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High-throughput single-molecule screen for small-molecule perturbation of splicing and transcription kinetics

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Abstract

In eukaryotes, mRNA synthesis is catalyzed by RNA polymerase II and involves several distinct steps, including transcript initiation, elongation, cleavage, and transcript release. Splicing of RNA can occur during (co-transcriptional) or after (post-transcriptional) RNA synthesis. Thus, RNA synthesis and processing occurs through the concerted activity of dozens of enzymes, each of which is potentially susceptible to perturbation by small molecules. However, there are few, if any, high-throughput screening strategies for identifying drugs which perturb a specific step in RNA synthesis and processing. Here we have developed a high-throughput fluorescence microscopy approach in single cells to screen for inhibitors of specific enzymatic steps in RNA synthesis and processing. By utilizing the high affinity interaction between bacteriophage capsid proteins (MS2, PP7) and RNA stem loops, we are able to fluorescently label the intron and exon of a β -globin reporter gene in human cells. This approach allows one to measure the kinetics of transcription, splicing and release in both fixed and living cells using a tractable, genetically encoded assay in a stable cell line. We tested this reagent in a targeted screen of molecules that target chromatin readers and writers and identified three compounds that slow transcription elongation without changing transcription initiation.

Keywords

transcription; splicing; small-molecule; RNA; fluorescence; fluctuation; single-molecule

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1. Introduction

Transcription of DNA into RNA in eukaryotes is a highly regulated process and can be divided into multiple steps, each involving the coordinated action of megadalton enzymatic complexes [1,2]. First, a pre-initiation complex consisting of both sequence-specific and general transcription factors is assembled on DNA. Next, RNA polymerase II (RNAPII) escapes the pre-initiation complex and begins to synthesize the nascent RNA. In higher eukaryotes, regulatory checkpoints exist which can stall RNAPII even after initiation and synthesis of nascent RNA [3]. Elongation of the nascent transcript occurs after RNAPII clears these checkpoints and moves along the gene body, synthesizing the complete pre-mRNA. After elongation, the nascent pre-mRNA is cleaved, poly-adenylated and released into the nucleus. Splicing can occur either co- or post-transcriptionally [4–6]. RNA synthesis and processing in eukaryotes thus relies on a wide range of enzymes, including polymerases, kinases, histone acetyltransferases and deacetylases, histone methyltransferases and demethylases, topoisomerases, helicases, ubiquitin ligases, and many more [7]. An emerging model of gene regulation is that the kinetic balance between these competing enzymatic processes can control RNA fate [8].

Many of the enzymes regulating RNA processing are prime targets for small molecule inhibition. For example, it has been shown that elongation can be slowed by inhibiting topoisomerase I with camptothecin (CPT) [9], RNAPII with alphaamanitin [10], or by disrupting the interaction between Bromodomain-containing protein 4 (BRD4) and acetylated histones using JQ1 [11]. Similarly, spliceostatin A can disrupt splicing by binding SF3B, a component of U2 snRNP [12]. One might therefore expect that perturbation of the kinetics of transcription, processing and splicing would result in changes in gene expression. Previous efforts to develop high-throughput screens for inhibitors of transcription use post-transcriptional readouts like fluorescent protein products [13–15] or *in vitro* splicing assays [16] that are not sensitive to changes in kinetics. There is not, to our knowledge, a systematic large-scale approach for discovering small molecules that perturb the kinetics of RNA synthesis. In this article, we describe a high-throughput cellular imaging assay for screening a library of compounds for their effects on transcription kinetics.

When designing this high-throughput imaging assay, which extracts kinetic information from population measurements, there were several important experimental criteria that needed to be met. First, we desired an *in vivo*, cellular screen which would capture the interplay of factors in the complex environment of the nucleus. Second, we desired a primary screen which could be done on fixed cells and then verified by live-cell imaging. Measurements on fixed cells enable higher throughput but are inherently ambiguous for determining kinetics [17]. Live-cell imaging is more cumbersome but can be used to precisely identify the underlying kinetic change. Third, we sought a perturbation strategy that would reduce indirect effects. Finally, we sought a cellular screening strategy that relied on stable cell lines with a genetically encoded readout in order to improve accuracy and reduce consumable reagent requirements.

To meet these criteria, we utilized an approach which relies on direct observation of nascent RNA in the fluorescence microscope. The system is based on DNA cassettes that encode for

