

# Single-molecule imaging for investigating the transcriptional control

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## ABSTRACT

Transcription is an essential biological process involving numerous factors, including transcription factors (TFs), which play a central role in this process by binding to their cognate DNA motifs. Although cells must tightly regulate the kinetics of factor association and dissociation during transcription, factor dynamics during transcription remain poorly characterized, primarily because of the reliance on ensemble experiments that average out molecular heterogeneity. Recent advances in single-molecule fluorescence imaging techniques have enabled the exploration of TF dynamics at unprecedented resolution. Findings on the temporal dynamics of individual TFs have challenged classical models and provided new insights into transcriptional regulation. Single-molecule imaging has also elucidated the assembly kinetics of transcription complexes. In this review, we describe the single-molecule fluorescence imaging methods widely used to determine factor dynamics during transcription. We highlight new findings on TF binding to chromatin, TF target search, and the assembly order of transcription complexes. Additionally, we discuss the remaining challenges in achieving a comprehensive understanding of the temporal regulation of transcription.

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## INTRODUCTION

Transcription is a highly regulated process typically initiated by transcription factors (TFs) or activators that bind to cognate-binding motifs (Lambert et al., 2018). The classic model of transcriptional activation posits that TFs and coactivators recruit chromatin remodelers and histone-modifying enzymes to reorganize nucleosomes, thereby increasing chromatin accessibility (reviewed in Roeder, 2019). Transcriptional activation ultimately facilitates the assembly of the RNA polymerase II (Pol II) preinitiation complex (PIC), which is capable of initiating RNA synthesis at the transcription start site within the core promoter (reviewed in Hahn, 2004; Orphanides et al., 1996; Schier and Taatjes, 2020; Thomas and Chiang, 2006). The Pol II PIC consists of Pol II and general transcription factors (GTFs) including TBP, TFIID, TFIIA, TFIIB, TFIIF, TFIIIE, and TFIIH. GTFs confer Pol II promoter specificity (reviewed in Schier and Taatjes, 2020; Thomas and Chiang, 2006). The prompt recruitment and release of each factor is crucial for the precise and timely synthesis of transcripts. However, the kinetics of factor association and dissociation during transcription remain largely uncharacterized.

Most models explaining the mechanisms of transcriptional regulation are based on ensemble experiments that measure

the population-averaged behavior of heterogeneous molecules. For example, although an invaluable assay for studying TF-chromatin interactions, chromatin immunoprecipitation quantifies the average signal of TF-DNA interactions across a population of molecules (Park, 2009). This can mask molecule-to-molecule variability and heterogeneity, which are important for understanding stochastic processes, such as transcription (Coulon et al., 2013; Levsky and Singer, 2003; Liu and Tjian, 2018; Swift and Coruzzi, 2017).

Recent advances in single-molecule fluorescence imaging have revealed the movement dynamics of individual molecules during transcription with unprecedented resolution (Basu et al., 2024; Chen et al., 2014; Cisse et al., 2013; Donovan et al., 2023; Gebhardt et al., 2013; Lerner et al., 2020; Pomp et al., 2024; Tang et al., 2022; Versluis et al., 2024). These findings have challenged the classic view of TF dynamics (Perlmann et al., 1990) and have provided new insights into the mechanisms underlying transcriptional regulation. While TF binding to its cognate DNA sequences is typically viewed as stable, single-molecule fluorescence imaging has uncovered transient interactions of TFs with chromatin, occurring in the order of seconds (Chen et al., 2014; Dufourt et al., 2018; Loffreda et al., 2017). In this review, we describe in vitro and in vivo single-molecule

**Table 1.** Kinetic information obtained from single-molecule fluorescence imaging approaches

Approach	Measurements	Kinetic information
CoSMoS	Colocalization of interacting molecules	Association/dissociation kinetics
smFRET	FRET efficiency	Binding orders Intra- and intermolecular distance Conformational changes
SMT	2D or 3D trajectory of molecules	Diffusive fraction Diffusion coefficient Residence time Trajectory radius
FRAP	Rate of fluorescence recovery (Recovery half-time)	Displacement anisotropy Diffusion kinetics Mobile and immobile fractions Association/dissociation kinetics
FCS	Fluctuations in fluorescence intensity	Diffusion kinetics

CoSMoS, colocalization single-molecule spectroscopy; smFRET, single-molecule fluorescence resonance energy transfer; FRET, fluorescence resonance energy transfer; SMT, single-molecule tracking; FRAP, fluorescence recovery after photobleaching; FCS, fluorescence correlation spectroscopy.

