Protocol for TimeLapse-seq

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Abstract

RNA sequencing \(RNA-seq) offers a snapshot of cellular RNA populations, but not temporal information about the sequenced RNA. Here we report a protocol for TimeLapse-seq, which uses oxidative-nucleophilic-aromatic substitution to convert 4-thiouridine into cytidine analogs, yielding apparent U-to-C mutations that mark new transcripts upon sequencing. The steps of the protocol are \(1) treating samples with 4-thiouridine and isolating the total RNA, \(2) a simple chemical treatment of the labeled RNA with an oxidant and amine under optimized conditions, and \(3) isolation of the converted RNA which can then be subjected to sequencing. TimeLapse-seq is a single-molecule approach that is adaptable to many applications.

Introduction

The dynamics of RNA populations can be investigated by several techniques. One well established approach is to use metabolic labeling to incorporate a chemical handle on a non-canonical nucleoside, and then use this handle to enrich new transcripts \(e.g., TT-seq[1] and s^4U-seq[2,3]). These techniques require large amounts of input sample and extensive handling, and they present challenges when normalizing enrichment and estimating contamination. To capture temporal information about RNA directly in a sequencing experiment without biochemical enrichment, we developed TimeLapse-seq, a method in which cells are exposed to a noncanonical nucleoside that becomes incorporated into only new transcripts. Rather than enriching the metabolically labeled RNAs, we developed chemistry that recodes the hydrogen-bonding pattern of the uridine analog 4-thiouridine \(s^4U) to match the hydrogen-bonding pattern of cytosine, thereby causing mutations in a sequencing experiment \(Fig. 1). The s^4U base itself leads to low levels of U-to-C transitions upon reverse transcription[4], but does so at levels too low to robustly identify new transcripts. While recent applications of s^4U have focused on the thione as a nucleophile[2], or for UV cross-linking[5,6], we pursued less explored reactivity—transforming s^4U using oxidative-nucleophilic-aromatic substitution7. We developed new conditions that allow the oxidation of s^4U, providing a convertible nucleoside intermediate that is converted into an analog of cytosine by aminolysis. When performed before RNA-seq analysis, this reaction reveals sites of s^4U incorporation through T-to-C mutations stably introduced in the cDNA. The following protocol describes a TimeLapse-seq experiment using optimized metabolic labeling conditions to study mRNA turnover in K562 cells \(4 h, 100 μM s^4U treatment)[8]. s^4U treatment conditions will vary based on cell type and desired application \(discussed in Schofield et al. Nature Methods, 2018), however the TimeLapse protocol described below can be applied to any standard s^4U metabolic labeling pipeline.

Reagents

K562 cells HyClone RPMI 1640 Media Fetal bovine serum \(FBS), certified Penicillin-Streptomycin, 100X solution Phosphate buffered saline \(PBS), 1X 6-well tissue culture plates 4-thiouridine DEPC-treated water Filter pipette tips—10 μl, 20 μl, 200 μl, and 1000 μl Qiagen RNeasy Mini kit 2-mercaptoethanol \

(BME) Ethanol, 200 proof, molecular biology grade 3 M sodium acetate, pH 5.2 2,2,2-trifluoroethylamine \(TFEA) 0.5 M EDTA, pH 8 Sodium periodate \($NaIO_4$) 0.2 mL PCR tube strips 1.5 mL microcentrifuge tubes, PCR clean 15 mL conical centrifuge tubes 1 M dithiothreitol \(DTT) 22-gauge disposable needle Luer slip syringe Agencocurt RNAClean XP beads SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian

Equipment

Magnetic PCR tube separation rack PCR Thermocycler Nanodrop spectrophotometer Mini centrifuge for PCR strips Benchtop microcentrifuge Analytical balance Bioanalyzer

