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October 21, 2018

Dr. Xiaoyan Cao
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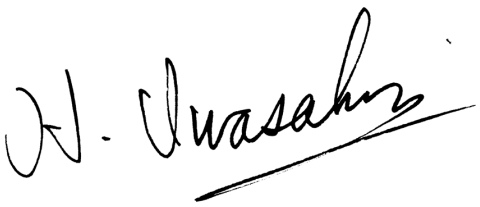
RE: JoVE59073

Dear Dr. Xiaoyan Cao:

I am sending a revised manuscript of our manuscript entitled, "**Real-time observation of the DNA strand exchange reaction mediated by Rad51**", by Ito, Argunhan, Tsubouchi, and Iwasaki (JoVE59073).

Yours sincerely



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TITLE:

Real-time Observation of the DNA Strand Exchange Reaction Mediated by Rad51

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KEYWORDS:

Homologous recombination, DNA strand exchange reaction, Rad51, Swi5-Sfr1, real-time observation, fluorescence resonance energy transfer (FRET)

SUMMARY:

Fluorescence resonance energy transfer-based real-time observation systems of the DNA strand exchange reaction mediated by Rad51 were developed. Using the protocols presented here, we are able to detect the formation of reaction intermediates and their conversion into products, while also analyzing the enzymatic kinetics of the reaction.

ABSTRACT:

The DNA strand exchange reaction mediated by Rad51 is a critical step of homologous recombination. In this reaction, Rad51 forms a nucleoprotein filament on single-stranded DNA (ssDNA) and captures double-stranded DNA (dsDNA) non-specifically to interrogate it for a homologous sequence. After encountering homology, Rad51 catalyzes DNA strand exchange to mediate pairing of the ssDNA with the complementary strand of the dsDNA. This reaction is highly regulated by numerous accessary proteins *in vivo*. Although conventional biochemical assays have been successfully employed to examine the role of such accessory protein *in vitro*, kinetic analysis of intermediate formation and its progression into a final product has proven challenging due to the unstable and transient nature of the reaction intermediates. To observe these reaction steps directly in solution, fluorescence resonance energy transfer (FRET)-based real-time observation systems of this reaction were established. Kinetic analysis of real-time observations shows that the DNA strand exchange reaction mediated by Rad51 obeys a three-step reaction model involving the formation of a three-strand DNA intermediate, maturation of this intermediate, and the release of ssDNA from the mature intermediate. The Swi5-Sfr1 complex, an accessary protein conserved in eukaryotes, strongly enhances the second and third steps of this reaction. The FRET-based assays presented here enable us to uncover the

molecular mechanisms through which recombination accessary proteins stimulate the DNA strand exchange activity of Rad51. The primary goal of this protocol is to enhance the repertoire of techniques available to researchers in the field of homologous recombination, particularly those working with proteins from species other than *Schizosaccharomyces pombe*, so that the evolutionary conservation of the findings presented herein can be determined.

INTRODUCTION:

Homologous recombination (HR) facilitates the shuffling of genetic information between two different DNA molecules. HR is essential for two fundamental biological phenomena: the generation of genetic diversity during gametogenesis[1] and the repair of DNA double-strand breaks (DSBs)[2] during mitosis. DSBs are the most severe form of DNA damage and constitute a breakage in the chromosome. Incorrect repair of DSBs can cause extensive chromosomal rearrangements and genomic instability, which are both hallmarks of cancer[3].

The DNA strand exchange reaction is the central phase of HR. The Rad51 protein, which is a member of the highly conserved RecA-type family of recombinases, is the key protein that catalyzes this reaction in eukaryotes[4,5]. In this reaction, Rad51 binds to single-stranded DNA (ssDNA) generated by nucleolytic processing of the DSB end and forms a helical nucleoprotein complex termed the presynaptic filament. This filament catches intact double-stranded DNA (dsDNA) nonspecifically to search for a homologous sequence. When the filament finds a homologous sequence, a reaction intermediate containing three-stranded DNA is formed and the Rad51 filament mediates strand exchange within this structure[6-8]. To accomplish this reaction efficiently, Rad51 requires several kinds of accessory proteins such as BRCA1 and BRCA2, products of breast cancer susceptibility genes[9,10].

Understanding how accessory factors regulate Rad51 is an integral step in uncovering the causes of genomic instability during tumorigenesis. Although much research is concerned with the effects of these factors on presynaptic filament formation and stability[11-16], the contribution of these factors to formation of the three-strand intermediate and its processing into the final product is still unclear. Observing these reaction steps through conventional biochemical experiments is very difficult because the three-strand intermediate is unstable and prone to collapse by common experimental manipulations such as deproteinization of samples or electrophoresis.

To overcome this problem, we adapted two previously developed real-time observation systems of the DNA strand exchange reaction using fluorescence resonance energy transfer (FRET): the DNA strand pairing and DNA strand displacement assays[17,18] (**Figure 1**). In the DNA strand pairing assay, Rad51 forms a presynaptic filament with fluorescein amidite (FAM)-labeled ssDNA and then homologous carboxy-x-rhodamine (ROX)-labeled dsDNA is added to initiate the strand exchange reaction. When the filament catches the ROX-labeled dsDNA and forms the three-strand intermediate, the two fluorophores come into close proximity and fluorescence emission of FAM is quenched by ROX (**Figure 1A**). In the DNA strand displacement assay, a presynaptic filament formed on unlabeled ssDNA is incubated with FAM and ROX double-labeled dsDNA. When strand exchange is completed and the FAM labeled ssDNA is

released from the three-strand intermediate, the emission of FAM increases because FAM is no longer in close proximity to ROX (**Figure 1B**). These assays enable us to observe the formation of three-strand intermediates and their processing into final products in real-time without any disturbances to the reaction.

Using this real-time observation system, we found that the DNA strand exchange reaction mediated by Rad51 proceeds in three-steps including the formation of the first reaction intermediate (C1), transitioning of the first intermediate into a second intermediate (C2), and release of ssDNA from C2[19]. We also found that fission yeast (*S. pombe*) Swi5-Sfr1, which is an evolutionarily conserved Rad51 accessory protein complex[13,16,20-22], stimulates the C1-C2 transitioning and release of ssDNA from C2 in a manner that is dependent on ATP-hydrolysis by Rad51[19].

