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Monitoring transcriptional activity by RNA polymerase II in vitro using single molecule co-localization

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ABSTRACT

RNA polymerase II (Pol II) transcribes eukaryotic mRNA genes. To initiate transcription, pre-initiation complexes (PICs) containing Pol II and general transcription factors (GTFs) form on the core promoters of target genes. In cells this process is regulated by transcriptional activators, co-activators, and chromatin modifying complexes. Reconstituted in vitro transcription systems are important tools for studying the enzymology and fundamental steps in the transcription reaction. In these systems, studying transcription can be complex due to the heterogeneous mixture of transcriptionally active and inactive complexes that assemble at promoters. Accordingly, we developed a technique to use single molecule microscopy to resolve this heterogeneity and distinguish transcriptionally active complexes from inactive complexes. This system uses fluorescently-labeled promoter DNA and a minimal reconstituted transcription system consisting of purified human Pol II and GTFs. Here we describe the materials, methods, and analysis required to study Pol II transcription at the single molecule level. The flexibility of our single molecule method allows for adaptation to answer diverse mechanistic questions about transcription that would otherwise be difficult to study using ensemble assays.

1. Introduction

To mount a global response to a variety of developmental and environmental stimuli, cells must embark on a journey to alter gene expression. The first step of eukaryotic gene expression is the process of transcription during which RNA polymerase II (Pol II) makes mRNA copies of the DNA in genes. In response to cellular signals, the transcription of specific genes is up- or down-regulated. The process of transcription can be divided into multiple steps, with each step subject to various means of regulation via protein-protein and protein-nucleic acid interactions. Transcription begins with the formation of preinitiation complexes (PICs). At protein coding genes, PICs are composed of Pol II, along with the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH assembled on the core promoter DNA [1]. While GTFs play a significant role in the recruitment and positioning of Pol II at the promoter, a vast array of additional transcription factors and large regulatory complexes control recruitment of GTFs and Pol II in cells [2–4]. After PICs form, RNA synthesis initiates and complexes progress through early stages of transcription, ultimately transitioning into elongation complexes, which complete synthesis of the mRNA. Ultimately, transcription is terminated and the RNA transcript and Pol II are released from the DNA.

A powerful tool to study individual steps in transcription is the use of in vitro systems; indeed, these systems have revealed valuable insight into the role of GTFs and how Pol II moves through each stage of the transcription cycle. However, an issue with in vitro Pol II transcription assays is that a low percentage of PICs are able to transcribe once provided with NTPs, whether the transcription system consists of crude nuclear extract or purified protein factors [5–7]. Typically, ~80–95% of complexes assembled on promoter DNA are inactive [5–8]. Interestingly, live cell imaging has also shown only a small fraction of the interactions between Pol II and promoter DNA result in productive transcription [9]. This heterogeneity complicates studies aimed at understanding how active PICs form and transcribe using ensemble biochemical techniques. By contrast techniques that visualize single molecules can be used to focus only on transcriptionally active PICs, thereby overcoming the complications caused in ensemble studies by the predominance of inactive complexes [8,10,11].

Here we will describe our single molecule approach to study the transcriptional activity of PICs in a reconstituted minimal human Pol II transcription system. The approach uses total internal reflection fluorescence microscopy (TIRF) for data collection and single molecule fluorescent co-localization (smFCo) for data analysis. The promoter DNA consists of a biotinylated fluorescent heteroduplex DNA construct that is assembled from three DNA strands (Fig. 1). The template strand consists of a 77 nt DNA oligo labeled with a Cy3 dye molecule on the 3′ end and a Cy5 dye molecule on the 5′ end. The non-template strand is composed of two oligos: an upstream 70 nt oligo containing a biotin molecule on its 5′

