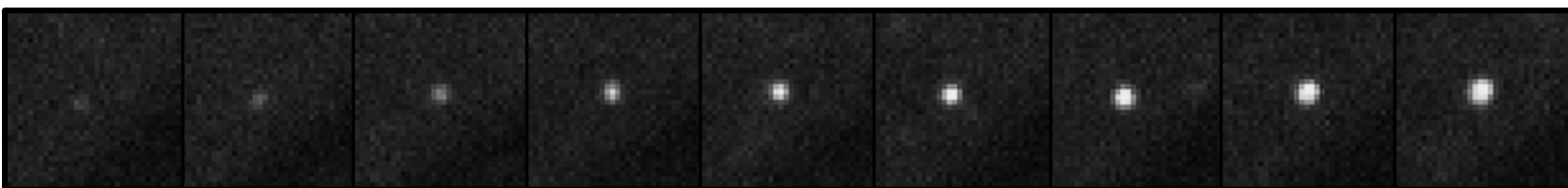


TransTrack 1.0

Manual



Author: Stijn Sonneveld
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1.1 Introduction

TransTrack is a software written in MATLAB with the primary goal to track translation sites visualized with live cell microscopy. Translation sites can be tracked over time and intensities in different channels can be measured at the same time. Furthermore, TransTrack aims to have a combination of automatic and manual functions that help the user to speed up the analysis while keeping a high quality by enabling the user to make corrections. Finally, although TransTrack is designed for tracking translation sites, it can also be used as a more general tool where spot tracking and intensity measurements are required.

2.1 Installing TransTrack

TransTrack 1.0 was built in the MATLAB environment with version R2018b (TransTrack was also tested in MATLAB version R2012b, where it was also functional except for the measure function, section 4.1). Take the following two steps to install TransTrack:

1. Download TransTrack from Github (<https://github.com/TanenbaumLab>) and save on computer (see Appendix I for a list of all the functions that should be in the TransTrack folder).
2. Add the saved folder to MATLAB's search paths. This can be done by clicking on *Set Path* (in MATLAB's main window) and selecting the correct folder. Note: always use the option *Add with Subfolders*, as this will also add the folders that are in the TransTrack folder. Important is that all folders that contain functions that are used by TransTrack are in MATLAB's search paths. Otherwise MATLAB will not be able to locate the specified functions.

2.2 Starting TransTrack and an initial overview

To start TransTrack, go to the folder where TransTrack is saved and open the *TransTrack.m* function in the MATLAB editor. Next, TransTrack is started by clicking on the *Run* button in the MATLAB editor (when the *TransTrack.m* file is selected).

After starting TransTrack, a new window is opened. This is TransTrack's main window and will display the movie that is loaded in TransTrack. All functions in TransTrack are reached through the menu bar at the top of the main window. In the remainder of the manual, the use of all menu options will be described in full detail. The manual is ordered in such a way that it follows the typical workflow of an analysis. To give an overview of all menu options see table 1. Here you can find: (1) all menu options, (2) potential short keys to reach these options in TransTrack, (3) a short description of the menu option, and (4) a reference to the section where a more detailed explanation is given.

Menu	Option	Short key	Short description	Section
File	Open	Ctrl + O	This opens a new movie in TransTrack.	2.1
	Load – Track Data	Ctrl + L	Load	4.2
	Load – ROIs		Load ROIs.	4.2
	Save – Track Data	Ctrl + S	Save results from the current analysis.	4.1
	Save – ROIs		Save the ROIs that are currently selected.	4.1
Image	Display Settings	Ctrl + C	Select brightness and contrast of channels.	2.2
	Channels	Ctrl + Z	Select color and visibility of channels.	2.2
Analysis	Measure	Ctrl + M	Measure intensity in different channels at a spot selected by the user.	3.1
	Photobleach correction – Status		Check which channels have been corrected for photobleaching.	3.2
	Photobleach correction – Correction		Activate or inactivate photobleach correction for each channel.	3.2
	Single channel tracking	Ctrl + A	Start analysis where tracks can be made in a single user selected channel, and tracks are analyzed.	3.3
	Find track	Ctrl + F	Find a specific track in the analysis.	3.4

Table 1. Overview of all menu options in TransTrack.