Procedure

Metabolic Labeling 1. Culture K562 cells at 37°C to approximately 50% confluence in RPMI media supplemented with 10% FBS and 1% P/S \(e.g., 5 mL of $5x10^5$ cells/mL in 6 well tissue culture plates). 2. Dissolve 4-thiouridine in a minimal volume of water \(e.g. 25 mM stock solution) and add to 5 mL cultured cells at 100 µM final concentration. Agitate cells gently to evenly distribute the metabolic label. Incubate 4 h at 37°C. Note: Take steps to protect 4-thiouridine treated cells and RNA in all subsequent steps from light exposure. Note: 4-thiouridine treatment time and concentration will vary by cell type and desired application. We provide an example above for K562 cells to examine relatively long RNA half-lives. 3. Transfer cells from each well to separate 15 mL conical Eppendorf tube and place on ice for 1 min. 4. Pellet cells for 3 min at 300xg, 4°C in a pre-chilled centrifuge. Aspirate cell culture medium from cell pellet. 5. Rinse pellet with ice-cold 1X PBS then pellet and aspirate as in step 4. Proceed immediately to modified RNeasy isolation \(recommended), or store cell pellet at -80°C. **Modified RNeasy isolation** 6. Resuspend cell pellet in 350 µl RLT buffer supplemented with 35 µl BME \(1% final). Pass cell suspension through a 22-gauge needle 5 times to lyse the cells and transfer the mix to a 1.5 mL Eppendorf tube. 7. Add 350 µl freshly prepared 70% ethanol to the lysis mixture and mix well by inversion. Transfer 700 µl of mixture to an RNeasy spin column, and centrifuge >10,000 RPM for 15 sec. Discard flow through. 8. Add 700 µl buffer RW1 to column and centrifuge >10,000 RPM for 15 sec. Discard flow through. 9. Add 500 µl buffer RPE supplemented with 50 µl BME \(1% final) to column and centrifuge >10,000 RPM for 15 sec. Discard flow through. 10. Add 700 µl freshly prepared 80% ethanol to column and centrifuge at maximum speed for 2 min. Transfer column to fresh collection tube and centrifuge at maximum speed for an additional 5 min. 11. Transfer column to 1.5 ml Eppendorf tube and add 30 µl DEPC-treated water directly to column membrane. Let column stand 1 min, then centrifuge >10,000 RPM for 1 min. Assess RNA concentration by nanodrop. Note: Incubating spin column at 65°C for 5 min prior to centrifugation increases total RNA yield. **TimeLapse chemistry** 12. Dilute RNA to 2 µg in 8.7 µl DEPC-treated water \(less RNA may be used if desired, 10 ng of total RNA is recommended for library preparation after TimeLapse chemistry). 13. Create a master mix by combining the following reagents \(15 µl total per sample, multiplied by number of samples, +10% to account for pipetting errors) on ice: 0.84 µl 3M sodium acetate, pH 5.2; 12.7 µl DEPC-treated water; 0.2 µl 0.5M EDTA, pH 8; 1.3 µl TFEA.

