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Corresponding Author:	Zhi Qi Peking University Beijing, CHINA
Corresponding Author's Institution:	Peking University
Corresponding Author E-Mail:	zhiqi7@pku.edu.cn
Order of Authors:	Zhi Qi
	Linyu Zuo
	Jiawei Ding
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TITLE:

Single-molecule Imaging of EWS-FLI1 Condensates Assembling on DNA

AUTHORS AND AFFILIATIONS:

Linyu Zuo[1,†], Jiawei Ding[1,†], Zhi Qi[1]

[†]Equal contribution

[1]Center for Quantitative Biology, Peking-Tsinghua Center for Life Sciences, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China

Email addresses of co-authors:

Linyu Zuo (zuolinyu1994@pku.edu.cn)

Jiawei Ding (djw20@stu.pku.edu.cn)

Corresponding author:

Zhi Qi (zhiqi7@pku.edu.cn)

SUMMARY:

Here, we describe the use of the single-molecule imaging method, DNA Curtains, to study the biophysical mechanism of EWS-FLI1 condensates assembling on DNA.

ABSTRACT:

The fusion genes resulting from chromosomal translocation have been found in many solid tumors or leukemia. EWS-FLI1, which belongs to the FUS/EWS/TAF15 (FET) family of fusion oncoproteins, is one of the most frequently involved fusion genes in Ewing sarcoma. These FET family fusion proteins typically harbor a low-complexity domain (LCD) of FET protein at their N-terminus and a DNA-binding domain (DBD) at their C-terminus. EWS-FLI1 has been confirmed to form biomolecular condensates at its target binding loci due to LCD-LCD and LCD-DBD interactions, and these condensates can recruit RNA polymerase II to enhance gene transcription. However, how these condensates are assembled at their binding sites remains unclear. Recently, a single-molecule biophysics method—DNA Curtains—was applied to visualize these assembling processes of EWS-FLI1 condensates. Here, the detailed experimental protocol and data analysis approaches are discussed for the application of DNA Curtains in studying the biomolecular condensates assembling on target DNA.

INTRODUCTION:

Transcriptional regulation is a crucial step for precise gene expression in living cells. Many factors, such as chromosomal modification, transcription factors (TFs), and non-coding RNAs, participate in this complicated process[1-3]. Among these factors, TFs contribute to the specificity of transcriptional regulation by recognizing and binding to specific DNA sequences known as promoters or enhancers and subsequently recruiting other functional proteins to activate or repress transcription[4-7]. How these TFs manage to search for their target sites in the human genome and interact with DNA coated with histones and non-histone DNA-binding proteins has perplexed scientists for decades. In the past few years, several classical models for the target

search mechanism of TFs have been built to describe how they "slide," "hop," "jump," or "intersegment transfer" along the DNA chain[8-11]. These models are focused on the searching behavior on the DNA of one single TF molecule. However, recent studies show that some TFs undergo liquid-liquid phase separation (LLPS) either alone in the nucleus or with the Mediator complex[12]. The observed droplets of TFs are associated with the promoter or enhancer regions, highlighting the role of biomolecular condensate formation in transcription and the three-dimensional genome[13-15]. These biomolecular condensates are linked to membrane-lacking compartments *in vivo* and *in vitro*. They are formed via LLPS, in which modular biomacromolecules and intrinsically disordered regions (IDRs) of proteins are two main driving forces of multivalent interactions[16]. Thus, TFs not only search DNA but also function synergistically within these condensates[4,17,18]. To date, the biophysical property of these transcription condensates on DNA remains unclear.

Therefore, this study aimed to apply a single-molecule method—DNA Curtains—to directly image the formation and dynamics of the transcription condensates formed by TFs on DNA *in vitro*. DNA Curtains, a high-throughput *in vitro* imaging platform to study the interaction between proteins and DNA, has been applied in DNA repair[19-21], target search[22], and LLPS[17,23,24]. The flowcell of DNA Curtains is coated with biotinylated lipid bilayers to passivate the surface and allow the biomolecules to diffuse on the surface. The nanofabricated zig-zag patterns limit the movement of DNA. Biotinylated Lambda DNA substrates can align along the barrier edges and be stretched by the oriented buffer flow. The same starting and ending sequences of all the molecules allow the tracking of the protein on DNA and describe the position distribution of the binding events[25,26]. Moreover, the combination of DNA Curtains with total internal reflection fluorescence microscopy (TIRFM) helps minimize the background noise and detect signals at a single-molecule level. Thus, DNA Curtains could be a promising method to investigate the dynamics of transcription condensate formation on DNA motifs. This paper describes the example of an FUS/EWS/TAF15 (FET) family fusion oncoprotein, EWS-FLI1, generated by chromosomal translocation. Lambda DNA containing 25× GGAA—the binding sequence of EWS-FLI1[27]— was used as the DNA substrate in the DNA Curtains experiments to observe how EWS-FLI1 molecules undergo LLPS on DNA. This manuscript discusses the experimental protocol and data analysis methods in detail.

PROTOCOL:

1. Preparation of the lipid bilayer master mix

1.1. Rinse glass vials with double-distilled water (ddH_2O) and 99% ethanol and dry them in a 60 °C drying oven.

1.2. Make the lipid master mix by dissolving 1 g of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 100 mg of polyethylene glycol-reacted (PEGylated) lipids (18:1 of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (PEG2000 DOPE) and 25 mg of biotinylated lipids (18:1 of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt) (Biotinyl Cap DOPE)) into 10 mL of chloroform.

1.3. Prepare 1 mL aliquots of the lipid master mix per vial and store them at -20 °C in clean glass vials.

NOTE: Store the dissolved lipid in glass vials and cover the vial cap with parafilm during storage.

2. Preparation of liposome solution

2.1. Rinse a 2 mL glass vial with ddH_2O and 99% ethanol and dry it thoroughly in a 60 °C drying oven.

2.2. Rinse a 250 µL glass syringe with chloroform and then transfer 200 µL of the lipid master mix into the dry glass vial.

2.3. Use a nitrogen spray gun to flow N_2 gently while continuously rotating the vial in one direction.

NOTE: The N_2 will help evaporate the chloroform, and the residual lipids will form a thin film on the wall of the glass vial. Adjust the nitrogen stream to be very gentle at first and increase the flow when a white film appears in the vial; complete this evaporation process within 5 min.

