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Visualizing transcription: key to understanding gene expression dynamics

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Visualization of transcription in living cells has taught us that genes are often transcribed in bursts, with periods of gene activity interspersed by periods of inactivity. Recently, technological advances in live-cell imaging have provided a more detailed picture of the characteristics of transcriptional bursts, and have allowed direct visualization of the upstream regulatory steps of bursting at single-molecule resolution. In this review, we highlight the latest insights into transcription dynamics and we discuss recent developments in understanding the regulation of transcriptional bursting through the binding kinetics of transcription factors, enhancer–promoter interactions and clustering/phase separation of the transcriptional machinery.

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Gene expression is a dynamic and stochastic process, resulting in heterogeneity in an isogenic population. Countless efforts have been made to grasp the causes and consequences of this heterogeneity, but a complete picture is lacking. Recent advances in imaging technologies allow for the visualization of transcription dynamics and its regulatory factors at the scale of individual molecules. Here we provide an overview of recent developments that have been made towards understanding transcription dynamics in a quantitative manner.

Dynamics of transcription

The dynamics of nascent transcription can be visualized directly *in vivo* using RNA imaging techniques [1–3] or it can be inferred from (short-lived) protein reporters. The emerging view is that, for many genes, RNA molecules are not produced at a constant rate, but rather several RNAs are produced almost simultaneously, followed by a period of transcriptional inactivity. This phenomenon is known as transcriptional bursting. Mathematically, bursting is often described using a two-state model with an active and inactive promoter state [4] which can be characterized by the parameters burst duration, burst size (number of polymerases loaded) and burst frequency, and many studies have focused on the regulation of these parameters [5–8,9••]. However, this two-state model may be too simplified, and more states may be needed to accurately describe transcriptional dynamics. In general, each transcriptional state is a potential target for regulatory mechanisms and understanding the number of states is therefore crucial for understanding transcriptional regulation. Early studies reported deviations from the 2-state model by addition of a refractory period: after responding to a stimulus, the gene stays inactive for a period of time before being able to activate again [6,10–13]. Recent work has described other inactive periods, such as irreversible [14] or 'primed' [15] states, the latter allowing rapid switching to the active state. Entering the inactive state may also require multiple rate-limiting steps [16]. However, these states were inferred from the dynamics of short-lived protein reporters, which could be influenced by mRNA processing, translation or degradation dynamics, and it thus remains unclear whether these states reflect different transcriptional states. Nevertheless, studies that image transcription directly also report bursting characteristics beyond the two-state model. For the *Act5* gene in *Dictyostelium*, both initiation and elongation rates are not constant over the duration of a burst, but rather, these processes are described by a continuum of transcriptional states [17]. Bursting at different time scales ('multi-scale bursting') was observed for both the POLR2A and the Tat-activated HIV-1 promoter, where minute-scale fluctuations are controlled by the mediator complex and subhour fluctuations are controlled by TATA-box binding proteins (TBPs) interacting with TATA-boxes [18]. These slower fluctuations are not buffered by RNA and protein half-lives and thus lead to variations at the protein level, resulting in phenotypic variability.

A complementary view on transcription dynamics can be obtained when directly imaging polymerase II (PolII). FRAP-based measurements on endogenously tagged PolII revealed four distinct polymerase states: free diffusion, initiation, promoter pausing and productive elongation. PolII showed dynamic promoter-proximal pausing and low rates of complete RNA production due to the large number of polymerases that terminate

prematurely [19•]. In agreement, previous measurements of PolII dynamics estimated that only 1 in 90 interactions leads to a complete mRNA. In addition, long pauses on the gene body lower the effective RNA production rate [20]. Not only the initiation dynamics, but also pausing and elongation dynamics thus contribute to the dynamics of transcription.

