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Using Single Molecule FRET to Study Hepatitis C Virus NS3h and NS3-NS4A Helicase Unwinding

by

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Hepatitis C virus infection results in development of chronic liver diseases. A potential target for drugs to prevent the development of the virus is the NS3 protein that has helicase and primase activities. In the last few years several studies have described mechanistic aspects of the helicase, but the role of the interaction with NS4A cofactor in the NS3 function is not clear. In this paper, we describe the use of fluorescence and single molecule fluorescence resonant energy transfer (FRET) to understand the mechanisms of binding and unwinding of DNA-DNA substrates and RNA-DNA substrates by the NS3 helicase domain and the NS3-NS4A complex. Our results indicate that multiple units bind to single strand substrates with very high affinity and that unwinding of RNA-DNA occurs at faster rates than unwinding of DNA-DNA. Our results provide the basis for more complete and systematic studies that will help determine the mechanisms of these proteins.

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ABSTRACT

Hepatitis C virus infection results in development of chronic liver diseases. A potential target for drugs to prevent the development of the virus is the NS3 protein that has helicase and primase activities. In the last few years several studies have described mechanistic aspects of the helicase, but the role of the interaction with NS4A cofactor in the NS3 function is not clear. In this paper, we describe the use of fluorescence and single molecule fluorescence resonant energy transfer (FRET) to understand the mechanisms of binding and unwinding of DNA-DNA substrates and RNA-DNA substrates by the NS3 helicase domain and the NS3-NS4A complex. Our results indicate that multiple units bind to single strand substrates with very high affinity and that unwinding of RNA-DNA occurs at faster rates than unwinding of DNA-DNA. Our results provide the basis for more complete and systematic studies that will help determine the mechanisms of these proteins.

INTRODUCTION

Hepatitis C Virus

Hepatitis C virus (HCV) infects about three percent of the world population today [1]. The infection is mostly asymptomatic; however, without treatment, most infections become chronic and result in acute hepatitis, chronic hepatitis or liver cirrhosis [2]. Understanding the virus and its essential enzymes would help develop drugs that target them.

The HCV genome is composed of a single-strand RNA of about 9.6kb in length [3, 4]. The strand is made up of a 5’ nontranslated region that contains an internal ribosome entry site, a region coding for a precursor polyprotein and a 3’ nontranslated

region [3-5]. Once translated, cellular and viral proteases cleave the polyprotein into 10 different products, structural proteins (C, E1, E2, and p7) and nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [6-8].

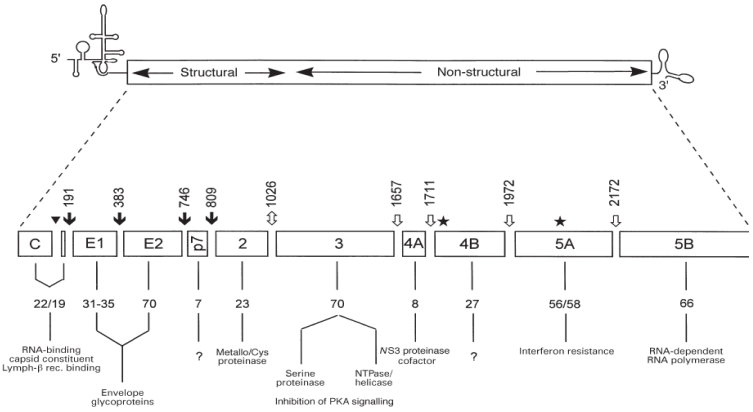

Figure 1. HCV Peptide [9].

Helicases

Helicases are essential motor proteins to all living organisms. Helicases help separate double-stranded nucleic acids such as dsDNA, dsRNA, and DNA-RNA into the single-stranded intermediates needed in processes such as replication, DNA repair, recombination, transcription, and translation [10, 11]. Helicases are motor proteins because of their ability to move along nucleic acids utilizing the chemical energy released from the hydrolysis of ATP or other nucleoside triphosphates [11]. Interestingly, one cell can express up to 12 different helicases, such as in *Escherichia coli* [10]. Initially, helicases are divided mechanistically into two classes. The first class requires a 3' free end of single-stranded nucleic acids in order to start unwinding, and the other class requires a 5' free end strand [10] [12].

Gorbalenya and Koonin recognized that helicase proteins share short, conserved amino acid sequence motifs, therefore, they classified helicase into five major groups

[12]. The groups are distinguished by both the number of distinct motifs and by differences in the consensus sequences for motifs that are shared by more than one group [10, 12]. Since helicases require energy, they have to bind to ATP and, accordingly, all of them carry the classical Walker 'A' (phosphate-binding loop or 'P-loop') and 'B' (Mg2+-binding aspartic acid) motifs [12].

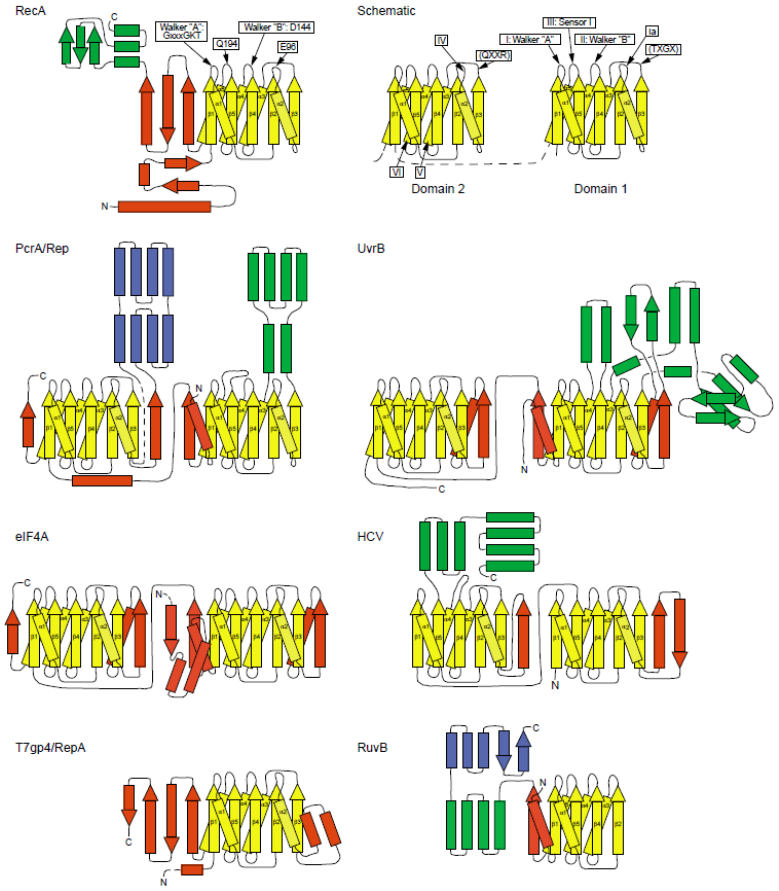

Figure 2. Examples of helicases in each different superfamily [12]. SF-1 (PrcA/Rep) SF2 (HCV helicase; UvrB). DnaB-like family (T7gp4/RepA). Topology diagrams of representative helicases. Yellow, conserved RecA-related 'core'; red, variable structural elements in domains with a RecA-like core; green and blue, additional structural domains [12]. The schematic on the upper right summarizes the positions in the topology of the RecA-like core of the seven conserved motifs defined by Gorbalenya and Koonin for the SF-1 and SF-2 helicases [12].

NS3-NS4A

The NS3 protein from HCV has two functions, a serine protease at the amino-terminus and a NTPase/helicase activity with 3' to 5' helicase activity [13-15] for translation and replication of the HCV genome at the carboxy-terminus [6]. The helicase domain of NS3 is also known as NS3h. The active site can be seen in Figure 3 [16].

NS3 usually binds with NS4A to form NS3-4A complex. NS3/NS4A is a multifunctional enzyme with both serine protease and helicase activities that help hepatitis C viral replication [17]. It has been shown that NS4A acts as a cofactor for NS3 in order to increase NS3 unwinding activity [18]. NS3 can unwind both DNA and RNA [19]. The major role of NS3would likely be unwinding of the RNA genome of HCV, and it is possible that activity toward the host DNA plays a role in viral function [16]. Recently, a mechanism has been proposed for NS3 unwinding on DNA, based on single molecule studies of the full length NS3 protein [16]. There is evidence that *in vivo* NS3 interacts with NS4A and they co-localize in membrane bound complexes. How this interaction affects the helicase activity is not well understood and the focus of this study.

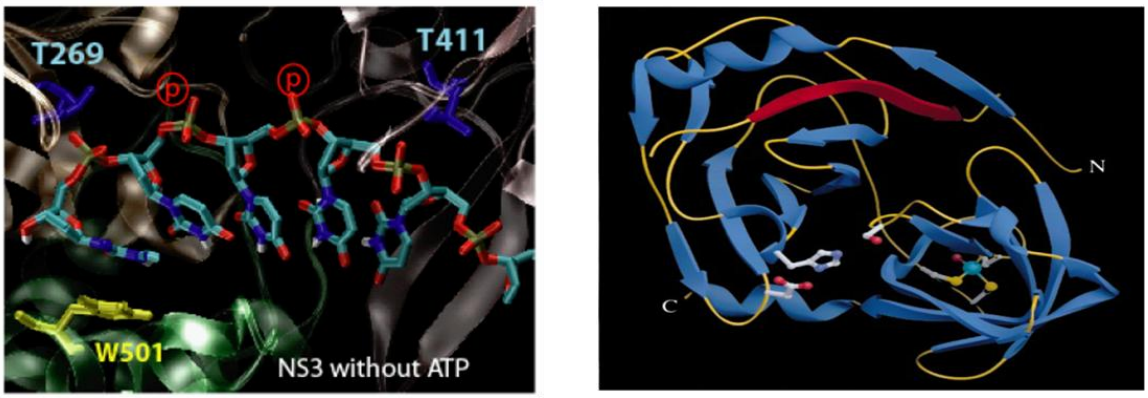

Figure 3. NS3 Helicase [16]. NS3 crystallized without ATP shows two well conserved threonine residues (T269, T411 in blue) contacting backbone phosphate 3 nucleotides apart. W501 (yellow), also known as "gatekeeper" is at the 3' end of the bound nucleotide.

Fluorescence Resonance Energy Transfer (FRET)

Fluorescence resonance energy transfer (FRET) is used to monitor macromolecular interactions and conformational changes by monitoring inter-molecular distances[20]. FRET is most sensitive in the 30-80 Å range [21]. It is a process where the excited-state energy of one chromophore molecule (donor) is nonradiatively transferred to a neighboring chromophore (acceptor) in the ground state via an induced-dipole, induced-dipole interaction [20, 21]. The efficiency of energy transfer (E) is equal to $1/[1 + (R/R_0)^6]$ where R is the distance between the donor and the acceptor and R_0 is a constant for given experimental conditions at which 50% of the energy is transferred. It is a function of the spectral overlap of the donor emission and acceptor absorption, refractive index of the medium, quantum yield of the donor and a factor κ^2 that depends on the relative orientation in space between the transition dipoles for donor and acceptor [21]. As the distance between the donor and acceptor fluorophores increases, the efficiency of energy transfer decreases. The efficiency of energy transfer (E) can be estimated experimentally by using $I_A/(I_D + I_A)$ where I_A and I_D are the fluorescence intensity of the acceptor and donor, respectively [21].

