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Maria Manich, Aleix Boquet-Pujadas, Stéphane Dallongeville, Nancy Guillén,  
Jean-Christophe Olivo-Marin

## ► To cite this version:

Maria Manich, Aleix Boquet-Pujadas, Stéphane Dallongeville, Nancy Guillén, Jean-Christophe Olivo-Marin. A Protocol to Quantify Cellular Morphodynamics: From Cell Labelling to Automatic Image Analysis. Nancy Guillén. Eukaryome Impact on Human Intestine Homeostasis and Mucosal Immunology, Springer International Publishing, pp.351-367, 2020, 978-3-030-44825-7. 10.1007/978-3-030-44826-4\_25 . pasteur-02773115

**HAL Id: pasteur-02773115**

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Submitted on 4 Jun 2020

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# Chapter 25

## A Protocol to Quantify Cellular Morphodynamics: From Cell Labelling to Automatic Image Analysis

Maria Manich, Aleix Boquet-Pujadas, Stéphane Dallongeville, Nancy Guillén, and Jean-Christophe Olivo-Marin

1 **Abstract** Cellular morphodynamics can be used as markers for many physiological  
2 and pathological processes. This protocol provides a step-by-step guide to identify  
3 variations in motility and morphology within (or across) cell populations using  
4 non-invasive live imaging and reproducible image analysis techniques such as segmentation  
5 and tracking. Detailed instructions cover all the way from cell culturing and labelling  
6 to automatic image and statistical analyses, including the definition of  
7 multiple descriptors that characterise the shape and movement of cells in a quantitative  
8 manner. All methods are available as free open-source software and illustrated  
9 by video tutorials.

10 **Keywords** Cell biology · Image analysis · Software · Segmentation · Tracking ·  
11 Shape descriptors

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**Electronic supplementary material** The online version of this chapter ([https://doi.org/10.1007/978-3-030-44826-4\\_25](https://doi.org/10.1007/978-3-030-44826-4_25)) contains supplementary material, which is available to authorized users.

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M. Manich · A. Boquet-Pujadas · S. Dallongeville · N. Guillén · J.-C. Olivo-Marin (✉)  
Institut Pasteur, Bioimage Analysis Unit, 75015 Paris, France  
e-mail: [jcolivo@pasteur.fr](mailto:jcolivo@pasteur.fr)

M. Manich · A. Boquet-Pujadas · S. Dallongeville · J.-C. Olivo-Marin  
Centre National de La Recherche Scientifique, CNRS, UMR3691 Paris, France

A. Boquet-Pujadas  
Sorbonne Université, Paris, France

N. Guillén  
Centre National de La Recherche Scientifique, CNRS, ERL9195 Paris, France

© Springer Nature Switzerland AG 2020  
N. Guillén (ed.), *Eukaryome Impact on Human Intestine Homeostasis and Mucosal Immunology*, [https://doi.org/10.1007/978-3-030-44826-4\\_25](https://doi.org/10.1007/978-3-030-44826-4_25)

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## 12 Introduction

13 Advances in microscopy techniques and fluorescent probes have long been helping  
14 the scientific community determine the importance of cell movement and deformation  
15 in multiple biological processes. However, many studies remain qualitative, i.e.  
16 differences in shape or motility are assessed visually, adding subjectivity to potential  
17 biological conclusions. Conversely, using image analysis to assign numerical values  
18 to both shape and movement does not only guarantee the reproducibility of the  
19 conclusions but also opens the door to statistical analyses that allow classifying cell  
20 populations and phenotyping. Accordingly, we present a step-by-step manual that  
21 shows how to quantify cellular morphodynamics in a non-invasive and reproducible  
22 way using only confocal microscopy and fluorescent markers.

23 The present protocol details both biological and computational experiments. We  
24 first describe the necessary biological techniques, namely culturing the cells and fluorescently  
25 labelling the cytoplasm; next, we comment on how to perform non-invasive  
26 imaging using a confocal microscope; and, finally, we provide a ready-to-use image  
27 analysis workflow that goes all the way from raw images to biological conclusions  
28 in a reproducible manner. More specifically, we present automatic tools for cell segmentation  
29 and tracking that are freely accessible as modules in the Icy platform; as  
30 well as multiple descriptors that quantify cell shape and movement from the resulting  
31 contours and tracks. These descriptors serve as a basis from which to perform statistical  
32 tests and assess any possible correlation between morphodynamical variables.  
33 All the key steps of the protocol are available as video tutorials and are exemplified  
34 using a population of *Entamoeba histolytica*, a highly motile parasite that migrates  
35 through diverse human tissues, including the intestine and the liver.

## 36 Results

### 37 *Wet-Lab Protocol: Culturing Cells and Acquiring Images*

38 To quantify movement and deformation using image analysis (see dry-lab protocol),  
39 it is paramount to image the cells non-invasively (physiological relevance) and in  
40 good spatiotemporal resolution (easier analysis). To meet these two criteria, we label  
41 the cytoplasm with a fluorescent dye and use a spinning-disk confocal microscope.