Whether these findings are evolutionarily conserved remains unknown. This protocol is provided with the hope that researchers in the field of HR, especially those working with proteins from organisms other than *S. pombe*, may apply these techniques to determine the extent to which the molecular mechanism of Rad51-driven strand exchange is conserved. Furthermore, these techniques have proven highly successful in determining the role of *S. pombe* Swi5-Sfr1. Thus, it is a rational prediction that these techniques will be invaluable in uncovering the precise roles of other HR accessory factors.

PROTOCOL:

1. Preparation of Proteins and DNA Substrates

1.1. Purify *S. pombe* Rad51 and Swi5-Sfr1 proteins to homogeneity (judged by Coomassie staining), as previously reported[13,21].

1.2. Prepare oligonucleotide DNA substrates listed in **Table 1**[18].

NOTE: The oligonucleotides were purchased (see **Table of Materials**) and synthesized at HPLC grade. For the DNA strand pairing reaction, oligonucleotides 16FA(-), 16A(-)_40bp and 16AR(+)_40bp are required. For the DNA displacement assay, oligonucleotides 16A(-), 16FA(-)_40bp and 16AR(+)_40bp are required (**Figure 1** and **Table 1**). All DNA concentrations in this protocol refer to fragment concentrations as opposed to nucleotide concentrations.

1.3. To form donor dsDNA, mix equimolar amounts of complementary strands in a thin-walled PCR tube with annealing buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$), ensuring a total volume greater than 20 µL. Perform this mixing on a prechilled metal rack on ice (between 2 °C and 4 °C).

NOTE: The combination of oligonucleotides for the pairing assay are 16A(-)_40bp and 16AR(+)_40bp. For the displacement assay, anneal oligonucleotides 16FA(-)_40bp and 16AR(+)_40bp.

1.4. Heat the annealing mixture at 90 °C for 5 min and cool down over 3 h to 30 °C using a PCR machine. Store the annealed DNA at -20 °C.

2. DNA Strand Pairing and Displacement Assays

2.1. Perform DNA strand pairing assay.

2.1.1. Prepare 1.6 mL of reaction buffer A (30 mM HEPES-KOH pH 7.5, 1 mM dithiothreitol [DTT], 15 mM $MgCl_2$, 0.25 mM ATP, 0.1 mg/mL bovine serum albumin [BSA], and 0.0075% polyoxyethylenesorbitan monolaurate) containing 36 nM 16FA(-) in a 2.0 mL micro-centrifuge plastic tube (polypropylene) and pre-incubate it at 37 °C for 5 min.

2.1.2. To form Rad51-ssDNA filaments, add Rad51 protein to a final concentration of 1.5 µM in the pre-incubated reaction buffer and incubate it at 37 °C for 5 min.

2.1.3. Add Swi5-Sfr1 protein to the mixture to a final concentration of 0.15 µM and incubate it at 37 °C for a further 5 min.

2.1.4. Take 1.5 mL of the mixture and transfer it into a 1.0 x 1.0 cm quartz cuvette containing a magnetic stirrer and set the cuvette into a spectrofluorometer. Configure the peltier temperature controller of the spectrophotometer to 37 °C and set the magnetic stirrer to 450 rpm to ensure rapid mixing of the injected sample.

2.1.5. Start measuring the fluorescence emission of FAM at 525 nm (bandwidth: 20 nm) upon excitation at 493 nm (bandwidth: 1 nm). Collect data every second.

2.1.6. After starting the measurement for 100 s, inject ROX-labeled donor dsDNA to a final concentration of 36 nM into the mixture using a syringe and measure the change in emission at 1 s intervals for a further 30 min.

2.2. Perform DNA strand displacement assay.

2.2.1 Prepare 1.6 mL of reaction buffer A containing 36 nM 16A(-) in a 2.0 mL micro-centrifuge plastic tube and pre-incubate it at 37 °C for 5 min.

2.2.2 Form Rad51-ssDNA filaments in the presence of Swi5-Sfr1 at 37 °C, as described in steps 2.1.2. and 2.1.3.

2.2.3. Take 1.5 mL of the mixture and transfer it into the quartz cuvette containing a magnetic stirrer and set the cuvette into the spectrophotometer, as described in step 2.1.4.

2.2.4. Start measuring the fluorescence emission and after 100 s, inject FAM- and ROX-labeled donor dsDNA as described in step 2.1.6. Measure the change in fluorescence emission at 1 s intervals for a further 30 min.

3. Analyzing Experimental Data from the Pairing and Displacement Assays

3.1. Estimate maximum FRET efficiency.

3.1.1. Prepare 16FA(-) annealed with 16AR(+)_40bp and 16FA(-)_40bp annealed with 16AR(+)_40bp using the same procedure described in steps 1.3 and 1.4.

3.1.2. Prepare 130 µL of reaction buffer A containing 36 nM of either 16FA(-), 16FA(-) annealed with 16AR(+)_40bp, 16FA(-)_40bp annealed with 16AR(+)_40bp or 16FA(-)_40bp in a 0.2 x 1.0 cm quartz cuvette.

3.1.3. Set the cuvette into the spectrofluorometer and incubate at 37 °C for 5 min.

3.1.4. Measure fluorescence spectra from 500 to 600 nm upon excitation at 493 nm.

3.1.5. To test the effect of Rad51 on the emission of FAM and quenching of FAM by ROX, add Rad51 to a final concentration of 1.5 µM to the mixture and incubate at 37 °C for 5 min.

3.1.6. Measure fluorescence spectra from 500 to 600 nm upon excitation at 493 nm.

3.1.7. Calculate maximum FRET efficiency ($E_{maximum}$) using the equation described below:

$E_{maximum}$ = (Fluorescence intensity at 525 nm of dsDNA labeled with both FAM and ROX) / (Fluorescence intensity at 525 nm of FAM labeled ssDNA)

3.2. Analyze experimental data from the displacement assay.

3.2.1. To convert the change in fluorescence observed in the displacement assay to the change in the amount of final product, normalize raw experimental data obtained from this assay using the equation described below, where F_{raw} is fluorescence intensity from raw data and $F_{normalized}$ is the change in fluorescence calculated by the following equation.