RNA stem loops from either the MS2 or the PP7 bacteriophage (Fig. 1A). When a stem loop is transcribed, it is recognized and bound immediately by its corresponding bacteriophage coat protein which we have labeled with fluorescent proteins (either MS2-eGFP or PP7-mCherry). The transcription site (TS) appears as a bright punctate 'spot' in the nucleus when imaged on a fluorescence microscope (Fig. 1A). The result is a fluorescent signal which is sensitive enough to detect single molecules and is also proportional to the amount of RNA present. We used a reporter gene that has been previously described [18], consisting of the human β -globin sequence, with PP7 stem loops integrated in the terminal intron, and MS2 stem-loops integrated in the 3' untranslated region (UTR). The two orthogonal MS2 and PP7 RNA-labeling systems allow for simultaneous dual-color imaging of introns and exons of the same RNA. The ratio of intron fluorescence (mCherry, red) to exon fluorescence (eGFP, green) depends on the kinetics of RNA synthesis and processing. For instance, if splicing is fast, intron fluorescence will be small compared to exon fluorescence. On the contrary, if splicing and elongation are slow, intron fluorescence will be observed for much longer than exon fluorescence. Importantly, this ratio (red/green) can be measured both in a population of fixed cells and also in real time in single cells. This construct, along with the fluorescent coat proteins, is stably integrated into human cells and is entirely genetically encoded. Thus, this RNA-visualization approach uniquely allows both high-throughput screening in a fixed-cell primary screen and validation in a live-cell secondary single molecule assay.

As a proof-of-concept of this approach, we chose a targeted library of small molecule inhibitors that disrupt the activity of chromatin readers and writers. We specifically chose this library over siRNA knockdown because small molecule inhibitors can diffuse across the cell and nuclear membrane rapidly, whereas siRNA knockdowns typically have maximal effects two days post transfection [19]. This feature allows us to carry out the screen a few hours after addition of the drug, thus enriching for direct effects of the drug over indirect effects. Furthermore, we focused our screen to search for small molecules that perturb post-initiation kinetics (i.e. elongation, splicing, or release) and not transcription initiation itself. Drugs that inhibit transcription initiation might have general toxicity, and the power of this screen is the ability to separate such non-specific effects from those which target a particular step during RNA synthesis. Here, we show that our screen is indeed capable of selectively identifying factors that change the elongation rate of RNAPII by up to 50%. We identify bromodomain inhibition as a general strategy for perturbing elongation rates without affecting initiation rates. Overall, this single-cell approach represents a new strategy both for identifying drugs that perturb the kinetics of transcription, and also for rapidly determining the specific kinetic step that is altered.

2. Materials and methods

2.1. Cell line and DNA constructs

The reporter gene and cell line were described previously [18]. Briefly, the reporter gene was constructed using the coding sequence of the human β -globin gene under the control of a Tet-on promoter. A cassette containing 24 PP7 stem loops was inserted in the terminal intron of the β -globin gene, approximately half way between the 5' splice site (5'ss) and the branch point. An additional cassette containing 24 MS2 stem loops was inserted in the 3'

UTR of β -globin, followed by the bovine growth hormone polyadenylation sequence (BGH-PolyA, Fig. 1A). This vector was co-transfected into U2-OS cells with a puromycin resistance plasmid. Transfected cells were treated with puromycin, resistant cells were isolated, and cells positive for integration of the reporter gene were confirmed using RNA fluorescent *in situ* hybridization (FISH) [18]. Clonal cell lines positive for the reporter were subsequently infected with two lentiviruses, both under the control of the ubiquitin promoter. One vector constitutively expresses the PP7 bacteriophage coat protein fused to the mCherry fluorescent protein (PP7-mCherry) while the other constitutively expresses the MS2 bacteriophage coat protein fused to eGFP (MS2-eGFP). Both fusion proteins also contain a nuclear localization signal (NLS). Each PP7 or MS2 stem loop is specifically bound by a dimer of PP7-mCherry or MS2-eGFP respectively, resulting in 48 fluorophores bound to each cassette. Previous reports show that it is likely the PP7 cassette is fully occupied by 48 fluorophores, while the MS2 cassette is occupied by approximately 26 fluorophores [20]. TSs are visible above background as diffraction-limited spots resulting from the high number of bound fluorophores. The cell line selected for this assay has three insertion sites on chromosomes 3, 8, and 21 that were mapped by paired end sequencing, with a total number of four to seven copies of the reporter distributed among the three insertion sites [18]. Only one of these three insertions is likely to be in an active region since the cells rarely show multiple TSs. To further optimize this cell line for high-throughput imaging, cells were seeded at an average density of less than one cell per well into a 96-well plate, and grown up into colonies. A single clone that had both high levels of transcription and high contrast of TS to background was selected.

2.2. Tissue culture and pharmacological treatments

All tissue culture was conducted using Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% Tetracycline-free fetal bovine serum (FBS, Clontech Laboratories) and 1% penicillin streptomycin (Life Technologies). Cells were maintained at 37°C and 5% CO₂.

For the screening studies reported here, we analyzed a library of 24 compounds from the Cayman Chemical Epigenetics Screening Library, made up of molecules reported in the literature to inhibit chromatin-interacting proteins (Cayman Chemical Company). In addition, as positive controls we analyzed camptothecin (CPT; Topoisomerase I inhibitor which reduces initiation and slows elongation), 5,6-dichloro-1- β -D-ribofuranosyl-1H-benzimidazole (DRB; Cdk9 inhibitor which reduces RNAPII pause release) and herboxidiene (splicing inhibitor which disrupts U2snRP assembly) whose effects on transcription have been previously reported [21,22]. For imaging on the PerkinElmer Opera high-throughput spinning disk confocal microscope, 10,000 cells were seeded per well in PerkinElmer 96-well CellCarrier plates (Fig. 1B). The cells were allowed to go through one cell cycle (24hr) before the reporter gene was induced with 10 μ M of doxycycline (Sigma Aldrich) for another 24hr. Pharmacological treatments were then administered in the same complete media with a final concentration of 0.4% DMSO. CPT was administered at 10 μ M, DRB was administered at 100 μ M [23] and herboxidiene was administered at 40nM [24]. All other compounds were administered at 40 μ M, a concentration routinely used in a number of small molecule screens [25]. Four hours after pharmacological treatment, cells were fixed by