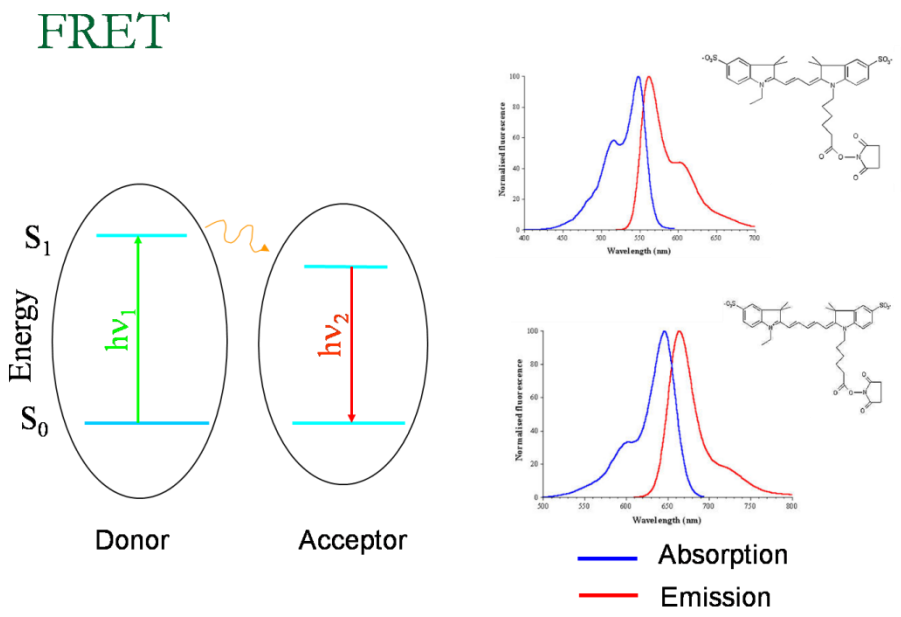

Figure 4. [22]. Energy levels diagram showing FRET transfer between donor and acceptor. On the right absorption (blue) and emission (red) spectra for donor (top) and acceptor (bottom). Also shown the chemical structure of the donor-acceptor pair used in this work (Cy3 top and Cy5 bottom).

Single Molecule FRET (smFRET)

There are two primary methods of performing single molecule FRET (smFRET) measurements: in solution and on the surface [21]. Solution experiments are often used and completely alleviate concerns about surface interactions [21, 23-25]. However, their diffusion limited temporal window leaves them inadequate for investigation of slower (>10ms) phenomena [21]. Surface immobilization provides an extended observation window limited only by photobleaching, but requires careful attention because of potential artifacts induced by surface interactions.

To be able to detect the emission of single molecule dyes it is necessary to reduce the excitation volume to reduce background light from the sample; there are two ways commonly used to reduce the excitation volume [22]. In confocal microscopy, a laser

beam is focused to a diffraction limited spot resulting in a small excitation volume. In total internal reflection microscopy the excitation beam undergoes total internal reflection at the interface of the microscope slide and the sample. The beam is totally reflected but the evanescent wave penetrates into the sample at a distance of the order of 200 nm. In this case the sample is excited in a wide field of the order of 100 micrometers but only a thin layer of the sample is excited so that the background fluorescence is reduced.

Single molecule FRET has been used in the last decade to study biological systems. In general, it is necessary to attach a FRET pair (donor and acceptor) to the system under study so that it is possible to monitor the distance between the dyes. In particular, smFRET has been used to study the unwinding mechanisms of the *E. Coli* Rep helicase [26] and the full length NS3 helicase [16]. The technique has also been used to understand the binding kinetics of Rep helicase to DNA substrates.

OBJECTIVE

We want to understand the mechanisms of binding and unwinding of NS3h and NS3-NS4A from HCV by using single molecule fluorescence methods.

MATERIALS AND METHODS

Experimental Setup

Single molecule FRET experiments were performed using an inverted Olympus IX-71 microscope modified for total internal reflection excitation. Excitation was done using a Nd:YAG laser at 532 nm through a prism on top of the sample. Labeled DNA molecules were immobilized on a sample cell (see below). Fluorescence emission from the single molecules was collected with a water immersion high numerical aperture objective (60 x, NA 1.20). Scattered laser light was blocked with a long pass filter (LP

550) and the fluorescence emissions from donor and acceptor were separated using dichroic mirrors and recombined after shifting the images laterally and focused on a CCD camera (Andor iXon). In this way, donor and acceptor are imaged on separate halves of the CCD camera. Images on the CCD are stored in a PC at a rate of 30 frames per second. Software allows detecting individual diffraction limited spots corresponding to donor and acceptor molecules using an average of the first 10 frames. After identification of all Donor-Acceptor pairs the software calculates the total intensity associated with the spots for each frame and allows one to calculate FRET efficiency using $E = I_A/ (I_A+I_D)$ where I_A is the intensity of the acceptor and I_D is the intensity of the donor.

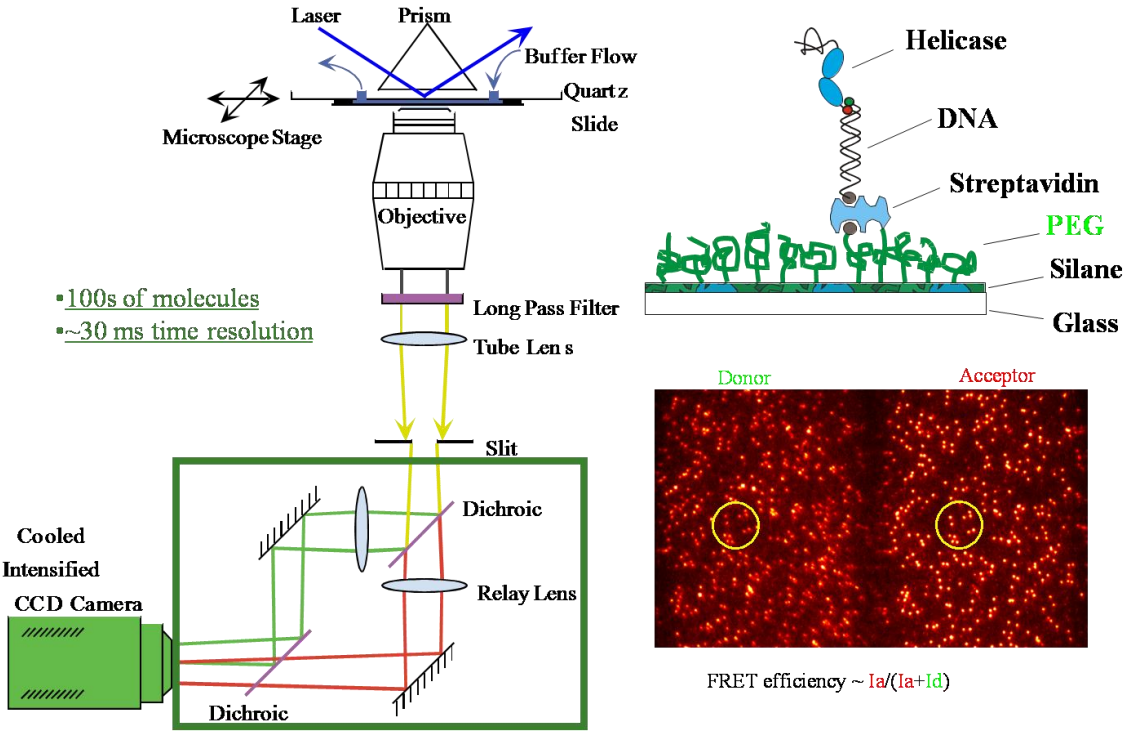

Figure 5. Experimental setup for smFRET [27]. The left panel shows the direction of light from the laser to excitation of the sample, and from the sample to the camera. The top right panel shows a schematic of the samples used for single molecule observation. The bottom right panel shows how the software sees one spot of immobilized DNA but with two views: donor emission on the left and acceptor emission on the right.

DNA substrates.

Oligonucleotides were purchased from IDT DNA. Substrates were annealed by mixing the complementary single strands at a final 10 μM concentration in a buffer containing 500 mM NaCl, heating at 80 C for three minutes and the subsequent mixture was cooled down slowly to room temperature.

Sequences

Top strand (common) - 5' **1** GCC TCG CTG CCG TCG CCA biotin 3'

Where **1** = 5'amino modifier C6 (for labeling with Cy5)

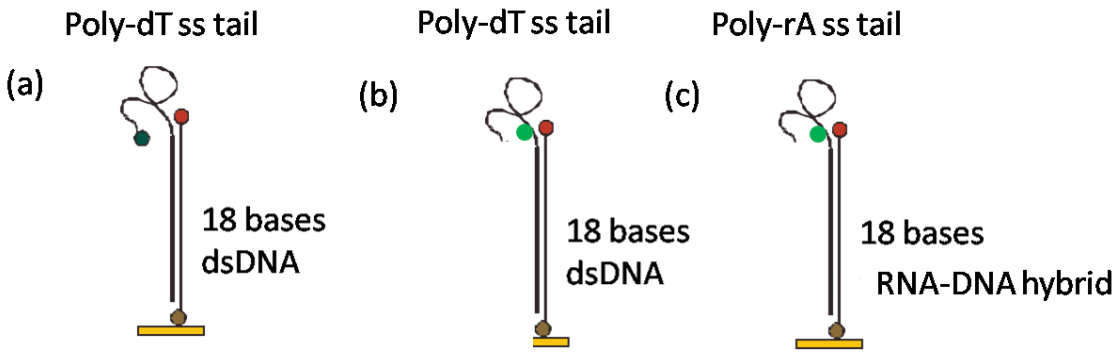

Figure 6. Substrates used in this work. Acceptor indicated with red circle, donor indicated with green and biotin indicated with yellow. All substrates contain a single stranded tail 3'-5' to provide a binding site for the protein. (a) Substrate used to determine binding dynamics (b) (c) Substrates used for unwinding experiments.

Bottom strand (binding experiments)

5'-TGG CGA CGG CAG CGA GGC TTT TTT TTT TTT T **2** 3'

Bottom strand (unwinding experiment) dsDNA

5'-TGG CGA CGG CAG CGA GGC **2** TTT TTT TTT TTT TTT TTT 3'

Bottom strand (unwinding experiment) hybrid

5’ rUrGrG rCrGrA rCrGrG rCrArG rCrGrA rGrGrC **2** rArArA rArArA rArArA rArArA rArArA 3’

Where **2** = amino modifier C6 dT (for labeling with Cy3)

Bulk Solution Experiments

Ensemble experiments were done using a fluorometer (Jobin Yvon) to measure fluorescence emission of the donor and acceptor. Excitation was done at 540 nm, slit width was 2 nm, and labeled substrate concentration was 5 nM using a total volume of 800 μL for each experiment.

PEG Slide

To perform single molecule experiments with immobilized substrates, microscopes slides must be treated with PEG. PEG (poly(ethylene glycol)) is attached covalently (protocol in appendix) to the slides after aminosilanization forming a polymer brush reducing protein absorption to the slide surface [21].