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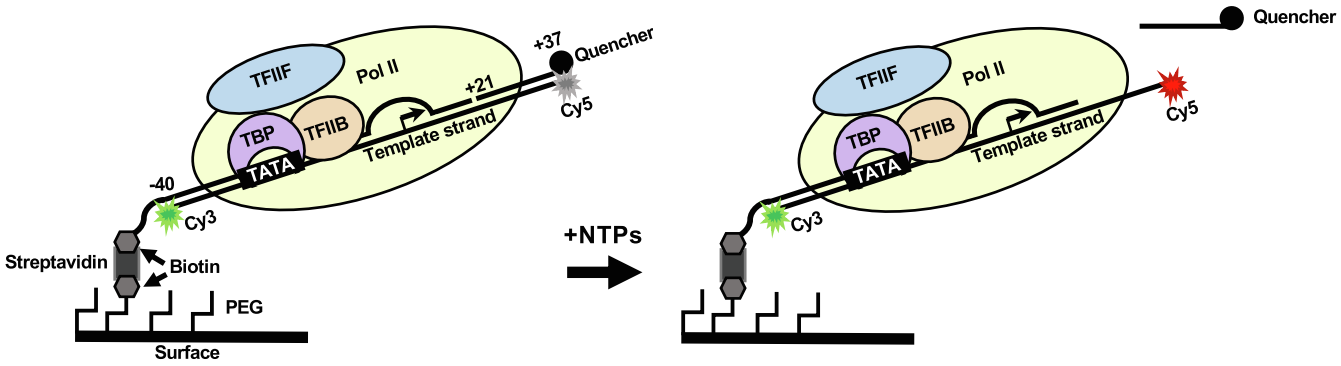

Fig. 1. Transcriptional activity of surface-immobilized PICs is observed through the co-localization of Cy3 and Cy5 fluorescence after the addition of NTPs. PICs can be immobilized on a streptavidin treated surface using a biotinylated DNA construct containing a Cy3 dye molecule and a Cy5 dye molecule. The emission of the Cy5 dye is quenched by the Iowa Black RQ-SP quencher molecule in the absences of NTPs. The addition of NTPs allows transcription, which displaces the quencher oligo resulting in the appearance of Cy5 fluorescence.

end and a 17 nt oligo containing an Iowa Black RQ-SP quencher on its 3′ end. Once assembled the promoter DNA contains a 12 nt mismatched region from positions −9 to +3 (with respect to the transcription start site at +1), which resembles the melted region of DNA that exists after PICs transition to open complexes prior to the initiation of transcription. The use of this heteroduplex DNA bypasses the need for TFIIE and TFIIH in the transcription system [12–14]. The template strand of the DNA extends contiguously from −40 to +37, whereas the non-template strand has a single nick between positions +20 and +21. Importantly, the Iowa Black RQ-Sp will quench the emission of the Cy5 dye on the template strand in the assembled construct. The three-piece construct can be immobilized on a streptavidin coated surface of an imaging slide via the biotin on the upstream 5′ end, which is connected to the non-template DNA via a 10 nt single stranded DNA linker. The minimal Pol II transcription system consists of highly purified native human Pol II, and recombinant TBP, TFIIB, and TFIIF. Upon PIC formation on immobilized DNA, the addition of NTPs allows Pol II to initiate transcription. As Pol II transcribes, the short non-template strand oligo containing the quencher is displaced, revealing emission from the Cy5 dye on the template strand (Fig. 1). By using a TIRF microscope and co-localizing the Cy3 and Cy5 signals before and after the addition of NTPs, we can identify active and inactive PICs in a population. Only active complexes will obtain a Cy5 signal in an NTP-dependent manner. The ability to visualize transcription complexes at the single molecule resolution can be used to obtain key insight into the inherent heterogeneity and potential dynamic behavior of steps in the transcription reaction.

2. Materials and methods

2.1. Required materials and equipment

The following materials and equipment are used for observing transcription at the single molecule level.

