# REAL-TIME FISH USING OPTICALLY DRIVEN MICROSPHERES FUNCTIONALIZED BY THE HOMOLOGOUS RECOMBINATION PROTEIN, RecA

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## ABSTRACT

Here, we demonstrate the active probing of a specific sequence without thermal denaturation of a target double-stranded DNA by using a recombination protein, RecA. A microsphere with single-stranded DNA-RecA complexes on its surface was optically manipulated and slid onto the target DNA, and a strong interaction between the target DNA and the microspheres at the specific position of the target DNA was observed. This result shows the potential use of recombination proteins to facilitate detection of specific DNA sequences with spatial information, i.e., real-time FISH.

## KEYWORDS

DNA, FISH, Optical tweezers, RecA

## INTRODUCTION

Detecting specific sequences with spatial information along a target DNA strand is an important technique in DNA analysis because it facilitates the assembly of genomic sequences, genetic identification, and other various DNA-related applications. A well-known current method is fluorescence in situ hybridization (FISH). In FISH, a target double-stranded DNA (dsDNA) is first thermally or chemically denatured to open up the double strand and expose the bases present within. Then, a fluorescently labeled oligonucleotide probe is added and allowed to bind to its complementary site, the location of which is visualized by fluorescence microscopy. [1] However, due to the helicity of the dsDNA, the denaturation is only partial, and the probe must find its denatured target position by diffusion. In addition, immobilization of the target dsDNA on a surface can cause steric hindrance during denaturation and hybridization, whereas excessive denaturation by heating can damage the target dsDNA. [2] As a result, FISH is often a very inefficient and time-consuming process with lack of reproducibility, requiring highly trained people.

In contrast, a highly efficient DNA sequence-detection system is present in cells; this process is known as homologous recombination. A protein called RecA plays a role in repairing damaged dsDNA by using the homologous sequence in its diploid counterpart. As shown in Figure 1, RecA first binds to the enzymatically exposed single-stranded (ss) portion of the broken DNA, migrates to the counterpart dsDNA in search of the complementary position to promote recombination, and reproduces the lost portion. The process takes place at physiological temperature and is completed within 5–10 minutes. [3]

We previously proposed the use of RecA for FISH and reported a single-molecule observation of the binding of the fluorescently labeled ssDNA-RecA complex onto dsDNA that was electrostatically stretched-and-positioned onto microelectrodes. [4] However, the because of nonspecific binding, the signal-to-noise ratio (S/N) of the measurement was impractically low. In this study, we proposed a novel method of FISH using a microsphere on which the ssDNA-RecA complexes are immobilized. Here, the target dsDNA is stretched-and-fixed at both ends and suspended on the cover slip to prevent steric hindrance, which is different from conventional FISH. When the microspheres were in contact with the suspended target dsDNA and were induced to scan along it by using optical tweezers, we observed a strong interaction between specific positions of the target dsDNA and the microsphere.

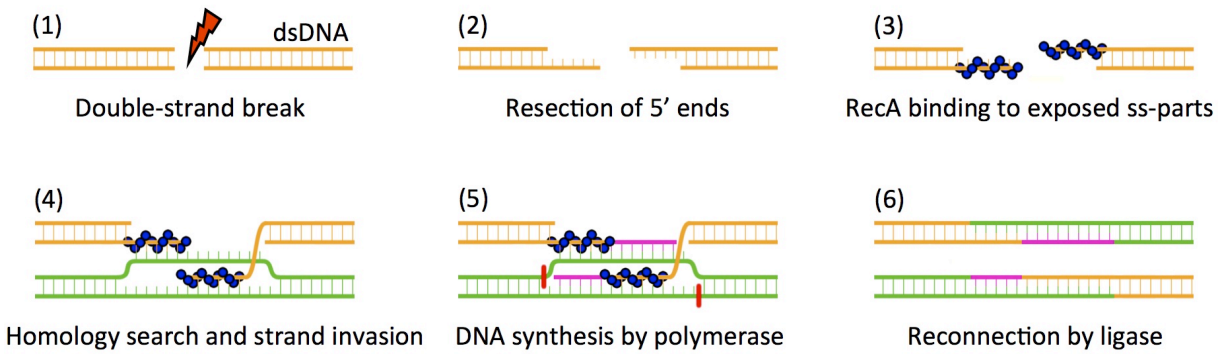


*Figure 1. Common pathway of homologous recombination*

## EXPERIMENTAL

We used 2 oligonucleotides (Japan Bio Services) as ssDNA in this study, with the following sequences:

Oligo-1: 5′-GCC TAG TGA TTT TAA ACT ATT GCT GGC AGC ATT CTT GAG TCC AAT ATA AAA GTA TTG TGT ACC TTT TGC TGG GTC AGG TT-3′ and

Oligo-2: 5′-AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AA-3′.

