni-NTA agarose is pre-equilibrated by stir-mixing with 10ml buffer A, centrifuging at 1500rpm for 10 sec (LWS M24 Combo). This is repeated 2-3 times). The Ni column and supernatant mix were stir-mixed for 1 hr 30min at 4°C. Such batch mixing enhances binding of His-tagged protein to Ni column greatly. Buffer A is composed of 50mM Tris-HCl, pH 7.67 at R/T, 150mM NaCl, 25% v/v glycerol.

**Column washing:**
40ml of buffer A was added to the batch mix (same 50 ml tube). The tube was centrifuged at 2000 rpm (5,000g with JA-20, Avanti J-E, Beckman Coulter) for 10-15 min at 4°C. Supernatant was discarded gently to avoid losing the Rep bound column. This step was repeated twice to remove free proteins or non-specifically bound proteins off of Ni column.
**Column loading:**
The Ni column was transferred to an empty disposable 10ml column (Poly-Prep chromatography column, Bio-Rad). Column was further washed with 10x column volume (10 cv) of Buffer A at 4°C.

The column was treated with 1 cv of 0.1mM TCEP (tris(2-carboxyethyl)phosphine, 10mM stock, diluted in buffer A) to reduce any disulfide bonds that may have formed between Rep molecules, which would hinder labeling with maleimide PEG. Reductants such as βME and DTT (dithiothreitol) are not to be included in any buffers used in this preparation since it would prevent labeling. After 10min TCEP treatment (at room temperature), the column was washed thoroughly with 20 cv of buffer A.

**Elution with Imidazole:**
The elution buffer was prepared by adding a high purity imidazole powder (e.g. Cat# 56749 (Fluka), Sigma-Aldrich) into a buffer A for the final concentration of 200 mM. The pH of the solution should be adjusted to 7.67 at room temperature since the imidazole significantly increases the pH, which impairs the proper elution.

0.5 cv of the elution buffer was added to the Ni column. The first elution does not contain any protein. 0.5cv of the elution buffer was added several times (~10 cv in total) and the eluant was collected in 0.25 cv aliquots. The concentration of the Rep protein (extinction coefficient: 76,800 cm⁻¹M⁻¹ at 280nm) was measured in an absorption spectrometer. Typically the maximum concentration was around 25-50 µM. Since Rep protein aggregates when the concentration is higher than 25 µM, the concentration should be adjusted accordingly, for long term storage. The eluted protein is shown in (Figure D.1, lanes 6-7).
D.2 PEG-Mediated Dimer

D.2.1 Dimerization

We use PEG polymer (MW 3,400Da) that has maleimide groups at both ends. Since the Rep mutant we use has only one Cys (at the 333rd amino acid residue) (Rasnik et al., 2004), when the conjugation occurs between the PEG molecules and the proteins, we get some monomers that are cross-linked with each other (Figure D.2A).

To start, we degas the solution that has the proteins eluted from Ni-column, to enhance the reaction efficiency. Then we mix the protein with a specific amount of a freshly-made PEG solution (1 mM in water). We incubate the mixture at 4°C with gently stirred/rotated overnight.

From SDS-PAGE (1hr, 100V, 10%, pre-cast gel, Bio-Rad) (Figure D.2B), we find that there are three major populations with different ratios between the PEG molecules and the protein. The lowest band represents the unmodified monomers (see the lane 9 for control). The middle band is a PEG-tethered monomer that does not have another monomer cross-linked (see the lanes 7 and 8 for control). The highest band must be the PEG-mediated dimer based on its molecular weight. There are other extra thin bands shown, which are likely monomers cross-linked with other small protein not removed.

The maximum efficiency of cross-linking is achieved when the ratio is 1:1 between the PEG molecules and the protein (Figure D.2B, lane 3). When the ratio is lower (i.e. less amount of PEG molecules than proteins), the cross-linking efficiency drops and the population of the PEG-tethered monomers almost disappears (Figure D.2B, lanes 1 and 2), as expected. On the other hand, when the ratio is higher (i.e. more amount of PEG molecules than proteins), the population of the PEG-tethered monomers increases (Figure D.2B, lanes 4, 5, and 7), reducing the yield of obtaining PEG-mediated dimer, which is also predictable.

As a control, when we have reacted PEG with a maleimide group only at one end with the Rep mutant, we observe three populations (Figure D.2B, lanes 7 and 8). The dominant population is the PEG-tethered monomers, as expected. The appearance of a small population of dimers is not explainable at this moment.
D.2.2 Size Exclusion Purification

To separate the dimer from the monomer we carry out size-exclusion purification with a sephacryl column (HiPrep 16/60, Sephacryl S-100, GE Healthcare) that is suitable for differentiating a range of proteins from 10 to 100kDa.

The column (cv =120ml) is first pre-equilibrated with 2 cv of buffer A (with 150mM NaCl), which is monitored by the change in conductivity of the column. For the buffer A that contains 25% glycerol, we set the flow rate 0.20-0.25 ml/min while keeping the pressure lower than 0.15 MPa, which is carried out overnight (>15 hours). Insufficient pre-equilibration leads to poor resolution in purification. Also, note that the buffer needs to have ionic strength higher than 150mM NaCl to avoid non-specific interaction. All the solutions are filtered with 0.2 µm filter and degassed by 20 min sonication for FPLC (fast protein liquid chromatography) system.

Then we load ~1 ml of the protein sample (25 µM) into the column. The higher the concentration and the smaller the volume, the higher the purification resolution. We set the flow rate at 0.2 ml/min; the lower the flow rate, the higher the resolution. After ~1/3 CV of buffer A, the protein sample is pushed through the column, then loaded proteins start to come out. As shown in Figure D.3B, three blurry peaks are observed in a UV trace when the PEG-mediated dimers are eluted, while only one major peak is observed when a normal monomer is eluted (Figure D.3A). Note that the protein is 10x diluted than before loading. After purification, the column is washed with 4cv water for short term storage and then 4cv 20% ethanol for long term storage. For a frequent use, the column may be stored with buffer solution inside.

We confirmed the purification quality by running a SDS-gel (1hr, 100V, 10%, pre-cast gel, Bio-Rad) (Figure D.3C). As expected, the first peak in a UV trace corresponded to the PEG-mediated dimer; the second, the mixture of the dimer and the PEG-tethered monomer; and the last was the unmodified monomers.
D.3 Unwinding with PEG-Mediated Dimer

We have tested the unwinding activity of PEG-mediated dimer with ensemble FRET measurements. In buffer W (20mM Tris-HCl pH7.5, 6mM NaCl, 1.7mM MgCl$_2$, 5mM βME, 10% v/v glycerol), we add 1 mM ATP and 5 nM 3’-partial dsDNA with (dT)$_{40}$ tail and 18mer dsDNA stem (5’-Cy3-TGG CGA CGG CAG CGA GGC with the tail and its complementary strand 5’-GCC TCG CTG CCG TCG CCA-Cy5-3’). We initiate the unwinding reaction by adding Rep protein into the mixture. FRET efficiency is very high before unwinding but the total intensity is very low since the two dyes are right next to each other (Figure D.4A). Unwinding leads to decrease in FRET and increase in intensity.