1. An objective based TIRF microscope coupled to a CCD camera(s). The optical setup will be unique to each microscope. This has no direct bearing on the method described as long as images of green excitation/emission and red excitation/emission can be separately collected. Our TIRF system is composed of a Nikon TE-2000U microscope equipped with a 1.49 NA immersion objective and a piezo nanopositioning stage, a 532 nm and a 640 nm laser that each lead to an EMCCD camera, and all accompanying mirrors, lens, and filters.
2. Solutions needed for the functionalization of microscope slides and coverslips. 1% alconox in water, 100% denatured ethanol, 100% methanol, 1 M potassium hydroxide, a mixture containing 0.38% w/v biotin-PEG-SC (MW 5 kDa, Laysan Bio Inc.) and 20% w/v mPEG-SVA (MW 5 kDa, Layan Bio Inc) dissolved in 0.1 M sodium bicarbonate, and 2% aminosilane (N-(2-aminoethyl)-3-Aminopropyltrimethoxysilane, United Chemical Technologies) in 100% methanol.
3. Equipment needed for the functionalization of microscope slides and coverslips. Precleaned glass microscope slides (25 × 75 × 1.0 mm), micro glass cover slips (VWR, No. 1.5 thickness, 24 × 40 mm, 1 oz), glass microscope slide rack with glass container, plastic cover slip rack with glass container, water bath sonicator, microwave, oven capable of heating at 110 °C, humidity chamber, Scotch permanent double-sided tape, degassing chamber, and ultrapure nitrogen gas. A food vacuum sealing system and packages of sealed desiccant packs are optional to store finished functionalized flow chambers to prevent any moisture buildup.
4. Solutions and equipment for annealing DNA oligos. 10X annealing buffer (200 mM Tris pH 7.9, 20 mM $MgCl_2$, 500 mM KCl) and a thermocycler.
5. Solutions for single molecule assays that can be prepared ahead of time and stored in aliquots at −20 °C until ready to use. 1 mg/mL stock of streptavidin dissolved in resuspension buffer (10 mM Tris pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol), 3.4 mg/mL stock of catalase dissolved in storage buffer (100 mM Tris pH 7.9, 50 mM KCl, 50% glycerol), 44 mg/mL stock of glucose oxidase dissolved in storage buffer, 10% w/v stock of D-glucose dissolved in T-50 buffer (10 mM Tris pH 7.9, 50 mM KCl, syringe filter sterilize with a 0.22 μm filter), TE-Low Buffer (10 mM Tris pH 7.9, 0.1 mM EDTA), 25 mM stock of NTPs dissolved in TE-Low buffer (25 mM each of UTP, ATP, CTP, and GTP, ThermoFisher), and 50 ng/μL stock of poly (dG:dC) competitor DNA.
6. Buffers for single molecule assays that should be prepared fresh before each experiment. DB buffer (20 mM Tris pH 7.9, 100 mM KCl, 20% glycerol, 1 mM DTT, 0.1 mg/mL BSA), RM buffer (20 mM HEPES pH 7.9, 8 mM $MgCl_2$, 1 mM DTT), 100 mM Trolox (dissolved in 110 mM NaOH), DB/RM buffer (10 mM Tris pH 7.9, 10 mM HEPES pH 7.9, 50 mM KCl, 4 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.1 mg/mL BSA), DB/RM + Trolox buffer (final concentrations are the same as DB/RM but with the addition of 3.45 mM Trolox), and Imaging buffer in DB/RM + Trolox (1.02 mg/mL of glucose oxidase, 0.04 mg/mL of catalase, and 0.83% D-glucose added to the DB/RM + Trolox).
7. Software to perform co-localization analysis of recorded emission movies. Images can be analyzed with Image J or our in-house developed IDL software that is open source. A portable version of our in-house software and the raw code are available on SourceForge, along with example data and instructions (https://sourceforge.net/projects/smf-colocalize/).

2.2. Functionalization of microscope slides and coverslips

Prior to starting, ensure that the stock bottles of aminosilane, biotin-PEG-SC, and mPEG-SVA are warmed to room temperature in the dark for at least an hour. After every use, all three chemical stock bottles

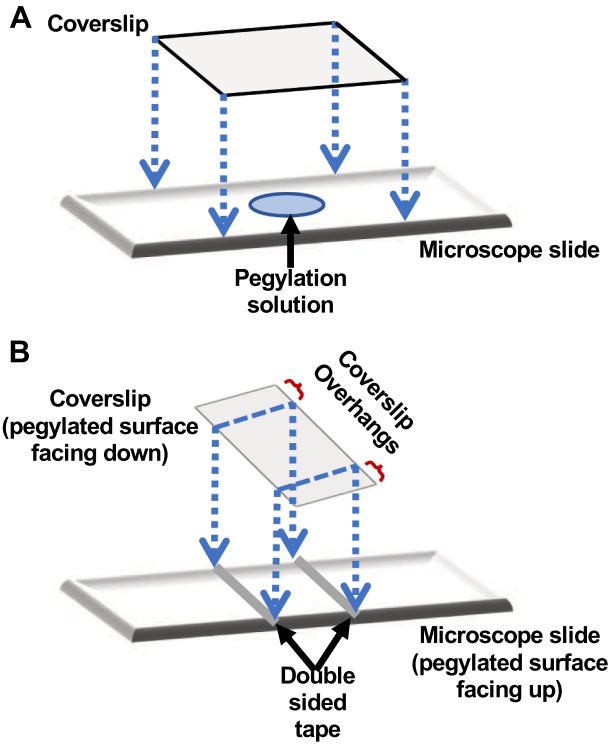

Fig. 2. Orientation of the coverslip to the microscope slide for (A) the incubation with the pegylation solution and (B) the assembly of the flow chamber.

should be degassed with ultrapure nitrogen gas before storage at −20 °C in a desiccant container. Ensure the oven for drying slides and coverslips is set to 110 °C, and the humidity chambers that will be used for the pegylation step contain the appropriate amount of MilliQ water.