3.1 How to open and navigate through a movie

Type of files recognized by TransTrack

TransTrack only recognizes .tif files. Furthermore, TransTrack takes each .tif file it encounters as a separate position. Finally, each .tif file should be organized in the following order: frame – channel. For example, a 2-frame and 2-channel movie should be organized as follows: frame 1 - channel 1, frame 1 - channel 2, frame 2 - channel 1, frame 2 - channel 2.

How to open a movie

A new movie is opened in TransTrack by selecting *File/Open* (shortcut: Ctrl + O). This will prompt a popup-menu where the user is asked to give the following information:

- number of channels: give the amount of channels in the movie.

- first frame: first frame in movie from which the user wants to start (if not given, TransTrack will open the movie from the first frame).
- last frame: last frame in movie until the user wants to open (if not given, TransTrack will open the movie until the last frame).

The first input is required to open the movie, the latter two inputs are not required and can be given by the user if he/she doesn't want to open the whole movie but only a part.

Next, a new dialog window is opened where the user can select the folder that contains the actual movie he/she wants to open (select the folder that contains the .tif files). After this, TransTrack will start opening the movie while updating on its progress through a progress bar. Note that all other functions in TransTrack require an image to be opened in TransTrack. Therefore, the other functions will not work as long as there is not an image opened.

Navigating through a movie

Once TransTrack has opened the movie, the first image of the first position will be displayed in TransTrack's main window. Furthermore, a number of sliding bars appear that allow the user to navigate through the movie. Here, the function of these sliders will shortly be described:

- Position slider. With this slider the user can scroll through the different positions. The position can also be controlled by listing a position in the box next to it.
- Frame slider. With this slider the user can scroll through the different frames. The frame can also be controlled by listing a frame in the box next to it.
- Zoom slider. With this slider the user can zoom into the image. The zoom can also be controlled by listing the amount of zoom in the box next to it. A 'zoom factor' of 1 means that the entire image will be visible. The maximum 'zoom factor' can be 10.
- x and y sliders. The x and y sliders are located on the left and lower edge of the image, respectively. With the two sliders the user can change the center of the image left-right (with x slider) and up-down (with y slider). This is useful when zooming in, since the whole image will not be visible anymore once zoomed in.
- Reset button. Small button with letter *R*, located in the lower left-right corner of the image. This button automatically centers the image (this re-sets the x and y slider to the middle).

Opening new movies

At any point a new movie can be opened in TransTrack by clicking on the *Open* option in the *File* menu. This will delete the previous movie along with any analysis done with this movie.

3.2 How to set image display settings

There are two different options to set the display settings for visualizing the image in TransTrack. First, brightness and contrast can be set by selecting *Image/Display Settings* (shortcut: Ctrl + C) and the color and visibility of the channels can be set by selecting *Image/Channels* (shortcut: Ctrl + Z). Here both will shortly be described.

Setting the brightness and contrast

After selecting *Image/Display Settings*, a new window will appear. Here, the brightness of each channel (select the channel through the sliding bar or the edit box at the top of the window) can be tweaked by changing the minimum, maximum, brightness, and contrast values. Furthermore, a histogram of the intensities in the selected channel is displayed as a visual aid to choose the most optimal settings.

Setting the channel colors and visibility

After clicking on *Image/Channels*, a new window will appear. Here, a display color can be chosen for each channel by clicking on the colored box next to the channel that the user wants to change. Also, the user can toggle on/off the visibility of a specific channel by checking/unchecking the checkbox.

4.1 Measure intensities in movie

The measure function enables the user to quantify the intensities in all channels at a specific user-defined location in the image. The measure function can be started by selecting *Analysis/Measure* (shortcut: Ctrl + M). Next, the user has to select a point inside the image in TransTrack's main window. TransTrack will determine the following intensities at the location given by the user:

- *Peak intensity*. This is the intensity at the selected location.
- *Mean intensity*. This is the mean intensity at the selected location. This is calculated by taking the mean intensity of a box drawn around the given location (the box size used is the same as in further analyses, see section 5.1). Note: the mean intensity is NOT background subtracted!
- *Total intensity*. This is the sum intensity of all pixels in the current frame that is displayed.

All three intensities are determined for each channel present in the image. The data will be stored in a table to which any new measurement is added. When the table is closed all measurements are deleted, and a new table (without any previous measurements) will be opened when a new measurement is performed.

