Chapter 6

Quantification of mRNA Turnover in Living Cells: A Pipeline for TREAT Data Analysis

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Abstract

mRNA turnover plays an important role in the regulation of post-transcriptional gene expression. While many protein factors involved in mRNA degradation have been identified, we still lack a basic understanding of the principles that regulate the spatiotemporal dynamics of mRNA turnover within single cells. To overcome this limitation, we have developed the TREAT biosensor, which allows for discrimination of intact reporter transcripts and stabilized decay intermediates using single RNA imaging. Here, we present an image analysis pipeline that performs semiautomated detection and tracking of individual mRNA particles. It colocalizes tracks and applies the colocalization information to quantify the number of intact transcripts and degradation intermediates. Based on the analysis of control data, the workflow further determines detection efficiencies and uses them to correct RNA particle numbers.

Key words mRNA turnover, TREAT, MS2/PP7 stem-loops, Fluorescence microscopy, Live cell imaging, Single-molecule

1 Introduction

RNA turnover is a highly regulated process that controls cellular mRNA levels and eliminates aberrant RNAs. Canonical mRNA decay is mediated by 5′-3′ and 3′-5′ exonucleases, which are activated after deadenylation, decapping, and/or endonucleolytic cleavage steps [1]. The cytoplasmic 5′-3′ exoribonuclease 1 (XRN1) has been shown to be the main 5′-3′ exonuclease responsible for the turnover of cytoplasmic mRNAs [2].

Ensemble measurements have contributed to our understanding of the mechanisms and protein factors involved in mRNA degradation, however, the inherent instability of degradation intermediates has prevented detailed mechanistic studies of the spatiotemporal dynamics of mRNA turnover. To overcome this limitation, we have developed an assay that applies XRN1-resistant pseudoknots to generate stabilized degradation intermediates (Fig. 1a) [3]. These pseudoknots (PKs) inhibit XRN1-mediated

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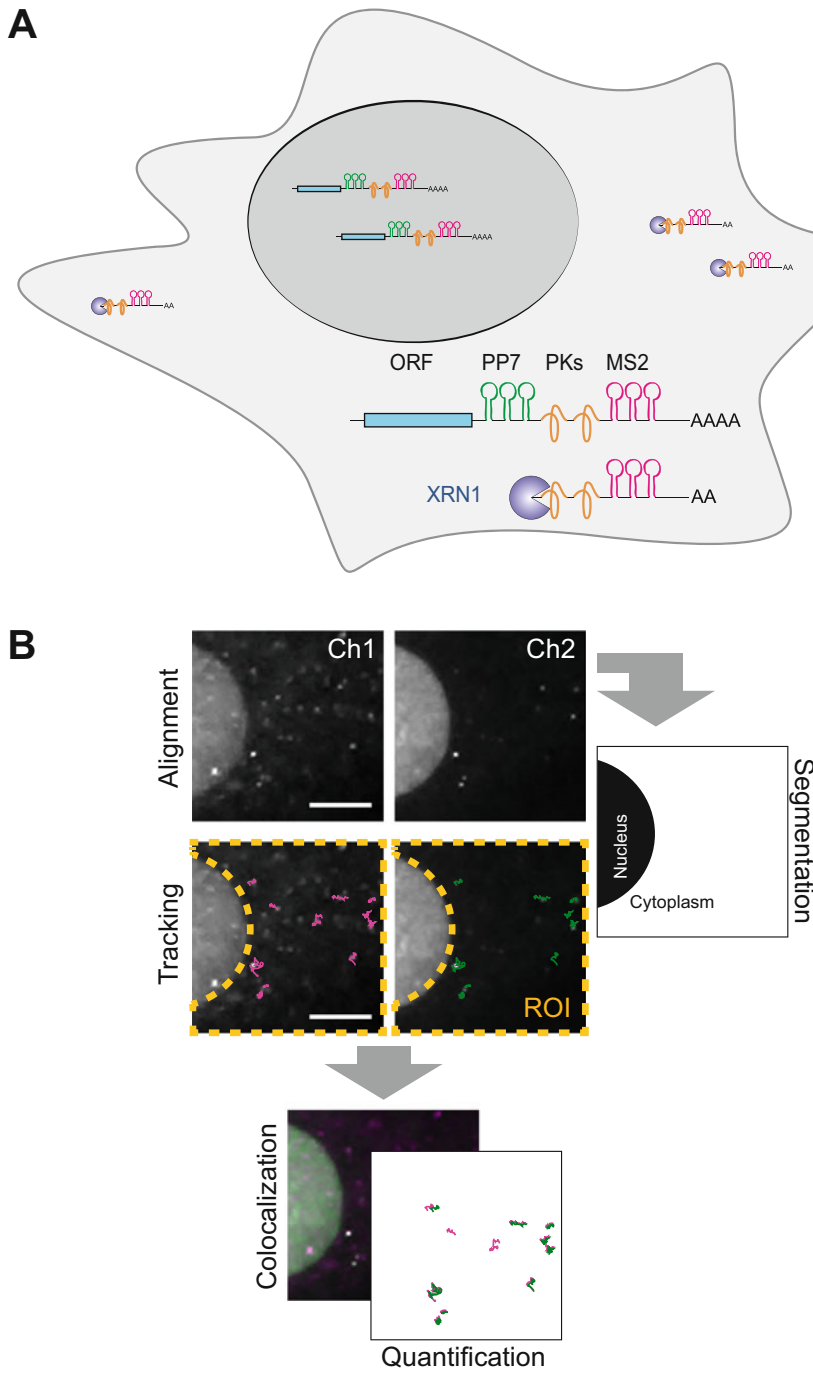