addition of 8% Paraformaldehyde (PFA, Electron Microscopy Sciences) added 1:1 directly to the media of each well, and incubated at room temperature for 30min (Fig. 1B), resulting in a final concentration of 4% PFA. Cells were then washed twice with phosphate buffered saline (PBS, Corning), and then stored in PBS at 4°C for imaging.

For live-cell imaging, cells were seeded in 20mm glass bottom cell culture dishes (Nest Biotechnology). The reporter gene was induced 24hr before imaging with 10 μ M of doxycycline. Pharmacological treatments were performed in Leibovitz's L-15 phenol red-free medium (Life Technologies) supplemented with 10% Tetracycline-free FBS and 10 μ M doxycycline. Treatments were done at 40 μ M with a final concentration of 0.4% DMSO, and were administered 2hr before imaging. Multiple traces were acquired in the same dish, resulting in measurements ranging from 2hr to 10hr after administration of drugs.

2.3. High-throughput confocal measurements and data analysis

Each TS consists of multiple fluorescently labeled RNAs appearing as a diffraction-limited spot in the microscope (Fig 1 A, D). For high-throughput imaging with the Opera spinning-disk microscope (Perkin Elmer), separate exposures were acquired using 488nm and 561nm excitation at 8mW for 400ms, and 5mW for 800ms, respectively. Images were acquired with a 40x water immersion objective with an NA of 0.9. Five z-planes ($z=1\mu$ m) were acquired for each field, with a total of 100 fields of view imaged for each well (Fig. 1C). While 5 z-planes may not capture the entire thickness of the nucleus, we observed that the number of TSs increased marginally from 3 z-planes to 7 z-planes (Fig. 1C). Compounds were tested in two technical replicates and three biological replicates, resulting in a total of ~ 6,000 points for each condition.

Images were processed using a custom script written with the Acapella software package (PerkinElmer). First, cell nuclei were segmented using only images from the eGFP channel. Then, areas within nuclear boundaries were searched for TSs using images from the mCherry channel (Fig 1D). An initial intensity threshold finds the candidate TS, and spot finding is further refined with size and intensity contrast filters to exclude nuclear structures. After TSs are selected, the background intensity (the average intensity of the 14 surrounding pixels) is subtracted from the image of the spot in each channel. A 9-pixel kernel is fitted to the spot to extract the TS fluorescence intensity in each channel.

For each well, we generate a scatter plot showing the eGFP and mCherry fluorescence intensities of all the TSs (Fig 1E). A linear regression is fit to the scatter plot using the 'robustfit' function in MatLab (The MathWorks), to extract the y-intercept and slope for each well, and the average values from six replicates are calculated for each pharmacological compound. The center of mass of each scatter plot, or the average eGFP and mCherry intensity is extracted, and these values are averaged for each compound. Cell count and spot count are also measured in each well, and the ratio is the percent of cells transcribing.

2.4. Live-cell single-molecule measurements

Images of live cells were acquired using a custom built AxioObserver inverted wide-field microscope (Zeiss). PP7-mCherry and MS2-eGFP were excited simultaneously with 488 nm and 594 nm lasers (Spectra Physics). A Zeiss 63× C-Apochromat objective with an NA of 1.2 was used for wide-field excitation as well as collection of emission signals. Excitation and emission signals were separated by a custom dual band pass filter (Chroma). TSs were imaged at non-saturating power levels. Photobleaching between start and end of a timelapse acquisition was no more than 25%. A 590nm long pass dichroic mirror (Chroma) was used to separate the eGFP and mCherry emission signals and these were further filtered by a 535/70 or a 650/70 band pass filter just before they were imaged onto two EMCCD cameras (Photometrics). Images were acquired every 10s using 11 z-steps ($z=0.5\mu\text{m}$) with a typical exposure time of 150ms for 512 frames. MicroManager [26] software was used to collect the raw images. Maximal intensity projections were computed with ImageJ software (<http://imagej.nih.gov/ij>) and used for tracking of the TSs. A stage top incubator (Tokai Hit) maintained temperature and humidity during image acquisition.

Fluorescence time traces were corrected for photobleaching as described previously [18], and fluctuations in eGFP and mCherry fluorescence at the TS were extracted using custom software written in Interactive Data Language (IDL, Exelis Visual Information Solutions). The eGFP and mCherry fluorescence intensity was cross- and auto-correlated at varying time delays and the cross- and autocorrelations were averaged over multiple traces before being fit to a model to extract elongation rates, splicing time, co-transcriptional splicing fraction and 3' end retention time of the reporter transcripts, as described earlier [18]. Correlation analysis of the fluorescent time traces enables the measurement of transcription kinetics with single transcript resolution.

