Simple method of DNA stretching on glass substrate for fluorescence imaging and spectroscopy

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Abstract. We demonstrate a simple method of stretching DNA to its full length, suitable for optical imaging and atomic force microscopy (AFM). Two competing forces on the DNA molecules, which are the electrostatic attraction between positively charged dye molecules (YOYO-1) intercalated into DNA and the negatively charged surface of glass substrate, and the centrifugal force of the rotating substrate, are mainly responsible for the effective stretching and the dispersion of single strands of DNA. The density of stretched DNA molecules could be controlled by the concentration of the dye-stained DNA solution. Stretching of single DNA molecules was confirmed by AFM imaging and the photoluminescence spectra of single DNA molecule stained with YOYO-1 were obtained, suggesting that our method is useful for spectroscopic analysis of DNA at the single molecule level. © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.19.5.051210]

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1 Introduction

Using x-ray crystallography and molecular modeling, Watson and Crick[1] proposed the double helical structure of DNA in 1953, and Fujiyoshi and Uyeda[2] visualized this structure by using high resolution electron microscopy in 1981. Nowadays, the DNA is essential to many research fields, such as studying chronic diseases,[3,4] tracing criminals,[5] sensing of different gases, and chemicals.[6,7] For more molecular insight, the DNA researchers are focusing at the single molecule level by using various microscopy and spectroscopy tools on adsorbed and stretched DNA molecules.

Several techniques have been developed for the adsorption and stretching of the DNA molecules: atomic force microscopy (AFM) cantilever technique, optical or magnetic tweezers technique, flowing liquid, and flowing gas techniques have been reported.[8–12] But most of these techniques are limited to stretching only a single DNA molecule at a time. More productive techniques, such as flowing liquid and gas techniques for stretching multiple number of DNA molecules are rather sophisticated and require the trained skills. After the development of the molecular combing technique, stretching of many DNA molecules at a time has become more convenient.[13,14] This technique requires chemical modification of the substrate surface. Cetyltrimethyl ammonium bromide coating,[15] gas phase silanization and liquid phase silanization coating[16] are mostly used for these surface modifications. Thus, with the introduction of this combing technique, many advanced research areas, such as investigating DNA replication,[17] fluorescence situ hybridization,[18] and genomic physical mapping have been made possible.[19] A modified liquid phase silanization technique using a novel solvent/ silane combination has also been established, which showed better performance for stretching of more DNA molecules at a time.[20] And polydimethyl-siloxane stamping combined with soft lithography was also used for stretching DNA, but this process tends to somewhat overstretch the DNA molecules.[21]

Here, we demonstrate a simple and fast method for stretching DNA molecules on a glass substrate. In this method, the substrate is first modified with piranha and RCA[22] treatment, followed by spin-coating DNA molecules stained with a dye. This surface modification of substrate can also be used for the precise controlling of the cationic or anionic polyelectrolyte film thickness in monomolecular level.[22,23] This protocol disperses and stretches the DNA molecules to their full lengths. Fluorescence imaging and AFM imaging were used to confirm the isolation of single DNA molecules. We also performed photoluminescence (PL) spectroscopy of stretched single DNA molecule stained with dye.

2 Experiments

2.1 Cleaning and Surface Treatment of the Cover Glasses

Cover glasses $(22 \times 40 \times 1 \text{ mm}^3)$ were first sonicated for 30 min in 200 mL of 1-M KOH then for 15 min in acetone to remove all inorganic and organic residues. They were then sonicated 4 times for 10 min each in distilled water and finally dried with nitrogen gas. The cleaned and dried cover glasses were taken into 250-mL beaker and 200-mL piranha solution was added to it, which was prepared by mixing of strong sulfuric acid (98.08%) with hydrogen peroxide (30%) at a ratio of 7 : 3 while maintaining constant temperature using ice bath to avoid explosions.[24] It was then sonicated for 1 h at 75°C temperatures.

