J Fluoresc (2013) 23:635–640
DOI 10.1007/s10895-013-1210-1

ORIGINAL PAPER

# Direct Observation of Fluorescently Labeled Single-stranded λDNA Molecules in a Micro-Flow Channel

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Received: 2 November 2012 / Accepted: 24 February 2013 / Published online: 8 March 2013


**Abstract** We developed two labeling methods for the direct observation of single-stranded DNA (ssDNA), using a ssDNA binding protein and a ssDNA recognition peptide. The first approach involved protein fusion between the 70-kDa ssDNA-binding domain of replication protein A and enhanced yellow fluorescent protein (RPA-YFP). The second method used the ssDNA binding peptide of *Escherichia coli* RecA labeled with Atto488 (ssBP-488; Atto488-IRMKIGVMFGNPETTTGGNALKFY). The labeled ssλDNA molecules were visualized over time in micro-flow channels. We report substantially different dynamics between these two labeling methods. When ssλDNA molecules were labeled with RPA-YFP, terminally bound fusion proteins were sheared from the free ends of the ssλDNA molecules unless 25-mer oligonucleotides were annealed to the free ends. RPA-YFP-ssλDNA complexes were dissociated by the addition of 0.2 M NaCl, although complex reassembly was possible with injection of additional RPA-YFP. In contrast to the flexible dynamics of RPA-YFP-ssλDNA complexes, the ssBP-488-ssλDNA complexes behaved as rigid rods and were not dissociated even in 2 M NaCl.

**Keywords** Single molecule · Single-stranded DNA · Single-stranded DNA recognition peptide · Replication protein A · Micro-flow channel

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## Introduction

Millions of molecules have been submitted to conventional molecular biological analysis methods, such as electrophoresis and the incorporation of radioisotope-labeled nucleotides. Such techniques report the average behaviors of a large number of molecules. The real behaviors of individual biomolecules and the elementary processes of the reactions cannot be elucidated by conventional biological analysis. Therefore, the steps involved in DNA metabolic reactions, such as binding rates and interactions with single-stranded binding proteins, remain unknown. Techniques that involve single-molecule resolution, such as the visualization of fluorescently labeled DNA and/or protein molecules under fluorescence microscopy, permit observations of individual molecular behavior and reveal elementary reaction processes and phenomena. For example, the sliding motion of fluorescently labeled RNA polymerase on dsDNA has been observed directly [1, 2], dsDNA hydrolysis by RecBCD helicase has been analyzed using a direct single-molecule fluorescence imaging system [3], and exonuclease III activity was revealed by controlling DNA form or tension [4–6]. Moreover, single-molecule measurements have dissected the interactions among RecBCD, Rad54, and Tid1 proteins and various DNA states (e.g., duplex DNA unwinding, DNA degradation, DNA packaging) [7]. These studies have

also described the accumulation of fluorescently labeled ssDNA-binding proteins on the ssDNA product of DNA helicase [8] and have elucidated as to how Rad51 filament formation initiates on dsDNA at multiple nucleation points [9]. Direct molecular observation techniques have been most effective in analyzing the mechanisms of DNA-protein interactions involved in DNA metabolic reactions. These methods are also being used to clarify the specific molecular behaviors involved in DNA replication, DNA repair, DNA recombination, and transcription.

Micro-flow channels are effective for the dynamic analysis of DNA and/or protein behavior at single-molecule resolution. Direct observation of single molecules using fluorescence microscopy has also been successful in studies on DNA behavior and DNA-protein interactions [10, 11]. However, random-coiled DNA in aqueous solution fluctuates in shape due to Brownian motion, and it is difficult to analyze such DNA along its length. To date, several DNA stretching techniques have been developed to measure DNA length stably. These approaches facilitate the analysis of DNA-enzyme interactions (e.g., DNA binding proteins) because DNA form can be regulated by controlling buffer flow. In our previous study, ssλDNA molecules were visualized directly using a fusion protein consisting of the 70-kDa DNA-binding domain of replication protein A and enhanced yellow fluorescent protein (RPA-YFP) [12]. However, RPA-YFP was limited by large molecular size of the fluorescent component and by low solubility of the fusion protein. We subsequently designed a chemically labeled fluorescent peptide that recognizes ssDNA. Sugimoto reported that the 24-residue peptide, IRMKIGVMFGNPETTTGGNALKFY, derived from *Escherichia coli* RecA could recognize ssDNA using BIAcore [13]. We developed a direct observation method for ssDNA using this peptide. In the present study, we characterize this labeling method by observing the behaviors of ssDNA molecules in micro-flow channels.