Fig. 1 Quantification of mRNA turnover via the TREAT assay. (**a**) Schematic representation of the TREAT assay: Reporter transcripts are transcribed in the nucleus and exported to the cytoplasm. They can be labeled using orthogonal PP7 and MS2 stem loop cassettes (green, magenta) that are included in the 3′ UTR and separated by viral pseudo knots (PKs, orange). XRN1 degrades mRNAs via 5′-3′ exonucleolytic digest until it is blocked by the PK sequences. This produces stabilized decay intermediates, which can be detected as single labeled

decay by sequestering the phosphorylated 5′ end of the RNA substrate from the nuclease active site [4] thereby increasing the half-life of the degradation intermediate and enabling its detection by standard biochemistry techniques [5].

Additional integration of PP7 and MS2 stem loop cassettes flanking the PKs in the 3′ UTR of a reporter construct allows detection of single mRNAs using live cell imaging techniques. This design, which relies on the protection of the MS2 stem loops from XRN1-mediated digest by PKs positioned upstream of the cassette, enables discrimination of intact transcripts (PP7 and MS2) and stabilized degradation intermediates (MS2 only). Detection of reporter mRNAs in living cells is mediated by fluorescently labeled PP7 and MS2 coat proteins (PCPs and MCPs) that specifically recognize each stem loop cassette and are fused to spectrally distinct fluorophores. To increase labeling efficiency synonymous (identical amino acid, but slightly different codon usage) tandem PCP fused to synonymous tandem GFP (stdPCP-stdGFP) and synonymous tandem MCP fused to Halo (stdMCP-Halo) were used [6]. In addition, both fusion proteins contain nuclear localization signals (NLS-stdPCP-stdGFP and NLS-stdMCP-Halo) to sequester unbound proteins in the nucleus.

Quantification of single- and dual-labeled particles allows assessment of mRNA decay dynamics at any point in time after induction of mRNA reporter expression. Since this technique relies on the quantification of stabilized 3′ decay intermediates, we refer to it as Three(3′)-RNA End Accumulation during Turnover (TREAT).

The following chapter first provides instructions on how to perform TREAT experiments using reporter and colocalization control transcripts. It briefly describes how to acquire TREAT imaging data and then focuses on step-by-step instructions on how to analyze the images using a custom KNIME data analysis workflow that allows semiautomated image processing as well as batch-tracking of single mRNAs through application of user-defined tracking parameters.