2.4. Transfer the glass vial to a vacuum drying oven at room temperature (RT) for 16–24 h for complete removal of the chloroform from the lipids.

2.5. Add 2 mL of fresh lipid buffer (10 mM Tris-HCl (pH 7.5) and 100 mM NaCl) to the glass vial and dissolve the lipids at RT for 2 h. Vortex the solution several times and transfer it to a 5 mL polypropylene culture tube.

2.6. Sonicate the lipid solution on ice with a micro-tip sonicator to form small unilamellar vesicles, using the following settings: 6 s on–12 s off (20% amplitude) for 6 cycles, then 6 s on–6 s off (30% amplitude) for 3 cycles, finally 10 s on (40% amplitude).

NOTE: The temperature increase during sonication could result in the bursting of foam and destroy the liposomes. Therefore, check the state of the foam and the temperature and replenish the ice frequently.

2.7. Filter the liposomes through a 0.22 µm nylon syringe filter, aliquot, and store it at 4 °C.

NOTE: As their mobility decreases with prolonged storage, the liposomes must be used in 2–3 months or prepared freshly before use.

3. Sequence cloning and biotinylation of Lambda DNA

3.1. Insertion of the binding motifs into Lambda DNA

3.1.1. Digest the target fragment containing the binding motifs and Lambda DNA with NheI and XhoI. Ligate the target fragment with Lambda DNA using T4 ligase overnight at RT.

3.1.2. Culture *E.coli* SCV257 cells in antibiotic-free LB medium until the OD_{600} reaches 0.6. Store the bacteria at 4 °C for later use.

3.1.3. Heat the ligation solution at 65 °C for 20 min to denature the T4 ligase.

3.1.4. Thaw the Lambda phage extract, mix it with the ligated product gently by pipetting with blunt tips, and incubate the mixture at RT for 2 h.

3.1.5. Add 500 µL of SM buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 8 mM $MgCl_2$) and 20 µL of chloroform into the packaging mixture from step 3.1.4, mix well, and centrifuge the solution.

NOTE: The activity of the packaging extract can be maintained at 4 °C for prolonged periods.

3.1.6. Prepare the reaction mixture by mixing 10 µL of packaging extract with 90 µL of SM buffer (100 mM NaCl, 50 mM Tris-HCl, pH=7.5, 8 mM $MgSO_4$, filtered through a 0.22 □m filter) and 100 µL of the SCV257 cells (from step 3.1.2) together, and incubate the mixture at 37 °C for 15 min.

NOTE: The packaging solution concentration depends on the density of the phage plaques on the following day; it can be diluted or added as needed.

3.1.7. Take ~5 mL of melted top agar (22 g/L NZCYM broth with 15 g/L agar) in a 15 mL tube. As soon as the top agar cools down to ~42 °C, add the reaction mixture (from step 3.1.6) slowly using a blunt-end pipette and pour the liquid onto an antibiotic-free LB agar plate. Keep the plate in a 37 °C incubator overnight.

3.1.8. Confirm the positive plaques among the transparent phage plaques on the top agar plate by PCR. Transfer the positive plaques to a new top agar plate with a pipette and keep the plate in a 37 °C incubator overnight.

3.1.9. Add 400 µL of SCV257 cells into 400 µL of buffer containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$, inoculate one plaque into this cell mixture, and incubate it in a 37 °C shaker at 250 rpm for 15 min.

3.1.10. Add 800 µL of this suspension containing the phage plaque to 200 mL of NZCYM broth in a 2 L flask and incubate it in a 37 °C shaker at 125 rpm.

3.1.11. Measure OD_{600} of the bacterial suspension after ~4 h of cultivation. Keep in mind that the OD_{600} value will rise first and then drop to a trough at ~0.2. Stop the incubation when the OD_{600} value is about to rise again, add 500 mL of chloroform, and shake at 80 rpm for another 5 min.

NOTE: The increase in OD_{600} after the trough will occur quickly; hence, the OD_{600} value must be measured more often when it decreases to 0.3 to avoid excessive SCV257 cell growth in the culture.

3.1.12. Transfer the phage culture to a 500 mL bottle, add 11.7 g of NaCl, and shake the bottle. Incubate the bottle on ice for 10 min.

3.1.13. Transfer the suspension equally into four 50 mL tubes. Centrifuge the tubes at 12,000 × *g* for 10 min.

NOTE: All these centrifugation steps in section 3.1 need to be done at 4 °C.

3.1.14. Transfer the supernatants to four new 50 mL tubes and centrifuge again at the same speed for 5 min.

3.1.15. Collect the supernatants, add 10% (w/v) PEG8000 powder, and rotate at 4 °C to incubate for 30 min.

3.1.16. Centrifuge the suspensions from step 3.1.15 at 12,000 × *g* for 15 min to obtain the phage pellets.

3.1.17. Resuspend the pellets in 1 mL of phage dilution buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$) in four tubes and pour all 4 mL of these mixtures into a 50 mL tube.

3.1.18. Add RNase A (10 mg/mL) and DNase I (4 mg/mL) to 20 µg/mL and 5 µg/mL final concentrations and incubate at 37 °C for 30 min to degrade excess nucleic acids.

3.1.19. Add 4.5 mL of 300 mM Tris-HCl (pH 7.5), 2.76 mL of 0.5 M ethylenediamine tetraacetic acid (EDTA), 1.67 mL of 10% sodium dodecylsulfate (SDS), and 75 µL of proteinase K (10 mg/mL) into the tube, and incubate at 65 °C for 10 min.

3.1.20. Add 4.5 mL of pre-cooled 3 M potassium acetate, and incubate on ice for 10 min.

3.1.21. Centrifuge at 8,000 × *g* for 10 min, and spin the supernatant for an additional 5 min at 10,000 × *g*.

3.1.22. Add 70% volume of isopropanol into the collected supernatant (from step 3.1.21) with sufficient mixing, incubate at RT for 2 min, and then centrifuge at 8,000 × *g* for 10 min. Discard the isopropanol and spin again for 5 min.

NOTE: The DNA pellet will be smeared before the first 10 min centrifugation; the pellet will be concentrated at the bottom of the tube after the second 5 min centrifugation.

3.1.23. Wash the precipitated DNA with 2–3 mL of 70% ethanol and spin down again for 1 min. Dry the pellet at 4 °C for 2 h, and then resuspend the DNA in 500 µL of TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) overnight at RT.