2.2.1. General cleaning of slides and coverslips

1. Set up a water bath sonicator and place microscope slides and coverslips in their racks and then place the racks in their glass containers. Our racks hold 10 slides or coverslips.
2. Boil at least 1 L of 1% alconox. Add enough boiling solution to each reaction container to completely submerge the slides and cover slips. Sonicate in the water bath for 20 min.
3. Rinse the containers, slides, and coverslips with MilliQ water. Handle using the handles on the racks. Avoid directly touching the slides and coverslips. Place the racks holding the slides and cover slips back in the glass containers.
4. Add 100% denatured ethanol to the glass containers and sonicate for 60 min. Rinse the containers, slides, and coverslips with MilliQ water as stated in step 3. Place the racks back into the glass containers.
5. Add MilliQ water to the glass containers and sonicate for 5 min. Discard the water.
6. Add 1 M KOH to the containers and sonicate for 20 min. Pour the used 1 M KOH into a second set of empty glass slide containers and set aside until step 8. Rinse with MilliQ water as stated in step 3.
7. Gently rinse slides and cover slips in the racks with 100% denatured ethanol and immediately microwave for 90 s on high to dry. Place each dry rack with the slides and coverslips in a clean and dry glass container and allow to cool to room temperature (15–30 min).
8. In the meantime, sonicate the second set of empty glass containers from step 6 in 1 M KOH for 20 min. Rinse the containers with MilliQ water.
9. Once the racks with the slides and coverslips have cooled to room temperature, place them in the second set of glass containers from step 8.

2.2.2. Coating slides and coverslips with aminosilane

1. Add 100% methanol to the pre-cleaned empty set of glass containers and sonicate for 5 min to remove any residual water. Discard the methanol and set containers aside.
2. Add 100% methanol to the containers with the slides and coverslips and sonicate for 5 min to remove any residual water. Discard the methanol and transfer the racks with the slides and coverslips back to the pre-cleaned empty set of containers from step 1. Set the newly emptied containers aside.
3. Make a fresh solution of 2% aminosilane in methanol and immediately add it to the containers with the slides and coverslips. Incubate at room temperature for 10 min. Carefully sonicate for 1 min, making sure to prevent any water from mixing with the aminosilane solution. Incubate at room temperature for 10 min.
4. Discard the 2% aminosilane and rinse the containers, slides, and coverslips with 100% methanol. Place the racks with the slides and coverslips back into the containers.
5. Add 100% methanol to the containers and incubate at room temperature for 5 min.
6. Discard the methanol and rinse the containers, slides, and coverslips with 100% methanol.
7. Repeat steps 5 and 6.
8. Transfer the racks with the slides and coverslips to the oven and bake at 110 °C for 10 min.
9. Remove the slides and coverslips from the oven and let cool to room temperature before continuing to step 2.2.3.
10. Rinse the reaction containers used in steps 4–6 with methanol followed by MilliQ water. Add fresh 1 M KOH and sonicate empty containers for 20 min to remove residual aminosilane. Wash all glass containers with 1% alconox and rinse with MilliQ water before putting away for storage.

2.2.3. Pegylation of slides and coverslips

1. Make 1 mL of a 0.38% w/v biotin-PEG-SC, 20% w/v mPEG-SVA solution in 0.1 M sodium bicarbonate. This mixture should be made fresh and used immediately.
2. Add 70 μL of the mixture to the top of one microscope slide.
3. Slowly and gently place a coverslip on top of the slide to avoid bubbles. The longest edge of the cover slip should be parallel to the longest edge of the slide (Fig. 2A). Re-do the placement if bubbles form.
4. Repeat steps 2 and 3 for each microslide and coverslip pair.
5. Place all slide/coverslip pairs in a humidity chamber for 4–16 h. Any makeshift humidity chamber that can hold the slide/coverslip pairs without the pairs directly touching the MilliQ water can be used to ensure the biotin-PEG-SC and mPEG-SVA solution does not evaporate.
6. Gently take apart one slide/coverslip pair and rinse both sides of the slide and coverslip with MilliQ water. The surface of the slides and cover slips that contacted the pegylation solution should be noted with care as these surfaces will have to face one another when assembling the flow chambers. Place the slide and coverslip into racks in containers filled with MilliQ water to prevent the surfaces from drying as the rest of the pairs are rinsed with MilliQ water.
7. Repeat step 6 for each slide/coverslip pair.
8. Individually remove slides and coverslips from water and carefully dry both sides of each slide and coverslip by holding only the edges and blowing the surfaces with ultrapure nitrogen gas. Place in dry racks.