### 42 Cell Culture and Staining

43 Trophozoites of the *Entamoeba histolytica* strain HM1: IMSS were grown overnight  
44 at 37 °C in TYI-S-33 medium (Diamond et al. 1978). Medium was then replaced  
45 by incomplete TYI-S-33 medium (serum/vitamines-free) (TYI). Cells were labeled  
46 with Cell Tracker™ Red CMTPX, a fluorescent dye that is well suited for monitoring

47 cell movement and displacements (Petropolis et al. 2014). The dye has low cytotoxicity, does not affect cell viability nor proliferation, and its fluorescence was stable  
48 during the entire imaging process, allowing us to track cellular movements with a red  
49 excitation/emission spectra (577/602 nm). In this case, we have used the fluorescent  
50 dye that emits in the red spectra because *Entamoeba histolytica* autofluoresces at  
51 488 nm. Since the forthcoming image analysis methods are based on accurate cell  
52 segmentation, in this paper we used non-confluent cultures. Other image analysis  
53 tools are required to deal with confluent cell cultures, but they are not the focus of  
54 this paper.  
55

56 Cells were incubated for 45 min at 37 °C, and then washed with TYI pre-warmed  
57 at 37 °C by reversing the tube and simply discarding the medium. No centrifugation  
58 is required because amoebas are adherent cells and remain attached to the glass  
59 tube during the process. Trophozoites were gently suspended in pre-warmed TYI by  
60 shaking the tube and then seeded on 35 mm glass-bottomed imaging Ibidi dishes,  
61 obtaining an estimate of  $5 \times 10^3$  cells.

## 62 Microscopy Experiment

63 Images were taken with a spinning disk confocal microscope (25 × objective) inside  
64 an incubator at 37 °C to keep the parasites at a physiological temperature where  
65 they are specially motile. Indeed, at these temperature *E. histolytica* parasites can  
66 move at up to 1 μm/s in 2D culture conditions (Dufour et al. 2015). Fortunately, with  
67 the spinning disk microscope, images can be acquired at very high frame rates with  
68 minimal illumination and photo-bleaching of the living samples.

69 Videos were recorded for four minutes at an imaging rate of one frame per second  
70 (i.e. a total of 240 frames) and at a pixel size of 0.48 μm. The fields of view were  
71 taken to be of around 512 × 512 pixels, corresponding to 246 × 246 μm<sup>2</sup>, which  
72 typically contained around 2–6 cells. The *z* position was set at a height of around  
73 2 μm from the glass.

74 Both pixel size and frame rate are necessary for the posterior image analysis,  
75 for example to obtain the speed in real units, and therefore need to be stored. They  
76 are typically stored automatically in the metadata of the image files by the software  
77 associated with the microscope, but we recommend to double-check that this is  
78 indeed the case. In our case, all images were acquired with the Volocity 3D image  
79 analysis software (Perkin Elmer, USA) and the files and their associated metadata  
80 stored in the mvd2 format.

81 There are no potential dangers involved in the experiments, neither because of  
82 laser beams nor of parasite pathogenicity. However, a P2-class laboratory is needed  
83 to handle the living trophozoites. The protocol was set up according to the guidelines  
84 provided by the Safety Authorities and the Image Microscopy Facility platform of  
85 Institut Pasteur.

## 86 *Dry-Lab Protocol: Analysing Images*

87 The motility of a cell population can be studied quantitatively using image analysis.  
 88 In this context, each individual cell in a video sequence is first singled out of the  
 89 background in a process called segmentation. Cell segmentation not only allows  
 90 to delimit the borders of the cells present in an image, but also to calculate their  
 91 centroid and thus to track the displacement of the cells over time. On the one hand,  
 92 digitising the contours of the cell opens the door to characterising the cell shape with  
 93 descriptors such as roundness; on the other hand, the time tracks contain information  
 94 on the movement of the cell such as its speed or the straightness of its trajectory,  
 95 which shed light on the reasons behind its migration (random, directed chemotaxis,  
 96 etc.). Therefore, these data enable a rich quantification of both cell morphology and  
 97 motility that ideally translates into cell phenotyping when complemented with an  
 98 extensive statistical and correlation analysis.

99 The three main steps (segment, track and statistical assessment) are visited in detail  
 100 in respective Sects. “[Hierarchical K-Means](#)”, “[Active Contours](#)”, “[Cell Tracking  
 101 with Track Manager](#)” and are automatised by bioimaging softwares such as Icy (de  
 102 Chaumont et al. 2012; Wiesmann et al. 2015), which we address immediately in  
 103 Sect. 25.2.2. All steps are available as video tutorials.

### 104 **Bioimage Analysis Software**

105 To quantify cell motility, we present Icy, a free and open-source platform for bioim-  
 106 age analysis that provides multiple resources to visualize, annotate and quantify  
 107 bioimaging data (<http://icy.bioimageanalysis.org>).

108 Icy provides a user-friendly approach to new and classical image analysis tech-  
 109 niques alike: filtering, segmentation, tracking.... They are all available under different  
 110 modules called plug-ins who all share the same graphical interface. Examples of seg-  
 111 mentation plug-ins are Thresholding, Active Contours, Parametric Snakes, Potts Seg-  
 112 mentation, Spot Detector (Olivo-Marin 2002) and HK-Means (Dufour et al. 2008);  
 113 whereas plug-ins such as Spot Tracking, Track Manager and Kymograph Tracker  
 114 provide different approaches to tracking. In this protocol, we will focus on HK-  
 115 Means (Sect. “[Hierarchical K-Means](#)”), Active Contours (Sect. “[Active Contours](#)”),  
 116 and Track Manager (Sect. “[Cell Tracking with Track Manager](#)”) in order to provide  
 117 a step-by-step guide on how to analyse cell shape and motility.

118 The graphical interface integrates 2D and 3D visualisation resources, as well  
 119 as a series of tools to easily crop and cut through time series, z-stacks or multi-  
 120 channel sequences. Also intuitive is the management of so-called Regions Of Interest  
 121 (ROIs), i.e. delimited areas of the image that are of special interest and that might  
 122 want to be analysed aside, for example a cell segmented from the image. In the Icy  
 123 platform, ROIs are superimposed over the original image and can be manipulated  
 124 as independent objects on which common operations such as “copy/paste” (ctrl +  
 125 c/v) or “delete” can be applied, allowing to easily combine analyses performed on

126 different channels or sequences. ROIs are deeply integrated into Icy so that any  
127 analysis or quantification from them is automatic and straightforward. For instance,  
128 cell segmentation results are represented as ROIs from which multiple descriptors  
129 such as area, mean fluorescence intensity or roundness can be directly accessed in  
130 the ROI menu and exported into Excel files for further data analysis.