$F_{normalized}$ = ([F_{raw} at time x] - [F_{raw} at time 0]) / (([F_{raw} at time 0] / $E_{maximum}$) - [F_{raw} at time 0])

F_{raw} at time 0 is the average fluorescence monitored for the first 5 s after the dead time (*i.e.*, the time required for mixing after an injectant is introduced into the cuvette).

3.2.2. To exclude the effects of photobleaching and spontaneous displacement, subtract $F_{normalized}$ without protein from $F_{normalized}$ of the sample to obtain F_D, which is the change in the amount of final product in this assay.

F_D = [$F_{normalized}$ of sample] - [$F_{normalized}$ without protein]

3.3. Analyze experimental data from the pairing assay.

3.3.1. Normalize raw experimental data obtained from the pairing assay using the equation described below, where F_{raw} is fluorescence intensity from raw data and $F_{normalized}$ is the change in fluorescence calculated by the following equation.

$F_{normalized}$ = (F_{raw} at time x) / (F_{raw} at time 0)

F_{raw} at time 0 is the average fluorescence monitored for the last 20 s before initiating the reaction by injecting the dsDNA substrate.

3.3.2. To convert change in fluorescence into change in amount of substrate and exclude the effects of photobleaching and spontaneous pairing, normalize $F_{normalized}$ of sample using the equation described below, where F_P is the change in amount of substrate in this assay.

F_P = 1 - (([$F_{normalized}$ without protein] - [$F_{normalized}$ of sample]) / $E_{maximum}$)

3.3.3. To examine the reaction kinetics of DNA strand exchange, perform non-linear last-square regression analysis of the pairing reaction using the analysis program[23] (see **Table of Materials**).

3.3.3.1. Prepare a file in .txt format containing time course data of F_P.

3.3.3.2. Start the program and paste the script in the **Supplemental Code File** into a window of the program:

3.3.3.3. Start non-linear last-square regression analysis. The results of this analysis will be displayed in the same window.

REPRESENTATIVE RESULTS:

In order to effectively analyze the experimental data from the pairing and displacement assays, it is necessary to define how a change in fluorescence emission of FAM corresponds to a conversion of DNA substrates into products. To achieve this, the relevant range of fluorescence intensity must be determined. For the pairing assay, the fluorescence emission of 16FA(-), which corresponds to the ssDNA substrate, is compared with the emission of 16FA(-) annealed with 16AR(+)_40bp, which corresponds to the final products of this reaction (**Figure 2A**). This equates to the maximum FRET efficiency and hence the maximum reduction in fluorescence intensity that would be expected if all ssDNA substrate was converted into the dsDNA product. For the displacement assay, the emission of 16FA(-)_40bp annealed with 16AR(+)_40bp, which corresponds to the substrate, is compared with the emission of 16FA(-)_40bp, which corresponds to the final product (**Figure 2B**). In this case, the maximum increase in fluorescence intensity of FAM conveys a scenario in which all of the dsDNA substrate is converted into ssDNA

product. *S. pombe* Rad51 did not affect fluorescence emission of FAM or quenching efficiency of FAM by ROX in both assays (**Figure 2**). The maximum FRET efficiency should be re-measured with each new preparation of oligonucleotides as it is dependent on the labeling efficiency of oligonucleotides.

Representative data of DNA strand pairing and displacement reactions are shown in **Figure 3**. The effects of spontaneous reactions between substrate DNAs and photobleaching were small in both assays, as revealed by the negligible changes seen in the emission of FAM without Rad51 compared to the substantial changes seen with Rad51 (**Figure 3A** and **Figure 3B**). Based on the data shown in **Figure 3A** or **Figure 3B**, the change in fluorescence was converted into the change in amount of substrate (F_P) or final product (F_D), respectively, using the equations described in steps 3.2.2 or 3.3.2 (**Figure 3C** and **Figure 3D**).

The pairing reaction was simulated using a sequential three-step reaction model, consisting of the formation of the first three-strand intermediate (C1), transitioning of the first intermediate into the second intermediate (C1 to C2), and the release of ssDNA from the second intermediate to form the two products (D + E) (**Figure 3E**). To test whether the simulation using a sequential three-step reaction model fit the experimental data, residuals between experimental data of the DNA strand pairing assay and a theoretical curve obtained by the simulation were calculated (**Figure 3F**). In addition, residuals between the pairing reaction and a theoretical curve generated using a sequential two-step reaction model were also calculated (**Figure 3G**). The residuals for the pairing reaction and the two-step model show a systematic deviation in the early stage, whereas the residuals for the pairing reaction and the three-step model do not show such a deviation. This indicates that the three-step model is a better fit than the two-step model for simulating the pairing reaction.

To test whether the three-step model is consistent with the displacement reaction that detects the late step of DNA strand exchange, we generated a theoretical curve of the displacement reaction using the kinetic parameters obtained from simulation of the pairing reaction shown in **Figure 3C** and compared it with the experimental data of the displacement reaction shown in **Figure 3D** (**Figure 3H**). The theoretical curve fit the experimental data of the displacement assay. From these results, we conclude that simulation using the three-step model is able to reasonably evaluate the DNA strand exchange reaction mediated by Rad51.

Representative data of the DNA strand pairing reaction containing Rad51 and the Swi5-Sfr1 complex, an accessary protein of Rad51, are shown in **Figure 4A**. The Swi5-Sfr1 complex strongly stimulated the pairing activity of Rad51. As was seen in the absence of Swi5-Sfr1, the pairing reaction better fit the three-step model than the two-step model in the presence of Swi5-Sfr1 (**Figure 4B**). Through simulation of the reaction using the three-step model, reaction equilibrium constants of each reaction step with or without Swi5-Sfr1 were calculated. The reaction equilibrium constants indicated that the Swi5-Sfr1 complex does not stimulate the first reaction step (**Figure 4C**, **panel a**), in which a three-strand intermediate is formed, but strongly stimulates C1-C2 transitioning (**Figure 4C**, **panel b**) and the release of ssDNA from the C2 intermediate (**Figure 4C**, **panel c**).