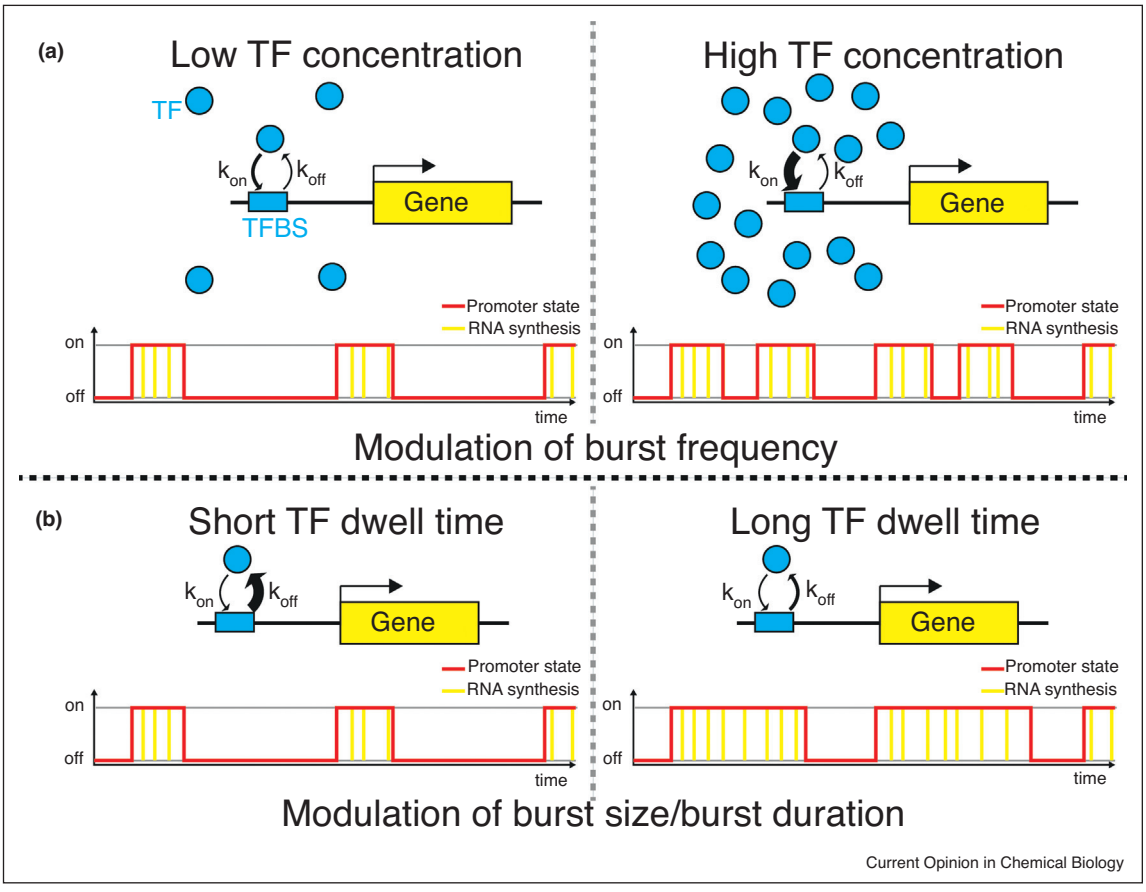
Analysis of transcription dynamics has so far only been performed on reporter genes and a small number of endogenous genes. As more data become available on the bursting characteristics of endogenous genes, we will be able to dissect the rules of how different states are coupled to their regulatory mechanisms. In the next section of this review, we focus on recent insight for some of these regulatory processes. Specifically, we discuss transcription factor dynamics, 3D genome architecture (specifically through enhancer–promoter interactions) and clustering of the transcriptional machinery. Besides these, many other cellular aspects (such as chromatin accessibility, nucleosome positioning and DNA topology) could play a role in the regulation of transcriptional dynamics, but for these we refer the reader to previous literature reviews [21–24].

Transcription factor dynamics

Recent developments in *in vivo* fluorescence microscopy have made it possible to directly 'see' the binding and diffusion of individual transcription factors (TFs) in the nucleus of living cells. TFs were found to bind to DNA on the timescale of seconds, which has led to a dynamic view of transcription regulation [22,25–29].

Since TFs show high turnover, it has been proposed that TFs bind target sites with high frequency to ensure sufficient binding site occupancy. The activity of target genes is dependent on the TF on-rate and thus sensitive to (local) TF concentration. In the simplest view, the frequency of TF binding directly correlates with the transcriptional burst frequency (Figure 1a). In agreement, burst frequency regulation by modulating TF concentration was reported for steroid-receptor reporter

Figure 1



Models of the regulation of transcriptional bursting by TF binding.
(a) The local concentration of TFs around the gene affects the on-rate of the TF to the transcription factor binding site (TFBS), which regulates the frequency of transcriptional bursting. **(b)** Dwell time of the TF at TFBS is determined by the off-rate of the TF, which regulates the size and duration of transcriptional bursts. Figure assumes a two-state model for simplicity.

genes [30] and endogenous genes (such as c-Fos [31]), and for targets of developmental TFs in Drosophila such as Bicoid [32•,33]. However, this correlation is not general, as, the concentration of the light-induced TF VP-EL*222* in yeast modulates burst duration, but does not affect burst frequency [34].