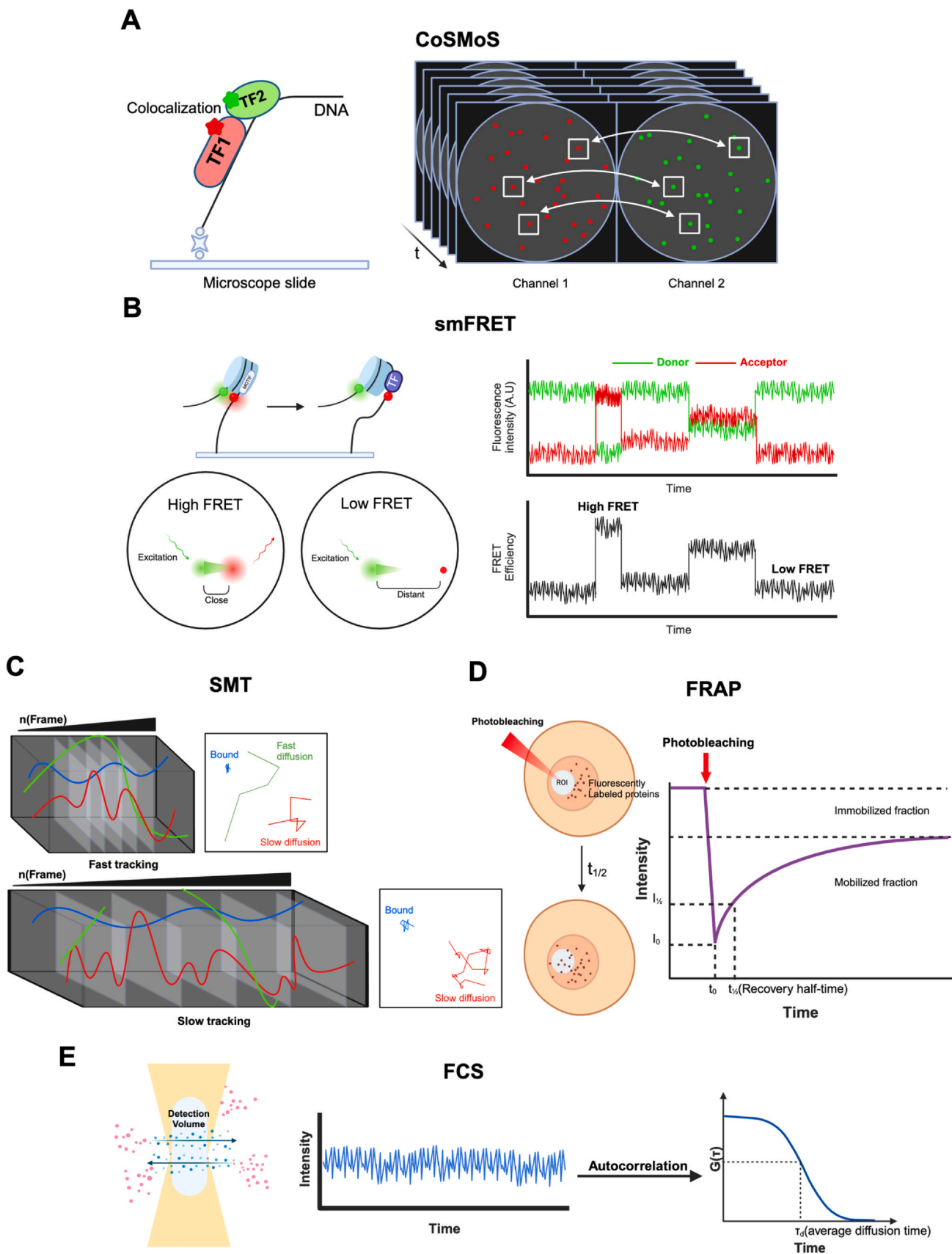
fluorescence imaging techniques that are currently used to elucidate the dynamics of factor association and dissociation during transcription and highlight key findings on TF kinetics. We also address the assembly order of the Pol II PIC, as revealed by single-molecule fluorescence imaging. We conclude with questions that need to be further investigated to better understand the kinetics of transcriptional regulation.