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental design of DNA strand pairing and displacement assays. Schematics of DNA strand pairing (**A**) and displacement (**B**) assays. Yellow circles represent Rad51 monomers. Green circles containing "F" and red circles containing "R" represent fluorescein amidite (FAM) and carboxy-X-rhodamine (ROX), respectively. Black DNA strands are identical in sequence and complementary to blue DNA strands. Thin black lines with arrowheads point to the name of each oligonucleotide, as depicted in **Table 1**. This figure has been adapted from Ito *et al.*[19] and modified.

Figure 2: Measurements of the maximum FRET efficiency of the pairing and displacement assays. (**A**) Comparison of fluorescence spectra between the ssDNA substrate, 16FA(-), and the dsDNA product, 16FA(-) paired with 16AR(+)_40bp, of the pairing assay. Blue and red lines represent the fluorescence spectra of the substrate without and with Rad51, respectively. Green and purple lines show the fluorescence spectra of the final product without and with Rad51, respectively. (**B**) Comparison of fluorescence spectra between the dsDNA substrate, 16FA(-)_40bp paired with 16AR(+)_40bp, and ssDNA product, 16FA(-)_40bp, of the displacement assay. Blue and red lines represent the fluorescence spectra of the final product without and with Rad51, respectively. Green and purple lines show the fluorescence spectra of the substrate without and with Rad51, respectively. This figure is adapted from Ito *et al.*[19] and modified.

Figure 3: DNA strand pairing and displacement reactions mediated by Rad51. (**A**) Time course of the normalized fluorescence of the pairing reaction with or without Rad51. (**B**) Time course of the normalized fluorescence of the displacement reaction with or without Rad51. (**C**) Time course of the change in the amount of substrate in the pairing reaction with Rad51. (**D**) Time course of the change in the amount of substrate in the displacement reaction with Rad51. (**E**) A schematic of the sequential three-step reaction model. A and B correspond to the presynaptic filament and donor dsDNA. C1 corresponds to the first (immature) three-strand intermediate. C2 corresponds to the second (mature) three-strand intermediate. D and E correspond to a heteroduplex and ssDNA released from C2. (**F** and **G**) Residuals between experimental data of the DNA strand pairing assay and a theoretical curve obtained by simulation using either the three-step (**F**) or two-step (**G**) model. (**H**) Red dots indicate experimental data from the displacement reaction with Rad51 shown in panel **D**. Blue line indicates the theoretical curve of the final products. The theoretical curve was generated by simulation using the reaction rate constants obtained from the pairing assay shown in panel **C**. This figure is adapted from Ito *et al.*[19] and modified.

Figure 4: Swi5-Sfr1 stimulates the second and third steps of the DNA strand exchange reaction. (**A**) Time course of the change in the amount of substrate in the pairing reaction with or without Swi5-Sfr1 (S5S1). (**B**) Residuals between experimental data of the DNA strand pairing assay with Swi5-Sfr1 and a theoretical curve obtained by simulation using the two-step (blue line) or three-step (red line) model. (**C**) The pairing reaction shown in **Figure 4A** was simulated

by the three-step model using the analysis program[23] (see **Table of Materials**). The reaction equilibrium constants of each reaction step, K1 (**a**), K2 (**b**), and K3 (**c**), were obtained from the simulation. This figure is adapted from Ito *et al.*[19] and modified.

Table 1: A list of oligonucleotides used in the DNA strand pairing and displacement assays. Where applicable, the positions of fluorophores (fluorescein amidite, FAM; carboxy-x-rhodamine, ROX) are indicated in square parentheses.

DISCUSSION:

Here, we have described a detailed protocol that utilizes FRET to measure Rad51-driven DNA strand exchange in real time. Importantly, these measurements allow for the determination of reaction kinetics. While the descriptions provided above are sufficient to reproduce our published results, there are several critical points that will be described in this section. Furthermore, the advantages and disadvantages of FRET-based methodologies for studying DNA strand exchange will be discussed, along with the application of such techniques to study other aspects of DNA metabolism.

As with all biochemical reconstitutions, ensuring that all reaction substrates are of high purity is essential. It is negligent to assume the absence of contaminating activities based solely on the apparent purity of a protein preparation judged by Coomassie staining. In particular, the presence of trace amounts of nucleases or helicases can drastically affect the results of the pairing and displacement assays. Thus, we strongly encourage testing for such activities each time a new batch of protein is purified. Furthermore, it is prudent to check the purity of synthesized DNA substrates by native polyacrylamide gel electrophoresis. Despite many companies guaranteeing the purity of oligonucleotides, we have often found through our own testing that purity of the synthesized DNA can vary between batches.

It is important to consider the following two points when conducting experiments with quartz cuvettes. Firstly, some proteins are prone to bind quartz cuvettes nonspecifically. To counter this, BSA and polyoxyethylenesorbitan monolaurate are included in the reaction buffers. Secondly, temperature has a drastic effect on the reaction speed and fluorescence intensity. To minimize this effect, the quartz cuvette should be pre-incubated at 37 °C prior to use.

Although conventional biochemical assays have been hugely useful in studying DNA strand exchange, they have several drawbacks. In a typical time-course experiment, a reaction is incubated at a certain temperature and aliquots are withdrawn at desired timepoints and deproteinized by treatment with detergent and protease to terminate the reaction. Upon completion of the time-course, samples are then subjected to electrophoresis to separate DNA substrates from products. The major advantage of the method described here is that it allows for the real-time observation of the reaction without any disturbance. Any timepoint during the reaction can be inspected without disruption to the reaction itself and there is no need to deproteinize samples or subject them to the potentially disruptive forces of electrophoresis. This is especially relevant when monitoring labile DNA structures.

Despite these strengths over conventional assays, the method described here does have some disadvantages. While the use of oligonucleotide DNA substrates for strand exchange simplifies interpretation of the results, it is important to remember that such substrates do not resemble the DNA substrates involved in HR in the cell. Some conventional assays utilize plasmid-sized DNA substrates, which are more likely to reflect the number of base-pairs that are exchanged *in vivo*. Furthermore, the use of topologically constrained circular dsDNA substrates in a subset of conventional assays can at least partially recreate the tensions in physiological DNA.