An additional mode of regulating transcriptional output is by modulation of the TF off-rate or dwell time (Figure 1b) [35]. TF dwell time may affect the number of polymerases that are recruited during one binding event (burst size and burst duration). The binding kinetics of the tumor suppressor p53 in lung-cancer cells correlate with transcription and depend strongly on the C-terminal acetylation of p53 [36•]. Acetylation increases the residence time of p53 to its binding sites and thereby increases burst duration of one of its target genes (*CDKN1a)*. In addition to acetylation, TF residence time may be determined by other factors such as the affinity of the TF binding site or cooperative binding of other regulators [25,37–40].

Despite many efforts in characterizing TF binding kinetics, the causative relationship between TF binding events and transcriptional bursting remains unknown. The main reason for this is the technical difficulty of measuring both TF binding and target gene activity simultaneously at a single locus in the same cell, which should be facilitated by future technical advances. Even though TF binding events are short-lived, they may result in long-lasting effects by recruiting complexes that change the chromatin landscape. In addition to TFs, bursting is also affected by the stability of other components of the transcription machinery such as the pre-initiation complex. Mutations in the TATA box that reduce TBP binding result in altered fluctuations on the subhour time scale [18]. However, for the majority of the transcription machinery, the kinetics and effect on gene expression dynamics are unknown. Lastly, it will be interesting to identify how these isolated biomolecules act as parts of larger multimolecular structures, which will require technical advances in single-molecule *in vivo* imaging to quantify the dynamics of multiple factors as well as their interactions simultaneously.