Fig. 1 (continued) (MS2 only) particles. TREAT turnover can be assessed via quantification of dual and single labeled reporter transcripts per cell. (**b**) Overview of the analysis steps included in the image processing pipeline. The workflow first aligns image series from both channels according to a user defined input. It then generates a nuclear segmentation to generate the cytoplasmic region of interest (ROI) for single-particle tracking. Next, spot detection and tracking are performed based on a user defined input of tracking parameters and for both channels independently. Track coordinates are colocalized to identify dual and single labeled particles and the fraction of intact transcripts and stabilized decay intermediates is quantified after correction for detection efficiencies

2 Materials

2.1 Sample Preparation

1. Cell lines stably expressing TREAT reporter and control transcripts as well as fluorescently labeled MCP and PCP. To this aim, we have integrated TREAT reporter and control constructs (Addgene: 104095, 104097) via flp recombinase-mediated cassette exchange into HeLa 11ht cells [7] so that constructs can be expressed from a single doxycycline-inducible locus. In addition, the cell lines stably express NLS-stdPCP-stdGFP and NLS-stdMCP-Halo that were genomically integrated via lentivirus-mediated transduction (Addgene: 104098, 104099). The cell lines are available upon request from the Chao laboratory.
2. Cell culture incubator.
3. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) tetracycline-free FBS and 1% (v/v) penicillin and streptomycin (pen/strep).
4. Automated cell counter and counting slides.
5. Imaging dish (μ-Dish), 35 mm, high, glass bottom.
6. Fluorobrite™ DMEM imaging medium + 10% FBS.
7. 1 μg/mL Doxycycline in Fluorobrite™ DMEM imaging medium + 10% FBS.
8. 100 nM Halo-labeling Janelia Fluor 549 (JF_{549}; Tocris) [8] in DMEM + 10% FBS.
9. Phosphate-buffered saline (PBS).

2.2 Image Acquisition

1. Inverted multipoint confocal spinning disk microscope (e.g., Olympus IX81). We use one equipped with a CSU-X1 scanhead (Yokogawa) and two back-illuminated EvolveDelta EMCCD cameras (Photometrics). Images are collected using a 100 × 1.45 NA PlanApo TIRFM oil immersion objective (Olympus), green (Semrock, FF01-617/73-25) and red (Semrock, FF02-525/40-25) emission filters, beam-splitter between cameras (Chroma, 565DCXR), solid-state lasers (100 mW 491 nm and 100 mW 561 nm; Cobolt), and motorized X,Y,Z-Piezo controlled stage (ASI).
2. Incubation chamber for live cell imaging that provides heating and CO_2 regulation. Before starting the experiment, equilibrate incubation chamber and microscope to 37 °C and 5% CO_2.
3. Multicolor calibration slide for channel alignment (e.g., Argolight, type SLF-001). Before starting the experiment apply calibration slide to make sure that cameras are precisely aligned.

2.3 Data Processing and Analysis

1. Fiji [9] including the TrackMate plugin [10].
2. KNIME Analytics Platform (version 3.7.0 or later) [11]. Configure additional update sites (*File* > *Preferences* > *Install/Update* > *Available Software Sites* (using the **Add...** button)) to add the FMI software site (https://community.knime.org/download/ch.fmi.knime.plugins.update/). Then add the FMI KNIME Plugins via *Help* > *Install New Software...* and selecting *FMI KNIME Plugins* from the newly added update site.
3. R (version 3.5.2) (https://cran.r-project.org/) with the rserve, ggplot2 and cairo packages installed. To install R in KNIME save R locally and configure KNIME to use it (*File* > *Preferences* and under *KNIME* > *R* provide the path to your R home directory).
4. Analysis workflow and training datasets available from the Chao lab (https://data.fmi.ch/PublicationSupplementRepo/?group=gchao). Download workflow and install (*File* > *Import KNIME workflow...*).