As shown in Figure D.4A, 10nM of PEG-mediated dimers (i.e. 20nM monomers in total) unwinds faster than 20nM monomers do. Such enhancement is universally observed at different protein concentrations (Figure D.4B). In contrast, the unwinding amplitude is the same regardless of whether monomers or dimers are used as long as the total monomer concentration is the same. The enhancement in the unwinding rates becomes less dramatic with a partial dsDNA with (dT)$_{20}$ tail (data not shown).

We have also carried out single-turnover experiments (Cheng et al., 2001). This time, we first incubate Rep protein and 5 nM partial dsDNA for 3 min in buffer W. Then we add 1 mM ATP together with 30 µM unlabeled 18mer ssDNA (5’-TGG CGA CGG CAG CGA GGC-3’). The large amount of the unlabeled ssDNA works as a protein “trap” thereby makes the unwinding reaction occur only once and only for the DNA bound with proteins before initiation. As shown in Figure D.4C, the higher the protein concentration, the bigger the unwinding amplitude. The unwinding amplitude saturates with less amount of dimers (~50nM) than monomers (~300nM) indicating the enhanced unwinding activity. The enhancement is less dramatic with (dT)$_{20}$ tail (data not shown).
References


Figure 1.1 FRET
(A) Shown is the distance dependence of FRET efficiency. Cy3 (donor, green circle) transfers half of its energy to Cy5 (acceptor, red circle) when they are separate by $R_0$ (6nm).
(B) The change of FRET is the most prominent around $R_0$. 

$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$
Figure 1.2 Single-Molecule FRET Study
(A) FRET in ensemble represents only the average value from many molecules.
(B) The time traces of individual molecules reveal the true FRET states of different conformations in real time.
(C) Events under study can be post-synchronized during data analysis.
Figure 1.3 Recombination-Mediated DNA Repair

(A) Shown is the RecBCD-mediated pathway. (a) When a replication complex encounters a nick, (b) the replication fork collapses. (c) RecBCD processes the dsDNA break into a partial dsDNA, loads RecA onto the nascent ssDNA and (d) the RecA filament mediates the homologous strand exchange. (e) RuvAB branch-migrates the structure forming Holliday junction (Cox et al., 2000).

(B) Shown is the RecFOR-mediated pathway. (a) When the replication complex encounters a lesion, (b) it stalls. (c) RecFOR loads RecA. (d) The same as [Ad]. (e) The same as [Ae]. (Cox et al., 2000)
Figure 1.4 Structure, Formation and Dynamics of a RecA Filament

(A) The structure of a RecA filament is shown (a) in an X-ray crystal structure adapted from Rajan and Bell (2004) (b) as a schematic and (c) by electron microscopy (Courtesy of C. Lei and Jiajie Diao in UIUC).

(B) Illustrated is an ill-featured model based on ensemble studies. (a) A RecA filament nucleates on ssDNA randomly. (b) Once nucleated, it extends towards the 3’-end of the DNA very rapidly. (c) At the same time, RecA dissociates from the 5’-end of the DNA when hydrolyzing ATP. We call the stable one as the 3’-extending end and the other as the 5’-disassembly end.
Figure 1.5 Structure and Dynamics of Holliday Junction

(A) X-ray crystal structure of the anti-parallel stacked conformer, adapted from Ho and Eichman (2001)

(B) Holliday junction has two stacked conformations (isoI and isoII), which are inter-changeable through an open form.

(C) Initially, the structure of the stacked conformations was predicted to be parallel, in which the strands cross each other at the core of the junction.
Figure 2.1 Objective-Type TIR Microscopy

(A) (a) The schematic shows how the TIR occurs at the interface between a coverslip and water (side view).
(b) The photo shows how the expanded beam is introduced into a microscope through a back port.

(B) (a) In the two-color emission optics, the collimated beam goes through a dichroic mirror where the donor signal is reflected. After passing through a lens (M2), the donor and acceptor images are projected into one half of the CCD screen each. (b) Shown is a photo of the two-color setup (bird’s eye view).
Figure 2.2 Prism-Type TIR Microscopy

(A) (a) The schematic shows how the total internal reflection occurs at the interface between a quartz slide and water (frontal view). (b) The photo is a bird’s eye view of the excitation optics.

(B) (a) Shown are components used for temperature control: a prism holder (bottom, center), its securing bar (left), a stage plate that holds the sample cell (top) and a home-built brass collar on the objective (center) (b) Shown is the microscope equipped with the components listed in [Ba] and tubing.
Figure 2.3 Confocal Microscopy
Shown is the schematic of the three-color confocal microscopy (Courtesy of Sungchul Hohng). The confocal setup was made on an inverted microscope (Olympus, IX50). A solid-state laser (CrystaLaser) excites a sample at 532-nm through a 100x oil-immersion objective. Emission from three dyes is collected by the same objective and separated by dichroic mirrors and filtered out by band-pass filters. Identities of symbols are D1, Chroma dichroic 545DRLP; D2, Chroma dichroic 650 DRXR; D3, Chroma dichroic 690DCLP; F1, Chroma filter HQ580/60 m; F2, Chroma filter HQ670/40 m; F3, Chroma filter HQ715/30 m; M, mirror; L, lens; P, pinhole; PBS, polarizing beam splitter; λ/2, half-waveplate; and APD, avalanche photodiode. Note that the two-color setup does not have the optics of D3, F3 and the corresponding APD installed.
Figure 2.4 Fluorophore

(A) (a-b) Drawn are the molecule structures of Cy3 and Cy5 dyes, respectively.

(B) (a) The emission spectrum of Cy3 overlaps with the absorption spectrum of Cy5. (b) The emission spectra of Cy3 and Cy5 are well-separated.

(C) (a) Cy3 and Cy5 are placed in such way that the distance between them becomes shorter than $R_0$ (left) and farther than $R_0$ (right) in two different conformations. (b) A dye is placed in the middle of ssDNA for the optimal separation between Cy3 and Cy5.
Figure 2.5 Sample Immobilization

(A) On a naked glass surface, biotinylated BSA is deposited and then neutravidin is conjugated with biotinylated BSA. A biotinylated specimen (here DNA) is then immobilized via biotin-neutravidin binding.

(B) On the amine-modified glass surface, the NHS-ester form of PEG is covalently conjugated. Neutravidin specifically binds to a fraction of PEG molecules that have biotin at the end of them.