2.2.4. Assembly of flow chambers

1. Assemble flow cell chambers using double sided tape to adhere the pegylated side of a microscope slide to the pegylated side of a

coverslip (Fig. 2B). The longest edge of the coverslip is placed perpendicular to the longest edge of the slide. This will allow wicking of solutions from one end of the coverslip into the flow chamber.
2. Gently wrap each individual flow chamber with parafilm and store in a light-blocking container at room temperature. Desiccant packages can be added to the container. We recommend placing the container in a plastic bag and vacuum sealing.

2.3. DNA and protein factors for in vitro transcription

Native Pol II and recombinant TBP, TFIIB, and TFIIF are prepared as described previously [6,15–17]. The DNA construct used is generated by annealing three separate DNA oligos (Fig. 1). The sequence of the promoter has been optimized to maximize template usage [10]. The doubly labeled template strand oligo, which contains a 5′ Cy5 molecule and a 3′ Cy3 molecule, has the sequence 5′CCTGAGGTTAGTGTGAGTAGTGATTAAAGATAGTGATGAGGACGAACGCGCCCCCACCCCCTTTTATAGCCCCCCTT 3′ (Eurofins Scientific). The non-template strand upstream oligo, which contains a 5′ biotin molecule, has the sequence 5′TACGAGGAATAAGGGGGGCTATAAAAGGGGGTGGGGGCGCGAAGCAGGAGTAGACTATCTTTAATCACTA 3′ (Integrated DNA Technologies). The downstream non-template strand oligo, which contains a 3′ Iowa Black RQ-Sp quencher, has the sequence 5′CTCACACTAACCTCAGG 3′ (Integrated DNA Technologies). Oligos ordered from IDT have the option of being HPLC and/or PAGE purified; we recommend at least one of the purification options. Oligos can be gel purified by the user to increase purity prior to annealing the three-piece construct; however, this will generally result in some loss as it is difficult to recover all the oligo from the gel.

2.3.1. Annealing the 3-piece DNA construct

1. Mix 100 pmol of the template strand, 100 pmol of the non-template strand containing the biotin, and 1 nmol of the non-template strand containing the quencher to a final volume of 50 μL of 1X annealing buffer.
2. Using a thermocycler: heat for 5 min at 95 °C; cool to 60 °C at a rate of 0.1 °C per second; incubate for 60 min at 60 °C; cool to 45 °C at a rate of 0.1 °C per second; incubate for 60 min at 45 °C; cool to 4 °C at a rate of 0.1 °C per second.
3. Store annealed 2 μM DNA stock solution in small aliquots at −80 °C.

2.4. Single molecule in vitro transcription assay

Prepare the following buffers fresh prior to starting the experiment: DB, RM, DB/RM, 100 mM Trolox, and DB/RM + Trolox. 100 mM Trolox should be nutated for 30–60 min to ensure that Trolox is resuspended into solution prior to syringe filtering with a 0.22 μm filter. Add enzymes and D-glucose to DB/RM + Trolox to make the imaging buffer right before the solution is added to the flow chamber. There are two methods to immobilize the DNA on the surface, as described below in Sections 2.4.1.1 and 2.4.1.2. Either method will result in the same transcriptional activity and is based on individual preference. All protein samples should be kept frozen until needed, at which time they can be quickly thawed and diluted into the appropriate buffer. Diluted protein samples should be placed on ice but should be used soon after they are made. Once solutions are mixed and flowed into the chamber, incubation should be at room temperature.