3. Results

3.1 Transcription kinetics can be extracted from combined scatter plot and linear regression

Each site of transcription in the nucleus reflects multiple nascent RNA being transcribed from a single gene. Because the intronic cassette (red) is transcribed before the exonic cassette (green) and may also be spliced out before release of the transcript, the relative brightness of the red and green signals is a reflection of elongation, splicing, and release kinetics. We have previously demonstrated how the kinetics of transcription elongation, splicing and termination can be extracted from single live cells by correlating the fluorescence intensity fluctuations of the TS in time [18]. We show here in this section that a scatter plot of red vs. green intensities from thousands of TSs in fixed cells contains analogous kinetic information that can be extracted through a linear regression.

In the case where the relative levels of intron and exon RNA are determined only by elongation and release (i.e. no splicing), the fluorescence intensity in both channels is the following:

$$G = c \left(\frac{L_G}{v} + T \right) \quad \text{Eq. 1}$$

$$R = c \left(\frac{L_G + \Delta}{v} + T \right) \quad \text{Eq. 2}$$

where R and G are the fluorescence intensity from the red intronic and green exonic channel in arbitrary units; c is the transcription initiation rate (min^{-1}); L_G is the distance from the center of the exon cassette until the end of the gene (bp); v is the velocity of the polymerase (bp/min); T is the 3' processing and release time (min); Δ is the distance between the intronic red cassette and the exonic green cassette (bp). Note that R is greater than G because of the longer dwell time, represented by Δ , i.e. the increased distance the polymerase must elongate. Eqs. 1 and 2 can be substituted and re-arranged to give:

$$R = G + \frac{c\Delta}{v} \quad \text{Eq. 3}$$

expressing the red signal R as a function of the green signal G , which corresponds to the experimental scatter plot.

Because RNA synthesis and processing varies over time due to stochastic fluctuations, a population of cells will contain an ensemble of TS intensities. The mean field relationship between the intron and exon fluorescence intensity is captured by Eq. 3 and shows an effective linear relationship between R and G with a slope of unity and an intercept which depends on the initiation rate (c), the distance between stem loop cassettes (Δ) and the polymerase elongation rate (v). Therefore changes in elongation rate between two conditions (i.e. drug and vehicle), will manifest as a change in the y-intercept in the linear regression fit to the experimental scatter plot. This model assumes a completely processive polymerase, with no abortive elongation between the intron and exon.

We tested this simple model *in silico* using Monte Carlo simulations of transcription. Experimentally realistic parameters were chosen for initiation and elongation, resulting in a typical polymerase occupancy curve as shown in Fig. 2A. Transcription has been observed to occur in two general ways: constitutive and bursting transcription [27]. In the former, delay between initiation events is exponentially distributed, resulting in a Poisson distribution of transcripts at the TS [28]. In this case, the gene is always on, and the polymerase occupancy fluctuates around an average level (Fig. 2A). In the bursting case, the promoter toggles between an active and inactive state [29]. In the active state, many polymerases are initiated consecutively, resulting in large, cooperative bursts of RNA synthesis (Fig. 2D).

The scatter plot generated from constitutive transcription for ~ 5,000 TSs is shown in Fig 2B. The blue circles correspond to an *in silico* polymerase elongation rate of 1.3 kb/min, and the pink circles correspond to an *in silico* elongation rate of 3.9 kb/min. As shown by the linear regression fits, the intercept decreases with increasing velocity (Fig. 2B, solid lines).

Therefore, the change in intercept calculated analytically (Eq. 3) is reproduced by Monte Carlo simulations of constitutive transcription.

This simulation was carried out over a range of polymerase elongation rates, as shown in Fig. 2C. The black circles correspond to Monte Carlo simulation results, and the red curve is the analytical result in Eq. 3. The actual value of the y-intercept is arbitrary based on a number of experimental parameters (efficiency of photon collection, filter configuration, exposure time, numerical aperture of the objective, laser power, etc.) but in the case of simulated data these factors can be collapsed into a single, known parameter. Taking this factor into account, there is near perfect agreement between the analytical theory and the Monte Carlo simulations.

We next turned to the case of transcriptional bursting (Fig. 2D). Again, increasing velocity from 1.3 kb/min (Fig. 2E, blue circles) to 3.9 kb/min (Fig. 2E, pink circles) results in a noticeable shift of the linear regression intercept. However, as in the case of constitutive transcription above, the slope is unchanged.

Although the linear regression does not capture the complexity of the scatter plot in the bursting scenario, the intercept still faithfully captures changes in elongation rate. Fig. 2F shows the y-intercept values from Monte Carlo simulations for different elongation rates (black circles). The analytical result (Eq. 3, Fig. 2F, red dashed line) shows a consistent offset from the data, indicating that the theory does not adequately account for bursting. However, the y-intercept values from the Monte Carlo simulations are well fit with a $1/v$ dependence multiplied by a constant scaling factor. The concordance between the Monte Carlo points and the $1/v$ fit indicates that the y-intercept is a robust measure of elongation changes, even in the presence of complicated initiation dynamics.

