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Transcription Factor Dynamics: One Molecule at a Time

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Abstract

Cells must tightly regulate their gene expression programs and yet rapidly respond to acute biochemical and biophysical cues within their environment. This information is transmitted to the nucleus through various signaling cascades, culminating in the activation or repression of target genes. Transcription factors (TFs) are key mediators of these signals, binding to specific regulatory elements within chromatin. While live-cell imaging has conclusively proven that TF–chromatin interactions are highly dynamic, how such transient interactions can have long-term impacts on developmental trajectories and disease progression is still largely unclear. In this review, we summarize our current understanding of the dynamic nature of TF functions, starting with a historical overview of early live-cell experiments. We highlight key factors that govern TF dynamics and how TF dynamics, in turn, affect downstream transcriptional bursting. Finally, we conclude with open challenges and emerging technologies that will further our understanding of transcriptional regulation.

Contents

INTRODUCTION

Chromatin: a DNA–protein complex consisting of DNA wrapped around a histone octamer composed of histones H2A, H2B, H3, and H4

Euchromatin: a lightly packed, largely transcriptionally active chromatin state typically found in the nuclear interior

Heterochromatin: a condensed, largely transcriptionally inactive chromatin state, typically found near the nuclear periphery and nucleolus

Most cells within multicellular eukaryotes contain the same DNA sequence. However, different cells within the same organism express distinct sets of genes and proteins, with each set finely tuned to the cells' developmental trajectory. Tight regulation of the spatiotemporal dynamics of gene expression is essential for normal development and to maintain cellular homeostasis (Cardoso-Moreira et al. 2019).

DNA within eukaryotic cells is packaged into chromatin as a series of hierarchical structures, and genome organization provides the first layer of control over gene expression. At least four different features of chromatin are associated with gene regulation: (*a*) Chromatin accessibility is closely associated with gene expression, with active genes primarily occupying regions with higher accessibility (Klemm et al. 2019); (*b*) specific histone and DNA modifications mark active and inactive promoters and enhancers (Bannister & Kouzarides 2011); (*c*) long-range interactions bring *cis*-regulatory elements that are separated in genomic distance within close physical proximity of each other (Kim & Shendure 2019); and (*d*) within each chromosome, active and inactive genes generally occupy different spatial compartments. The A compartment broadly contains transcriptionally active regions, and the B compartment contains transcriptionally inactive regions, roughly corresponding to euchromatin and heterochromatin, respectively. However, high-resolution Hi-C experiments have unveiled finer compartmentalization of active and inactive genes at much smaller scales within both A and B compartments (Rowley & Corces 2018). DNA-binding proteins such as histones, high-mobility group proteins, architectural proteins, and the DNA replication and repair machinery play a central role in regulating genome organization and, consequently, transcription.

Transcription factors (TFs) are sequence-specific DNA-binding proteins that provide an additional level of control over gene expression. TF binding to a specific site stochastically triggers

the recruitment of the preinitiation complex (PIC) and subsequently the Mediator complex and RNA polymerase II (RNA Pol II) (Spitz & Furlong 2012). TFs are modular proteins that contain a structured DNA-binding domain (DBD) that recognizes a 6–12-bp motif, typically within *cis*-regulatory elements such as promoter-proximal regions and distal enhancers (Spitz & Furlong 2012). The exquisite specificity with which TFs bind some of their regulatory elements (REs) but not others is surprising, especially considering that these motifs are highly degenerate.

Photobleaching: irreversible light exposure–induced photochemical change in the chemical composition of a fluorescent protein that leads to permanent loss of fluorescence

TF–chromatin interactions were initially studied indirectly by leveraging the propensity of a genomic region to be susceptible to cleavage by DNase I, micrococcal nuclease, or other DNA-modifying enzymes (Wu et al. 1979). Sites sensitive to DNase I cleavage were termed DNase I hypersensitive sites, and the measured TF footprint was used to infer the stability of the TF–DNA complex. The development of chromatin immunoprecipitation allowed for direct measurements of TF binding (Gilmour & Lis 1984). These approaches led to the hypothesis that TFs bind to specific motifs as part of a stable multicomponent complex that remains bound to chromatin for the entire duration of active transcription of the corresponding gene (Perlmann et al. 1990).

Biochemical approaches, while informative, suffer from two limitations. First, the addition of fixatives eliminates any inherent dynamics and prevents the study of steady-state TF–chromatin interactions. Second, these are population-level studies that can provide only ensemble averages. The discovery of the green fluorescent protein (GFP) (Chalfie et al. 1994) allowed researchers to monitor the dynamics of proteins in single living cells (Htun et al. 1996). The ensuing Green Revolution led to several paradigm-shifting findings. Chromatin, long believed to be a stable scaffold, was found to exhibit subdiffusive behavior in living cells (Marshall et al. 1997). This finding challenged the view that nuclear structures such as chromosomes were static and immobile, and raised questions about other nuclear proteins. Histone H1, also thought to be stably incorporated into nucleosome arrays, was shown to rapidly exchange with chromatin on the order of minutes, from both heterochromatin and euchromatin (Misteli et al. 2000), with many other nuclear proteins subsequently shown to be highly dynamic (Phair & Misteli 2000).

Because chromatin, the underlying substrate for TFs, was shown to be a dynamic polymer, the static TF–chromatin interaction model was called into question. Photobleaching experiments on GFP-fused glucocorticoid receptor (GR) showed that GR rapidly exchanges from a 200-copy tandem array of mouse mammary tumor virus (MMTV) promoters (henceforth termed the MMTV array), with a mean residence time of seconds (McNally et al. 2000). This and other studies showed that TF binding to specific sites is highly transient, with binding times on the order of several seconds (Hager et al. 2009). These early studies were conducted using either fluorescence recovery after photobleaching (FRAP) (Axelrod et al. 1976) or fluorescence correlation spectroscopy (FCS) (Magde et al. 1972). Advancements in microscopy as well as fluorophore chemistry have since allowed for tracking single TF molecules within living cells, providing unprecedented spatial and temporal resolution for the study of TF–chromatin interactions and aspects of transcription.

In this review, we begin with an introduction to single-molecule tracking (SMT) and discuss SMT in the context of FRAP and FCS, which have been workhorses of the protein dynamics field. We then describe imaging technologies frequently used for SMT. Next, we focus on two fundamental questions that SMT is well positioned to address: (*a*) Which mobility signatures does a TF exhibit, and (*b*) how long does a TF bind? Then, we discuss biochemical and biophysical cues within the local chromatin microenvironment that alter TF and chromatin dynamics. Finally, we link TF dynamics as measured through SMT with functional outcomes and conclude by summarizing the state of the field and highlighting open questions.

WHY SINGLE-MOLECULE TRACKING?

Diffusion: a mode of molecular transport arising due to thermal fluctuations, in which the mean-squared displacement scales linearly with time

Before the advent of SMT, FRAP and FCS were the go-to techniques to monitor protein dynamics using fluorescent markers.

FRAP experiments involve targeted photobleaching of a predefined area and subsequent monitoring of the recovery of fluorescence to prebleach levels (Axelrod et al. 1976) (**Figure 1*a*,*b***). A molecule that diffuses rapidly results in faster recovery compared with a molecule that diffuses slowly. FRAP, while involving careful mathematical modeling to obtain the relevant parameters such as binding times and diffusion coefficients, is technically accessible and hence has been widely adopted.

The accessibility of FRAP made it a popular tool in the field of molecular biology, with the early to middle 2000s being dominated by FRAP studies of various nuclear proteins. Initial FRAP studies showed that most TFs, such as GR (McNally et al. 2000, Schaaf & Cidlowski 2003, Schaaf et al. 2005, Stavreva et al. 2004), the androgen receptor (Farla et al. 2004, 2005), the progesterone receptor (Rayasam et al. 2005), the estrogen receptor (ER) (Sharp et al. 2006, Stenoien et al. 2001), p53 (Hinow et al. 2006), and the yeast TF Ace1p (Karpova et al. 2004), exhibit rapid dynamics both throughout the nucleus and at specific binding sites. The same is true for cofactors such as GRIP1 (Becker et al. 2002) and the chromatin-associated protein HMGB1 (Agresti et al. 2005). RNA Pol I and II showed slower exchange from ribosomal DNA and nonribosomal DNA, respectively, consistent with their processive enzymatic activity (Darzacq et al. 2007, Dundr et al. 2002, Kimura et al. 2002). In contrast, histones H3 and H4 exchanged slowly from chromatin, whereas histone H2B showed slightly faster kinetics (Kimura & Cook 2001).

FCS, on the other hand, requires specialized equipment and sophisticated mathematical analysis, which has hindered its widespread adoption. FCS relies on correlations in intensity fluctuations to extract biophysical parameters such as diffusion coefficients and reaction rates. Since FCS experiments involve measuring correlations buried in intensity fluctuations, the analysis requires careful calibration and mathematical modeling. A typical FCS experiment involves collecting fluorescence signal from a point scan (**Figure 1*c*,*d***). Molecular species that rapidly diffuse in and out of the illumination area lead to rapid fluctuations and thereby short temporal correlations. By contrast, molecules such as TFs interacting with chromatin diffuse more slowly and are likely to produce longer-lived correlations (Digman & Gratton 2011) (**Figure 1*e***). FCS was used to study the dynamics of GR and various GR oligomerization mutants within the interphase nucleus (Stortz et al. 2017). FCS experiments in developing mouse embryos showed that the kinetics of Sox2 (a key TF in the maintenance of pluripotency of stem cells) interactions with DNA regulate cell-fate decisions as early as the four-cell embryo (White et al. 2016).

FRAP and FCS, while powerful quantitative tools to investigate nuclear protein dynamics in vivo, are population averages. Further, both methods are indirect probes of molecular dynamics. The measurements (fluorescence recovery in the case of FRAP and fluorescence intensity fluctuations in the case of FCS) are fit to mathematical models, and the fit parameters are used to calculate residence times, diffusion coefficients, and reaction rates. The choice of mathematical model and associated boundary conditions can significantly alter the interpretation of the experiments (Mueller et al. 2010). Models can contain a variable number of bound states (both specific and nonspecific) and free diffusive states, with several models producing good fits to the same underlying data (Mazza et al. 2012, Sprague & McNally 2005). Careful scrutiny of the underlying assumptions and meticulous calibrations and controls are essential to reliably compare numbers obtained from each of these methods (Mueller et al. 2010).

