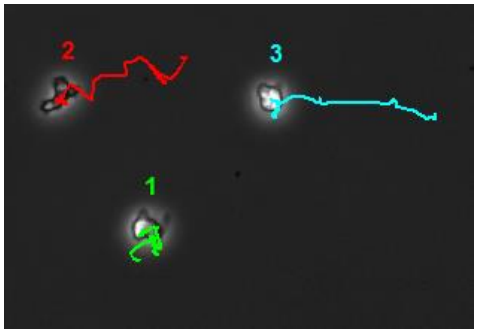
Dicty Tracking



a free, fast and easy-to-use standalone tool for
semi-automatic tracking of migrating *Dictyostelium discoideum* cells
from phase-contrast time-lapse image series
based on MATLAB®

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License

Quick Start Guide

Prior to start

- ⚠ **Critical** Check for fulfillment of the requirements for the usage of Dicty Tracking (page 4 f.)! ⚠
- ⚠ **Critical** Make sure, that `Dicty_Tracking.exe` and the image stack to analyze are saved in the same directory! ⚠

1. To start, double-click on `Dicty_Tracking.exe` and press *OK* in the appearing start window.
2. Select the image stack to analyze (e.g. `sample movie unconfined.tif`) and set cell detection parameters as described in subsection "Step 1: Cell detection" on page 5 ff.
3. Control cell detection by inspection of the generated image stack `sample movie unconfined_cell detection.tif` in Fiji/ImageJ. If cells were not detected properly, restart the program and adjust the parameters (see subsection "Step 2: Control of cell detection & selection of cells for tracking" on page 7 f.).
4. In the *Cell Selection Window*, select cells that should be tracked by clicking on them and press *Enter* when finished (see subsection "Step 2: Control of cell detection & selection of cells for tracking" on page 7 f.).
5. Enter time interval, time interval unit, pixel size and pixel size unit in the appearing window and click *OK*.
6. Check the generated image stack `sample movie unconfined_tracks.tif` for tracking errors using Fijj/ImageJ (see subsection "Step 4: Export of track data" on page 8 ff.).
7. Further analyze track data using the Excel workbooks `Dicty Tracking Evaluation.xlsm` and `MSD Calculation.xlsm` (see section "Evaluation of track data" on page 10 ff.)

1 Principle of Dicty Tracking

The tool detects cell bodies of migrating *Dictyostelium* cells from phase-contrast images using the Sobel operator implemented in MATLAB®'s Image Processing Toolbox as well as subsequent dilation and erosion steps. After quality checking of cell detection, the user selects cells that should be tracked and cells that should be excluded from tracking, e.g. colliding and dividing cells or cells that leave the field of view, via a graphical user interface. The Dicty Tracking algorithm tracks selected cells by connecting the centroids of a cell at each time point. Dicty Tracking generates a TIFF stack with differently colored cell tracks and exports position, instantaneous speed and various other parameters as an Excel sheet. Dicty Tracking comes with VBA-based Excel workbooks for further analysis of the motility data (see component list). The MATLAB® script as well as the VBA code is available at GitHub (https://github.com/ChristofLitschko/Dicty-Tracking/tree/v1.3).

2 Components of the Dicty Tracking package

The `Dicty-Tracking-1.3.zip` package contains the following files:

- `DictyTracking.exe`. A MATLAB® standalone application (see subsection "MATLAB® Runtime") performing cell detection, tracking and export of primary data to an Excel spreadsheet. It is important that the `Dicty_Tracking.exe` file and the image stack(s) that should be analyzed are saved in the same directory on the user's computer!
- `Dicty-Tracking-User-Guide.pdf`. The present user guide.
- `sample movie unconfined.tif`. A 8-bit, 46 frame sample image stack showing freely (unconfined) and randomly migrating Ax2 wild-type *Dictyostelium* cells at a time interval of 20s. The pixel size is 0.645 µm.
- `sample movie confined.tif`. A 8-bit, 46 frame sample image stack showing randomly migrating Ax2 wild-type *Dictyostelium* cells confined by an 0.17 mm thick, 1.5% agarose slice (agar-overlay technique) at a time interval of 20s. The pixel size is 0.645 µm.
  It is recommended to perform the steps described in the section "How to use Dicty Tracking" with at least one of the two sample movies to become familiar with the tool.
- `Dicty Tracking Evaluation.xlsm`. This VBA-based Excel workbook calculates mean speed and directionality ratio of all tracked cells of an image stack. Additionally, it shifts the trajectories of all tracked cells to the origin allowing easy generation of trajectory plots with Excel or standard graphing software (e.g. SigmaPlot).
- `MSD Calculation.xlsm`. This VBA-based Excel workbook calculates the mean squared displacement (MSD) of up to four different populations of migrating cells according to $MSD(t) = \langle \left(x_i(t) - x_i(t_0)\right)^2 + \left(y_i(t) - y_i(t_o)\right)^2 \rangle_{t,i}$, where angle brackets indicate averages over all times *t* and all cells *i*.
- `MTrackJ Evaluation.xlsm`. A VBA-based Excel workbook comprising the same features like `Dicty Tracking Evaluation.xlsm`. It can be used to calculate mean speed, directionality ratio and trajectories shifted to the origin from the *Points* output table of MTrackJ, a Fiji/ImageJ plugin for manual tracking (see https://imagescience.org/meijering/software/mtrackj/). This workbook is provided additionally and is not required for cell tracking and subsequent migration analysis using `Dicty Tracking.exe` and the two Excel workbooks described above. The workbook contains short user instructions and is not further described within this guide.
- Folders `demo-movies` and `source-code` as well as the files `LICENSE` and `README.md`. These files are not required for cell tracking as well as for subsequent migration analysis using the standalone tool `Dicty Tracking.exe` and associated Excel workbooks and can be deleted if desired. `demo-movies` comprises the two animated .gif files shown on GitHub. `source-code` comprises the MATLAB® script (`Dicty_tracking_v1_3.m`) as well as the VBA macro code of the Excel workbooks.