4.2 How to perform photobleach correction of movie

TransTrack can perform a photobleach correction in case this is desired by the user. When a photobleach correction is performed, the image displayed in TransTrack's main window will be updated accordingly and any intensity measurements in downstream analyses will be performed on the photobleach corrected image. The user can always check if a photobleach correction is applied to a channel by clicking on *Analysis/Photobleach correction/Status*. A dialog box will appear, listing for each channel if a photobleach correction has been applied.

To apply or remove the photobleach correction, the user can select *Analysis/Photobleach correction/Correction*. Per channel, the user will be asked to apply a photobleach correction (in case no correction has been applied yet for this channel) or to remove the photobleach correction (in case a correction has already been applied for this channel). It is possible to use a photobleach correction for only a subset of the channels.

Photobleach correction in TransTrack is done as follows: (1) the mean intensity of all pixels for each frame is measured (this is done per position) and (2) a decay curve is fitted to each mean intensity time trace. The following function is used to fit the mean intensity time traces:

$$\text{Mean Intensity}(t) = k_1 e^{-k_2 t} + k_3 \quad \text{Equation 1.}$$

Next, the mean intensity time traces and resulting fits are plotted and shown to the user, who can decide (based on visual inspection) to allow the photobleach correction or to reject it. When the user decides to allow the photobleach correction, all images are corrected per position according to their corresponding fit. Note that each position is photobleach corrected with its own fitted curve, and not with an average of all positions!

4.3 Single channel tracking analysis

The single channel tracking analysis enables the user to track spots in a single channel. To start the single channel tracking analysis the user has to select the *Analysis/Single channel tracking* (shortcut: Ctrl + A). This will open a new window containing all the steps of the analysis. The analysis consists of four steps that are in short:

1. User selects what to analyze (channel, which positions, ROIs per position).
2. Select a threshold to find spots (local maxima) and link them together to make tracks.
3. Select tracks in a manual or automated (based on different criteria; for example track length) fashion.
4. Optional: manually curate tracks.

During all the steps, the user can set a number of parameters that influence the analysis. Below an extensive description of all the steps and parameters involved is given. Note, that when the analysis is started, all parameters have initial values that could serve as a starting point of an analysis.

Step 1 | Select channel, positions and region of interests (ROIs)

Before starting any analysis the user has to choose what to analyze. There are three things to select: (1) the channel used for analysis, (2) the positions that you want to analyze, and (3) ROIs at each position to exclude parts of the image for analysis. While the ROI selection is optional, the analysis channel and the positions used for analysis have to be selected to proceed further with the analysis.

Choose analysis channel

The user has to select the channel that is used for the analysis (in which channel it has to find spots and make tracks). This selection is made in the top-left popup menu which says *Choose Channel*. It is obligatory to select a channel for the analysis to proceed further.

Choose positions to analyze

The positions that the user wants to analyze have to be listed in the *Positions* box. This can be done by typing in the numbers of the positions of interest (separate positions with an enter), or by typing “all” in case you want to analyze all positions. Note, for every analysis it is necessary to give a list of positions, it is not possible to proceed without giving any positions!

Select ROIs

It is possible to only analyze a part of each position by applying one or multiple ROIs to a position. An ROI that you select is only used for that particular position, and it is also possible to use ROIs for some positions, while analyzing the whole image for other positions. An ROI is added to the current position by clicking on the *Add ROI* button. This allows the user to draw an enclosed area in the image, which will be added to the ROI list for that position. There are two options to remove ROIs again. First, a single ROI can be deleted by clicking on the *Delete ROI* button. This allows the user to select an ROI that will be removed (click inside the ROI to remove it). The second option removes all the ROIs at the positions given by the user by clicking on the *Clear ROI* button. The positions where ROIs have to be cleared are given in the *Positions* box.

To use the ROIs during the downstream analysis, the user has to select the tick box called *Use ROIs*. In this case, TransTrack will take for each position the ROI(s) that were selected (if no ROI was selected at a position, it will analyze the whole image), and only tracks where the entire track remains within an ROI are taken along.

Step 2 | Find spots and make tracks

This step is performed when the user clicks on the *Track* button, and involves 3 sub-steps. All steps are shortly described below. Furthermore, the user can tweak different parameters during these steps and the use of each parameter is explained.

1. Filtering of the image (low and high bandpass filter).

During this step the image is 'cleaned' by using a low and high bandpass filter. The following parameters can be tweaked for the filtering step:

- *Noise size*: the pixel size of noise (this will filter out all small structures up to this size).
- *Object size*: slightly larger than the pixel size of the object of interest (the filter will filter out large structures).
- *Noise level*: use as a background subtraction.