3.1.24. Transfer the DNA to a 1.5 mL tube and spin it at 18,000 × *g* for 3 min. Transfer the DNA to a new 1.5 mL tube. Aliquot the purified Lambda DNA and store it at -20 °C.

3.2. Biotin-Lambda DNA preparation

3.2.1. Melt 50 µL of purified Lambda DNA with cloned motifs at 65 °C for 10 min.

NOTE: As Lambda DNA is ~48 kbp long, it must be heated before pipetting to avoid DNA breaks.

3.2.2. Mix 50 µL of Lambda DNA, 1 µL of Biotin primer (5' - (Phos) - AGG TCG CCG CCC - BIOTEG – 3') (100 µM) and 54 µL of ddH_2O together. Co-incubate the mixture at 65 °C for 5 min and leave it on the bench to cool down to RT.

3.2.3. Add 12 µL of 10x T4 ligase buffer and 3 µL of T4 ligase, and incubate at RT for several hours or overnight.

3.2.4. Add an equal volume of phenol-chloroform (1:1) mixture to the ligation product, mix well immediately, and centrifuge at 12,000 × *g* for 2 min at RT.

3.2.5. Transfer the upper aqueous phase containing the DNA carefully to a new tube, add an equal volume of chloroform, mix well, and then centrifuge at 12,000 × *g* for 2 min.

3.2.6. Transfer the upper aqueous phase to a new tube, add an equal volume of isopropanol and 10% of the volume of 3 M sodium acetate, and incubate at RT for 2 min. Centrifuge the mixture at 12,000 × *g* for 5 min.

3.2.7. Remove the supernatant, wash the pellet with 75% ethanol, and then centrifuge at 12,000 × *g* for 5 min.

3.2.8. Remove the 75% ethanol, air-dry the DNA at RT for 10 min, and use 120 µL of TE buffer to dissolve the DNA.

3.2.9. Add 60 µL of Buffer A (30% (w/v) PEG8000 and 30 mM $MgCl_2$) to 120 µL of the solution from step 3.2.8, and rotate at 4 °C for 20–24 h.

3.2.10. Centrifuge the mixture at 18,000 × *g* for 5 min, and remove the supernatant.

3.2.11. Use 180 µL of pre-cooled 75% ethanol to wash the pellet, and repeat step 3.2.10.

3.2.12. Dry the pellet at RT, and use 100 µL of TE150 buffer to dissolve the DNA.

4. Nanofabricated zig-zag barriers

4.1. Clean slides in acetone for 20 min by sonication and transfer them to a new glass container with 1 M NaOH for sonication for another 20 min. Mix 90 mL of H_2SO_4 with 30 mL of H_2O_2, and immerse the slides in this mixture for 30 min. Rinse the slides with acetone and isopropanol and dry the slides with N_2.

NOTE: Mixing H_2SO_4 and H_2O_2 is an exothermic process; take adequate safety precautions during the operation.

4.2. Coat the cleaned slide with polymethylmethacrylate (PMMA) 25 kDa, PMMA 49.5 kDa, and the top layer of conductive protective coating in succession. Use electron beam lithography to write the zig-zag barriers (**Figure 1A**).

4.3. Wash the slides with ddH_2O to remove the conductive protective coating and dry the slides with N_2.

4.4. Deposit a 30 nm layer of chromium (Cr) using a magnetron sputtering machine and remove the remaining PMMA layers with acetone.

NOTE: One flowcell can be reused for more than 20 DNA Curtains experiments. To reuse the flowcell, wash it with ethanol, detergent, and NaOH.

5. Purification of EWS-FLI1 protein

NOTE: The observation of 500 nM EWS-FLI1 on Lambda DNA with 25× GGAA motifs serves as a good example for the application of DNA Curtains to condensate formation. EWS-FLI1 is a fusion protein combining the N-terminal of EWSR1 (1–265) and the C-terminal of FLI1 (220–453). An mCherry tag was fused to the N-terminal of the EWS-FLI1 fusion protein for visualization.

5.1. Clone the EWS-FLI1 gene into a reconstituted pRSF vector or any other suitable prokaryote expression vector.

5.2. Transform the plasmid encoding 7x His-mCherry-EWSFLI1 into the *E.coli* BL21(DE3) strain; grow a single colony in 5 mL of LB medium at 37 °C overnight. When the OD_{600} value reaches 0.6–0.8 after transferring to 2 L of LB medium, add 0.5 mM IPTG and shake the cell culture at 18 °C for 16–18 h.

5.3. Centrifuge the culture to remove the supernatant and resuspend the cells with lysis buffer (50 mM Tris-HCl, pH 7.5; 1 M KCl; 1 M urea; 10 mM imidazole; 1.5 mM beta-mercaptoethanol (β-ME); and 5% glycerol).

5.4. After sonication and centrifugation at 18000 × *g* for ~30 min, load the supernatant onto Ni-NTA resin equilibrated in a 5-fold volume of lysis buffer, and then wash the resin with a 10-fold volume of washing buffer (50 mM Tris-HCl (pH 7.5), 1 M KCl, 1 M urea, 40 mM imidazole, 1.5 mM β-ME, and 5% glycerol).

5.5. Elute the bound protein with 15 mL of elution buffer (50 mM Tris-HCl (pH 7.5), 1 M KCl, 1 M urea, 500 mM imidazole, 1.5 mM β-ME, and 5% glycerol) and concentrate the elution to ~500 µL.

5.6. Load the concentrated protein solution onto a gel filtration column and analyze the products by SDS-polyacrylamide gel electrophoresis. Quickly freeze the purified protein in liquid nitrogen and store at -80 °C.

6. Preparation of a DNA Curtains flowcell

6.1. Prepare a flowcell with zig-zag nanofabricated barriers for a DNA Curtains experiment and determine the direction of flow. To do this, connect the input and output tubes in the correct direction.

6.2. Wash the flowcell with 2.5 mL of lipid buffer using two 3 mL syringes. Ensure there is no bubble in the flowcell.

6.3. Remove the syringe from the output, and inject 1 mL of the liposome solution (40 µL of liposome stock from section 2 and 960 µL of lipid buffer) three times with 5 min incubation after each injection.

6.4. For the healing step, wash the flowcell with 2.5 mL of lipid buffer slowly, and incubate at RT for 30 min.

NOTE: The healing time can be longer if necessary; if any bubbles appear, perform double-healing by repeating steps 5.3 and 5.4 to remove the bubbles.

