Automated Multi-Track Kymography (AMTraK): User Guide

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A. Using the executables

The AmtraK algorithm is implemented in MATLAB (2014b) supplemented with the Image Processing Toolbox and Statistics Toolbox. The executables supplied in combination with the MATLAB Compiler Runtime (MCR) R2014b (distributed freely by MATLAB), allow the user to run the software as a stand-alone application, and no MATLAB installation is required. The user needs to install the MCR using the MCRInstaller file provided.

A.1 Windows

In addition to the AmtraK User Guide, two files have been provided:

- AmtraK.exe (Stand-alone application)
- MCRInstaller.exe (Setup launcher)

On a 64-bit Windows system without MATLAB, the user first needs to install MATLAB Compiler Runtime (version R2014b) using the MCRInstaller file.
Running the AmtraK.exe file displays a GUI as well as a command window. Please refer to Section C for instructions on operating the GUI.

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A.2 Linux

In addition to the AmtraK User Guide, three files have been provided:

- AmtraK (Stand-alone application)
- AmtraK.sh (Shell script)
- MCRInstaller (Setup launcher)

On a 64-bit Linux system without MATLAB, the user first needs to install MATLAB Compiler Runtime (version R2014b) using the MCRInstaller file.

Open the **Terminal** in the directory where the downloaded executables are stored.

Now to run the shell script, type **./run_AmtraK.sh** *space* **path**

where **path** is the location of the directory where the MCR is installed on the machine.

This displays the AmtraK GUI. Please refer to Section C for instructions on operating the GUI.

B. Using the source-code

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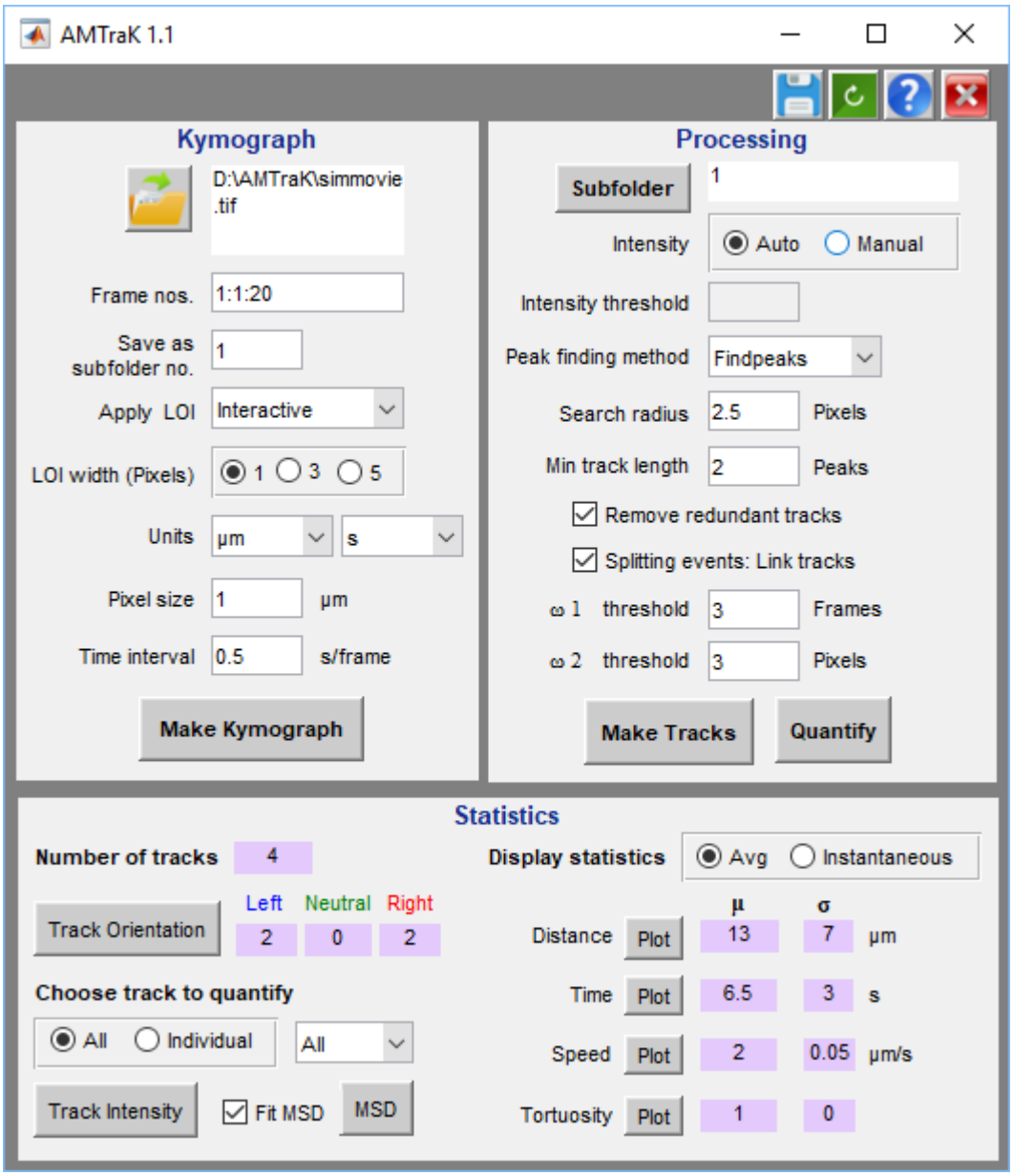
The AmtraK algorithm is implemented in MATLAB (R2014b) supplemented with the Image Processing Toolbox (ver. 7.3) and the Statistics Toolbox (ver. 7.3). The user needs these or more advanced versions of MATLAB and the toolboxes running on the machine. Invoke the 'AMTraK.m' file in the folder to run the software as a GUI (instructions in Section C). Note: Please close other figure tabs if open in MATLAB, before running AMTraK.