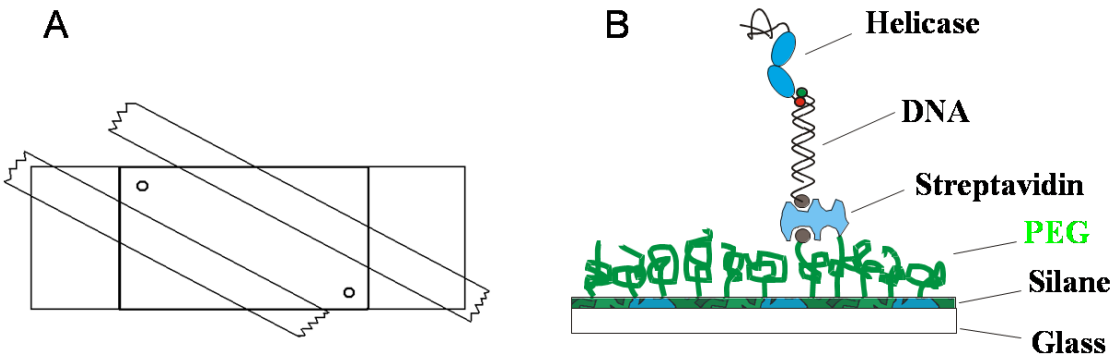

Figure 7. PEG Slide. A) The top view of the PEG slide. The two diagonals rectangles are tapes that are inserted between the slide and the cover slip providing a spacer of ~ 100 μm. . B) The schematic of what the experiment would look like. Nucleic acids containing biotin would bind to the Streptavidin, which is in turn bind to the biotin that is attached to some of the polyethylene glycol on the slide.

A fraction of the PEG molecules contain biotin allowing for specific immobilization of substrates. Briefly: Streptavidin, (that contains four binding sites for biotin) is added to the sample. Streptavidin binds to the biotinylated PEG and afterwards substrates containing biotin are added that bind to available sites in the Streptavidin. By using small a concentration of substrates, it is possible to control the density of substrates on the surface so that observation of single molecules is possible. Slides contain two small holes that allow for easy exchange of buffer solutions.

Data Analyses

Data obtained from the fluorometer is saved in ASCII files and analyzed in Origin.

Single molecule data obtained with the CCD camera is analyzed using homemade software in IDL that detects the individual molecules and stores the fluorescence intensity for each donor-acceptor pair as a function of time (traces). The single molecule traces are analyzed using homemade software in Matlab that allows one to determine the distribution of FRET efficiencies and to analyze individual traces.

RESULTS

SmFRET Binding Experiments

In these experiments a dsDNA substrate with a single stranded tail (poly-dT_{13}) was immobilized under conditions apt for single molecule observation. Data was taking in the absence of NS3 and in the presence of NS3 at different concentrations. These experiments are carried on in the absence of ATP, so that binding can be observed without unwinding of the substrate. The substrate is designed so that the donor acceptor pair is at the ends of the single strand. The single strand DNA is usually flexible, with

fluctuations in the end to end distance much faster than our time resolution (30 ms) so that we only observe an average distance. When protein binds to the single strand tail it reduces its flexibility resulting in an increase in distance and a decrease in the observed FRET efficiency. Data from different areas of the sample was analyzed, calculating the FRET efficiency for each donor acceptor pair. In the figure we show the distribution of FRET efficiency values among all analyzed molecules. In the absence of protein, we observe two peaks.

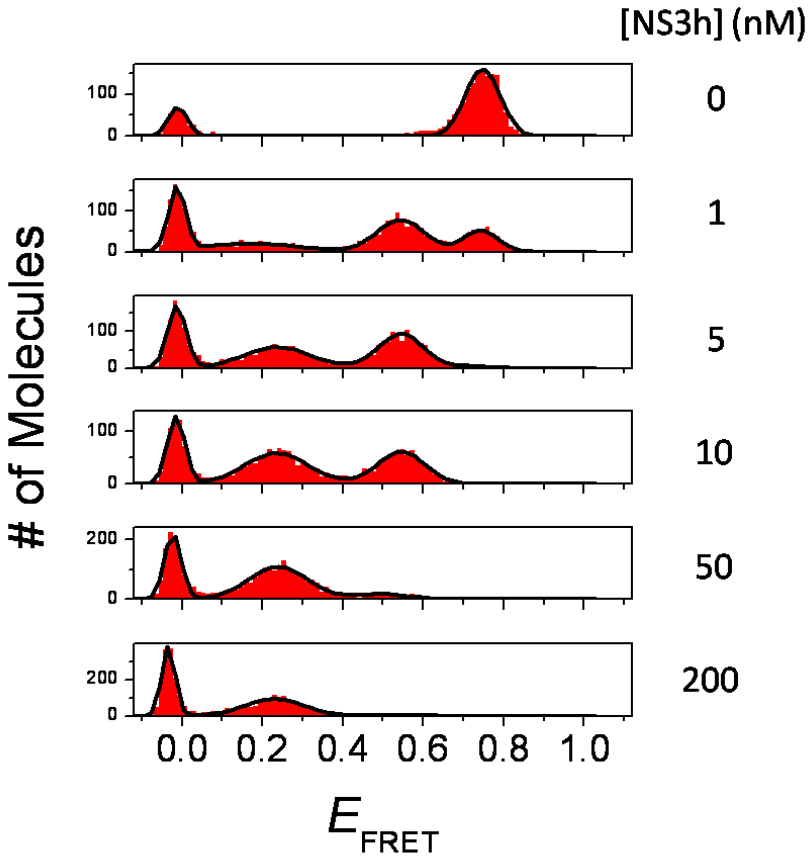

Figure 8. FRET efficiency histograms for binding substrates at different NS3 concentrations. The peak at zero FRET corresponds to inactive acceptor molecules. The histograms are obtained by determining the FRET efficiency for thousands of molecules.

The peak at zero FRET is due to DNA substrates that contain inactive acceptor dyes (so there is no transfer). This zero FRET population provides no information and it will not be further considered. The DNA substrates with active donor and acceptor show a peak at high FRET efficiency consistent with the flexible polydT$_{13}$. Surprisingly as the NS3 concentration is increased we observed two new peaks at intermediate and low FRET efficiency values. These two peaks may correspond to one and two NS3 molecules bound to the single stranded tail or to different conformations of the NS3-DNA complex. To determine the dependence of the population of each FRET efficiency state with concentration, we measure the area for each peak in the FRET efficiency histograms and plotted versus protein concentration after normalizing by the total area (excluding the zero FRET peak).

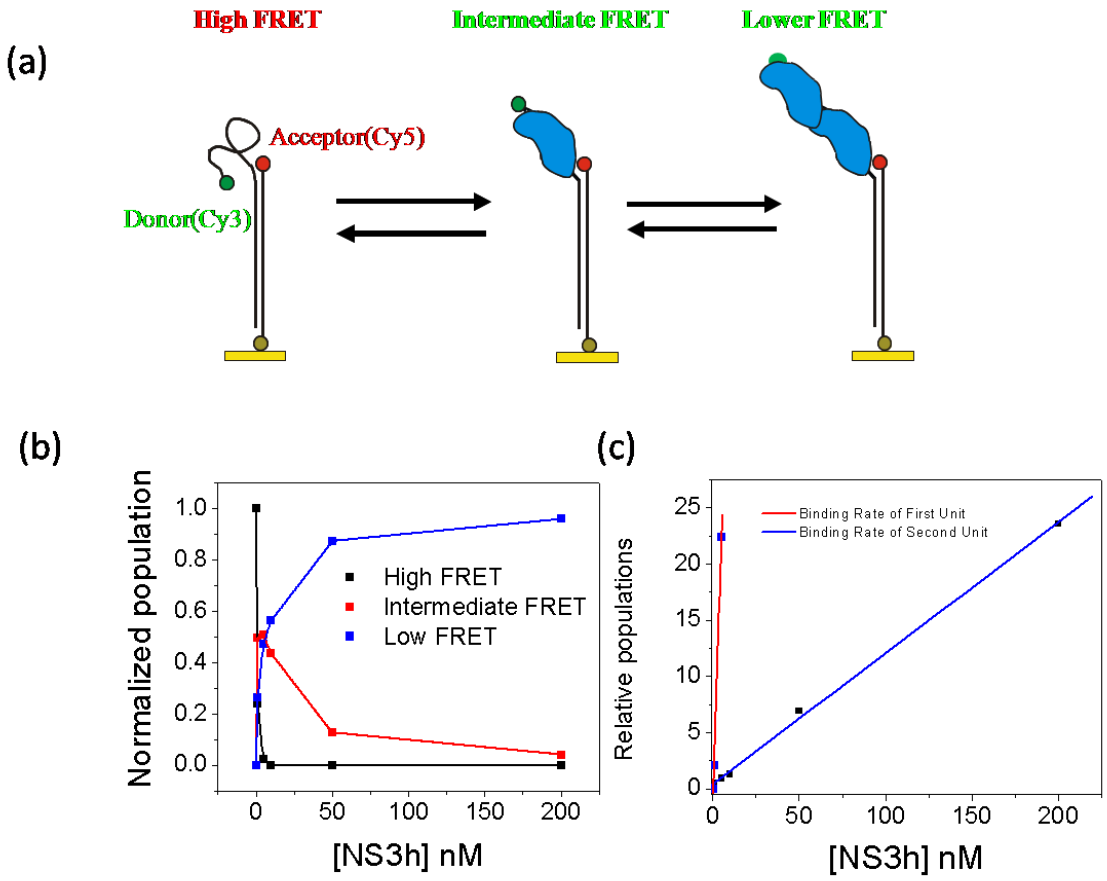

Figure 9. (a) Binding model for two NS3 molecules in successive steps. (b) Dependence on the population of the FRET states observed in the FRET efficiency histograms. (c) Relative populations of the low FRET efficiency state and intermediate state and intermediate and high states showing a linear dependence.

As expected the free DNA population decreases as the protein concentration increases. The intermediate FRET efficiency peak first increases and then decreases at higher protein concentrations and the lower FRET efficiency peak increases monotonically showing saturation at high protein concentrations. This behavior is consistent with a model in which two NS3 proteins bind in two steps. If we plot the population of the low FRET efficiency state divided by the population of the intermediate state we observe a linear dependence consistent with one and two bound proteins respectively. Experiments with NS3/NS4A show virtually identical results (data not shown) indicating that at least in solution the presence of the NS4A does not affect binding kinetics to the partial duplex substrates.

Unwinding Experiments

Unwinding experiments were performed using substrates in which the donor and acceptor are closely located at the junction of the partial duplex as shown in Figure 6 (b) and (c). Such substrates show 100% FRET efficiency. If unwinding occurs, the two fluorophores are moved apart from each other and the course of the unwinding reaction can be monitored by the decrease in FRET efficiency with sensitivity of few base pairs.

Ensemble Experiments

Ensemble experiments were performed in a fluorometer (Jobin Yvon) using an excitation wavelength of 540 nm. At this wavelength, Cy3 (donor) is excited directly but Cy5 (acceptor) is not. Emission spectra from the sample (10 nM labeled substrate) is taken between 545 nm and 750 nm, so that the emission of both Cy3 (with emission

maximum at 565 nm) and Cy5 (emission maximum at 656 nm) can be observed. Spectra in the absence of protein show strong emission from the acceptor (Cy5) but virtually no emission from the donor (Cy3) which is excited directly. This indicates very strong FRET transfer consistent with the design of the substrates. When protein is added in the absence of ATP, no changes in the spectra are observed. The binding experiments described in the previous section indicate that protein is binding to the single stranded portion of the substrate, but because the two fluorophores are so close together binding does not introduce changes in FRET efficiency.