While we have learned a lot from FRAP and FCS studies, following individual molecules as they search for target sites within the nucleus provides the only direct measurement of TF dynamics (**Figure 1*f*,*g***). By tracking single proteins, we can obtain a direct readout of their

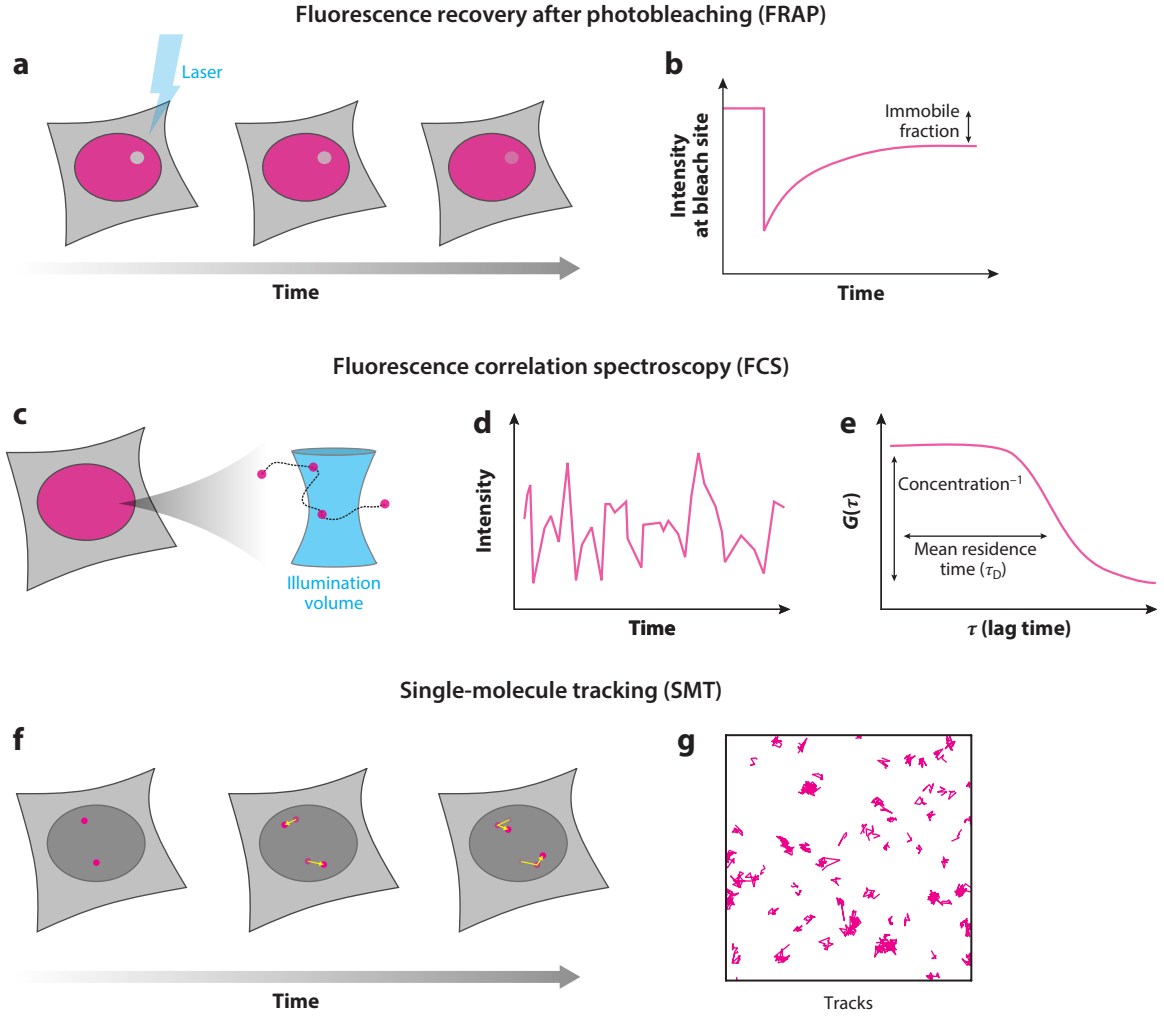

Figure 1

Live-cell imaging techniques to monitor transcription factor dynamics. (*a*,*b*) Fluorescence recovery after photobleaching (FRAP). (*a*) Schematic of a FRAP experiment. A targeted region of interest (ROI) of the nucleus is photobleached. The ROI intensity is then monitored over time. Molecules diffusing into the bleached ROI lead to recovery of fluorescence. (*b*) Representative FRAP curve. (*c*–*e*) Fluorescence correlation spectroscopy (FCS) measures correlations in fluorescence intensity at a spot in the nucleus. (*c*) Schematic depicting a cell with fluorescently labeled protein in the nucleus (*magenta*). (*Inset*) A protein diffusing through the illumination field. (*d*) Example intensity trace obtained from an FCS experiment. (*e*) Autocorrelation function $G(\tau)$ calculated from the FCS time series. The height of $G(\tau)$ at $\tau = 0$ is inversely proportional to the concentration of fluorescently labeled molecules. The width of $G(\tau)$ is proportional to the average residence time of a molecule within the illumination volume. (*f*,*g*) Single-molecule tracking (SMT). (*f*) Schematic of an SMT experiment. The protein of interest is visualized as diffraction-limited spots within the nucleus that are tracked over time. (*g*) Representative tracks obtained from an SMT experiment.

spatiotemporal dynamics. The heterogeneity in molecular kinetics that is typically masked in the ensemble averages measured by FRAP and FCS provides important information about the motion of TFs and how it varies within distinct subnuclear compartments.

SMT presents its own challenges. The photophysics of fluorescent tags and integration times of cameras can limit both the localization precision of single molecules and the imaging rates

accessible to SMT. For example, typical SMT experiments have frame rates of 1 to 125 Hz. In comparison, FCS experiments can sample as fast as 50,000 Hz (Price et al. 2021), enabling the study of dynamics at the microsecond scale. To minimize background, specialized optics that generate thin light sheets are required, whereas FRAP experiments can be performed on conventional confocal microscopes. Ideally, these techniques should be combined to obtain information across different timescales and length scales. FCS can provide insight into fast diffusion kinetics of TFs, and SMT can probe the residence times and mobility of slowly diffusing and bound molecules. In the next section, we discuss technological advances that led to the development of SMT.

TECHNOLOGIES FOR SINGLE-MOLECULE TRACKING

SMT has been used to study the diffusion of membrane proteins for over 30 years. While membrane receptors can be labeled with gold nanoparticles (Kusumi et al. 1993), quantum dots (Dahan et al. 2003), cell-impermeable fluorescent ligands (Schütz et al. 2000), and antibodies (Wilson et al. 1996), the adaptation of these techniques to the study of individual TFs was hampered by the need to deliver fluorescent probes into the nucleus with minimal physiological perturbation and subsequent imaging deep inside the cell. Single-molecule imaging inside the nucleus remained a challenge for two main reasons: (*a*) Precise localization of individual molecules requires sparse labeling of the protein of interest, and (*b*) the molecule to be tracked must be labeled with a bright fluorophore with an intensity at least twofold higher than the local background.

The first SMT study of TFs in live cells leveraged the low copy number of the *lac* repressor (*lac*I) in *Escherichia coli* cells to achieve sparse labeling. Fusing *lac*I to Venus (a derivative of the yellow fluorescent protein) and mutating the *lac* operon to further reduce *lac*I–Venus expression allowed for localization of individual molecules using widefield microscopy (Elf et al. 2007). In eukaryotic cells, however, achieving a low copy number of tagged molecules is challenging.

Advances in in situ labeling technologies have helped achieve the sparse labeling required for SMT within eukaryotic cells. The development of photoactivatable (Patterson & Lippincott-Schwartz 2002) and photoconvertible (Gurskaya et al. 2006, Wiedenmann et al. 2004) genetically encoded fluorescent tags with high photon counts allowed for stochastic activation of a few molecules at a time within the nucleus by a technique called single-particle tracking photoactivated localization microscopy (Manley et al. 2008). The invention of a novel protein labeling technology called HaloTag (Los et al. 2008) together with cell-permeable organic fluorophores that bind specifically to HaloTag-protein chimeras allowed for precise control over the labeling density without artificially controlling protein expression levels and photoactivation/photoconversion efficiencies (Grimm & Lavis 2022, Grimm et al. 2015).

The large fraction of unbound TFs results in a high background fluorescence intensity that reduces the overall signal-to-noise ratio (SNR) of individual fluorophores, preventing their detection and tracking with epi-illumination. To overcome this, typical SMT experiments use some form of light sheet microscopy to illuminate only a thin section of the sample, thereby minimizing background and enabling visualization of single proteins in the nucleus with high SNR. High-powered lasers are essential to achieve good SNR, but laser powers and exposure times must be optimized not only to visualize single molecules but also to image them long enough to obtain particle trajectories over relevant timescales. Highly inclined and laminated optical sheet (HILO) microscopy (Tokunaga et al. 2008) is the most commonly used microscopy technique in SMT. HILO can be implemented on a commercial total internal reflection fluorescence microscope by changing the angle of the laser so that it is no longer in total internal reflection mode but produces an inclined light sheet through the sample (**Figure 2*a***). Other microscopy techniques frequently used in SMT are reflected light sheet microscopy (Gebhardt et al. 2013) (**Figure 2*b***), light sheet

