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Video Article

Visualizing Single-molecule DNA Replication with Fluorescence Microscopy

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Abstract

We describe a simple fluorescence microscopy-based real-time method for observing DNA replication at the single-molecule level. A circular, forked DNA template is attached to a functionalized glass coverslip and replicated extensively after introduction of replication proteins and nucleotides (Figure 1). The growing product double-strand DNA (dsDNA) is extended with laminar flow and visualized by using an intercalating dye. Measuring the position of the growing DNA end in real time allows precise determination of replication rate (Figure 2). Furthermore, the length of completed DNA products reports on the processivity of replication. This experiment can be performed very easily and rapidly and requires only a fluorescence microscope with a reasonably sensitive camera.

Video Link

The video component of this article can be found at <http://www.jove.com/video/1529/>

Protocol

Before performing a single-molecule replication experiment, a few materials need to be prepared in advance.

1. DNA Replication Template

The substrate for the replication reaction is a biotinylated, tailed M13 rolling circle prepared using standard molecular biology techniques^{1,2}.

Materials: M13mp18 single-stranded DNA, Biotinylated tail oligonucleotide primer (5'-Biotin-T₃₆AATTCGTAATCATGGTCATAGCTGTTTCCT-3'), T7 DNA polymerase, Heat block, Phenol/Isoamyl Alcohol/Chloroform

1. Anneal the biotinylated tail oligo to the M13 by adding a 10-fold excess of oligo in TBS buffer (330 nM tail oligo, 33 nM M13).
2. Heat the mixture to 65 °C in the heat block. Once heated, turn the heat block off and allow slow cooling to properly anneal the primer.
3. Add the primed M13 to a mixture of 64 nM T7 DNA polymerase and T7 replication buffer containing dNTPs and MgCl₂.
4. Incubate the reaction at 37 °C for 12 minutes and quench with 100 mM EDTA.
5. Purify the filled-in product DNA with phenol/chloroform/isoamyl alcohol extraction and dialyze into TE buffer or other suitable storage buffer and determine DNA concentration (Back-extract each organic phase to recover any residual primed M13).
6. One iteration of template preparation can easily make several milliliters of nanomolar concentration template, enough for hundreds to thousands of single-molecule experiments.

2. Functionalized Coverslips

For attaching the DNA to the glass coverslip, the glass is first functionalized with an aminosilane, which is then coupled to biotinylated PEG molecules. This coating helps to reduce the nonspecific interactions of DNA and replication proteins with the surface^{1,3}.

Materials: Glass coverslips, Staining jars, 3-aminopropyltriethoxysilane, Methoxy-PEG5k-NHS ester, Biotin-PEG5k-NHS ester, Acetone, 1M KOH, Ethanol, Oven, Bath sonicator

1. Thoroughly clean the glass coverslips by placing them in staining jars and filling the jars with ethanol. Sonicate for 30 minutes and rinse out the jars and fill with 1 M KOH. Sonicate for 30 minutes Repeat both washes.
2. For the first functionalization reaction, all traces of water need to be removed. Fill the jars with acetone and sonicate for 10 min. Rinse the jars again with acetone.
3. Prepare a 2 % v/v solution of the silane reagent in acetone. Add to the staining jars and agitate for 2 minutes. This reaction couples the alkoxy group of the aminosilane to the glass, leaving a reactive amine for the next coupling step. Quench the reaction with a large excess of water.
4. Dry the coverslips by baking them at 110 °C in the oven for 30 minutes.

5. Prepare a 50:1 methoxy:biotin PEG mixture in 100 mM NaHCO₃ pH 8.2. Aim for around 0.2 % w/v biotinylated PEG.
6. Pipette 100 μ L of the PEG mixture onto a dry silanized coverslip and place another coverslip on top. Including a glass coverslip spacer will allow separation of the coverslips.
7. Incubate the coverslips in the PEG solution for 3 hours, then separate each pair of coverslips and wash extensively with water. Be careful to keep the coverslips functionalized side up as only once side will be coated with PEG.
8. Dry the coverslips and store under vacuum. The surfaces remain stable for at least a month, so dozens of coverslips can be made in a batch and used as needed.

These materials should be made ahead of time, and then used for each experiment. To start every single-molecule experiment, begin by assembling the flow chamber.

3. Flow Chamber Preparation

The experiment is performed using a simple flow chamber constructed with a functionalized coverslip, double-sided tape, a quartz slide and tubing. One flow chamber is prepared for each single-molecule experiment^{1,4}.