3 Requirements for the usage of Dicty Tracking

MATLAB® Runtime 2017a (9.2)

To use Dicty Tracking, a MATLAB® installation is not needed, as the tool is provided as a standalone application (for more information see https://mathworks.com/products/compiler.html). To run Dicty Tracking, it is necessary to download and install MATLAB® Runtime

2017a (9.2), which is a free set of libraries enabling the execution of compiled MATLAB applications on computers that do not have MATLAB installed. It is available at https://mathworks.com/products/compiler/mcr.html.

Fiji/ImageJ

ImageJ is necessary for the usage of Dicty Tracking as it allows the conversion of 12-bit camera images to 8- or 16-bit images. Additionally, the cell detection control step (see subsection "Step 2: Control of cell detection & selection of cells for tracking" on page 7 f.) as well as the final control of cell tracking (see subsection "Step 4: Export of track data" on page 8 ff.) are performed in ImageJ. Fiji, a flavor of ImageJ optimized for the life sciences, is available at https://imagej.net/Fiji.

Bit depth

Dicty Tracking requires full range 8-bit (0-255) or 16-bit (0-65535) greyscale TIFF stacks. Note, that microscope cameras often generate 12-bit (0-4095) images that are saved as 16-bit images! These images naturally do not cover the full 16-bit range and have to be converted either to full range 8-bit or to 16-bit images using Fiji/ImageJ prior to cell tracking with Dicty Tracking. To convert such a 12-bit image stack to a full range 8-bit stack, open it in Fiji/ImageJ and click *Image* → *Type* → *8-bit*. To convert it to 16-bit stack suitable for Dicty Tracking click *Image* → *Adjust* → *Bright-ness/Contrast* → *Apply*.

4 How to use Dicty Tracking

After installation of the MATLAB® Runtime, download of Fiji/ImageJ and, if necessary, conversion of the bit depth (see above) Dicty Tracking can be started. ⚠ Critical It is important that the `Dicty_Tracking.exe` file and the image stack(s) that should be analyzed are saved in the same directory on the user's computer! Otherwise Dicty Tracking won't work! ⚠

Start Dicty Tracking

To start Dicty Tracking, double-click on the `Dicty_Tracking.exe` file. A start window containing some basic information about the program will appear. Press the *OK* button to continue. Next, a window will open that allows the user to select an image stack for analysis. Select an image stack and click *Open* to continue.

Step 1: Cell detection

After selection of the image stack, a window for setting the cell detection parameters will appear (Figure 1). These seven parameters influence the accuracy of cell detection and, at least in part, have to be adjusted for each image stack.

- *Threshold for binarization*. This value defines a threshold for binarization (edge detection) by the Sobel algorithm and normally has a value between 0.12 and 0.17. A good starting point is 0.15 which is also suitable for the unconfined sample movie. Use 0.12 for the confined one.
- *1st dilation parameter*. This parameter defines the size of a line-shaped structuring element used for shape expansion (dilation) of detected objects within the generated binary image. A higher value will increase the size of objects. This parameter normally has a value between 3 and 6 for unconfined *Dictyostelium* cells and between 6 and 10 for cells confined by the agar-

overlay technique, respectively. Use 4 for the unconfined and 8 for the confined sample movie.

- *1st erosion parameter*. This parameter defines the size of a disk-shaped structuring element used for shape shrinkage (erosion) of objects within the generated binary image. A higher value will decrease the size of objects. This parameter normally has a value between 4 and 6 for unconfined cells and between 7 and 11 for confined cells, respectively. Use 5 for the unconfined and 9 for the confined sample movie.
- *Threshold for halo removal*. This parameter represents a grey scale intensity value used to exclude cell halos from detected shapes. All pixels of the input image with an intensity equal or higher than the threshold value are set to zero in the generated binary image. This parameter normally has a value between 150 and 220 for 8-bit images and between 38000 and 56000 for 16-bit images. Use 150 for the unconfined and 230 for the confined sample movie.
- *2nd dilation parameter* and *2nd erosion parameter*. After halo removal, the object shapes within the binary image are dilated and eroded again. These parameters define the sizes of the line- and disk-shaped structuring elements, respectively, used for these operations. See corresponding paragraphs above for the values of these parameters. For the unconfined sample movie, use 3 and 5, respectively, for the confined one 7 and 8, respectively.
- *Area threshold (px)*. This parameter defines an area size in pixels. Objects within the generated binary image that are equal or smaller than this value normally do not represent cells, but some dirt or debris on the glass surface, and they are removed from the image by setting the corresponding pixels to zero. It turned out that 150 is a good threshold size to remove most of these unspecific objects.