If we now include splicing in our mathematical model, Eq. 2 can be rewritten

$$R = c \left(\frac{L_G + \Delta}{v} + T - s \right) \quad \text{Eq. 4}$$

where s represents the average time between the disappearance of the red signal (splicing or release of the RNA, whichever comes first) and disappearance of the green signal (release of the RNA). Note here that since s is visualized here as a loss in the red signal, we actually measure time of release of the intron-containing lariat, and not the chemistry of exon ligation. In this case, we find that

$$R = \left(1 - \frac{s}{\frac{L_G}{v} + T} \right) G + \frac{c\Delta}{v} \quad \text{Eq. 5}$$

i.e. R still shows a linear dependence on G , with the same y-intercept, but with a slope that depends on the splicing kinetics. We illustrate this dependence with a full Monte Carlo simulation, which includes splicing in addition to elongation and termination/release kinetics. Splicing is represented here as a stochastic process, happening with an

exponentially distributed delay after the 3' splice site (3'ss) has been transcribed. The presence of splicing increases the spread of points in the scatter plot (Fig. 2G). However, linear regression fits to the scatter plot indicate that when splicing time increases from 4 minutes to 8 minutes after transcription of the 3'ss, the slope increased from 0.5 to 0.7 (Fig. 2G, pink and blue, respectively). The intercept was unchanged as predicted by the mathematical model. For splicing times ranging from 1 minute to 40 minutes, the mathematical model (Eq. 5) also predicts accurately the slope and y-intercept of the Monte Carlo simulations (Fig. 2H, gray lines). We note that s is an implicit function of several parameters, including not only the splicing time, but also the elongation rate, termination time, and position of the stem loops within the intron and is therefore *not* the same quantity as the abscissa in Fig. 2H. Thus, it appears that for this particular kinetic scheme, elongation rate changes and splicing rate changes have easily distinguishable results on the regression fit: elongation changes intercept, and splicing changes slope.

In summary, scatter plots of intron vs. exon fluorescence contain kinetic information when analyzed by linear regression. Although static measurements across a population are not able to distinguish all kinetic steps (for example, initiation rate and elongation rate changes both affect the regression y-intercept), under certain circumstances the regression is highly informative. For example, elongation and splicing changes show differential changes on the y-intercept and slope, respectively.

3.2 High-throughput imaging identifies small molecules that perturb elongation kinetics

Using the scatter plot analysis coupled with simple linear regression fitting, we screened a targeted library of small molecules that interfere with chromatin readers and writers. This library consisted of molecules that target bromodomains, histone deacetylases, and sirtuins. In addition, we included several controls such as CPT, which inhibits topoisomerase I and reduces initiation and slows elongation [30], DRB, which inhibits carboxyl-terminal domain kinases and blocks RNAPII pause release [31], and herboxidiene, which blocks U2snRNP assembly and inhibits splicing [24]. Compounds were assessed for activity based on three criteria: (1) RNAPII elongation rate, inferred from the y-intercept from a scatter plot of red-green fluorescence intensities (Fig. 3A), (2) cytotoxicity, inferred from the mean cell count and fraction of cells that show a TS (Fig. 3B), and (3) large changes in absolute levels of fluorescence intensities in the red and green channels, which are potentially indicative of abortive transcription or general artifacts like autofluorescence of a compound (Fig. 3C).

Using our primary assay, we measured the effects of 24 known epigenetic-modulating compounds on transcription initiation, elongation, and splicing at a fixed dose (40 μ M) and time point (4 hr). From this analysis, we identified four compounds that caused a significant increase in y-intercept (Criteria 1), indicating inhibition of transcriptional elongation rate (Table 1 and Fig. 3A). These compounds included SGC-CBP30, which specifically binds the CREB-binding protein (CBP) bromodomain [32], PFI-1, which inhibits the bromodomain 4 (BRD4)-histone interactions [33], and Tenovin-6 and Tenovin-1, which inhibit the histone deacetylase SIRT-2 [34]. Assessing these hits based on our second criteria, total cell count and total TS number, we found that Tenovin-6 causes a reduction in cell number consistent with toxicity. In contrast, the positive controls CPT and DRB

decrease the fraction of cells with a TS, without causing large changes in cell count (mean >1500). The ability to triage mechanistically distinct inhibitors indicates that using total cell count and total TS number as a cut-off may be useful to identify compounds which affect post-initiation kinetics without inhibiting transcription initiation. Finally, PFI-1 and Tenovin-1 also showed significant separation by criteria 3, absolute fluorescence, indicating possible changes in retention time (Fig. 3C).

