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 MgCl2 (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 MgCl2 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.