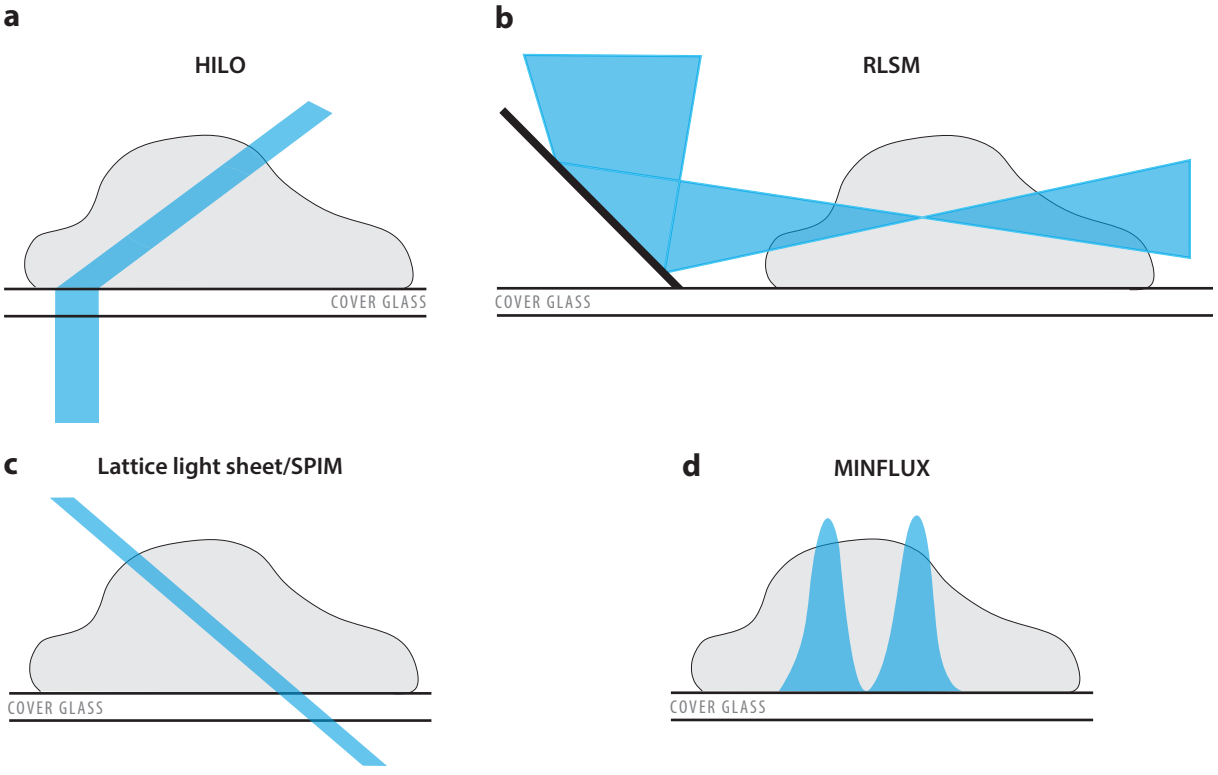

Figure 2

Microscopy techniques used in single-molecule tracking. (*a*) Highly inclined and laminated optical sheet (HILO) microscopy. (*b*) Reflected light sheet microscopy (RLSM). (*c*) Light sheet techniques such as lattice light sheet microscopy and selective plane illumination microscopy (SPIM). (*d*) MINFLUX (minimal photon fluxes) nanoscopy.

techniques such as lattice light sheet microscopy (B.C. Chen et al. 2014) (**Figure 2*c***) and selective plane illumination microscopy (Huisken et al. 2004) (**Figure 2*c***), and more recently, MINFLUX (minimal photon fluxes) (Balzarotti et al. 2017) (**Figure 2*d***). However, these techniques require more sophisticated instrumentation compared with HILO.

ANALYZING SINGLE-MOLECULE TRACKING DATA

After acquisition of an SMT movie, the next step involves detecting and tracking single molecules. Movies are typically filtered to improve the SNR, and molecules are detected based on their intensity, subject to an intensity threshold (Hansen et al. 2018, Lelek et al. 2021, Mazza et al. 2013, Presman et al. 2017). This step provides a rough estimate for the locations of the single molecules. To achieve subpixel localization, one fits the point-spread function to the intensity profiles of the point sources that were detected in the first step. Maximum likelihood estimation (MLE) algorithms are often used for localizing single molecules and approach the theoretical limit for localization precision at high SNR (called the Cramér–Rao lower bound) (Mortensen et al. 2010). Starting with the center of the pixel identified in the first detection step, MLE algorithms compute the *x*-*y* coordinates of the point emitter that maximizes the probability of obtaining the observed image (i.e., the likelihood function) (Lelek et al. 2021). This is repeated for every frame of the time-lapse movie.

Anomalous diffusion: a mode of molecular transport characterized by nonlinear scaling of the mean-squared displacement with time

Once all the molecules have been detected, consecutive localizations of the same molecule need to be stitched together to form tracks. This step is critical since all downstream analysis is performed on the track coordinates. Tracking errors can occur if the density of labeled molecules is too high or if the molecule that is being studied diffuses over long distances on the timescales of measurement (i.e., the imaging interval) or if the tracked particle blinks due to either fluorophore photophysics or local SNR changes. Thus, tracking algorithms that make use of all the available information and efficiently solve the assignment problem of linking localizations across time are critical for effective SMT (Jaqaman et al. 2008, Serge et al. 2008; for an extensive review, see Manzo & Garcia-Parajo 2015). The use of photoactivatable or photoconvertible dyes allows for precise control over the number of fluorescent molecules, with some studies using as few as one molecule per cell per frame to minimize tracking errors (Hansen et al. 2018). We should note that most SMT experiments are performed in two dimensions. Fast-diffusing molecules rapidly disappear from the focal plane, resulting in their preferential underrepresentation, compared with more stably bound molecules, and methods have been developed to account for this in downstream analyses (Hansen et al. 2018).

Two main questions repeatedly arise in the study of TF–chromatin interactions using SMT: (*a*) Which mobility signatures does a TF exhibit, and (*b*) how long is a TF bound to chromatin?

WHICH MOBILITY SIGNATURES DOES A TRANSCRIPTION FACTOR EXHIBIT?

TF consensus motifs are only 6–12 bp in size. In contrast, the typical human genome contains about 3 billion bp. The probability that a TF will find its cognate motif within the billions of nonspecific sites is vanishingly small (Mazzocca et al. 2021). How, then, are TFs able to respond to acute stimuli and modulate specific genes with such exquisite specificity?

The TF search problem cannot be resolved solely by TFs undergoing Brownian motion. Simple back-of-the-envelope calculations show that a TF simply diffusing in the nucleus would take up to 3 days to find a single motif, much longer than the life cycle of a single cell (Mazzocca et al. 2021). Combinatorial control, in which clusters of motifs that recruit multiple TFs serve as attractors for other molecules, can help significantly boost the odds of a TF finding its target site (Dror et al. 2016) (see the section titled What Regulates the Spatiotemporal Dynamics of Transcription Factors?). SMT has provided several answers to how this process occurs in bacteria. Since the bacterial genome is not packaged inside a nucleus, TFs have fewer obstacles to overcome to find their binding sites and employ a combination of simple diffusion and facilitated diffusion to find their targets (Elf et al. 2007, Hammar et al. 2012, Mazzocca et al. 2021). Can SMT provide similar mechanistic insight into the eukaryotic TF search process?

A description of the TF search process would be incomplete without first addressing the question of chromatin mobility. Correlation analysis of the motion of bulk chromatin together with biophysical modeling has shown that chromatin is a viscoelastic polymer, exhibiting different dynamics over short and long timescales (Bronstein et al. 2009). Chromatin exhibits transient anomalous diffusion at short timescales (the mean-squared displacement has a scaling exponent of 0.3) and normal diffusion at long timescales (Bronstein et al. 2009). This is consistent with theoretical modeling of chromatin as a polymer with an energy landscape that allows for long-range interactions due to loop formation by cohesin and is regulated by epigenetic modifications (Di Pierro et al. 2018, Shi et al. 2018). Analysis of fast SMT data on H2B has shown that chromatin can exhibit multiple mobility states on timescales shorter than 500 ms. These mobility states exist in spatially separated domains on these short timescales (Ashwin et al. 2019, Lerner et al. 2020). On longer timescales, however, nucleosomes can exist in one of two low-mobility states

and chromatin can dynamically switch between both states with no obvious spatial patterning (Wagh et al. 2023) (**Figure 3*a***). This finding underscores the importance of performing SMT studies across multiple timescales.

TFs must dynamically interact with chromatin to find binding sites. To speed up the target search, TFs must reduce the dimensionality of their search space. Theoretical models have proposed that TFs employ 3D diffusion to rapidly scan the entire nucleus but then rely on sliding, hopping, and facilitated diffusion once they localize close to their target sites (Mazzocca et al. 2021, Suter 2020) (**Figure 3*b***). While these models have been proven to some extent in bacteria, experimental evidence in support of these models in eukaryotic cells is lacking.

Intrinsically disordered region (IDR): region of a protein that lacks ordered 3D structure

Recent SMT studies of eukaryotic cells have shed some light on how TFs navigate the complex nuclear microenvironment. Examination of the mobility patterns of two transcriptional proteins, the TF c-MYC and the general transcriptional cofactor positive transcription elongation factor b (P-TEFb), showed that each of these TFs employ different target search strategies. c-MYC is a global explorer that undergoes noncompact exploration (**Figure 3*c***), in which c-MYC rapidly and repeatedly samples a large volume, eventually scanning the entire nucleus. This search mode prioritizes rapid search for distant targets at the expense of local target search. By contrast, P-TEFb exhibits a compact exploration strategy, in which it repeatedly samples its local chromatin microenvironment, optimizing the search in favor of local target sites rather than far-away targets (**Figure 3*c***). From single-molecule trajectories, one can compute the anisotropy index, which is the ratio of backward jumps to forward jumps. Noncompact exploration presents an increased propensity of backward jumps, whereas compact exploration, or for that matter Brownian motion, exhibits more balanced forward and backward jump probabilities (Izeddin et al. 2014).

Two factors involved in the same process can also exhibit distinct target search strategies. Both CTCF and cohesin are important for the formation of chromatin loops (Davidson & Peters 2021). For effective loop extrusion, CTCF must bind to specific sites, and the cohesin complex must be loaded to chromatin by accessory proteins (Davidson & Peters 2021). Despite being actors in the same process, CTCF spends relatively short time in search of CTCF motifs (~1 min), whereas cohesin spends over 30 min in search of its targets (Hansen et al. 2017). Using theoretical modeling along with SMT, researchers proposed that CTCF accelerates its search process by combining diffusion with transient trapping within presumed CTCF clusters via an internal RNA-binding domain in a process called guided exploration (**Figure 3*d***). If these clusters occur near CTCF binding sites, this could represent a targeting mechanism that increases the specificity of CTCF binding (Hansen et al. 2020). We should note, however, that whether these CTCF clusters occur within the vicinity of CTCF binding sites remains to be shown.