Materials: Double-sided tape, Razor blade, Quartz slide with holes for tubing, Quick-dry epoxy, Functionalized coverslip (see above), Streptavidin solution (25 μ L of 1 mg/mL in PBS), Tubing, Blocking buffer (20 mM Tris pH 7.5, 2 mM EDTA, 50 mM NaCl, 0.2 mg/mL BSA, 0.005% Tween-20)

1. Begin by placing 20-25 mL of blocking buffer in a desiccator to remove any air bubbles for later steps.
2. Take a PEG-functionalized coverslip and spread 125 μ L of PBS-diluted streptavidin solution over the surface. Leave this to incubate for 20 minutes while preparing other parts of the chamber, allowing streptavidin to bind surface biotins.
3. Cut a piece of double-sided tape to match the size of the quartz slide. Mark the position of the tubing holes on the tape with a pencil so that an outline of the flow chamber can be drawn on the tape (1 mg/mL).
4. Make a 2 mm-wide rectangle around the holes. This will serve as the flow channel, so be sure to make straight sides and leave room around the tubing inlet and outlet. If desired, multiple channels or different sizes can be used.
5. Cut along the drawn outline, making straight, clean cuts to make sure no adhesive protrudes into the channel.
6. Clean the quartz thoroughly using acetone or ethanol to remove adhesive from the previous flow cell construction.
7. Find the best alignment of the channel outline, remove one side of the adhesive backing and carefully place the tape onto the quartz slide. Be careful to align the tape properly, as the inlet and outlet holes need to remain unblocked.
8. Cut lengths of tubing for the inlet and outlet of the flow cell. It helps to cut the end of the tube at about a 30° angle so that the tube will not press flat against the chamber bottom. Place the tubes on some sort of support to suspend them for easy attachment to the flow cell in the next steps.
9. Rinse the streptavidin-coated coverslip thoroughly with water and dry using compressed air. Remember that only one side is functionalized; be careful not to turn the coverslip over. Remove the other side of the tape backing and place the quartz slide onto the coverslip.
10. Lightly press on the coverslip to push out any air trapped in the adhesive. This will help avoid any air bubbles getting into the flow channel.
11. Seal the sides of the chamber with epoxy. Insert the cut tubing into the holes of the quartz and seal in place with epoxy. This needs to dry for a few minutes.
12. Once dry, begin blocking the surface by pulling some of the degassed blocking buffer through one of tubes. A 21-gauge needle fits perfectly inside the 0.76 mm tubing. Flush out a few times to remove air bubbles, and allow the chamber to incubate for at least half an hour.

4. Single-Molecule Replication Experiment

Materials: Prepared flow chamber, SYTOX Orange, TIRF microscope, 532 nm laser, CCD camera, Computer with image acquisition software, Rolling-circle DNA substrate, Replication proteins

1. After the flow cell has been blocked, everything is ready to begin the single-molecule experiment.
2. Take the flow cell and place it on the microscope stage. Hold the chamber in place with stage clips and be sure that the flow channel is positioned straight along the y-axis.
3. Connect the flow cell outlet tube to the syringe pump using a larger diameter connector tubing or a needle. Place the inlet in blocking buffer and pull back on the syringe to remove any air in the tube. A gentle flick of the outlet tube will help clear any air bubbles trapped in the flow cell.
4. Dilute the stock DNA template to 25 pM in 1 mL of blocking buffer. Flow into the chamber at moderate flow rate to allow good surface coverage of DNA. 0.025 mL/min for 30 minutes works well. This can be varied based on how many DNA molecules are on the surface for each batch of coverslips or how many are desired for the experiment.
5. Once DNA is in, wash out excess DNA using blocking buffer. Wash for at least 200 flow-cell volumes to get rid of all the free DNA.
6. Begin degassing the replication buffer. For the *E. coli* replication experiments, do this at 37 °C to reduce the risk of bubbles in the heated flow chamber.
7. Turn on the laser and camera. The cooled CCD needs to reach temperature before it can be used.
8. If performing a 37 °C experiment, turn the heater on. Be sure the objective is in contact with the flow cell or it will be a heat sink later.
9. After washing and degassing, the reaction can be prepared. Mix nucleotides, DTT, and SYTOX in the replication buffer. Then add proteins and mix gently.
10. Flow the reaction mixture into the flow cell. This flow speed needs to be sufficient to stretch dsDNA. For a 2-mm wide, 100- μ m tall flow channel 0.04 mL/min works well.
11. Allow time for the mixture to enter the chamber and begin imaging. A good tip is to move the field of view to the side of the channel and focus on the adhesive material. This will ensure you are near the surface for focusing.