Piranha solution is a strong oxidizing agent; it hydroxylases the surface of cover glass by adding OH groups and thus making the glass extremely hydrophilic.[24] Those cover glasses were then washed several times with distilled water to remove all sulfuric acid residues and to get uniform distribution of reactive hydroxyl groups. Afterward, they were again dried with nitrogen gas and then sonicated for 1 h at 75°C in 200 mL of RCA solution. This RCA solution was prepared by mixing distilled water, hydrogen peroxide (30%), and ammonium solution (27% to 31%) in the ratio of 5:1:1.[25] The RCA treatments enriches the hydroxyl and hydroxylate groups on the cover glasses, which develops negative charge and extremely hydrophilic surfaces.

Figure 1(a) shows a schematic sketch of how piranha solution hydroxylases the substrate and how the RCA solution develops negative charges over its surface. Figure 1(b) describes the intercalation of YOYO-1 dye to DNA strands. The molecular structure of DNA and YOYO-1 molecules are correctly fit for this purpose. The alkyl functional group on the YOYO-1 dye molecule and the amine functional group contained on DNA molecule support intercalation because of hydrogen bonding to each other. Here, we chose a negatively charged, modified glass substrate for stretching DNA molecules stained with YOYO-1. The negative charges distributed over the substrate surface and the positively charged nitrogen ions of the YOYO-1 molecules form ionic bonds. Figure 1(c) describes the process of DNA dispersion and stretching by spin coating. The strategy used for this approach is the development of competition between the centrifugal force caused by spin coating and the electrostatic force between the negatively charged
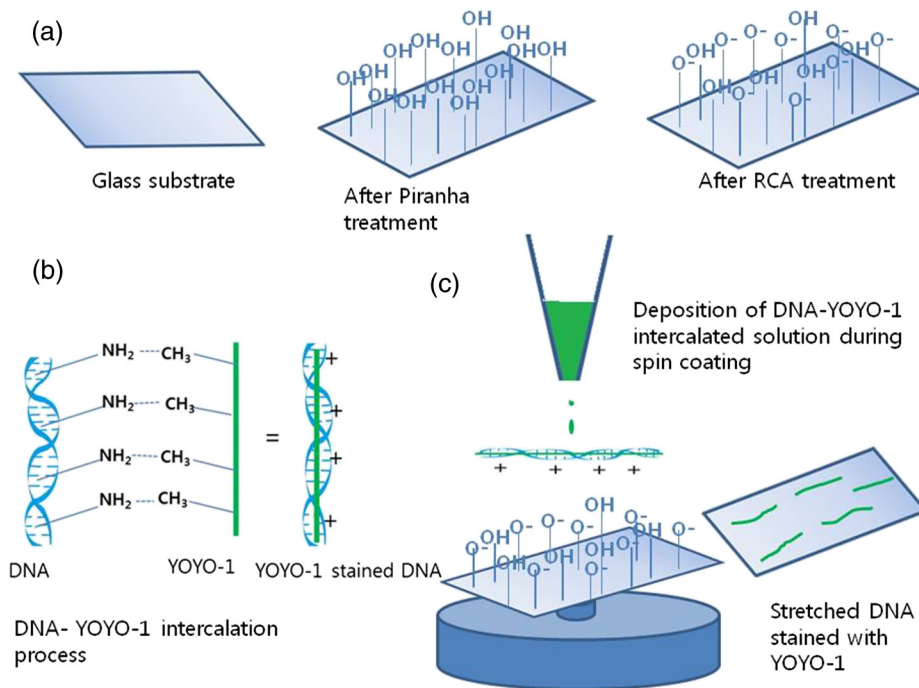

Fig. 1 Schematic diagram for DNA stretching: (a) surface modification of glass substrate after piranha and RCA treatments; (b) DNA and YOYO-1 dye intercalation process; (c) stretching of DNA molecules stained with YOYO-1 dye.
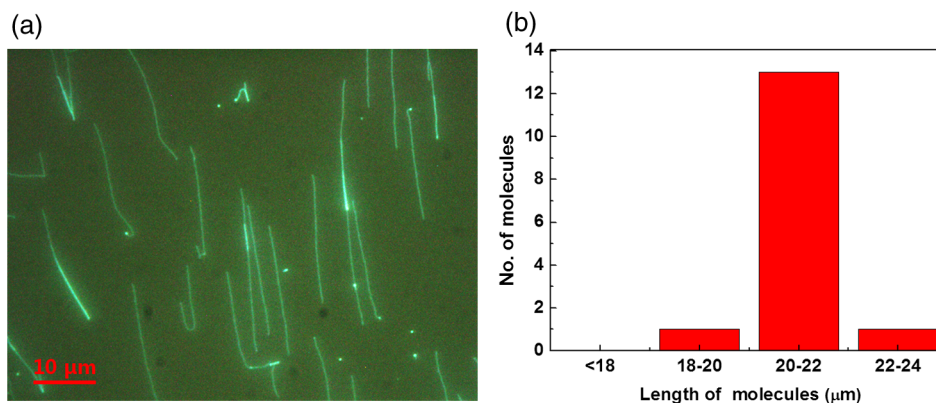