6.5. Prepare 30 mL of bovine serum albumin (BSA) buffer (40 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mg/mL BSA for surface passivation). After 30 min of healing, wash the flowcell with 2.5 mL of BSA buffer from the output side.

NOTE: The BSA stock is a 20 mg/mL solution stored at 4 °C and must be used within 1–2 weeks. The BSA concentration in the buffer can be adjusted according to the surface condition.

6.6. Inject 800 µL of streptavidin buffer (10 µL of a 1 mg/mL streptavidin stock, 790 µL of BSA buffer) into the flowcell from the input side in two steps with 10 min of incubation after each step.

NOTE: The streptavidin anchors the DNA onto the lipids due to its multivalency by bridging

between the biotinylated lipids and the biotinylated DNA.

6.7. Wash the flowcell with 2.5 mL of BSA buffer to remove all free streptavidin molecules.

6.8. Dilute the Biotin-Lambda DNA with cloned motifs from section 3 using BSA buffer (2.5 µL of DNA and 998 µL of BSA buffer). Inject Lambda DNA 4 times slowly at 5 min intervals.

6.9. Turn on the microscope and the scientific Complementary Metal Oxide Semiconductor (sCMOS) system during the injection. Wash the tubing with 10 mL of ddH_2O. Rinse the prism and the tubing connector with water, 2% liquid cuvette cleaner, and 99% ethanol. Prepare the imaging buffer by adding KCl and double-stranded DNA dye into the BSA buffer to obtain final concentrations of 150 mM and 0.5 nM, respectively, and take up at least 20 mL of the imaging buffer into a 30 mL syringe.

6.10. Set up the flowcell on the microscope stage and connect it to the microfluidic system.

6.11. Use a flow rate of 0.03 mL/min to flush the DNA molecules to the barrier for 10 min, incubate for 30 min with the flow stopped, and then switch it off to let the DNA diffuse laterally.

NOTE: The purpose of this 30 min incubation is to let the DNA molecule distribute more evenly in front of the barrier through simple diffusion because DNA molecules tend to accumulate on the side of the barrier that is closed to the input where they are flushed in. The incubation time can be adjusted as necessary.

7. Imaging of EWS-FLI1 condensation formation on DNA Curtains

7.1. Open the imaging software, find and mark the positions of the 3 × 3 zig-zag patterns under bright-field.

7.2. Turn on the flow at 0.2 mL/min to stain the DNA with the double-stranded DNA dye for 10 min.

7.3. Dilute mCherry-EWS-FLI1 with the imaging buffer to the concentration of 100 nM in 100 µL in the tube.

7.4. Load the protein sample through the valve with a 100 µL glass syringe, and change the flow rate to 0.4 mL/min.

7.5. Turn on the 488 nm laser and pre-scan each region to check the DNA distribution state; select the region in which the DNA molecules distribute evenly. Set the laser power to 10% for the 488 nm laser and 20% for the 561 nm laser. Use the power meter to measure the real laser power near the prism: 4.5 mW for the 488 nm laser and 16.0 mW for the 561 nm laser.

7.6. Image acquisition

7.6.1. Start acquiring images at 2 s intervals with both 488 nm and 561 nm lasers simultaneously.

7.6.2. Change the valve from manual mode to injection mode to let the imaging buffer flush the protein sample into the flowcell after 60 s.

NOTE: This process will take ~30 s, and the field-of-view will be covered with mCherry signals as soon as the EWS-FLI1 proteins reach the flowcell.

7.6.3. To remove free EWS-FLI1, keep washing the flowcell with the imaging buffer for 5 min with only the 561 nm laser switched on. Stop the flow and incubate at 37 °C for 10 min.

7.6.4. Turn on the flow at 0.4 mL/min to let the DNA extend, and acquire images at 2 s intervals between different frames to obtain high-throughput data of EWS-FLI1 condensate formation.

8. Intensity analysis for mCherry-EWS-FLI1

8.1. Import the data as image sequences into ImageJ software, pick out the puncta at 25× GGAA sites in an 8× 8 pixels square, and save the image in .tif format.

8.2. Calculate the intensity as the summation of the intensity in the 8×8 pixels square, including the whole puncta, and remove the background by subtracting the intensity of background in an area of the same size near the 8 x 8 pixels square.

REPRESENTATIVE RESULTS:

The schematic of DNA Curtains is shown in **Figure 1A, Figure 1B**, and **Figure 1D**. The cloned target sequence containing 25 uninterrupted repeats of GGAA is found in the *NORB1* promoter in Ewing sarcoma. This target sequence is crucial for EWS-FLI1 recruitment[28]. EWS-FLI1 molecules were visualized by detecting the mCherry-labeled EWS-FLI1 signals obtained with a 561 nm laser (**Figure 1C** and **Figure 1E**). After a DNA Curtains experiment was set up, the *in vitro* formation of biomolecular condensates of EWS-FLI1 at the 25× GGAA sites of the DNA substrate could be directly visualized (**Figure 1B–E**). The specificity of the mCherry-EWS-FLI1 used in DNA Curtains was confirmed by an electrophoretic mobility shift assay using a DNA template containing 25× GGAA and without GGAA separately (**Figure 2A**). Additionally, the concentration of EWS-FLI1 was titrated from 20 nM to 500 nM, and the intensity of the puncta at the 25× GGAA sites was determined. Compared with the intensity of mCherry-FLI1DBD, the intensity of EWS-FLI1 increased dramatically, whereas the change in the intensity of FLI1DBD was negligible when the proteins were saturated to cover the 25× GGAA sites. Therefore, these results strongly suggest that EWS-FLI1 formed condensates on DNA (**Figure 2B–E**).

FIGURE AND TABLE LEGENDS:

Figure 1: EWS-FLI1 condensate formation on Lambda DNA containing 25× GGAA motifs. (**A**) Schematic of DNA Curtains. **(B** and **D)** Two strategies for detecting EWS-FLI1 condensates (500 nM) assembling on Lambda DNA: (**B**) Keep washing for 10 min; (**D**) incubate for 10 min. **(C** and **E)**

Representative wide-field total internal reflection fluorescence microscopy image of DNA Curtains: (**C**) Detection strategy in **B**; (**E**) Detection strategy in **D**. **C (ii)** and **E (ii)** DNA is zoomed in to show the distinct puncta formed on a single DNA substrate. DNA substrates are Lambda DNA with 25× GGAA motifs. Numbers "1, 2, 3, 4" represent different positions of puncta on one Lambda DNA, and "3" is where the 25× GGAA microsatellite sequence was cloned. Scale bars = 4.9 µm (**C, E (i)**) and 0.7 µm (**C, E (ii)**). This figure has been modified from [24]. Abbreviation: dsDNA = double-stranded DNA.