2.4.1. Preparation of flow chambers for DNA immobilization

1. Wash the flow chamber by adding 200 μL of water. This is done by holding a piece of Whatman paper at the end of the chamber to allow excess liquid that is pipetted on the other end to wick out of the chamber. Stop once all the liquid has just entered the chamber. Repeat. Do not over wick solutions at any step. Approximately 50 μL of any solution that was added should remain within the chamber until the next solution is added to prevent drying of the chamber.
2. Wash the chamber two times with 200 μL room temperature DB/RM buffer.
3. Make a streptavidin mixture containing 0.2 mg/mL streptavidin and 0.8 mg/mL BSA diluted in DB/RM buffer to a final volume of 50 μL. Flow this solution into the chamber. Incubate for 5 min.
4. Wash the chamber two times with 200 μL DB/RM buffer, leaving buffer in the chamber when done.
5. Dilute the 2 μM stock of the three-piece DNA construct to a final concentration of 10 nM DNA in 1X annealing buffer via serial dilution. Place on ice.

2.4.1.1. Formation of PICs on the slide (option 1).

1. Prepare room temperature RM + DNA solution by adding 1 μL of 10 nM DNA to 50 μL of RM buffer.
2. Mix 50 μL of room temperature DB buffer with 50 μL of RM + DNA solution from step 1 for a final concentration of 0.1 nM DNA.
3. Flow the DB + RM + DNA mixture into the chamber and incubate for 10 min.
4. Wash the chamber two times with 200 μL DB/RM buffer.
5. Make a protein mixture on ice with a final volume of 30 μL in DB buffer and the following final concentrations: 23 nM TBP, 67 nM TFIIB, 13 nM TFIIF, and 13 nM Pol II.
6. Dilute the poly(dG:dC) competitor DNA to 0.5 ng/μL in DB/RM for a final volume of 85 μL at room temperature. Add 15 μL of the protein mixture from step 5 for a final volume of 100 μL and immediately flow the solution of protein and poly(dG:dC) into the flow chamber and incubate for 20 min.
7. Wash the flow chamber two times with 200 μL DB/RM buffer and proceed to Section 2.4.2.

2.4.1.2. Formation of PICs in an eppendorf tube (option 2).

1. Prepare a RM + DNA solution by adding 1 μL of 10 nM DNA to 50 μL of RM buffer and set aside at room temperature.
2. Make a protein mixture on ice with a final volume of 50 μL with DB buffer and the following final concentrations: 3.5 nM TBP, 10 nM TFIIB, 2 nM TFIIF, 2 nM Pol II, and 0.5 ng/μL poly(dG:dC) competitor DNA.
3. Mix the 50 μL of DNA + RM from step 1 with 50 μL of the protein mixture from step 2 and incubate in the tube for 20 min at room temperature.
4. Flow the solution from step 3 into the flow chamber and incubate for 10 min.
5. Wash the flow chamber two times with 200 μL DB/RM buffer and proceed to Section 2.4.2.

2.4.2. Imaging PICs

1. Make at least 100 μL of imaging buffer with a final concentration of 1.02 mg/mL of glucose oxidase, 0.04 mg/mL of catalase, and 0.83% D-glucose in DM/RM + Trolox buffer.
2. Immediately flow the imaging buffer into the chamber.
3. Mount the flow chamber on the stage of the TIRF microscope.
4. Excite with the 532 nm laser and collect a green emission movie with a 500 ms exposure time. For the same region, excite with the 640 nm laser and collect a red emission movie with a 500 ms exposure time. Repeat this over multiple different regions of the slide. We will refer to these as “PIC” movies. The green and red emission movies on one specific region are best obtained sequentially rather than simultaneously to avoid emission blead through into the incorrect channels. Note that how the red and green emission movies are collected (e.g. one camera, two cameras, manual switching, motorized switching, etc.) will be unique to each microscope, and has no bearing on the overall method described.

5. If the TIRF microscope is equipped with a piezo stage and nanopositioning system, it is possible to collect green and red emission PIC movies with a 500 ms exposure time over specific regions that will then be revisited in subsequent imagining steps.

2.4.3. Initiating transcription with addition of NTPs

1. Make a 100 µL solution of NTPs in DB/RM buffer by diluting the 25 mM NTP solution to a final concentration of 625 µM.
2. Flow the NTP solution into the flow chamber and incubate at least 5 min and up to 30 min to allow transcription to occur. Do not wash after the incubation.
3. Make at least 100 µL of imaging buffer with a final concentration of 1.02 mg/mL of glucose oxidase, 0.04 mg/mL of catalase, and 0.83% D-glucose in DM/RM + Trolox buffer.
4. Immediately flow the imaging buffer into the chamber.
5. Excite with the 532 nm and 640 nm lasers and collect both green and red emission "NTP" movies, respectively, with a 500 ms exposure time over different regions (as described in Section 2.4.2). It is best to do this sequentially as opposed to simultaneously.
6. If a piezo stage and nanopositioning system was used to collect PIC movies, return to the same regions and collect new green and red NTPs emission movies for a direct comparison of the same DNAs before and after the addition of NTPs.