(C) On the surface of [B], a biotinylated lipid-vesicle is immobilized, which contains labeled molecules such DNA inside.
Figure 2.6 Single-molecule Image
(A) Shown is an image from a blank chamber. The image is split into donor and acceptor channels, each 25 µm x 50 µm. A few fluorescent junk molecules are visible.
(B) (a) Shown are Cy3 molecules immobilized on the same chamber which are distinctly brighter. (b) Shown is an example fluorescence time trajectory from two selected spots.
Figure 2.7 Conformational Dynamics in Equilibrium

(A) Shown is the scheme of two-state dynamics whose examples are in [B-C] below.

(B) (a) A sm-time trace shows a two-state fluctuation between high and low FRET states. (b) FRET histogram from the data in [Ba]. (c-d) The dwell time distribution of low and high FRET states, respectively. $\tau_A$ is equal to $1 / k_{A \rightarrow B}$ and $\tau_B$ to $1 / k_{B \rightarrow A}$. Figures are adapted from Hohng et al. (2004b).

(C) (a) The fluctuation between two states is very fast. (b) FRET histogram from the data in [Ca]. (c) Cross-correlation between the donor and acceptor time traces in different salt concentrations.

(D) When there are more than two states observed (here six including photoblinking of acceptor), an advanced algorithm is required for reliable data analysis. The fit (green line) is from HaMMy analysis.
Figure 2.8 Single-Molecule Three-Color FRET

(A) A FRET pair attached to the points 1 and 2 reports on the distance between 1 and 2 but no information can be obtained on the position of the point 3.

(B) Three-color FRET are classified into the following categories (a) two-donors and one-acceptor, (b) two-step FRET, and (c) one-donor and two-acceptors.

(C) (a) Normalized emission spectrum of Cy3 (green) and absorption spectra of Cy5 (red) and Cy5.5 (navy).

(b) Normalized emission spectra of Cy3 (green), Cy5 (red) and Cy5.5 (navy).

(D) Cy5 signal shows up in one conformation (right) and Cy5.5 in the other (left).

(E) In the TIR setup, the donor signal is separated first, then the other two colors are later split in the same way as in Figure 2.1B. They are then combined by another dichroic mirror and projected into CCD.
Figure 3.1 Filament Nucleation on Short ssDNA Tails

(A) A dsDNA with a 3’-(dT)_n ssDNA tail is immobilized on a surface. FRET between fluorescent labels report on the changes in the average end-to-end distance of the ssDNA.
(B) Fluorescence spectra with (green) and without (red) RecA and ATPγS; and with RecA and ATP (black)
(C) FRET histograms (a) High FRET ($E \sim 0.55$) is from DNA-only (white, 51 molecules) and low FRET ($E \sim 0.1$) is with RecA and ATPγS (green, 57 molecules, after 5 min incubation) (b) RecA with ATP ($E \sim 0.1$ and 0.55—114 molecules, after 5 min incubation). Data from donor-only molecules or periods were excluded.
(D) ssDNA-length dependent FRET efficiency without (gray) and with (black) RecA and ATPγS
(E) Time trace with RecA and ATP (100 ms resolution). FRET stays high ($E \sim 0.55$) but briefly drops at $t = 75$ sec. Acceptor blinking (left arrow) and bleaching (right arrow) are distinguishable from the low FRET state.
(F) (a) Normalized integrated dwell-time histograms for the low-FRET state vs. ssDNA length (115, 119 and 228 events for 17, 19 and 21 nt) and exponential fits. S.D. are from three data sets. (b) Resulting nucleation frequency (602, 614 and 350 molecules from left)
(G) (a) At different temperatures with (dT)_19 (62, 54 and 70 events for 15, 23, 35 °C). (b) Nucleation frequency (479, 212 and 344 molecules from left)
Figure 3.2 Rapid Nucleation in a Confined Volume

(A) DNA and RecA encapsulated inside porous vesicle (not to scale)

(B) Either (a) binding of an oligomer or (b) simultaneous binding of monomers will lead to the nucleation.

(C) Time trace from an encapsulated molecules in presence of 1mM ATP together with RecA.

(D) (a-b) Dwell time plot of the low-FRET (filament assembly) and high-FRET states (filament disassembly)
    of the 103 ssDNA with the exponential decay fits, respectively.

(E) Comparison of transition frequencies between encapsulated molecules (navy) and surface tethered partial dsDNA with (dT)_{19} tail with 1, 2.5, 5 and 10 µM RecA (black).
Figure 3.3 Dynamics at the 5'-Disassembly End

(A) FRET reports on the dynamics at the 5'-disassembly end

(B) FRET histograms (a) from DNA-only ($E \approx 0.65$, green) and with RecA and ATPγS ($E \approx 0.15$, white, after 2 min incubation) and (b) with RecA and ATP (after 30 min incubation). Vertical gray lines mark $E=0$.

(C) Trace with RecA and ATP (100 ms resolution). The FRET trace (black) is fitted by HaMMy (green) then four states are assigned $M_0$, $M_1$, $M_2$ and $M_3$ based on the TDP in [Da]. Donor bleaching at $t \approx 255$ s (arrow).

(D) Transition density plot (TDP) (a) This pseudo-3D plot is constructed by adding a Gaussian peak for each transition. There are four states with $E \approx 0.15$, 0.3, 0.5 and 0.7. (b) Rates and frequencies (inside circles) of each transition, $M_i \rightarrow M_j$, presented in the same scheme.

(E) (a) Binding and (b) dissociation rates vs. RecA concentration for different transitions (based on 12,204 transitions from 172 molecules at 0.1 μM; 23,580 from 196 at 0.25 μM; 17,203 from 165 at 1 μM). (c) corrected dissociation rates. The errors are standard deviations of three data sets each.
Figure 3.4 Dynamics at the 5'-Disassembly End
(A) TDP and kinetic rates (a and b) with (dT)$_{10+49}$ tail based on 8,637 transitions from 104 molecules ($E$~0.35, 0.6 and 0.8). (c) Shown are the time traces taken with RecA and ATP (200 ms time resolution). The fit by HaMMy is in green, overlaid on each FRET trajectory in black.
(B) TDP and kinetic rates (a and b) with (dT)$_{5+54}$ based on 5,917 transitions from 92 molecules ($E$~0.8 and 0.9) and (c) time traces.
Figure 3.5 Stable Formation of $<\text{dsDNA}|\text{ATP}\gamma S>$ as a Nucleation Cluster

(A) (a and b) Filament formation on a 3'-(dT)$_{13}$ tail of a partial dsDNA shows lower FRET ($E\sim0.2$, white, after 20 min incubation) than DNA-only ($E\sim0.8$, green). (c and d) $<\text{ssDNA}|\text{ATP}\gamma S>$ disassembles in ~5 min upon RecA and ATP$_\gamma S$ removal from solution and FRET is restored to the DNA-only value ($E\sim0.8$, after 10 min incubation). (e and f) When RecA and ATP are added, stable $<\text{ssDNA}|\text{ATP}>$ forms quickly, assisted by $<\text{dsDNA}|\text{ATP}\gamma S>$ and FRET returns to a low value ($E\sim0.2$, after <1 min incubation).