Most TFs, in addition to their structured DBD, contain intrinsically disordered regions (IDRs) that harbor transactivation domains (Lambert et al. 2018). Transactivation domains contain binding motifs for the recruitment of cofactors, leading to the assembly of the PIC (Chen & Pugh 2021). In addition to this, hydrophobic interactions between IDRs have been proposed to drive the formation of biomolecular condensates (Wagh et al. 2021a). IDRs are also key players in guiding the specificity of TFs. Biochemical studies have shown that TFs, in the absence of their DBDs, can still localize within close proximity of their cognate motifs due to their IDRs (Brodsky et al. 2020, Gera et al. 2022) (**Figure 3*e***). This allows them to rapidly sample their vicinity for consensus motifs using their DBDs, thereby speeding up the search (Jana et al. 2021). Consistent with this, IDR-swap experiments on the hypoxia-inducible factor (HIF) family of TFs showed that the IDRs of HIF-1α and HIF-2α regulate the specificity of their binding to distinct REs even though their DBDs are highly conserved (Chen et al. 2022). However, whether IDRs direct TF binding specificity through biomolecular condensates or by direct IDR–DNA interactions remains to be elucidated (Brodsky et al. 2021).

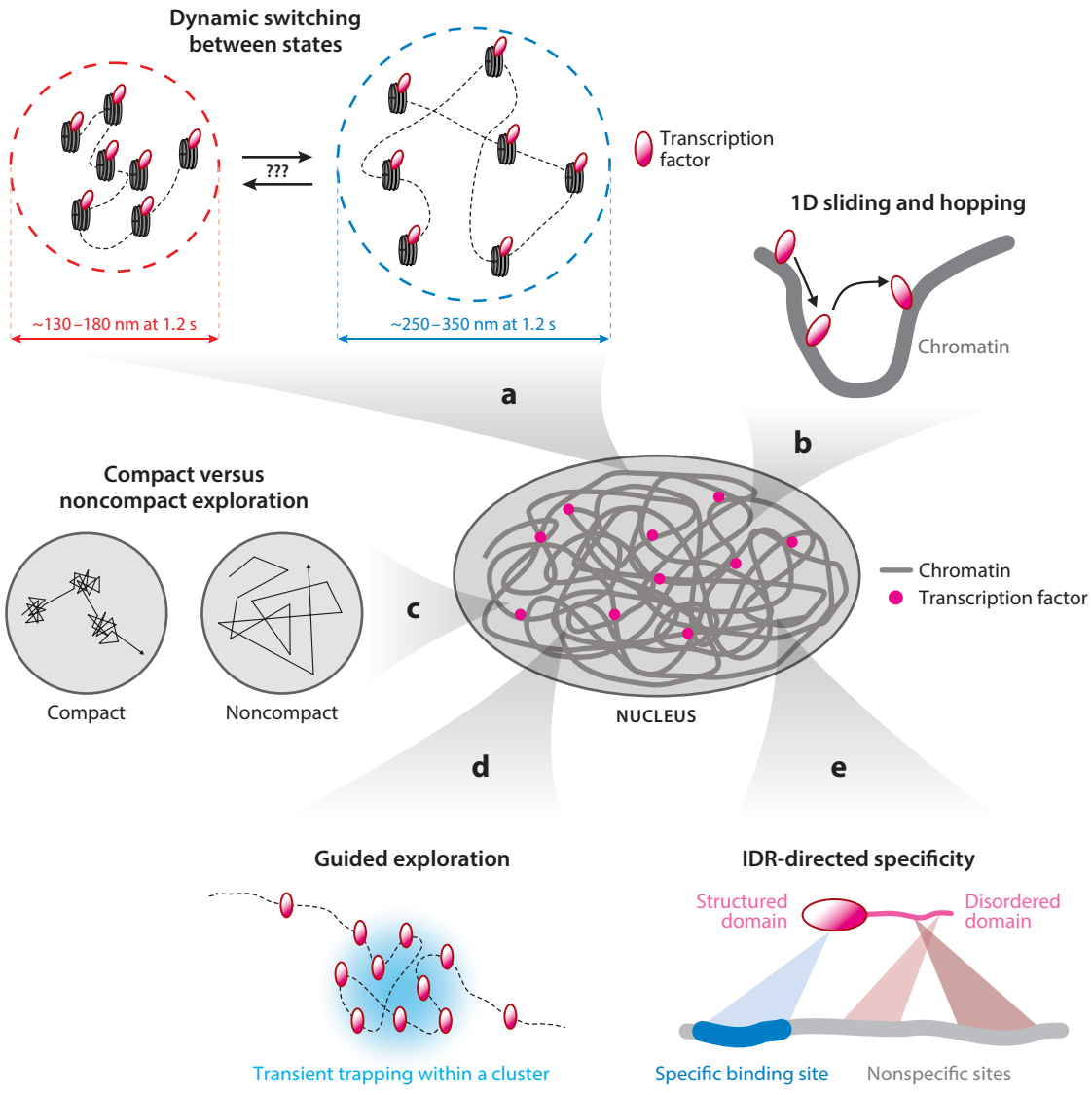

Figure 3

Spatial mobility of transcription factors (TFs). (*a*) When bound to chromatin, TFs dynamically switch between two distinct mobility states, through mechanisms to be determined (*question marks*). The lower-mobility state has an exploration diameter of ~130 to 180 nm at 1.2 s, and the higher-mobility state has an exploration diameter of ~250 to 350 nm at 1.2 s. (*b*) TFs undergo 1D sliding and hopping in search of their target sites. (*c*) TFs employ distinct target search strategies within the nucleus. Some TFs undergo compact exploration, exhaustively scanning local sites at the expense of distant ones. Others employ noncompact exploration, rapidly sampling the entire nucleus, which allows them to search for distant sites at the expense of missing nearby ones. (*d*) TFs can get transiently trapped within dynamic clusters. This allows them to rapidly sample the entire volume of the cluster, thereby speeding up the search process. (*e*) While the structured DNA-binding domain recognizes a specific motif within the binding site, intrinsically disordered regions (IDRs) allow the TF to localize close to the specific site, reducing the search space. The TF can then perform a local search to find its specific binding site. Panel *a* adapted from Wagh et al. (2023). Panel *e* adapted with permission from Brodsky et al. (2020).

Once a TF has found its binding site, what spatial mobility signatures do bound TF molecules exhibit? By combining SMT with a machine learning–based systems-level analysis, Wagh et al. (2023) showed that multiple TFs, coregulators, remodelers, and other cofactors exhibit two distinct low-mobility states, state 1 (the lower of the two mobility states) and state 2 (the higher of the two mobility states). These states reflect the underlying mobility of chromatin on a timescale of 1.2 s. Strikingly, H2B as well as transcriptional regulators dynamically switch between these states (**Figure 3*a***). The activation of steroid receptors with their respective hormones results in a dramatic increase in the population fraction of state 1. Mutagenesis experiments on PPARγ2 showed that binding in this lowest-mobility state requires an intact DBD as well as dimerization domains. These results indicate that the lowest-mobility state for TFs represents the binding of an active TF with potential functional outcomes (Wagh et al. 2023). The cohesin loader NIPBL was recently shown to be recruited by GR to specific genomic sites in a hormone-dependent manner. Acute depletion of the cohesin complex using an auxin-inducible degron system substantially reduced the GR bound fraction, residence time, and population fraction of state 1, suggesting that long-range interactions are essential to regulate GR dynamics (Rinaldi et al. 2022). Deletion of the disordered N terminus of GR resulted in the loss of state 2, suggesting that binding in state 2 may result from interactions mediated by the IDRs of the TF, through biomolecular condensates, multivalent interactions with cofactors, or direct interactions between the IDRs and chromatin (Garcia et al. 2021b).

The TF search process is highly complex and multifactorial. For the amount of time that a TF spends searching for its target sites, how much time must it actually spend bound to its RE to effectively regulate target genes?

HOW LONG IS A TRANSCRIPTION FACTOR BOUND TO CHROMATIN?

Binding of a TF to sites within enhancers or promoter-proximal regions leads to the recruitment of cofactors, eventually activating or repressing target genes. This is a highly stochastic process, and many TF binding events do not lead to productive transcription (Rodriguez & Larson 2020). Initial biochemical studies predicted that TFs, such as the ligand-dependent steroid receptor GR, remain bound to chromatin for hours in the presence of hormone (Perlmann et al. 1990). This long residence time of GR was believed to be essential to maintain a substrate for the recruitment of transcriptional machinery. FRAP studies challenged this view by demonstrating that GR only transiently interacts with REs (McNally et al. 2000). However, FRAP recovery times are not direct measures of the residence time of the TF, and the choice of model can significantly influence these numbers (Mazza et al. 2012, Mueller et al. 2010). SMT can bridge this gap by directly observing bound molecules.

To measure residence time distributions from SMT experiments, one needs to address the challenge of photobleaching. Photobleaching, while our ally in FRAP experiments, prevents the detection of long binding events in SMT. A bound TF molecule can disappear from the focal plane either because it diffuses away from the focal plane (unbinds from chromatin) or because the fluorophore photobleaches (Garcia et al. 2021a, Hansen et al. 2018, Kuhn et al. 2021). One cannot distinguish between these two scenarios while tracking molecules in two dimensions. However, one can measure the rate of photobleaching and develop appropriate methods to correct for it.

The photobleaching rate is determined by the photostability of the fluorophore along with the laser power and exposure time of the experiment, and this sets the upper limit for the temporal range that can be explored by SMT (Kuhn et al. 2021, Presman et al. 2017). The binding affinity of a TF sets the timescale of TF–chromatin interactions, but if the photobleaching rate is faster,

one cannot resolve binding events longer than the photobleaching time constant. On the other hand, if the photobleaching rate is slower than the timescale of TF binding, then one can resolve the entire temporal range of TF–chromatin interactions.