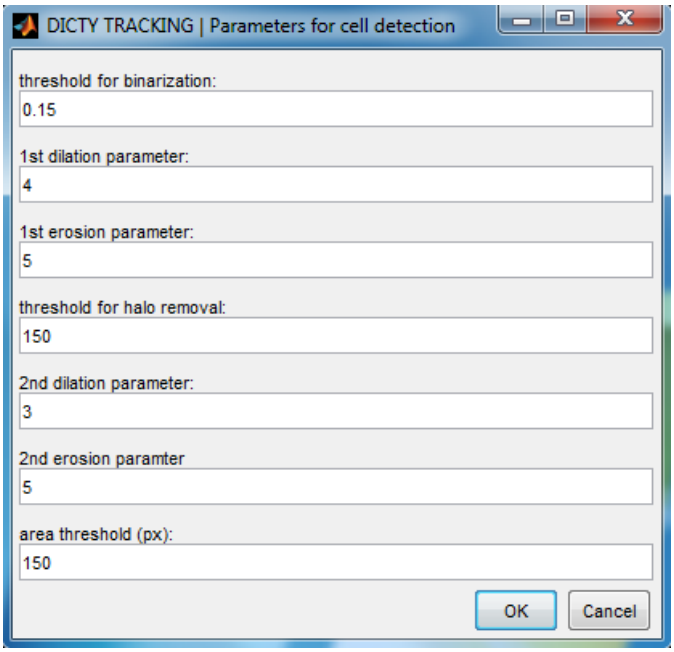


Figure 1 | Window for setting of the cell detection parameters. Entered values are suitable for `sample movie unconfined.tif`.

After entering all values, click *OK* and Dicty Tracking will start detecting cells within the selected image stack. A progress bar appears showing the current status. The cell detection parameters used are saved in the same directory as an excel file that is named for example `sample movie unconfined_params.xls` in case of the unconfined sample movie. The cell detection results are

saved as image stack termed `sample movie unconfined_cell detection.tif`.

Step 2: Control of cell detection & selection of cells for tracking

After finishing cell detection, a window containing instructions and a window named *Cell Selection Window* will open (Figure 3) allowing the user to select cells for tracking. ⚠ **Critical** However, it is necessary to check for proper cell detection before selecting cells for tracking! ⚠ Therefore open the `…_cell detection.tif` image stack with Fiji/ImageJ. Do not close the *Cell Selection Window* or the window containing instructions, as this will stop Dicty Tracking! The images of the `…_cell detection.tif` stack consist of the original input image (top) and the corresponding binary image showing the cell detection results (bottom, see Figure 2). Carefully check the whole stack for proper cell detection either through animating the stack or by using the scrollbar. Click on the *Start Animation* button (▸) to start the stack animation. The animation speed can be adjusted through right-clicking onto ▸. ⚠ **Critical** Especially when a cell is not detected in one or more frames this leads to severe errors in cell tracking. ⚠ If cells are detected properly (as shown in Figure 2), continue as described below. If cell detection is insufficient close and delete the `…_cell detection.tif` stack and also close the *Cell Selection Window* as well as the instruction window (this will stop the program). Start Dicty Tracking again as described above and perform cell detection with altered parameters.

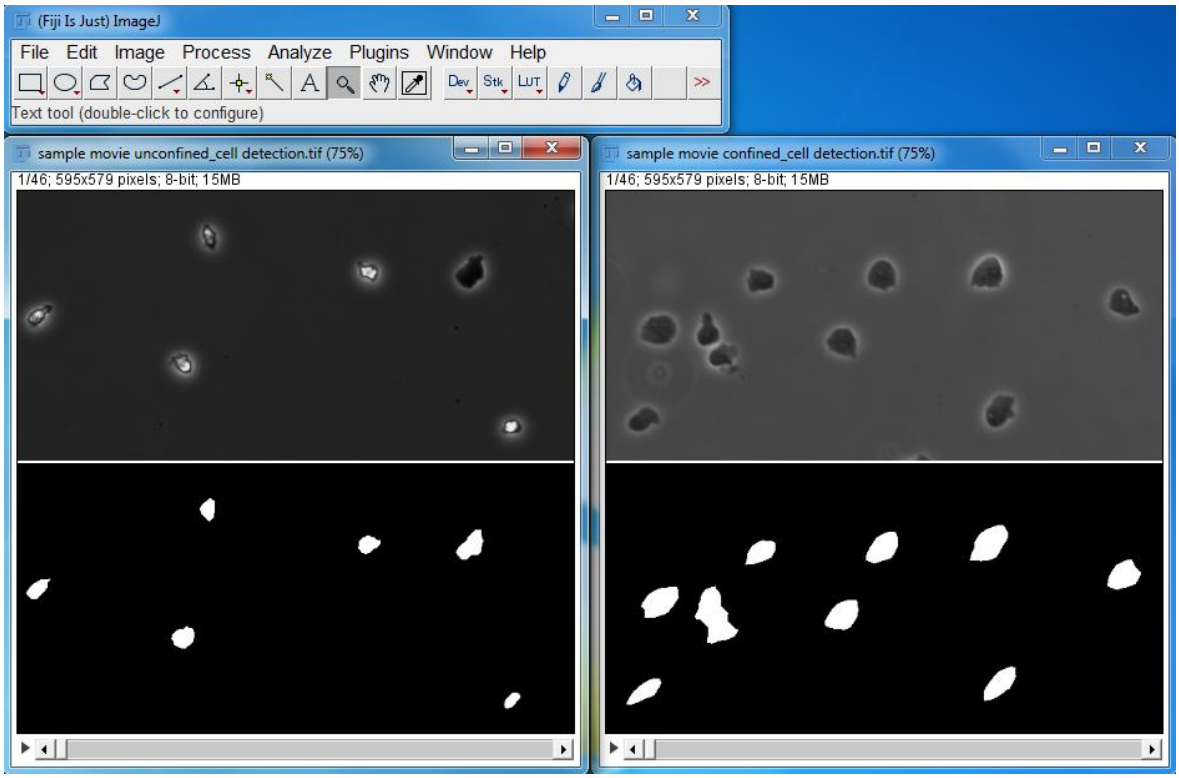


Figure 2 | Control of cell detection using Fiji/ImageJ. Frame 1 of corresponding `…_cell detection.tif` stacks for the unconfined (left) and confined (right) sample movie generated with parameters stated in subsection "Step 1: Cell detection" are shown. Each frame of a `…_cell detection.tif` stack consists of the original input image (top) and the binary image showing detected cells (bottom).