(B) Disassembly of $<\text{ssDNA}|\text{ATP}\gamma S>$ from (dT)$_{13}$ and (dT)$_{10}$ ssDNA tails. (a) After [Ab], upon removal of RecA from solution but with ATP$_\gamma S$ kept in solution, monotonic stepwise increase in FRET is observed with (dT)$_{13}$. Time traces show (a) five and (b) four different FRET states during disassembly.

(C) The same assay as (B) but with (dT)$_{10}$ after 60 min incubation with RecA and ATP$_\gamma S$. Time traces show (a) four and (b) three different states during disassembly.

(D) (a) Filament formation on the dsDNA of a partial dsDNA results in $E\sim0.1$ (green, 15 min incubation) compared to $E\sim0.25$ of DNA-only (white). (b) $<\text{dsDNA}|\text{ATP}\gamma S>$ does not disassemble up to 1 hour after removal of RecA and ATP$_\gamma S$ but does slowly afterwards.
Figure 3.6 Dynamics at the 3'-Extending End

(A) FRET reports on the dynamics at the 3'-extending end. The stable <dsDNA|ATPγS> facilitates the formation of <ssDNA|ATP>.

(B) Time traces with ATP and 8 nM RecA (100 ms time resolution).

(C) (a) TDP shows five FRET states (E~0.2, 0.3, 0.55, 0.75 and 0.85) based on 4,635 transitions from 82 molecules. (b) The binding and dissociation rates and frequencies (inside circles) of each transition.

(D) (a) Binding and dissociation rates and (b) corrected dissociation rates. The errors are the standard deviations from two data sets each.
Figure 3.7 Revised Model on RecA Filament Formation and Dynamics

Our sm-data suggests a new model. A RecA filament nucleates by binding of a pre-assembled oligomer. Then the filament extends toward 5’- as well as 3’-end of ssDNA. Once it reaches the equilibrium, the dissociation of monomers occurs at either end; however, the rapid binding makes the 3’-extending end very stable, while the 5’-disassembly end experiences continuous binding and dissociation of monomers.
Figure 4.1 Model for Double-Strand Break Repair Process In Vivo

- Helicase
- Exonuclease
- SSB

process:

- double-strand break
- 3' ssDNA tail
- RecA-loading protein
- unknown mechanism by RecA-loading protein
Figure 4.2 RecA Displaces SSB Only When Supported by a Pre-formed Nucleation Cluster

(A) (a) Inability of RecA to displace SSB bound to (dT)$_{70}$ (b) DNA-only ($E \approx 0.1$). (c) With 10 nM SSB, DNA wraps around protein ($E \approx 0.7$, after 5 min incubation). (d) When RecA and ATP are added replacing SSB in solution, there is no change observed in FRET ($E \approx 0.7$, after 1 hour incubation).

(B) (a) RecA displaces SSB efficiently, given a pre-formed nucleation cluster. (b) DNA-only ($E \approx 0.1$) (c) A RecA filament forms around the dsDNA as well as the ssDNA ($E \approx 0.0$, after 15 min incubation). (d) After adding 10 nM SSB and removing RecA and ATPyS, <ssDNA|ATPyS> is effectively removed by binding of SSB ($E \approx 0.7$, after 15 min incubation) (d) When RecA and ATP are added replacing SSB in solution, RecA quickly forms a filament displacing SSB efficiently ($E \approx 0.0$, after <1 min incubation).

(C) Time traces show the transitions from SSB bound form ($E \approx 0.7$) to RecA filament ($E \approx 0.0$) (30 ms time resolution). After solution exchange ($t = 0$), $E$ drops through diverse pathways: FRET changes (a) without any intermediate state and (b) with one or (c) more intermediate state(s) were found with comparable frequencies. To distinguish the low FRET ($E \approx 0$) from fluorescence signal with inactive acceptor, the activity of acceptor was checked by directly exciting acceptor using 633 nm laser at $t > 38$ (sec) (arrows). (d) A group of FRET traces (57 molecules for 1 μM RecA; 26 for 0.1 μM) are averaged and fitted by exponential decay curves.
Figure 4.3 Formation of <ssDNA|ATP> in the Presence of <dsDNA|ATPγS>

(A) (a) Extension of a RecA filament from <dsDNA|ATPγS> into <ssDNA|ATP> in the absence of DNA-bound SSB (b-d) The same procedures as Figures 4.2Bb-d (e) When 100 nM (dC)₈₀ is introduced, SSB is transferred to (dC)₈₀ within a minute, leaving ssDNA tail naked but keeping <dsDNA|ATPγS> intact (after 2 min incubation). (f) When RecA and ATP are added in solution, a filament extends rapidly assisted by <dsDNA|ATPγS> (after <1 min incubation).

(B) (a and b) Time traces recorded during the transition from [Ae] to [Af]. RecA and ATP are added at t = 0. To distinguish the low FRET (E~0) from photobleaching of Cy5, the activity of acceptor was checked by directly exciting acceptor at t > 12 (sec) (arrows). (c) The transition rate is 0.8 (sec⁻¹), determined from 55 molecules.
Figure 4.4 <ssDNA|ATPγS> is Removed by SSB

(A) During the procedure in Figures 4.2Bb-d, <ssDNA|ATPγS> is displaced by SSB when RecA and ATPγS are removed from solution and 10 nM SSB is added.

(B) Typical sm-traces observed during the displacement show gradual change in FRET over several seconds.

(C) The change in the population of SSB-bound ssDNA is calculated from the change in FRET value. Each data point is based on the normalized average FRET value from more than 100 molecules (recording for 2 seconds). Different areas were imaged for individual data points. $t = 0$ when SSB is introduced and RecA is removed from solution.
Figure 4.5 Model for the Interaction Between SSB and RecA In Vivo
Figure 4.6 RecA Filament Dynamics Around a Junction

(A) Model for the filament dynamics in vivo (a) in the presence of a RecA-loading protein and (b-c) in its absence with >1 µM and <1 µM RecA, respectively.

(B) (a) Schematic of a dsDNA with (dT)_{16+43} tail. (b) Time traces with RecA and ATP in solution (200 ms time resolution). They stay in low FRET, most of the time, with infrequent visit to high FRET. (c) A FRET histogram (after 1 min incubation).