After incubation of the substrates with protein, we added 4 mM ATP and took the emission spectra of the sample at different times after the addition. We observed that the emission peak corresponding to the donor fluorophores starts to increase in amplitude while the emission peak of the acceptor fluorophores starts to decrease. This corresponds to a decrease in the overall FRET efficiency of the sample consistent with unwinding. The reaction is performed under multiple turnover conditions, which the helicase proteins may dissociate and bind again multiple times. By analyzing the decay rate in the FRET efficiency, we can determine the average unwinding rate for our substrates under the conditions of the experiment. These experiments are important also as control experiments to compare with biochemical assays done in other laboratories and to determine that the presence of the fluorophores does not affect the unwinding rate.

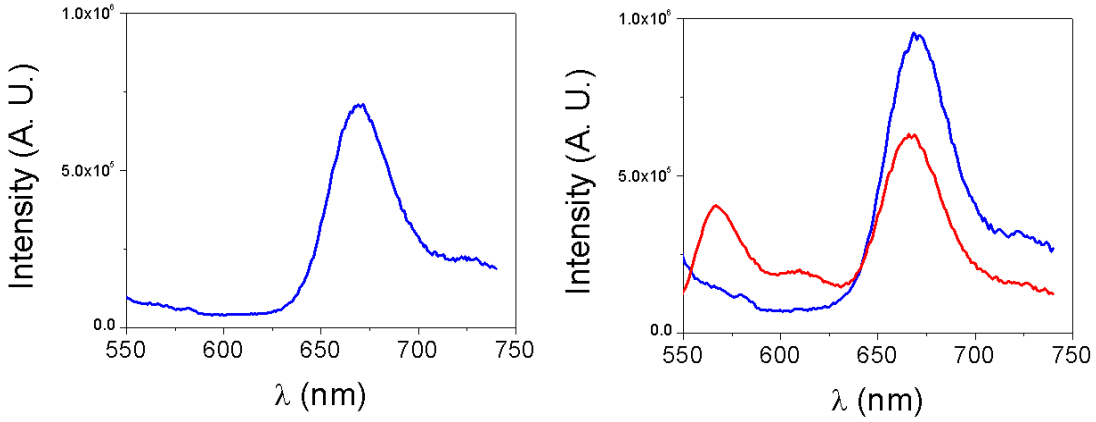

Figure 10. Unwinding experiments in ensemble. Left panel, substrate only showing high FRET efficiency. Right panel, after incubation of 10 nM substrate with 100 nM protein (blue curve) we added 4 mM ATP to initiate unwinding. Data shown (red curve) is 30 seconds after ATP addition, showing increase in donor emission and decrease in the acceptor emission consistent with decrease in FRET efficiency.

For each time, we determine the FRET efficiency for the ensemble experiments. In Figure 11 we show the intensity of the donor and acceptor emission versus time and the FRET efficiency versus time for experiments with NS3-NS4A with two different unwinding substrates, one consisting of duplex DNA and the other a DNA-RNA hybrid. Both experiments were done under identical conditions. Exponential fits to the FRET efficiency decay curves give us a decay time of 28 seconds for the hybrid substrate and 141 seconds for the duplex DNA.

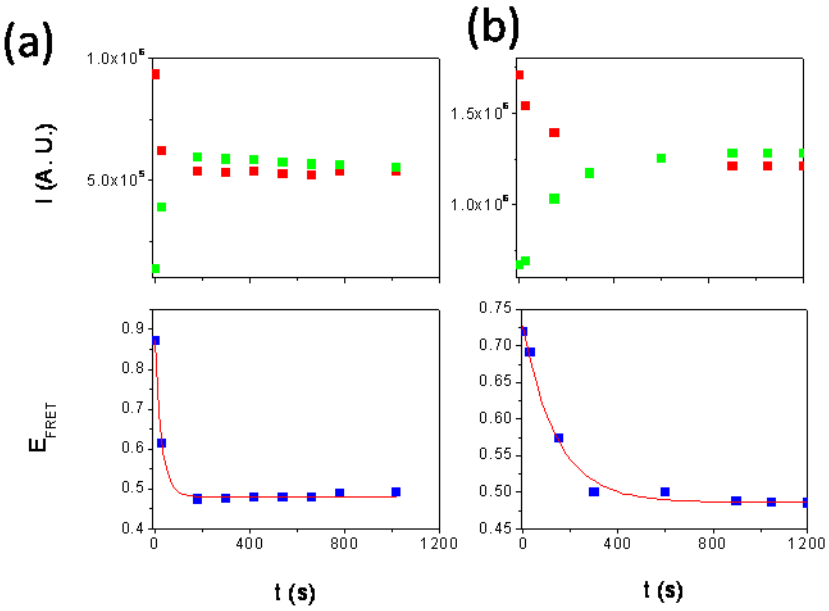

Figure 11. Unwinding experiments in ensemble, 10 nM substrate concentration, 100 nM NS3-NS4A, and 4 mM ATP. Acceptor intensity in red, donor intensity in green (top panels) and FRET efficiency in blue (lower panels) (a) RNA-DNA substrates (b) DNA-DNA substrate.

DISCUSSION

Single molecule FRET binding experiments show that both NS3h and NS3-NS4A bind to single stranded substrates with high affinity. We observed binding of multiple units. Analyses of the population dependence on protein concentration indicate that each binding step is proportional to the free protein concentration in solution, which is consistent with the model shown in Figure 9a. Quantitative analyses of the model allow one to determine the equilibrium constants for the binding steps from the slope of Figure 9c and equations shown below. The equilibrium constant for the first unit is 4.3×10^{9} M^{-1} and for the second unit is 1.2×10^{8} M^{-1}. The much smaller equilibrium constant for the second binding is probably associated with the fact that the single strand binding site is only 13 bases long. Therefore, after the first unit is bound it becomes more difficult to

bind a second one. Binding experiments with substrates of different lengths using the analyses previously described may help to determine if binding of multiple units when binding site length is not a constraint shows cooperativity.

$$N_1 = \frac{N_0}{1+\alpha+\alpha\beta} \qquad \alpha = \frac{k_1}{k_{-1}}[NS3],\ \beta = \frac{k_2}{k_{-2}}[NS3]$$

$$N_2 = \alpha \cdot N_1 \qquad \frac{N_2}{N_1} = \frac{k_1}{k_{-1}}[NS3],\ \frac{N_3}{N_2} = \frac{k_2}{k_{-2}}[NS3]$$

$$N_3 = \alpha \cdot \beta \cdot N_1$$

Equation 1. Population distribution as a function of concentration for the model in Figure 9a.

Unwinding experiments show that under multiple turnover and identical experimental conditions, the unwinding of RNA-DNA substrates in which the RNA strand is the 3'-5' strand is much faster than unwinding of DNA-DNA substrates. This could be a consequence of duplex stability or because translocation along DNA and RNA strands occur at different rates. The time scale of unwinding of 18 base pairs duplex is compatible with typical observation times for single molecule experiments, indicating single molecule unwinding experiments can be carried on.

We have shown that it is possible to realize single molecule FRET experiments to understand mechanisms of NS3h and NS3-NS4A. We have developed the methods to analyze quantitatively binding kinetics of the helicase to the substrates and find conditions in which unwinding occurs at rates compatible with single molecule fluorescence experiments. We have shown that even at low protein concentration the binding affinity for single strand substrates is very high. In the future these techniques could be applied systematically to better understand binding kinetics and unwinding mechanisms to compare NS3h and NS3-4A. In addition, it would be interesting to

determine the effect of localization on the membrane of the NS3-NS4A. So far, all characterization experiments of helicase activity have been done with proteins in solution.

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Appendix

Experimental Protocols

Preparing T50 Buffer:

10 mM Tris pH 8.0 + 50 mM NaCl + purified (nanopurified by Purelab Ultra) water

From the stock solution in the refrigerator, get 1 M Tris (pH 8.0) and 5 M NaCl.

Put 300 μL Tris and 300 μL NaCl into a 50 mL container and fill the container up to the 30 mL line with purified water. Store the solution in the refrigerator.

Preparing Saturated Trolox:

Trolox (6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, 97%) should be prepared ever 4 weeks.

1. Get the trolox out of the refrigerator and let it warm up to room temperature. It is in a brown bottle with a red top labeled:

   Aldrich

   6-Hydroxy02,5,7,8-tetramethyl.

   i) Put this much of the powder out of the little bottle and put it in a 50 mL tube, filling the rest of the tube up to the 40-45 mL line:

3. Let the solution sit in the dark (in a drawer) for awhile so all the powder will dissolve.
4. Get out one of the big fat syringes with the filter. Prewash the syringe and filter by flowing purified water through it slowly.
5. Pour the solution into the syringe. Rinse off the outside of the syringe.
6. Let the first few drops go into the sink; then, flow the liquid from the syringe into a clean, orange-topped 50 mL container. Throw away the syringe.

7. Label the container as saturated Trolox solution. It is light sensitive so don't leave it in the light.
8. Refrigerate the solution.

Preparing Gloxy:

Ingredients (in amount per each slide):

10 mg GOD (glucose oxidase) → stored at -20° (From the freezer: Open a little square white box)

20 μL catalase → stored at 4° (range 2-8°) (From the refrigerator)

100 μL T50 buffer

Anytime you get something from the freezer, make sure it gets to room temperature before you use it.

1. From the freezer: Open a little square white box, and get GOD out.
2. From the refrigerator: Get out the catalase; make sure you invert it slowly.
3. Weigh out 10 mg of GOD in the shared lab. Put it in a tube. Put the GOD back into the freezer.
4. Put 20 μL catalase into the tube that the GOD was put in.
5. Add 100 μL T50 buffer to the tube.
6. Shake the solution well. It is hard to get it to dissolve. It is helpful to mix the solid that has settled at the bottom with a pipet tip. Spin it ~30 seconds. Put it on ice and let it sit awhile.
7. Put the catalase back in the refrigerator.
8. Label the new gloxy solution. Put it on ice or in the refrigerator.

For the gloxy to work, we have to use (20%) glucose 0.4% w/v.

The element that is the triplet quencher is: BME (ß- mercaptoethanol, see below in Preparing Imaging Buffer Section) (1%) + trolox (the problem with trolox is that the solubility is very low, therefore we have to use a saturated trolox solution (2 mM).

Preparing BSA-biotin:

BSA-biotin comes in a little brown bottle in the refrigerator (Sigma. Albumin, biotin labeled, bovine). It is in powder form, and there are 10 mg of the substance in the bottle. Put 1 mL T50 buffer into the bottle to make 10 mg/mL concentration.

We use1 mg/mL BSA-biotin in our experiments. Get a big tube, label it "1 mg/mL BSA-biotin," and add 50 µL BSA-biotin stock and 450 µL T50 buffer. Refrigerate.

Preparing the Imaging Buffer:

The 1x Buffer Contains:

10 mM Tris pH 8.0

50 mM NaCl

0.4% glucose

Trolox

0.1 mg/mL BSA

We want to prepare a 5x buffer, which we can use for a year.