While theory and simulation results indicate that we should observe changes in splicing kinetics as changes in the slopes of our regression fit, we were not able to detect statistically significant differences in slope between samples and the control (data not shown). We attribute the lack of sensitivity to several reasons. First, the value of the slope harbors information not just about splicing, but also the kinetics of initiation and termination. Thus, changes in one or more of these parameters may obscure changes in splicing kinetics when measured in a static intensity-based screen. Second, our technique is sensitive to splicing only when it happens prior to release from the TS. Previous live cell measurements on our reporter gene [18] indicated that co-transcriptional splicing happens for only ~16% of the transcripts, and thus any changes in splicing may not be in the kinetic window to be detected by a static intensity-based screen for this construct. If more TSs are imaged per well, this indicator may become more robust and provide additional information about splicing kinetics. Lastly, limitations in the choice of excitation power and wavelength in the Opera system resulted in imaging that was better optimized in the green channel than in the red. This hardware limitation meant that we were not utilizing the full dynamic range of readout in the red channel. Since splicing is detected only by changes in the intron (red) signal, this imaging limitation could also lead to insensitivity to splicing changes. We note however that these limitations are not fundamental and could be corrected in future versions of the screen.

In summary, our cell reporter system, in combination with the three criteria outlined above, gives us a primary screen that can be used to dissect the effect of drugs on transcriptional processes (e.g. initiation, elongation, etc.) and prioritize small molecules for in-depth study.

3.2 Live-cell single-molecule imaging validates high-throughput static measurements

Based on the results of our primary screen, we prioritized SGC-CBP30, PFI-1, and Tenovin-1 for further study as agents that perturb post-initiation kinetics (elongation, splicing, termination/release) without altering transcription initiation rates. Since kinetic changes cannot be unambiguously resolved with static measurements, these compounds were validated by live-cell imaging and correlation analysis. Live-cell single-molecule imaging coupled with correlation analysis can be used to reveal transcription kinetics by adding a time-resolved component to fluorescence information extracted from the reporter gene. In other words, instead of sampling many TS at a single snapshot in time, one can sample a single TS over many time points. Fig. 4A shows an example of such a time-resolved trace from a TS. The fluctuations in red and green fluorescence intensity over time reflect the underlying kinetic processes which occur during RNA synthesis and processing. This approach has been described in detail in [18].

To extract data from these time traces, we used a correlation approach. Approximately 20 auto- and cross-correlation curves were averaged (Fig 4B) before being fit to a model to

obtain kinetic information (Fig 4C). The autocorrelation reflects the dwell time of either the intron or the exon individually. Crosscorrelation describes the temporal relationship between intron and exon and contains information about both splicing and elongation. The delay between time zero and the onset of the decay of the red to green (rg) cross-correlation (Fig. 4D, blue curve) reflects the time taken by the polymerase to transcribe between the two stem loop cassettes. The slope of the rg cross-correlation after time zero is a measure of the fraction of transcripts that are spliced before the RNA is released from the TS. The time taken to release the RNA after the polymerase reached the 3' end of the gene can be extracted from the decay of the rg and green to red (gr) cross-correlations (Fig 4D, pink curve). Therefore, correlation analysis allows one to dissect how each kinetic step might have changed under drug treatment.

Since two of the initial three hits in the screen were bromodomain inhibitors, we chose to examine the effects of an additional bromodomain inhibitor JQ1 in our live cell screen. JQ1 specifically inhibits BRD4 and has been shown to lower RNA levels for target genes which depend on acetylation of *cis*-acting regulatory elements [35,36]. Even though JQ1 did not reach statistical significance ($p=0.152$; Table 1 and Fig. 3A), it is unique in that it has an inactive control compound, (-)JQ1, that is commercially available and can be used to calibrate the ability of our assay to resolve small molecule-induced transcriptional perturbations. As visible on Figs. 4E–I, all compound tested in the live-cell screen, with the exception of (-)JQ1, showed alteration of the cross-correlation functions at short delays when compared to vehicle-treated cells, indicating an effect on the kinetics of elongation and/or splicing. Vehicle-treated cells showed an average elongation rate of 2.5kb/min, in agreement with previously reported results [18]. SGC-CBP30 and Tenovin-1 treated cells showed an elongation rate of approximately 1.8kb/min while PFI-1 showed a rate of 1.42kb/min (Fig 4E–G), which is up to ~50% slower than control (Table 2). Correlation analysis revealed that (+)JQ1 resulted in a 34% reduction in elongation rate (Table 2 and Fig 4H). (+)JQ1 also showed a small increase in the fraction of splicing happening before release, which is consistent with slower elongation rates.

Overall, live-cell imaging validated all small molecules shown to affect elongation from the primary screen. In addition, we observed that (+)JQ1 induced a 34% reduction in elongation rate even though it was not identified in the primary screen with statistical significance. Thus, even with this limited library we were able to identify and validate inhibitors of elongation.

4. Concluding remarks

The high-throughput single-molecule imaging screen described here has been used to successfully identify and validate small molecules that alter transcription kinetics. Changes in elongation rate could be identified in the primary screen by changes in the eGFP and mCherry fluorescence intensity scatter plot. This measurement is only possible when thousands of TSs are measured in single cells, which is why the primary screen failed to identify CPT (which also decreases initiation rate) despite previous studies showing its perturbative effect on elongation [18,23]. Indeed, one of the remarkable aspects of this approach is that we are able to robustly visualize and analyze single TSs with a few RNA in

a high-throughput spinning disk microscope. A secondary measurement based on live-cell single-molecule imaging and correlation analysis validated results from the high-throughput static imaging screen. All of the hits from the primary screen were validated by live-cell imaging and showed up to ~50% reduction in elongation rates. Changes in 3' retention times were observed in SGC-CBP30 treated cells.