These oligonucleotides were 80-mers and biotinylated at the 5′-end by the supplier. Oligo-1 was designed to be complementary to λ DNA (48.5 kb, 16.5 μm) from the nucleotides 26122 to 26201. On the other hand, Oligo-2, i.e., 80-mer poly-A, was complementary to λ DNA of a maximum length of 8 nucleotides. RecA protein was purchased from New England Biolabs, and ATP was purchased from Roche. As the target dsDNA, λ DNA concatemer (several λ DNAs linked in series) was purchased from Bio-Rad, and the nucleic acid stain SYBR Gold was purchased from Invitrogen. Streptavidin-coated polystyrene microspheres (2 μm in diameter) for the probing microsphere and carboxylate polystyrene microspheres (6 μm in diameter) for immobilizing target dsDNA were purchased from Polysciences, Inc. Poly-L-lysine (PLL, $M_n$ = 300,000) was purchased from Sigma-Aldrich.

Microspheres for sequence probing (hereafter, probing microspheres) were prepared as follows: First, streptavidin-coated microspheres and biotinylated ssDNA were incubated in 20 mM HEPES-KOH (pH 7.5) at room temperature for 1 h. Then, RecA was added to form ssDNA-RecA complexes on the microspheres by incubation in a buffer containing 20 mM HEPES-KOH (pH 7.5), 1 mM ATP, 10 mM dithiothreitol, and 1 mM $MgCl_2$ at 37°C for 10 min. Figure 2 shows the schematic diagram for the preparation of the probing microspheres. Meanwhile, PLL-coated microspheres for target dsDNA immobilization were prepared by coupling of primary amines of PLL with carboxyl groups on the carboxylate microspheres.

Figure 3 shows a schematic side view of the experiment of the real-time FISH. First, PLL-coated microspheres were scattered on a cover slip and immobilized randomly, and then, the target dsDNA (λ DNA concatemer) molecules were added so that they could be stretched hydrodynamically and fixed at both ends on the PLL-coated microspheres in a random manner. Subsequently, probing microspheres were introduced, and one of them was picked up and moved along the fixed target dsDNA by using optical tweezers. The interaction between the target dsDNA and the probing microsphere was then examined. If RecA functions as expected, the probing microsphere should have higher binding affinity to the complementary position, and therefore be arrested there.

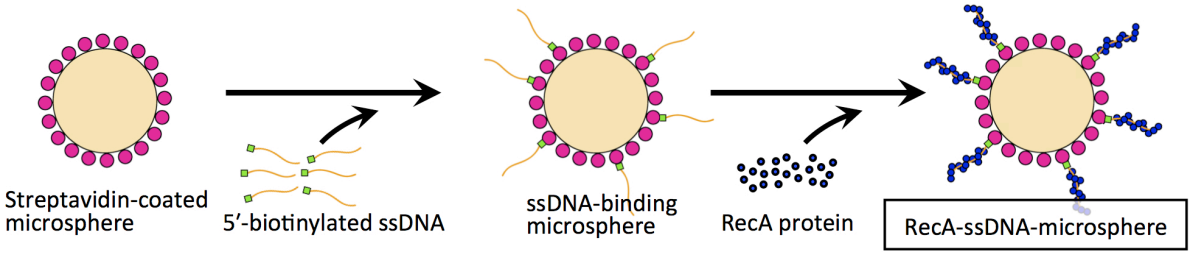


*Figure 2. Preparation of the RecA-ssDNA-microsphere probe, i.e., probing microsphere Streptavidin-coated polystyrene microsphere was coupled with 5′-biotinylated ssDNA, followed by the binding of RecA to the ssDNA. The diameter of the polystyrene microspheres was 2 μm.*