Fig. 2 (a) Fluorescence images showing the representative result-stretched λ-DNA molecules obtained with the concentration of 7.5 ng/μL DNA-dye solutions. (b) Column diagram illustrates the statistic distribution of stretched lengths of the DNA molecules.

surface of the substrate and the positively charged YOYO-1 dye of the stained DNA molecules. The YOYO-1 dye has good intercalating properties with DNA and becomes highly fluorescent after intercalating with the DNA molecules.[26]

2.2 Solution Preparation

Initially, 5 μL of YOYO-1 (1-mM solution in dimethyl sulfoxide) dye was mixed with 500 μL of distilled water and the mixture was sonicated for 2 min to make a homogeneous solution. Then, 200 μL of this solution were mixed with 5 μL of λ-DNA

(0.3 μg/μL) solution and the mixture was sonicated for 10 to 15 min at room temperature for effective intercalation of DNA with the YOYO-1 dye. Human placenta DNA (1 mg/mL) solution was also prepared by using the same proportions of DNA and dye as for the λ-DNA, but the mixture was kept overnight at −4°C and only 2 min of sonication was performed for the protection from its shearing. Finally, 20 to 30 μL of the DNA-dye solution was spin-coated over the surface of the modified glass substrate with the rotating speed of 4550 rpm for first 20 s and 5750 rpm for an additional 10 s for optimal dispersion.
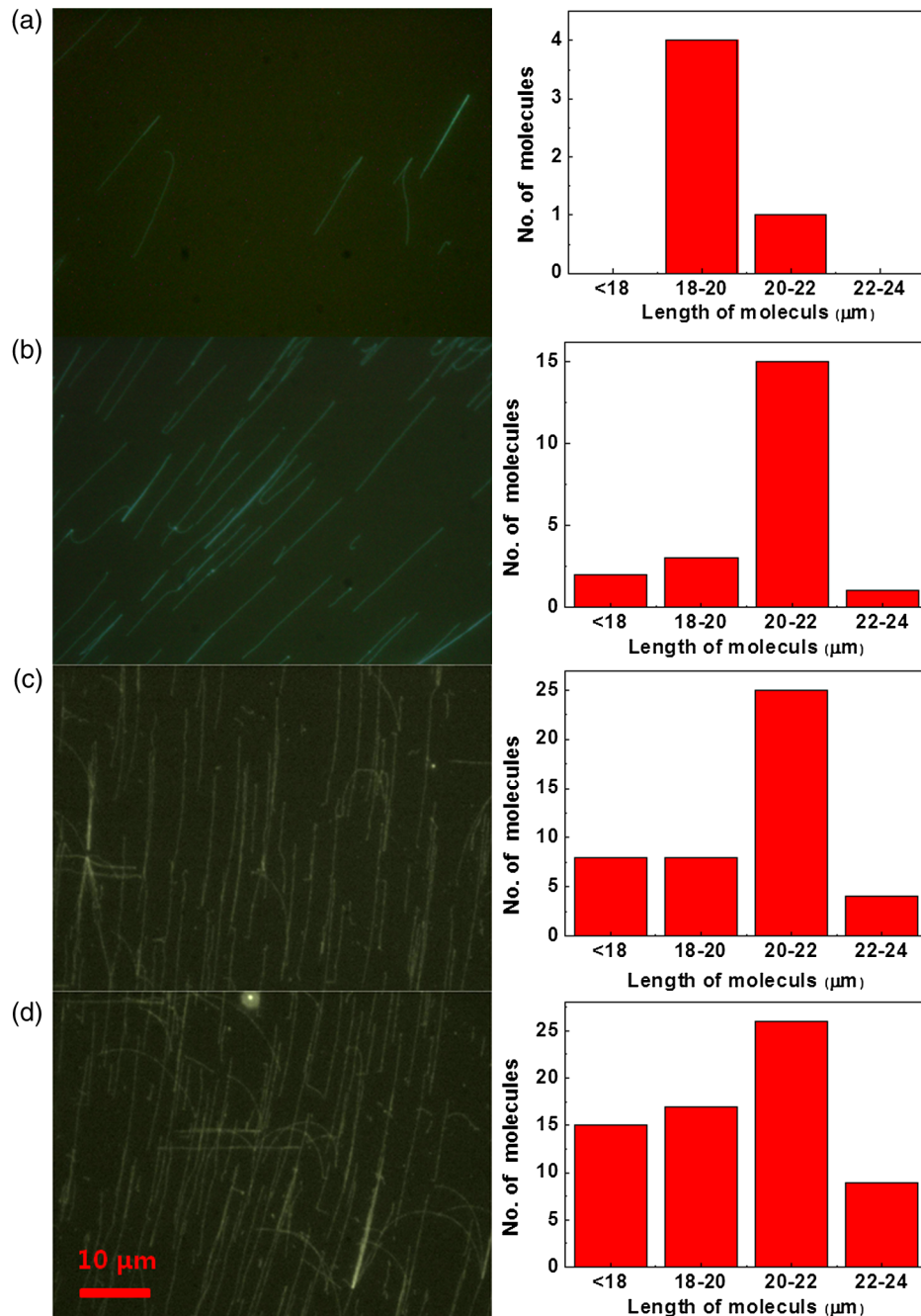