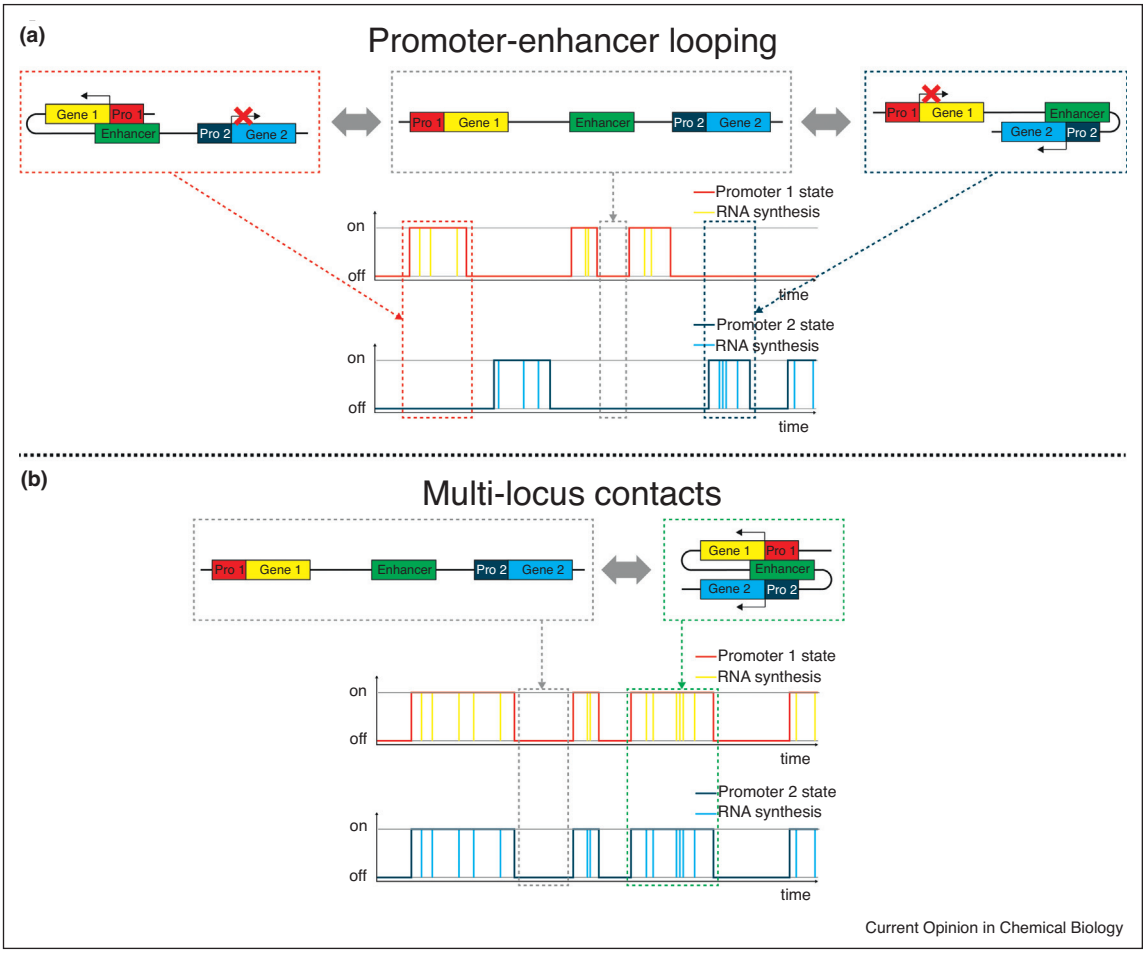
Enhancers

In higher eukaryotes, transcription is regulated by distant DNA elements called enhancers. Enhancers can be positioned hundreds of kilobases away from the target gene, and recent efforts have focused on the mechanisms by which enhancers regulate transcription in the context of the 3D architecture of the nucleus. Perhaps the simplest model to link promoter–enhancer contacts to transcriptional bursting is a model where each contact between a promoter and an enhancer initiates a transcriptional burst (Figure 2a). This model is in line with studies of the beta-globin locus control region (LCR), the enhancer regulating the beta-globin gene. Forced chromatin looping – achieved by specifically targeting the enhancer to a beta-globin promoter in a developmentally silenced region – was shown to activate transcription at this locus by increasing the frequency of bursting [9••,41]. Likewise, live-cell imaging of promoter–enhancer contacts in *Drosophila* embryos revealed that promoter–enhancer proximity is required for the initiation of a transcriptional burst [42•]. In both systems, transcriptional bursting of two different target genes of the same enhancer is mutually exclusive, suggesting that target genes compete for enhancer contacts. Each enhancer may thus only be able to contact one promoter at the time, which suggest the formation of a specific promoter–enhancer loop.

In contrast, other studies report that enhancers are able to simultaneously activate multiple target resulting in coordinated bursting of these genes [8]. During the process of transvection, where enhancers on one homolog activate transcription on the other homolog, a single shared enhancer can co-activate two reporters on different homologs [43••]. These findings challenge the simple promoter–enhancer looping model and suggest a more complex model allowing for multi-loci interactions (Figure 2b). Novel tri-loci interactions are also detected by systematic genome-wide approaches that map chromatin interactions [44–46]. These three-way contacts are mostly found in regions with super enhancers and in highly transcribed regions. A key example of a multi-contact locus is the cluster of enhancers that forms the *Igk* super enhancer [44,47]. Here, enhancer-sharing directly affects transcription, with deletion of one enhancer reducing contact frequency among the other elements of the cluster, suggesting multi-way enhancer–enhancer contacts [47]. However, in a strong super enhancer in erythroid cells, individual enhancers act independently without showing synergistic effects [48], thus providing no evidence for multi-loci contacts.

Chromatin contacts are regulated by several structural proteins, such as CTCF and cohesin. CTCF is important for maintenance of the 3D genome organization through mediating loop formation (together with its binding partner cohesin) and for creating topologically associating domain (TAD) boundaries, which prevents enhancers in one TAD from contacting promoters in different TADs [49–51]. CTCF binding directly correlates with enhancer activity and promoter–enhancer contacts [49]. Deletion of CTCF sites in enhancer regions results in an increase in cell-to-cell variability of gene expression, which suggests a change in either the burst duration or the burst frequency [49]. However, depletion of CTCF affects gene expression only long after its depletion [52], suggesting this might be due to a secondary effect. In addition, the transcription factor YY1 has been reported to facilitate interactions between promoters and