131 As a last remark, in its most recent version 2.0, Icy has introduced a new image  
132 handling engine that allows working with big video sequences, be it either because  
133 they are long or because they were taken at very high spatial and/or temporal resolu-  
134 tion. The idea behind the new engine is that only a portion of the image sequence is  
135 loaded into the local RAM memory, while the rest is stored on hard-disk at the price  
136 of longer processing times.

137 We have used the sequence called  $25 \times 40$  to illustrate this protocol (Movie 1)  
138 over its several steps. The Time Stamp Overlay plugin (Tutorial 1) can be used to  
139 stamp the elapsed time onto the video.

## 140 Cell Segmentation with Hierarchical K-Means and Active Contours

### 141 Hierarchical K-Means

142 Hierarchical K-Means (HK-means or HKM here) is a segmentation method based  
143 on a K-Means clustering of the image histogram, i.e. an algorithm that divides the  
144 different intensity values in the image into groups according to a similarity mea-  
145 sure. Ideally, these groups correspond to the different cells and to the background.  
146 However, the K-means algorithm requires the number of groups to be specified in  
147 advance. To tackle this problem, a hierarchical strategy is introduced. In this way, the  
148 algorithm attempts to find the ideal number of groups using a bottom-up approach.  
149 This process can be helped if the user specifies a value for the expected minimum  
150 and maximum size of the cells.

151 HKM is a fundamental tool in image processing; it is one of the go-to algorithms if  
152 the user wants to segment cells in a quick, ready-to-use and quasi-automatic manner.  
153 And precisely because of its hierarchical clustering approach, it performs better  
154 than classical clustering and thresholding algorithms. However, HKM suffers from  
155 three main drawbacks. First, it has difficulty telling apart cells that are in contact  
156 with each other. Second, big intensity heterogeneities inside the cell might trigger  
157 multiple detections. And third, since the resulting segmentations are groups of pixels  
158 rather than polygonal contours, some accuracy may be lost when computing shape  
159 descriptors.

160 A step-by-step guide to the HKM plug-in in Icy can be found in Fig. 25.1 and is  
161 accompanied by Tutorial 2.

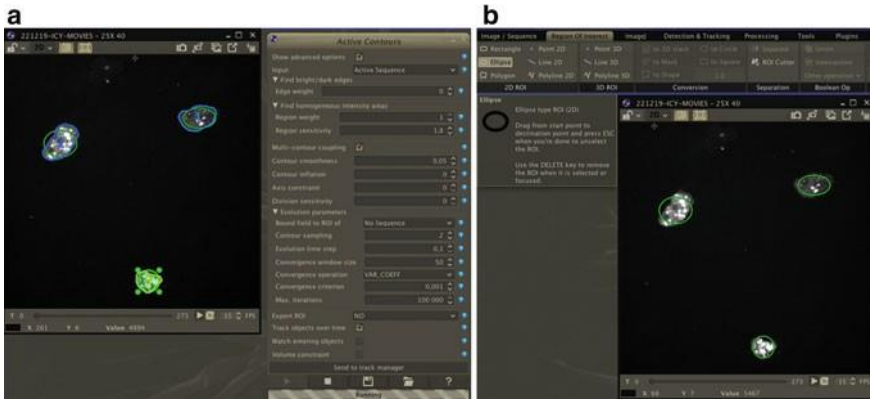


**Fig. 25.1 Cell segmentation with HK-Means and ROI color selection.** HK-Means can be opened from the detection tab or from the search bar. The first step is to choose which frames we are interested in segmenting (here we select frame 0, but ‘ALL’ is also a possibility). We have to specify the number of intensity classes (see the text for an explanation on HK-Means) in which the histogram is to be split. That is the number of different intercellular intensities (e.g. if all cells are the same intensity, two classes are enough). When in doubt, we recommend choosing higher values, but we also remark that it comes at a computational price. To aid the segmentation of the image into the different classes the user can also input an expected minimum and maximum size for the cells so as to eliminate possible groups that are respectively too small (e.g. debris) or too big to possibly be a cell (e.g. cell clusters). Notice that these sizes are required in pixels, to have a rough idea of the cell size in pixels draw a ROI around the cell and check its size (“Interior” descriptor in the ROI tab at the right-hand side). Finally, applying the Gaussian pre-filter can help improve the segmentation of noisy images. Since the segmentation output are ROIs, we can obtain any descriptor directly from the ROI tab. Here we show the perimeter, the area, the mean intensity, the roundness and the homogeneity inside the ROIs, but many more shape descriptors can be selected using the “gear” button. ROI colors can be chosen (see Tutorial 2)

## 162 Active Contours

163 Active Contours (Zimmer et al. 2002) (AC) are well adapted to study cell morpho-  
 164 dynamics; they provide accurate cell contours and are capable of segmenting cells  
 165 that are in close contact, as well as cells with inner heterogeneities. However, in  
 166 contrast to the more classical segmentation methods, AC need to be initialised. The  
 167 user has to specify an approximative initial contour (ROI) around the cell so that the  
 168 algorithm can pick up on it. This initialization can be done manually, by drawing  
 169 the ROI over the object of interest, or automatically, using other segmentation tools  
 170 (e.g. the above-described HKM). The initial ROI contours are then refined by the AC  
 171 method, which slowly deforms the contour. In this way, the contour is progressively  
 172 fitted to the cell shape in an attempt not only to separate the image into multiple  
 173 intensities, but also to find the edges of the cells in the gradient of the image. When  
 174 the segmentation spans a whole video sequence, the ROI resulting from segmenting  
 175 a given frame can be used as an initial ROI for the following frame (see ‘*track objects*  
 176 *over time*’ in the AC plug-in). Therefore, if the image acquisition is relatively fast,  
 177 initialising the ROIs at the very first frame is enough to segment the entire sequence.