Fig. 3 Fluorescence images showing the result of the dispersion density control of stretched λ-DNA molecules with the variation of concentration of the DNA. Stretching of DNA molecules with the concentration of (a) 3, (b) 7.5, (c) 22.5, and (d) 37.5 ng/μL. Column diagrams are displayed along with the fluorescence images to show the statistic distribution of stretched lengths of λ-DNAs.

2.3 Microscopy

Epi-fluorescence microscopy was performed using the 435-nm excitation light from Hg lamp and a color CCD camera was used for the fluorescence imaging of stretched DNA molecules stained with YOYO-1 dye. For the AFM imaging, we used a commercial AFM (PSIA, XE 120 system) that stood on a lab-made confocal microscope which was built around an inverted optical microscope and shared the sample-scanning stage with AFM. The AFM imaging was operated in noncontact mode. The PL spectra and laser confocal fluorescence image were obtained with the confocal microscope.[27] We used an oil-immersion objective lens with a numerical aperture (NA of 1.4) to focus the laser light onto the sample. For PL excitation, the 458-nm line of an argon ion laser was used. The spot size of the laser beam focused on the sample was estimated to be less than 300 nm.[27] The same objective was used to collect PL signals which was guided to a 30-cm long monochromator (equipped with a cooled CCD) through multimode optical fiber having 100-μm core diameters, which acted as a confocal detection pinhole. The laser power incident upon the sample and the acquisition time for each PL spectrum were fixed at 10 μW and 3 s, respectively.

3 Results and Discussion

Figure 2(a) displays a representative result of stretching of λ-DNA, double stranded linear DNA having 48,502 base pairs. The column diagram shown in Fig. 2(b) shows that most of DNA strands have a length between 20 and 22 μm. The observed lengths of λ-DNA are somewhat longer than the expected crystallographic size of 16.2 μm of λ-DNA suggesting the possibility of overstretching. Overstretching of DNAs has been mostly due to the nature of the hydrophobic surface.[21] Here, piranha and RCA treatment develops the hydrophilic nature on the surface therefore minimizing the possibility of overstretching. This lengthening might be due to the relaxation of DNA strands, caused by the intercalation with the YOYO-1 dye molecules.