3 Methods

TREAT is a single-molecule technique that distinguishes intact mRNAs from stabilized decay intermediates by assessing the presence of two spectrally distinct fluorescent labels bound to stem loop sequences in the 3′ UTR of a reporter transcript (Fig. 1a). Dual-labeled reporter transcripts (NLS-stdPCP-stdGFP and NLS-stdMCP-Halo) are intact mRNAs while particles exhibiting only a single label (NLS-stdMCP-Halo alone) are stabilized decay intermediates. Quantification of decay dynamics therefore relies on correct identification of mRNA particles as either intact transcripts or degradation intermediates, which in turn depends on the accurate detection of both fluorescent labels.

The following protocol provides a general description of how to obtain and quantify TREAT data using a custom semiautomated image processing pipeline in KNIME (Fig. 1b). The workflow incorporates quantification of the detection efficiencies for both fluorescent labels based on image acquisition of a colocalization control reporter (lacking PKs) that is always labeled by both fluorophores. In depth discussion of colocalization control reporter design and the principles underlying detection efficiency calculation have been described previously [12].

Subheadings 3.1 and 3.2 give an overview of how to perform TREAT experiments for acquisition of experimental and colocalization control data. Step-by-step instructions on how to design reporter constructs, generate stable cell lines and acquire images have been described by Horvathova et al. (2017) [3]. Subheading 3.3 then provides basic instructions on how to determine batch

tracking parameters using the Fiji plugin TrackMate [10]. For more detailed guidelines on how to perform single-molecule tracking of mRNA reporters using TrackMate refer to Voigt et al. (2018) [13]. Finally, Subheading 3.4 focuses on how to perform semiautomated tracking and track colocalization analysis using the custom KNIME workflow mentioned above. All analysis software as well as sample experimental and control data sets are available from the Chao lab publication repository (https://data.fmi.ch/PublicationSupplementRepo/?group=gchao).

3.1 Performing a TREAT Experiment

1. Approximately 48 h before starting the experiment, seed 30,000 HeLa cells stably expressing NLS-stdMCP-Halo, NLS-stdPCP-stdGFP as well as the TREAT or control reporter construct in 2 mL DMEM + 10% FBS + 1% Pen/Strep into a 35 mm imaging dish. Make sure that cells are homogeneously distributed within the dish (*see* **Note 1**).
2. Grow cells for approximately 2 days at 37 °C and 5% CO_2. Depending on the cell line, longer or shorter incubation times are possible.
3. Before starting the experiment, prewarm PBS and Fluorobrite™ DMEM + 10% FBS to 37 °C.
4. For fluorescent labeling of Halo-tags incubate with 1 mL 100 nM JF_{549} dye in DMEM + 10% FBS for 30 min at 37 °C and 5% CO_2.
5. Take medium off cells to remove dye. Wash three times with PBS.
6. Add prewarmed Fluorobrite™ DMEM + 10% FBS + 1 μg/mL doxycycline to induce TREAT or control reporter expression (*see* **Note 2**).
7. Consider the timing of induction and image acquisition to guarantee particle densities that are suitable for single-particle tracking (SPT). To characterize reporter transcript expression profiles over time quantify particle densities in the cytosol via imaging trials at different time points after induction. For the HeLa cell lines used here, TREAT and control transcripts are transcribed ~20 min after doxycycline induction and are exported into the cytoplasm ~25 min later in most cells. Shorter or longer expression times are possible depending on the experiment performed (*see* **Note 3**).

3.2 Image Acquisition

1. Begin image acquisition at the appropriate time point after doxycycline induction. Expression times can vary depending on the reporter used and experimental set up. Consider removing doxycycline from the imaging dish at defined time points to stop induction and limit the particle density in the cytoplasm.

2. Use the red channel (NLS-stdMCP-Halo) to find suitable cells for image acquisition as Halo+JF_{549} bleaches slower than GFP. Make sure to use low laser intensities to reduce photobleaching prior to the experiment and select cells with well-resolved spots (high SNR) at densities that permit SPT (*see* **Note 4**).
3. Acquire images in both channels simultaneously. Use acquisition settings suitable for SPT. In particular, choose exposure times $\leq$50 ms that allow for unambiguous assignment of fast moving particles in subsequent frames and laser powers that achieve maximum spot intensities without excessive bleaching of the sample. Adjust camera gains accordingly to yield an optimal SNR without detector saturation.
4. Collect short image series in a single plane using the streaming mode in both channels simultaneously. Acquire 5–50 frames per field of view.
5. Make sure to perform image acquisition for both TREAT and colocalization control reporter expressing cell lines using identical acquisition settings in several independent replicates.