178 In summary, whereas HKM is fast and does not need to be initialised, it is most  
 179 performant when image quality is good and cells are well separated; otherwise AC  
 180 take over at the price of initialisation and speed. In fact, we remark that a good



**Fig. 25.2 Manual drawing of approximative ROIs and automatic cell segmentation with Active Contours.** **a** The first step before running Active Contours is to draw approximative ROIs around each of the cells; we do it manually here (green ellipses), but one could use HK-Means to initialize the method automatically. **b** Active Contours can be opened from the detection tab or from the search bar. In order to segment and track all of the time frames make sure to activate “tracks objects over time”. Perhaps the two most important parameters of the plug-in are the edge and region weights, which control the balance between the importance of (1) the big intensity differences that are expected at the border of the cell and (2) the homogeneous intensity that is expected inside the cell as opposed to that of the outside. In addition, “contour inflation” might help compensate for a lack of contrast between cell and background by adding an artificial expansion rate. On the other hand, the set of evolution parameters are more technical but can help speed up the process and/or make the final contours more accurate. All parameter settings can be readily stored and loaded using the save icon on the bottom. The results of the segmentation are also presented as ROIs (see text and Tutorial 3)

181 approach is to combine the two; that is to use HKM (only) on the first frame to  
 182 automatically set the initial ROIs required by AC. However, in this protocol we have  
 183 found it more pedagogic to set the initial contours manually.

184 A step by step guide to the AC plug-in in Icy can be found in Fig. 25.2 and is  
 185 accompanied by Tutorials 2, 3 and 4. Movie 2 shows the segmented cells with Active  
 186 Contours.

## 187 Cell Tracking with Track Manager

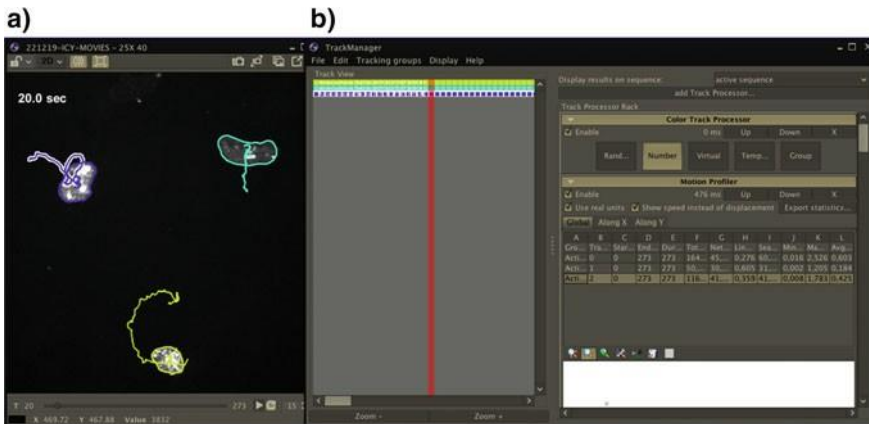
188 Using either of the segmentation plug-ins on a video sequence results in a time-series  
 189 of ROIs that can potentially be linked together to generate the track of a cell, i.e.  
 190 to draw the path that the cell followed. At our spatiotemporal resolution, it suffices  
 191 to associate a ROI at a given time point with the closest ROI at the following time  
 192 point to accurately track cells; more precisely, it is the centroids of the successive  
 193 ROIs that are concatenated into a cell track. However, more advanced tracking tools  
 194 such as Multiple Hypothesis Tracking (Chenouard et al. 2013) become necessary  
 195 for high-speed particle tracking. In either case, the resulting tracks can be analyzed



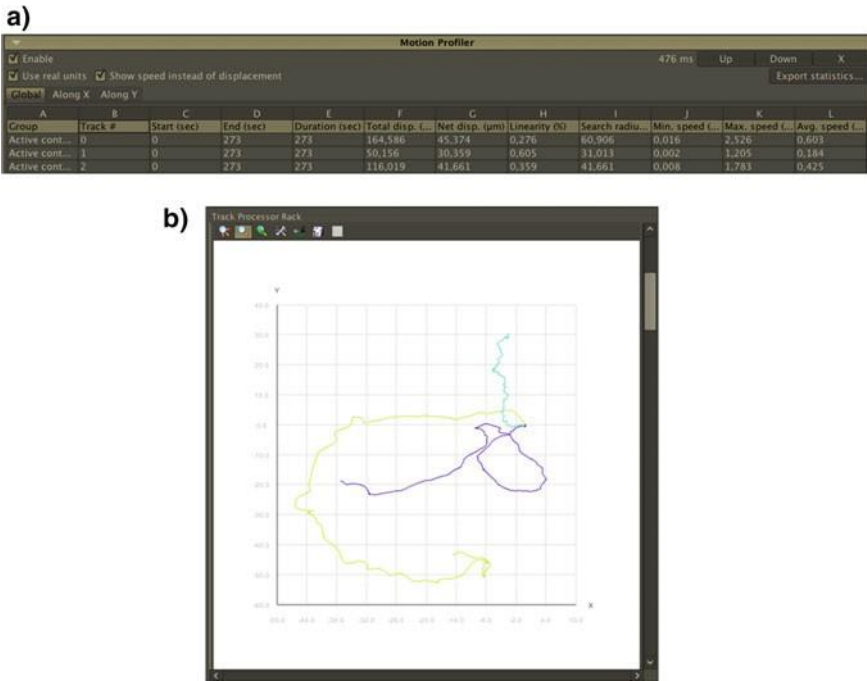
196 with the Track Manager (TM) module in Icy, which is readily invoked from the very  
 197 segmentation plug-ins using the ‘*send to track manager*’ button.