We found rotating speeds of spin coater and its time periods are the controlling keys for the good stretching of DNA molecules on the glass substrate. Rotating speed of 4550 rpm for first 20 s and 5750 rpm for an additional 10 s was the optimum condition for good stretching. We found that dominating electrostatic attraction force brings coiling and aggregations of DNA molecules and dominating centrifugal force tend to cause the shearing of the DNA molecules. In our techniques, we first chose so that the attractive electrostatic force is somewhat dominating to give good attachment of DNA molecules on the substrate and subtle adjustment of the rotating speed in an additional 10 s removed the coiling and aggregation.

We found that density of dispersed DNA molecules depended upon the solution concentration, dispersed volume, viscosity, and rotating speed and time. In our technique, we controlled the density of stretched DNA molecules by varying the DNA concentration in DNA-dye solution. We made four different concentrations of λ-DNA solutions with the concentration of 3, 7.5, 22.5, and 37.5 ng/μL and carried out the stretching of molecules under the same rotating speed and the time. The number of DNA molecules was estimated to be 8.5×10^4, 3.5×10^5, 7.6×10^5, and 1.1×10^6 cm^{-2} for 3, 7.5, 22.5, and 37.5-ng/μL concentration, respectively. The result of controlling the dispersion density is shown in Fig. 3.

Stretching of shorter DNA molecules has been more difficult than the long DNA molecules. We investigated the stretching of human placenta DNA molecules by applying our method. The approximate crystallographic length of these molecules having ~25,000 base pairs ranges from 6.6 to 9.9 μm. By applying the same spin coating speed and volume of drop casting as was determined in stretching of λ-DNA molecules, we were able to obtain some number of stretched DNA molecules in the range of 8 to 12 μm, as shown in Fig. 4. Some overstretching is noticed in the fully stretched DNA molecules, and the yield for stretching of short-length DNA strands is not as high as stretching λ-DNA. However, we believe that we showed a reasonable success considering the technical difficulty of stretching shorter DNA molecules.

An AFM image of a strand of λ-DNA is shown in Fig. 5(a). Line profiles across the DNA strand are displayed in Fig. 5(b), where the height of the DNA strand is only ~2.5 nm, which confirms the stretching of a single strand of λ-DNA without the aggregation of multiple DNA strands. The height of 2.5 nm is somewhat bigger than previously reported heights of a single λ-DNA molecule (~1 nm).[28–30] The intercalated YOYO-1 molecules with DNA may have increased the height of a single DNA strand. In the AFM image, some bright spots are also observed along the DNA strand, which are believed to be the aggregated YOYO-1 molecules.
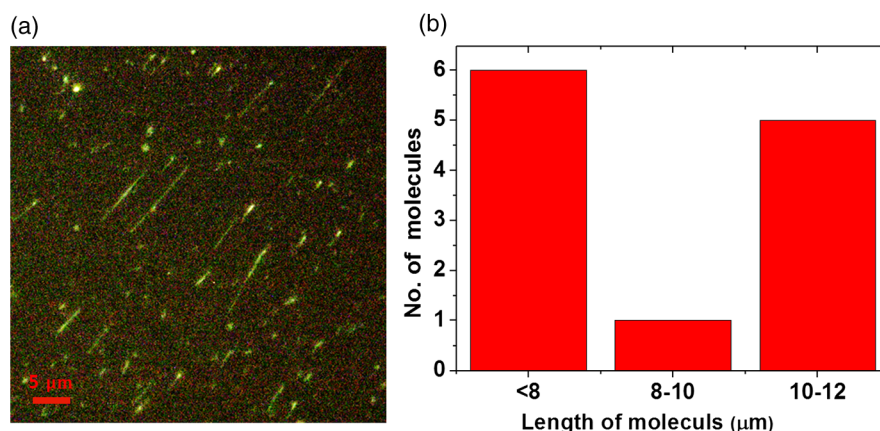