To minimize photobleaching in the experimental setup, researchers typically use low laser powers coupled with long (∼0.5–1 s) exposure times. This has the dual advantage of blurring out fast-diffusing molecules and visualizing only those molecules that are bound (or remain in the same spot) (Garcia et al. 2021a, Presman et al. 2017). The residence time distributions obtained from SMT experiments must be corrected for photobleaching to obtain the true underlying binding time distributions. To identify the best photobleaching correction method, one can acquire SMT data for the same TF with different photobleaching rates by tuning the laser power and exposure times (Garcia et al. 2021a, Kuhn et al. 2021). Regardless of the imaging parameters, the affinity landscape of the nucleus for the TF of interest should be unchanged, and therefore, so should the survival distribution. Several methods to measure and correct for photobleaching exist in the literature, but care should be taken when applying these methods to ensure that the photobleaching-corrected survival distributions obtained under different acquisition conditions remain invariant (Garcia et al. 2021a).

Methods that rely on estimating the photobleaching rate by counting the number of detected molecules in every frame, fitting the decay curve to a bi-exponential function, and dividing the raw survival distribution by this exponential tend to underestimate the photobleaching rate. This is because fast-diffusing molecules that remain in the focal plane for only one or two frames dominate the decay curve.

Other photobleaching correction methods use histones to measure the photobleaching rate, because histones, when incorporated into chromatin, exchange slowly, on timescales much longer than the measured photostability of organic fluorophores commonly used in SMT (Kimura & Cook 2001). The photobleaching rate can be extracted by fitting the survival distribution of histones to a multiexponential function. Irrespective of the functional form of the TF survival distribution, photobleaching correction can be done by dividing the raw TF survival distribution by the slowest exponential component that fits the histone survival distribution. A recent study has shown that this latter method most faithfully reproduces the same survival distribution independent of the acquisition conditions (Garcia et al. 2021a).

Finally, all survival distributions are constructed from the dwell times of those TF segments that are considered bound, as measured by their motion compared with that of histones, with the assumption that TFs bound to chromatin present the same motion as incorporated histones (Garcia et al. 2021a, Mazza et al. 2012).

Textbook models present a black-and-white picture of TF–chromatin interactions in which TFs bind specific sites via their DBDs with high affinity and interact with chromatin nonspecifically with low affinity. If the binding affinities to these sites are indeed well separated and narrowly distributed in vivo, then the resulting TF survival distribution can be phenomenologically modeled by a bi-exponential survival distribution (J. Chen et al. 2014, Hansen et al. 2017, Morisaki et al. 2014, Paakinaho et al. 2017, Presman et al. 2017, Swinstead et al. 2016, Tang et al. 2022) (**Figure 4*a***). The fast component of the bi-exponential fit is interpreted as the time constant of nonspecific binding, and the slow component reflects that of specific binding (Presman et al. 2017).

However, the nucleus is highly heterogeneous, with several subnuclear compartments, membraneless organelles, and biomolecular condensates, which would impose constraints on TF mobility. These constraints, combined with the multiplicities of DNA binding sequences of varying affinities, should present a rough energy landscape for the TF to navigate, implying that TF–chromatin associations should exhibit more complex temporal characteristics. Accordingly, the *Tet* repressor, which has no specific binding sites within the eukaryotic genome, exhibited a

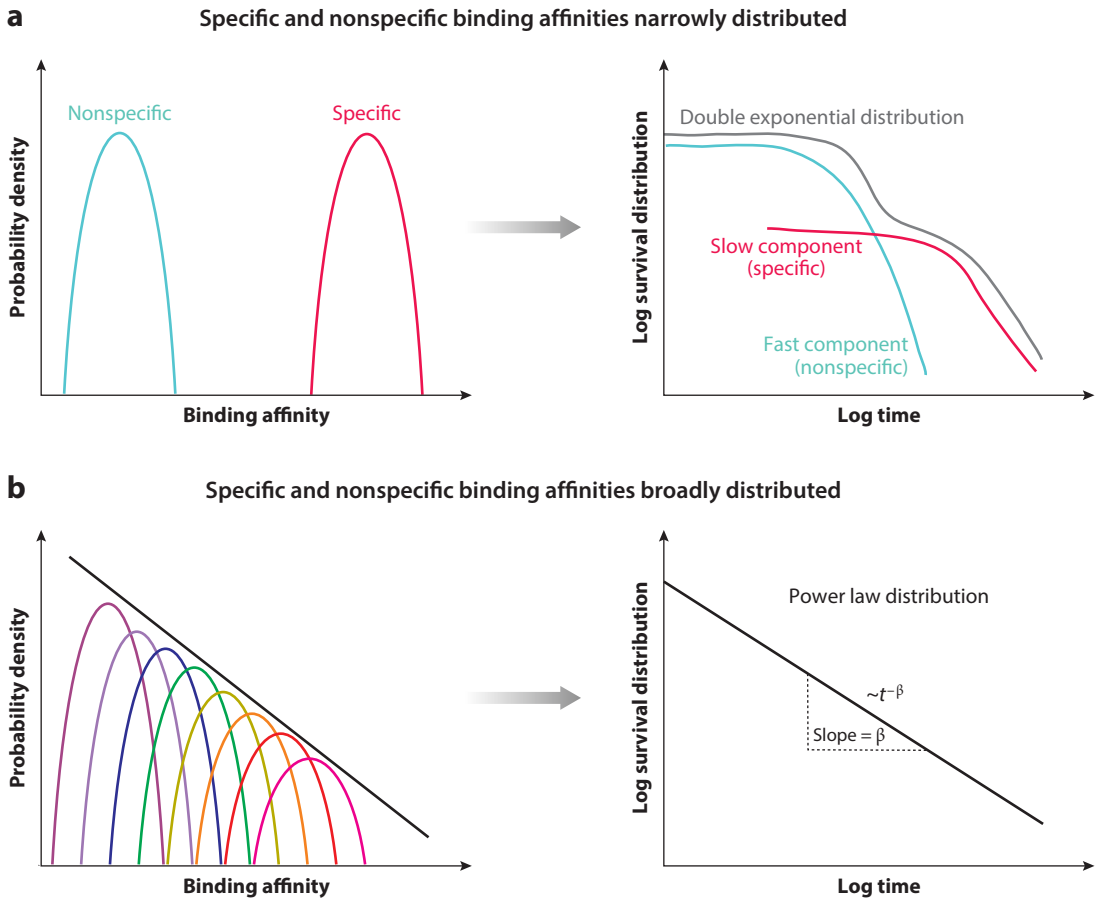

Figure 4

Temporal dynamics of transcription factors. (*a*) Well-separated and narrowly distributed specific and nonspecific binding affinities lead to a double exponential survival probability distribution of transcription factor residence times. The fast component is typically interpreted as reflecting nonspecific binding, and the slow component reflects specific binding. (*b*) A broad distribution of binding affinities arises due to the heterogeneity within the nucleus. This leads to a power law distribution of residence times. The power law exponent can be calculated as the slope of the survival distribution on a log-log plot. Figure adapted from Garcia et al. (2021a) (public domain).

power law survival distribution (Normanno et al. 2015), as did *lac*I (Caccianini et al. 2015). This broad distribution of residence times cannot be reconciled with the assumed narrow distribution of nonspecific binding affinities. Furthermore, SMT experiments on endogenous and ectopically expressed mammalian TFs revealed that, upon appropriate photobleaching correction, most TFs exhibit a power law distribution of dwell times, suggesting that specific chromatin interactions, much like nonspecific ones, have broad affinity distributions (Garcia et al. 2021a, Johnson et al. 2023, Stavreva et al. 2019). Mathematically, one can model nuclear heterogeneity (as experienced by a TF in search of its target site) as a rough energy landscape, which naturally results in a power law survival distribution (Garcia et al. 2021a) (**Figure 4*b***). Indeed, in vitro high-throughput measurements of TF–DNA binding affinity have revealed a continuum of DNA binding affinities without a clear separation between specific and nonspecific binding (Kribelbauer et al. 2019). These findings imply that the distinction between specific and nonspecific binding is nuanced and

Power law: functional relationship between the independent variable x and dependent variable $f(x)$, where $f(x) \sim x^a$

complex, with affinity not necessarily correlated with binding specificity (Rastogi et al. 2018). Furthermore, binding to a specific site is necessary but not sufficient to elicit a functional response (Rodriguez & Larson 2020). While most studies focus on distinguishing between specific and nonspecific binding, the central challenge in the field is to tie binding (specific or nonspecific) to functional outcomes, and further experimentation is required to address this question.

WHAT REGULATES THE SPATIOTEMPORAL DYNAMICS OF TRANSCRIPTION FACTORS?

To get a better understanding of how TFs regulate gene expression, we must first understand the factors that regulate the spatiotemporal dynamics of TFs at REs, especially their residence times.

ATP-Dependent Remodeling Complexes

Dissociation of TFs from response elements could be either an equilibrium process, which means that this step does not require the expenditure of energy, or an active process that requires ATP. The first clue that unbinding of a TF might require ATP came from in vitro biochemical experiments. GR binds to the in vitro reconstituted MMTV promoter sequence. Ultrafast UV laser cross-linking experiments showed that the recruitment of the SWI/SNF complex by GR is dependent on DNA sequence, and in the absence of GR the complex is still recruited to chromatin but to random positions (Nagaich et al. 2004). The resulting remodeling activity of the SWI/SNF complex results in ATP-dependent eviction of GR from the MMTV template (Fletcher et al. 2002) (**Figure 5*a***).

In vivo, depletion of ATP by sodium azide and deoxyglucose slowed down the exchange of GR from the MMTV array as observed by FRAP (Stavreva et al. 2004). GR and BRG1, a subunit of the SWI/SNF complex, exhibit identical dwell time distributions as measured by SMT (Garcia et al. 2021a). While not causative, this indicates that the temporal dynamics of GR and BRG1 are intimately coupled as well. ER and BRG1 also colocalized at a prolactin array, which contains tandem repeats of ER response elements (Sharp et al. 2006). Like GR on the MMTV array, ER also rapidly exchanged from the prolactin array in the presence of remodeling complexes. ATP depletion, however, led to the immobilization of ER.

