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Single molecule approaches for quantifying transcription and degradation rates in intact mammalian tissues

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ABSTRACT

A key challenge in mammalian biology is to understand how rates of transcription and mRNA degradation jointly shape cellular gene expression. Powerful techniques have been developed for measuring these rates either genome-wide or at the single-molecule level, however these techniques are not applicable to assessment of cells within their native tissue microenvironment. Here we describe a technique based on single molecule Fluorescence *in-situ* Hybridization (smFISH) to measure transcription and degradation rates in intact mammalian tissues. The technique is based on dual-color libraries targeting the introns and exons of the genes of interest, enabling visualization and quantification of both nascent and mature mRNA. We present a software, TransQuant, that facilitates quantifying these rates from smFISH images. Our approach enables assessment of both transcription and degradation rates of any gene of interest while controlling for the inherent heterogeneity of intact tissues.

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1. Introduction to the technique

The levels of cellular mRNA are governed by two highly controlled processes – transcription and mRNA degradation. The balance between these processes dictates not only the steady state transcript levels but also the gene's response time [1,54,63,64,79] and susceptibility to noise [3,57]. Measurements of degradation rates of endogenous mRNAs have been performed in a number of ways, including usage of transcriptional inhibitors such as actinomycin D, which interferes with transcription by intercalating into DNA [44,54], and 5,6-dichloro-1 β -D-ribofuranosylbenzimidazole (DRB), which interacts directly with the RNA polymerase II transcription apparatus [12,21]. Genome-wide measurements of gene expression at sequential time points following such transcription inhibition enable inference of mRNA lifetimes. A limitation of this methodology is that transcription inhibition often introduces significant changes to cell physiology, e.g. to the transcription of components of RNA degradation machinery [6,26,61]. As a result, mRNA lifetimes obtained in such experiments may not necessarily reflect the true stability of the mRNAs.

Other techniques to estimate rates of transcription and degradation rely on combined measurements of both newly transcribed RNA molecules and total RNA. These include techniques that capture the RNA bound to actively transcribing Pol2, such as

Nascent-seq [35,45,60], NET-seq [15,43,50] and GRO-seq [16]. A complementary approach to estimate these rates on a genome-wide basis is metabolic labeling of RNA with 4-thiouridine (4sU) or 5'-bromo-uridine (BrU), modified uridines that enable specific pull-down of recently transcribed RNA from the overall RNA population, with minimal interference to normal cell growth [30,54,55]. These powerful techniques provide a genome-wide view of transcription and degradation; however, they work on bulk measurements, thus providing an average picture of these rates and potentially missing the variability between sub-populations. Accounting for such variability is important when samples are heterogeneous [27,32]. In such cases techniques that enable single-cell measurements are required.

A complementary set of strategies to infer transcription and degradation rates that focus on single cells apply imaging techniques to follow individual RNA molecules inside living cells. The MS2-GFP technique [7,10,24,53] uses a modified RNA that contains multiple tandem sequences recognized by the MS2 bacteriophage coat protein. A simultaneously expressed MS2-GFP fusion protein localizes on the tandem repeats yielding a bright fluorescent spot, which can be followed in time within the cells. Another method that allows imaging of individual mRNA is by hybridization of molecular beacons (MBs) to multiple tandem repeats engineered into the desired mRNA [11,48,75,76]. Molecular beacons are hairpin shaped molecules with an internally quenched fluorophore, the fluorescence of which is restored when they bind to a target nucleic acid sequence. Although these techniques offer

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unprecedented spatial and temporal resolution they require manipulations such as transfection of constructs that incorporate the modified RNA (MS2–GFP) or microinjection of the fluorescent probes into the cells, thus applicable only to living cultured cells.

Extracting the rates of transcription and degradation of cells that reside within their natural tissue microenvironment is an outstanding challenge. This is particularly important in heterogeneous tissues, which invariably contain different cell types and diverse sub-populations. Tissues are often polarized by morphogens or blood flow and thus the location of cells within a tissue is a key determinant of their gene expression states [27]. Accounting for this spatial variability requires techniques for quantitative single-cell analysis without dissociating the tissue. The single molecule Fluorescence *in-situ* Hybridization (smFISH) technique facilitates visualization of mRNA molecules in fixed cells or tissues [19,34,39,40,42,51,57,58,71,78]. This technique relies on the specific hybridization of libraries of short DNA sequences that are complementary to a specific target RNA sequence that are all coupled to the same fluorophore. Binding of multiple probes to the same transcript yields a bright dot, indicative of a single mRNA transcript. This method has been used in bacteria [42,65,66], yeast [42,71,80], mammalian cells [34,57] and recently even in intact tissues [3,28,29,39,78]. Here we describe a modification of this technique [3] that enables not only measurements of the amount of mRNA per cell but also quantification of the rates of transcription and mRNA degradation at the single-molecule, single-cell level in intact tissues. We provide a simple software, TransQuant, that implements our method for estimating these rates from smFISH microscopy images.