The application of the method described here has started to unravel the molecular mechanisms of Rad51-driven DNA strand exchange. Nonetheless, there are many interesting questions that remain to be answered. There is clear evidence that HR during meiosis requires both Rad51 and Dmc1, the meiosis-specific RecA-type recombinase in eukaryotes[24]. However, the lack of major biochemical differences between these two recombinases has baffled researchers in the field for years. Moreover, the roles of numerous distinct groups of recombination accessory factors has been a focal topic of research in the field of HR. In addition to elucidating the biochemical differences between Rad51 and Dmc1, we aim to investigate and compare the effects of different recombination accessory factors on the kinetics of DNA strand exchange in the immediate future. Finally, it is important to stress that the FRET-based methodology described here is not limited to the study of DNA strand exchange. With relatively minor modifications, we envision many kinds of applications for this technique in investigating functionally diverse proteins involved in DNA metabolism[25-28]. We hope the developments described here will provide further options to researchers belonging to many different disciplines.

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DISCLOSURES:

The authors have nothing to disclose.

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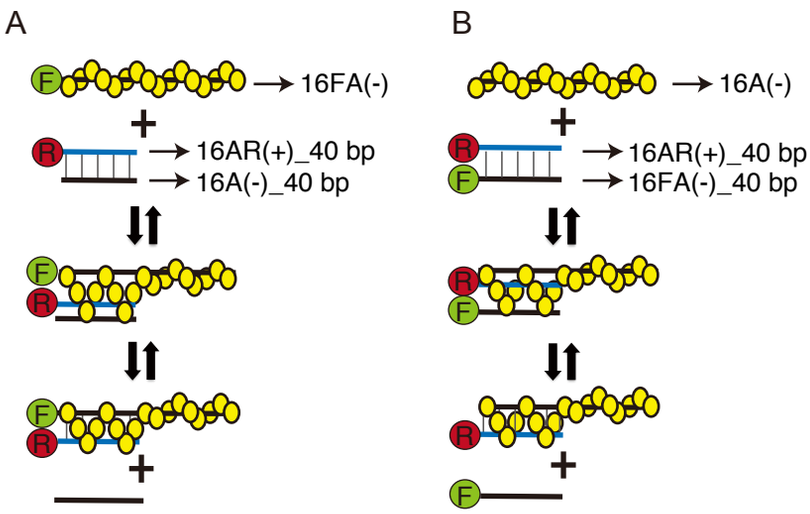

Fig. 1. Ito et al.

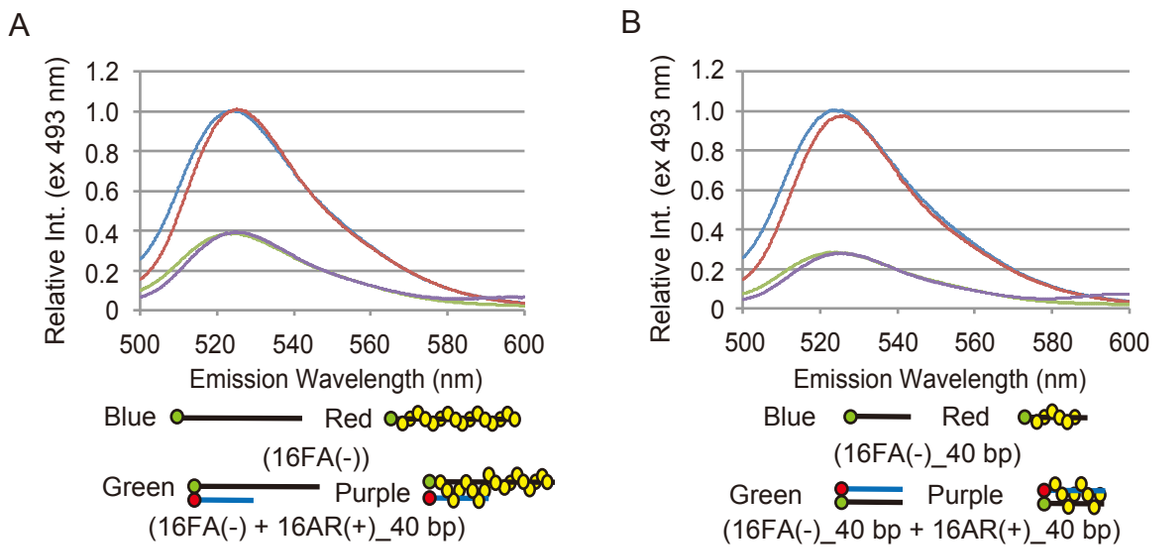

Fig. 2. Ito et al.