This coupling between TFs and chromatin remodelers is not unique to mammalian cells. The yeast remodeler RSC2 was shown to be necessary for unbinding the TF Ace1p by both FRAP (Karpova et al. 2004) and SMT (Mehta et al. 2018). However, other remodelers were dispensable for this process, since their deletion did not affect Ace1p binding kinetics (Karpova et al. 2004). The specificity is further reinforced by fluorescence resonance energy transfer (FRET) experiments that showed a direct interaction between Ace1p and RSC2 (Karpova et al. 2008). Moreover, in vitro DNA nucleosome unzipping experiments showed that the TF Gal4 is evicted from chromatin by the SWI/SNF complex but not by ISW1a (Li et al. 2015). Yeast remodelers themselves exhibit fast kinetics, with a mean residence time of 4 to 7 s (Kim et al. 2021). However, more studies of the role of chromatin remodeling complexes in TF eviction are needed to examine the generality of this phenomenon.

Proteasome and Chaperones

Chromatin remodelers are but one species of complexes that consume ATP. The proteasome is a multicomponent complex that degrades misfolded or otherwise unnecessary proteins through its proteolytic activity (Bard et al. 2018). Conversely, chaperones such as Hsp90 and Hsp70 help fold proteins and degrade misfolded proteins (Genest et al. 2019). Both of these ATP-dependent complexes play a role in regulating TF kinetics.

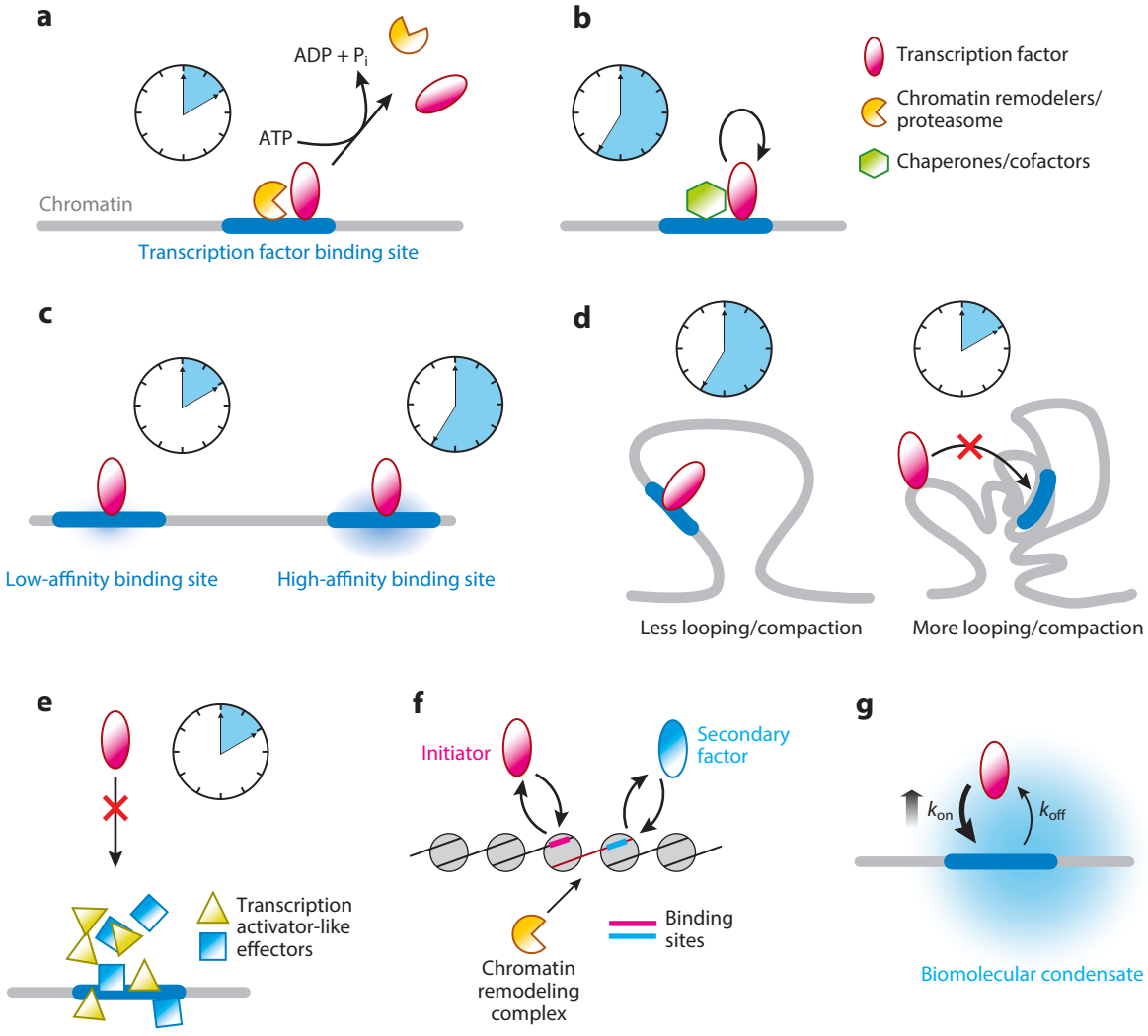

Figure 5

Regulation of transcription factor (TF) residence times. (*a*) Chromatin remodelers and the proteasome evict TFs from chromatin, shortening their residence times. (*b*) Chaperones and other cofactors can stabilize TFs, extending the residence times. (*c*) TFs bind to low-affinity binding sites for shorter times and to high-affinity binding sites for longer times. (*d*) In the presence of a small number of loops, chromatin is less compacted, and TFs can find their target sites within the loop. In the presence of several loops, chromatin is highly compacted, and TFs can bind only to the surface of the chromatin globule due to volume exclusion, with short-lived nonspecific interactions. (*e*) Transcription activator-like effectors bound to the regulatory element can inhibit the binding of TFs. (*f*) Dynamic assisted loading: An initiator TF (*magenta*) binds to a closed chromatin site and recruits chromatin remodeling complexes. Dynamic binding of the initiator and remodeler leads to a transient increase in the accessibility of the cognate motif of a secondary TF (*cyan*), which is sufficient for the (assisted) loading of the secondary factor. Notably, the initiator and secondary factor can reverse roles depending on the local chromatin context. (*g*) An increase in the local concentration of TFs within biomolecular condensates increases the concentration-dependent ON rate (k_{on}) for TFs within the condensate. Panel *f* is adapted with permission from Swinstead et al. (2016).

Chaperones Hsp90 and Hsp70 along with the 19S subunit of the proteasome localized along with GR at the tandem MMTV array (Stavreva et al. 2004). Inhibition of Hsp90 with the antibiotic geldanamycin resulted in faster FRAP recovery, which implies shortened GR residence time at the MMTV promoter. On the other hand, inhibition of the proteasome with MG-132 slows down

FRAP recovery and immobilizes GR to the MMTV array (Stavreva et al. 2004). The inhibition of the proteasome also immobilizes ER at target promoters (Reid et al. 2003, Stenoien et al. 2001). Proteasomal inhibition increases GR transactivation (Deroo et al. 2002) but has the opposite effect on ER (Lonard et al. 2000), indicating that the proteasome may play a specific role in regulating GR-mediated activation and a more general role in evicting TFs from chromatin (**Figure 5*a***). To further support the proteasome-mediated cycling of TFs from chromatin, an NF-κB mutant insensitive to proteasomal degradation exhibited a higher mobile fraction than did wild-type NF-κB (Bosisio et al. 2006). Thus, chaperones and proteasomes regulate TF kinetics in opposite ways: Chaperones stabilize TF binding to specific sites (**Figure 5*b***), whereas proteasomes speed up the dissociation of TFs from chromatin (Stavreva et al. 2004) (**Figure 5*a***). One takeaway is clear: The unbinding of TFs from their REs is an ATP-dependent process.

Ligand and Motif Affinity, Crowding

The transactivation potential of ligand-dependent TFs depends strongly on the affinity of the ligand for the TF. For GR, the high-affinity synthetic hormone dexamethasone (Dex) is far more potent at transactivation than its natural hormone, corticosterone (Cort) (Stavreva et al. 2019). Both Dex- and Cort-liganded GR exhibit power law distributed dwell times, with GR-Cort binding longer than GR-Dex (Stavreva et al. 2019). Washing out Cort from the cells still produces a power law distribution of dwell times, underscoring the broad distribution of nonspecific binding affinities within the nucleus (Garcia et al. 2021a, Stavreva et al. 2019). However, the overall dwell time distribution upon Cort washout is faster than that for either Cort- or Dex-liganded GR (Stavreva et al. 2019). Similarly, unliganded ER binds for shorter times than ER activated by 17β-estradiol (E_2) (Paakinaho et al. 2017). Somewhat paradoxically, the antagonist ICI-182,780 immobilizes ER, resulting in little FRAP recovery (Stenoien et al. 2001). An SMT study of the serum response factor found that it can bind for as long as 1 min in serum-starved (quiescent) cells. Serum stimulation increased the population fraction of these long-bound molecules and the longest dwell time (Hipp et al. 2019).

The affinity of the binding site also plays an important role in determining the residence time of a TF at that site. By mutating a Gal4 binding site in yeast, protein-induced fluorescence enhancement experiments showed that Gal4 binds for longer times to high-affinity motifs than to low-affinity motifs with consequences for transcriptional outcome (Donovan et al. 2019) (**Figure 5*c***).

TF binding can be hindered because of competition from other TFs binding to the same site and also because of volume exclusion, either due to high chromatin density or due to crowding by other factors (Cortini & Filion 2018) (**Figure 5*d***). Artificial TFs, termed transcription activator-like effectors (TALEs), can be used to examine the role of multiple nucleotide recognition repeat domains in determining TF dwell times. Changing the DBD affinity for a GR response element led to a sixfold difference in TALE residence time (Clauss et al. 2017). Since these TALEs were binding to GR response elements, the longer TALE residence times interfered with GR binding, resulting in downregulation of the target gene, *SGK1* (Clauss et al. 2017) (**Figure 5*e***).