2. Find spots (local maxima) in the filtered image (pixel higher than every other neighbor).

Spots in the filtered image are determined based on (1) the pixel intensity has to be higher than the threshold and (2) it has to be a local maximum (pixels surrounding it have to have a lower pixel intensity). The following parameter can be tweaked during this step:

- *Threshold*: threshold value to find a local maximum (in other words: pixel intensity has to be higher).

3. Link the spots together to make tracks.

During the final step, the spots found in the filtered image are linked together to form tracks. To find the optimal configuration of linked spots, the Hungarian algorithm is used. The following parameters can be tweaked during this step:

- *Tracking distance*: this sets a maximum to the distance a spot can move from one frame to the next. So, two spots in subsequent frames that are separated by a greater distance than this value, can never be combined in one track.
- *Frame skipping*: Once all the tracks are formed, the algorithm has the possibility to connect two tracks that are not connected in time, but would be in space (the distance between the last frame of the first track and the first frame of the second track is smaller than the *Tracking distance*). So, this gives the possibility to make one track, if several frames are missing. Here, the user can define the amount of frames between two tracks, where it is still possible to connect the two tracks.

Tip: Typical values for *Noise size* and *Object size* are 1 and between 6-8, respectively.

Tip: Usually, the values for *Noise level* and *Threshold* are kept the same. To find an optimal value, try one position and lower the *Noise level* and *Threshold* until you are satisfied with the result (click the *Track* button every time you lower the values to see how it affects spot detection).

Tip: Values for *Tracking distance* and *Frame Skipping* are very dependent on the type of imaging data (for example: how much do spots move from one time frame to the other, how much spots are present in the image, etc.).

Step 3 | Track selection

During the third step the user can select the tracks of interest from all the tracks that were made in the previous step. There are two ways of selecting tracks: (1) manual selection and (2) automatic selection. Manual selection of tracks is done by clicking on *Add Track*. Next, the user has to select tracks by clicking (left mouse click!) on the desired tracks in the image. Multiple tracks can be added at once by clicking on multiple tracks, and the user has to press the enter button to stop track selection (this can also be done before clicking on any track if the user accidentally clicked on *Add Track*).

The user can delete a track that was added by clicking on *Delete*. Next, the user has to select the track that needs to be deleted by clicking (left mouse click!) on this track. In case the user accidentally clicked on *Delete*, the user can exit by clicking with a right mouse click. Next to this manual selection of tracks, the user can also choose for an automatic selection by clicking on *Make Tracks*. Initializing this option will remove all current selected tracks at the positions listed in the *Positions* box and select tracks based on the criteria given at those same positions. Note that it is possible to combine an automatic and manual selection, however the user should always start with the automatic selection as all manual selected tracks will be removed once automatic selection is started. Furthermore, the user can also delete tracks that were selected through the automatic track selection by clicking on *Delete*.

The user has a number of criteria he/she can set for the automatic track selection. The four basic criteria are:

- *Start*: the starting frame of analysis, if ticked on all frames before the starting frame will not be analyzed.
- *End*: the last frame of analysis, if ticked on all frames after the end frame will not be analyzed.
- *Overlap*: this option is only used if either *Start* or *End* or both *Start* and *End* are ticked on. In case *Overlap* is ticked on, all tracks that are partially within the analysis boundaries (as given by the user through *Start* and/or *End*) will be taken along and will be clipped according to the *Start* and/or *End* settings. In case *Overlap* is not ticked on, only the tracks that are completely within the analysis boundaries will be taken along.
- *Minimum Track Length*: the minimum length of a track, if ticked on all tracks that are shorter than this length will be removed.

Next to these four basic criteria there are two more advanced criteria which are: (1) co-localization of a spot (local maximum) in another channel and (2) segmentation of image based on another channel. Both these criteria will be described below.

The user can select tracks based on co-localization with a spot (local maximum) in another channel. If the user ticks on the *Colocalization* option, only tracks that have a co-localization spot (local maximum) in another channel for a certain amount of time will be taken along. The user has to define the following parameters for TransTrack to determine for each track if there is a colocalization spot:

- *Channel*: choose a channel in which TransTrack has to search for a colocalization spot (this has to be a different channel than the analysis channel).
- *Threshold*: the threshold value for finding spots (local maxima) in the channel selected by the user. For each track, TransTrack will only search in a small area around the track for spots in the 'colocalization channel'. Next, only if a spot is found within 3 pixels of the center of the track it will call that a colocalization spot was present in that frame. This is done for each frame of the track, and in each frame it is indicated if a colocalization spot was present.
- *Length*: the maximum length of the colocalization signal. Based on the threshold set above, TransTrack will determine for each frame if there is a colocalization spot present. Next, it will

determine the maximum number of consecutive frames where a colocalization spot was present during the track. If this number is smaller than the value given by the user (in the *Length* edit box), it will not be regarded as a colocalization event.