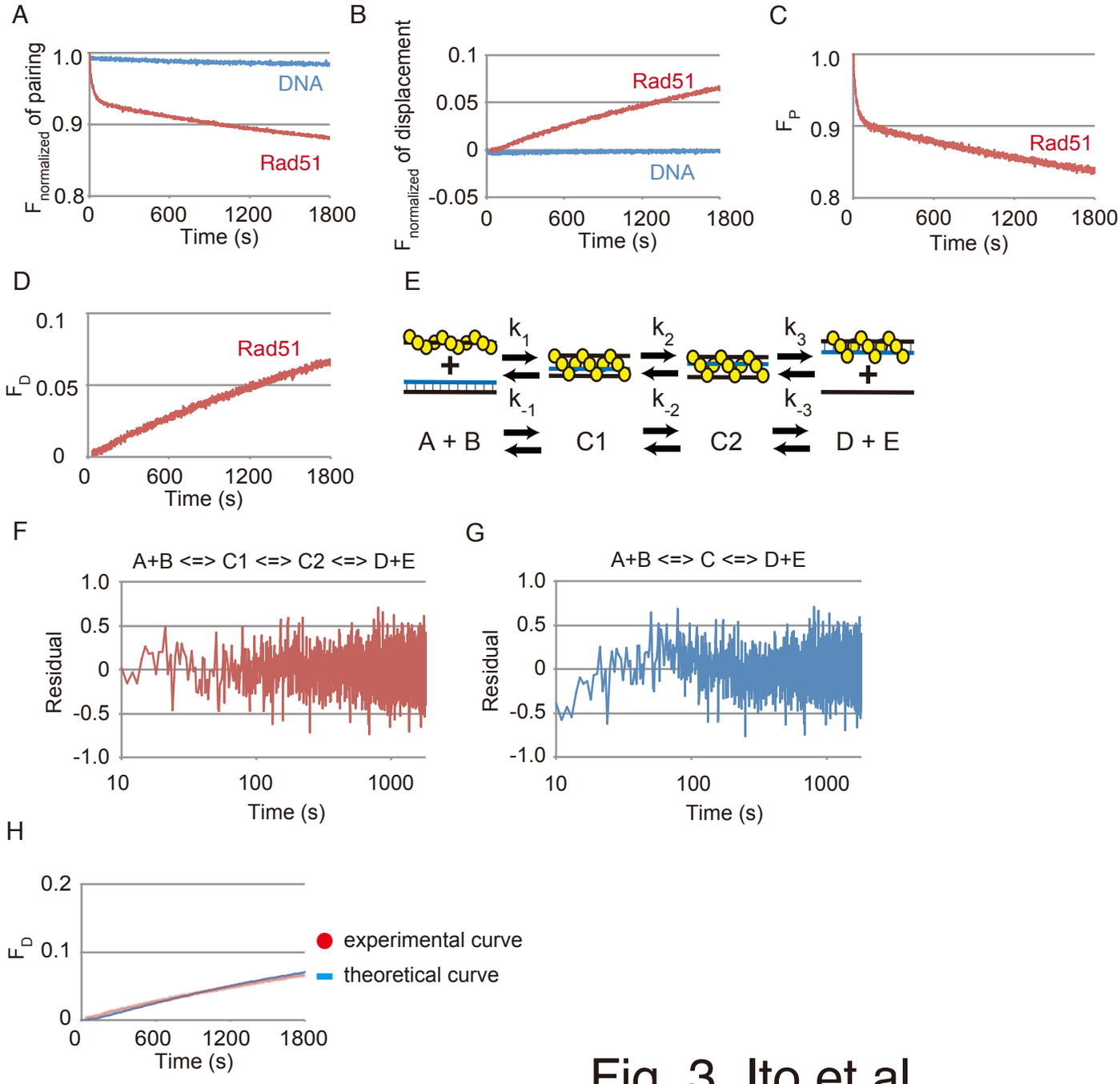

Fig. 3. Ito et al.

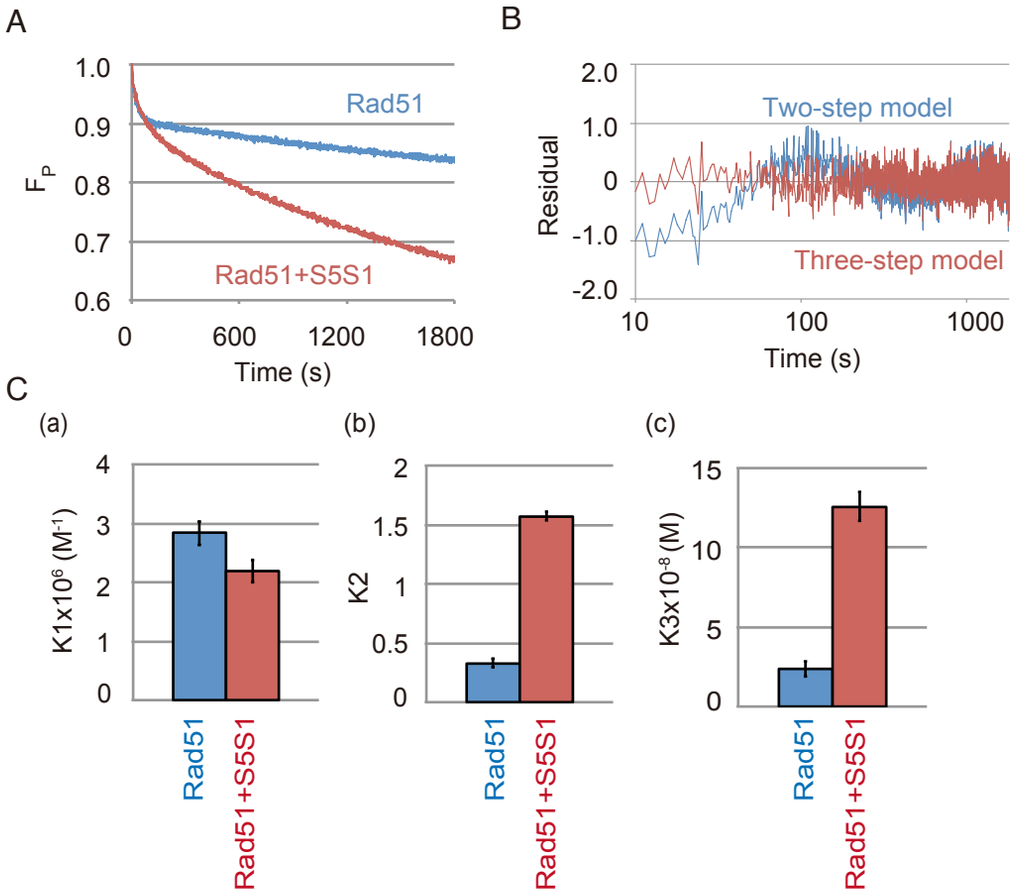

Fig. 4. Ito et al.