Dynamic Assisted Loading

While TFs and other competing nuclear proteins can hinder TF binding in some cases, TFs can also assist the binding of another TF. In the dynamic assisted loading model, the binding of an initiator TF to nucleosomal DNA leads to the recruitment of chromatin remodeling complexes, which creates a transient open chromatin state at the RE. This transient open chromatin state provides a short temporal window for a secondary TF to bind to its cognate motif, which is now

accessible (Biddie et al. 2011, Goldstein et al. 2017, Grontved et al. 2013, Miranda et al. 2013, Swinstead et al. 2016, Voss et al. 2011) (**Figure 5*f***). In contrast to the pioneer factor model, wherein only a special class of pioneer TFs can penetrate closed chromatin to increase accessibility for other nonpioneer TFs, dynamic assisted loading is symmetric. The initiator and secondary factor can help each other bind chromatin, depending on the local chromatin context. Highly dynamic TF–chromatin interactions are sufficient to facilitate the binding of the secondary factor, and the recruitment of ATP-dependent remodeling complexes is essential (Voss & Hager 2014).

Intermolecular Interactions

The IDRs within transactivation domains have been implicated in the formation of biomolecular condensates through various mechanisms. The binding of a TF to a specific site can nucleate site-specific transcriptional condensates (Kent et al. 2020, Wei et al. 2020) that can serve as attractors for other TFs of the same species, leading to their local accumulation near an enhancer or promoter of a target gene. This accumulation increases the concentration-dependent ON rate, leading to more frequent TF binding within this condensate (Garcia et al. 2021b) (**Figure 5*g***). This hypothesis has also been proposed in a developmental context in *Drosophila melanogaster* embryos, where the TF Bicoid forms a morphogen gradient that determines various cell fate decisions. Bicoid forms transient hubs in both high- and low-Bicoid-expressing cells. This clustering has been proposed to enhance the binding of Bicoid to low-affinity targets by selectively enriching its concentration at these sites (Mir et al. 2017, 2018).

These transactivation domains also recruit cofactors, which can modulate TF binding kinetics. For example, overexpression of MYC leads to an increase in the overall binding times of general TFs such as TBP and SPT5 while reducing the dwell time of others such as MED1 (Patange et al. 2022). GR and its sister receptor, the mineralocorticoid receptor (MR), interact on chromatin in a site-specific manner. The presence of GR significantly extends the residence time of MR in the presence of the GR-activating hormone Cort (Johnson et al. 2023).

HOW DO CHANGES IN TRANSCRIPTION FACTOR DYNAMICS AFFECT TRANSCRIPTION?

Transcription is a stochastic process in which almost all eukaryotic genes transcribe in bursts, exhibiting ON times on the order of minutes to hours and even longer OFF periods (de Jonge et al. 2022, Lammers et al. 2020, Rodriguez & Larson 2020). Researchers have used MS-2/PP7 stem-loop reporter systems to examine hundreds of genes, and one can now confidently state that most genes transcribe in bursts (Chubb et al. 2006, Donovan et al. 2019, Larson et al. 2011, Lee et al. 2019, Rodriguez et al. 2019, Stavreva et al. 2019, Suter et al. 2011, Wan et al. 2021). TF dynamics can modulate transcriptional kinetics through changes in the average occupancy of TFs at REs, the residence time of TFs, or the local and global concentrations of TFs within the cell (de Jonge et al. 2022, Rodriguez & Larson 2020). Three important parameters characterize the kinetics of any gene: burst frequency (how often a gene turns on), burst duration (the length of time a gene is on), and burst amplitude (the amount of RNA produced during the ON period). In this section, we examine evidence in support of the role of TF dynamics in regulating each of these kinetic parameters.

Using super-resolution microscopy, researchers have shown that RNA Pol II and the Mediator complex form highly dynamic clusters, lasting only ~5–10 s before dissipating (Cho et al. 2018, Cisse et al. 2013). Enhancer–promoter loops bring distal REs in close physical proximity of target genes. The transcriptional factory model posits that clustering of multiple enhancers boosts transcription by enhancing the local concentration of TFs and other regulatory factors near genes

(Wagh et al. 2021a). However, single-molecule techniques have shown that these CTCF-mediated loops themselves form for short periods of time (~10–30 min) (Gabriele et al. 2022). Even the assembly of the PIC can occur within seconds (Nguyen et al. 2021). How, then, do multiple events occurring on the timescale of a few seconds to minutes lead to transcription bursts that can last for over 1 h? To answer this question, we first must understand how TF dynamics contribute to transcriptional bursting.

Transcriptional bursting: dynamic property of transcription characterized by finite ON periods interspersed with long OFF periods

Synthetic TFs are a powerful model system to dissect the role of the DBD and transactivation domains in modulating TF dwell times and, consequently, bursting parameters. One study using synthetic TFs with transactivation domains of varying strengths showed that the TF residence time on a promoter primarily regulates burst duration, with longer residence times translating to longer ON times for the oncogene *c-FOS* (Senecal et al. 2014). The strength of the transactivation domain did not affect residence time but tuned the initiation rate of the polymerase. Other studies with synthetic TFs also found that the number of messenger RNA (mRNA) detected by single-molecule fluorescence in situ hybridization was positively correlated with TF residence times. However, this increase in the number of mRNA arises from a residence time–dependent modulation of burst frequency, not burst duration (Popp et al. 2021). As discussed above, transactivation domains of TFs can play two distinct roles. They can serve as substrates for the recruitment of cofactors or promote the formation of biomolecular condensates through liquid-liquid phase separation (LLPS) mediated by IDRs or other multivalent interactions. By fusing modular DBDs to transactivation domains with differential potential for recruiting cofactors along with optogenetic control of droplet formation, researchers showed that multivalent interactions that drive the recruitment of cofactors and prolong TF–chromatin interactions boost transcription, but in a mechanism that is independent of LLPS (Trojanowski et al. 2022). Condensates formed through IDR–IDR interactions modulate transcription in a concentration-dependent manner. Within an optimal range of IDR expression, condensates increase transcription of target genes. However, high IDR expression leads to the formation of large condensates far away from target sites. These condensates sequester TFs away from their REs, leading to a decrease in overall transcription (Chong et al. 2022).

Extending these results to bona fide TFs, the residence time of GR was positively correlated with the ON time of an MMTV reporter gene. Longer residence times of GR led to longer bursts, and higher bound fractions led to more frequent initiations of transcription (Stavreva et al. 2019). Activation of GR by two different hormones changed both their residence times and bursting kinetics. Cort-liganded GR has longer dwell times and longer bursts, and Dex-liganded GR has a higher bound fraction and more frequent bursts (Stavreva et al. 2019). Similarly, 2 h after DNA damage induced by ionizing radiation, the tumor suppressor protein p53 exhibits longer residence times and this correlates with an increase in the number of nascent transcripts of a p53 target gene, *CDKN1a* (Loffreda et al. 2017). Upregulation of *CDKN1a* and longer binding of p53 were dependent upon acetylation of p53's C-terminal domain (CTD) (Loffreda et al. 2017). In yeast, too, the residence time of Gal4 dictates the ON time and burst size of Gal4 target genes but does not affect OFF times (Donovan et al. 2019). However, residence time alone is not sufficient to modulate transcription. Deletion of the transactivation domain of p65 does not significantly change its residence time but does significantly decrease the level of transcription of a p65 reporter (Callegari et al. 2019). This finding implies that although the DBD determines the residence time of p65, it is not sufficient to upregulate transcription in the absence of the transactivation domain.

How does the expression level or concentration of TFs affect transcription? In studies using synthetic TFs, the concentration of the TF modulated the burst frequency of *c-FOS* but not the other bursting parameters (Senecal et al. 2014). Changing the concentration of TALE TFs by dose-dependent degradation using an auxin-inducible degron system showed that concentration

does not significantly affect the burst size or burst frequency (Popp et al. 2021). On the other hand, MYC expression levels tune global transcriptional levels: Overexpression of MYC leads to a global increase in transcriptional burst durations (Patange et al. 2022). These findings suggest that TF concentration could affect transcription, but in a TF- and cell-type-specific manner.

While all these studies have presented somewhat confounding pieces of evidence about whether and how TF dynamics regulate transcription, one common thread emerges: the longer a TF is bound, the higher the transcriptional output of its target genes (Donovan et al. 2019; Loffreda et al. 2017; Stavreva et al. 2004, 2019). Since most TFs exhibit power law distributions of dwell times, there are indeed subpopulations of molecules that likely remain bound for long times. These molecules might represent the most productive binding events of the entire population. Indeed, analysis of TF binding times within regions of the nucleus enriched for active RNA Pol II shows that TFs bind longer within clusters of active RNA Pol II than outside the clusters (Liu et al. 2014, Morisaki et al. 2014).

INTEGRATING TRANSCRIPTION FACTOR DYNAMICS INTO THEORETICAL MODELS OF TRANSCRIPTION

Transcription is a complex process consisting of several steps, such as nucleosome eviction, TF binding, recruitment of coactivators, formation of the PIC, and RNA Pol II recruitment, each of which is a highly stochastic process. How, then, are cells able to respond rapidly to acute stimuli with such specificity?

Live-cell studies have shown that residence time is singularly coupled with transcriptional activation or repression. ATP-dependent remodelers and the proteasome are essential for the eviction of TFs from chromatin, whereas chaperones serve to stabilize at least some TFs (see the section titled What Regulates the Spatiotemporal Dynamics of Transcription Factors?) (**Figure 5**). How can these findings be integrated into theoretical models of gene activation?

Several biological processes within cells must be executed with high fidelity, trading efficiency for specificity: for example, translation of mRNA by ribosomes and aminoacyl-tRNA synthetases, DNA replication by DNA polymerases, and splicing by the spliceosome. Equilibrium thermodynamics and detailed balance set a limit on the efficiency of these processes, called the Hopfield barrier, which can be overcome with the expenditure of energy (Wong & Gunawardena 2020). Kinetic proofreading (Hopfield 1974; reviewed in Boeger 2022) has been proposed as a nonequilibrium model to explain the substrate recognition specificity of several biological processes. The kinetic proofreading framework has been recently extended to model gene regulation as well (Blossey & Schiessel 2008, Schiessel & Blossey 2020, Shelansky & Boeger 2020, Shelansky et al. 2022).