## SINGLE-MOLECULE METHODS TO VISUALIZE FACTOR DYNAMICS DURING TRANSCRIPTION

Transcription is a stochastic process that is driven by a heterogeneous population of molecules with dynamic interactions. Single-molecule fluorescence imaging assays enable us to directly measure the kinetics of molecular interactions at a single-molecule resolution, allowing us to identify the mechanisms underlying transcriptional regulation (Table 1). Various single-molecule fluorescence imaging assays using total internal reflection fluorescence (TIRF) microscopy have been developed (Kudalkar et al., 2016; Reck-Peterson et al., 2010; Tokunaga et al., 1997). TIRF uses an evanescent wave generated when an excitation light is totally internally reflected at the solid-liquid interface (Axelrod, 2008). The evanescent wave has the same wavelength as the excitation light. Because the intensity of an evanescent wave decays exponentially with the distance from the interface, the thickness of the excitation depth is limited to approximately 100 nm, which restricts the illumination space and thus enhances the signal-to-noise ratio (Reck-Peterson et al., 2010). This background elimination feature of TIRF enables the acquisition of images at a single-molecule resolution. TIRF is a useful tool for studying cellular processes occurring at or near the plasma membrane, such as cytoskeleton assembly, cell-cell interactions, and vesicle trafficking (Li et al., 2015; Pellinen et al., 2006; Rappoport and Simon, 2003; Yamada and Nelson, 2007). With the development of in vitro systems that recapitulate biological reactions in the nucleus, TIRF has been used to study transcription. One in vitro single-molecule imaging approach utilizing TIRF is colocalization single-molecule spectroscopy (CoSMoS) (Friedman and Gelles, 2015) (Fig. 1A). The CoSMoS measures the interaction kinetics between individual

molecules at the single-molecule level. It has been used to elucidate the assembly mechanisms of macromolecular complexes involved in biological processes such as transcription, cotranscriptional processes, and DNA replication (Baek et al., 2021; Friedman and Gelles, 2012; Hoskins et al., 2011; Revyakin et al., 2012; Ticau et al., 2015; Zhang et al., 2016). TIRF has also been used to perform single-molecule fluorescence resonance energy transfer (smFRET) (Ha et al., 1996) (Fig. 1B). The smFRET assay on a TRIF microscope measures the dynamics of the intra- or intermolecular interactions of the surface-immobilized molecules within the 10 nm range (Holden et al., 2010). The smFRET TIRF studies identified the structural dynamics of TFs, Pol II, and nucleosomes during transcription (Andrecka et al., 2008; Chen et al., 2021; Crickard et al., 2017; Kilic et al., 2018; Malkusch et al., 2017; Xiong et al., 2024). Collectively, in vitro single-molecule imaging approaches have provided important kinetic information on the interactions of TFs with chromatin and the assembly of transcription complexes (Table 1).

To overcome the limitations of limited depth of illumination in TIRF, a single-molecule imaging technique called highly inclined and laminated sheet (HILO) microscopy has been developed (Tokunaga et al., 2008). HILO microscopy illuminates a cell with a thin and inclined sheet of a laser beam, thus enabling the acquisition of single-molecule images of nuclear proteins in living cells. Over the past decade, HILO microscopy has been widely used to perform single-molecule tracking (SMT) (or called single-particle tracking to elucidate the kinetics of TFs and transcription complexes in live cells (reviewed in Dahal et al., 2023)). The development of super-resolution microscopy techniques, such as photoactivated localization microscopy and stochastic optical reconstruction microscopy, has further expanded the capabilities of SMT assays (Betzig et al., 2006; Fujiwara et al., 2023; Klein et al., 2011; Rust et al., 2006; Shroff et al., 2008). In SMT, individual molecules visualized as fluorescent spots are monitored over time, and a temporal sequence of spot locations or a 2D trajectory is generated. Recent advances in determining the axial position using feedback-tracking methods have enabled the generation of 3D trajectories (Hou et al., 2020; Nguyen et al., 2023).



