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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.

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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 deactylases, 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 campothecin (CPT) [9], RNAPII with alpha-amanitin [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.

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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 florescence. 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

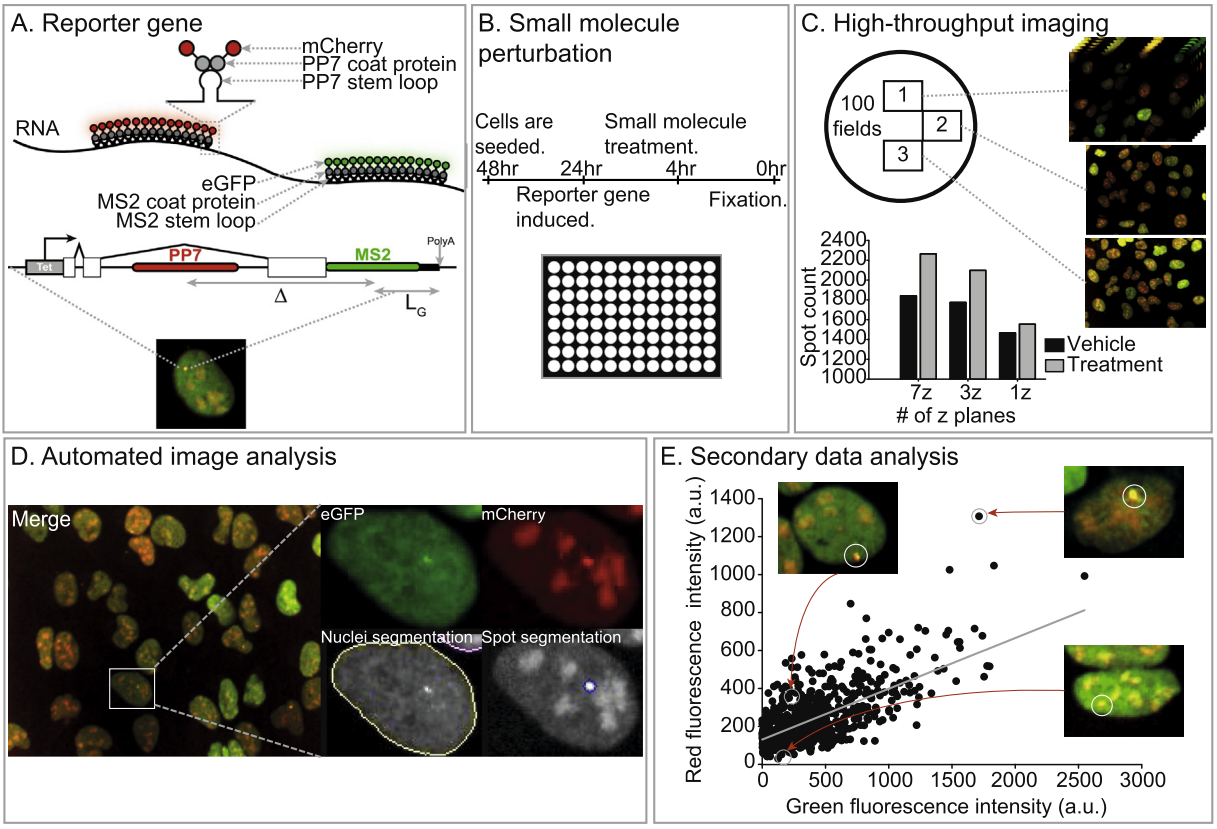

Fig. 1. Work flow for the imaging-based small-molecule screen. (A) Transcription and splicing reporter gene, with MS2 and PP7 cassettes, stably integrated in U2-OS cells. (B) Experimental protocol performed before imaging. (C) Fully automated high-throughput confocal imaging. The inset shows that the 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.

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