Figure 2



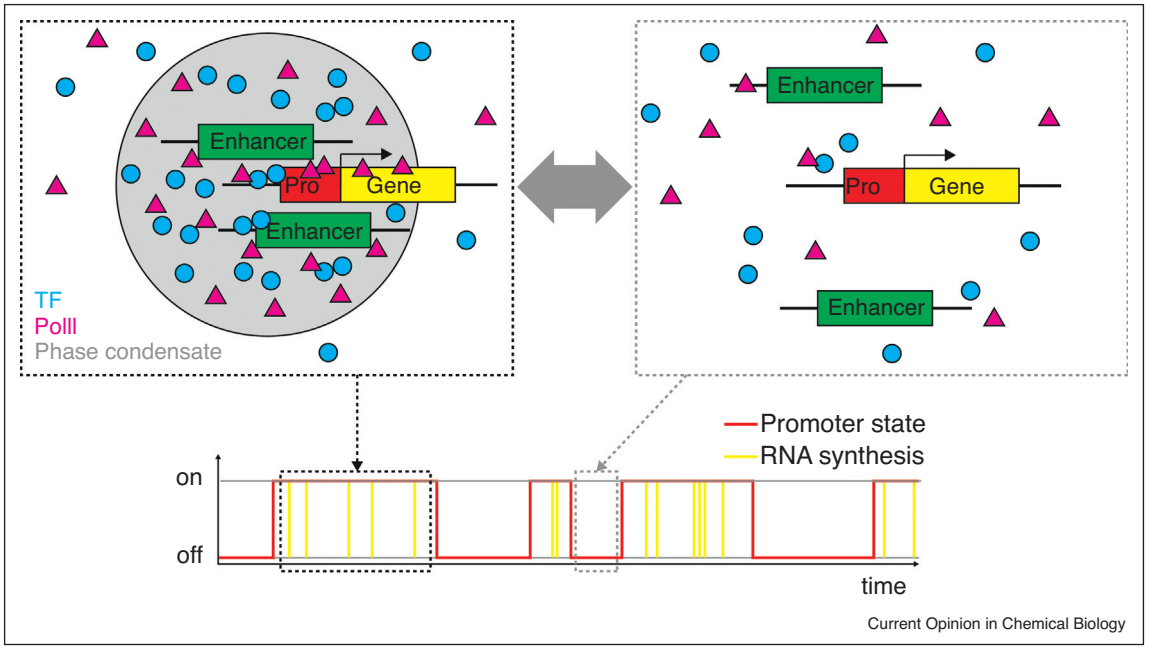
Regulation of transcriptional bursting through promoter–enhancer contacts.
(a) A single enhancer (green) may regulate multiple genes (yellow/cyan) by contacting their respective promoters (red/dark blue). In the promoter–enhancer looping model, the enhancer contacts are mutually exclusive, resulting in transcription of either gene 1 or gene 2. **(b)** Alternatively, a single enhancer could contact multiple promoters simultaneously, resulting in simultaneous bursting of both genes. Figure assumes a two-state model for simplicity.

enhancers [53] and it will be interesting to understand how this factor affects transcriptional bursting.

Clustering and phase separation

The findings above suggest a more complex model to understand the effect of 3D nuclear organization on transcriptional bursting than the simple promoter–enhancer looping model. Recently, a novel framework has been suggested that could greatly shift the view on nuclear organization: phase-separation [54•,55]. In this model, similar biomolecules cluster, thus locally increasing their concentration. Above a critical concentration, these molecules interact with each other to form a macromolecular assembly that isolates itself from its surroundings in a process known as phase separation. In the context of transcription, highly frequent interactions might occur between different components of the transcription machinery that contain low complexity domains (LCDs), leading to efficient loading of several polymerases onto the gene and a burst of transcription (Figure 3). Several criteria are often listed as 'requirements' for clusters to be classified as phase separated. First, molecules move dynamically within these clusters, showing that they are not solid aggregates (as is often shown using fluorescence recovery after photobleaching (FRAP)) [56]. Movement in between two phases is significantly slower [57]. Second, these clusters fuse in a manner reminiscent of liquid droplets fusing [56]. Finally, the interactions are disrupted by 1,6-hexanediol [56]. If not all the above 'requirements' have been shown to apply, macromolecular assemblies are often referred to as hubs or clusters instead.

Figure 3



Transcriptional bursting regulation in the phase separation model.
The proximity of promoters and enhancers facilitates the formation of dynamic phase condensates with local high concentrations of TFs and PolII, which results a burst of transcription. Figure assumes a two-state model for simplicity.