(C) (a) A dsDNA with (dT)_{19+35} tail (b) Time traces with RecA and ATP in solution (100 ms time resolution). They stay in low FRET, most of the time, with infrequent visit to high FRET. (c) A FRET histogram display a majority of the population in low FRET (after 1 min incubation).
Figure 4.7 Potential Effect of RecA Filament Dynamics on Other DNA-Interacting Proteins

(A) The filament dynamics around the junction may interfere with the binding of other DNA-interacting proteins such as helicases.

(B) The RecA filament slows down the unwinding activity of Rep helicase. Without RecA, it unwinds with a time scale of 109 sec. When the ssDNA is incubated with 1 µM RecA for 3 min before Rep is introduced, the unwinding reaction is delayed and there appear two phases during the unwinding.

(C) The RecA filament also slows down the unwinding activity of UvrD helicase. Without RecA, it unwinds with a time scale of faster than 24 sec. When the ssDNA is incubated with RecA for 3 min before UvrD is introduced, the unwinding reaction is delayed. The higher the RecA concentration, the slower the reaction.

(D) The inhibition shown in [C] is also observed with sm-assays. Moreover, the presence of <dsDNA|ATPγS> further slows down the unwinding activity.
Figure 4.8 Potential Effect of Repetitive Shuttling of Rep Helicase

(A) FRET detection of RecA filament formation on (dT)$_{40}$, which may be hindered by Rep.

(B) Time-dependent smFRET histograms of the DNA shown in [A] after adding RecA and ATP. Dashed lines denote the FRET values for the DNA only and the RecA filament. Also shown are the Lorenzian fits.

(C) Same as in [B], except for the inclusion of 1 nM Rep. The shift to higher FRET is due to Rep activity.
Figure 5.1 Model for Homologous Strand Exchange

(A) (a) When a ssDNA is surrounded by a RecA filament, it has the capability of selectively binding to a homologous dsDNA. (b) The exact structure of the pairing complex is not resolved yet. (c) The strand transfer occurs locally first, then (d) propagates sideways. (e) The strand exchange is completed when the filament disassembles. All the reactions are reversible, mediated by a RecA filament except the one between [d] and [e].

(B) The ternary structure in [Ab] might be only the same as [Ac]. That is, the pairing might occur by the weak interaction from the partial strand transfer.
Figure 5.2 Construct that Detects Pairing and Strand Transfer During Strand Exchange

(A-B) (a) Before pairing there is no signal observed (state I). (b) Upon the pairing, a signal appears and the FRET is either very low or very high (state II). (c) After the strand transfer, the FRET becomes very high. (C) The histogram is the distribution of the dwell time taken for the strand transfer to be completed. (D) We do not see any significant difference in the dwell time of [C] (a) when two opposite ends were observed, (b) when different lengths of ssDNA were tested, (c) when different sequences were designed and (d) when we tried different ATP factors. (E) We do see the dwell time become longer when a heterologous sequence is introduced at either end of the dsDNA.

(E) (a) With a construct in which it is the ssDNA-filament that floats around and binds to an immobilized dsDNA, (b) the dwell time of [C] becomes significantly longer and broader.
Figure 5.3 Construct that Detects the Separation of the Incoming dsDNA

(A-B) (a) Before pairing there is no signal observed (state I). (b) Upon the pairing, a high-FRET signal is observed (state II). (c) What follows is the fluctuation in FRET (state III), which we interpret as the post-strand transfer state. (d) Once the displaced strand leaves the complex, there is no FRET observed (state IV).

(C) The combined dwell time of states II and III shows strong dependence on free magnesium ions. Such strong dependence disappears when SSB is introduced.
Figure 5.4 Construct that Shows Tentative FRET During Strand Exchange

(A-B) (a) Before pairing there is no signal observed (state I). (b) Upon the pairing, a portion of molecules show a high-FRET signal (state II). (c) Eventually, the FRET becomes low (state III). Donor signal is dynamic for tens of seconds before stabilized, which indicates the delayed disassembly of the filament after strand transfer. (C) The integrated dwell-time histogram follows a single exponential curve with a 2.3 sec-characteristic time.
Figure 5.5 Three-Color FRET Assay

(A-B) Shown is a three-color FRET assay that is the combination of the assays in Figures 5.2 and 5.6. (a) Before pairing (state I), there is no signal observed. (b) The pairing leads to appearance of a signal and a high FRET between Cy3 and Cy5 (state II). (c) Afterward, FRET between Cy3 and Cy5.5 replaces the former FRET (state III).

(C) Illustrated is another possible three-color FRET construct.
Figure 5.6 Pairing Assay in Bulk

(A) A similar pairing assay as Figure 5.2 was carried out in bulk.

(B) (a) Strand exchange was observed with a wild-type RecA (green and blue with and without free magnesium ions, respectively) and with a truncated RecA mutant, RecAΔC17 (black and red with and without free magnesium ions, respectively). (b) The strand exchange reaction was compared with annealing reaction (black and red with and without free magnesium ions, respectively).
Figure 5.7 Pairing Assay with Archaeal RadA

(A) The same experiment as Figure 5.8 was carried out with an archaeal protein, RadA. Different concentrations of RPA1 were added in each reaction. The rate was calculated with the first four curves.

(B) (a-b) The same experiment was carried out with C-terminus and N-terminus truncated RPA1 mutants, respectively. The rates shown were calculated only with the C-terminus and N-terminus truncated RPA1 mutants, respectively.
Figure 6.1 Conformational Polymorphism in a Four-Way DNA Junction Studied Using FRET

The four arms of the junction are named B, H, R and X sequentially around the point of strand exchange. Cy3 (green circle) and Cy5 (red circle) fluorophores are attached to the ends of two arms. Vectors are named according to the arms carrying donor and acceptor (in that order).

(A) The XB vector of junction 1. With divalent metal ions, the structure folds into the stacked X-structure. There are two possible anti-parallel conformers that differ in the stacking partners ($iso$ I and $iso$ II).

(B) The positions of attachment of fluorophores are reversed in the BX vector compared to the XB vector, but should exhibit the same variation in fluorescence in response to the transition between $iso$ I and $iso$ II.

(C) XR vector. The fluorophores will only be close in the form of $iso$ II, in contrast to the XB and BX vectors.
Figure 6.2 Conformational Dynamics Between Stacking Conformers

(A-B) The interconversion of the XB vector of junction 1 between two stacking conformers (8 ms bin time). It shows a time record of donor (green) and acceptor (red) fluorescence signals of a single molecule in the presence of 30 mM Mg²⁺ and 50 mM Na⁺. And shown at right is the histogram of $E_{\text{app}}$ from 41 molecules. The high FRET ($E_{\text{app}} \approx 0.69$) state corresponds to isoI and the low FRET ($E_{\text{app}} \approx 0.15$), isoII.

