Chapter 25

Detection of the First Round of Translation: The TRICK Assay

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Abstract

Quantitative fluorescence microscopy techniques are frequently applied to answer fundamental biological questions. Single-molecule RNA imaging methods have enabled the direct observation of the initial steps of the mRNA life cycle in living cells, however, the dynamic mechanisms that regulate mRNA translation are still poorly understood. We have developed an RNA biosensor that can assess the translational state of individual mRNA transcripts with spatiotemporal resolution in living cells. In this chapter, we describe how to perform a TRICK (*t*ranslating *R*NA *i*maging by *c*oat protein *k*nock-off) experiment and specifically focus on a detailed description of our image processing and data analysis procedure.

Key words mRNA translation, TRICK, MS2/PP7 stem-loops, Coat proteins, Quantitative fluorescence microscopy in live cells, Single-RNA imaging

1 Introduction

Ribosomes translate mRNAs to produce protein. mRNA translation is a highly regulated process that allows appropriate protein production in response to specific cellular needs. Ensemble measurements have contributed to our understanding of translation regulation by assessing ribosome occupancy and protein abundance on a genome-wide scale [1, 2]. These methodologies, however, can provide only limited insight into complex regulatory mechanisms that rely on the spatial or temporal distribution of individual molecules within the same cell. Single-molecule fluorescence microscopy, on the other hand, can image individual mRNAs in living cells [3] and has been used to investigate transcriptional dynamics in both prokaryotes and eukaryotes [4, 5].

In order to deepen our understanding of the spatiotemporal regulation of translation, we have established a dual-color single RNA imaging-based assay that can assess the translational state of individual mRNA molecules in living cells [6]. The TRICK (*t*ranslating *R*NA *i*maging by *c*oat protein *k*nock-off) assay takes advantage of the fact that the translating ribosome removes all proteins

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associated with the coding sequence of an mRNA transcript during the first round of translation (Fig. 1a). A TRICK reporter transcript contains translatable PP7 stem-loops in the coding sequence and MS2 stem loops in the 3′ UTR. Since these orthogonal RNA stem-