50 mM Tris pH 8.0

250 mM NaCl

2 % glucose

0.5 mg/mL BSA

Making the Trolox Buffer:

Using the 5x prepared buffer, when we want to make the 1x buffer, mix:

2 mL 5x buffer + 8 mL saturated trolox

Making the BME (ß- mercaptoethanol) buffer:

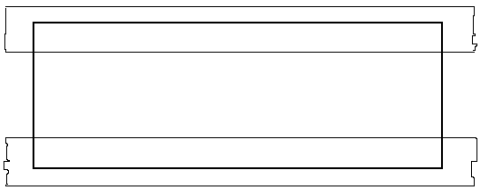
Combine: 2 mL 5x prepared buffer + 8 mL purified water.

Add 98 µL of the above solution to 1 µL gloxy and 1 µL BME.

*The problem with the BME buffer is that it gives lots of long-scale blinking, but it would be interesting to use both buffers in our experiments.

To Prepare a Bead Sample:

1. Get a glass slide.
2. Get a cover slip.
3. Put tape on the edges of the slide, as shown in the figure below:



4. From the refrigerator, get the deep red fluorescent beads.
5. Get 10 µL of the beads in the tip of a pipet.
6. Put the small volume on the surface in the center of the slide.
7. Spread them around with a pipet tip so there will be a uniform density.
8. Wait for the buffer to evaporate.
9. In the same container from the refrigerator, find the mounting medium.
10. Put a couple of drops of the mounting medium on the center of the slide. It is very viscous. Use a different tip for each drop so there is no cross contamination.
11. Put the coverslip on the slide. If there is air in the slide, the slide will not work right because the refractive index will be off.

12. Use a razor to cut off the excess tape.
13. Look at the slide under the microscope. If it works, seal it with epoxy and it will work for years.

Cleaning Slides:

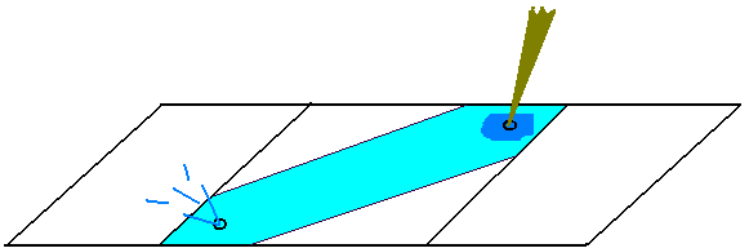
1. Let the slides sit in water so that the epoxy will begin to dissolve.
2. Put the slides in a mixture of 80% water/20% detergent (1/2 ounce of Dri-Clean detergent for Labware in 1 gallon of water).
3. Put slides and coverslips in the microwave for ~2 minutes.
4. Use a razor to take off the coverslips and excess epoxy.
5. Rinse slides with water and go over them with the razor one more time while putting them into the slide holder beaker (We will call this as glassware).
6. Rinse slides with nanopurified (by Purelab Ultra) pure water, fill the glassware with 80% water 20% detergent.
7. Put the slides in the sonicator for 15 minutes.
8. Rinse the slides with pure water. Fill the glassware with pure water.
9. Put the slides in the sonicator for 5 minutes.
10. Rinse the slides with acetone. Fill the glassware with acetone.
11. Put the slides in the sonicator for 15 minutes.
12. Rinse the slides with pure water. Fill the glassware with pure water.
13. Put the slides in the sonicator for 5 minutes.
14. Get coverslips (make sure they are labeled 24x40 with 1.5 thickness) from drawer. Put them in the coverslip glassware. Fill the glassware with 1 M KOH.

15. Fill the slide glassware with 1 M KOH. Sonicate the slides and coverslips for 20 minutes.
16. Rinse both slides and coverslips with nanopurified H_20.

Preparing Slides:

-Use 60 µL of the T50 buffer to clean the slides; use different tips for each slide.

1. Put a little buffer over the hole you intend to put the pipet in before flowing buffer through it—this will help keep deadly air bubbles out.
2. Flow the buffer through. See diagram below.



-Mixing epoxy: make sure you mix it in two linearly independent directions.

-Put the epoxy on the edges of the slides; wait on it to dry (10-15 minutes).

Procedures for preparing PEG slides:

Set the temperature of the sonicator to 55° for later.

Clean slides according to the normal protocol and then:

1. Get the little brown bottle of 3-Aminopropyltrimethoxysilaine (UCT Specialties. Amino-silaine) out of the freezer so it can warm up to room temperature.
2. Put gloves on. Put methanol in the blue-labeled glassware.
3. Rinse both the slides and the coverslips very well with pure water. Fill the slide and coverslip glassware up with pure water and cover with the lid. This is a good

place to stop if one does not intend to carry out the entire procedure on a given day. If equipped to proceed, continue on.

4. Pour the methanol out of the labeled glassware (for the slides and coverslips). Shake the methanol out of the labeled glassware.
5. Take slides/coverslips over to the drying table.
6. Start with coverslips:
    a. Rinse each coverslip well with pure water.
    b. Dry each coverslip with filtered air.
    c. Swipe each coverslip across the propane flame 4 times (2 swipes for each side of the coverslip). If you leave the glass coverslip over the flame too long, it will break.
    d. Place coverslips in the labeled coverslip glassware. Warning: Watch your face. The methanol residue could burn your face if you point the opening of it towards you. That could make you pretty busted.
7. Proceed to quartz slides:
    a. Rinse each slide well with pure water.
    b. Dry each slide with filtered air.
    c. Burn each slide with the propane flame. Make sure you do this very well, for this surface must be free of impurities.
    d. Place slides in the blue-labeled slide glassware. See warning in 18e.
8. Dump the methanol out of the remaining flask.
    a. Put 100 mL of methanol into the flask.
    b. Put 5 mL of purified water into the flask.

c. Put 1 mL of acetic acid, glacial (EMD) (from the flammable cabinet) into the flask. Make sure you use a glass tip over the plastic tip when you are getting the acetic acid.

This mixture of acid, water, and methanol is your solvent.

d. Put 2 mL of amino-silaine from freezer in the flask (use a glass pipet tip for this). This step must be done quickly so that it doesn't oxidize.

9. Make sure that the mixture in the labeled flask is mixed well (swirl it).
10. Pour the solution into the blue-labeled glassware containing the slides and coverslips. Put the lids on the glassware. Immediately, put the labeled flask in the sink and rinse it well with water.
11. Put the glassware in the 50 C hot bath for 10 minutes. (It might be a good idea to get the PEG out of the refrigerator at this point so it can warm up to room temperature)
    a. Put the jar of amino-silaine in the vacuum chamber.
    b. Make the sodium bicarbonate buffer ($NaHCO_3$)
        i. In the shared lab, measure out ~84 mg of the $NaHCO_3$ powder with a clean spatula.
        ii. Put it in the container with the orange lid.
        iii. Fill the container up with purified water to the 10 mL line.
        iv. Label the container.
        v. *Note* You must use the sodium bicarbonate buffer the same day you make it.
12. Sonicate the glassware for 1 minute.

13. Leave the glassware in the hot bath for another 10 minutes.
    a. Seal the amino-silaine bottle:
        i. Put N_2 nozzle in the vacuum and fill the chamber with N_2.
        ii. Open the chamber quickly and put the lid on the amino-silaine.
        iii. Seal the lid with parafilm. Put it back in the freezer.
14. Remove the flasks from the sonicator. At this point the aminocyline should be attached to the surfaces.
15. To both the coverslips and the slides:
    a. Dump out the solution.
    b. Rinse two times with methanol.
    c. Rinse many times with purified water.
    d. Fill the flasks with purified water.
16. Preparing the slides:
    a. Start with the coverslips:
        i. Rinse coverslip with purified water from the squirt bottle.
        ii. Dry the coverslip with filtered air.
        iii. Put the coverslip in a covered box.
        iv. Repeat for each coverslip.
    b. Proceed with the slides:
        i. Rinse the slides with purified water from the squirt bottle.
        ii. Dry the slides with filtered air.
        iii. Put the slides in a covered box.
        iv. Repeat for each slide.

Do these steps relatively quickly; the quicker the slides and coverslips are prepared, the cleaner they will be.

The following steps are very critical:

17. From the environment-controlled container in the freezer, get the M-SPA-500 PEG with biotin and without biotin. Let the vials warm up to room temperature.
    a. Weigh out 80 mg of the M-SPA-500 PEG (Regular PEG. Nektar. Biotin – PEG – NHS – 5000) in the shared lab and put it in a little tube.
    b. Weigh out 1-4 (~3) mg of the M-SPA-500 PEG with biotin and put it in the little tube.
    c. Now we have to work quickly. Put 320 μL of $NaHCO_3$ buffer into the test tube. Flick it. Centrifuge it for one minute. The solution is highly viscous, so we must spin it to eliminate bubbles. After one minute of spinning, you should have a clear solution.
18. Put 70 μL on top of each slide and put a coverslip over each slide. By this method, both internal surfaces are getting coated by the PEG.
19. Put water in the boxes to keep the slides from drying out and put the lids on the boxes. Put the boxes in a dark place (a drawer) so the polymer will not get hydrolyzed by the light.
20. Throw out the $NaHCO_3$ buffer.
21. Wait 3 hours for the slides to set.
22. Put the PEG vials in the vacuum for 30 minutes. Break the vacuum with N_2 and quickly close the lids and seal the vials with parafilm.

~3 Hours Later~

23. Rinse off the coverslips with purified water.
24. Dry the coverslips with filtered air, and put them on a covered rack PEG-side up.
25. Rinse off the slides with purified water.
26. Dry the slides with filtered air, and put them on a covered rack PEG-side up.

It is important to get the slides and coverslips very dry because the polymer hydrolyzes in the presence of water, so if you keep them dry, they last longer.

Make sure the symmetry of the human body is exploited to keep track of where the PEG surface is. (AKA: Be very careful to keep track of which side of the slides/coverslips the PEG is on.)

27. Assemble the slides with double-sided tape, cut off the excess tape, and put the slides in foil to protect them from dust. Put them in the environment-controlled chamber under the cabinet for later use.

Preparing DNA Samples on PEG Slides:

You should have your DNA and buffers prepared before beginning the experiment.

Start with clean PEG slide:

1. Flow 50 μL T50 buffer through the slide.
2. Epoxy the edges of the slide. Wait for epoxy to dry.
3. Once the epoxy is dry, flow another 50 μL T50 buffer through the slide.
4. Mix 1 nM DNA with 1 μL gloxy (check this) to check for non-specific binding. Look at the slide under the microscope.
5. Wash the slide with 100 μL T50 buffer.

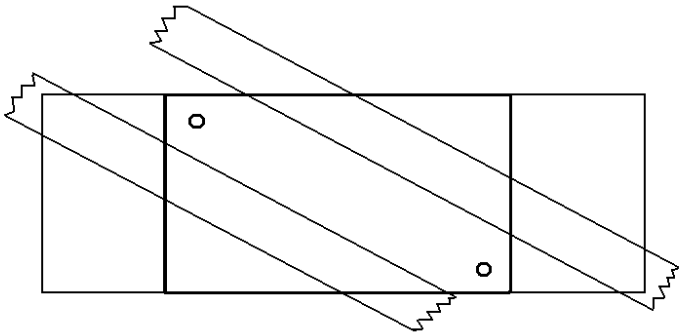
6. Combine 57 μL of the T50 buffer with 3 μL neutravidin (5 mg/mL concentration) into a little tube. Flow the solution into the sample and wait 10 minutes.
7. Wash the slide with 100 μL T50 buffer to remove all the neutravidin that is not bound.
8. Add the DNA solution; put it in the slide and wait 5 minutes.
9. Wash with 100 μL T50 buffer.
10. Create PG7 buffer:
    a. 50 mM MOPS pH 7
    b. 5 mM MgCl
    c. Trolox
    d. 5 mM DTT
    e. 0.1 % Tween20
11. Put a mixture of the PG7 buffer (99 μL) and gloxy (glucose, oxidase, and catalase; this eliminates oxygen from the solution) (1 μL) into the slide.
12. Now the slide is ready. Put the slide on the microscope stage.

