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smFRET experiments of the RNA polymerase II transcription initiation complex

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ABSTRACT

Single-molecule fluorescence and in particular single-molecule Förster Resonance Energy Transfer (smFRET) is a powerful tool to provide real-time information on the dynamic architecture of large macromolecular structures such as eukaryotic transcription initiation complexes. In contrast to other structural biology methods, not only structural details, but dynamics transitions are revealed thus closing in on the underlying molecular mechanisms. Here, we describe a comprehensive quantitative biophysical toolbox which can be used for rigorous analysis of dynamic protein-nucleic acid complexes and is applied to the study of eukaryotic transcription initiation. We present detailed protocols for the purification of all essential protein components of the minimal eukaryotic transcription initiation complex. Moreover, we demonstrate how elaborate strategies for site-specific protein labeling can be used to produce complexes with dye molecules attached to arbitrary desired positions. These complexes are then used for smFRET measurements. Moreover, we describe the Nano-Positioning System (NPS) which allows us to quantitatively use the results from a network of smFRET measurements to obtain structural information. With this we provide a toolbox to answer open questions which could not be addressed using methods like X-ray crystallography or cryo-electron microscopy.

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1. Introduction

In the past decades, our mechanistic understanding of eukaryotic transcription has improved dramatically due to a plethora of experiments probing the biochemistry, structure and molecular mechanism of transcription. In particular studies using X-ray crystallography [1] or cryo-electron microscopy [2] we have obtained detailed blueprints of the large assemblies involved in transcription initiation and elongation. On the other hand single-molecule methods have been applied to investigate the dynamics of multisubunit RNA polymerases from bacteria [3–5], archaea [6,7] as well as eukaryotes [8–13]. These experiments can be divided into two classes, force spectroscopy experiments, where the kinetics of transcription elongation and its control by transcription factors such as TFIIS, TFIIF, TFIIE or even nucleosomes are investigated, or fluorescence experiments, in particular single-molecule Förster Resonance Energy Transfer (smFRET), where typically structural and conformational information is obtained. In particular, studies where distances obtained from smFRET networks are analyzed to yield both structural and dynamic information about transient states during transcription elongation or initiation are of great importance, since they provide bridging information between classical structural biology and biochemical data. The Nano-Positioning System (NPS) was developed for this purpose [14–16] and its application to transcription initiation complexes will be described here.

In the following we will give detailed protocols on how to purify and label the proteins involved in yeast minimal transcription initiation complexes, how to assemble minimal promoter open complexes, how to perform smFRET experiments on these complexes and on how to analyze data. Moreover, we provide a brief introduction into NPS and its application to obtain mechanistic insight into transcription initiation in eukaryotes.

2. Purification of proteins

In order to assemble minimal open promoter complexes for smFRET measurements the twelve subunit RNA polymerase II (Pol II[1]), TFIIF (two subunits), TFIIB and TBP have to be purified. While the latter three can be expressed recombinantly in bacteria, Pol II needs to be isolated from yeast directly. While protocols for obtaining highly pure protein material have in the past decades optimized for crystallization purposes, where large quantities of pure Pol II needed to be obtained, for single-molecule experiments a rather modest amount of Pol II can be used. However, in order to label Pol II site specifically (see Section 3.1) a number of specific variants need to be expressed. Thus, a concise protocol using equipment available in a large number of biochemical labs was developed.

2.1. Isolation of RNA polymerase II

Pol II was isolated from yeast cultures using the *Saccharomyces cerevisiae* (*S. cerevisiae*[2]) strain BJ5464 Rpb3 His-Bio (gift from P. Cramer, Göttingen). This is a protease deficient yeast strain where the subunit Rpb3 is tagged with a His-Bio tag at the amino terminal end [17]. The expression culture was inoculated using three precultures. For the first preculture 2 × 5 ml yeast extract-peptone-dextrose (YPD[3]) including 2% glucose and 0.1 mg ml^{-1} ampicillin were inoculated from a YPD agar plate and incubated at 30 °C and 160 rpm overnight. For the second preculture 2 × 20 ml and for the third preculture 1 × 1000 ml YPD was used with identical incubation procedures. The final Expression culture of 12 × 2 L was inoculated in 5 L flasks to OD_{600} = 0.3 and grown at 30 °C and 160 rpm overnight. The cells were harvested by centrifugation at 7920*g* (Fiberlite™ F9-4 × 1000y, *ThermoFisher*) and 4 °C for 15 min and washed with cold HSB 150 buffer (50 mM Tris-Cl pH 7.9, 150 ml KCl, 1 mM EDTA, 10 µl $ZnCl_2$, 10% glycerol, 10 mM DTT). Typically, 24 L yeast culture yielded 200 ml cell pellet. Cell pellets were resuspended using HSB 150 buffer including 1 × PI-mix (1 mM Phenylmethylsulfonyl fluoride (PMSF[4]), 2.7 µM Benzamidine, 2 µM Pepstatine, 0.6 µM Leupeptine) to a final volume of 450 ml and lysed using a homogenizer (PandaPLUS 2000, *GEA Niro Saovi*) at 1500 bar and 4 °C within 12 runs. If preferred cells can also be lysed using a bead beater, however the described procedure is about twofold faster. After centrifugation at 35000*g* and 4 °C for 30 min, a white colored cell pellet indicates efficient cell lysis. Following ultracentrifugation at 200,000*g* and 4 °C for 90 min, the clear aqueous phase between pellet at the bottom and viscous phase at the top was collected using a 50 ml syringe. The yield of the aqueous phase was typically 350 ml.