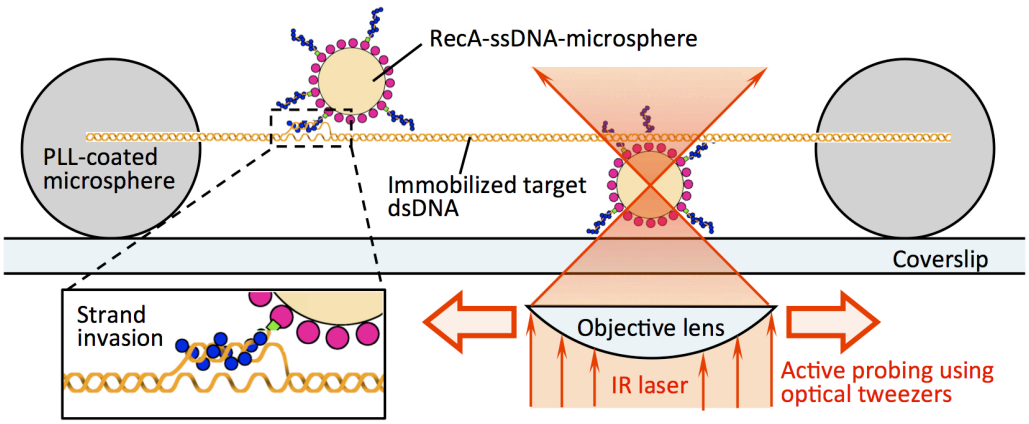


*Figure 3. Schematic side view of the real-time FISH experiment: searching a specific sequence along the target DNA with the RecA-ssDNA-microsphere probe driven by optical tweezers (not to scale). If the RecA-ssDNA complex on the microsphere is functional, it is expected that the RecA-ssDNA complex invades the dsDNA, and the probe is fixed on the target DNA where a complementary sequence exists. Experiments were performed at room temperature.*

## RESULTS AND DISCUSSION

When the poly-A (Oligo-2)-immobilized microsphere was moved at a constant speed (ca. 1 μm/s) along the target DNA, it slid smoothly and no stiction was observed, indicating that non-specific binding does not affect the microsphere's movement with optical tweezers. In contrast, when we used the probing microsphere with complementary ssDNA (Oligo-1), sticking occurred along the target DNA, and under these conditions the microsphere escaped the optical trapping and remained in the position. When the arrested microsphere was retrapped by the optical tweezers and moved perpendicularly, the target DNA was observed with bending at the location. Figure 4 shows a representative image, where 4 probing microspheres are arrested on the target DNA. The arrested positions appeared intermittently along the target λ DNA concatemer, and their intervals were comparable to the contour length of λ DNA. This indicates that complementary bindings occur between Oligo-1 and the central part of each λ DNA in the λ DNA concatemer.

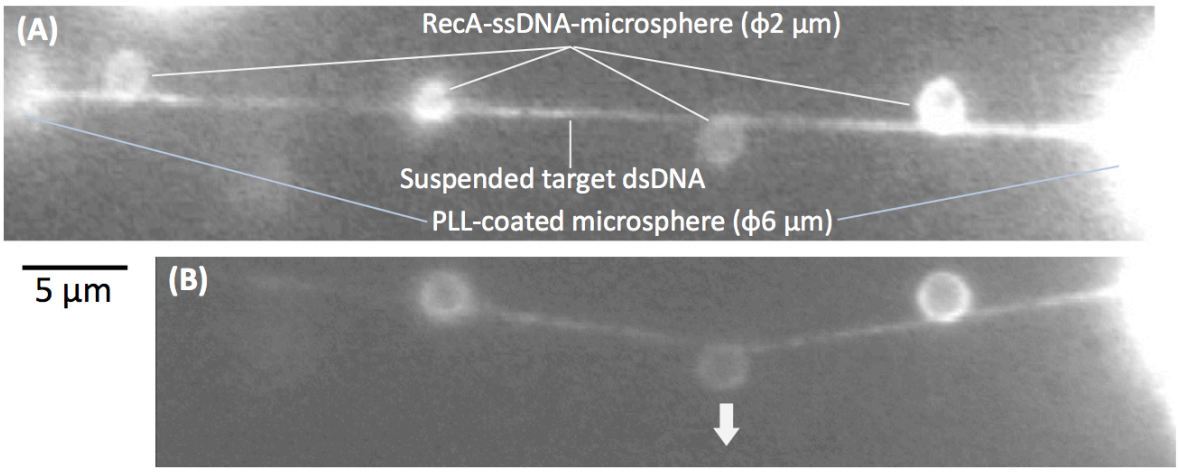


*Figure 4. (A) Representative fluorescence image of RecA-ssDNA-microsphere probes binding to the target λ DNA concatemer that is stretched-and-fixed at both ends with PLL-coated microspheres. (B)The binding affinity was sufficiently strong that it prevented detachment of the binding probes by optical tweezers. The white arrow indicates the direction of pulling with optical tweezers. Target DNA was visualized using SYBR Gold.*

## CONCLUSION

In conclusion, we successfully demonstrated a novel real-time FISH method with the use of optically driven, RecA-functionalized microspheres. This method does not require thermal denaturation of the target dsDNA and facilitates specific DNA sequence detection with spatial information.


## ACKNOWLEDGEMENTS

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