198 TM displays the resulting tracks directly overlaid on the original sequence. The  
 199 tracks can also be analyzed through an accessible interface, for instance to investigate  
 200 motility parameters such as cell speed or mean squared displacement (MSD) and  
 201 compare them between populations or correlate them with other descriptors, for  
 202 example of cell morphology (see below). All these quantifications tasks are done  
 203 through so called Track Processors (TPs). Each TP has a specific function: from  
 204 filtering unwanted tracks, to quantifying movement, passing by a myriad of display  
 205 functionality such as color-coding the tracks (“TP Color”). In this protocol, we have  
 206 used several TPs. Briefly, (i) “Motion Profiler” computes multiple motion descriptors  
 207 such as the average speed or the linearity/persistence of the tracks; (ii) “Instant Speed”  
 208 displays the speed of the cell as a time curve; whereas (iii) “ROI Statistics” (ROIS)  
 209 displays time curves of several shape descriptors as is described in Sect. “[Statistical  
 210 Tests with R](#)”.

211 A step-by-step guide to the TM plug-in in Icy can be found in Figs. [25.3](#), [25.4](#)  
 212 and [25.5](#) and is accompanied by Tutorial 5. Movie 3 shows the segmented cells with  
 213 Active Contours and their centroid tracks.



**Fig. 25.3** Cell track analysis with Track Manager and Track Processors. **a** Track Manager can be opened from the tabs or directly from most segmentation plug-ins (e.g. HK-Means or Active Contours, see respective Figs. [25.1](#), [25.2](#) and Tutorial 4). The tracks for each of the cells are automatically overlaid on the video sequence in the corresponding colors. **b** Each track is a separate entity and can be filtered or quantified by adding Track Processors. Here we are displaying the Color and Instant Speed Track Processors, but many others are available (e.g. see Figs. [25.4](#), [25.5](#) and [25.6](#)). The red vertical bar displays the current time point, and can be dragged to navigate the time sequence. Tracks can be saved into an.xml file

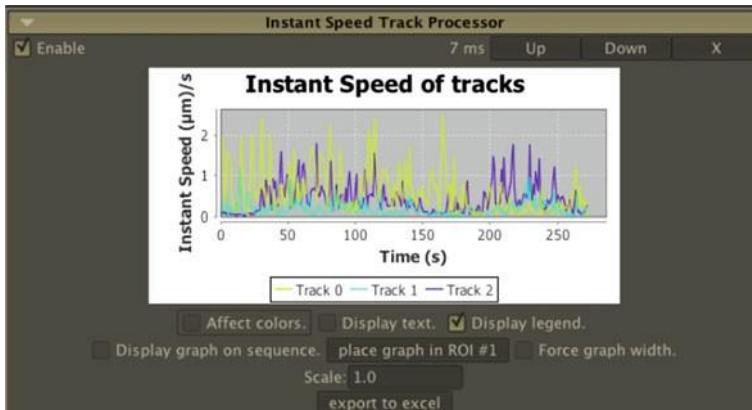


**Fig. 25.4 Descriptors and visualisation of cell tracks with the Motion Profiler Track Processor in Track Manager.** **a** By selecting Motion Profiler from the track processors in Track Manager, we obtain multiple descriptors of the three tracks. For example, we can see typical minimum, maximum and average speed values; and we can also quantify how straight the cells are moving with the measures of linearity and search radius. In addition, the processor can take the metadata into account to offer the values in real units. All these results can be exported to an Excel file. **b** Motion Profiler also provides a graphical representation of all the tracks from a common origin, from where we can visually assess whether motion is random or directed

214 **Morphological Descriptors and Statistical Tests with ROI Statistics and R**

215 **Cell Descriptors with ROI Statistics**

216 Different cell populations might be characterised by different morphologies. Given a  
 217 time sequence of already segmented cells in the form of ROIs (e.g. with AC), the ROI  
 218 Statistics (ROIS) processor in TM provides a wide range of geometrical properties  
 219 that describe the shape of each ROI. Together with a posterior statistical analysis,  
 220 these descriptors may help tell apart different populations or be used for phenotyping.  
 221 Many such descriptors are available in ROIS; in this study, we only consider the fol-



**Fig. 25.5** Time curves of cell speed with the Instant Speed Track Processor in Track Manager. By selecting Instant Speed from the track processors in Track Manager, we obtain the time curves of the speed at each time point along each of the cell tracks. These data can be used to explore the cycles of acceleration and deceleration of the cells for example. The curves can be exported to an Excel file

222 lowing: area ( $\mu\text{m}^2$ ), perimeter ( $\mu\text{m}$ ), roundness (%), mean intensity values (a.u.) and  
 223 homogeneity (a.u.). “Perimeter” measures the perimeter of the ROI in micrometers  
 224 (here the scaling information is extracted automatically from the metadata). Equiv-  
 225 alently, “Area” measures the ROI area in micrometers squared. “Mean intensity”  
 226 averages the intensity values inside the ROI, whereas “Homogeneity” highlights the  
 227 internal variations of the intensity distribution within the cell. Lastly, “Roundness”  
 228 is a measure of how similar to a circle the ROI is. These data are displayed directly  
 229 in Icy, but can also be exported to an Excel file (Table 25.1) for further analysis,  
 230 for example to perform statistical tests that assess the correlation between each of  
 231 the descriptors. For instance, we study the correlations between the temporal mean  
 232 of all these parameters and the Speed ( $\mu\text{m/s}$ ) resulting from TM. Alternative shape  
 233 descriptors can be extracted by rewriting the cell shape in different mathematical  
 234 basis such as Fourier (2D) or Spherical Harmonics (3D); these work well to separate  
 235 populations, but often lack biological interpretability (Ducroz et al. 2012).