*The DNA and gloxy should be put on ice.

Preparing Slides:

1. Dry slides and coverslips with filtered air. Hold the slides/coverslips with the tweezers and only blow them sideways so they will not break. Use less air when drying the coverslips—they are more fragile!

2. Put double sided tape on the slide and put the coverslip on top of the slide and tape, as shown in the figure below. Use a pipet tip to press down and seal the coverslip to the tape. Then, cut the excess tape with a razor blade.
3. Store slides in a covered box in a drawer.



Using the Total Internal Reflection (TIR) Microscope:

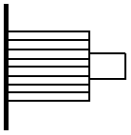
Setting up the stage:

1. Put a drop of water on the objective.
2. Carefully, put the slide on the stage, epoxy side down.
3. Put a couple of drops of oil on the top of the slide.
4. Secure the slide with the clips.
5. Make sure all the oil is off of the prism (wipe it with a kimwipe).
6. Secure the prism on the top of the slide.
7. Make sure the microscope is set on the viewing option.
8. Make sure it is set on local control.
9. Turn the microscope on.
10. If you aren't using the microscope for relatively short periods of time, turn the laser off.

11. When you are done using the microscope, take the slide off the stage and absorb the water from the objective by putting a kimwipe to the side of the objective. Just let the water absorb—do not touch the kimwipe to the objective (that will scratch the objective).

Adjusting the Microscope:

1. On the right side of the microscope, there are two knobs: the larger on is the course adjustment and the smaller one is the fine adjustment.



2. Clockwise movements of these knobs lower the objective and counterclockwise movements of these knobs raise the objective. Exercise caution in moving the course objective counterclockwise (up)—careless raising of the objective could break it.

Beam Adjustment:

1. The top knob affects the beam's position.
2. The left knob affects how big the spot is.
3. Do not monkey with the front knob.

*When focusing the microscope, take note of where the starting numbers are on the adjustment stage before touching them.

Using the Camera:

1. Manually focus the microscope on the sample.

2. Plug in the camera.
3. Open the program Single → Mod → TIR
4. Wait for the camera to get down to -90°
5. The black uniblitz box should be set on remote, normally open.
6. Turn the microscope to the camera option.
7. Once everything is ready, click Open Camera.
8. Click the red shutter to saturate (photobleach) an area.
9. If you click on 2-color, you can see the overlap of the donor and acceptor images (one is red and one is green).

When looking at the bead samples, the settings should be:

Gain = 1

Background = 390

Exposure time (s) = 0.032

Flash (ms) = 0

Preparing BSA-Biotin Slides:

BSA-biotin samples are less stable than PEG samples. They can be used for a few hours.

1. Get a clean slide and wash it with the T50 buffer.
2. Epoxy the edges of the slide and wait for it to dry.
3. Wash the slide again with the T50 bufer to make sure it is sealed properly.
4. Add 60 mL of 10 mg/mL BSA-biotin (6 μL BSA-biotin + 54 μL T50) or use 50 μL of BSA-biotin solution
5. Wait 10 minutes.
6. Wash the slide with 100 μL T50 buffer.

7. (optional) To check for non-specific binding, add 1 nM DNA and look at it under the microscope (From 100 nM concentration, make 1 nM solution). Observe the fluorescent on the slide.
8. Add streptavidin (3 μL stock streptavidin + 57 μL T50). The streptavidin is in the refrigerator; use it right out of the refrigerator. (5 mg/mL dilute in T50).
9. Wait 10 minutes.
10. Wash the slide with 100 μL T50 buffer.
11. Add DNA (want 20-50 pM DNA concentration for specific binding)

i.e. to get 20 pM concentration of DNA, with 60 μL final volume, we need:

2 μL 1 nM stock solution of DNA (from freezer) + 58 μL T50 buffer.

12. Wait 5 minutes.
13. Get the imaging buffer out of the refrigerator.
14. Put 100 μL T50 buffer to wash out the slide. The samples are now ready, in principle.
15. Combine 99 μL trolox imagining buffer with 1 μL gloxy (1 μL gloxy should cover about ½ of a pipet tip).
16. Put the above mixture into the slide and look at it with the microscope.

Note: the camera should be on the following settings:

Exposure time: 0.032

Data scaler: 6000

Background sub: 390

Gain: 240

Shutter: check

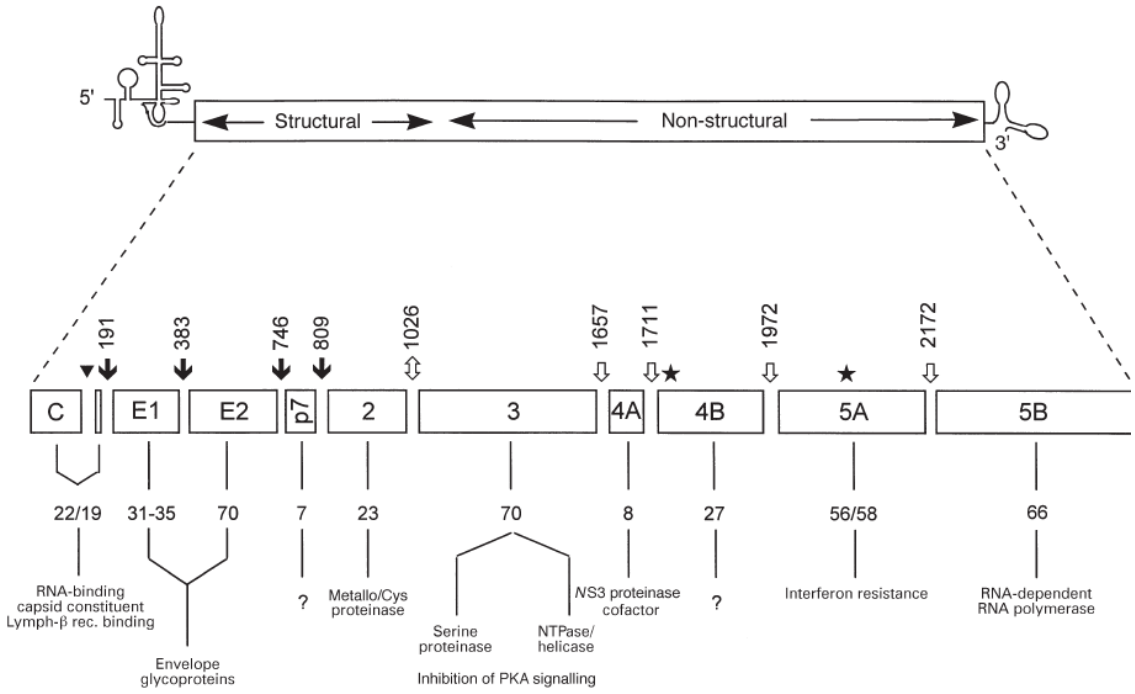


Figure 1. HCV Peptide [9].

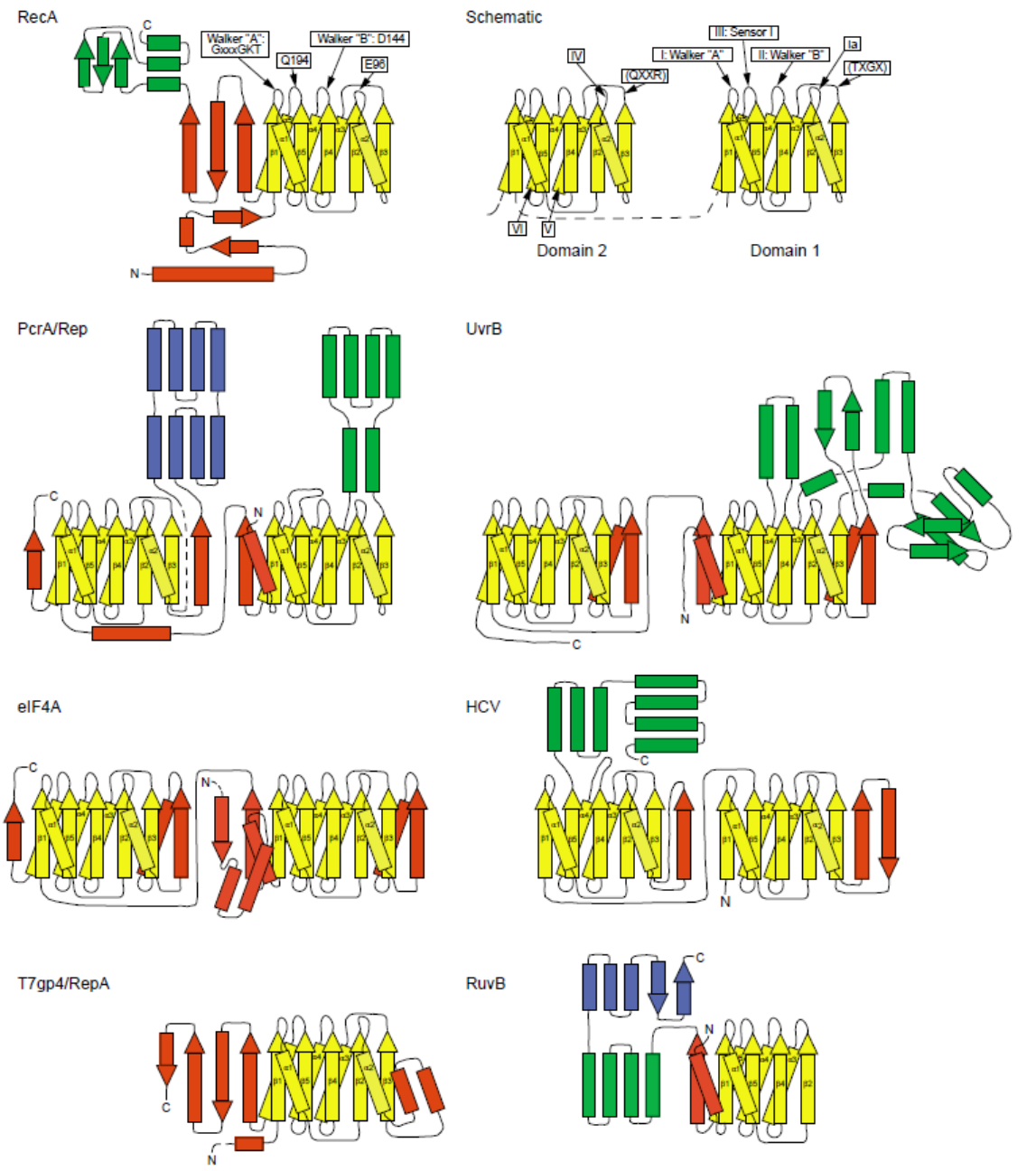


Figure 2. Examples of helicases in each different superfamily [12]. SF-1 (PrcA/Rep) SF2 (HCV helicase; UvrB). DnaB-like family (T7gp4/RepA). Topology diagrams of representative helicases. Yellow, conserved RecA-related 'core'; red, variable structural elements in domains with a RecA-like core; green and blue, additional structural domains [12]. The schematic on the upper right summarizes the positions in the topology of the RecA-like core of the seven conserved motifs defined by Gorbalenya and Koonin for the SF-1 and SF-2 helicases [12].