12. Adjust laser power, microscope focus, and TIRF angle. Keep the power low to avoid any photocleavage of the stained DNA. Acquire at 1-5 frames/second for several minutes depending on the rate of DNA replication. If desired, repeat to get longer replication trajectories or move to a new field to see more molecules.
13. After the reaction mixture is almost empty, it is a good idea to flow in more buffer with SYTOX to see other fields of view. Taking multiple images of different replicated molecules provides statistics for processivity determination.

5. Representative Results

Actively replicating molecules are easily seen as growing lines of stained DNA. In our experiments, flowing 25 pM of the M13 substrate gives 100-1000 molecules in a 60x, 125 μm x 125 μm field of view (Figure 1). Performing replication experiments using the *E. coli* replisome proteins at 37 °C (see Materials for details) gives 5-50 replicating molecules per field of view. At equivalent concentrations of the T7 replisome, which initiates on the substrate much more efficiently, we observe >70% of the molecules in a field replicate. These conditions yield a product density so high that individual molecules are difficult to resolve and analyze, so the T7 experiments are performed at lower protein concentrations.

Data Analysis: To obtain rate data, simply plot the endpoint of the DNA vs. time and calculate the slope (Figure 2). For processivity, determine the total length of the DNA. Both of these numbers will need to be converted to base pairs. Before performing the experiment, you should know the pixel size of the camera at the proper magnification. For basepair conversion, a good estimation is simply converting based on the crystallographic length of DNA, 2.9 bp/nm. However, laminar flow will not completely stretch the DNA, so a DNA length calibration should be performed by taking a known length DNA, e.g. 48,502 bp λ DNA, attaching it to the flow cell with a biotinylated oligo and measuring its SYTOX-stained length at the flow rate used for replication experiment. Determining the number of base pairs/pixel will allow accurate calculation of replication rates and processivities. Taking numerous images and movies will provide large numbers of product molecule, allowing plotting of rate and processivity distributions (Figure 2)¹.

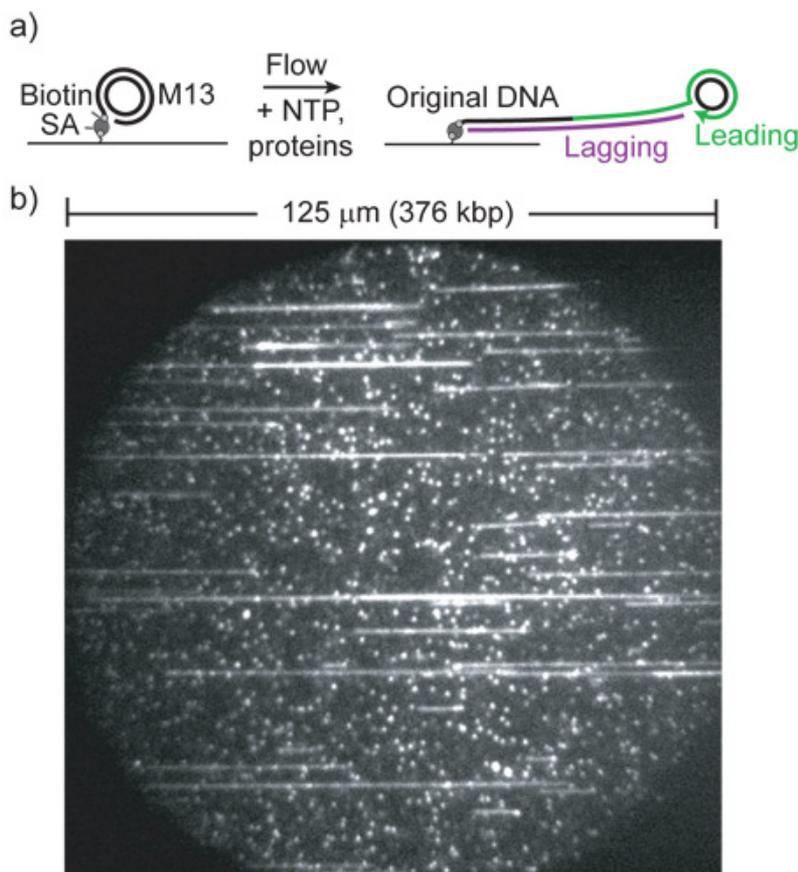


Figure 1: (From Ref. 1) **a)** Cartoon of the rolling-circle replication assay. (SA, streptavidin). Leading-strand synthesis extends the tail linking the circle and surface. The tail is converted to dsDNA by the lagging strand polymerase and stretched by laminar flow.