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**Fig. 1.** Single-molecule fluorescence imaging assays for investigating factor dynamics during transcription. (A) Colocalization single-molecule spectroscopy (CoSMoS) measures interaction kinetics between individual molecules at the single-molecule level. (B) Single-molecule fluorescence resonance energy transfer (smFRET) measures the distance between donor and acceptor fluorophores. The distance is determined by the FRET efficiency. (C) Single-molecule tracking (SMT) measures the 2D and 3D trajectories of individual molecules over time. (D) Fluorescence recovery after photobleaching (FRAP) measures the fluorescence recovery rate (ROI: region of interest). (E) Fluorescence correlation spectroscopy (FCS) measures fast-diffusive molecules over time scales ranging from microseconds to seconds.

Multiple types of kinetic information can be obtained from SMT trajectories, including the number of diffusive states, diffusion coefficients of each state, fractions of each state, residence time, trajectory radius, and displacement anisotropy. The type of kinetic information on molecular diffusion depends on the image acquisition parameters. High-speed measurements with a short interval of milliseconds (fast tracking) capture fast-diffusing molecules, whereas low-speed imaging (slow tracking) measures subpopulations bound to chromatin or other nuclear structures (Fig. 1C). In general, TFs exhibit 3 diffusive states: an unbound state, in which TFs freely diffuse in the nucleoplasm, a nonspecific bound state, in which TFs search for their target via transient interactions with chromatin, and a specific bound state, in which TFs stably bind to chromatin (Lu and Lionnet, 2021). The frequency and duration of TF occupancy can be extracted from slow-tracking data. Histones in a nucleosome are typically used as references for the static state to measure the average diffusion coefficient of bulk chromatin (Hansen et al., 2017; Izeddin et al., 2014). Evaluating the TF movements under various image acquisition conditions is important for avoiding any bias arising from technical limitations. For example, fast tracking can underestimate the residence time owing to photobleaching; whereas slow tracking can lead to overestimation of the residence time of chromatin-bound molecules by missing transiently bound molecules.

Other widely used single-molecule fluorescence imaging assays for transcriptional studies include fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) (Axelrod et al., 1976; Magde et al., 1974; Peters et al., 1974). FRAP and FCS typically use confocal microscopy (Braeckmans et al., 2007; Kubitschek et al., 1998; Wedekind et al., 1994; Yu et al., 2021). FRAP measures the diffusion dynamics of fluorescently labeled proteins in living cells (Fig. 1D). Specifically, a small area of interest in the cell is photobleached using a high-power laser, and the fluorescence recovery of the photobleached area is monitored over time. The scanning laser beam and pinhole of a confocal microscope enable photobleaching of a specific area within a cell with high spatial resolution. This precise photobleaching capability is beneficial for FRAP experiments. The rate of fluorescence recovery represents the mobility of the labeled proteins, as determined by diffusion and binding kinetics (Wachsmuth, 2014). FRAP has been successfully used to quantify the dynamics of TFs and chromatin regulators in living cells, including the immobile fraction, recovery half-life, diffusion coefficient, and association and dissociation constants (Cheutin et al., 2003; Raccaud et al., 2019; Stenoien et al., 2001; Versluis et al., 2024; Voss et al., 2011). FCS is a complementary approach to FRAP in that it is effective for measuring fast diffusion dynamics ranging from microseconds to seconds (Elson, 2011). FCS detects fluctuations in the fluorescence intensity within a limited volume

at the femtoliter level (Elson, 2011) (Fig. 1E). The diffusion of fluorescently labeled proteins through focused light causes changes in the fluorescence intensity. The confocal beam and pinhole provide a confined detection volume and ensure that only the emission light from this focused detection volume reaches the detector. FCS quantifies the diffusion coefficients and different diffusing populations of proteins from the temporal autocorrelation of the fluorescence intensity over time traces (Singh et al., 2017; White et al., 2016). FRAP and FCS are useful to cross-validate their measurements (Stasevich et al., 2010).