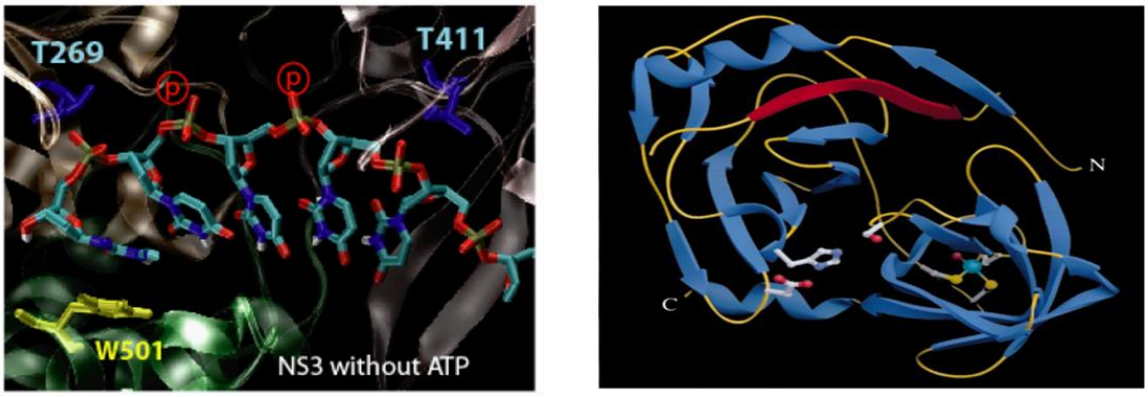


Figure 3. NS3 Helicase [16]. NS3 crystallized without ATP shows two well conserved threonine residues (T269, T411 in blue) contacting backbone phosphate 3 nucleotides apart. W501 (yellow), also known as "gatekeeper" is base stacked at the 3' end of the bound nucleotide.

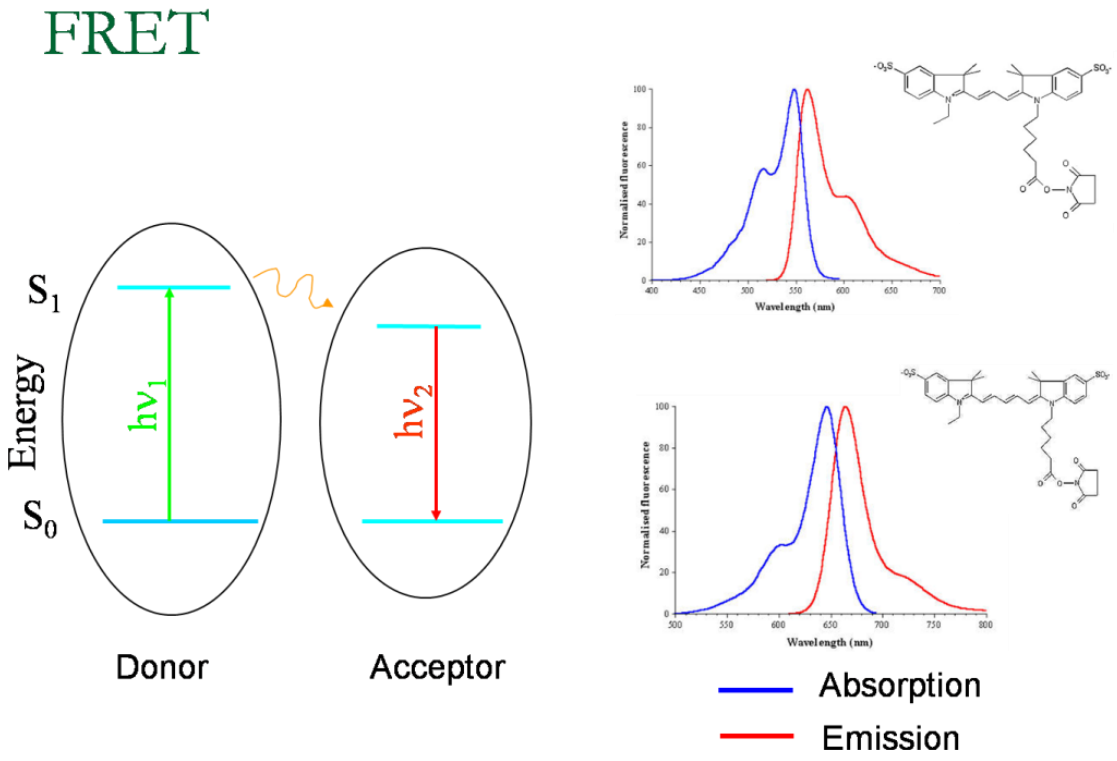


Figure 4. [22]. Energy levels diagram showing FRET transfer between donor and acceptor. On the right absorption (blue) and emission (red) spectra for donor (top) and acceptor (bottom). Also shown the chemical structure of the donor-acceptor pair used in this work (Cy3 top and Cy5 bottom).

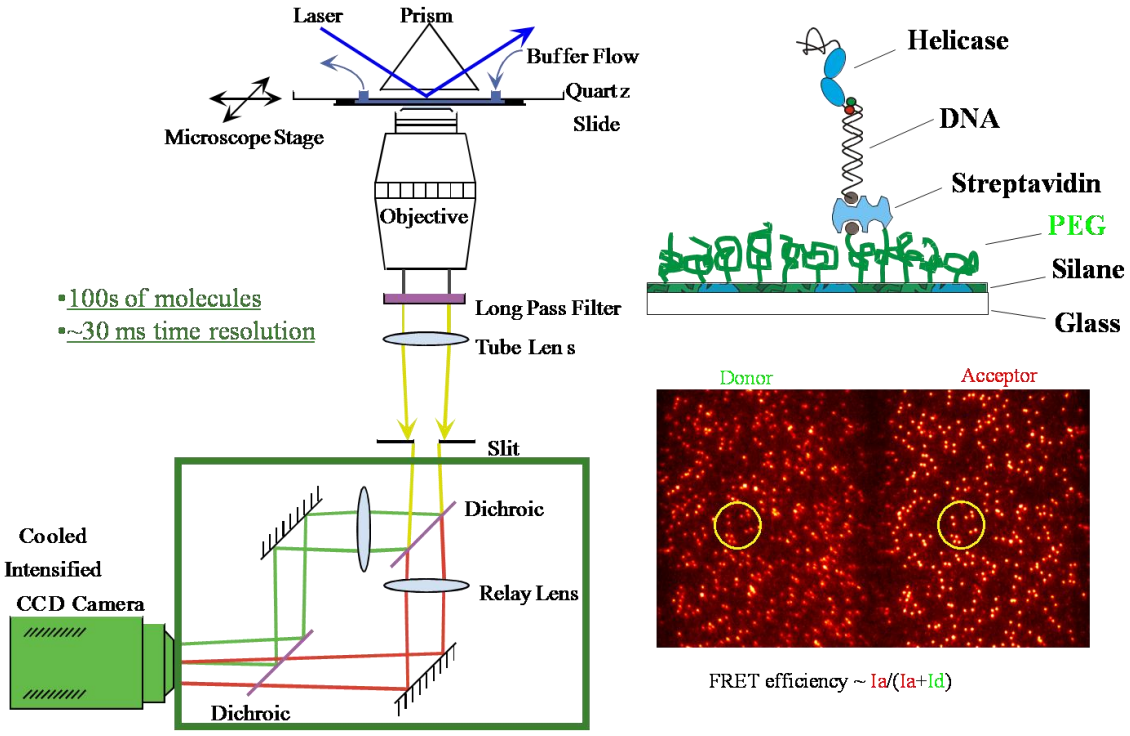


Figure 5. Experimental setup for smFRET. The left panel shows the direction of light from the laser to excitation of the sample, and from the sample to the camera. The top right panel shows a schematic of the samples used for single molecule observation. The bottom right panel shows how the software sees one spot of immobilized DNA but with two views: donor emission on the left and acceptor emission on the right.

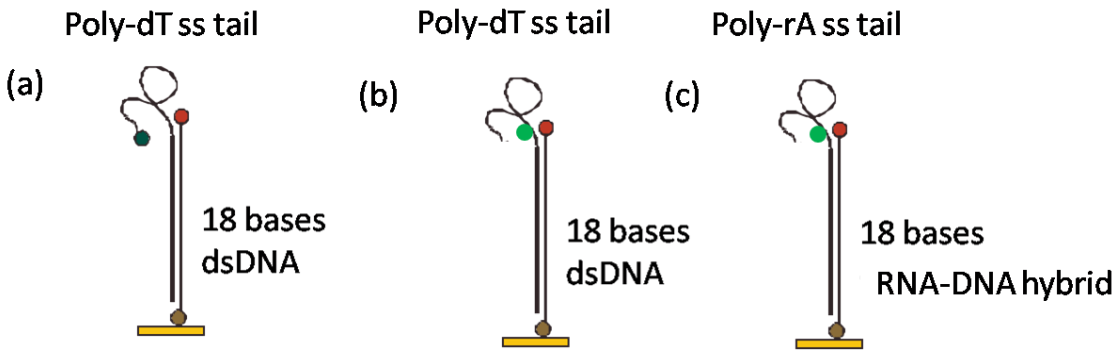


Figure 6. Substrates used in this work. Acceptor indicated with red circle, donor indicated with green and biotin indicated with yellow. All substrates contain a single stranded tail 3'-5' to provide a binding site for the protein. (a) Substrate used to determine binding dynamics (b) (c) Substrates used for unwinding experiments.

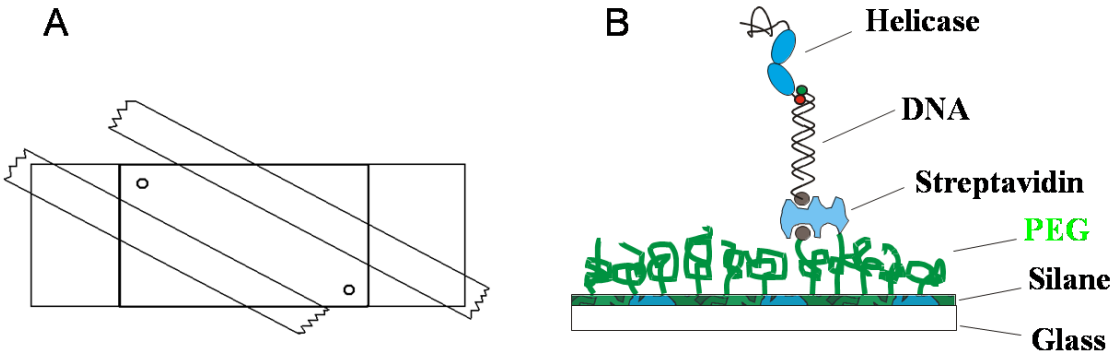


Figure 7. PEG Slide. A) The top view of the PEG slide. The two diagonals rectangles are tapes that are inserted between the slide and the cover slip providing a spacer of ~ 100 μm. . B) The schematic of what the experiment would look like. Nucleic acids containing biotin would bind to the Streptavidin, which is in turn bind to the biotin that is attached to some of the polyethylene glycol on the slide.