Combine well by vortexing. Note: TFEA is volatile. Pipette up and down several times prior to dispensing the reagent to ensure vapor pressure equilibration and accurate volumes. 14. Add 15 µl master mix from step 13 to RNA sample from step 12 in a 0.2 ml PCR tube. Combine well by flicking PCR tubes, and briefly spin to collect sample at bottom of tube. 15. Add 1.3 µl of 192 mM solution of $NaIO_4$ in DEPC-treated water \(freshly prepared, 10 mM final concentration). Combine well by flicking PCR tubes several times, and briefly spin to collect sample at bottom of tube. 16. Incubate samples at 45°C for 1 h in a pre-heated PCR thermocycler with a heated lid. Cool sample to 4°C after incubation. 17. Add an equal volume \(e.g. 25 µl) of RNAClean beads to each sample and gently vortex to combine. Incubate at room temperature 10 min. Note: An aliquot of RNAClean beads should be brought to room temperature for 30 min prior to use. 18. Briefly spin to collect sample at bottom of tube, and place on a magnetic isolation rack until solution is clear \(~5 min). 19. Carefully remove supernatant without disturbing bead pellet, and wash beads twice with 200 µl of freshly prepared 80% ethanol. 20. After removing second ethanol wash, briefly spin tubes, and recapture beads on magnetic isolation rack for 1 min. Remove residual ethanol with a pipette and allow bead pellet to dry \(2-4 min). Notes: A small crack will appear when beads are dried. Do not over-dry beads. 21. Add 18 µl of DEPC-treated water to dried beads. Flick tubes until beads are completely resuspended, and allow to rehydrate for 2 min. 22. Briefly spin tubes, and place on magnetic isolation rack until solution is clear \(~2 min). 23. Carefully collect the supernatant and transfer to a fresh PCR tube strip. 24. Prepare a 10X reducing master mix \(100 µl recipe): 10 µl 1 M Tris-HCl, pH 7.4; 10 µl 1 M DTT; 20 µl 5 M NaCl; 2 µl 0.5 M EDTA, pH 8; 58 µl DEPC-treated water. 25. Add 2 µl master mix from step 24 to 18 µl of supernatant from step 23 and mix well to combine. Spin tubes briefly, and incubate samples at 37°C for 30 min. 26. Add an equal volume \(e.g. 20 µl) of RNAClean beads to each sample and gently vortex to combine. Incubate at room temperature 10 min. 27. Repeat steps 18-20. 28. Add 12 µl of DEPC-treated water to dried beads. Flick tubes until beads are completely resuspended, and allow to rehydrate for 2 min. 29. Repeat steps 22-23. 30. Assess concentration and quality of RNA by bioanalyzer. 31. Proceed to RNA-seq library preparation using the SMARTer Stranded Total RNA-Seq Kit-Pico Input Mammalian.

Timing

TimeLapse-seq can be performed over two days: Day one 4-thiouridine treatment—time dependent on cell type/application, typically 1-4 h RNA isolation—1 h TimeLapse chemistry and cleanup—3 h Day two Library preparation with SMARTer Stranded Total RNA-Seq Kit—5 h

Troubleshooting

Improper handling can lead to loss of s^4U RNA prior to chemical treatment. While low levels of s^4U-RNA-specific loss can be corrected for bioinformatically \(see Schofield et al. 2018), it is preferable to handle the RNA in a manner that maximizes retention of the s^4U-RNA. One indication of specific loss of s^4U RNA is a depletion of fast turnover transcripts \(e.g., intronic signal and mRNA such as _MYC_) are relative to other RNA species. This loss can be avoided by handling the RNA in the dark, including reducing reagents

\(e.g., BME) when handling the RNA, and using the tips such as heated elution \(see Step 11) described in the protocol above.

Anticipated Results

RNA before and after chemical treatment should appear similar in size distribution \(i.e., the chemistry should not lead to RNA degradation). Bioanalyzer analysis of a successful TimeLapse-seq library results in a curve with a peak size of ~400 bp \(including sequencing adapters). RNA-seq analysis using standard bioinformatic tools should demonstrate that the total transcript counts from each sample are highly correlated irrespective of s^4U exposure \(comparison of plus and minus s^4U treatment) or chemical treatment \(expected Pearson's $r \geq 0.95$). A specific and reproducible increase in T-to-C mutations dependent on both metabolic labeling with s^4U and chemical treatment should be apparent. When separated by T-to-C mutation count, RNA-seq tracks display differences in mutations based on RNA turnover. The T-to-C mutation counts should be substantially higher for fast-turnover transcripts \(e.g., _MYC_ and _FOSL1_), compared to more stable transcripts \(e.g., _ACTB_). An enrichment of T-to-C mutations should be observed in intronic reads \(consistent with the fast turnover of intronic RNA). TimeLapse-seq can be applied to various experimental designs depending on desired application. It can be used to examine differential transcript stability \(including RNA half-lives), induced changes in gene expression \(e.g., heat shock), and the transient transcriptome \(e.g., TT-TimeLapse-seq).