Figure 2: Binding events of the detached domain of mCherry-EWS-FLI1 on 25× GGAA repeats. (**A**) (i) mCherry-EWS-FLI1. (ii) Electrophoretic mobility shift assay of mCherry-EWS-FLI1: 306-bp dsDNA labeled with 5' Quasar670 was incubated with mCherry-EWS-FLI1 at different concentrations under room temperature for 30 min in reaction buffer containing 40 mM Tris-HCl (pH 7.5), 150 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 0.2 mg/mL bovine serum albumin. The samples were loaded and run on 1.3% agarose gel for 25 min, 120 V. (**B–D**) Wide-field total internal reflection fluorescence microscopy images of 25× GGAA with DNA Curtains after incubation with 500 nM mCherry (**B**), 500 nM mCherry-EWSLCD (**C**), or 500 nM mCherry-FLI1DBD (**D**). (**E**) Intensity distribution of EWSFLI1 (cyan) and FLI1DBD (black) signals at the target site (25× GGAA) region vs. protein concentration. This figure has been modified from [24]. Abbreviations: LCD = low-complexity domain; DBD = DNA-binding domain.

DISCUSSION:

As single-molecule approaches are extremely sensitive to the contents of the reaction system, extra effort must be invested to ensure good quality of all the materials and solutions during the DNA Curtains experiments, especially the lipids prepared in sections 1 and 2 and the buffers used in section 5. Reagents of higher purity must be used to prepare buffers, and buffers must be freshly prepared for the single-molecule assay

When 500 nM mCherry-labeled EWS-FLI1 was flushed into the chamber, several magenta puncta appeared on Lambda DNA containing the 25× GGAA sequences. Notice that there was a consecutive non-specific distribution of magenta signals throughout the entire DNA even after 10 min of washing with blank buffer (**Figure 1B,C**). Interestingly, EWS-FLI1 molecules were rearranged on DNA during the 10 min incubation without the buffer flow and gathered into several puncta (**Figure 1D,E**). One of these puncta was formed at the cloned 25× GGAA site, while all others were formed in the regions containing a high density of consecutive GGAA motifs. This phenomenon strongly suggests that the no-flow incubation procedure allows EWS-FLI1 molecules to search the loci and assemble on DNA faster.

Several control experiments must be performed to clarify how these condensates formed on DNA Curtains. We purified mCherry, mCherry-EWSLCD, and mCherry-FLI1DBD and followed the same procedure to inject these proteins into the chamber. Neither mCherry (**Figure 2B**) nor mCherry-EWSLCD (**Figure 2C**) left any signals on DNA, indicating that the FLI1DBD of EWS-FLI1 was necessary for the interaction with DNA. To confirm that phase separation occurred in DNA Curtains at such low protein concentrations, the concentration of mCherry-EWS-FLI1 was titrated from 25 nM to 500 nM, and the intensity of each puncta on one DNA molecule was determined

at the clone site (**Figure 2E**). A comparison with the intensity of fusion TF FLI1DBD labeled with mCherry revealed that although the puncta intensities of EWS-FLI1 and FLI1-DBD were similar at low protein concentrations, the intensity of EWS-FLI1 increased dramatically while that of FLI1DBD remained low even when the concentration reached 500 nM. These results suggest that EWS-FLI1 molecules bind to the 25× GGAA sequence and assemble into a condensate on it through LCD interactions. A single FLI1DBD can bind 2x GGAA motifs, and higher-order oligomers bind to highly repetitive low-affinity sequences[27]. The mCherry-FLI1DBD signal on the 25× GGAA sequence was from a protein cluster rather than an individual protein molecule. Although mCherry-FLI1DBD could bind the 25× GGAA sites, it failed to assemble as a condensate on DNA, confirming that the LCD-LCD interaction was necessary for phase separation (**Figure 2D,E**).

Single-molecule methods enable researchers to study the dynamics inside transcription factor condensates. The DNA Curtains method has some advantages compared with other single-molecule methods such as single-molecule fluorescence resonance energy transfer (smFRET)[29], super-resolution imaging[30], and optical tweezer[31,32]. First, the DNA Curtains method allows for the reconstitution of the transcription machinery on long genomic DNA *in vitro* and the real-time observation of transcription condensate formation with high-throughput data acquisition. Second, aligned DNA molecules allow the mapping of the position of condensates on each DNA strand. Thus, the preferred DNA sequences for puncta formation can be easily determined.

Moreover, long-term acquisition is feasible with DNA Curtains, allowing for the measurement of the on-rate (k_{on}) and off-rate (k_{off}) of one punctum. Nevertheless, DNA Curtains has some inherent technical defects, necessitating the evidence from different methods to be collectively examined. On the one hand, the resolution of DNA Curtains can only reach 0.18 μm or ~1,000-bp because the long Lambda DNA template has a restriction with respect to the diffraction limit, which can hinder the differentiation of two neighboring fluorescence signals. On the other hand, the flow is used to extend double-strand DNA (dsDNA), and the force applied on the biomolecule may influence the diffusion property of the proteins binding to the DNA. Double-tethered DNA Curtains can anchor both ends of dsDNA and record the movement of proteins without flow, which is a noteworthy solution[33]. To summarize, deepening our understanding of the dynamic assembly of biomolecular condensates on DNA in real-time will shed light on not only the biophysical mechanism of LLPS but also on the basic biology of LLPS-related cellular processes, such as gene transcription regulation[24].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no conflicts of interest.