## Materials and Methods

### Proteins and Chemical Reagents

RPA-YFP was prepared using the pET32a-eYFP vector and Rosetta (DE3), as described in our previous report [12]. The fluorescently labeled ssDNA-recognizing peptide, Atto488-IRMKIGVMFGNPETTTGGNALKFY (ssBP-488), was obtained from Sigma Aldrich (St. Louis, MO, USA). Water was purified using a Millipore Milli-Q Water System (Billerica, MA, USA). Other reagents used in this study were analytical grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma Aldrich.

### Fabrication of the Micro-flow Cell for Single-molecule Observation

Single-molecule experiments were performed in micro-flow channels prepared from polydimethylsiloxane (PDMS)-sealed coverslips. A schematic of the micro-flow channel is given in Fig. 1. Each coverslip was treated with dichlorodimethylsilane for one-end immobilization of individual thiol-modified ssλDNA molecules [4–6]. PDMS was prepared as described previously [4–6]. Small holes (3 mm in diameter) were drilled into the PDMS using a borer to produce an inlet and an outlet. The inlet port (NanoPort N-124S; Upchurch Scientific, Oak Harbor, WA, USA) was bond to the inlet hole using silicone adhesive (Shin-Etsu Chemical, Tokyo, Japan). PEEK tubing (I.D. 100 μm, O.D. 360 μm, 1571-12X, Upchurch Scientific) was used to connect the inlet port to a 250-μl syringe (Hamilton, Reno, NV, USA), which was controlled by a syringe pump (KD Scientific, Holliston, MA, USA).

### Thiol-modification of λDNA for Single-molecule Observation

Thiol-modification of λDNA molecules was conducted as described in our previous report [4–6]. In brief, 0.55 pmol of λDNA (48,502 bp; Nippon Gene, Tokyo, Japan); 0.4 nmol of 27-mer oligonucleotides modified with phosphate groups and disulfide chains at the 5′-ends and 3′-ends, respectively (5′P-GGG CGG CGA CCT AGA TAG GAC ACT ACG 3′-$(CH_2)_3$-S-S-$(CH_2)_3$-OH); and 0.4 nmol of 15-mer oligonucleotides modified with disulfide chains at the 5′-ends (OH-$(CH_2)_3$-S-S-$(CH_2)_3$-5′ CGT AGT GTC CTA TCT 3′) (Sigma Aldrich), were combined in ligation buffer [66 mM Tris–HCl (pH7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.1 mM ATP]. The solution was incubated at 65 °C for 5 min and was cooled rapidly on ice to prevent linking among λDNA molecules. One recessed end of the λDNA was linked specifically to the two oligo-DNAs by overnight incubation at 16 °C in ligation buffer containing 13.6 U/μl (final concentration) of T4 DNA ligase (Takara, Shiga, Japan). The DNA solution then was incubated for 5 min at 65 °C to inactivate the T4 DNA ligase. The sample was purified through a gel filtration column (MicroSpin Columns; GE Healthcare, Little Chalfont, Buckinghamshire, England) to remove free oligo DNA. The disulfided λDNA was reduced with 40 mM DTT in 0.17 M sodium phosphate buffer (pH8.0) for 16 h at 30 °C to convert the disulfide bonds into sulfhydryl groups. Finally, the DNA sample was purified by removing DTT using gel filtration (NAP-5 Column; GE Healthcare) in a column equilibrated with TE buffer [10 mM Tris–HCl (pH8.0), 1 mM EDTA]. The concentration of the thiol-modified λDNA solution was determined by electrophoresing the sample in parallel with λDNA of known concentration. The thiol-modified λDNA sample was stored at −20 °C.