C. Graphical User Interface (GUI) operation



C.1. Constructing a kymograph

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Parameters

Open file  **:** Select the image time-series on which kymography is to be performed. Output subfolders will be stored in this parent directory.

The accepted format for the image time-series is given below:

Format	**Abbreviation**	**Extension**	**Accepted Bit-depth (bits)**

Tagged Image File Format	TIFF	.tif	8,12,16,24,32,36,48,64

Example- movie1_fl.tif

Frame nos.: It is the range of images in the time-series which are to be analysed. All the frames are selected by default. The number can be edited in the GUI to choose a subset of the series and/or skip frames in between by specifying a step size.

Notation- a: n: b

where a= Start image number

b= End image number

n= Step size

Example-

5:1:20 (All images between 5 and 20, skipping none in between)

1:2:20 (Images between 1 and 20, skipping alternate frames)

Save as subfolder no. : The output from AmtraK is stored in a subfolder with this index number (amtrak-#). This is especially useful in case the time-series is to be analysed using multiple Lines of Interest and the data from multiple subfolders is to be pooled.

Apply LOI: The kymograph is constructed based on a Line Of Interest (LOI), which can be selected in two ways:

1. **Interactive**

   On clicking the 'Make Kymograph' button, the user is prompted to interactively select a segmented Line of Interest on the maximum-intensity projection image of the image-series, with the help of a mouse. Use normal left clicks to add segments to the line. A shift-, right-, or double-click adds a final point and ends the selection. Pressing Return or Enter ends the line selection without adding a final point. Pressing Backspace or Delete removes the previously selected point from the LOI.

2. **From file**

   On clicking the 'Make Kymograph' button, the user is prompted to select a text file containing coordinates of Line of Interest which can be applied on the maximum-intensity projection image of the image-series. This is especially useful in case of multi-channel images where the same LOI needs to be applied. In this case, the user first needs to generate kymographs for one channel using interactive selection. The LOI coordinate-file is automatically in the subfolder as 'LOIselection.txt'. In order to apply this LOI onto another fluorescence channel, the user needs to first select the new time-series, adjust the subfolder number and relevant parameters and then click 'Make Kymograph'.

LOI width: The width of the LOI in pixels.

Distance and time units: The units in which displacement and time are to be quantified.

Pixel size: Dimension of each pixel for converting the image into previously selected distance units.

NOTE: Raw microscopy images have implicit pixel sizes in µm. If the user selects distance units other than µm to make the kymograph, he/she needs to ensure that the pixel size is adjusted accordingly.

Time interval: The time interval between two consecutive image-frames, in terms of units selected previously.

C.2 Processing a Kymograph

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Subfolder: Interactively choose the subfolder (s) to process, named specifically as 'amtrak-#' in the parent directory. In case of multi-subfolder selection, in the dialogue box, choose multiple folders > **Add** > **Done**.

Peak Detection Parameters

Intensity: The kymograph is segmented based on intensity or gray values of pixels in any of the following two ways:

1. Auto: Automated thresholding by Otsu's method (Otsu, 1979).
2. Manual: The user chooses to provide the segmentation threshold manually.

Intensity threshold: When the 'Intensity' parameter is on 'Manual' mode, the user needs to provide an intensity threshold (a fraction in the range 0-1) for segmenting the kymograph.

Peak detector: The user can select any of the three methods for detecting bright points in the kymograph, namely Findpeaks, Watershed and Canny edge detection.

Tracking Parameters

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Search radius: The threshold distance value (in pixels) for linking peaks to make a track.

Minimum track length: The minimum number of peaks that a track should contain in order to be considered for analysis. Default value = 2.

Remove redundant tracks: Spurious tracks generated during track-detection are eliminated. It is recommended to keep this option on while making tracks.