The primary bottleneck in the assay is the imaging time. In total, the imaging time is ~110 minutes for ~15,000 cells (6 replicates) for each drug treatment. The cells were imaged at ~80% confluency, and ~30% of cells had TSs. Since image analysis and data analysis for the entire plate took only a few minutes, imaging represents the most time-intensive step in the screen. Thus, total experiment time should vary linearly with the number of compounds tested and with automated liquid handling for cellular manipulations, scaling up the screen should be relatively straightforward.

There are limitations in the described high-throughput imaging screen including noise in the y-intercept analysis of scatter plots. For example, variability in both the vehicle control (DMSO) data and the (+)JQ1 data may have resulted in failure to identify JQ1 as a hit in the primary screen. Critically, greater sensitivity can be achieved in future screens by creating reporter genes with a longer distance between the PP7 and MS2 cassettes. Increasing the distance between the cassettes will increase the time when only one signal is visible, which will enhance the ability to detect changes in elongation rates. However, if the distance between the cassettes is too large, both cassettes may never be observed in the same RNA. This uncoupling would happen if the delay between transcription of the two cassettes is longer than the splicing time. Computational modeling as executed here will be helpful in designing next-generation reporter genes for kinetic screens. Alternatively, it is now possible to tag endogenous genes using the same criteria, opening up the possibility of screening for gene-specific factors.

Some of the inhibitors used in this study had previously been shown to affect splicing *in vitro*. For example, HDAC or HAT inhibitors like SAHA, splitomicin and garcinol have been shown to perturb splicing by inhibiting the formation of a stable spliceosome complexes [37]. However, we were not able to detect changes in splicing due to these compounds using our primary screen. These misses could be false negatives, but as previously described in the methods and results section, one limitation of the screen presented here appears to be its weak sensitivity to splicing, even though theoretical analysis and simulations predict that changes in splicing time could have been observed. We speculate that this insensitivity is due to limitations in the imaging system. Future experiments will improve the system by using better fluorophores, imaging with longer exposure times, or correcting for noise by imaging fluorescent beads. With these improvements, we should be able to reduce the rate of false negatives.

One important outcome of this study is an approximate rule of thumb about the number of TSs one needs to observe to detect a certain kinetic change. In fact, both the primary screen and live-cell imaging behave similarly in this respect. Approximately 2,500 cells were imaged in each replicate of the high-throughput assay, resulting in approximately 15,000 cells in total. In the live-cell assay, a typical time trace is ~ 512 frames, and each correlation

curve is generated from 15 – 25 cells, resulting again in about 15,000 cell images in total. These statistics allow one to identify 50% changes in elongation rate, which is approximately the range we have observed in this study. To identify smaller changes would most likely require an altered reporter gene. In any case, this rule of thumb demonstrates the need to image large numbers of cells to draw conclusions about transcription kinetics.

Despite its limitations, the primary screen identified compounds which change elongation kinetics. In fact most compounds which target bromodomain proteins showed significant effects in the primary screen. This clustering may be a biological result that indicates the importance of bromodomains in transcription elongation through chromatin. It is known that BRD4 interacts with acetylated histones and facilitates the elongation of RNAPII [11]. Conversely, this observation may be due to the conditions of the screen. For example, drugs that take longer than 4hr to show efficacy will not show up as positive hits in our screen. Similarly, drugs with IC₅₀ values higher than the 40μM used in this screen may not show up as positive hits. We tested a number of HDAC inhibitors and only Tenovin-1 showed activity in the primary screen even though other studies have shown strong effects of the HDAC inhibitor Trichostatin A on burst size and duration [38]. However the strong effects of Trichostatin A were observed at 2μM treatments over 24hr, which is vastly different from the dose and time regime explored here.

Taken together, the primary screen and live-cell validation revealed up to 50% changes in elongation. Others have shown similar changes in elongation rates on endogenous genes using complementary methods such as GRO-seq [39]. It is possible that this is the maximum effect that can be observed in cell-based assays. Or perhaps two-fold changes are the physiological limit of change cells can tolerate before transcription check points are activated and transcription shuts down. Here we have described a high-throughput imaging screen that was able to detect compounds that alter elongation rates. Live-cell imaging validated all compounds that target elongation identified by the primary screen. Subsequent versions of the screen will be designed to improve sensitivity in these measurements.

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Fig. 1.

Work flow for the imaging-based small-molecule screen. (A) Transcription and splicing reporter gene, with MS2 and PP7 cassettes, stably integrated in U2-OS cells. (B) Experimental protocol performed before imaging. (C) Fully automated high-throughput confocal imaging. The inset shows that the number of TSs detected does not differ much when 3 or 7 z planes are imaged (5 planes are used in the rest of the study). (D) Automated primary data analysis. Cell nuclei are detected in the green channel, TS in the red channel, and both red and green fluorescence intensity are recorded at each TS. (E) Semi-automated secondary data analysis to extract kinetic information from fluorescence intensities. A scatter plot of mCherry vs eGFP intensities for each TS is plotted and fit with linear regression.