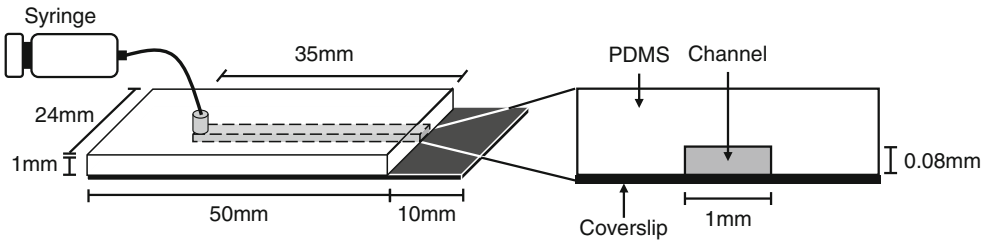


**Fig. 1** Overview of the micro-flow cell used in this study. The micro-flow cell was prepared from poly(dimethylsiloxane) (PDMS)-sealed coverslip that was treated with dichlorodimethylsilane for one-end immobilization of thiol-modified ssλDNA. The inlet port was bond to the inlet hole using silicone adhesive. PEEK tubing (I.D. 100 μm, O.D. 360 μm) was used for connecting the inlet port to a 250-μl syringe that was controlled by a syringe pump

### Preparation of ssλDNA and Oligonucleotide-annealed ssλDNA

Two types of the thiol-modified ssλDNA molecules were prepared. One was prepared without oligonucleotide and the other was annealed with 25-mer oligonucleotides on the 3′ terminal end. The 25-mer oligonucleotide sequence was 5′-CGT AAC CTG TCG GAT CAC CGG AAA G-3′ (Japan Bio Services; Saitama, Japan). The reaction mixtures contained 1× TE, 1× rTaq Buffer [10 mM Tris–HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$; Takara], and 4 fmol of thiol-modified λDNA with/without 100 pmol of 25-mer oligonucleotide. The heat denaturation program was 94 °C for 6 min, 58 °C for 1 min, and cooling to 4 °C.

### Optical Setup and Analysis

DNA molecules were observed under a fluorescence microscope (Eclipse TE2000U; Nikon, Tokyo, Japan) equipped with a 100×, 1.4 NA oil immersion objective lens (Plan Apo; Nikon). The excitation and emission wavelengths were selected using filter set B-2A (Blue excitation light, EX470-40, DM505, and EM520; Nikon). Fluorescent images of ssλDNAs stained with YFP (excitation 513 nm, emission 527 nm) and Atto488 (excitation 501 nm, emission 523 nm) were visualized using a high-sensitivity Watec Monochrome CCD camera (WAT-120 N+; Watec, Yamagata, Japan) and were recorded with an mAgic TV5 video capture board (I-O DATA, Ishikawa, Japan). The lengths of individual ssλDNA molecules were measured using the image processing software, ImageJ. All experiments were controlled at 37 °C using an Imaging ThermoPlate (MATS-U502RA26, Tokai Hit).

### Visualization of Single ssλDNA Molecules in Micro-flow Channels

Micro-flow channel inlets were connected to a syringe containing common buffer [25 mM HEPES (pH8.0), 1 mM EDTA, 10 % glycerol, 0.1 % Tween20, 2.5 % 2-mercaptoethanol] that dispensed buffer solution through a syringe pump. In the following experiments, the flow rate was adjusted to 25 μl/h unless otherwise stated. The experimental procedure used in the analysis of single-molecule ssλDNA behavior was with/without 25-mer oligonucleotides. Thiol-modified ssλDNA molecules were introduced into the micro-flow channel for 25 min. The ends of the thiol-modified ssλDNA molecules were immobilized on the glass surface during this period. Common buffer containing 1 % bovine serum albumin (BSA) then was injected into the micro-flow channel for 20 min to block the glass substrate, and free ssλDNA molecules were removed by washing. Aliquots of common buffer (200 μl) containing 13 pmol ssBP-488 or 0.5 nmol RPA-YFP were injected to the micro-flow channel for 30 min. During this period, RPA-YFP molecules bound to the ssλDNA molecules. Common buffer then was injected into the micro-flow channel to remove excess fluorescence due to ssBP-488/RPA-YFP so that signals from free fluorescent molecules did not interfere with observation of the complexes. Common buffer was injected continuously into the micro-flow channel for 45 min during observation of ssλDNA under a single microscopic field. Images of the ssλDNA molecules were captured every 5 min for 30 s each.