3.3 Determination of Batch Tracking Parameters Using TrackMate

To assess mRNA turnover via live cell imaging of single mRNAs, it is essential to reconstruct mRNA trajectories using SPT. The Fiji plugin TrackMate [10] first assesses the positions of individual mRNAs in all frames of the input image series (spot detection) and then links spot positions in successive frames into trajectories (tracking). The semiautomated image processing pipeline described below uses a custom plugin, which runs TrackMate and applies defined spot detection and tracking parameters within the KNIME environment. To this aim, it is necessary to first perform manual SPT of several image series in order to determine parameters that allow accurate tracking of the entire dataset using fixed parameter settings.

1. Select a small number of image series (3–10 frames) obtained from at least three independent experiments for manual SPT. If image quality varies in between image series and replicates, analyze more data until the heterogeneity in image quality can be estimated. Exclude outlier datasets that are heterogeneous with respect to spot intensity, size or SNR.
2. Compare input data to the training data sets provided for this manuscript on the Chao group publication repository (https://data.fmi.ch/PublicationSupplementRepo/?group=gchao), where example experimental and colocalization control data sets are available under Live_TestData/RawData and Live_ControlData/RawData respectively.
3. Determine if image series are calibrated correctly. If needed, use the "Properties" command to calibrate global image

parameters in Fiji. For the training data provided here, the pixel size equals 0.09 μm.

4. Define channel offsets for all datasets by inspection of superposed spots in both channels and align them by using, for example, the "Translate" command in Fiji. For the TREAT and colocalization control image series provided here, the operation that superposes both channels most closely requires a pixel shift from channel 1 to channel 2 of −2/−8 (x/y) for the test data and of 0/0 (x/y) for the colocalization control data. Take note of the channel offsets to be able to apply them during the batch analysis below.
5. Decide how many frames to include in the analysis. To perform accurate track colocalization, not more than 3–5 frames are needed. Limit the number of frames to a minimum since this facilitates more accurate manual tracking (less data points to be manually inspected) and reduces the processing time during the analysis steps.
6. Perform manual SPT using the TrackMate GUI in Fiji. For detailed instructions on how to perform SPT of mRNA transcripts in TrackMate consult Voigt et al. (2018) [13]. Determine the spot intensity "Threshold" for each channel as well as the spot size ("Estimated blob diameter" in the TrackMate GUI) that allows accurate spot detection in all inspected image series. Further identify the "Linking max distance," allowed frame "Gap size" and "Gap-closing max distance" that allow most accurate tracking in all image series (*see* **Note 5**). For the training datasets provided here, these parameters are listed in Table 1.

Table 1
Spot detection and tracking parameters

TrackMate parameters	Values used to obtain results provided along with training datasets
Threshold Ch1 control [AU]	550
Threshold Ch2 control [AU]	650
Estimated blob diameter/spot radius [μm]	0.38/0.19
Linking max distance [μm]	0.4
Gap size [frames]	2
Gap-closing max distance [μm]	0.8
Frame interval [s]	0.05
Threshold Ch1 test [AU]	550
Threshold Ch2 test [AU]	1200

3.4 High-Throughput Image Processing in KNIME

To quantify the number of intact reporter mRNAs and stabilized degradation intermediates, it is necessary to accurately track and colocalize the tracks obtained from a large number of image series. The image processing pipeline described in the following paragraph is designed to enable nonexpert users to quantify the results of a TREAT experiment in a reasonable amount of time. Since it performs high-throughput tracking and track colocalization of control and test datasets it aims to reduce the user input necessary to perform robust SPT.