3. Analysis and interpretation of co-localization data

The level of transcriptional activity in the system can be determined from the number of NTP-dependent co-localized pairs in the emission movies. Since each spot pair in the NTP emission movie describes a PIC that transitioned into a transcriptionally active complex after addition of NTPs, it is expected that the NTP emission movies will have significantly more spot pairs than the PIC emission movies. The greater the difference between the number of spot pairs from the NTP emission movies to that of the PIC emission movies, the greater the level of transcriptional activity being observed in the system of interest.

The following describes using our in-house software to analyze the co-localization between spots in the green and red emission movies collected for PICs and after adding NTPs. The same data analysis workflow can be performed with or without the use of a piezo stage. Detailed instructions on the technical specifics of the smF-colocalize software, use of the software, and how to generate offset files to align emission movies from two or more cameras can be found on SourceForge at https://sourceforge.net/projects/smf-colocalize/. Fig. 3 illustrates a general overview of the data analysis work flow. The following steps 1–6 are repeated for each pair of PIC and NTP movies.

1. Analyze a green emission movie and a red emission movie collected from the same region of the surface using the option *Colocalize spots in 2 images with rotated control* in the smF-colocalize software. The first input file is brought up in the software as the *left* summed image and the second input file is brought in as the *middle* summed image. The first input file is subsequently rotated 90° and shown as the third, *right* image. See Fig. 3 where green movies were the first input file and red movies were the second (panel A shows the PIC movies and panel B shows the NTP movies).
2. Apply the appropriate threshold estimates and square width setting such that the software identifies most of the spots visible in the green and red movies. The rotated *right* image will reflect the same settings used for the *left* image.
3. The software will identify all potential spot pairs between each pair of images. The output screen will reflect a value called *Vol* to describe the number of co-localized spot pairs. This is calculated from the volume of a gaussian that is fitted over a plot of X and Y locations of potential spot pairs. The greater the *Vol* value, the greater number of spot pairs and thus greater co-localization between a pair of movies.
4. If spot pairs do not co-localize well, the software will be unable to fit a gaussian over the plot of X and Y locations of potential spot pairs. The output in this case will state *No gaussian found.* For example, in Fig. 3A there was no gaussian found because in PIC movies there should be very few red spots to co-localize with green spots.