236 A step-by-step guide to the TM plug-in in Icy can be found in Fig. 25.6.

## 237 Statistical Tests with R

238 In order to assess whether any trend or correlation exists between the extracted  
 239 descriptors we perform a visual pairwise comparison educated with Spearman’s  
 240 rank correlation coefficient. So-called Spearman’s “rho” attempts to quantify the  
 241 monotonicity of the relationship between a pair of variables, irrespectively of its  
 242 linearity. The coefficient spans the interval  $[-1, 1]$ , where the extremes correspond  
 243 to perfectly monotonic functions, respectively decreasing or increasing (i.e. functions

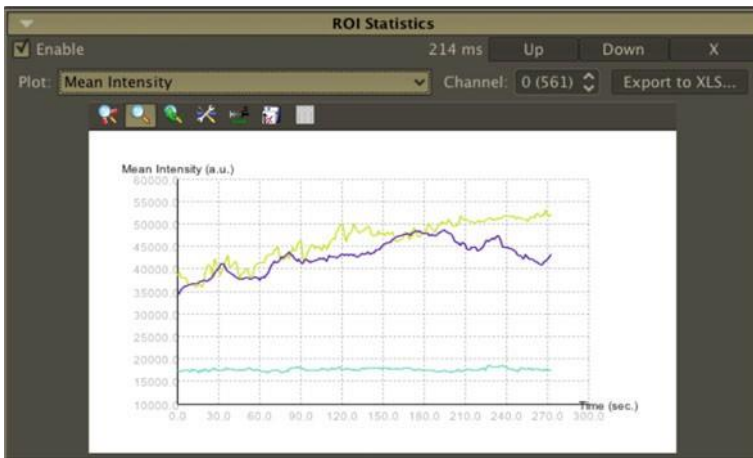
Name	Area	Homogeneity	Intensity	Perimeter	Roundness	Speed
	( $\mu\text{m}^2$ )	(561)	(561)	( $\mu\text{m}$ )	(%)	( $\mu\text{m/s}$ )
25 $\times$ 54	316.08	0.08	24,203	75.58	40.05	1.16
	2407.56	0.12	19,189	196.18	59.33	0.08
25 $\times$ 49	2779.13	0.13	24,801	208.46	59.11	0.24
	308.78	0.15	15,070	69.42	48.16	1.21
25 $\times$ 47	771.28	0.13	16,320	119.90	34.82	0.17
	411.95	0.13	21,341	81.62	49.3	0.43
25 $\times$ 44	760.11	0.10	25,160	114.79	45.55	0.40
	684.88	0.10	25,544	100.88	68.20	0.12
	545.58	0.11	21,754	90.64	58.77	0.97
	689.35	0.10	23,625	108.82	48.97	0.25
	658.80	0.10	23,308	109.13	46.66	0.49
	929.79	0.10	23,857	119.42	61.02	0.16
25 $\times$ 40	713.25	0.18	42,758	109.02	54.33	0.42
	438.46	0.32	46,123	88.07	42.48	0.60
	773.17	0.15	17,600	118.27	36.11	0.18
25 $\times$ 38	362.51	0.11	18,333	73.27	59.32	1.05
	1045.82	0.10	22,956	132.80	37.29	0.10
	814.01	0.10	21,460	113.92	54.76	0.33
25 $\times$ 36	1093.23	0.10	21,429	135.41	52.52	0.14
	525.07	0.08	27,100	92.00	50.12	0.31
	2713.55	0.10	36,252	233.99	29.84	0.10
	600.94	0.11	18,237	95.02	60.56	0.61
25 $\times$ 35	329.77	0.1	22,166	73.21	47.95	1.10
	630.77	0.1	24,413	101.25	51.82	1.37
	681.54	0.1	21,612	107.25	48.32	0.14
	466.57	0.1	33,879	85.38	53.27	0.23
	617.05	0.11	19,998	104.93	41.30	0.22
25 $\times$ 34	656.30	0.34	49,554	108.70	43.97	0.32
	663.35	0.21	10,132	103.12	47.39	0.29
25 $\times$ 33	207.05	0.07	24,868	56.05	52.87	0.62
	595.10	0.13	16,759	97.52	52.17	0.86
25 $\times$ 32	523.20	0.13	16,121	94.08	45.30	0.54
	653.94	0.11	19,977	105.08	41.45	0.14
25 $\times$ 30	1084.28	0.11	23,447	130.04	30.10	0.15

**Table 25.1 Cell shape descriptors and speed for the 42 cells of the experiment.** (25  $\times$  40 illustrates the protocol, values are means)

(continued)