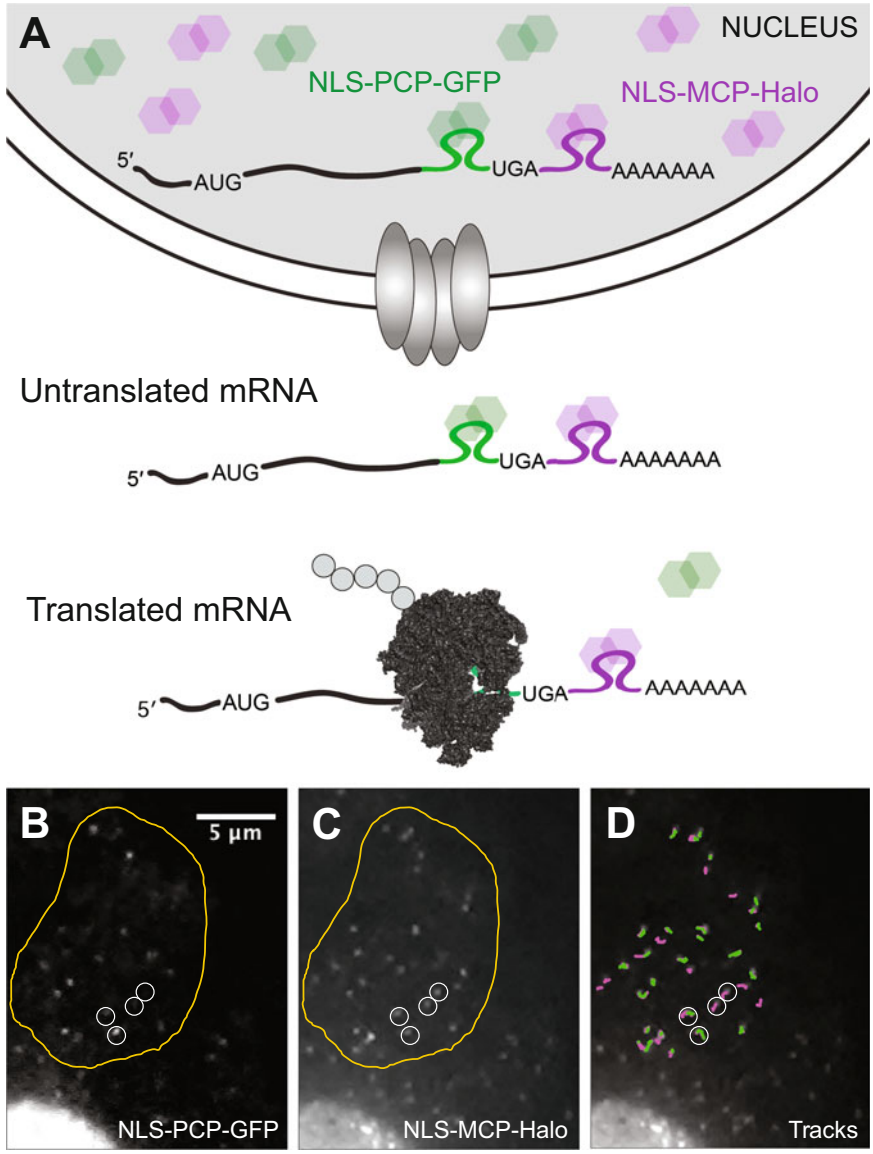

Fig. 1 Detecting the first round of translation. (**a**) Schematic representation of the TRICK assay. The translating ribosome (*black*) removes GFP-labeled PP7 coat proteins (NLS-PCP-GFP, *green*) from the coding region. Halo-labeled MS2 coat proteins (NLS-MCP-Halo, *magenta*) remain bound to the 3′ UTR. Figure modified from [6] and reprinted, with permission, from The American Association for the Advancement of Science © 2015. (**b**, **c**) Representative live cell images of a HeLa cell expressing NLS-PCP-GFP, NLS-MCP-Halo and a TRICK reporter. **b** = GFP, **c** = Halo labeled with JF_{549}. The ROI is shown in *yellow*. Several segmented spots are marked by *white circles*. (**d**) Superposition of tracks acquired for both channels after SPT in five frames

loops can be bound by PP7 and MS2 coat proteins (PCP and MCP) fused to spectrally distinct fluorescent proteins, an untranslated mRNA will be dual-labeled [7, 8]. Upon the first round of translation, the translating ribosome displaces all proteins bound to the coding region of the reporter mRNA including the fluorescent signal arising from the PCP without displacing the MCP signal. Since both MCP and PCP fluorescent fusion proteins contain nuclear localization sequences rebinding of PCP to the transcript after translation does not occur. TRICK measures the translational state of a transcript as a function of the colocalization of the PCP and MCP fluorescent labels. This chapter briefly explains how to perform a TRICK experiment and focuses on the subsequent image and data analysis.

2 Materials

2.1 Sample Preparation

1. A cell line is needed that stably expresses fluorescently labeled MCP and PCP as well as a TRICK mRNA reporter transcript. Specifically, we use the HeLa 11HT cell line [9], which contains a single doxycycline-inducible locus for recombinase-mediated cassette exchange of the TRICK reporter and stably expresses NLS-PCP-GFP and NLS-MCP-Halo. It is available upon request from the Chao laboratory. Alternatively, lentiviral transfer plasmids encoding NLS-PCP-GFP and NLS-MCP-Halo are available from Addgene (Addgene IDs 64539, 64540, 64544, and 84443). TRICK expression and colocalization control plasmids that can serve as cloning templates are also available on Addgene (Addgene IDs 84443, 84444) [6].
2. Cell culture incubator.
3. Gibco™ Dulbecco's Modified Eagle's 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 (e.g., Bio-Rad).
5. Imaging dish, 35 mm, high, glass bottom (e.g., ibidi μ-Dish).
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}; HHMI Janelia Research Campus) in DMEM + 10% FBS.
9. Phosphate-buffered saline (PBS).

2.2 Image Acquisition

1. Multipoint confocal spinning disk microscope, e.g., an Olympus IX81 inverted microscope equipped with a CSU-X1 scanhead (Yokogawa) and Borealis modification (Andor) featuring

a dichroic beam-splitter in the scanhead (Semrock Di01-T488/568—13 × 15 × 0.5), 100× 1.45NA PlanApo TIRFM oil immersion objective (Olympus), two back-illuminated EvolveDelta EMCCD cameras (Photometrics), 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 around the microscope to provide heating and CO_2 regulation.
3. Multicolor calibration slide for channel alignment (e.g., Argolight, type SLF-001).