### Dissociation of Fluorescent Label from ssλDNA

The ssλDNA molecules annealed with the 25-mer oligonucleotides were visualized using ssBP-488/RPA-YFP as described in the previous section. To dissociate ssλDNA molecules from the fluorescent proteins, NaCl was added to the common buffer and was increased stepwise with time, as follows: 0.1, 0.2, 0.5, 1, and 2 M. Images of the fluorescently labeled ssλDNA molecules were captured at 1-min intervals by opening the shutter and exposing samples to the excitation light. To examine complex reassembly, buffer containing the fluorescent proteins (ssBP-488/RPA-YFP) was injected into the micro-flow channel for 30 min. The micro-flow channel then was washed with common buffer for 30 min to remove excess fluorescence. At this time, we also observed re-complex formation between the fluorescent proteins and ssλDNA molecules prepared by stripping the fluorescent proteins from the fluorescent protein-ssλDNA

complex. During the incubation and washing out, and the fluorescent protein-ssλDNA re-complex was held on the same location in the microscope field. The fluorescent-labeled ssλDNA re-complex was observed and captured by opening the shutter and exposing samples to the excitation light for 2-min durations at 3-min intervals. For the direct visualization of ssBP-488-ssλDNA and RPA-YFP-ssλDNA behaviors in the absence of buffer flow, capture of the fluorescent images started after 15 min from termination of flow.

### Other Methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed through 10 % gels by the standard Laemmli method. Following electrophoresis, gels were stained using Coomassie Brilliant Blue R-250 (Wako Pure Chemical Industries) or an EzStain Silver Kit (Atto, Tokyo, Japan). Protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA, USA) with BSA as the standard.

## Results and Discussion

### Molecular Behaviors of ssλDNA with/without Annealed Oligonucleotides Complexed with ssBP-488 or RPA-YFP

The ssλDNA were prepared by the heat denaturation method and were visualized using the two methods (ssBP-488 or RPA-YFP) in micro-flow channels (Fig. 2). The complexes of ssBP-488-ssλDNA behaved differently from those of RPA-YFP-ssλDNA under identical flow conditions. As a result of ssλDNA visualization using ssBP-488, ssBP-488-ssλDNA complexes were stably observed for 45 min (Fig. 2a). The ssBP-488 molecules were tightly associated with ssλDNA, and the molecules of ssBP-488-ssλDNA complexes behaved as thick, rigid rods in the flowing buffer. During buffer flow, the ssBP-488-ssλDNA complexes were maintained in the fully stretched form in the flow, and those stretched length were kept about 15 μm. These results suggested that ssBP-488-ssλDNA complexes were sufficiently stable to maintain the full-length stretched form of the complex in the flow and facilitated measurement of full-length of ssλDNA.

While visualizing ssλDNA using RPA-YFP, RPA-YFP-ssλDNA complexes also were observed using fluorescence microscopy, but the behaviors of RPA-YFP-ssλDNA molecules differed substantially from those of ssBP-488-ssλDNA complexes. When buffer without RPA-YFP was injected, the fluorescent regions of the stretched RPA-YFP-ssλDNA complexes reached 15 μm, which is comparable to full-length ssλDNA molecules (~16 μm). As time progressed, the fluorescent regions of the RPA-YFP-ssλDNA complexes shortened gradually to 4 μm from the free end of ssλDNA molecules (Fig. 2b). These results suggest that RPA-YFP molecules bound cooperatively to ssλDNA via the RPA domains, formed clusters on the DNA molecules and were released from the termini of the clusters. The 4-μm fluorescent regions remained even 40 min after the initiation of flow. In this micro-flow channel, the Reynolds number was quite low; therefore, the flow was a laminar flow where the local flow velocity gradually increases with distance from the wall of the micro-flow channel. As a result, local flow velocity at the free end might decrease with decreasing length of RPA-YFP-ssλDNA. These results suggested that the terminal RPA-YFP complex was released from the cluster of RPA by hydraulic force when the local flow velocity exceeded a critical value.

Furthermore, to investigate effects of annealed oligonucleotides at the 3′-terminal, the ssλDNA molecules with the 25-mer oligonucleotide were prepared by annealing the 25-mer oligonucleotide at the free end of ssλDNA and fluorescently visualized using ssBP-488 or RPA-YFP in the micro-flow channel. As a result of ssBP-488 molecules, no obvious change was observed even though the oligonucleotides were annealed at the 3′-terminal (data not shown). However, the complex of RPA-YFP-oligonucleotide annealed ssλDNA showed quite different behaviors from that of RPA-YFP-ssλDNA under the same flow condition. In the case of the complexes of RPA-YFP-oligonucleotide annealed ssλDNA, the complexes were fully stretched in the flow, and those stretched lengths were kept at approximately 15 μm for 45 min (Fig. 2c). These results showed that RPA-YFP-oligonucleotide annealed ssλDNA complexes were sufficiently stable to maintain the fully stretched form of the complex in the flow. Different behaviors of the complexes between ssBP-488/RPA-YFP and the ssλDNA molecules annealed with the 25-mer oligonucleotide in the flowing buffer containing NaCl.