Kinetic proofreading in the context of transcription has one main requirement: energy expenditure by ATP hydrolysis (Trojanowski & Rippe 2022). Chromatin remodelers and the proteasome, both of which consume ATP, readily fulfill this requirement. The recruitment of a remodeler to a site bound by a TF can result in either the dissociation of the TF by the remodeler or, if the proofreading step is accepted, the eviction of the nucleosome, thereby making it easier for RNA Pol II to initiate transcription (**Figure 6**). For proofreading to occur, the TF must not dissociate before the proofreading agent (chromatin remodeler) gets recruited to the RE, necessitating sufficiently long residence times. While TFs rapidly exchange from REs, general TFs such as TBP bind for much longer times than other TFs (Sprouse et al. 2008), potentially bookmarking specific sites for the recruitment of RNA Pol II. Therefore, another requirement of the kinetic proofreading model is that rejection by the proofreading agent should result in restoration of the native promoter or RE state (**Figure 6**). This means that failure of the proofreading step should wipe the slate clean, removing any memory of the TF binding. For TBP, the ATPase activity of

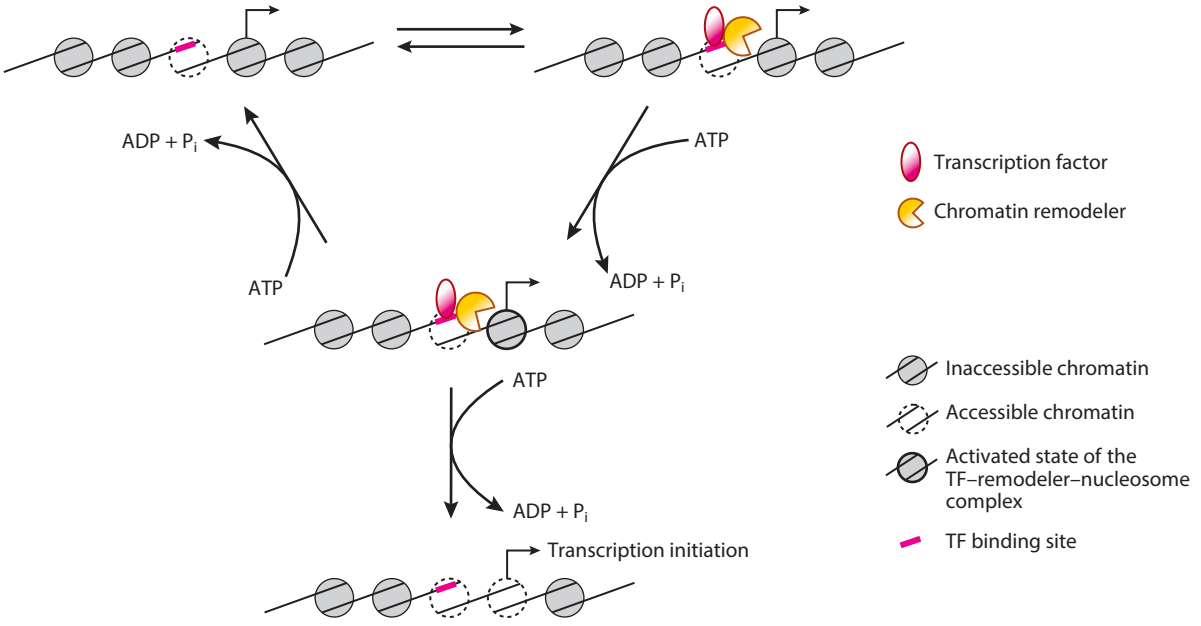

Figure 6

Kinetic proofreading. Promoters wrapped around nucleosomes are inaccessible to the transcriptional machinery. Transcription factors (TFs) and ATP-dependent remodelers (or other proofreading agents) can reversibly assemble at regulatory elements within 3D proximity of the occluded promoter. If the TF does not unbind from chromatin (i.e., it has a sufficiently long residence time), ATP hydrolysis transitions the TF–remodeler–nucleosome complex to an activated state. From here, two possible ATP-dependent transitions exist: (*a*) Failure of the proofreading step results in the entire complex dissociating, causing a transition back to the native state, or (*b*) eviction of the nucleosome by the remodeler grants access for the recruitment of the transcriptional machinery and subsequent initiation of transcription.

the Mot1 enzyme readily satisfies this requirement by evicting TBP from the RE (Auble et al. 1997).

After transcriptional initiation, two ATP-dependent steps further regulate transcription. Phosphorylation of Ser5 in the CTD of RPB1 (a subunit of RNA Pol II) leads to initiation of transcription (Jonkers & Lis 2015). After transcribing a short stretch of DNA, RNA Pol II pauses ~30–60 nucleotides downstream of the transcription start site (Core & Adelman 2019). Phosphorylation of Ser2 by P-TEFb releases paused RNA Pol II, allowing for productive elongation. FRAP studies of endogenous RPB1 showed that ~23% of RNA Pol II are paused for ~40 s, and only 1 in 13 paused RNA Pol II transition to productive elongation (Steurer et al. 2018). Thus, phosphorylation of RNA Pol II CTD serves as an additional nonequilibrium regulatory step in transcriptional regulation.

CONCLUSIONS AND OUTLOOK

Live-cell experiments over the past 20 years have shifted the paradigm away from stable long-term binding of TFs toward a picture where TF dynamics are intimately coupled with nuclear architecture and gene expression. Biochemical studies and theoretical modeling have helped identify mechanistic links between TF binding and transcriptional output. However, we still have little experimental evidence for how TFs navigate the 3D genome over time in search of their target sites in eukaryotic cells. Various theoretical models for how this process occurs have been proposed

but technical limitations in terms of spatial and temporal resolution of imaging and photobleaching have prevented experimental validation of these models. Advances in microscopy now allow researchers to track single molecules at a spatial resolution of 2 nm and a temporal resolution of hundreds of microseconds using MINFLUX nanoscopy (Balzarotti et al. 2017, Deguchi et al. 2023, Eilers et al. 2018, Gwosch et al. 2020, Schmidt et al. 2021, Wolff et al. 2023).

Similarly, we have largely correlative evidence for how changes in TF residence time, bound fraction, and concentration affect transcriptional bursting. However, to test theoretical models, we must image TF dynamics at specific gene loci. The sparse labeling required for SMT makes the probability of visualizing such interactions at a diffraction-limited transcription site vanishingly small. A new microscopy technique called orbital tracking (Kis-Petikova & Gratton 2004, Levi et al. 2005) allows for simultaneous visualization of TF dynamics and transcriptional output within an optical volume. Autocorrelation and cross-correlation analyses of the fluorescence traces reveal the complex interplay between regulatory factors and transcription (Donovan et al. 2019, Stavreva et al. 2019).

The chromatin scaffold with which TFs interact is itself a highly dynamic viscoelastic polymer. Recent studies have shown that active processes within the nucleus such as transcription and loop extrusion can significantly reduce the ensemble mean-squared displacement of chromatin (specifically nucleosome-incorporated histone H2B) (Nagashima et al. 2019, Nozaki et al. 2017). However, direct measurements of chromatin dynamics during and after a transcriptional burst are essential to put chromatin dynamics in a functional context. Emerging evidence from a study combining static measurements of local 3D chromatin organization along with gene expression suggests that transcription confines the local chromatin polymer (Bohrer & Larson 2023). Tracking these specific loci labeled by CRISPR-based technologies (van Tricht et al. 2023), under activation or repression of transcription along with polymer physics models, will provide insight into how genome packaging inside the nucleus is affected by TFs and architectural factors (Shin et al. 2022). The binding of a TF and subsequent recruitment of the transcriptional machinery affect not just the biochemical makeup of chromatin but also its biophysical properties. Yet how the mobility state of chromatin affects TF dynamics and search kinetics is often overlooked.

Last, mechanical cues in the environment of cells change cell shape, nuclear architecture, and gene expression programs (Wagh et al. 2021b). Cells must be able to sense and respond to extracellular cues within their microenvironment. To get a complete picture of how TF dynamics regulate pathophysiological processes such as development, cancer progression, and aging, one must study TF dynamics and real-time gene regulation within complete embryos or organoids (Kuhn et al. 2022; Mir et al. 2017, 2018) in physiologically relevant environments that best recapitulate biophysical cues that cells experience within their native tissue.

SUMMARY POINTS

1. Single-molecule tracking (SMT) allows for direct measurement of transcription factor (TF) diffusion coefficients and the entire distribution of TF dwell times.
2. Intrinsically disordered regions help the TFs localize within close proximity of their binding sites, and the structured DNA-binding domain (DBD) then rapidly searches for the specific motif.
3. Chromatin-bound TFs dynamically switch between two distinct mobility states, with binding in the lower-mobility state requiring an intact DBD as well as oligomerization domains.

4. Photobleaching prevents tracking TFs for both short (<10 ms) and long (>1 min) times.
5. Most TFs exhibit power law distributed dwell times, blurring the line between specific and nonspecific binding.
6. The residence time of TFs is positively correlated with transcriptional output.
7. Eviction of TFs from chromatin is an energy-/ATP-dependent process.
8. Kinetic proofreading can explain the specificity with which TFs bind some of their binding sites but leave most others unoccupied.

FUTURE ISSUES

1. Development of tools to track single TF molecules binding to specific loci within the nucleus at high spatial and temporal resolution will help researchers test theoretical models of the TF search process.
2. Simultaneous visualization of chromatin and transcriptional bursting at a particular locus will provide direct evidence for the modulation of chromatin mobility by transcriptional activity.
3. Visualization of TF dynamics at a *cis*-regulatory element along with a readout of downstream transcriptional bursting is essential to identify the timescales of TF binding and associated mobility signatures that contribute to productive binding events.
4. Measuring dynamics of TFs and chromatin remodelers or other ATP-dependent protein complexes at specific loci (along with transcriptional bursting) will provide direct evidence for (or against) the kinetic proofreading model.
5. Performing SMT experiments within organoids and intact organisms is critical for understanding the role of TF dynamics in regulating development, cancer progression, and other pathological conditions.

DISCLOSURE STATEMENT

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