In the second advanced criteria, the user can select based on the segmentation of the image performed in another channel than the analysis channel. The segmentation of the image is a simple one, where the image is divided in a high and low intensity region based on a channel given by the user. For example, in case of a DAPI staining, the user can segment the image in a DAPI high (the nuclei) and a DAPI low (the cytoplasm) region. Next, if the user ticks on the *subROIs* option only tracks that are either in the high or in the low region will be taken along. The user has to define the following parameters:

- *Channel*: choose a channel in which TransTrack has to perform the segmentation of the image.
- *Region*: choose if tracks in the high or low intensity region should be taken along in the analysis.
- *Threshold*: the threshold value for the segmentation. All pixels with a value lower than the threshold will be put in the intensity low region and all pixels with a value higher than the threshold will be put in the intensity high region. After thresholding a filter is applied to smoothen the segmented image.

Step 4 | Track repair

In the final step the user has the option to manually curate tracks that are incorrect. Note that this is an optional step that gives the user the opportunity to improve track quality if this is necessary. Furthermore, this step can also be performed while selecting tracks. For example, the user can select a new track through *Add Track*, inspect if the track is correct and repair it if necessary, and continue with selecting a new track. Only take care that in the case of automatic track selection, all selected tracks will be removed and new ones will be selected and this will also remove all track repairs done up to that point. Therefore, in case of automatic track selection, track repair can only be done after the automatic selection.

There are four different ways of repairing a track that will be described below.

1. Replace the location of a single frame.

With the replace option the user can change the location of a track in the current frame that is displayed. This option is started by entering the track number of the track that needs to be replaced (it will find the track in the current frame that is displayed) in the edit box (named #) and by clicking on *Replace*. Next, the user has to select the new coordinates of the track manually by clicking (left-mouse click) in the image at the correct location. In case the user accidentally clicked on *Replace*, the user can exit with a right-mouse click.

2. Extend a track.

With the extend option the user can extend a track manually either at the start of the track or at the end of the track. To select a track, a track number has to be entered (in the same edit box as for the replace option). For extending the start of the track, the user has to click on the *B* button and for extending the end of the track the user has to click on the *F* button. Next, the frame either before the first frame (in case of clicking on the *B* button) or the frame after the last frame (in case of clicking on the *F* button) will be displayed and the user has to select the location of the spot by clicking with a left-mouse click. The user can extend the track as far as he/she wants by continuously clicking with a left-mouse click at the location of the spot in subsequent frames. The user can stop extension by clicking with a right-mouse click. Furthermore, extension will automatically stop once the first or last frame has been reached.

3. Clip a track.

With the clip option the user can trim a track either at the start or at the end or at both sides at the same time. The user needs to enter a track number (in the edit box named *#*) and give a lower bound (in the edit box named *lb*) and upper bound (in the edit box named *ub*) that will be used to trim the track. If no lower bound is given, no trimming will occur at the start of the track. Similarly, if no upper bound is given, no trimming will occur at the end of the track. Finally, the trimming of the track is performed by clicking on the *Clip* button.

4. Merge two tracks.

With the merge option the user can merge two tracks together. Track numbers of both tracks have to be given in the edit boxes (named *#*). It is important that the track which will form the first part of the merged track is listed as first (in the edit box named *1.*) and the track which will form the second part of the merged track is listed as second (in the edit box named *2.*). So, it is NOT important if the first track has a higher or lower track number than the second track. Furthermore, (optionally) the user can give limits for which part of the tracks have to be taken in the merged track. When the user selects a limit for the first track, TransTrack will automatically take the first track from the first frame until this limit. When the user selects a limit for the second track, TransTrack will automatically take the second track from the limit until the last frame of the second track. Therefore, the limit of the first track cannot be higher than the limit of the second track. If no limits are given by the user, TransTrack will take the entire two tracks.