Each single-molecule fluorescence imaging assay offers unique capabilities for measuring distinct characteristics of factor dynamics during transcription. TIRF-based CoSMoS and smFRET are specialized in measuring the dynamics of molecular interactions. CoSMoS is useful for studying the assembly kinetics of macromolecular complexes, whereas smFRET is beneficial for detecting conformational and structural dynamics. However, these assays have limited applications in vivo. SMT is a powerful technique for measuring in vivo diffusion kinetics of TF and chromatin interactions. While SMT tracks individual molecules, FRAP and FCS are useful for studying molecular dynamics in a specific region within living cells. A limitation of FRAP is the use of high laser power for photobleaching, which can lead to photodamage. FCS, on the other hand, captures rapid diffusion more effectively but is highly susceptible to background signals.

## REGULATION OF TF BINDING STABILITY ON CHROMATIN

TFs are master regulators of gene expression. Understanding the high-resolution kinetics of TF behavior can provide mechanistic insights into TF-mediated transcriptional activation. Single-molecule fluorescence imaging techniques have revealed that TFs interact transiently with chromatin rather than exhibiting stable binding (Callegari et al., 2019; Chen et al., 2014; Mir et al., 2018; Paakinaho et al., 2017; Raccaud et al., 2019; Sugo et al., 2015). The average residence time of TFs on chromatin is in the order of seconds (Lu and Lionnet, 2021). For example, p53 has an average residence time of 3.1 s (Loffreda et al., 2017). Pioneer factors, such as FoxA1, Sox2, and Oct4, have residence times of approximately 10 s (Chen et al., 2014; Swinstead et al., 2016; Teves et al., 2016). The *Drosophila* pioneer factor Zelda shows an even more transient interaction with chromatin with a residence time of 0.696 s (Mir et al., 2017). However, some TFs remain bound to the chromatin for longer periods. For instance, the GAGA factor (GAF), a *Drosophila* pioneer-like factor, has a residence time of 130 s, which is several times longer than the typical TF residence times (Tang et al., 2022). This prolonged residence time is likely attributable to the multimerization of GAF, which increases the

local concentration of its DNA-binding domain, thereby increasing binding stability. Similarly, dimerization of c-Fos in MDA-MB-231 cells enhances its binding stability to chromatin (Shao et al., 2021). Transient interactions between individual TFs and their target sites might allow TFs to respond to changes in local concentrations or cellular states with high sensitivity. Although not yet generalizable to all TFs, several studies have demonstrated a positive correlation between TF binding stability and transcriptional output (Donovan et al., 2019b; Hipp et al., 2019; Popp et al., 2021). For example, in human U2-OS cells, an increase in VP64 concentration and residence time was associated with higher transcription bursting frequency and transcription output (Popp et al., 2021). Notably, the efficiency of transcription activation was more affected by VP64 residence time rather than by its concentration. Further studies examining the functional consequences of changes in TF dynamics on transcriptional output will provide a more precise connection between TF dynamics and function.

Recent single-molecule imaging studies have demonstrated that TF binding stability is dynamically regulated by cellular states and environmental factors. In mouse fibroblasts, chromatin-bound serum response factor (SRF) is classified into 3 populations based on its residence time: short-, intermediate-, and long-bound (Hipp et al., 2019). Each fraction responds differently to upstream signaling pathways. The residence time of long-bound SRF increases upon growth factor stimulation, whereas the inhibition of Rho-GTPase signaling reduces the residence time of the long-bound fraction. Inhibition of MAPK signaling decreases the residence time of intermediate-bound SRF. This differential regulation of SRF dissociation rates by different signaling pathways results in differential gene activation (Hipp et al., 2019). Similarly, in mouse mammary carcinoma cells, different types of glucocorticoid differentially affect the residence time of activated glucocorticoid receptors (GRs) on chromatin (Stavreva et al., 2019). Cortisol stimulation leads to a longer residence time of the GR than dexamethasone stimulation. The glucocorticoid-dependent GR residence time is positively correlated with the duration of transcriptional bursting (Stavreva et al., 2019). Additionally, the GR residence times vary depending on the activation phase, being longer during the early phase than during the later phase. Signaling pathways and extrinsic ligand cues dynamically regulate the stability of TF-chromatin interactions to modulate gene expression.