3.4.1 Data Selection and Input

1. Assemble raw data files from TREAT and colocalization control experiments in two separate folders. For the training datasets provided here, these can be found under Live_TestData/RawData and Live_ControlData/RawData for the example experimental and colocalization control datasets respectively.
2. Open the "Batch-Track-Colocalization-Analysis.knwf" workflow in KNIME (*see* **Note 6**). Throughout the workflow, nodes that require user input are framed in red and labeled according to the order of their execution. All nodes framed in orange are visualization and quality control nodes, which are designed to allow the user to evaluate the successful execution of the analysis steps following a required input. Output nodes are shown in blue and allow export of the results of the analysis.
3. Configure and execute the "Load image data" metanode (INPUT 1). To this aim, choose the folder that contains the TREAT data as "Input directory" and the folder that contains the colocalization control data as "Input directory for control data." Enter the "Image file extension" (nd for the training data provided here) and choose to "Override pixel spacing metadata" if raw data are not properly calibrated. If this is the case, also set "New xy pixel spacing" (0.09 μm for training data provided here) to provide the pixel size for recalibration. Choose the "Number of frames to load" for both, test and colocalization control data (load five frames to reproduce the results provided along with the training datasets) and select "Swap Z and Time in input data" in case the image series is mislabeled as a Z-stack.
4. Execute and view the loaded images using the "Image Viewer" (VISUALIZATION 1) downstream of the "Load image data" metanode in order to make sure that image files are loaded correctly.

3.4.2 Channel Alignment and ROI Generation

1. Configure and execute the "Correct channel shift and segment nucleus" metanode (INPUT 2). It aligns channel 1 to channel 2 by translating channel 1 images according to the pixel shifts provided by user input during configuration (and as defined in Subheading 3.3). The metanode then generates cytoplasmic

ROIs for SPT by segmentation of the cell nuclei using a global thresholding algorithm that applies the Huang method.

2. Execute the "Image Viewer" node (VISUALIZATION 2) directly connected to the INPUT 2 metanode in order to inspect the segmentation results provided in the last column of the image table.

3.4.3 Detection Efficiency Calculation from Control Data

The detection efficiencies for channel 1 and channel 2 labels can be derived via quantification of colocalization control data, in which all mRNA transcripts should appear dual-labeled [3, 12]. The branch described in the following paragraph performs SPT and track colocalization through application of user-defined input parameters. It then quantifies the numbers of single- and dual-labeled particles and applies them to calculate detection efficiencies that will later be used to correct the particle numbers obtained through track colocalization in the TREAT dataset (Subheading 3.4.4).

1. Configure and execute the "Tracking and colocalization parameters—Control data" metanode (INPUT 3). Insert batch tracking parameters for the colocalization control dataset as determined under Subheading 3.3 or provided for the training control dataset in Table 1. Further configure the "Distance cutoff" below which a spot pair is classified as colocalizing (default = 0.3 μm), choose the "Minimum track length" to be included in the analysis (default = 3 frames) and the "Minimum number of (spot) colocalizations" necessary to define a track as colocalizing (default = 2 events). Last, choose "channel 1 and 2 plotting colors" from the scroll down menus (default channel 1 = red and channel 2 = green).
2. Execute the "Plot tracks per cell" metanode (VISUALIZATION 3). The branch leading to this node performs spot detection, SPT and track colocalization for the control data. For details of the track colocalization algorithm, see Voigt et al. (2018) [13]. Briefly, track colocalization relies on pairwise spot colocalization, which depends on distance measurements of all spots in the first channel to all spots in the second channel. The workflow defines colocalizing pairs of spots by recursively selecting mutual nearest neighbors and only classifies spot pairs as colocalizing if their distance is below the user-defined cutoff provided above. Next, tracks are defined as colocalizing if they contain at least the user-defined number of colocalizing spot pairs. If multiple tracks from one channel colocalize with one or multiple tracks from the other channel (in different frames), the track pair with the largest number of spot colocalizations is considered genuine (recursive track pairing). Noncolocalizing tracks are called orphans.