Merging of two tracks is done by clicking on *Merge*. Next, TransTrack will merge the two tracks. However, often either the two tracks don't connect perfectly which results in an information gap or the two tracks overlap which results in conflicting information. Both of these problems have to be manually resolved. In the case where the two tracks don't connect perfectly, the user has to manually select the location of the track in the frames with missing information after clicking *Merge*. The displayed image will automatically go to the first frame with missing information and ask the user to click at the correct location. This will continue until all frames with missing information are filled. In the case where the two tracks overlap, the user has to manually select the location of the track in the frames where both tracks are present after clicking *Merge*. Again, the displayed image will automatically go to the first frame with conflicting information and ask the user to click at the correct location for all frames with conflicting information. When all frames with missing or conflicting information have been resolved, the two tracks will be merged and given the track number of the first track.

Visualization of the analysis

Different steps during the analysis can be visualized. In the visualization panel (top left) the user can switch on/off the visualization of these steps. The following steps can be visualized:

- ROIs: display the ROIs that are selected by the user at the current position (shows blue outline of the ROI).
- Peaks: display the spots found during step 2 (shows magenta circles centered on the spot).
- Track paths: display the tracks made during step 2 (shows blue lines that follow the track path).
- Selected tracks: display the tracks that were selected during step 3 (shows green circles centered on the spot; will overlay the magenta circles if both are selected at the same time).
- Tracknumber: display the track numbers of the selected tracks during step 3.

Clear and close analysis

If a user wants to re-start an analysis or delete an analysis, he/she can either click on the *Clear* button (located in the top) or close the analysis. When the user selects the *Clear* button, only the positions listed in the *Positions* box will be cleared. When the user closes the analysis, all analysis information will be deleted (including ROIs and analysis settings).

4.4 Find back a track from the analysis

To find a specific track that was selected in the analysis (during step 3 of the analysis, see section 4.3), the user can select *Analysis/Find track* (shortcut: Ctrl + F). This will prompt a popup-menu asking the user:

- position: the position of the track of interest.
- track number: the number of the track of interest.

If the track exists, TransTrack will automatically go to the first frame of the track, and a magenta colored circle will be drawn around the track of interest.

5.1 Save ROIs and results from the analysis

Both ROIs that were made as well as results from the single channel tracking analysis (see section 4.3) can be saved. Both procedures will be described below.

Save ROIs

To save ROIs that were made during the analysis, the user has to select *File/Save/ROIs*. A popup-menu will appear asking the user a location for saving and a name for the file. The final output file is a .mat file (which can be opened in MATLAB) and contains information about the outlines of all ROIs at the different positions. This file can also be loaded back into TransTrack if requested by the user (see section 5.2).

Save Track data

To save the results from the single channel tracking analysis (see section 4.3), the user has to select *File/Save/Track data* (shortcut: Ctrl + S). This will open a new window, which enables the user to select the exact output that the user wants to extract from the analysis. When the user clicks on the *Save* button (located at the bottom right of the window), a popup-menu will appear asking the user a location for saving and a name for the file. The final output is a .xlsx file and always contains a tab with the metadata used during the analysis and a tab with the general track information. It is important that these two tabs are never modified (contents as well as the title of the tabs). Otherwise, it is not possible anymore to load track data back into TransTrack (see section 5.2).

1. Overview of metadata tab

The metadata tab in the .xlsx file contains the following information:

- Row 1: shows if a photobleach correction (see section 4.2) was applied to the different channels when saving the analysis (1 = yes and 0 = no).
- Rows 2-5: general info about the movie that was analyzed (number of positions, channels, frames, and dimensions).
- Row 6: which type of analysis was performed (1 = single channel tracking analysis)
- Rows 7-28: settings that were used during the analysis. Corresponds to settings in the analysis window. For all filters, 1 = checkbox was on and 0 = checkbox was off. Row 26: subROI selection reports if tracks in high intensity region (=1) or low intensity region (=2) were taken along (see section 4.3).
- Rows 29-34: settings that were used for saving. Corresponds to settings in the save window.

2. Overview of general track info tab

The general track info tab in the .xlsx file contains the following information:

- Column 1: shows from which position track originates.
- Column 2: shows track number (at each position numbering starts again at 1).
- Column 3-5: x, y, and time coordinates of track.

- Column 6-end: intensities at track coordinates for all different channels. Given are an intensity trace, a background intensity trace (local background of the track), and a background subtracted trace. See for detailed description how these intensities are obtained in *Intensities* section below.