**Table 25.1** (continued)

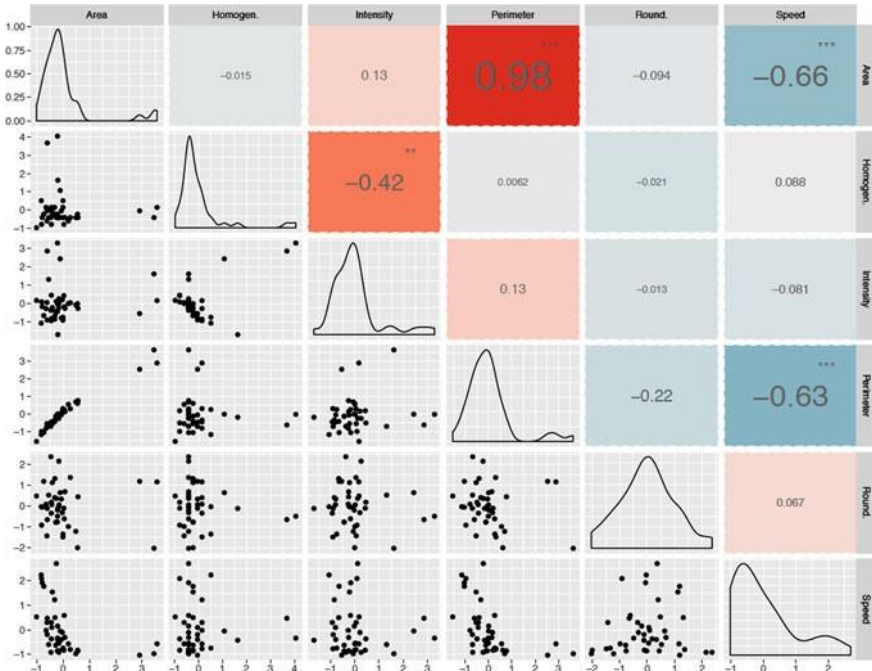
Name	Area ( $\mu\text{m}^2$ )	Homogeneity (561)	Intensity (561)	Perimeter ( $\mu\text{m}$ )	Roundness (%)	Speed ( $\mu\text{m/s}$ )
	887.42	0.09	23,499	128.19	39.44	0.24
	738.61	0.12	17,261	107.66	51.55	0.30
	791.83	0.08	26,903	115.63	47.52	0.15
	515.45	0.10	20,549	85.64	70.17	0.12
25 × 29	430.99	0.09	23,945	90.56	35.33	0.54
	532.69	0.12	17,155	93.70	49.06	0.47
	437.21	0.12	16,415	85.08	47.43	0.37
	799.27	0.10	22,078	118.07	42.83	0.64



**Fig. 25.6** Cell shape descriptors with the ROI Statistics Track Processor in Track Manager. By selecting ROI Statistics from the track processors in Track Manager, we obtain different descriptors of cell shape (perimeter, roundness, etc.) for each time point along a cell track. Here are presented the fluorescence average intensity values inside the ROIs. The data can be exported to an Excel file

244 that always go down, or up, without fluctuations); and 0 indicates a lack of correlation.  
 245 Precisely, the p-value associated with the coefficient results from testing whether this  
 246 coefficient is significantly different from 0.

247 Statistical analysis software can directly read the output values exported from Icy.  
 248 Here, we use a short R routine that can automatically generate the pairwise graphics  
 249 showing possible trends, as well as the correlation values and their corresponding  
 250 p-values (Fig. 25.7). This program uses some functionality from the ggplot2 library.  
 251 While it is not the aim of the paper to provide in-depth statistical insight, we remark  
 252 that it is important to check whether your data satisfies all the assumptions made  
 253 during the statistical analysis. For illustrative purposes, in Fig. 25.7 generated by



**Fig. 25.7 Statistical analysis of cell descriptors shows a significant correlation between cell speed and size.** The figure is a matrix quantifying the correlation between pairs of descriptors for  $n = 42$  cells from 13 different movies. On the upper triangular side, Spearman's correlation values resulting from descriptor pairs are displayed accompanied by their significance in the form of stars. The values are also displayed using a color gradient (red positive, blue negative) to facilitate the analysis. On the lower triangular side, we plot descriptor pairs on a normalised scale to show any possible trend. The diagonal contains univariate density plots

254 the R routine, we display univariate descriptor density plots (diagonal) and pair-  
 255 wise descriptor plots (lower diagonal), but directly compute the pairwise correlation  
 256 coefficients and their associated tests (upper diagonal) with no prior analysis.

257 **Example to Illustrate the Proposed Protocol for Image Analysis**

258 During the in vitro growth of *E. histolytica*, it is common to observe diverse phen-  
 259 types regarding the size of the cells, their mobility, the heterogeneity of fluores-  
 260 cence during labeling, etc. We wondered whether the protocol proposed here could help  
 261 us identify any correlations between these phenotypes. After acquisition of video-  
 262 microscopies of *E. histolytica* seeded on glass, the image analysis was performed on  
 263  $n = 42$  cells from 13 different video sequences. The data highlights several relation-

ships: the obvious correlation between area and perimeter, a less evident correlation between cell fluorescence intensity and homogeneity (as the image saturates), and a strong and significant (\*\*\*) correlation between the size of the cell and its mean speed (Table 25.1 and Fig. 25.7). For instance, the five smallest cells moved at  $62 \pm 14 \mu\text{m}/\text{min}$ , whereas the five largest cells moved at  $9 \pm 4 \mu\text{m}/\text{min}$ . Therefore, this experiment allows to conclude that the smaller cells have a higher average speed in the amoeba population moving on glass. This original observation opens the door to further studies on the molecular mechanisms sustaining the correlation between size and speed of *E. histolytica* when moving on a planar and neutral surface such as glass.