Next, the behaviors of RPA-YFP-oligonucleotide annealed ssλDNA complexes and ssBP-488-oligonucleotide annealed ssλDNA complexes were observed in the flowing buffer. The ssλDNA molecules annealed with the 25-mer oligonucleotide were sequentially treated with the buffer containing different concentration (0.1, 0.2, 0.5, 1 and 2 M) of NaCl in ascending order (Fig. 3). After the visualizing ssλDNA by using ssBP-488, ssBP-488-oligonucleotide annealed ssλDNA complexes were stably visualized in the buffer flows containing any concentrations of NaCl, and these complexes were kept stretched lengths of approximately 15 μm (Fig. 3a). After buffer flow was stopped, the stretched ssBP-488-oligonucleotide-annealed ssλDNA complexes retained rod-like shapes with small transverse vibrations. RecA protein has been observed to polymerize radially to form a twist

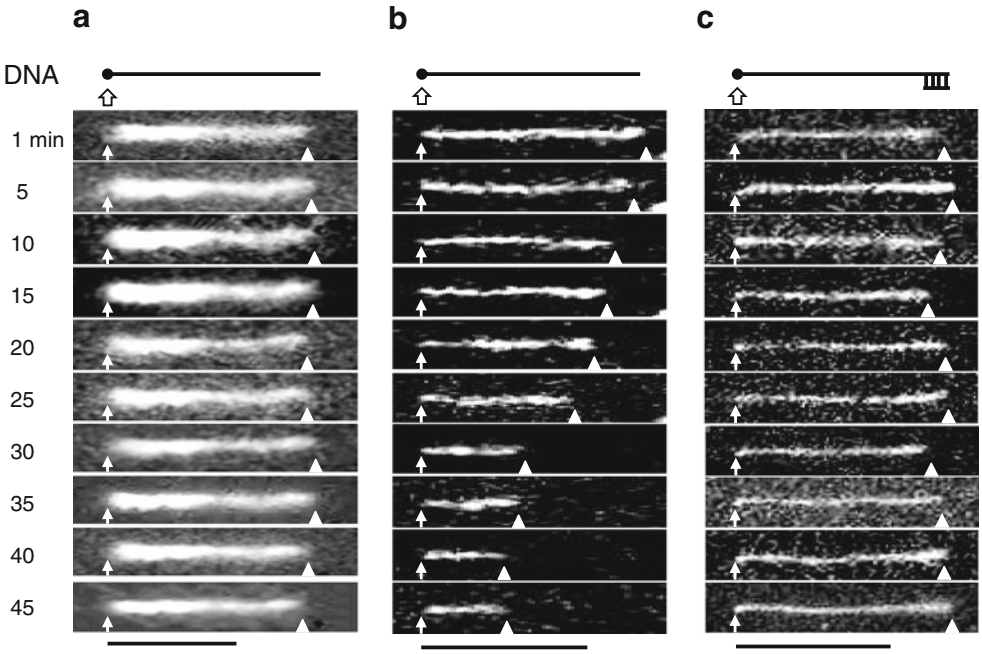


**Fig. 2** Sequential photographs of the time-course observation of the ssBP-488-ssλDNA (**a**), RPA-YFP-ssλDNA (**b**), and RPA-YFP-oligonucleotide-annealed ssλDNA (**c**) complexes during buffer flow. **a:** behaviors of ssBP-488-ssλDNA complex, **b:** behaviors of RPA-YFP-ssλDNA complex, and **c:** behaviors of RPA-YFP-oligonucleotide annealed ssλDNA complex. *White arrows and white triangles* show the positions of immobilized and free ends of stretched ssλDNAs, respectively. Scale bar=10 μm

around ssλDNA [14]. The rod-like forms we observed for ssBP-488-ssλDNA complexes might be attributed to characteristics of the cooperative binding of RecA.