Click *OK* on the instruction window to begin with the selection of cells that should be tracked. This window just serves as a reminder of how to proceed after cell detection has been completed. Then click on all cells in the *Cell Selection Window* (Figure 3) that should be tracked. The *Cell Selection*

Window shows the first frame of the input stack. ⚠ Critical Do not select cells for tracking that divide, collide, leave or enter the field of view or neighboring cells that are recognized as a single object, as this will lead to severe tracking errors! ⚠ Scroll through the `…_cell detection.tif` stack opened with Fiji/ImageJ to identify cells that are not suitable for tracking. Press *Enter* when finished.

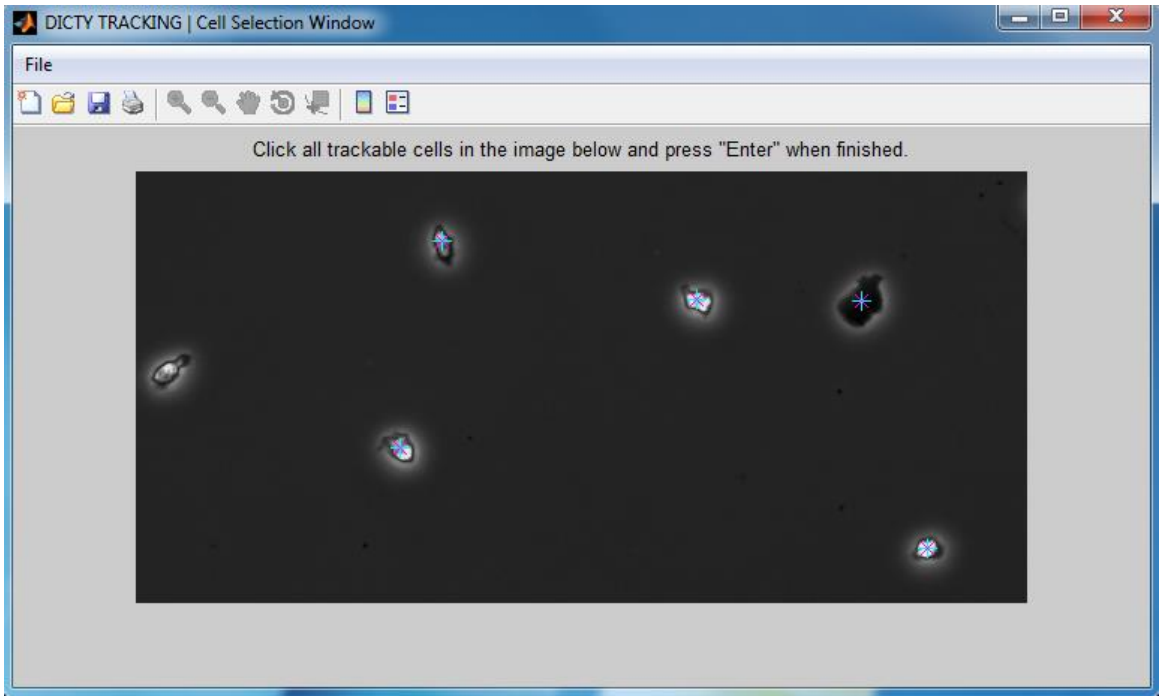


Figure 3 | The *Cell Selection Window* of Dicty Tracking. The *Cell Selection Window* shows the first frame of the input stack (here of `sample movie unconfined.tif`). Cells selected for tracking by mouse clicks are marked with an asterisk. In case of the sample movie all cells were selected except of the cell at the left image border which leaves the field of view.

Step 3: Generation of the trajectory stack

After selection of cells Dicty Tracking automatically tracks the selected cells and generates a TIFF stack named for example `sample movie unconfined_tracks.tif` showing the trajectories of the tracked cells in alternating colors (Figure 4). This stack is saved in the same directory as the input stack. Windows will automatically pop up and immediately close again during this process. The number of these pop up events is equal to the number of frames. A status bar shows the current progress of the process. No user input is required.

Step 4: Export of track data

The last step comprises the export of the track data consisting of

- the x and y position
- the distance to the previous point (D2pP, step size)
- the overall track length (Len)
- the direct distance to the starting point (distance to start, D2S)
- the instantaneous velocity (v)
- the instantaneous angle (angle between the displacement vector and the x-axis, inst. angle) given through $\alpha_t = \tan^{-1}\left(\frac{y(t)-y(t-1)}{x(t)-x(t-1)}\right)$

- the turning angle (difference of the instantaneous angles α_t and α_{t-1})
- the cosine of the turning angle (cos(turn. angle))

of each tracked cell (track) at each time point (frame). This data is saved to an Excel file named `…_track data.xls` (Figure 5). To calculate and export these data, Dicty Tracking asks the user to enter the time interval, the time unit (standardly set as 's'), the pixel size and the pixel size unit (standardly set as 'µm'). After finishing the export of the track data, Dicty Tracking asks the user to check the tracking results for potential errors by inspection of the `…_tracks.tif` stack in Fiji/ImageJ (Figure 4).