TFs mediate gene activation in concert with their interacting partners, such as other TFs, cofactors, and coactivators. These interactions influence the binding kinetics of each partner. In mouse embryonic stem cells, an enhanceosome assembles in a stepwise manner, with Esrrb binding after Sox2 and Oct4 (Xie et al., 2017). Sox2 depletion reduced the residence time of Esrrb in chromatin (Xie et al., 2017). Coactivators also influence TF residence times. In the secretory cells of *Drosophila* larval salivary glands, the TF Scalloped (Sd) interacts with the coactivator Yorkie (Yki) at active transcription sites (Manning et al., 2024). Increased Yki expression doubled the Sd residence time, resulting in higher gene expression than in cells with normal Yki expression.

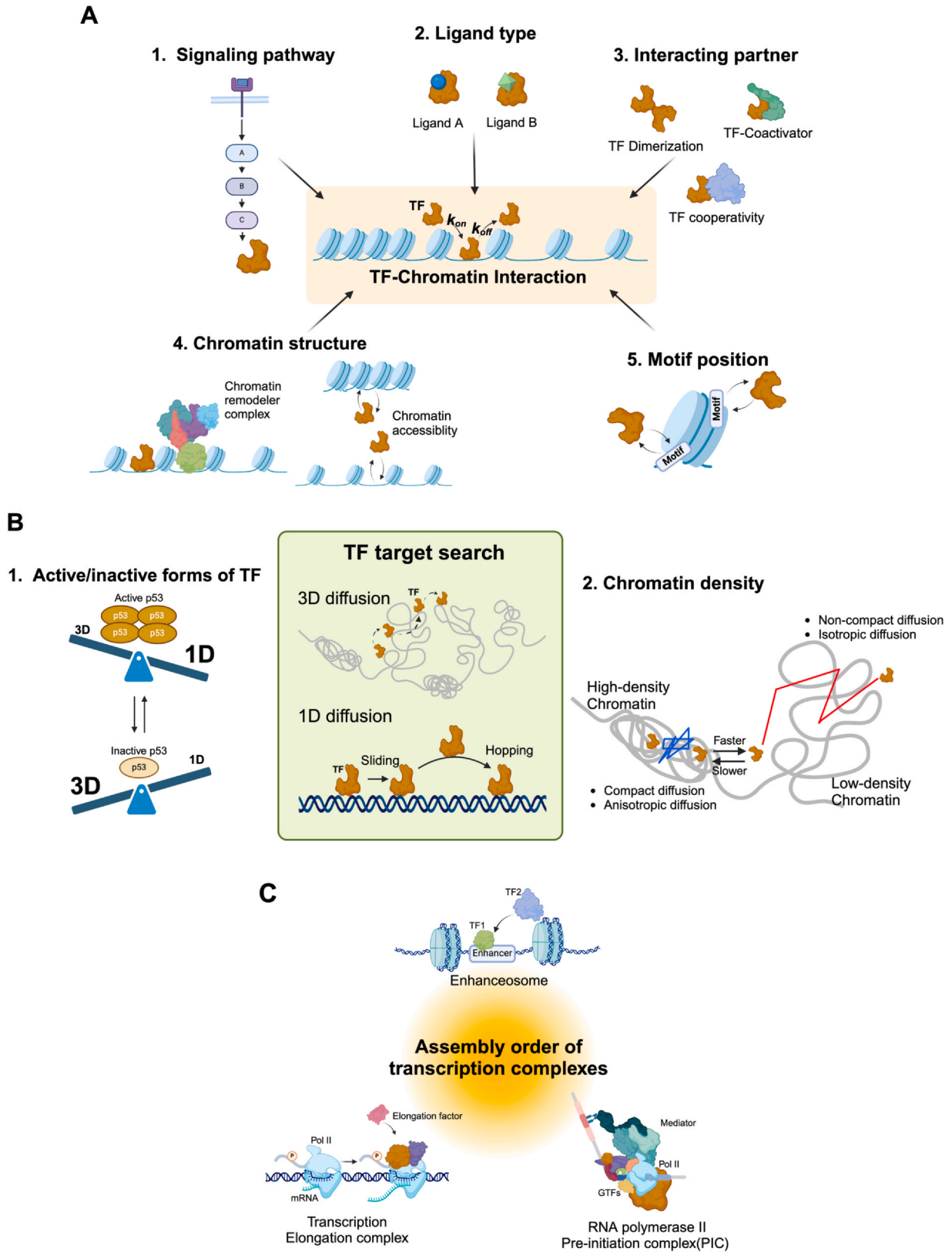
Chromatin states surrounding TF binding sites are another factor that regulates TF binding stability. In yeast, the RSC