Fig. 2.

Monte Carlo simulations reveal kinetic information that can be determined from intron vs. exon scatter plots. (A) RNAPII occupancy of a constitutively expressed gene. (B) *In silico* simulations of constitutive transcription with RNAPII velocities of 1.3kb/min (blue circles) and 3.9kb/min (pink circles) are fit by linear regression. The fits show different y-intercepts but the same slope (blue, pink solid lines). The black dotted line indicates the position of the y-intercept. (C) Simulated Monte Carlo results (black circles) agree with analytical result (red dashed line) from Eq. 3 in the case of a constitutively expressed gene. There are no free parameters in the analytical result. (D) RNAPII occupancy of a bursty gene. (E) *In silico* simulations, for bursty transcription, of RNAPII velocities of 1.3kb/min (blue circles) and 3.9kb/min (pink circles) show different y-intercepts. (F) Simulated Monte Carlo results (black circles) display consistent offset with analytical results (red dashed line) from Eq. 3 in

the case of a bursty gene. The solid black line is a fit with I/v dependence and adjustable scaling parameter. (G) *In silico* simulations of 4 min (pink circles) and 8 min (blue circles) splicing times show different slopes. (H) *In silico* simulations of splicing times ranging from 1 to 40 min show a relationship between splicing time and slope but not splicing time and y-intercepts. The analytical model for slope and intercept is shown as dashed and solid gray lines, respectively.

Fig. 3.

High-throughput imaging to identify molecules which change RNA synthesis kinetics. (A) Plots of the y-intercept extracted from the mCherry vs. eGFP scatter plot of each well. *p-value<0.05 **p-value<0.005 (two-tailed t-test vs control). Each column shows the 6 replicates for each treatment (3 biological with 2 technical replicates each). (B) Number of cells per well and fraction of cells transcribing for each compound tested. (C) Center of mass extracted from the mean intensity of eGFP and mCherry in each well. On panels B and C, the red point is the DMSO control and error bars are SEM.

Fig. 4.

Live-cell single-molecule measurements validate high-throughput imaging results. (A) A representative transcriptional time trace in control conditions (DMSO), where fluorescence is represented in both channels on the y-axis. (B) Control DMSO auto- and cross-correlation curves. (C) Average DMSO cross-correlation fit to a previously described mathematical model [18]. (D) Parameters extracted from cross-correlation curves. (E) DMSO data overlaid with SCG-CBP30 data. (F) DMSO data overlaid with PFI-1 data. (G) DMSO data overlaid with Tenovin-1 data. (H) DMSO data overlaid with (+)JQ1 data. (I) DMSO data overlaid with (-)JQ1 data.

Table 1

Compounds selected from primary screen and validated by live-cell single-molecule imaging

Small-molecule	Target	Specific effect	HIT t-test ^a	Validated by live cell
PFI-1	BRD2/4	Blocks interactions of bromodomains with acetylated histone tails	p=0.0003	Yes
SGC-CBP30	CBP	Competitive inhibitor for acetyl-histone-binding pocket	p=0.0206	Yes
Tenovin-1	SIRT	Inhibits the protein deacetylase activities of SIRT1, SIRT2 and SIRT3	p=0.0029	Yes
Tenovin-6	SIRT	More water-soluble analog of Tenovin-1	p=0.0148	-
(+)JQ1	BRD4	Competitive inhibitor for acetyl-histone-binding pocket	p=0.1520	Yes
(-)JQ1	BRD4	Inactive isomer of JQ1	p=0.5860	No

^a calculated from two-tailed t-test vs control

Table 2

Kinetics of transcription and splicing revealed by single-molecule imaging and correlation analysis

Small molecule	Elongation rate (kb/min)	3' end retention time (sec)	Splicing time (sec)	Pre-release splicing (%)
DMSO	2.64 ± 0.20	149.49 ± 9.04	358.75 ± 23.75	12.97 ± 4.02
SGC-CBP30	1.85 ± 0.08 **	190.87 ± 8.20 **	387.08 ± 11.85	19.62 ± 2.94
PFI-1	1.42 ± 0.09 **	118.78 ± 7.01 *	237.36 ± 4.93 **	31.27 ± 5.06 **
Tenovin-1	1.74 ± 0.14 **	147.72 ± 11.36	511.94 ± 79.70	5.41 ± 4.38
(+)JQ1	1.74 ± 0.09 **	160.05 ± 10.19	364.53 ± 14.46	17.02 ± 3.91
(-)JQ1	2.14 ± 0.23	108.46 ± 7.20 **	272.82 ± 12.70 **	14.86 ± 4.58

The table shows results from correlation functions fit to the model [18]. Error bars are propagated SEM from the functions.

*
p-value < 0.05,

**
p-value < 0.005 (two-sided z test vs control)