2.3 Data Processing and Analysis

1. FijiImageJ [10] including the TrackMate plugin [11].
2. KNIME Analytics Platform (version 3.2.1, [12]).
3. RStudio (version 0.99.896) with the ggplot2 package installed [13].

3 Methods

TRICK is a single-molecule technique that distinguishes untranslated from translated mRNAs by assessing the presence of two spectrally distinct fluorescent labels bound to the open reading frame (ORF) and 3′ UTR of a reporter transcript. Subheadings 3.1 and 3.2 briefly explain how to perform a TRICK experiment. More detailed instructions on how to design reporter constructs, generate stable cell lines and best acquire images have been described by Halstead et al. [14].

Subsequently, Subheadings 3.3 and 3.4 provide detailed protocols on how to perform the image processing and data analysis part of a TRICK experiment. They focus on the challenges and advantages of multiple-channel single-particle tracking (SPT) using the Fiji plugin TrackMate [10, 11, 15] and provide detailed instructions on how to employ a KNIME [12] data processing pipeline for spot-based track colocalization. The analysis pipeline and a sample data set are available for download on the Chao lab web site (http://www.fmi.ch/research/groupleader/?group=132).

3.1 Performing a TRICK Experiment

1. To generate a TRICK reporter construct, integrate a PP7 cassette into the coding sequence and an MS2 cassette into the 3′UTR of the RNA of interest. This can be done by excision and gel extraction of the complete PP7-stop codon-MS2 fragment via SalI and ClaI restriction enzyme sites from the Renilla TRICK reporter construct (Addgene ID 84443) described by Halstead et al. [6]. Alternatively, the PP7 (SalI/XhoI) and MS2

(BamHI/ClaI) cassettes can be individually cloned into reporter constructs. Conventional PCR amplification of the PP7 and MS2 cassettes described here is not recommended due to the repetitiveness of the DNA sequences. A co-localization control plasmid (*see* Subheading 3.4.3) can be generated by introduction of a stop codon in front of the PP7 cassette using site-directed mutagenesis.

2. Seed 30,000 HeLa cells stably expressing NLS-MCP-Halo, NLS-PCP-GFP and the TRICK reporter construct in 2 mL DMEM + 10% FBS + 1% Pen/Strep into a 35 mm imaging dish ~48 h prior to the experiment. Take care that cells are homogeneously distributed within the dish to improve cytoplasmic imaging of mRNAs (*see* **Note 1**).
3. Incubate cells for 2 days at 37 °C and 5% CO_2. Longer or shorter incubation times are possible depending on the cell line being used.
4. Immediately prior to the experiment, prewarm PBS and Fluorobrite™ DMEM + 10% FBS to 37 °C.
5. Halo-label cells by incubation with 1 mL 100 nM JF_{549} in DMEM + 10% FBS for 30 min at 37 °C and 5% CO_2.
6. Remove medium from cells, wash three times with PBS and add prewarmed Fluorobrite™ DMEM + 10% FBS + 1 μg/mL doxycycline to induce TRICK reporter expression.
7. Since TRICK measures the first round of translation, it is important to consider the timing of induction and the acquisition of images. For the HeLa cell line we use, TRICK transcripts begin to enter the cytoplasm approximately 45 min after doxycycline addition.

3.2 Image Acquisition

1. Equilibrate the incubation chamber of the microscope to 37 °C and 5% CO_2.
2. Align cameras prior to image acquisition using a multicolor calibration slide.
3. Identify suitable cells for image acquisition using the red laser (MCP-Halo channel) at low intensities to reduce photobleaching prior to the experiment. Choose cells with well-resolved (high signal/noise) diffraction-limited mRNA particles (diameter approximately 4 pixels or 0.36 μm for optical settings described above) at densities that facilitate SPT.
4. Image cells in both channels simultaneously using laser powers, camera gain and exposure time compatible with SPT. Exposure times should be no longer than 50 ms to ensure that fast moving particles can be unambiguously tracked between subsequent frames. Laser power should be adapted to yield maximum signal intensity while limiting bleaching the samples,

which would prevent accurate spot detection in the last frames of the image series. Camera gain can be adjusted with respect to the laser power and should result in optimal signal-to-noise ratios without saturation of the detector.