The behaviors of RPA-YFP-oligonucleotide annealed ssλDNA complexes were clearly different from those of ssBP-488-oligonucleotide annealed ssλDNA in Fig. 3b. The ssλDNA molecules annealed with the 25-mer oligonucleotide were stably visualized by RPA-YFP in the flowing buffer without NaCl, and then sequentially treated with the buffer containing different concentration (0.1, 0.2, 0.5, 1 and 2 M) of NaCl in ascending order. Upon treatment with 0.2 M NaCl for 20 s, the fluorescently labeled region of RPA-YFP-ssλDNA shortened rapidly by dissociating RPA-YFP from the oligonucleotide-annealed ssλDNA molecules. This fluorescence quenching was neither due to cleavage of the ssλDNA molecules nor due to removal of ssλDNA molecules from the glass surface because the quenched region of the ssλDNA molecules annealed with the 25-mer oligonucleotides could be re-visualized by re-injecting RPA-YFP molecules (Fig. 3b). When buffer flow was stopped, the stretched RPA-YFP-oligonucleotide-annealed ssλDNA complexes contracted due to entropic force. This strongly suggests that the RPA-YFP-ssλDNA complexes were more flexible than the ssBP-488-ssλDNA complexes and that the cooperative binding of RPA-YFP to ssλDNA was much lower than that of ssBP-488.

Our results demonstrate that ssBP-488 molecules stably and tightly associate with ssλDNA with/without annealed 25-mer oligonucleotides and that ssBP-488-oligonucleotide-annealed ssλDNA complexes maintain highly stable and

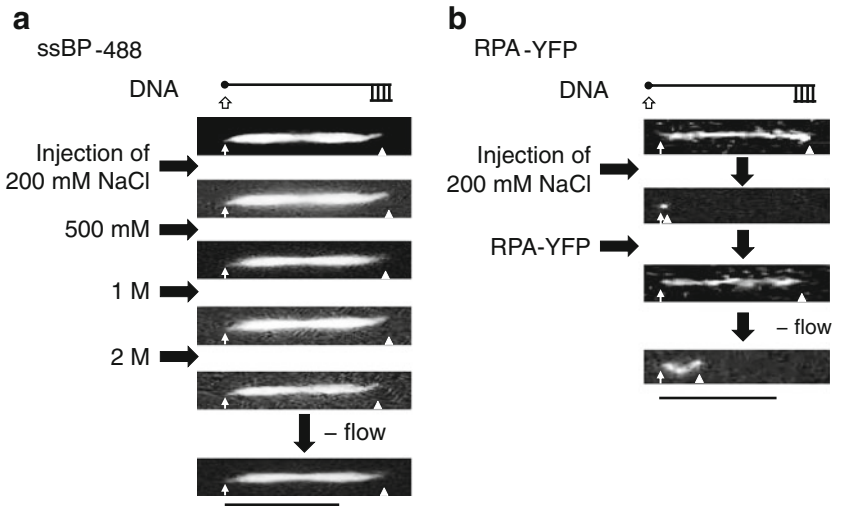


**Fig. 3** Sequential photographs of the time-course observation of the complexes between ssBP-488/RPA-YFP and ssλDNA molecules annealed with the 25-mer oligonucleotide in the flowing buffer containing each NaCl concentration ranging from 0.1 to 2 M. **a:** Behaviors of ssBP-488-oligonucleotide annealed ssλDNA complex, and **b:** behaviors of RPA-YFP-oligonucleotide annealed ssλDNA complex. *White arrows and white triangles* show the positions of immobilized and free ends of stretched ssλDNAs, respectively. Scale bar=10 μm

rigid forms under high-salt conditions. Owing to the high affinity of ssBP-488 for ssDNA, ssBP-488 can be applied to discriminate ssDNA regions from dsDNA regions. Therefore, ssBP-488 molecules are suitable for the analysis of the products of DNA metabolic reactions because this labeling method does not require demineralization to reduce salt. However, the RPA-YFP-ssλDNA complexes were much more flexible than the rigid ssBP-488-ssλDNA complexes. Flexibility is a disadvantage for measurements of ssDNA length. Of note, ssλDNA molecules were easily relabeled by the re-injection of RPA-YFP after stripping RPA-YFP from the complex. This characteristic is advantageous for real-time microscopic observation of ssDNA regions.

**Acknowledgments** This work was partially supported by the Nakatani Foundation of Electronic Measuring Technology Advancement (to S.K.). M.O. was supported by a Grant-in-Aid for Young Scientists (B: 24770164).

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