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597

Figure 1

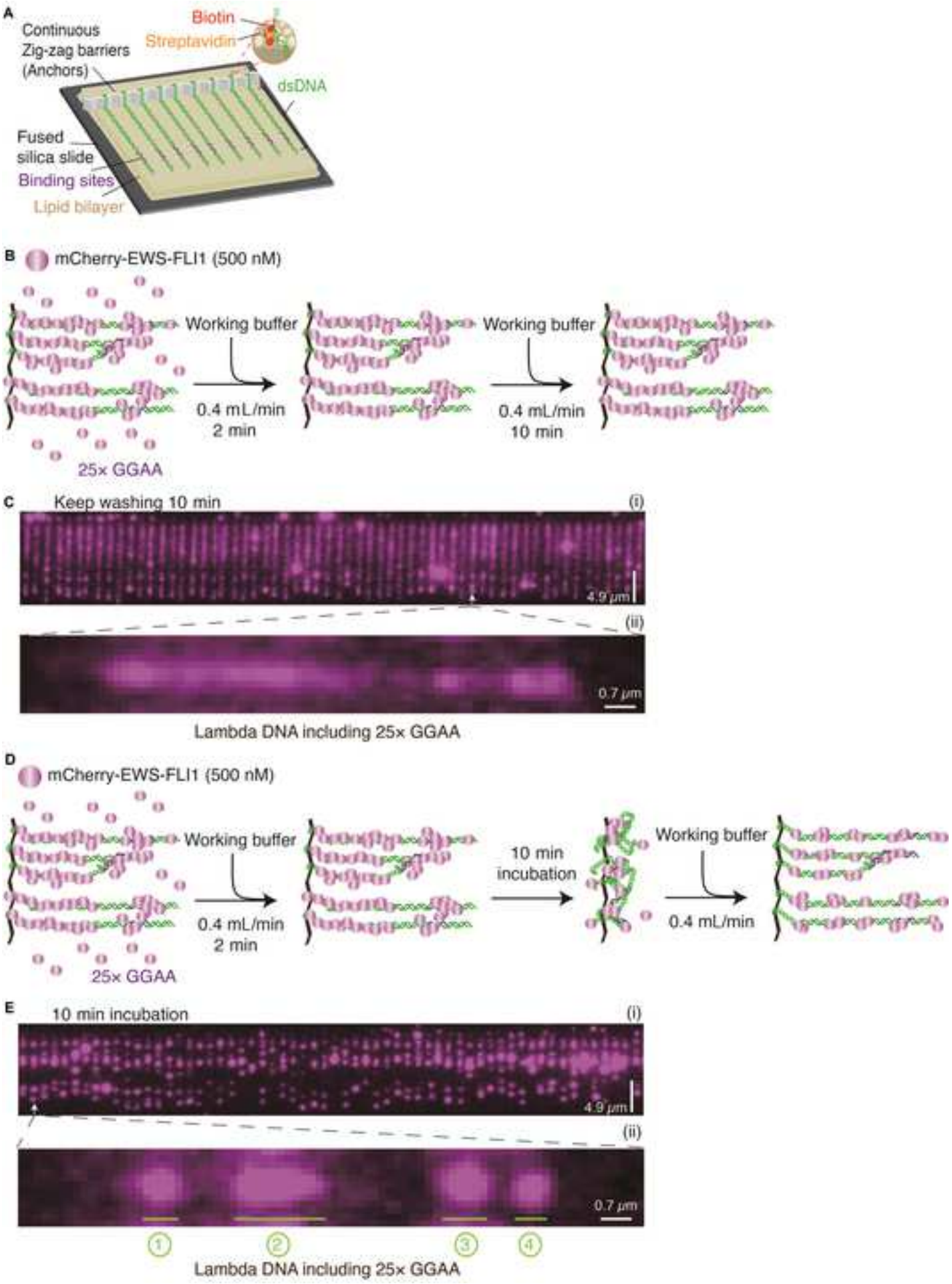
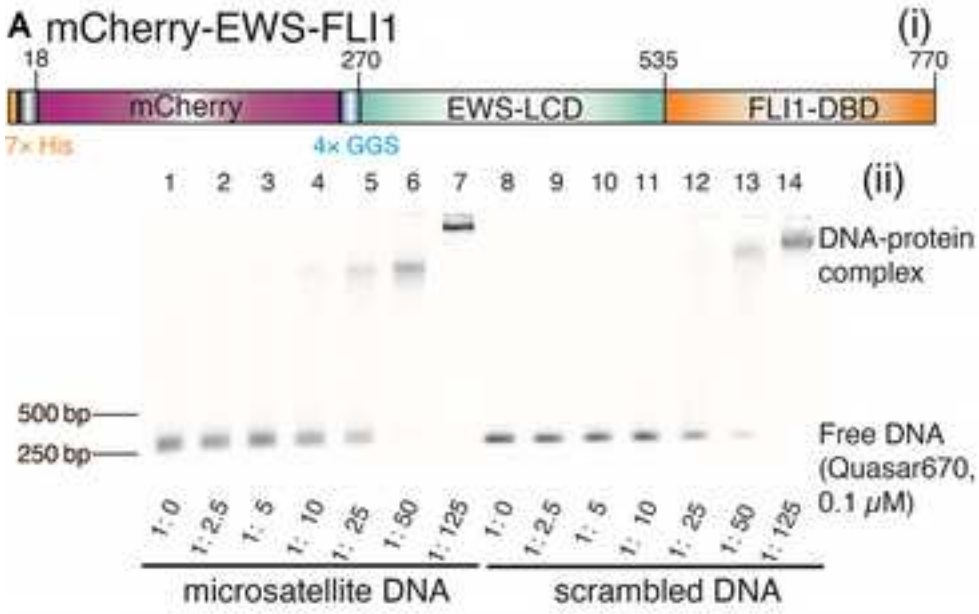
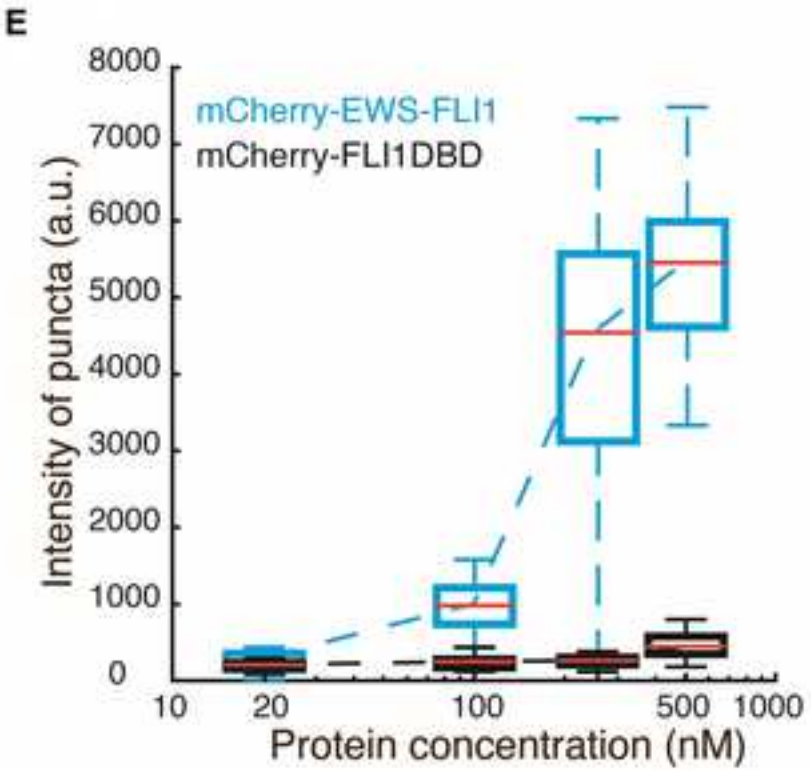