(C-D) Time record in the presence of 1 mM Mg²⁺ and no Na⁺. The acceptor photobleached at 2.6 s. And shown at right is the histogram of $E_{\text{app}}$ from 10 molecules.

(E-F) Time record in the presence of 0.1 mM Mg²⁺ and no Na⁺. The histogram of $E_{\text{app}}$ is from 13 molecules.

(G) Cross-correlation analysis on BX vector of junction 1. The correlation time, $\tau$, changes over different Mg²⁺ ion concentrations. $\tau$ is 2.1, 21.2 and 71.3 ms in the presence of 0.5 (circles), 10 (squares) and 100 mM (triangles) Mg²⁺, respectively.

(H) Conformer transition rates $k_{\text{CT}}$ of BX, XB and XR vectors of junction 1 as a function of Mg²⁺ ion concentration. Each $k_{\text{CT}}$ value is the average over about 10 molecules. Error bars on the graph represent the heterogeneity in $k_{\text{CT}}$ values between molecules.
Figure 6.3 Dynamics of Stacking Conformer Exchange of Junction 7 in the Presence of Na$^+$ and Hexammine Co (III) Ions

Data were obtained from the junction 7 HB vector.

(A-B) Fluorescence signals with time resolution of 4 ms, in the presence of 1.5 M Na$^+$. The acceptor bleached at 3.25 s. The histogram of $E_{\text{app}}$ is from 13 molecules. $E_{\text{app}}$ fluctuates between ~0.66 (isoII) and ~0.19 (isol).

(C-D) Time record in the presence of 2 mM [Co(NH$_3$)$_6$]$^{3+}$ and 50 mM Na$^+$. The acceptor photobleached at 2.25 s followed by the donor photobleaching at 2.35 s. The histogram of $E_{\text{app}}$ is from 16 molecules.

(E) The conformer transition rate $k_{\text{CT}}$ as a function of Na$^+$ concentration.

(F) $k_{\text{CT}}$ as function of [Co(NH$_3$)$_6$]$^{3+}$ concentration in the presence of 50 mM Na$^+$. Each rate is an average from ~10 different molecules, and error bars reflect the heterogeneity in $k_{\text{CT}}$ values between molecules.
Figure 6.4 Competition and Cooperation Between Monovalent and Divalent Ions

The dependence of transition rates for junction 7 on Na⁺ concentration in the presence of three different background Mg²⁺ concentrations of 2 (triangles), 5 (circles) and 20 mM (squares). Each rate is an average of ~5 different single junction molecules. Error bars reflect the heterogeneity in $k_{CT}$ values between molecules.
Figure 7.1 Estimating the Population of the Open Structure

(A) The XB vector of junction 1. In the absence of metal ions the junction takes the open form (low FRET). On addition of divalent metal ions, the structure folds into the stacked X-structure of isoI and isoII (high and low FRET, respectively). For the transition between the two stacked structures, the junction has to go through an open form, which is not well-characterized.

(B) XR vector. The fluorophores will only be close in the antiparallel form of isoII, in contrast to the XB vector. The open form still exists in low FRET.

(C) The XB (circles) and XR (squares) vectors of junction 1 have been studied as a function of Mg$^{2+}$ concentration. $E_{\text{app}}$ values averaged over ~20 molecules are plotted as a function of ion concentration. The averaged $E_{\text{app}}$ of the XB vector is close to the $E_{\text{app}}$ of isoI above 100 µM Mg$^{2+}$. Below 10 µM Mg$^{2+}$, the junction exists primarily in the open structure with $E_{\text{app}}$ value significantly below that of isoI. In between, a clear and gradual transition in the average $E_{\text{app}}$ is observed. In contrast, no significant change in $E_{\text{app}}$ is observed in the XR vector.
Figure 7.2 Parallel Forms of the Junction are not Detected

(A) The donor-acceptor pair for the BR vector of junction 1 should come into close proximity only in the parallel forms. The XH vector (not shown) would similarly detect the parallel form.

(B-G) Typical sm-time records with 5-6 ms time resolution are shown for the BR vector of junction 1 in (B) 30 mM Mg²⁺, (D) 1 mM Mg²⁺ and (F) for the XH vector in 50 mM Mg²⁺. In each case, donor and acceptor fluorescence signals do not exhibit any anti-correlated two-state fluctuations except in the case of photoblinking/photobleaching events of the acceptor. (C), (E) and (G) show the cross-correlation analysis from about 30 molecules in each case, which supports non-existence of parallel forms.
Figure 7.3 No Parallel State Observed with a Three-Color FRET Assay
(A) Three-color FRET experiment on X3H5B5.5
(B) Time traces of X3H5B5.5. FRET fluctuation between Cy3 and Cy5.5 is clear and we conclude this is a “real” molecule. No high FRET state was observed for Cy5, indicating the absence of stable parallel states.
Figure 7.4 Holliday Junction Dynamics Using One Donor and Two Identical Acceptors

(A) Two-color FRET experiment on X³R⁵B⁵

(B) When both of the two acceptors are active, the acceptor signals are almost constant, which supports the model depicted in [A]. After one of the two acceptors bleaches (dotted red lines), time traces show kinetics similar to X³B⁵ (top) or X³R⁵ (bottom), depending on which acceptor bleaches.
Figure 7.5 Holliday Junction Dynamics Using One Donor and Two Different Acceptors

(A) Three-color FRET experiment on X₃R³B₅.₅
(B-C) Intensity time traces of X₃R³B₅.₅. All signals are corrected for bleed-through. When the three dyes are active, anti-correlation between Cy5 and Cy5.5 signals are clear. After one of the two acceptors photobleaches, time traces show the behavior of [B] X₃B₅ or [C] X₃R₅ depending on which acceptor bleaches first.
(D) Relative distribution of apparent Cy5 FRET efficiency (E₅) and apparent Cy5.5 FRET efficiency (E₅.₅). Data were collected from ~ 60 molecules.
(E) Histograms of transition time difference between Cy5.5 and Cy5 signals.
Figure A.1 Sample Chamber

(A) A sample chamber is made by putting a microscope slide and a coverslip together with a double-sided tape and by sealing with epoxy. The holes on the slide are used for the inlet and outlet of solution exchange.