Next to the metadata and general track info tab, there are several options that analyze the tracks in more detail. The following options can be selected: (1) *Intensities*, (2) *Total # spots*, (3) *Spot appearances*, and (4) *Colocalization*. These options are selected with the respective checkboxes in the *Select files to save* panel.

Intensities

The *Intensities* option provides a tab in the .xlsx file with all background subtracted intensity traces from one channel (each channel gets an own tab). The traces are ordered by position number and track number. The background subtracted intensities are obtained as follows: first, an intensity trace is made for each track by taking the average pixel intensity of a box drawn around the center of the track. The size of the box can be given by the user at the *Box size* edit box in the *Save settings* panel. Next, a background intensity trace is made for each track by taking the average pixel intensity of boxes (with the same *Box size*) drawn around the central box. Here, only boxes are taken along that have a homogeneous intensity profile (the maximum/minimum pixel value ratio in a box has to be smaller than 4) to exclude boxes that also contain some signal. Finally, the background intensity is subtracted from the intensity trace to get a background subtracted intensity trace. In the intensity tabs for the different channels only the background subtracted intensity trace is listed. The intensity trace and background intensity trace can be found in the general track information.

Total # spots, spot appearances, and colocalization

The *Total # spots* option will provide a tab in the .xlsx file with the amount of tracks present in each frame of the movie. The *Spot appearances* option will provide a tab in the .xlsx file with the amount of tracks that appeared in each frame of the movie (every track is now only counted once, namely when it appears). The *Colocalization* option will provide a tab in the .xlsx file with information about the number of tracks that have a colocalization spot in another channel for a certain amount of time (specified by user). To determine the number of tracks that have a colocalization spot, the user has to select a number of inputs (which are located in the *Save settings* panel at the *Colocalization* option):

- *Choose channel*: select the channel that TransTrack has to use to look for colocalization spots.
- *Threshold*: the threshold value for finding spots (local maxima) in the channel selected by the user. For each track, TransTrack will only search in a small area around the track for spots in the 'colocalization channel'. Next, only if a spot is found within 3 pixels of the center of the track it will call that a colocalization spot was present in that frame. This is done for each frame of the track, and in each frame it is indicated if a colocalization spot was present.
- *Length*: the minimum length of the colocalization signal. The length of the colocalization signal is determined by taking the maximum number of consecutive frames where a colocalization spot was found during the track.

The results of the colocalization can also be visualized in the image by selecting the *View colocalization* checkbox. For each track, a green dot (the spot has colocalization with the current settings) or a red dot (the spot has no colocalization with the current settings) will be placed in the center of each track. This visualization can also be used as an aid to find the best settings.

For all three options (*Total # spots*, *Spot appearances*, and *Colocalization*) it is required to select which parts of the image the user wants to use to obtain the data. There are two different options: (1) the user wants to determine this for the entire image or (2) the user wants to determine this per ROI (basically:

each ROI is treated as a separate position). If any of the three options (*Total # spots*, *Spot appearances*, and *Colocalization*) is selected, it is therefore necessary that the user selects one of the two options (whole image or per ROI) or both. If the user wants to have the information for the entire image, the user has to select the *FOV* checkbox located at the top of the *Save settings* panel. If the user wants to have the information per ROI, the user has to select the *ROIs* checkbox located at the top of the *Save settings* panel. **IMPORTANT:** at least one of the two has to be selected to proceed with saving the data.

Finally, there is an option to split the information (*Total # spots*, *Spot appearances*, and *Colocalization*) in the whole image or per ROI even further. This can be done by selecting the *subROIs* checkbox located at the top of the *Save settings* panel. When the user selects the *subROIs* checkbox, the image will be segmented in a high and low intensity region based on the settings selected by the user. The user has to select the following settings in the *Save settings* panel at the *subROIs* option:

- *Channel*: choose a channel in which TransTrack has to perform the segmentation of the image.
- *Threshold*: the threshold value for the segmentation. All pixels with a value lower than the threshold will be put in the intensity low region and all pixels with a value higher than the threshold will be put in the intensity high region. After thresholding a filter is applied to smoothen the segmented image.

The resulting segmentation with the settings selected by the user can be visualized by selecting the *View subROIs* checkbox. In this way the user can choose a good threshold value based on visual inspection.