Figure 2





Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please provide an email address for each author.
3. Please increase the word count of the abstract to be 150-300 words.
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Hellmanex; YOYO-1 etc

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
8. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.
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been modified from [citation]."
11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.
13.Please add all materials to your Table of Materials (e.g., Hellmanex) to your Table of Materials and then sort the Materials Table alphabetically by the name of the material.
We thank all these editorial comments, and we revised the manuscript by following all these comments.

Reviewer #1:
Manuscript Summary:
In the manuscript by Zuo, authors describe a method of imaging EWS-FLI1 protein forming condensates on DNA. They provide details of visualizing the molecular interactions by using DNA curtains and total internal reflection fluorescence microscopy. Overall, the manuscript provides enough technical details to perform DNA curtain experiments and combined with video recording, users will be able to perform similar experiments with provided instructions. Several topics need to be addressed before manuscript can be published.
We thank the reviewer for these important comments and questions, and we added the control experiments and more details about DNA Curtains into the new manuscript. We used the "Track changes" function of Microsoft Word to track all changes in the new manuscript.

Major Concerns:
1. Authors have chosen the imaging of EWS-FLI1 interaction with DNA for their title and the main representative data. However, they do not provide any information about the source of the protein. EWS-FLI1 is not listed in the materials table. That is extremely unlikely that the EWS-FLI1 protein is available as a commercial product. Therefore, it is critical to describe how the EWS-FLI1 protein was purified or where was it acquired from. The quality of the data will be dependent on the quality of the protein. Authors can have the option of writing the paper on "DNA curtains" and not mention EWS-FLI1. They can use a different commercially available DNA binding protein as an example.

We thank the reviewer for this comment. EWS-FLI1 we used here is a fusion protein from FET family, and we inserted the gene into an *E.coli* expression plasmid, then the protein was purified from *E.coli* cells *in vitro*. The fused sequence of EWS-FLI1 and purification method have already added into the methods part of the manuscript (page 6- page 7).

2. It is likely that EWS-FLI1, being an intrinsically disordered protein, will have tendency to undergo liquid-liquid phase separation. However, since there is no experimental validation of phase separation happening in the DNA curtains, authors should refrain from implying that the observed condensates have this property. Since the selected method cannot accurately measure how many EWS-FLI1 proteins are present on a single DNA molecule, it is not even clear if the observed signal is coming from a protein condensate or individual protein molecules.

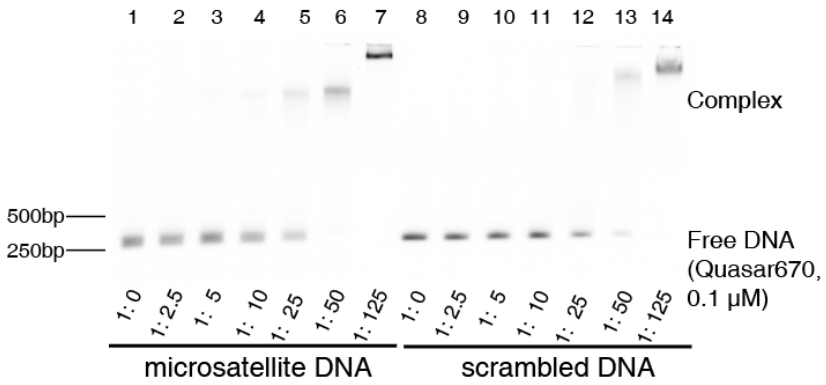
We thank the reviewer for this comment. In order to confirm that the LLPS happen on DNA Curtains with such low protein concentration, we titrated the concentration of mCherry-EWS-FLI1 from 25 nM to 500 nM and determined the intensity of each puncta on one DNA molecule at the clone site (Fig 2d). Compared with the intensity of fusion TF FLI1DBD labeled with mCherry, the puncta intensities of EWS-FLI1 and FLI1DBD were similar at low protein concentrations, nevertheless, the intensity of EWS-FLI1 increased significantly while that of FLI1DBD remained low even the concentration reached 500 nM. These evidence strongly suggested EWS-FLI1 not only bind on the 25x GGAA repeats, but also concentrated on it through LCD interactions. Besides, as it has been well-studied that single FLI1DBD can bind 2x GGAA motifs or higher order oligomers binds to highly repetitive low-affinity sequences[1], the signal of mCherry on 25x GGAA sequence was from a protein cluster rather than individual protein molecule.

3. Since there is no data on EWS-FLI1 or the condensates searching on DNA, the title is misleading. DNA curtains can provide such data but the manuscript does not provide any details on how to perform the specific experiment of data analysis nor it provides any representative data for it. Title may be revised to better reflect the true content of the manuscript or additional text may be added to support analysis of the protein searching on DNA.

We thank the reviewer for this question. The reason why we promote the hypothesis of 3D or 2D search model of EWS-FLI1 condensate is because we observed the difference of the condensate distribution before and after incubation. However, single-tethered DNA Curtains requires flow to extend Lambda DNA, so that we cannot acquire the whole incubation process during which the DNA molecule was not well-extended. We have changed the title because we cannot provide more direct data to prove our hypothesis.

4. To prove that the observed interactions are specific the representative data has to have a negative control DNA. The best negative control would have been the same Lambda DNA containing mutated GGAA sites but the least could have been Lambda DNA without any GGAA sites. The experiment also needs a negative control protein, which can be another intrinsically disordered protein transcription factor with the same mCherry label.