(B) A syringe is connected to the chamber through tubing and a pipette tip that contains a solution is snugly plugged into an inlet hole. When the syringe is pulled, the solution is introduced into a chamber.
Figure B.1 Fluorescence and Absorption Spectra of Cy3 and Cy5 that are Conjugated to DNA
(A) Formation of a filament on a dsDNA with 3' ssDNA tail
(B) (a) While (dT)$_{13}$ ssDNA tail alone shows high FRET (red), the filament formation with RecA and ATP$_{γ}S$ results in 60% decrease in FRET (green, after 10 min incubation) (b) The same DNA but with Cy3 only. Fluorescence of Cy3 becomes enhanced when a filament forms with ATP$_{γ}S$, by 70% (after 10 min incubation). (c) Fluorescence signal of Cy5 decreases when the filament forms with ATP$_{γ}S$, by 30% (after 10 min incubation) (d) Absorption spectra of Cy3 and Cy5 molecules without and with a RecA filament formed, with ATP$_{γ}S$ (after 10 min incubation)
(C) Time traces (100 ms time resolution) with RecA and ATP$_{γ}S$ shows photobleaching of Cy5 at $t = \sim 30-90$ and $\sim 95-130$ (sec) before photobleaching of Cy3 at $t = \sim 150$ (sec) (arrows).
(D) Time traces (100 ms resolution) taken by alternative excitation of green and red laser light. A dsDNA with (dT)$_{31}$ tail was excited by 532nm light for 9 sec and by 633nm for 1 sec, alternatively. Until Cy5 bleaching at $t = \sim 32$ (sec) (arrow), not only there is Cy5 signal by FRET ($\sim 0.1$) but also there is Cy5 signal when directly excited by 633nm light. After the Cy5 bleaching, there is neither FRET nor Cy5 signal by direct excitation.
(E) (a) Formation of a filament on a ssDNA with Cy3-labeled in the middle that was used in Figure 3.3A (b) Cy3 fluorescence becomes enhanced when a filament forms with ATP$_{γ}S$, by 50% (after 10 min incubation) (c) Absorption of Cy3 molecule without and with a RecA filament formed, with ATP$_{γ}S$ (after 10 min incubation).
Figure B.2 Filament Formation on Short ssDNA Tail with ATP and ATPγS

(A) Formation of a filament on a dsDNA with 3' tail

(B) 2-dimensional density plot of $E$ vs. ‘fluorescence intensity’ from 114 molecules

(C) FRET and total-intensity trajectories (100 ms time resolution) from (dT)$_{21}$ tail with RecA and ATP in solution. *Top:* FRET stays high ($E \approx 0.55$) most of the time but frequently becomes low ($E \approx 0.1$). *Bottom:* Accompanying the shift to $E \approx 0.1$ is an overall increase in the total fluorescence signal.
Figure B.3 Filament Formation on Short ssDNA Tail with Different Types of NTP Factors

(A) Formation of a filament on a dsDNA with 3’ tail

(B) FRET histograms with (dT)_{21} tail in the presence of RecA (a) with ATP (after 1 min incubation), (b) with ATPγS (after 1 min incubation), (c) with dATP (after 1 min incubation), and (d) without any NTP (after 1 min incubation)

(C) Time traces with a 100 msec time resolution (a) (dT)_{21} tail with RecA and dATP in solution. Several low-FRET states are observed until photobleaching of Cy5 (arrow). (b) (dT)_{17} tail with RecA and dATP. Formation of a nucleation cluster is observed several times (arrows). (c) (dT)_{21} tail with RecA only. Frequent two-state transitions between $E \approx 0.3$ and $\approx 0.55$ are observed until Cy3 and Cy5 photobleach, respectively (arrows).
Figure B.4 Formation of \(<\text{dsDNA}|\text{ATP}_{\gamma}\text{S}>\)

(A) (a) Filament formation on a dsDNA with 3’ (dT)$_{20}$ ssDNA tail. Cy5 is labeled in the middle of a dsDNA instead of at the junction of ss-dsDNA. (b) Change in a FRET histogram when RecA is added with \text{ATP}_{\gamma}\text{S} indicates a filament formation on the dsDNA (after 15 min incubation).

(B) Protection of dsDNA by \(<\text{dsDNA}|\text{ATP}_{\gamma}\text{S}>\) from an endonuclease (a) dsDNA with 3’ (dT)$_{13}$ tail was labeled with Alexa647 at the junction. It is incubated with RecA and \text{ATP}_{\gamma}\text{S} before cleavage by DNase I. (b) The 600nM DNA molecules were incubated with 10 μM RecA and 1 mM \text{ATP}_{\gamma}\text{S} for 15, 30, 60, 120 and 300 seconds before cleavage (lanes 1-5). The DNA (600 nM) was incubated with 10 μM RecA and 1 mM ATP (lane 7); and with 0, 2, 6 and 10 μM RecA and 1 mM \text{ATP}_{\gamma}\text{S}, for 10 minutes before cleavage (lanes 8-11). Lane 6 is for a DNA without any RecA and DNase I.

(C) (a) No filament formation on a dsDNA with blunt ends, which is labeled with Cy3 and Cy5. FRET histograms (b) without RecA and (c) with RecA and \text{ATP}_{\gamma}\text{S} in solution (after 2 hour incubation).
Figure B.5 Formation and Disassembly of <dsDNA|ATPyS> at 35°C

(A) The same experiment as Figure 3.5A but at high temperature (35°C). (a and b) Filament formation on a 3'- (dT)_{13} tail of a partial dsDNA results in low FRET (green, $E \approx 0.05$, after 30 min incubation), while DNA-only shows high FRET (white, $E \approx 0.75$). (c and d) <ssDNA|ATPyS> disassembles after RecA and ATPγS are removed from solution thus FRET is restored to the DNA-only value [Ab, white] (after 10 min incubation). (e and f) After 10 min-incubation in the condition of [Ac], RecA and ATP are added and a stable <ssDNA|ATP> forms on ~50% of the molecules, assisted by <dsDNA|ATPyS> (after <1 min incubation).

(B) The same experiment as Figure 3.5D but at high temperature (35°C). (a) Filament formation on a dsDNA with (dT)_{20} tail results in $E \approx 0.05$ (green, after 30 min incubation) compared to $E \approx 0.2$ of DNA-only (white). (b) About a half of <dsDNA|ATPyS> disassembles during the first 10 minutes after removal of RecA and ATPγS from solution (after 10 min incubation).
Figure B.6 Formation and Disassembly of $\langle$dsDNA$|$ATP$\gamma$S$\rangle$ Mediated by 5’ Tail ssDNA

(A) (a and b) Filament formation on 5’-(dT)$_{20}$ ssDNA tail of a partial dsDNA results in low FRET ($E\sim0.05$, green, after 30 min incubation), while FRET is high with DNA-only ($E\sim0.6$, white). (c and d) $\langle$ssDNA$|$ATP$\gamma$S$\rangle$ disassembles in 5 minutes after RecA and ATPγS are removed from solution (after 30 min incubation). (e and f) After 30 minute-incubation in the condition of [Ac], when RecA and ATP are added, $\langle$ssDNA$|$ATP$\rangle$ forms assisted by $\langle$dsDNA$|$ATP$\gamma$S$\rangle$ (after <1 min incubation). (g) Time trace (100 ms time resolution) in the condition of [Af]. Cy3 photobleached at $t \sim 130$ sec (arrow).