Splitting events (Optional): When checked, tracks within proximity will be linked depending on thresholds ω1 and ω2.

ω1 : A threshold in time (frames) to detect a splitting event.

ω2 : A threshold in space (pixels) to detect a splitting event.

Quantification

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This module produces both text-file outputs and plots of the dynamics estimated from the kymograph.

In the 'Statistics' panel, the user may choose to display statistics of 'All' tracks or a specific 'Individual' track picked from the drop-down menu.

Similarly, the flexible display allows the user choose between 'Average' and 'Instantaneous' values of motility parameters.

In case of the average statistics, the frequency distribution of Distance, Time, Speed and Tortuosity (i.e. directionality) are plotted if the button 'Plot' corresponding to these variables is pressed. Additionally, the mean (μ) and standard deviation (σ) of these variables are also generated in the text boxes.

Track Intensity: It plots the normalized (0-1) grey value intensity of each track as a function of the time.

Track orientation: It triggers a recolouring of the tracks in the kymograph based on the net direction of movement along the X-axis- blue (negative, left), red (positive, right) and green (stationary, neutral). The number of tracks following respective directions is displayed in the text boxes adjacent to the button.

MSD: Mean square displacement analysis of all detected tracks is performed. A window with the plot of the average MSD against increasing time-step is displayed. Ticking the 'Fit MSD' option *a priori* helps the user to extract the value of the average one-dimensional diffusion coefficient by fitting the entire or a fraction of the average MSD curve to the anomalous diffusion equation:

Mean square displacement (< r2 >) of particles was calculated as described in (Khetan and Athale, 2016) using the Cartesian coordinates (x,y) obtained from AMTraK, FluoreT and DICOT.

$$< r2\ (\delta t) > = [x\ (t + \delta t) - x\ (t)]^{2} + [(y\ (t + \delta t) - y\ (t)]^{2}$$

In the above equation, r is the displacement of the particle at two time-points separated by a time-step δt that ranges from the minimal time-step in experiment to 3/4th of the length of the trajectory.

Deff was calculated by fitting the average MSD profile (as a whole or its linear portion, based on user input) to the anomalous diffusion model as follows:

$<r^2> = 2Dt^{\alpha}$

Here, t is the time-step and α is the anomaly parameter that indicates the nature of diffusion. The motion is said to be purely diffusive if $\alpha = 1$, sub-diffusive or 'restricted' when $\alpha < 1$ and super-diffusive or 'transported-like' when $\alpha > 1$.

Batch Processing

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If multiple subfolders are selected for processing, the user is prompted to fill in the common parameters for processing each kymograph.

The batch-quantification output is stored in a subfolder named 'BatchPro' in the parent folder. The statistics can be displayed in the GUI panel.

C.3 Others

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Save Parameters  **:**

When pushed, the button allows input parameters to be saved in a text file in the given subfolder.

Reset  **:**

The parameters are set to blank/default.

D. Output Files

Outputs of the analysis are stored in multiple tab-delimited text files to enable reproducible analysis and are summarized in the table below:

Function	Sr. No.	Output file	Description
Make Kymograph	1	OutputKymo.txt	The kymograph matrix
	2	OutputKymo.tif	The kymograph image
	3	LOIselection.txt	(x,y) Coordinates of Line of Interest
	4	LOIselection.tif	LOI overlaid on projected image-stack
Detect Peaks	5	Peaks.tif	Peaks overlaid on the kymograph
	6	Brightcoords.txt	(x,y) Coordinates of peaks detected in the kymograph
Make Tracks	7	PlotContour.tif	Tracks overlaid on the kymograph
	8	Tracklist.txt	(x,y) Coordinates and intensity of tracks detected in the kymograph
	9	Branchpoints.txt	Intersecting tracks and their branch points

Quantify	10	OutputStats.txt	Summary of particle movement statistics
	11	USER_InstStats.txt	Instantaneous (stepwise) statistics of each track
	12	USER_TrackStats.txt	Averaged statistics of each track
Track Orientation	13	Track_Orientation.txt	Directions indicated against track numbers
	14	Track_Orientation.tif	Colour-coded tracks overlaid on kymograph, red=right, blue=left, green= neutral
Track Intensity	15	Track_Intensity.tif	Intensity profiles of each track
MSD	16	MSD_vs_Time.tif	Mean square displacement profiles
	17	AvgMSD_vs_Time.tif	Average MSD curve with fit
	18	ID_Time_MSD.txt	Trackwise MSD
Save Parameters	19	All_Parameters.txt	Record of kymograph making and processing parameters

Note: If the initial attempt at building and tracking the kymograph fails, it might have multiple causes. If image-noise is an issue, de-noising the image using a median filter in ImageJ can help. Alternatively, prominent image-drift can also lead to aberrations. We recommend using the ImageJ Stack Registration plugin to correct for translation.