Protein purification was performed in a four step protocol adapted from [17]. In a first step an ammonium sulfate precipitation was employed. Fine-ground ammonium sulfate was added to the protein solution (291 g per 1 L protein solution = 50% saturation) during a time period of 60 min at 4 °C and slow stirring (150 rpm). Precipitation was allowed to continue at 4 °C and 150 rpm overnight. After centrifugation for 45 min at 35,000*g* and 4 °C, the supernatant was discarded and the cell pellet weighted. A typical yield was ≈20 g. The pellet was resuspended up to 20% saturation using HSB 0/7 buffer (50 mM Tris-Cl pH 7.9, 1 mM EDTA, 10 µl $ZnCl_2$, 10% glycerol, 7 mM Imidazole, 2 mM DTT) including 1× PI-mix on ice. After centrifugation at 35,000*g* and 4 °C for 10 min, the supernatant was subjected to further purification.

The next purification step is a Nickel affinity chromatography, where the N-terminal His tag on Rpb3 is used. For this the supernatant solution was incubated with 15 ml of Ni-NTA agarose beads (*Qiagen*, pre-washed and suspended in HSB 0/7 buffer) for 45–60 min at 4 °C. Afterwards, the mixture was loaded onto a gravity flow column (glass econo-column, *BioRad*) and the beads were washed with 5 column volumes (CV[5]) of HSB 1000/7 buffer (50 mM Tris-Cl pH 7.9, 1000 mM KCl, 7 mM Imidazole, 1 mM EDTA, 10 µM $ZnCl_2$, 10% glycerol) and 3 CV Ni7 buffer (20 mM Tris-Cl pH 7.9, 150 mM KCl, 7 mM Imidazole, 10 µM $ZnCl_2$). The elution was performed using 3 CV Ni200 buffer (20 mM Tris-Cl pH 7.9 at 4 °C, 150 mM KCl, 200 mM Imidazole, 10 µM $ZnCl_2$).

Next, anion exchange chromatography was performed using a 8 ml MonoQ 10/100 GL column (*GE Healthcare*) with 0.5 ml/min flow rate and the buffers MonoQ0 (20 mM Tris-acetate pH 7.9, 10% glycerol, 0.5 mM EDTA, 10 µM $ZnCl_2$, 10 mM DTT) and MonoQ2000 (using 2000 mM KOAc). The column was equilibrated with 4 CV of 7.5% MonoQ2000 buffer (150 mM KOAc) prior to loading. The protein solution was filtered through a 0.22 µm filter and its conductivity was adjusted to that of 7.5% MonoQ2000 buffer (≈10 mS) using MonoQ0 buffer and loaded on the culomn. Bound proteins were eluted with 12 CV of a linear gradient from 7.5% to 75% MonoQ2000 buffer (150 mM–1.5 M KOAc) and fractions of 1 ml were collected. Pol II eluted at ≈1160 mM KOAc.

Last, Pol II was purified by gel filtration. The sample buffer was exchanged by adding 3 volumes of Pol II buffer (50 mM HEPES pH 7.25, 40 mM $(NH_4)_2SO_4$, 10 µM $ZnCl_2$, 10 mM DTT) and concentrating using a 10 kDa MWCO[6] filter device (Amicon Ultra-15) to 480 µl at 2800 g and 4 °C. The sample was purified on a previously

[1] RNA polymerase II.
[2] *Saccharomyces cerevisiae*.
[3] Yeast extract-peptone-dextrose.
[4] Phenylmethylsulfonyl fluoride.
[5] Column volume.
[6] Molecular weight cut-off.