## Conclusion

We expect this protocol to serve as a beginner's guide for cell biologists that would like to capture the morphodynamical characteristics of their live cell populations in a quantitative manner by using image analysis. The results are any potential correlations between multiple morphodynamical descriptors (in the present case, we found a link between cell size and speed), as well as the possible discovery of criteria that can tell apart subpopulations of cells.

## Materials and Basic Methods

### Biological Materials

- Trophozoites of *Entamoeba histolytica* strain HM1:IMSS growing in TYI-S33 media (Diamond et al. 1978).
- Cell Tracker™ Red CMTPIX (ThermoFisher, catalog number C34554, final concentration  $2.5 \mu\text{M}$ ). Before use, suspend the desiccated dye ( $50 \mu\text{g}$ ) in  $8.33 \mu\text{l}$  of DMSO to obtain a  $10 \text{ mM}$  stock solution. An intermediate dilution (1/200) has to be prepared to avoid aggregates of DMSO and Cell Tracker in the media.
- $35 \text{ mm}$  high glass-bottom Ibidi dish (catalog number 81158, Ibidi, France).

### Equipment

- Microbiological safety station with laminar flow to manipulate the cells; wearing a blouse and gloves is mandatory during the experimental steps.
- Spinning disk confocal microscope (UltraVIEW VoX, Perkin Elmer, USA; excitation:  $561 \text{ nm}$ ; objective:  $25\times$ ; temperature control set to  $37 \text{ }^\circ\text{C}$ ).

## 297 Softwares

298

- 299 – Volocity (Perkin Elmer, USA) to perform imaging.
- 300 – Icy (Institut Pasteur, France) to perform image analysis.

## 301 Summary of the Protocol

302 **Procedure**—The protocol can be summarised as a general workflow (Fig. 25.8) in  
303 the following steps: culture the cells and label them with a fluorescent cytoplasm dye;  
304 image the cells with a temporal resolution that is appropriate to the cell movement.  
305 Save the video sequences on the hard-disk; open the Icy software and allocate RAM  
306 according to the expected image size, open the sequence, and double-check the  
307 metadata; draw initial ROIs over the cells and run the Active Contours plug-in; send  
308 the resulting segmentation to the Track Manager, use the different track processors  
309 to analyse cell movement and shape and export them to Excel; perform statistical  
310 and correlation tests on the data, for example using R.

311 **Timing**—Cell labelling and preparation takes between one and two hours. Live  
312 imaging only involves setting up the sample on the microscope and taking multiple  
313 video sequences of around 240 frames (i.e. around 4 min). Segmentation and tracking  
314 takes a fraction of a second per frame. Statistical analysis takes well under an hour.

315 **Troubleshooting**—1. Check that the Java version in your computer is compatible  
316 with Icy. 2. From within the preferences tab in Icy assign RAM memory to the  
317 software according to the potential size of your images. 3. Check that your temporal  
318 resolution is adequate: if there are too many frames per second compared to the speed  
319 of the cells, remove frames in constant intervals in order to lift some computational  
320 burden. 4. All stages of the quantification can be saved in their corresponding formats.  
321 For example, image sequences can be saved in.tif, whereas ROIs and tracks are saved  
322 in.xml. This guarantees complete reproducibility, as slightly different ROIs can result  
323 in slightly different segmentations.

324 **Data availability**—All data presented in this protocol (files as.tif,.xml,.avi,.mov)  
325 and tutorials are available online (Manich 2020) so that any potential user can  
326 reproduce the results by following the protocol.

327 **Acknowledgements** We acknowledge Marion Louveaux for advice on image analysis repro-  
328 ducibility and data online availability. We are grateful to the technical unit of BioImagerie Pho-  
329 tonique of Institut Pasteur for their help with microscopy experiments. A.B.P. is part of the  
330 Pasteur-Paris University (PPU) International Ph.D. Program.

331 **Funding** This project has received funding from the European Union’s Horizon 2020 research and  
332 innovation program under the Marie Skłodowska-Curie grant agreement No 665807, the Institut  
333 Carnot Pasteur Microbes & Sante (ANR 16 CARN0023-01), the Labex IBEID (ANR-10-LABX-62-  
334 IBEID), France-BioImaging infrastructure (ANR-10-INBS-04) and the program PIA INCEPTION  
335 (ANR-16-CONV-0005).



Grow the cells until a pre-determined confluency  
Label the cells with fluorescent cytoplasmic marker

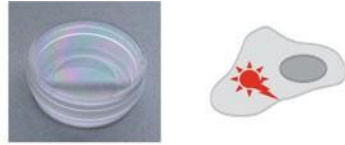
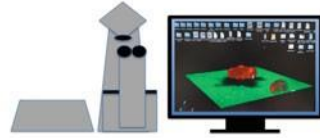


Image the cells with an appropriate microscope, ideally with optical sectioning



Open the Icy software, load the image sequences  
Check the image properties (e.g. time, pixel size) and the available RAM  
Pay special attention to the exposure time and the pixel size



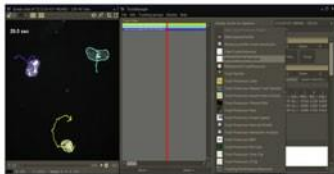
Initialize segmentation with HKM or draw approximative ROIs



Run Active Contours to get the contour of the cells and to track their centroids



Select Processors in the Track Manager  
Export data on Excel files



Perform statistical tests  
Interpret the results and raise a biological conclusion

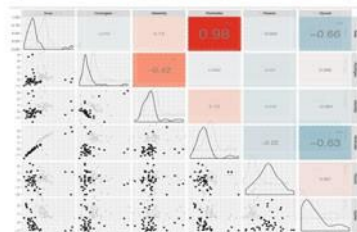


Fig. 25.8 Summary of the protocol. See text for a complete description of the protocole

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