Fig. 4 (a) Fluorescence image of human placenta DNAs stretched on glass substrate. (b) Column diagram illustrating the statistic distribution of stretched lengths of the DNA molecules.
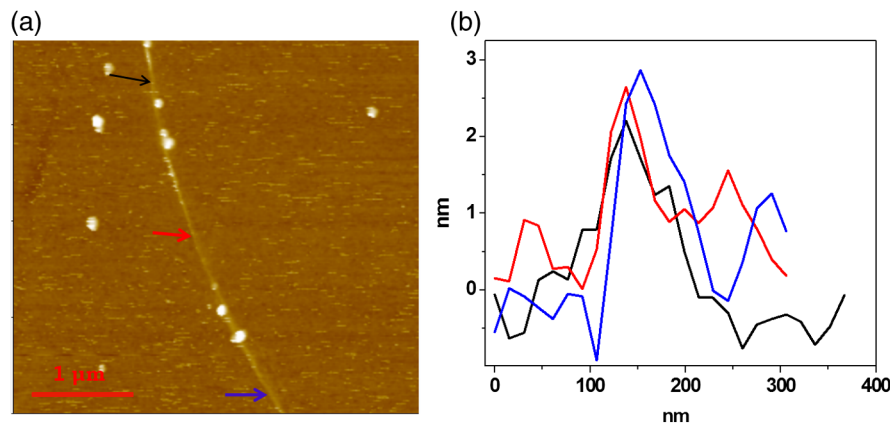
Fig. 5 (a) Atomic force microscopy image of stretched λ-DNA molecule stained with YOYO-1 dye. (b) Cross sectional profile that shows the height of a stretched λ-DNA molecule.
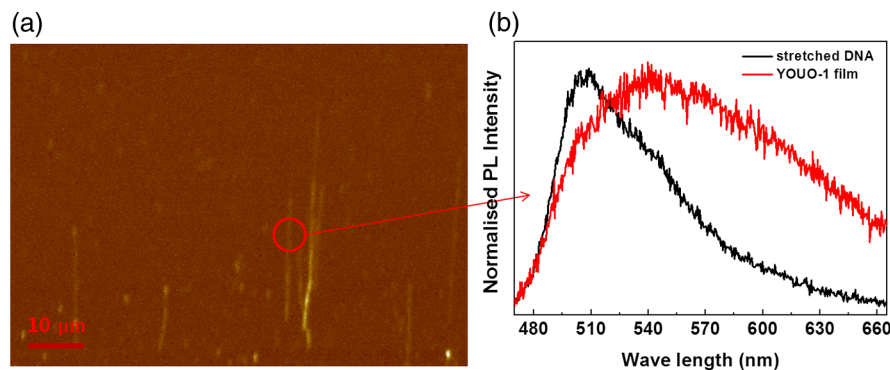

Fig. 6 (a) Laser confocal fluorescence image of stretched λ-DNAs stained with YOYO-1 dye. (b) Normalized photoluminescence spectra taken from a stretched stained λ-DNA molecules and YOYO-1 film.

We compared the PL spectrum of stretched λ-DNA stained with YOYO-1 dye molecule to PL spectrum of pristine YOYO-1. Figure 6(a) shows a confocal fluorescence image of stretched λ-DNA molecule stained with YOYO-1 and Fig. 6(b) shows a comparison between the PL spectra taken from the marked region of stretched single DNA molecule in Fig. 6(a) and the spin-coated film of YOYO-1. The stretched DNA molecule shows the different PL spectrum of which the peak position lies at 505 nm, while the PL peak position in spin-coated YOYO-1 film is at 550 nm, and the band width in stretched DNA molecule is narrower. This kind of PL spectral modification comes from the DNA-dye intercalation because it contains many stably bound chromospheres after the intercalation.[26]

4 Conclusion

We presented a simple and effective technique for DNA stretching on a glass substrate for AFM, fluorescence imaging, and PL spectroscopy. We used the piranha and RCA solutions to develop the negative charges on the glass substrate and DNAs were intercalated with YOYO-1 dye to make positive charge on them. Two competitive forces of the centrifugal force created during the spin coating process and the electrostatic force developed between the negatively charged surface and positive charge of the YOYO-1 molecules were optimally controlled to achieve the isolation of the fully stretched DNA molecules. The stretched DNA molecules were visualized and confirmed using fluorescence and AFM imaging. We also showed that the density of the stretched DNA molecules could be controlled by concentration of λ-DNA solutions. Our method also showed a reasonable success in stretching shorter DNA molecules having length <10 μm. The PL spectroscopic study of YOYO-1 stained single DNA molecule showed a different PL spectrum from YOYO-1 film showing the possibility of optical study in single DNA molecular level.

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