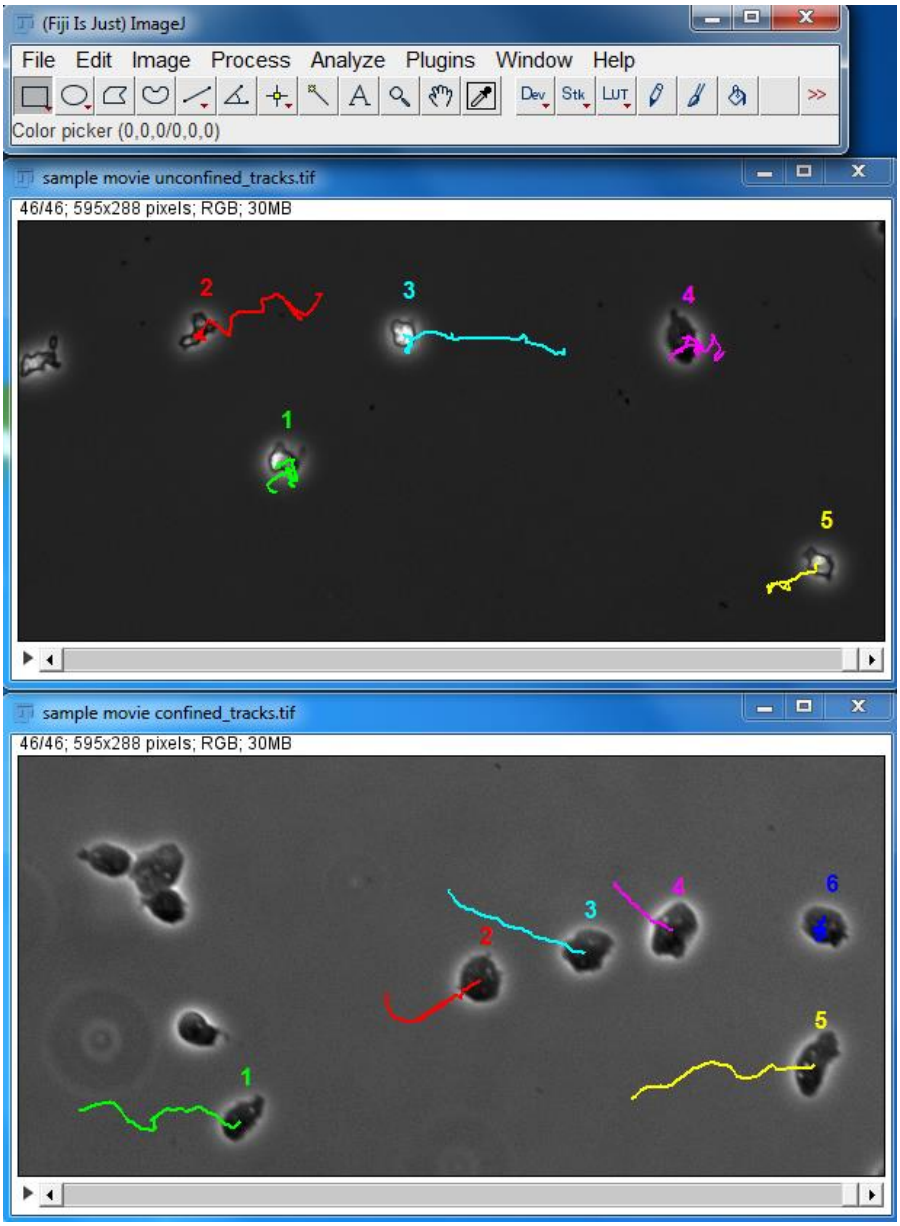
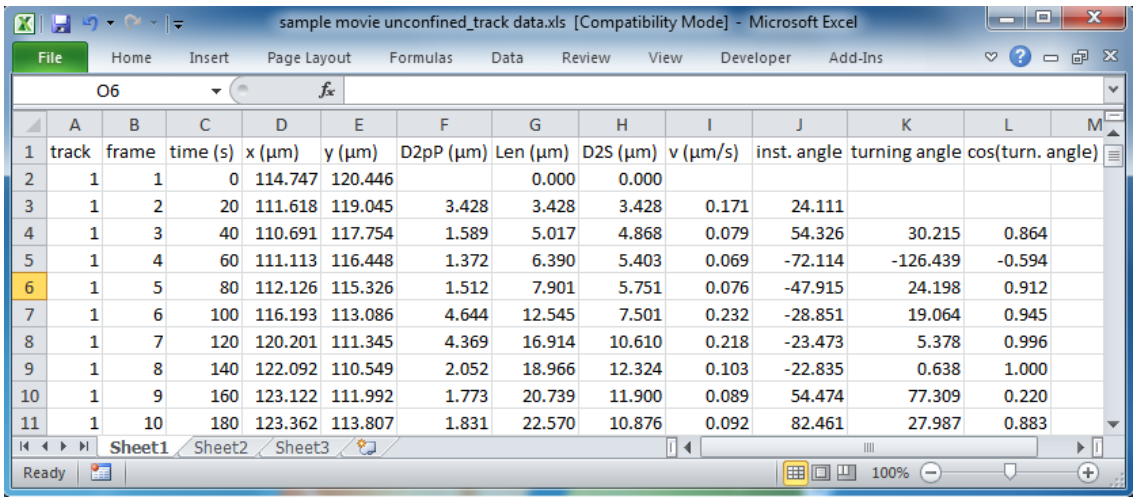


Figure 4 | The trajectory stack viewed in Fiji/ImageJ. The last frames of `sample movie unconfined_tracks.tif` and of `sample movie confined_tracks.tif`, respectively, generated with Dicty Tracking as described above and opened in Fiji/ImageJ are shown. Not all cells of the confined sample movie were tracked as some of them collide during the experiment (see also subsection "Step 2: Control of cell detection & selection of cells for tracking"). Note, that *Dictyostelium* cells confined by a thin sheet of agarose migrate much more directional than freely migrating (unconfined) cells.