complex remodels chromatin states, thereby destabilizing the interaction between the yeast TF Ace1p and chromatin (Mehta et al., 2018). The RSC-mediated dissociation of Ace1p allows transcription reinitiation, as Ace1p can rebind to the promoter. The knockdown and knockout of Rsc2p, a subunit of the RSC complex, increased the residence time of Ace1p. The delayed exchange between Rsc2p and Ace1p decreased transcription burst frequency. These results suggest that changes in the chromatin state by chromatin remodelers influence TF residence times on chromatin, contributing to the dynamic regulation of transcription activation. An *in vitro* study using reconstituted nucleosomes revealed that the position of a cognate DNA motif influences TF binding stability through position-dependent cooperativity of Sox2 and Oct4 (Li et al., 2019). In the study, when the Sox2:Oct4 motif was positioned at the end of the nucleosomal DNA, Oct4 stabilized Sox2 binding. In contrast, when the Sox2:Oct4 motif was located in the nucleosomal dyad, Oct4 did not affect the residence time of Sox2. To summarize, the binding stability of TFs is regulated at multiple levels, including their intrinsic binding affinities for binding sites, upstream signaling pathways, interacting factors, and chromatin states (Fig. 2A).

## ROLES OF CHROMATIN IN TF TARGET SEARCH

A current TF target search model proposes that TFs utilize a trial-and-error mechanism that involves a combination of 3D diffusion in the nuclear space and 1D diffusion, either sliding or hopping along the DNA, to locate their binding sites. TFs exhibit distinct 3D and 1D diffusive states depending on the biological conditions. For example, an *in vitro* single-molecule fluorescence imaging study using naked DNA revealed that upon activation, p53 exhibited a 4-fold reduction in 3D diffusion and a 1.5-fold increase in 1D diffusion (Itoh et al., 2016) (Fig. 2B). Activated p53 showed an increased search distance by intermittent hopping along the DNA groove rather than by continuous sliding (Subekti et al., 2020). Additionally, the kinetics of TF target search are influenced by chromatin density (Fig. 2B). Live-cell imaging studies have revealed that TFs such as p53 and p65 exhibit distinct behaviors related to chromatin density upon activation (Mazzocca et al., 2023). Specifically, p53 showed opposite diffusion patterns in the chromatin-dense and chromatin-poor regions. p53 molecules with slow diffusion, high anisotropy, and compact exploration were more prevalent in chromatin-dense regions than in chromatin-poor regions. These slow-diffusing p53 molecules in chromatin-dense areas had a higher chance of transitioning to the chromatin-bound state. Unlike p53, chromatin enrichment of p65 was not dependent on its diffusion speed, as both slow- and fast-diffusing p65 molecules were found in chromatin-poor regions.

The effects of nucleosomes on TF search kinetics are TF-specific. *In vitro* studies using reconstituted nucleosomes revealed that the yeast TF Rap1 displays no differences in association rates between naked DNA and nucleosomes, suggesting that Rap1 target search is independent of the nucleosomal presence (Mivelaz et al., 2020). In the study, unlike Rap1, Reb1 and Cbf1 showed approximately 50- to 100-fold reductions in association rates with reconstituted nucleosomes



**Fig. 2.** Kinetics of transcriptional control and their regulators. (A) Dynamic regulation of the TF-chromatin interaction. (B) Regulation of TF target search. (C) Assembly order of transcription complexes.

compared with naked DNA, whereas their residence time on nucleosomal DNA increased (Donovan et al., 2019a). The increased residence time of Cbf1 was attributed to direct interactions between its basic helix-loop-helix domain and histones (Donovan et al., 2023).