2. Theory and rational

The dynamics of mRNA production can be modeled as a first-order process [1]:

$$dX/dt = \beta - \delta X \quad (1)$$

where β is the cellular transcription rate, in units of mRNA/h, δ is the rate of mRNA degradation (1/h) and X the number of cytoplasmic mRNA molecules. Under steady state conditions, defined as conditions where β and δ have been constant for enough time so that temporal changes in X are negligible, the average number of mRNA per cell can be found by setting Eq. (1) to 0 ($dX/dt = 0$):

$$X_{st} = \beta/\delta \quad (2)$$

Eq. (2) indicates that a given level of cellular mRNA can be achieved by either high rates of both transcription and degradation or low rates of both transcription and degradation. Using Eq. (2) we can infer mRNA degradation rates δ from combined measurements of β and X_{st} :

$$\delta = \beta/X_{st} \quad (3)$$

The number of mRNA per cell in steady state, X_{st} , can be obtained using smFISH by identifying individual mRNA dots in 3D images and assigning them to cells. Obtaining the transcription rate, β , requires identification of the transcription sites (TS), and quantification of the average number of Pol2 molecules actively transcribing (termed ‘polymerase occupancy’, M). Assuming that Pol2 elongates at a constant rate v and immediately releases the nascent transcript at the 3' end of the gene, the rate of mRNA production from a TS, denoted by μ , is:

$$\mu = M \cdot v/L \quad (4)$$

where L is the length of the gene and M is the average number of Pol2 molecules on a typical locus (Fig. 1A). Transcription is generally a bursty process [3,4,8,9,13,17,20,24,33,49,52,56,69], and promoters are thought to stochastically switch between non-active

and active transcriptional states, so in general only a fraction f of the chromosomal copies in the cell will be active. As a result, the average transcription rate per cell that contains n chromosomal copies, β , is:

$$\beta = n \cdot f \cdot \mu = n \cdot f \cdot M \cdot v/L \quad (5)$$

where we have used Eq. (4) for μ . Eq. (5) assumes that the velocity of Pol2 is known (this will be discussed in the computational methods section) and that all nascent mRNA end up in the cytoplasm, neglecting nuclear degradation of improperly spliced mRNA [22].

Eq. (5) indicates that measurements of the cellular ploidy (n), the fraction of chromosomal copies that are transcriptionally active (f), and the average number of Pol2 molecules on a given TS (M) can be used to infer cellular transcription rate (β). Additionally measuring the average number of mRNA per cell at steady state (X_{st}) will facilitate inferring degradation rates using Eq. (3) (Fig. 1B). We will next describe our smFISH approach for imaging individual mature and nascent mRNA in intact mouse tissue sections and for inferring the rates of transcription and mRNA degradation.

3. Establishing image based analysis of transcription and degradation rates in intact mammalian tissues

The detailed protocol of single molecule mRNA detection and counting in mammalian tissues was previously published [41]; here we describe how to modify the smFISH technique in order to quantify the active sites of transcription and to extract dynamic gene expression properties in intact mammalian tissues (Fig. 1C). In order to visualize nascent mRNA, one must locate and quantify TS. A common approach for identifying active transcription sites using smFISH is to seek bright dots that reside in the nucleus [39,80]. Since several Pol2 molecules may be actively engaged in transcription of the target gene of interest, and since each Pol2 will carry a tail of partially transcribed mRNA (Fig. 1A), the local concentration of smFISH fluorescent probes will be higher in a TS compared to a single cytoplasmic mRNA, thus yielding a brighter dot. While this strategy seems to work well for organisms such as yeast and drosophila, the abundance of nuclear mRNA molecules and the low Pol2 occupancy in many of the endogenous genes prohibit unambiguous identification of TS in mammalian cells using this approach (Fig. 1D).

Identifying TS can be achieved by labeling not only the exons of the transcripts of interests but also the introns [38]. Introns are generally spliced and degraded co-transcriptionally [2,23,36,46,72], therefore intact stretches of introns only reside at the active sites of transcription. To utilize this fundamental property of mammalian transcription we use two smFISH probe libraries coupled to spectrally resolvable fluorophores; one that targets the exons of the gene of interest and a second library that targets the introns. The intronic dot facilitates unambiguous identification of the TS, whereas the exonic dot enables quantification of the average Pol2 occupancies (Fig. 1C and D). In the following sections we describe in details our protocol steps, summarized in Fig. 2.

4. Tissue processing

To obtain precise measurements of the gene expression parameters, RNA integrity must be preserved during the tissue handling. To this end, we excise the tissue immediately after sacrifice of the animal and place it in 4% PFA for fixation (as described in [41]). After cryopreservation, tissues are placed in OCT molds and stored frozen at -80°C . For visualization of full nuclei, thick tissue sections (25 μm) are mounted on poly-L lysine coated #1 coverslips. The sections are left to air-dry for around 10 min and placed on

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