5. Collect 10–50 frame image series per region of interest.
6. To facilitate SPT, take special care to image cells with low to medium spot density in the region of interest. High particle densities can often lead to false assignment of displacements in between neighboring particles and will complicate SPT.

3.3 Single-Particle Tracking Using TrackMate

To assess the translational state of individual mRNAs, it is necessary to reconstruct their trajectories using SPT. For an informative TRICK experiment, it is further essential to track the mRNA movement in both channels independently.

The Fiji plugin TrackMate performs SPT as a two-step process: First, it localizes individual particles (spot detection). Second, it links particle positions in consecutive frames into tracks (tracking). TrackMate outputs all positions assumed by a particle throughout the time course of the experiment as a list of coordinates that serves as input for the track colocalization pipeline described under Subheading 3.4.

3.3.1 Image Preparation in Fiji

1. Before starting TrackMate, make sure that the channels are precisely aligned. To correct potential offset between channels, use a suitable method for channel alignment, e.g., the "Translate" command or the descriptor-based registration plugin. Use the "Search" option within the "Help" menu to find Fiji commands mentioned throughout this chapter.
2. Decide how many frames to include in the analysis and, if needed, reduce the length of the image series accordingly using the "Slice Keeper" function. Accurate tracking is best performed on a small number of frames (typically 3–10) since this limits the number of data points that has to be visually inspected (*see* **Note 2**).
3. Optionally, reduce randomly distributed noise by application of the "Bandpass filter" (filtering small objects below three pixels).
4. Select a single region of interest (ROI, yellow in Fig. 1b and c) via the freehand selection tool (*see* **Notes 3** and **4**). Make sure to use the same ROI in both channels by using the "Restore Selection" function or the ROI Manager, but track both channels individually.

3.3.2 Spot Detection

1. Launch TrackMate while having the first image series selected. Check that image parameters are loaded correctly. If not, calibrate using the image "Properties" function in Fiji and "Refresh source" in the TrackMate graphical user interface

(GUI). Alternatively, edit the calibration settings in the TrackMate GUI. Make sure that both image series are calibrated the same way.

2. Detect spots using the "Laplacian of Gaussian" (LoG) detector. Alternatively, use the "differences of Gaussian" (DoG) detector as a quicker approximation of the LoG detector.
3. Optimize detector settings for the detection of single mRNA particles. Choose an "Estimated blob diameter" that is slightly bigger than the expected spot size (approximately four pixels or 0.36 μm in the imaging set-up described above) and an intensity "Threshold" that detects all, even weak intensity spots throughout the whole image series.
4. Visually inspect detected spots in all frames either using the "Preview" function or after spot detection has been performed. For inspection, choose the "Hyperstack Displayer" to superpose the segmentation results over the images (white circles in Fig. 1b–d).
5. Do not threshold or filter the detected spots. All filtering can be done later as part of the track colocalization pipeline.
6. Make sure that all visible spots are detected in all images. If not, go back and repeat the spot detection using a reduced intensity threshold or larger spot diameter. A slight overdetection is not a problem since all spots that cannot be linked to form tracks will be discarded during tracking.

3.3.3 Tracking

1. Choose the "Simple LAP tracker" as particle-linking algorithm since it allows gaps and prevents track merging or splitting events.
2. Set the "Linking max distance" to match physiological diffusion coefficients observed for individual mRNA particles, i.e., 0.1–0.8 μm to account for diffusion coefficients between 0.009 $\mu m^2/s$ [16] and 3.42 $\mu m^2/s$ [17] that have been observed for different types of transcripts in various subcellular localizations.
3. Set the "Gap-closing max frame gap" to 2 in order to allow single frame gaps in tracks. Gap size is defined as the frame difference, which is 2 if one frame is missing.
4. Set the "Gap-closing max distance" to a multiple of the "Linking max distance" that matches the allowed frame gap if particle density is low and correct tracking likely. Otherwise, reduce the "Gap-closing max distance" to values similar to the "Linking max distance" to allow gaps only for low mobility particles.
5. Perform tracking, do not filter tracks and click through the GUI until the "Display Options" interface is reached. Apply the interface to change track appearance to facilitate visual inspection.