Table

Oligonucleotides for DNA strand pairing assay	
16FA(-)	5’-[FAM]-AAATGAACATAAAGTAAATAAGTATAAGGATAATACAAAATAAGTA
16A(-)_40bp	5’-AAATGAACATAAAGTAAATAAGTATAAGGATAATACAAAA-3’
16AR(+)_40bp	5’-TTTTGTATTATCCTTATACTTATTTACTTTATGTTCATTT-[ROX]-3’
Oligonucleotides for DNA strand displacement assay	
16A(-)	5’-AAATGAACATAAAGTAAATAAGTATAAGGATAATACAAAATAAGTAAATGA
16FA(-)_40bp	5’-[FAM]-AAATGAACATAAAGTAAATAAGTATAAGGATAATACAAAA-3’
16AR(+)_40bp	5’-TTTTGTATTATCCTTATACTTATTTACTTTATGTTCATTT-[ROX]-3’

AATGAATAAACATAGAAAATAAAGTAAAGGATAT AAA -3’

ATAAACATAGAAAATAAAGTAAAGGATAT AAA -3’

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2 x 1.0 cm quartz cuvette	Hellma Analytics	105-250-15-40	
1.0 x 1.0 cm quartz cuvette	Hellma Analytics	101-10-40	
adenosine triphosphate (ATP)	Sigma	A2383	
DynaFit	BioKin, Ldt.		DynaFit is a program to analyze kinetics of biochemical re
Fluorescent labeled and non-labeled oligonucleotides	Eurofins Genomics		The sequences of oligos are listed in Table. 1.
Magnetic stirrer	Aisis (Japan)	CM1609	
PCR machine	TAKARA (Japan)	TP600	TAKARA PCR Thermal Cycler Dice
Spectrofluorometer	JASCO	FP8300	Contains a peltier temperature controller and magnetic st
Syringe	HAMILTON	1702RN 25ul SYR (22s/2"/2)	

actions.

irrer system

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Author(s):	Kentaro Ito, Bilge Argunhan, Hideo Tsubouchi and Hiroshi Iwasaki

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CORRESPONDING AUTHOR

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Institution:	Tokyo Institute of Technology
Title:	Professor
Signature:	H. Iwasaki
Date:	Sep. 7, 2018

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Editor's and Reviewers' comments are in black font. Our responses are written in blue font.

Before addressing the points raised by the editor, we would just like to highlight a discrepancy in the line numbering. We were instructed to make all revisions to the Word document that was returned to us with the track changes feature. While making these revisions, we noticed an inconsistency in the line numbering. For example, the last line of page 2 is 74 but the first line of page 3 is 82; there are several incidents of this glitch in line numbering. We eventually realized that this inconsistency was caused by the track changes feature. However, it is our understanding that these line numbers are primarily required to facilitate the editorial process, so such an inconsistency may not be important. Thus, we have continued using the track changes feature despite this disturbance in line numbering, as was requested by the JoVE editor. We hope this does not cause any problems.

We have also updated the reference list to include an additional paper about Swi5-Sfr1, which was just published in PNAS (DOI:10.1073/pnas.1812753115). This paper is now reference 16.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been subjected to extensive proofreading and any errors that were found have been corrected.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Having contacted the publishers of our previous publication, we have confirmed that we have permission to reuse any figures from that publication. This information will be uploaded to the Editorial Manager.

3. Figure 2: What does mer represent? Please insert a space between “40” and “mer” (i.e., 40 mer).
In the context of oligonucleotides, “mer” has the same meaning as “base” and describes the number of nucleotides in an oligonucleotide (i.e., a 40 mer is an oligonucleotide composed of 40 nucleotides). Although this is standard terminology in the field of molecular biology, we have changed the designation of these oligonucleotides to avoid confusion.

4. Please rephrase the Long Abstract to more clearly state the goal of the protocol.
The goal of this protocol is to allow researchers in the field who are working with proteins from model organisms other than fission yeast to replicate our experiments so that we may learn about the similarities and differences in homologous recombination between evolutionarily diverged species. A description of this has been added to the Long Abstract (lines 57-60).

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
We have changed all units to SI abbreviations.

6. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.
We have included spaces between all numerical values and their corresponding units.

7. Please rephrase the Introduction to include a clear statement of the overall goal of this method.
A paragraph describing the overall goal(s) of this method has been incorporated into the introduction (lines 116-122).

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We checked the manuscript and minimized the use of personal pronouns.

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
We have rewritten several points of the protocol in the imperative tense. Where this was not possible, the descriptions have been added as a note (e.g., lines 150-153).

10. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
We revised the NOTE sections described below. Line numbers in parenthesis refer to the revised document, whereas those not in parenthesis refer to the original document that was returned to us from JoVE with line numbers.
Lines 133-134 were moved to the discussion section (lines 664-665).
Lines 145-146 were moved to the discussion section (lines 665-667).
Lines 147-149 were merged with section 2.1.4. (lines 183-222).
Lines 167-179 were merged into representative results section (line 443-465), because the content was a better match for that section.
Two NOTE sections (lines 150-153 and 160-162) were created to clearly explain which oligonucleotides and combinations of oligonucleotides are necessary for the pairing or displacement assay (as was requested in point 1 of Reviewer 3).

11. 1.2: Please describe how to synthesize oligonucleotide.
Oligonucleotides were purchased from a company. Double-stranded oligonucleotides were prepared by annealing, as described in sections 1.3 and 1.4.

12. 1.3: What volume of annealing buffer is used?

The scale of the annealing reaction is dependent on the number of samples. From our experience, greater than 20 µL is better because manipulating very small volumes of liquid can increase the error margins.

13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Please see our response to point 15 below.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please note that calculations steps (3.1.7, 3.2, 3.3 and their substeps) are not appropriate for filming.

Please see our response to point 15 below.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have highlighted section 2 (and all subsections) in yellow as the essential steps for the video. This is the section of the protocol where the strand exchange reaction is conducted and monitored in real-time. We would like to note that the viewers of the video will not be able to distinguish section 2.2. (displacement assay) from section 2.1. (pairing assay) due to the nature of the experiment. Thus, although we have highlighted both sections 2.1. and 2.2., the inclusion of section 2.2. in the video will likely be overly repetitive.

16. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

These items have been sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This study of Rad51 DNA strand exchange is written very clearly. The protocols are straightforward and should be easy to duplicate. The analysis if fluorescence data is very clearly written. I can find very little wrong with this study and recommend publication.

We thank this reviewer for their encouraging words and are pleased they found our protocols easy to understand.