Although phase separation is difficult to study *in vivo*, several studies provide evidence towards the phase separation model. Probably the best example of a phase separated nuclear body is the nucleolus, which is sub-compartmentalized into multiple coexisting, immiscible liquid phases [58]. Similar phase separation has been proposed for heterochromatin [57,59,60], and super enhancers (SEs) [55,61••]. At a super enhancer, a cluster may form through interactions between the LCDs of transcription complexes such as TFs, Mediator, BRD4, and PolII, which results in phase separation at high density of LCDs. Clusters form in a single nucleation event and collapse upon deletion of specific chromatin factors [32•,62–65,66•,67]. For example, many TFs are recruited to clusters of Mediator, from which transcription is activated [61••]. MED1-TF clusters phase separate *in vivo* [61••], as well as clusters of the LCDs of MED1 and BRD4, which can concentrate PolII from nuclear extracts [64]. Although interesting, a full review of all literature on phase separation of nuclear bodies is beyond the scope of this paper and we refer the reader to the many review articles on this topic [54•,56,68–70]. Here, we focus on clustering of PolII and TFs and its effects on transcriptional bursting.

Using super resolution microscopy on living cells, PolII was shown to form clusters [65,66•,67]. The average cluster lifetime is ~8 s [66•], and the reported sizes vary approximately from below the diffraction limit [66•] to around 500 nm [65]. Because clusters co-localize with active genes, and cluster lifetime correlates with nascent mRNA output, it was proposed that during the lifetime of a cluster several polymerases are loaded on the gene to form a burst of transcription [66•]. The formation of PolII clusters may be mediated by the LCDs at the C-terminus [71,72]. The CTD contains 26–52 heptad repeats of the YSPTSPS consensus sequence that are phosphorylated during the transcription cycle. Whether PolII CTD alone undergoes phase separation is debated [71,72] but the CTD was shown to interact with liquid droplets of the CTD kinase cyclin T1 [72], the Mediator complex [73] and the LCDs of FET (FUS/EWS/TAF15) proteins [74]. Phosphorylation of the CTD may change its ability to interact with the different droplets, allowing for transition from initiation to elongation [71,72].

Clustering of TFs is also observed independently of PolII. Cluster formation is mediated by transient, weak interactions between LCDs that are often part of the transactivation domains of TFs. For the LCDs of the FET and SP1 TFs, high-concentration clusters are observed that stabilize DNA binding and facilitate rapid TF-PolII interactions that may activate transcription [74,75•]. These TF clusters do not undergo liquid–liquid phase separation at physiological concentrations and are therefore referred to as hubs. Interestingly, LCD–LCD

interactions are selective for binding partners: the LCDs of the three FET proteins interact among themselves but do not interact with the LCD of SP1. This selectivity suggests a mechanism for combinatorial control by TFs. In addition, the yeast TF Mig1 was shown to form spherical clusters of 7–9 monomers (~10 of nm) through interacting LCDs [62]. Similarly, clustering is observed in *Drosophila* embryos, where clusters of the TFs Ubx [63] and Bicoid [32•] colocalize with their respective gene loci and enhancers. Perhaps this leads to the formation of highly efficient transcription domains, which might facilitate TF binding to its low-affinity target sites and lead to a burst of transcription.

Together, these studies provide novel insights into how clustering and phase separation might be involved in regulation of transcriptional bursting. For genes where activity is regulated by TF on-rate (which regulates burst frequency), clustering may ensure high enough local TF concentration. Additionally, phase separation might explain multi-way contacts between one enhancer and multiple target genes by being in close proximity. However, a complete and consistent picture is still lacking, and both nomenclature and results regarding the effect of phase separation on transcription is often contradictory and confusing. Likely, phase separation and its role in transcription will be subject of many future studies, which will elucidate the process further and lead to a more solid understanding of this novel aspect of transcriptional control and how it affects transcriptional bursting.

Conclusion and outlook

Live-cell single-molecule imaging approaches have changed our view of gene expression, and have provided insight into the regulation of transcription dynamics. To further understand the regulatory mechanisms of transcriptional bursting, future studies will need to focus on how the kinetics of TFs and enhancers, as well as phase separation quantitatively contribute to the kinetic parameters of bursting. These analyses will be facilitated by technical advances in single-molecule technologies to image multiple TFs, their interactions, and their effect on transcriptional output at specific target genes in the same living cell. In addition, the combination of *in vivo* imaging with single-cell sequencing approaches and mathematical modelling will allow a more systematic analysis of bursting at multiple genomic loci. Correlation of bursting parameters to gene characteristics such as promoter architecture or 3D interaction profiles will help dissect the regulatory mechanisms of gene expression dynamics at the level of single molecules.

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Conflict of interest statement

Nothing declared.

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