3. Inspect the results from SPT and track colocalization in the output table generated by the VISUALIZATION 3 metanode. Scroll through the table to look at track maps of individual cells. Colocalizing tracks are depicted opaque while orphan tracks are shown semitransparent. The color scheme corresponds to the plotting color for each channel chosen above. Large numbers of colocalizing tracks indicate good detection efficiencies while equal track numbers (in both channels) combined with low colocalization rates might be due to suboptimal data quality even though spot detection and tracking is performed accurately. If many more (short, often 2-frame) tracks are depicted in one but not the other channel, this likely indicates suboptimal spot detection thresholds. Refer to Voigt et al. (2018) [13] for instructions on how to troubleshoot SPT parameters.
4. Execute the "Export results for control data" metanode (OUTPUT 1). It activates the branch calculating detection efficiencies for each channel and outputs all results obtained for the colocalization control dataset in a single excel file called "ResultsControlData.xlsx", which is automatically saved into the input data directory. Compare results obtained for the training control dataset with the results provided under Live_-ControlData/Results. Inspect the detection efficiencies calculated per cell (DetEff—per Cell) and on average (DetEff—Mean) in the corresponding tabs of the results file. For the control data provided along with this text, mean channel 1 and 2 detection efficiencies should equal 0.89 ± 0.08 and 0.94 ± 0.05 respectively. Values of ~90% indicate good data quality.

3.4.4 Quantification of TREAT Data Including Particle Number Correction

Quantification of single- and dual-labeled TREAT reporter mRNAs is performed analogous to the quantification of colocalizing and orphan control transcripts explained above (Subheading 3.4.3). In addition, the branch described in the following paragraph, uses the detection efficiencies determined above to correct the numbers of intact transcripts (colocalizing tracks) and stabilized decay intermediates (orphans) for the detection bias of the specific experimental setup.

1. Configure and execute the "Tracking parameters—Test data" metanode (INPUT 4). To this aim, define intensity thresholds for both channels of the TREAT dataset as determined under Subheading 3.3 or provided for the training test dataset in Table 1. All other parameters must be identical in the control and TREAT datasets and do not need to be entered again.

2. Execute the "Plot tracks per cell" metanode (VISUALIZATION 4) and inspect the SPT and track colocalization results for the TREAT dataset in the output table provided by this node. Interpret tracking results (analogous to the control data tracking results) as described above (Subheading 3.4.3).
3. Execute the "Export results for test data" metanode (OUTPUT 2). The branch leading to this node applies the detection efficiencies determined above (Subheading 3.4.3) to correct the colocalizing and orphan track numbers determined via colocalization analysis of the TREAT dataset. It then exports a single excel file called "ResultsTestData.xlsx", which is automatically saved into the input data directory. Compare results obtained for the training dataset with the results provided under Live_TestData/Results. Inspect the "Summary—Mean" tab within the results file. For the TREAT data provided here, the mean fraction of colocalizing particles corresponding to the fraction of intact mRNA transcripts should equal 0.48 ± 0.11 (*see* **Note 7**). This result (obtained from cells that were imaged approximately 2 h after induction of reporter expression) is in good agreement with the half-life time of the TREAT reporter transcript (1.63 ± 0.24 h).

4 Notes

1. If cells are seeded at lower densities and more than 48 hours before the experiment they can grow larger and have more time to widely spread out on the imaging dish. This can result in reduced cell thickness, which in turn increases SNR due to a reduction of overall background fluorescence originating from out-of-plane particles.
2. Consider using shorter induction times to reduce the number of particles in the cytoplasm, which will facilitate robust SPT.
3. Consider supplementing Fluorobrite imaging medium with 4 mM L-glutamine for long time course experiments.
4. In order to minimize photobleaching, try to reduce the overall radiation that cells are exposed to by adapting laser intensities and exposure times.
5. For determination of batch tracking parameters, make sure that tracking parameters yield robust results for all image series analyzed. If not, consider generating a more homogeneous cell population by sorting to uniformly low fluorescent protein levels.

6. For any questions about the tools employed (KNIME, Fiji, TrackMate) as well as about usage of the workflow, please consider asking on https://forum.image.sc.
7. Make sure the results obtained from the TREAT experiment match the half-life of the applied reporter transcript as obtained from an unrelated experiment (qPCR, smFISH).

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