We thank the reviewer for this comment. We have mapped the GGAA distribution on Lambda DNA and there are more than 400 GGAA motifs except for the cloned site, so it is hard to remove all the GGAA sites out of Lambda DNA. In order to confirm the specificity of mCherry-EWS-FLI1, we conducted the electrophoretic mobility shift assay (EMSA) using DNA template containing 25x GGAA and without GGAA separately (Response Fig1).



Response Fig.1. EMSA of mCherry-EWS-FLI1. 306-bp dsDNA labeled with 5' Quasar670 was incubated with mCherry-EWS-FLI1 in different concentrations under room temperature for 30 min in reaction buffer containing 40 mM Tris-HCl (pH 7.5), 150 mM KCl, 2 mM $MgCl_2$, 1 mM DTT and 0.2 mg/mL BSA. The samples were loaded and run on 1.3% agarose gel for 25 min, 120 V. Adapted with permission from Reference [2].

5. There is no information on data analysis. Manuscript should provide guidance to readers on how to evaluate simple protein binding versus protein searching on DNA.

We thank the reviewer for this suggestion. Since the resolution of DNA Curtains doesn't allow us to distinguish a single mCherry signal in the flowcell, and EWS-FLI1 binds on the DNA very quickly once after it reaching into the flowcell, instead we injected high concentration (for single-molecule experiments) of EWS-FLI1 to observe the condensate formation through the change of puncta formed on target. We have added the protocol of intensity analysis of mCherry-EWS-FLI1 condensate on DNA in the new manuscript (page 7).

Minor Concerns:

1. Discuss section should include a paragraph on proper negative and positive controls for this method.

We thank the reviewer for this comment. We have added the related controls for this method to prove that the condensate formed on DNA is resulted from both the interactions between EWS-FLI1 and the interactions between proteins (page 8, Fig 2 a-c).

2. Discussion section should contain a paragraph, comparing DNA Curtains to others biophysical methods that provide data on direct binding of proteins and DNAs and list advantages and limitations of the method.
We thank the reviewer for this suggestion. This discussion was added in the 4th paragraph in the original manuscript (page 8).

3. Page 2, Line 55. The sentence indicate that flow cell is coated with biotinylated lipid bilayers. However, the lipid bilayer is not biotinylated. The lipid bilayer contains streptavidin protein. What is biotinylated is the DNA. The difference should be clarified and the misleading sentence should be revised or removed.
We thank the reviewer for this question. Our liposome solution contains three ingredients, one of them is biotinylated lipids, and during the flowcell preparation we inject streptavidin so that it can bind to the biotinylated lipids, while one streptavidin molecule can bind with four biotin molecules. That is the reason why the biotinylated DNA can be anchored on the lipids and move to the barrier through the interaction between biotin and streptavidin. We added this information in the new manuscript to make it clearer (page 6, 5.6).

4. Page 2, Line 66, The reference cited here (#26) is not appropriate for NR0B1 and GGAA motives. Instead of citing a review article the authors should cite the original paper that discovered the GGAA microsatellites and NR0B1 as EWSFLI1 target.
We thank the reviewer for this suggestion. We have checked the reference and cite the original paper of GGAA microsatellites in the new manuscript.

5. Page 6, Line 222, Spell out "sCMOS"
We thank the reviewer for this question. “sCMOS” is referred to “scientific Complementary Metal Oxide Semiconductor” and we have added this message in the new manuscript.

6. Figure 1e has numbers (1, 2, 3,4) under the image but it is not described anywhere what those numbers mean.
We thank the reviewer for this question. “1, 2, 3, 4” represent different positions of puncta on one Lambda DNA, and “3” is where 25x GGAA microsatellite sequence was cloned. Related information have added in the new manuscript.

7. Figure 1e(i) has 6 protein condensates but the enlarged image that was shown in Figure 1e (ii) has only 4. It is not clear if the selected enlargement is

coming from the indicated DNA line.
We thank the reviewer for this question. We are sorry for this mistake and it has been correct in the new manuscript.

Reviewer #2:
Manuscript Summary:
This manuscript provides a detailed protocol for single-molecule imaging of transcription factor condensate formation and association with DNA in vitro. It details the method used in the author´s recent publication in Nature Communications, thus fulfilling JoVe´s requirements. It describes the DNA Curtain technology using fluorescently tagged EWS-FLI1 protein binding to DNA containing a GGAA microsatellite, but in the authors´ previous publication they have already shown that the same protocol works for other proteins in a similar way. The protocol is sufficiently detailed to allow reproduction.
We thank the reviewer for these important comments and questions, and we have added more details about the flowcell preparation and materials used in this assay.

Major Concerns:
The only protocol step that needs more detail is 4.1., the preparation of a flowcell with zig-zag nanofabricated barriers on it for DNA Curtains, unless this is a ready-made, purchased product.
We thank the reviewer for this comment. Nanofabrication needs the department have a clean room and some special equipment like electron beam lithography machine. The detailed preparation methods of nanofabricated barriers have been added to the new manuscript which some professional institutes or company can help to make it (page 5, part 4).

Minor Concerns:
Some additional information in part provided in the Nature Communication study should also be included in the protocol presented here, such as the structure of the fluorescently tagged protein (N- or C-terminally tagged EWS-FLI1), and the purpose of the use of YOYO-1.
Figure 1a should indicate the input and output sides of the flow cell.
Line 274: "regions containing high intensity….." should read "regions containing high density…."
We thank the reviewer for these questions. We have added the additional information of the materials we used in DNA Curtains like protein structure (EWS-FLI1 labeled with mCherry at N-terminal) and purification (page 6), also the use of YOYO-1 to stain double-stranded DNA in the new manuscript.

References

1 Gangwal, K., Close, D., Enriquez, C. A., Hill, C. P. & Lessnick, S. L. Emergent Properties of EWS/FLI Regulation via GGAA Microsatellites in Ewing's Sarcoma. *Genes Cancer* **1**, 177-187, doi:10.1177/1947601910361495 (2010).

2 Zuo, L. *et al.* Loci-specific phase separation of FET fusion oncoproteins promotes gene transcription. *Nature Communications* **12**, 1491, doi:10.1038/s41467-021-21690-7 (2021).

Loci-specific phase separation of FET fusion oncoproteins promotes gene transcription

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Publication: Nature Communications

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