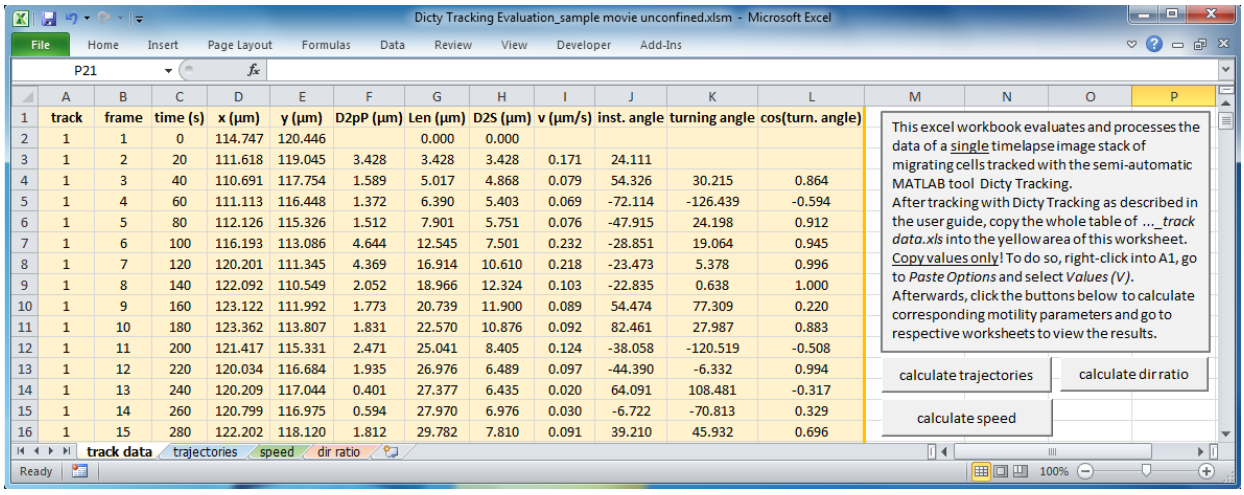
	A	B	C	D	E	F	G	H	I	J	K	L	M
1	track	frame	time (s)	x (µm)	y (µm)	D2pP (µm)	Len (µm)	D2S (µm)	v (µm/s)	inst. angle	turning angle	cos(turn. angle)	
2	1	1	0	114.747	120.446		0.000	0.000					
3	1	2	20	111.618	119.045	3.428	3.428	3.428	0.171	24.111			
4	1	3	40	110.691	117.754	1.589	5.017	4.868	0.079	54.326	30.215	0.864	
5	1	4	60	111.113	116.448	1.372	6.390	5.403	0.069	-72.114	-126.439	-0.594	
6	1	5	80	112.126	115.326	1.512	7.901	5.751	0.076	-47.915	24.198	0.912	
7	1	6	100	116.193	113.086	4.644	12.545	7.501	0.232	-28.851	19.064	0.945	
8	1	7	120	120.201	111.345	4.369	16.914	10.610	0.218	-23.473	5.378	0.996	
9	1	8	140	122.092	110.549	2.052	18.966	12.324	0.103	-22.835	0.638	1.000	
10	1	9	160	123.122	111.992	1.773	20.739	11.900	0.089	54.474	77.309	0.220	
11	1	10	180	123.362	113.807	1.831	22.570	10.876	0.092	82.461	27.987	0.883	

Figure 5 | Track data exported by Dicty Tracking. Track data of the unconfined sample movie (`sample movie unconfined_track data.xls`) opened in Microsoft Excel is shown.

5 Evaluation of track data

Dicty Tracking Evaluation.xlsm

To obtain parameters not calculated by Dicty Tracking like the mean speed or the mean directionality ratio (dir ratio) of all tracked cells of an image stack, a VBA-based Excel workbook termed `Dicty Tracking Evaluation.xlsm` is provided. This workbook furthermore shifts the trajectories of all tracked cells to the origin allowing am easy generation of trajectory plots with Excel or other standard graphing software (e.g. SigmaPlot or Origin). A detailed description of how to import the track data and how to use the workbook is included (grey box in Figure 6). ⚠ Critical As this workbook contains Excel macros written in Visual Basic it is necessary to press *Enable Content* to enable the macro execution! ⚠ It is recommended to use the original file as a template that is copied for every use. The mean speed calculation is exemplarily shown in Figure 7.



	A	B	C	D	E	F	G	H	I	J	K	L
1	track	frame	time (s)	x (µm)	y (µm)	D2pP (µm)	Len (µm)	D2S (µm)	v (µm/s)	inst. angle	turning angle	cos(turn. angle)
2	1	1	0	114.747	120.446		0.000	0.000				
3	1	2	20	111.618	119.045	3.428	3.428	3.428	0.171	24.111		
4	1	3	40	110.691	117.754	1.589	5.017	4.868	0.079	54.326	30.215	0.864
5	1	4	60	111.113	116.448	1.372	6.390	5.403	0.069	-72.114	-126.439	-0.594
6	1	5	80	112.126	115.326	1.512	7.901	5.751	0.076	-47.915	24.198	0.912
7	1	6	100	116.193	113.086	4.644	12.545	7.501	0.232	-28.851	19.064	0.945
8	1	7	120	120.201	111.345	4.369	16.914	10.610	0.218	-23.473	5.378	0.996
9	1	8	140	122.092	110.549	2.052	18.966	12.324	0.103	-22.835	0.638	1.000
10	1	9	160	123.122	111.992	1.773	20.739	11.900	0.089	54.474	77.309	0.220
11	1	10	180	123.362	113.807	1.831	22.570	10.876	0.092	82.461	27.987	0.883
12	1	11	200	121.417	115.331	2.471	25.041	8.405	0.124	-38.058	-120.519	-0.508
13	1	12	220	120.034	116.684	1.935	26.976	6.489	0.097	-44.390	-6.332	0.994
14	1	13	240	120.209	117.044	0.401	27.377	6.435	0.020	64.091	108.481	-0.317
15	1	14	260	120.799	116.975	0.594	27.970	6.976	0.030	-6.722	-70.813	0.329
16	1	15	280	122.202	118.120	1.812	29.782	7.810	0.091	39.210	45.932	0.696