With the segmentation performed, it is now possible to split the information (*Total # spots*, *Spot appearances*, and *Colocalization*) even further. In table 2 an example is given how the total number of spots can be quantified in different (sub)regions of the image (whole image, per ROI, high/low intensity region). The same holds true for the spot appearances and number of tracks having a colocalization spot. Table 2 also gives the checkboxes that need to be selected to get the specific tabs and also the final tab names in the .xlsx file.

Tab names	Checkboxes selected	Description of tab
FOV – All	<i>FOV</i>	The total number of spots in each frame for the whole image.
FOV – High	<i>FOV, subROIs</i>	The total number of spots in each frame in the high intensity region of the whole image.
FOV – Low	<i>FOV, subROIs</i>	The total number of spots in each frame in the low intensity region of the whole image.
ROI – All	<i>ROIs</i>	The total number of spots in each frame in each ROI.
ROI – High	<i>ROIs, subROIs</i>	The total number of spots in each frame in the high intensity region of each ROI.
ROI – Low	<i>ROIs, subROIs</i>	The total number of spots in each frame in the low intensity region of each ROI.

Table 2: Example of output tabs in the .xlsx file for the total number of spots. Similar output tabs can be generated for the spot appearances and colocalization. Also the checkboxes that need to be selected to generate the tabs are given.

5.2 Load data from a previous analysis

It is possible to load a previously saved TransTrack analysis session to continue with the analysis or inspect the tracks more closely. Furthermore, it is also possible to load an ROI file to re-use ROIs made and saved previously. Both options will shortly be explained below.

Load ROIs

A saved ROI file (saved with TransTrack as described in section 5.1) can be loaded into TransTrack by clicking on *File/Load/ROIs*. This will prompt a popup-menu asking the user for the ROI file that needs to be loaded. After selection of the file, the ROIs are loaded into the movie and can be used during further analyses. Note that the ROI file that is loaded should be from a movie with the same number of positions, otherwise it will not be able to load the file. Also, the order of the positions should be the same as at the time the ROI file was saved.

Load track data

A saved analysis file (saved with TransTrack as described in section 5.1) can be loaded into TransTrack by clicking on *File/Load/Track data* (shortcut: Ctrl + L). A popup-menu will appear where the user has to select the file that needs to be loaded. Loading an analysis file will close any active analysis at this moment, and load the data from the saved file instead. Furthermore, a saved file that is used needs to fulfill the following criteria:

- It contains a tab called 'Metadata' and 'General Track info'. These names need to be exact!
- The metadata file should contain the correct amount of entries (never change the metadata from the saved output!).

When all requirements are met, the track data will be loaded and a new analysis window will be started.

6.1 References

The organization and display of images in TransTrack is inspired by FIJI to make it easier for users familiar with FIJI to use TransTrack. The following functions were downloaded from Mathworks file exchange and are implemented in the TransTrack code:

- progressbar.m: shows the progress of a process taking place.

Written by: Steve Hoelzer

URL: <https://nl.mathworks.com/matlabcentral/fileexchange/6922-progressbar>

version: 1.2.0.0

- bpass.m and pkfnd.m: performs a bandpass filter to clean image (bpass.m) and finds local maxima in cleaved image (pkfnd.m).

Written by: saurosaum

URL: <https://nl.mathworks.com/matlabcentral/fileexchange/15765-sptrack1-0>

version: 1.0.0.0

- simpletracker.m (and functions used: hungarianlinker.m, munkres.m, nearestneighborlinker.m, TestSimpleTracker.m): makes tracks of spots by linking them using the Hungarian algorithm.

Written by: Jean-Yves Tinevez

URL: <https://nl.mathworks.com/matlabcentral/fileexchange/34040-simple-tracker>

version: 1.5.0.0

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Appendix I Required files for TransTrack

Table 3 shows all files that are required for TransTrack to run correctly. Part of the files are GUIDE objects and have therefore both a .m as well as a .fig file.

Guides (both .fig as well as .m file)
TransTrack.m / TransTrack.fig
DisplaySettings.m / DisplaySettings.fig
Channels.m / Channels.fig
SingleChannelTracking.m / SingleChannelTracking.fig
Save.m / Save.fig
Functions (only .m file)
displayim.m
intensity_tracker_single_channel.m
make_tracks.m
track_finder.m
bpass.m
pkfnd.m
progressbar.m
simpletracker.m
hungarianlinker.m
munkres.m
nearestneighborlinker.m
TestSimpleTracker.m

Table 3: overview of all functions involved in TransTrack