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Figures

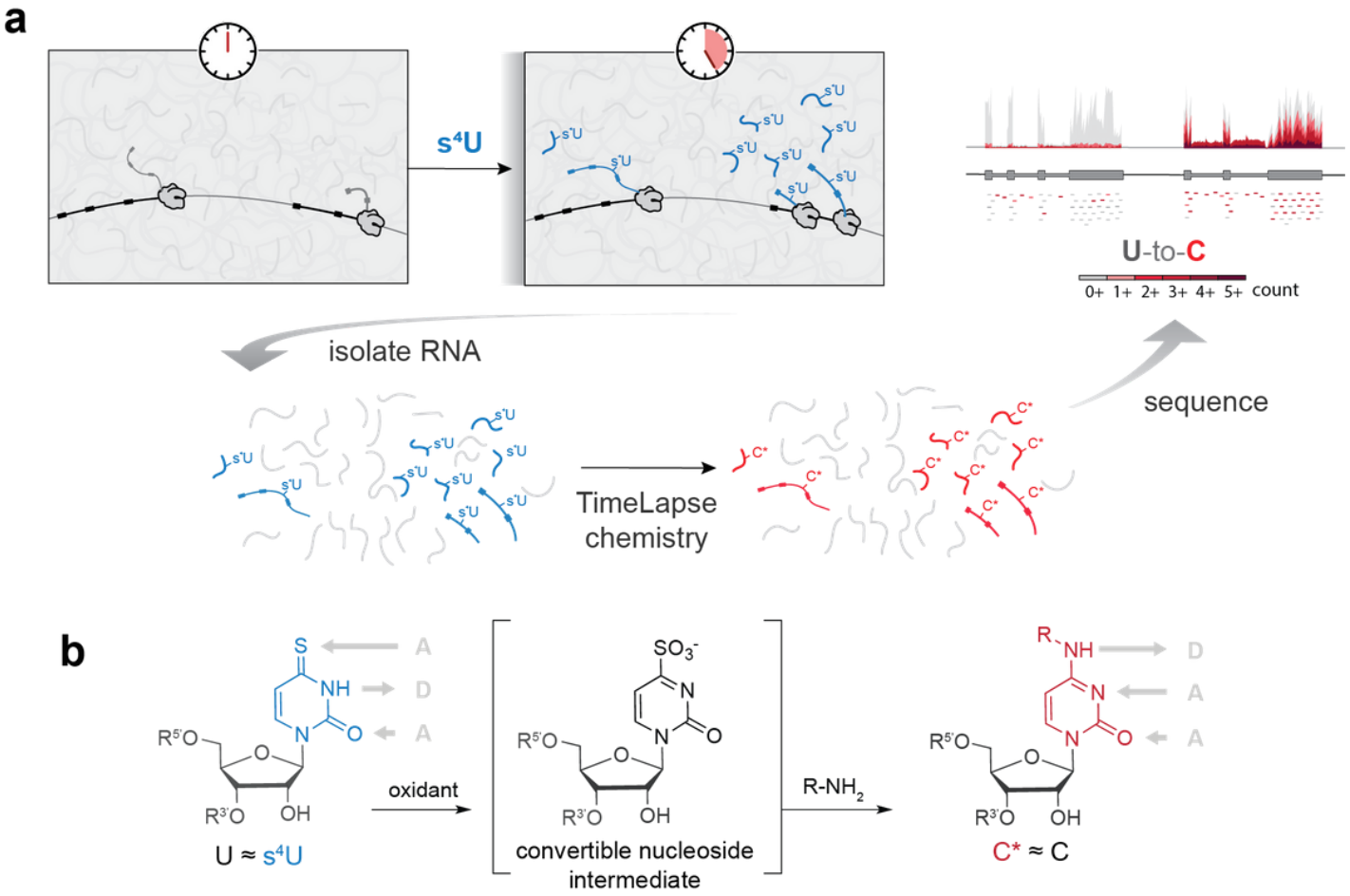

Figure 1

Overview of TimeLapse-seq TimeLapse-seq uses a convertible nucleoside approach to identify new transcripts in a sequencing experiment. (a) Scheme of TimeLapse-seq. (b) Oxidative-nucleophilic-aromatic substitution reaction that recodes 4-thiouridine into cytidine analogues.