This excel workbook evaluates and processes the data of a single timelapse image stack of migrating cells tracked with the semi-automatic MATLAB tool Dicty Tracking.
After tracking with Dicty Tracking as described in the user guide, copy the whole table of *..._track data.xls* into the yellow area of this worksheet.
Copy values only! To do so, right-click into A1, go to *Paste Options* and select *Values (V)*.
Afterwards, click the buttons below to calculate corresponding motility parameters and go to respective worksheets to view the results.

calculate trajectories | calculate dir ratio | calculate speed

Figure 6 | The Excel workbook `Dicty Tracking Evaluation.xlsm`. The worksheet *track data* with pasted data from `sample movie unconfined_track data.xls` is shown. The grey box on the right contains instructions of how to import track data and how to use the workbook. When clicked, the buttons beneath the grey instruction box will execute VBA macros calculating cell trajectories shifted to the origin, the mean speed and the mean directionality ratio. Results of these calculations are shown in the worksheets *trajectories* (blue), *speed* (green) and *dir ratio* (red).

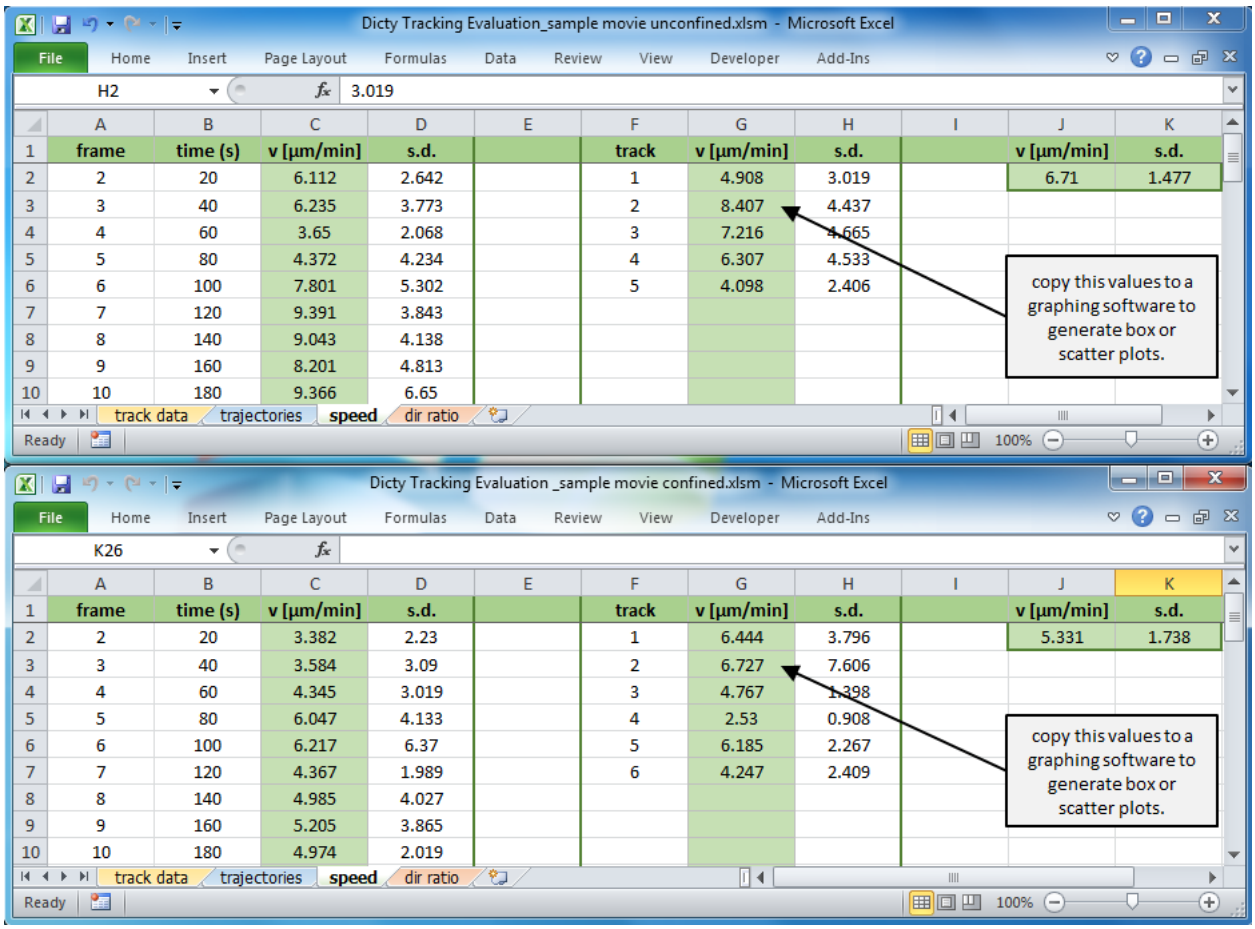


Figure 7 | Calculation of cell speed using `Dicty Tracking Evaluation.xlsm`. The *speed* worksheets of `Dicty Tracking Evaluation.xlsm` after execution of the *calculate speed* macro (see clickable buttons in Figure 6) are shown for the unconfined (top) and the confined sample movie (bottom), respectively. The macro calculates the mean speed of all analyzed cells at each time point (left) as well as the mean speed of every single cell/track (middle) and the mean speed of the whole sample (right). Standard graphing software like SigmaPlot, Origin or GraphPad Prism can be used to generate scatter or box plots of single cell mean speed values and for further statistical analyses.

