Supplementary Note

Important parameters in TimeLapse-seq.

TimeLapse-seq builds upon previous work using s^4U to metabolically label RNA, and many of the considerations when designing experiments are shared with previous work and have been discussed in depth elsewhere[22], including the time of s^4U treatment required to accurately estimate transcript half-lives. Considerations that are specific to TimeLapse-seq are discussed below.

Each read-pair in TimeLapse-seq data reports mutations that are present in a single molecule of RNA that was either made prior to the s^4U treatment, or was made after s^4U was added to the cells. For new RNA, there is an s^4U- and chemistry-dependent increase in the probability of a T-to-C mutation at each nucleotide. For any given region of an RNA molecule that is copied into a sequencing read of a given length (l_r), our ability to accurately identify whether the read pair is from a new RNA or not is dependent on the following: n_u, the number of uridine residues that could be substituted with s^4U; p_{new}, the probability a s^4U residue substitutes for U at each position; y_{chem}, the efficiency of the conversion from s^4U to C*; and p_{old}, the background mutation rate in untreated samples. At the population level, the accuracy of the estimates for the newly made faction of any feature (e.g., transcript, exon, etc.) depends on the read depth (n_{reads}).

The background mutation rates (p_o) are constrained by the methods and technology used for RNA-seq and estimated using negative controls. The number of uridines (n_u) in the read is dependent on the U-content of the RNA feature and on the read length (l_r) in the sequencing experiment (*e.g.*, single-end 75 nt reads vs paired end 150 nt reads). The probability of s^4U incorporation (p_n) depends on ratio of s^4UTP/UTP in the nucleotide pool, which is dependent on the s^4U concentration used in the feed, the cell line used and the time of the experiment. The rate of incorporation of s^4U into the UTP pool is quite fast. This is clear from the observation that many reads in the TT-TimeLapse-seq experiment have multiple mutations, suggesting that even within 5 min at 1 mM s^4U treatment, the nucleotide pool builds up substantial concentration of s^4UTP. There is also cell-type variability in the influence of s^4U treatment (*e.g.*, we found TimeLapse-seq in MEF cells worked best with 1 mM s^4U, whereas labeling of K562 cells was successful with 100 μM s^4U used in the 4h treatment). In practice 10 μM – 1 mM treatments have been successful. The chemical efficiency (y_{chem}) determines the number of s^4U residues that are converted to C, which we have estimated to be 80% (**Supplementary Fig.S4**).

To explore how deeply any RNA feature must be sequenced in order to detect changes in the new transcript pool by TimeLapse-seq, we simulated data according to the following model:

$$i \in \{1, 2, ..., n_{\text{reads}}\}$$

$$n_{u,i} \sim \text{Binom}(l_r; p = 0.25)$$

$$X_i \sim \text{Bernoulli}(\theta_n)$$

$$Y_i \sim \begin{cases} \text{Binom}(n_u;\ p_n \times y_{chem}) & X_i \in \text{new reads} \\ \text{Binom}(n_u;\ p_o) & \text{otherwise} \end{cases}$$

where the i^{th} read (out of n_{reads} total) with n_u uridine residues is determined to arise from either a new or old RNA according to a Bernoulli distribution with the fraction of new RNA (θ_n). If the transcript is new, it is modeled to have a number of mutations (Y_i) defined by a binomial distribution with n_u trials and probability of mutation the probability of s^4U incorporation (p_n) attenuated by the yield of the chemistry (y_{chem}). If the RNA is old, the number of mutations is modeled by a binomial distribution n_u trials and a background probability of mutation (p_o). The data from these simulated trials were treated as the output from a TimeLapse-seq experiment in which the fraction new was modeled as described in the methods (using likelihood maximization to estimate θ_n), and the number of new reads inferred using this estimate. Different fold changes in the new transcript pool (x) were modeled in duplicate, with duplicate controls to match the design we used in this manuscript. To provide a conservative estimate of the sensitivity of the approach, these counts were added to a real RNA-seq data set (from the differential expression of heat shock expression) and the significance determined using DESeq2 with default parameters. We favored this approach because the dispersion estimates used to determine the significance in the simulation are influenced by the distribution of real TimeLapse-seq data. This simulation was repeated 250 times for each set of parameters, and the average number of times the simulation provided a significant difference was plotted (**Supplementary Fig.S12**). For each simulation, conditions were held constant that were similar to (or more conservative than) the actual parameters for the MEF experiment presented in **Fig.2**.

In general, many conditions lead to reliable detection of differential expression when there are hundreds-to-thousands of reads. Under the conditions of these simulations, neither the chemical efficiency nor the read length have dramatic impact unless they are greatly reduced. One practical consequence of this observation is that improving the efficiency of the reaction from 80% would have very little impact, and even a drop to 50% yields would only have a small impact on the sensitivity of the experiment. On the other hand, to be able to sensitively detect changes, the fraction of new RNA must be large enough to detect (> 5%), but less than half the RNA. Large fold differences (>2) are straight forward to detect even at very low coverage, but much higher coverage is necessary to confidently detect transcripts that have a 1.5-fold induction in the new RNA pool. Both depletion and enrichment were detected, and the specificity was very high (the false positive rate by this metric was too low to detect). The background mutational rates (~0.1% in the samples presented in this manuscript) are predicted to have minimal impact unless they are increased five-to-ten-fold. While decreasing the amount of s^4U can decrease sensitivity, increasing it is predicted to only lead to a modest increase in sensitivity. In summary, experimental design regarding the timing of the s^4U treatment is critical.