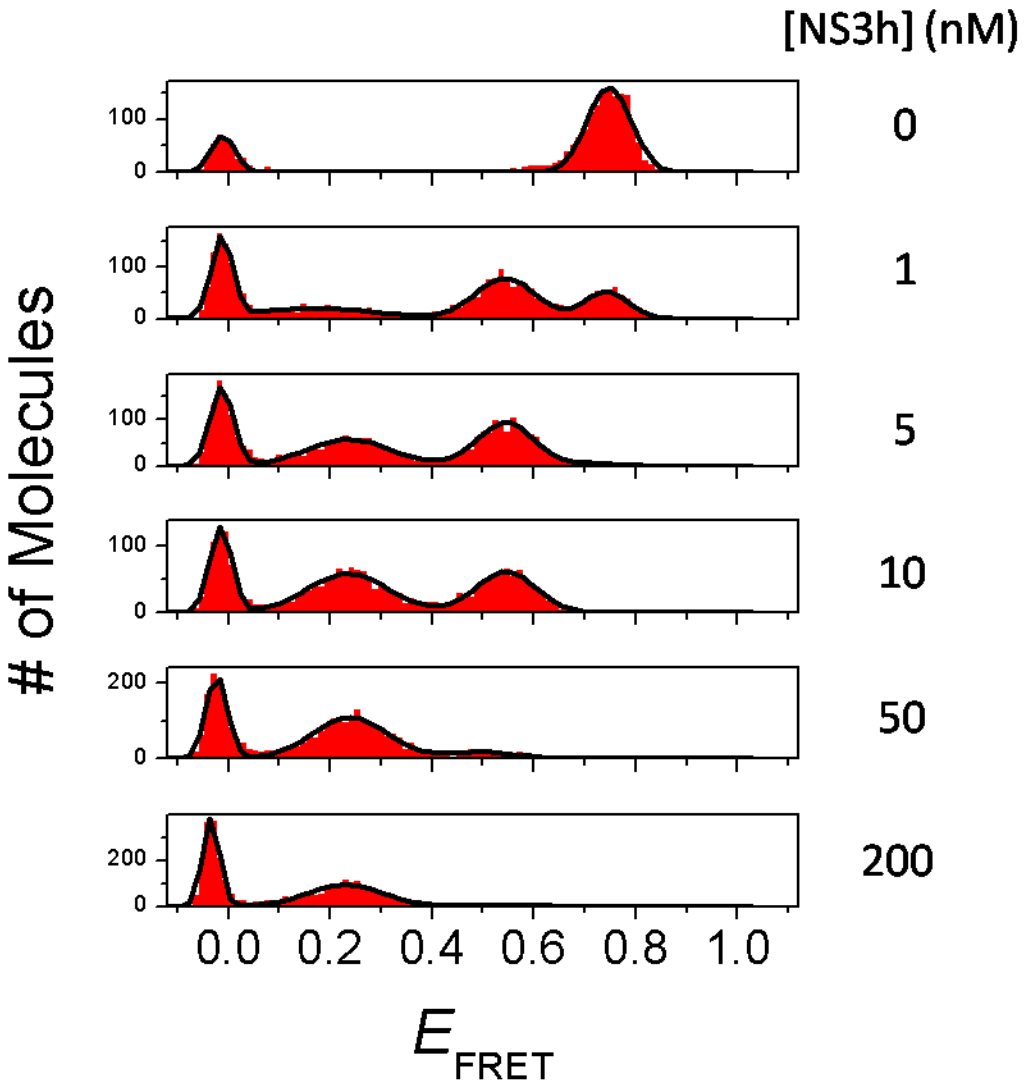


Figure 8. FRET efficiency histograms for binding substrates at different NS3 concentrations. The peak at zero FRET corresponds to inactive acceptor molecules. The histograms are obtained by determining the FRET efficiency for thousands of molecules.

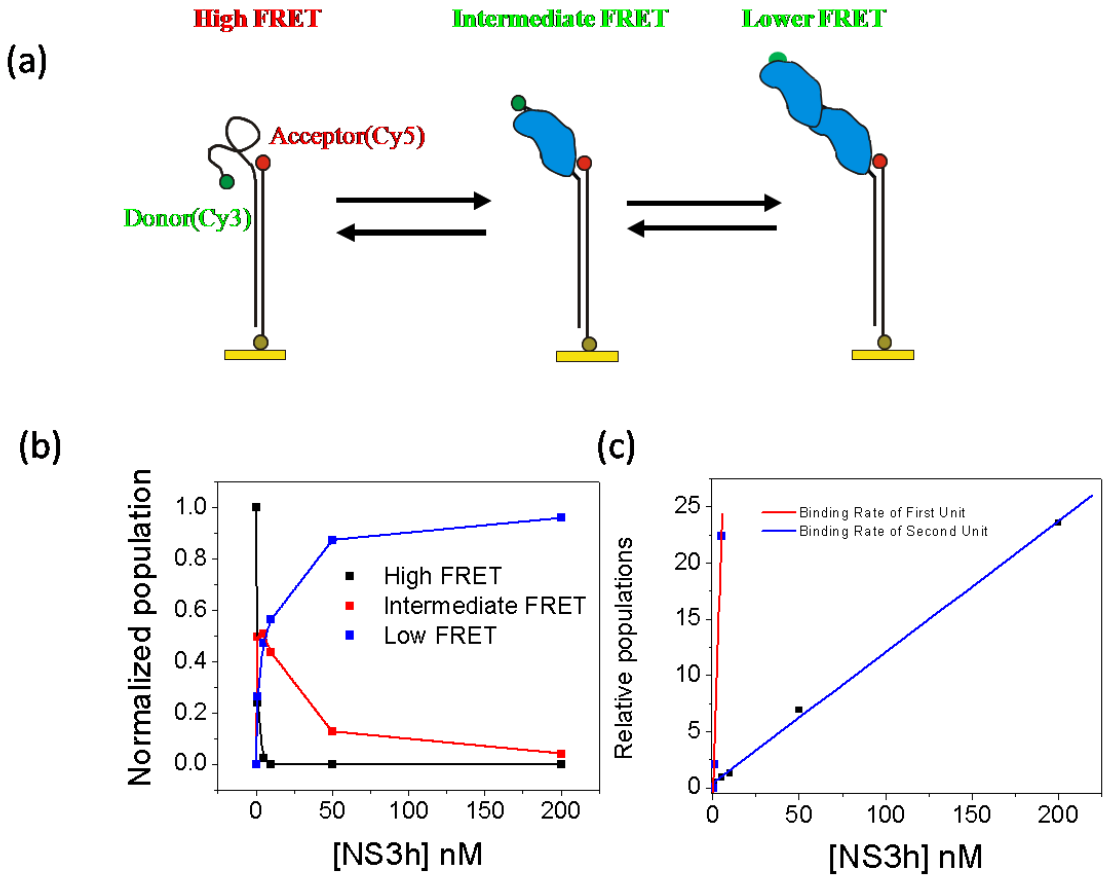


Figure 9. (a) Binding model for two NS3 molecules in successive steps. (b) Dependence on the population of the FRET states observed in the FRET efficiency histograms. (c) Relative populations of the low FRET efficiency state and intermediate state and intermediate and high states showing a linear dependence.

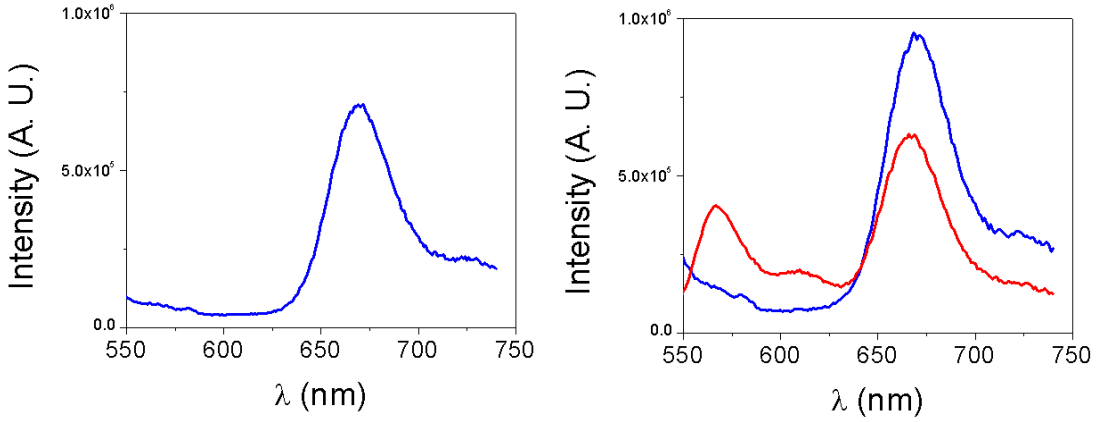


Figure 10. Unwinding experiments in ensemble. Left panel, substrate only showing high FRET efficiency. Right panel, after incubation of 10 nM substrate with 100 nM protein (blue curve) we added 4 mM ATP to initiate unwinding. Data shown is 30 seconds after ATP addition, showing increase in donor emission and decrease in the acceptor emission consistent with decrease in FRET efficiency.

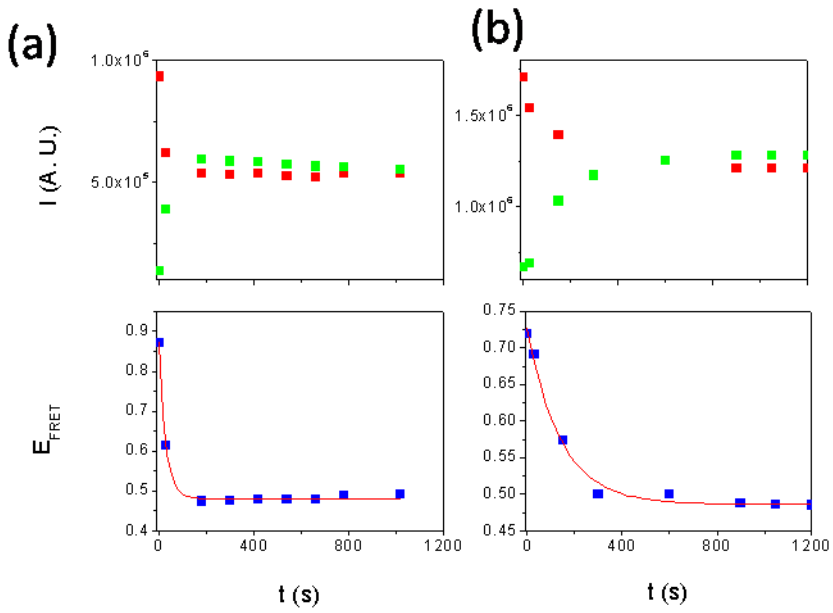


Figure 11. Unwinding experiments in ensemble, 10 nM substrate concentration, 100 nM NS3-NS4A, and 4 mM ATP. Acceptor intensity in red, donor intensity in green (top panels) and FRET efficiency in blue (lower panels) (a) RNA-DNA substrates (b) DNA-DNA substrate.