6. Examine all tracks to see whether they describe spot movement accurately. If not, go back and change tracking parameters accordingly.
7. Optimal tracking is always a trade-off between large linking distances that allow tracking of highly mobile particles and false linking of arbitrary spots resulting from spot detection errors. The choice of parameters should be optimized depending on the images being analyzed and their effect should be systematically evaluated.
8. Export the tracking results via the "Analysis" button in the "Display Options" interface. To this aim, save the "Spots in tracks statistics" table that pops up as an .xls file with exactly the same name as the image series used for tracking.
9. Repeat all steps from Subheadings 3.3.2 and 3.3.3 for the second channel using identical tracking but not necessarily identical spot detection parameters since each channel may have different signal-to-noise ratios.

3.4 Spot-Based Track Colocalization in KNIME

TRICK assesses the translational state of individual mRNA transcripts via detection of two spectrally distinct fluorescent labels and relies on the pairwise colocalization of tracks obtained after independent SPT in both fluorescent channels.

Pairwise colocalization analysis is performed by measuring the distances of all spots belonging to the first channel to all spots in the second channel. Using the measured distances, colocalizing pairs of spots are determined by selecting mutual nearest neighbors. To accommodate for cases where two spots in one channel have the same nearest neighbor in the other channel, the mutual nearest neighbor analysis is recursively performed on all unpaired spots until every spot is assigned to a pair. Spot pairs are classified as colocalizing if their distance is below a user-defined cutoff.

Tracks are classified as colocalizing if they contain a user-defined minimum number of colocalizing spot pairs (usually two). All tracks shorter than a user-defined minimum track length (usually three frames) that were not classified as colocalizing are excluded from the analysis. All other tracks are called orphans and represent translated mRNAs.

3.4.1 Data Preparation

1. Assemble both .xls files exported after SPT of the two channels along with both image series used for tracking in a single folder for each cell. Make sure that coordinate files and image series are named identically (except for the file extension).
2. Combine the folders containing tracking and imaging data for each cell in one or several parent folders.

3.4.2 Track Colocalization

1. Open the Track-based colocalization analysis workflow in KNIME.
2. Configure and execute the "Parameter input" node (#391) by choosing the parent folder that combines the tracking and imaging data folders as input directory. Enter the "File Extension" of the coordinate files (xls) and choose "Channel identifiers" that distinguish input files belonging to both channels. Tick "Load images for quality control" to load image files into the pipeline. Identify image files by providing the image file extension (e.g., tif). Define the distance cutoff below which a spot pair is classified as colocalizing (default = 0.3 μm). Enter the pixel size to calibrate imaging data (default = 0.09 μm, depending on microscope set-up). Choose "channel 1 and 2 plotting colors" from the scroll down menus in case defaults (channel 1 = red, channel 2 = green) do not apply.
3. Configure and execute the "Filtering parameters" node (#405). Choose the minimum track length that is to be included in the analysis (default = three frames) and the minimum number of spot colocalizations necessary to define a track as colocalizing (default = two events).
4. Execute and view the "Interactive Segmentation View" node (#454). It provides a control mechanism that allows superposition of the spot coordinates of channel 1 over the image files of the same channel. Open the interactive view panel by double-clicking an image in the (segmented) labeling column. Use the "Bounding Box Renderer" to visually check that spot coordinates match spot positions.
5. Execute and view the "JavaScript Table View" node (#427). The branches leading to this node perform the colocalization analysis. For quality control purposes, they generate line plots showing the tracks belonging to each channel in the colors defined under point 2. Tracks that are classified as colocalizing are depicted in opaque colors while orphan tracks are shown semitransparent (alpha = 0.2).
6. Inspect the line plots (Fig. 1d) that are generated for each cell and included in the output table of node #427. If colocalization analysis was performed satisfactory, continue with point 7. If not, refine the parameters to improve results quality. Specifically, test different cutoff values if track classification does not match visually determined colocalization. Return to Subheadings 3.3.2 or 3.3.3 to improve the tracking of individual cells in case orphan tracks appear systematically or track patterns repeatedly differ between channels.
7. Execute the "XLS Sheet Appender" node (#438) to activate the branch that calculates the statistics of the colocalization analysis.