(B) A FRET histogram when RecA and ATP are added, in the absence of $\langle$dsDNA$|$ATP$\gamma$S$\rangle$ (after 1 min incubation). Longer incubation does not change the shape of the distribution.

(C) (a) Time trace (100 ms time resolution) in the same condition and configuration as [Ag] but with 3’-(dT)$_{19}$ tail. Cy3 photobleached at $t \sim 120$ sec (arrow). (b) A FRET histogram with 3’ (dT)$_{19}$ tail. In contrast to [Af], no intermediate or high FRET states are observed.

(D) (a) Filament formation on the dsDNA with (dT)$_{20}$ tail results in $E\sim0.05$ (green, after 30 min incubation) compared to $E\sim0.2$ of DNA-only (white). (b) It takes more than two hours for $\langle$dsDNA$|$ATP$\gamma$S$\rangle$ to disassemble after RecA and ATPγS are removed from solution.
Figure C.1 Optical Trapping
(A) Shown is a transparent spherical bead trapped by a focused laser beam.
(B) Shown is an example of a beam path through a bead and the corresponding momentum change of the beam.
(C) Net force is generated when the trap center is offset from the bead center (a) horizontally and (b) vertically.
(D) When a trap moves toward the right, the bead follows behind due to the drag force. The distance between the bead and the trap centers is determined by the equilibrium between the drag and the trapping forces.
Figure C.2 Experimental Scheme of Studying Cell Response to External Force
(A) A cell is immobilized on surface and a bead is captured by an optical trap.
(B) The bead is brought to the cell and is attached to the cell surface.
(C) If the bead is shined by a trap when the bead center is offset from the trap center, force is exerted to the cell.
Figure C.3 Optically Conjugated System for Steering a Mirror

(A) The distance between L1 and L2 is kept as the sum of their focal lengths, to have the beam collimated after the two lenses.

(B) The distance between L1 and the steering mirror is the focal length of L1; the distance between L2 and the objective lens is the focal length of L2, which make the system optically conjugated.
Figure C.4 Optical Tweezers Combined with Fluorescence Microscope

(A) A parallel beam is focused by $L_{1\text{int}}$ then is modified by the other three lenses. Eventually the beam reaches the objective lens as focused.

(B) A focused beam is collimated by $L_{1\text{int}}$ then is modified by the other three lenses. Eventually the beam reaches the objective lens as collimated.

(C) To achieve the same system in Figure C.3, we do not need to consider the presence of $L_{2\text{int}}, L_{3\text{int}}$ and $L_{4\text{int}}$ but only $L_{1\text{int}}$. By placing $L_{1\text{ext}}$ and the piezo-mirror in proper positions as illustrated, an optically conjugated system is built. The focal lengths of $L_{2\text{ext}}$ and $L_{3\text{ext}}$ are 125 and 40mm each.
Figure C.5 Computer Control for Positioning an Optical Trap
(A) Shown is a user interface designed for controlling the piezo-mirror, consequently, the position of a trap. (B) (a) When the angle of the piezo-mirror changes by a small angle $\theta_x$ in a horizontal direction, that of the reflected beam changes by $2\theta_x$ if the beam was initially reflected by $90^\circ$. (b) It is $\sqrt{2} \theta_y$ for the angle change in a vertical direction in the otherwise same configuration.
Figure D.1 SDS-PAGE
SDS-PAGE was used to identify the proteins for each step of purification. Lane 1 was loaded with 2.5 µL sample before the cell lysis; lane 2, 2.5 µL after lysis with lysosome; lane 3, 2.5 µL after further lysis by sonication; lane 4, 5 µL marker (pre-stained SDS-PAGE standards, low range, Bio-Rad); lane 5, 5 µL flow-through before elution; lanes 6 and 7, 5 µL eluted Rep protein.
Figure D.2 PEG-Mediated Dimerization

(A) Shown are the three possible populations after reaction with PEG.

(B) SDS-PAGE was used to find out the cross-linking efficiency. In lanes 1 to 6, loaded are 5 µL of Rep proteins reacted with PEG that has two maleimide groups. The ratio between the PEG molecules and the proteins are written at the bottom. Proteins were also reacted with PEG that has only one maleimide group (lanes 7 and 8, 5 µL each). In lane 9, unmodified Rep (5 µL) was loaded as a marker. Between lanes 5 and 6, a marker (pre-stained SDS-PAGE standards, broad range, Bio-Rad) was loaded.
Figure D.3 Size Exclusion

(A) Size exclusion was carried out with 1mL of 25 µM monomers in buffer A (150mM NaCl, 50mM Tris-HCl, pH 7.67 and 25% glycerol)

(B) The same procedure was practiced with 0.4mL of 25 µM PEG-mediated dimers.

(C) SDS-PAGE was run to check the quality of size-exclusion. The eluted sample was 5 times concentrated before loading. 10 µL was loaded for each lane. The marker is pre-stained SDS-PAGE standards (broad range, Bio-Rad). Lane 5 was loaded with the sample before size-exclusion purification (5 µL).
Figure D.4 Enhanced Unwinding Activity with PEG-mediated Dimer

(A) Unwinding is observed through the decrease in FRET and the increase in fluorescence intensity (a) with monomers and (b) with PEG-mediated dimers. The reactions were initiated by adding Rep protein at \( t = 0 \).

(B) The unwinding rates and amplitudes are measured with 10, 20, 200nM monomers and 5, 10, 100nM PEG-mediated dimers.

(C) Shown are the unwinding amplitude with monomers and PEG-mediated dimers from single-turnover experiments.
Author’s Biography

Chirlmin Joo was born in a small and quite harbor, Mokpo, in Korea in 1976. He attended the Seoul National University in Korea receiving several scholarships along the way and graduated with a B.S. *summa cum laude* in physics in 2002. He came to the USA to begin his graduate study in the University of Illinois at Urbana-Champaign in the summer of 2002 and joined the lab of Prof. Taekjip Ha in early 2003. In 2006, he received a fellowship (Drickamer Award) from the Department of Physics (UIUC) for his significant research abilities and was also awarded for the best presentation in Annual Cell and Molecular Biology & Molecular Biophysics Research Symposium.

During his graduate study, he has given talks at:

- Condensed Matter Research Institute Seminar, Seoul National University, Korea (2007)
- Annual Cell and Molecular Biology & Molecular Biophysics Research Symposium, UIUC, USA (2006)
- Annual Biophysics and Computational Biology Symposium, UIUC, USA (2006)
- Biophysical Society Meeting, Salt Lake City, UT, USA (2006)

His publications include:


After graduation, he will begin a post-doctoral fellowship at the School of Biological Sciences in Seoul National University under the direction of Prof. V. Narry Kim. He will investigate the molecular mechanism of RNA interference.