MSD Calculation.xlsm

To calculate the mean squared displacement (MSD) of cells tracked from one or more image stacks, a VBA-based Excel workbook named `MSD Calculation.xlsm` is additionally provided. It calculates the MSD over time of up to four different cell populations. A detailed description of how to import data for MSD calculation (time, x and y positions of cells) and how to use the workbook is included (grey box, not shown in Figure 8). ⚠ **Critical** As this workbook contains Excel macros written in Visual Basic it is necessary to press *Enable Content* to enable macro execution! ⚠ It is recommended to use the original file as a template that is copied for every use.

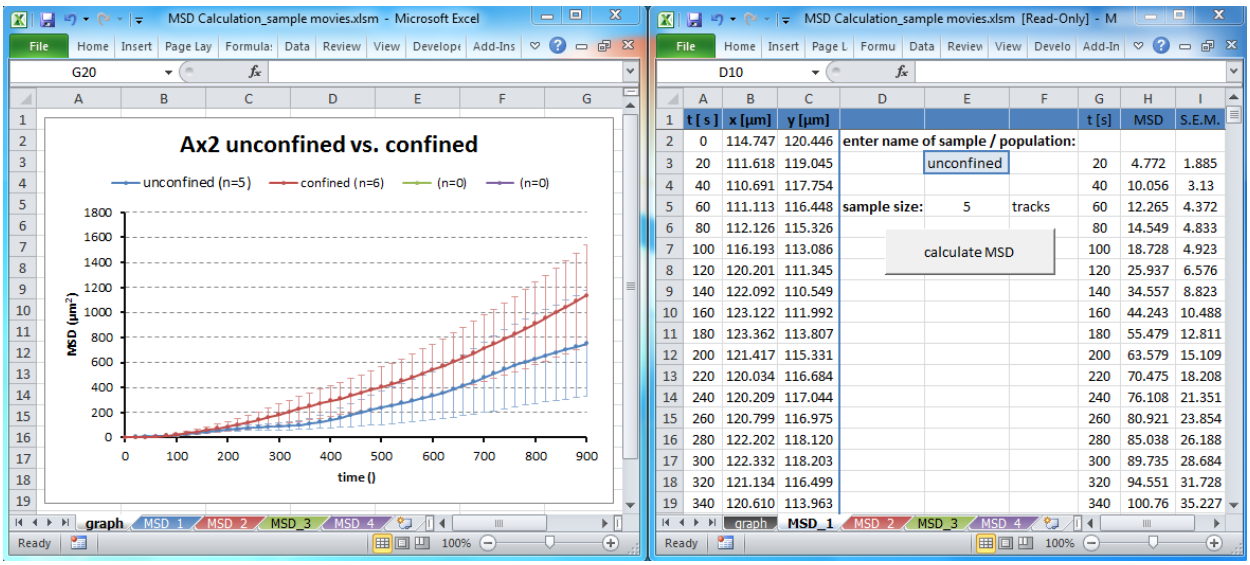
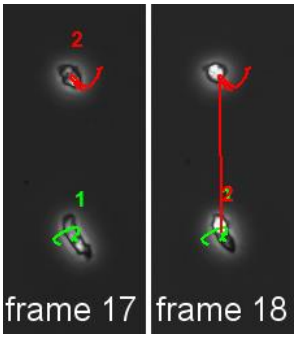


Figure 8 | `MSD Calculation.xlsm`. Calculation of the mean squared displacement (MSD) over time of the five and six cells from the unconfined and confined sample movie, respectively, is shown. Left, section of the *graph* worksheet of `MSD Calculation.xlsm` showing a chart of the calculated MSD of up to four different cell populations termed *MSD_1* to *MSD_4*. Right, worksheet *MSD_1*. These worksheets comprise an area to paste time and position data on the left, a field for the sample name and a clickable button to execute the VBA macro in the middle as well as columns for the calculated MSD and standard error values on the right. Note, that confined *Dictyostelium* wild-type cells, despite their lower migration speed (see Figure 7), cover a larger area due to their more directional migration behavior (see also Figure 4).

6 Troubleshooting

Problem	Reason and solution
No cell shapes are detected; lower image of the `…_cell detection.tif` stack is completely black.	Wrong bit depth of the input image stack. Convert bit depth of the image stack to full range 8- oder 16-bit as described in subsection “Bit depth”.
Tracking errors occur as shown in the image below. 	This tracking error occurs when a cell is not detected in a certain frame of the input image stack. In this case, cell 2 is not detected in frame 18 resulting in a fusion of cell tracks 1 and 2. Start Dicty Tracking again with modified cell detection parameters to ensure that all cells are detected at all time points (frames) of the stack. See also subsection “Step 2: Control of cell detection & selection of cells for tracking”.

Please do not hesitate to contact us, if you experience any other problems as described above.