8. Inspect the interactive table generated by "JavaScript Table View" node (#426). It contains the results of the colocalization analysis for each cell. Table columns depict: (a) the number of tracks discarded from the analysis based on the minimum track length and colocalization criteria defined in node #405 ("Excluded Tracks" for channel 1/2); (b) the number of colocalizing and orphan tracks for each channel; (c) the total number of tracks (= colocalizing + orphan) included in the analysis of each channel; (d) the ratio of colocalizing tracks over total included tracks for both channels ("Ratio Colocalized/Total" for channel 1/2).
9. Inspect the colocalization ratio for the green PCP-GFP channel (channel 2). Since green spots should never be present without red spots, this ratio defines the detection efficiency.
10. Inspect the colocalization ratio for the red MCP-Halo channel (channel 1). This ratio represents the untranslated fraction of mRNAs observed in each cell. Use the detection efficiencies defined above to normalize the translation ratios per cell.

3.4.3 Iterative Tracking and Analysis Cycles

Optimal tracking and colocalization is an iterative process (*see* **Notes 5–7**). Therefore, go back in the analysis pipeline and repeat individual steps as many times as it takes to refine parameters that give the best possible detection efficiency:

1. Before performing a TRICK experiment, it is important to assess the colocalization of a dual-labeled transcript that contains both PP7 and MS2 stem-loops in the 3′ UTR of the reporter construct (addgene ID 84444). Since the colocalization of the fluorescent signals of this transcript is not translation-dependent, this analysis is a measure of the maximum colocalization that can be achieved. Using the HeLa cell line and microscope setup described above, we achieve colocalization of 89 ± 7% in the red and 91 ± 6% in the green channel.
2. If orphan tracks accumulate in a specific area of the cell, this often indicates reduced spot detection efficiency due to high background fluorescence in perinuclear zones. Repeat SPT using a different ROI that excludes the problematic area (*see* **Note 8**).
3. If multiple short tracks localize close to each other, this can indicate too short linking distances. Repeat Subheading 3.3.3 using a larger "linking max distance".
4. If a large number of short tracks are excluded from analysis, this could be due to overdetection of spots that are arbitrarily linked to short tracks. Increase intensity "Threshold" or reduce "Estimated blob diameter" during Subheading 3.3.2.

3.4.4 Output

1. Inspect the results of the translation analysis that are saved in a "KNIME_Results.xlsx" file in the parent folder that served as input directory. The file contains two sheets, one giving a summary and one showing the results for each cell included in the analysis.
2. Find the line plots of colocalizing and orphan tracks for each cell as .png files (superposed to representative cell image in Fig. 1d) named after the channel 1 input files in each subfolder.

4 Notes

1. Consider seeding cells more than 48 h prior to the experiment (at lower densities) to yield larger cells that are widely spread out on the imaging dish. Reduced cell thickness can increase signal/noise via reduction of background fluorescence resulting from fluorescent objects above or below the focal plane.
2. To lessen intensity loss due to photobleaching try the "Bleach Correction" function in Fiji using histogram matching. Note that spot detection can get error-prone if long time series are analyzed.
3. Only analyze those regions in a cell that facilitate SPT, i.e., choose ROIs that exclude low signal/noise areas. However, make sure not to bias the analysis with repeated selection of similar ROIs.
4. Always select an ROI to prevent false-positive spot detection at image boundaries.
5. Good SPT is essential since it generates the data that will be used in the analysis. Refine SPT until the results match the physiological conditions as well as possible.
6. Best SPT results are achieved at low till medium particle densities. There are two options to reduce the average density of labeled particles dependent on the type of experiment performed: (1) use very short induction times, start imaging $\geq$30 min after induction and do not remove doxycycline from the medium; (2) use longer induction times (1–2 h), remove doxycycline from the medium and start imaging $\geq$1 h after transcription shut-off.
7. Reduced spot densities allow less stringent tracking parameters, i.e., larger gaps at increased gap closing distances. Set higher "Linking max distances" (Subheading 3.3.3) to allow tracking of highly mobile particles in low particle densities.
8. Overdetect spots to make sure not to miss any particles in noisy data sets.

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