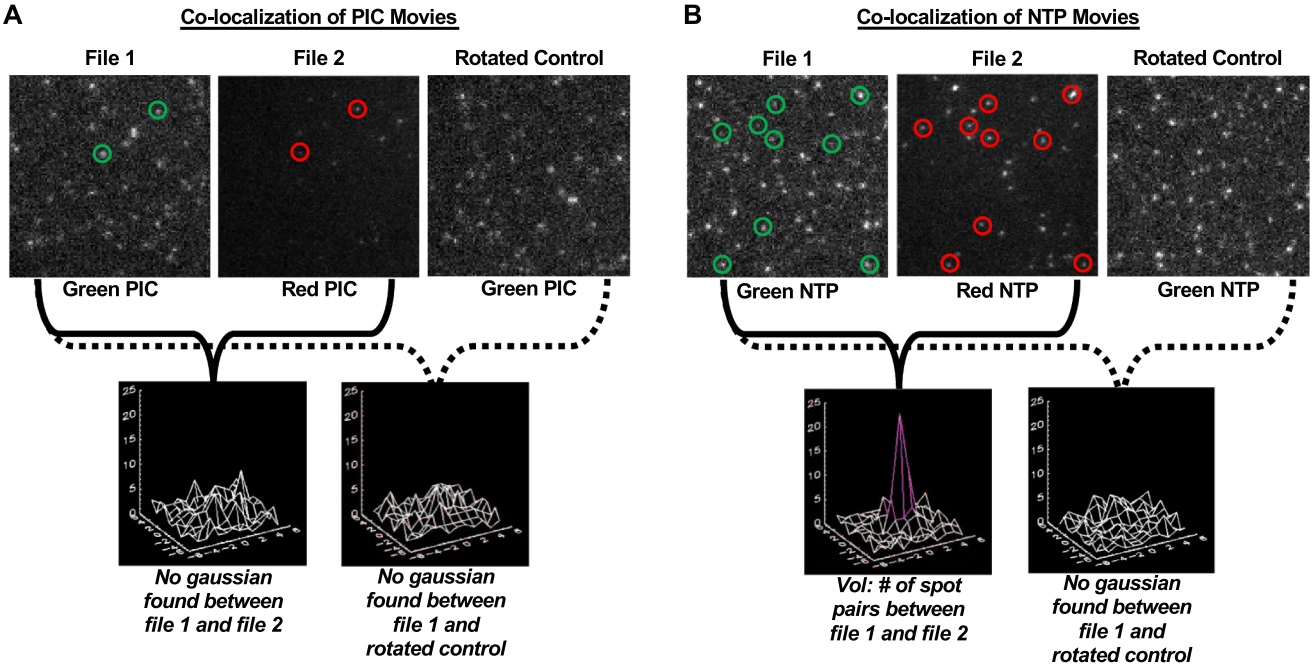

Fig. 3. Overview of the co-localization data analysis work flow of (A) PIC emission movies and (B) movies obtained after the addition of NTPs. The software identifies potential spot pairs between file 1 and file 2, and between file 1 and the rotated control. The output plots of X and Y locations of potential spot pairs visually displays the level of co-localization. The volume of a gaussian that is fitted over the plots reflects a value called *Vol,* which describes the number of co-localized spot pairs. See text for more details.

Table 1
The number of total red spots, spot pairs, and template usage increased substantially after the addition of NTPs to surface immobilized PICs, indicating robust transcriptional activity.

	PIC movies (n = 4)	NTP movies (n = 4)
Total Number of Green Spots	6414	6730
Total Number of Red Spots	662	2398
Total Number of co-localized spot pairs	298	1262
Template usage (%)	4.6	18.8

Observing a gaussian here likely indicates poor annealing of the quencher oligo. By contrast, in Fig. 3B for the NTP movies there is a co-localization gaussian for green and red spots.

5. The output from the co-localization of spots with the rotated image should typically yield *No gaussian found*. If a gaussian value is indeed calculated using the rotated image the *Vol* value should be substantially less than the *Vol* value obtained from co-localization of the *left* and *middle* images.
6. Selection of *Continue on to pair found spots* allows the user to manually check each spot pair identified by the software to either accept or reject the spot pair.

4. Results

PIC and NTP movies were collected using the method described in Section 2.4.1.2 and analyzed as described in Section 3. As shown in Table 1, the addition of NTPs to PICs caused the total number of observed red spots on the surface to increase from 662 spots to 2398 spots, a substantial increase. Importantly, the total number of co-localized spot pairs increased from 298 pairs to 1262 pairs. This reflects a high level of NTP-dependent displacement of the quencher oligo due to transcription. To quantify transcription levels, we calculated template usage, or the number of DNA templates that were transcribed (co-localized spot pairs) divided by the total number of templates (green spots). As shown in Table 1, the template usage increased from 4.6% to 18.8% in the presence of NTPs. We have previously published using this single molecule approach to perform transcription and showed that template usage was comparable to that obtained from ensemble transcription assays [10].

5. Conclusions

Here we described using a single molecule approach to directly measure the ability of Pol II to transcribe DNA after PIC formation. By using TIRF microscopy to observe PICs formed on immobilized doubly labeled fluorescent DNA, we determined the number of PICs that transition into actively transcribing complexes upon addition of NTPs. Since we can observe single active PICs within an entire population, our technique can deconvolute the level of heterogeneity that exists within a population of PIC complexes, which is not possible in ensemble assays. This approach could be used to determine how various parameters impact the fraction of active PICs within a population. For example, we previously investigated how assembly pathways impact PIC activity [10]. It is easy to image other extensions of this approach. For example, mutant GTFs could be utilized to explore how changes to the interaction interface between GTFs and Pol II/DNA modify the level of transcriptional activity. Additional factors (general or gene-specific) could be added to determine their effect. The DNA template could be constructed without a pre-melted region to evaluate how TFIIH-facilitated promoter melting impacts fractional activity of PICs. This approach could be further expanded to a three-color TIRF system to allow for the fluorescent labeling of a transcription factor. This would allow for the direct observation of the dynamic behavior of a protein during PIC formation and the subsequent transition to active complexes. The general ease of modifying the type of dye pairing used in either a two-color or three-color TIRF set up will enable testing many hypotheses and mechanistic inquiries about heterogeneity and the activity of transcription complexes.

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