Reviewer #2:

Manuscript Summary:

Homologous recombination (HR) is important for cells to repair DNA double-strand breaks (DSB) and to restart stalled/collapsed replication forks during replication stress. Moreover, the physical connection of homologous chromosomes through HR during meiosis I is a prerequisite for a proper chromosome segregation and generation of genetic diversity. RAD51 recombinase is the main player to trigger recombination reaction. RAD51 forms a nucleoprotein filament onto ssDNA and then engages duplex DNA for searching homology and subsequently DNA exchange. This RAD51-driven DNA strand exchange process has been well characterized by biochemical and biophysical analyses. However, the kinetics and reaction intermediates of this reaction remain largely unknown due to the lack of advanced methodology. Previously, Iwasaki's team has successfully established the fluorescence resonance energy transfer (FRET)-based DNA strand pairing and displacement assays to monitor this process in real-time. Importantly, his work clearly demonstrated that Rad51-driven DNA strand exchange is a three-step reaction mechanism. In this manuscript, the authors described a step-by-step protocol to perform this FRET-based system. This system now opens the door for scientists to address previously uncharacterized questions regarding how Rad51 accessory factors regulate Rad51-driven three-step reaction mechanistically. In conclusion, the detailed protocol of describing FRET-based real-time assay is an urgent need in this field and the authors nicely presented this methodology in the current

manuscript. Inclusion/explanation of below suggestions/questions may strengthen this manuscript.

We thank this reviewer for their constructive comments on our manuscript and agree with their assertion that the techniques described here will be useful for the study of Rad51 accessory factors.

Major Concerns:

1. Lines 128-137, why we need an excess amount of Rad51 (1.5 uM) for ssDNA (36 nM) if the ratio of protein and ssDNA nucleotide is 1:3. Or "36 nM" represents the number of whole ssDNA molecule. Please make this clear in the manuscript for the readers.

In this manuscript, DNA concentration means fragment concentration. We revised this section to explain clearly the definition of DNA concentration (lines 152-153).

Minor Concerns:

1. Protocol 1.2 (Lines 110-) the authors should consider organizing all the oligonucleotide information into a Table for readers to follow easily.

The oligonucleotides were renamed to improve clarity and the relevant information was listed in Table 1.

2. Protocol 1.2 (Lines 128-), is that correct of using the high concentration of $MgCl_2$ (15 mM) and no salts (such as NaCl and KCl) are required for the reaction?

This is correct. Salts such as KCl and NaCl have an inhibitory effect on the DNA strand exchange reactions employed here. In contrast, a high concentration of $MgCl_2$ is important for forming active and stable Rad51-ssDNA filaments in these assays.

Reviewer #3:

Manuscript Summary:

Ito and colleagues have presented a FRET-based protocol that assesses Rad51-mediated DNA strand exchange, which allows measurement of formation of the three-strand intermediate, maturation of the intermediate and release of the ssDNA

from the intermediate. This is a useful protocol and will be of interest to researchers in the field of DNA repair and protein dynamics.

We are grateful to this reviewer for their detailed comments and suggestions.

Major Concerns:

1. Section 1.2 - The oligos should be listed as a table. Specifying clearly what assay each is for.

The oligos have now been listed in a table (Table 1). Furthermore, to better convey which oligonucleotides are necessary for the pairing and displacement assays, we annotated Figures 1 and 2 with the names of the oligonucleotides. We hope this will avoid all ambiguity regarding which oligonucleotides are used in each assay.

2. Section 1.3 - Specify what temperature this step is done

Mixing of the complementary strands for annealing donor dsDNA is conducted on a prechilled metal rack on ice. Typically, the temperature of the metal rack is between 2 ˚C and 4 ˚C. This has now been clearly stated (lines 157-158).

3. Section 2.1 - Specify what volume and type of plastic tube used

A 2.0 ml micro-centrifuge plastic tube (polypropylene) is used. This has now been clearly stated (lines 173-174).

4. Note in Section 2.7 - I think it would be better to have a separate section for the displacement assay. Within this section state to follow the relevant steps and go through the procedure accordingly. I think it will be easier to follow as a protocol this way.

We made a new section for the displacement assay (section 2.2., lines 232-245). However, we have written this section with partial reference to the corresponding section for the pairing assay (e.g., section 2.2.2., “Form Rad51-ssDNA filaments in the presence of Swi5-Sfr1 at 37˚C, as described in sections 2.1.2. and 2.1.3.”). This was done to avoid giving the impression that the preparation of Rad51-ssDNA filaments etc are different in each assay. We would like to stress that these early steps are identical in both assays, with the only difference being in the DNA substrates employed (as stated clearly in 2.2.1.).

5. Section 3.1.2 - Table FMA/ROX labelled oligos to make it easy to follow depending on assay.

All the oligonucleotide information has now been listed in Table 1. In addition, we have included a clear description of what assay each oligonucleotide is for in a NOTE in section 1.2. (lines 150-153).

6. Section 3.3.3 - Reference to "the program" and to "see the Table of Materials". However, it is not clear what they are referring to in this table. This "program" is central to the protocol, and the authors need to demonstrate a step-by-step guide on how to use the program preferably with figures.

We apologize for this shortcoming in our original manuscript. We have now created new sub sections in section 3.3.3 to explain the data analysis using the program (lines 409-439).

7. Line 266 - do they mean to refer to D+E instead of C2 in the last bracket of the sentence?

Thank you. We changed it.

Minor Concerns:

1. Abstract, line 47 - the "F" in Fluorescence should not be capitalized.

Thank you. We changed it.

2. Introduction, line 63 - put a full-stop after "chromosome" and start a new sentence from "Incorrect"

Thank you. We changed it.

3. Make Figure 3F-a and b into separate figures 3F and 3G.

Thank you. We changed it.

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Adrianna Linbo-Terhaar
Senior Editorial Assistant, NSMB

```
[task]
data = progress
task = fit
[mechanism]
 A + B <===> C : k1 k2
C <===> D : k3 k4
D <===> E + F : k5 k6
[constants]
k1 = 0.000379 ?
k2 = 0.109 ?
k3 = 0.0126 ?
k4 = 0.00554 ?
k5 = 0.00132 ?
k6 = 0.0000972 ?
[concentrations]
A = 36
B = 36
[responses]
A = 0.0278
[data]
(Write a directory of input data here)
[output]
(Write a directory of output data here)
[end]
```