## ASSEMBLY ORDERS OF POL II PIC

Timely association and dissociation of various regulatory factors is essential for precise transcription processes. Identification of the association kinetics of each factor suggests binding order(s) during transcription complex assembly (Fig. 2C). Recent single-molecule fluorescence imaging studies have provided deeper insights into the stepwise assembly model of Pol II PIC. Sequential binding of TFIIA and TFIIB was confirmed by the *in vitro* study using a DNA template containing the consensus TATA, initiator motif, and downstream promoter elements, TFIID, TFIIA, TFIIB, TFIIF, and Pol II (Zhang et al., 2016). Interestingly, TFIIB binding was highly transient and repetitive, with an interval of 3 s. This transient on/off TFIIB binding was stabilized by the subsequent binding of Pol II and TFIIF. A single-molecule fluorescence imaging study using yeast nuclear extract identified branched pathways of TF-dependent PIC assembly, where Pol II and TFIIF initially bind to the upstream activating sequence (UAS) and then join the PIC at the core promoter (Baek et al., 2021). TFIIE can bind to a Pol II•TFIIF complex, either at the UAS or the core promoter. Additionally, TFIIF can simultaneously associate with or after Pol II binding. The assembly of the Pol II•TFIIF•TFIIE complex does not require a core promoter, but the core promoter makes the complex more stable. Unlike other factors, TFIIF binding to DNA requires the core promoter. This spatiotemporally coordinated PIC assembly has also been observed in living yeast (Nguyen et al., 2021). TBP, TFIID, TFIIA, and TFIIB reconfigure promoter structures for PIC formation. The mediator controls the recruitment of Pol II, TFIIF, and TFIIE. Interestingly, the observed association rates of Mediator and TFIID are  $0.01 \text{ s}^{-1}$  (or 1 event every 100 s) and the average time of association and dissociation of PIC components is a few seconds. These findings suggest that PIC formation is infrequent but occurs efficiently once TFIID and Mediator are nucleated.

## CONCLUSION

The development of single-molecule fluorescence imaging techniques, along with advances in fluorescent dyes and labeling methods, has enabled the identification of the kinetics of molecular interactions during transcription at a single-molecule resolution, providing insights that cannot be obtained from ensemble experiments. Single-molecule studies have uncovered unexpectedly short residence times of TFs on chromatin, as well as 1D and 3D target search modes, and the hierarchical and branched assembly of Pol II PIC. Additionally, various environmental cues and chromatin states dynamically regulate TF kinetics.

Despite the significant advances in our understanding of the temporal characteristics of heterogeneous populations of TFs and transcription complexes, several challenges remain unaddressed. Single-molecule fluorescence imaging results can vary based on experimental conditions such as dye labeling methods, image acquisition parameters, and experimental

specimens. Using orthogonal methods for cross-validation and conducting extensive control experiments concurrently is crucial. Another challenge involves data analysis and modeling, which can introduce biases in data interpretation. For example, the analysis of TF residence time distribution using a bi-exponential model assumes the presence of 2 TF subpopulations: specific and nonspecific binding fractions. However, this model is often too simplistic to capture the stochastic and complex nature of TF behaviors. Instead, a power-law model, which assumes a continuous distribution of TF residence times, could provide a more accurate analysis of TF residence times (Garcia et al., 2021a, 2021b). A comparison of single-particle tracking methods has revealed differences between algorithms (Chenouard et al., 2014). There is no universally best particle tracking method; rather, factor and data-specific approaches are necessary. These approaches should consider particle shape and size, frame rate, background uniformity, and photobleaching. The recent development of deep learning-assisted methods has enabled more precise analysis, including photobleaching correction, trajectory generation, and classification of TF diffusion models, allowing for more reliable interpretations of single-molecule imaging data (Liu et al., 2022). Other questions that remain unaddressed include the differential TF dynamics across various cell types and their contributions to gene expression variability, the roles of long noncoding RNAs in regulating TF dynamics, detailed kinetics of TF-TF interactions in a single enhancer region, and assembly dynamics of transcription elongation and termination complexes.

## AUTHOR CONTRIBUTIONS

**Inwha Baek:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Insung Choi:** Writing – review & editing, Writing – original draft.

## DECLARATION OF COMPETING INTERESTS

The authors have no potential conflicts of interest to disclose.

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