b) Example field of view with SYTOX Orange and 532 nm excitation. Small fluorescent spots are tethered, non-replicated substrates. Note the extreme length of the products and the large number of products per field (each flow cell contains >5000 such fields).

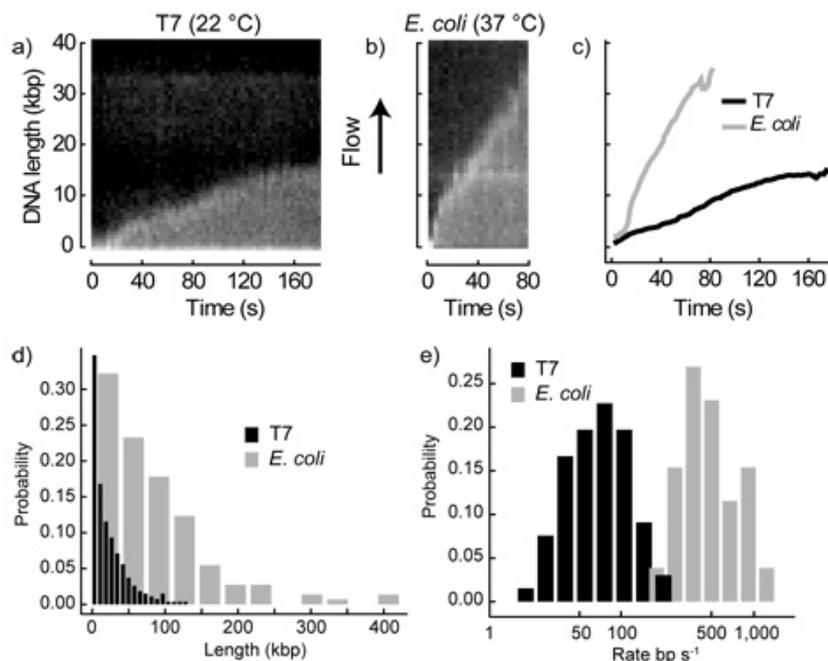


Figure 2: (adapted from Ref. 1) a,b) Kymographs of typical replicating molecules from T7 (a) and *E. coli* (b) experiments. c) Endpoint trajectories of molecules from a) and b) plotted vs. time. Trajectories are fitted with linear regression to obtain replication rates. Shown examples are 99.4 bp s⁻¹ and 467.1 bp s⁻¹. d) Length distributions of replication products, fit with single exponential decay to obtain processivity: 25.3 ± 1.7 kbp (T7), 85.3 ± 6.1 kbp (*E. coli*). e) Rate distributions of single molecule trajectories, fit with single Gaussians. Means: 75.9 ± 4.8 bp s⁻¹ (T7), 535.5 ± 39 bp s⁻¹ (*E. coli*).

Discussion

One critical control is examining the effect of the stain, SYTOX Orange, on the replication proteins of interest. A simple way to do this is to perform the replication experiment in the flow cell as described but with the omission of SYTOX. After the reaction mixture has flowed through the chamber, add buffer with SYTOX to stain DNA and examine the length distribution of replicated molecules. Alternatively, standard bulk reactions can be used to check any effect of SYTOX on replication rate and efficiency.

The experiment described here uses only a single flow channel. This can be changed easily by creating multi-channel flow chambers or using PDMS or similar microfluidic devices. Increasing the number of channels greatly facilitates screening of protein concentrations, mutants, or inhibitor molecules and increases the rapidity of replication data collection.

As mentioned, we perform the *E. coli* replication experiments at 37 °C using a home-built aluminum flow cell heater, a resistive heating element (cartridge heater) and variable power supply. This gives good temperature stability and avoids the purchase of an objective heater. To calibrate the heater, we simply drilled a hole in the center of a quartz flow cell top, inserted a thermocouple into the flow channel and flowed buffer as normal. Measuring the flow cell buffer temperature at increasing voltages allows accurate heating.

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