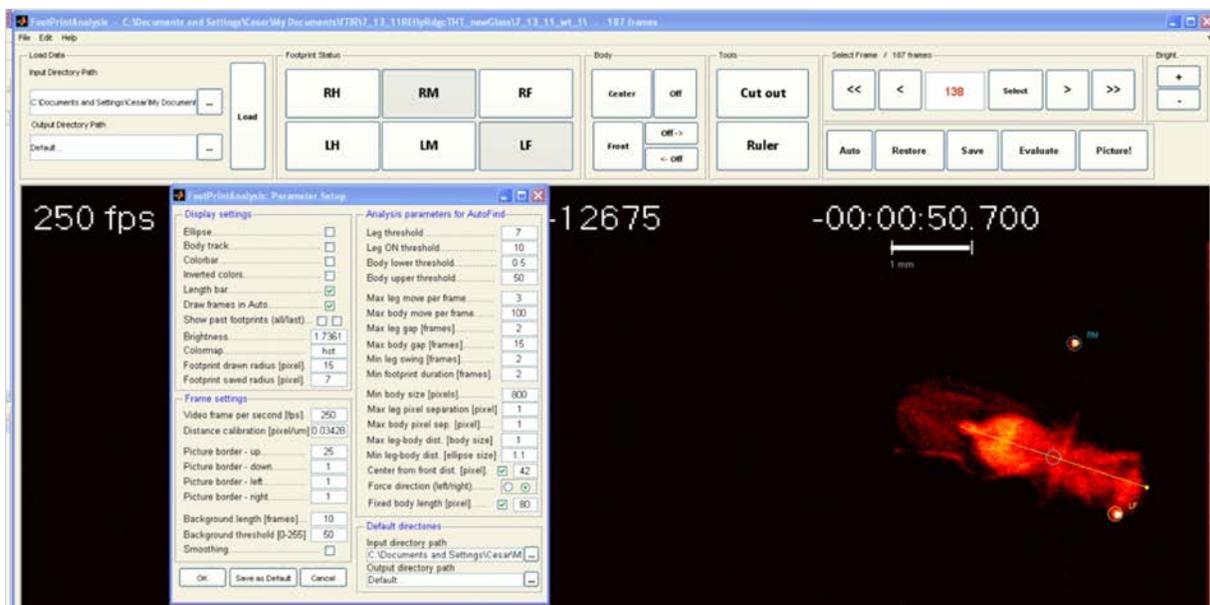


# FLYWALKER MANUAL



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## 1. Installation

FlyWalker can be downloaded from <http://biooptics.markalab.org/FlyWalker/>

Make sure to download the version that corresponds to your operating system.

If you have [Matlab™](#) or [Matlab Compiler Runtime \(MCR\)](#) installed on your computer, proceed downloading the standalone executable **FlyWalker.exe**.

If Matlab or MCR are not installed on your computer, download **FlyWalker\_install.exe**. This installation will first install MCR on your computer. After the program self-extracts, you can use the program running FlyWalker.exe.

## 2. Description

The FlyWalker program allows the tracking and quantification of the walking behavior of a hexapod using the frustrated Total Internal Reflection (fTIR) optical technique. The program can automatically identify and track footprints (FP), body center and body orientation. In addition, it allows the user to manually edit the tracked video so that the tracking is complete and properly done. Finally, it can statistically analyze the walking behavior by generating a large set of graphs and output the results in an excel spreadsheet with the quantification of gait and other parameters.

FlyWalker can analyze movies saved as image sequences. Note that only the sequence files can be present in the analyzed directory. The program can read the following graphic file extension formats:

BMP — Windows Bitmap	JPEG — Joint Photographic Experts Group	PNG — Portable Network Graphics
CUR — Cursor File	JPEG 2000 — Joint Photographic Experts Group 2000	PPM — Portable Pixmap
GIF — Graphics Interchange Format	PBM — Portable Bitmap	RAS — Sun Raster
HDF4 — Hierarchical Data Format	PCX — Windows Paintbrush	TIFF — Tagged Image File Format
ICO — Icon File	PGM — Portable Graymap	XWD — X Window Dump

Auto tracking is based on cluster identification created on the brightness of an image within preset thresholds, as well as information from preceding frames.

## I. Quick guide

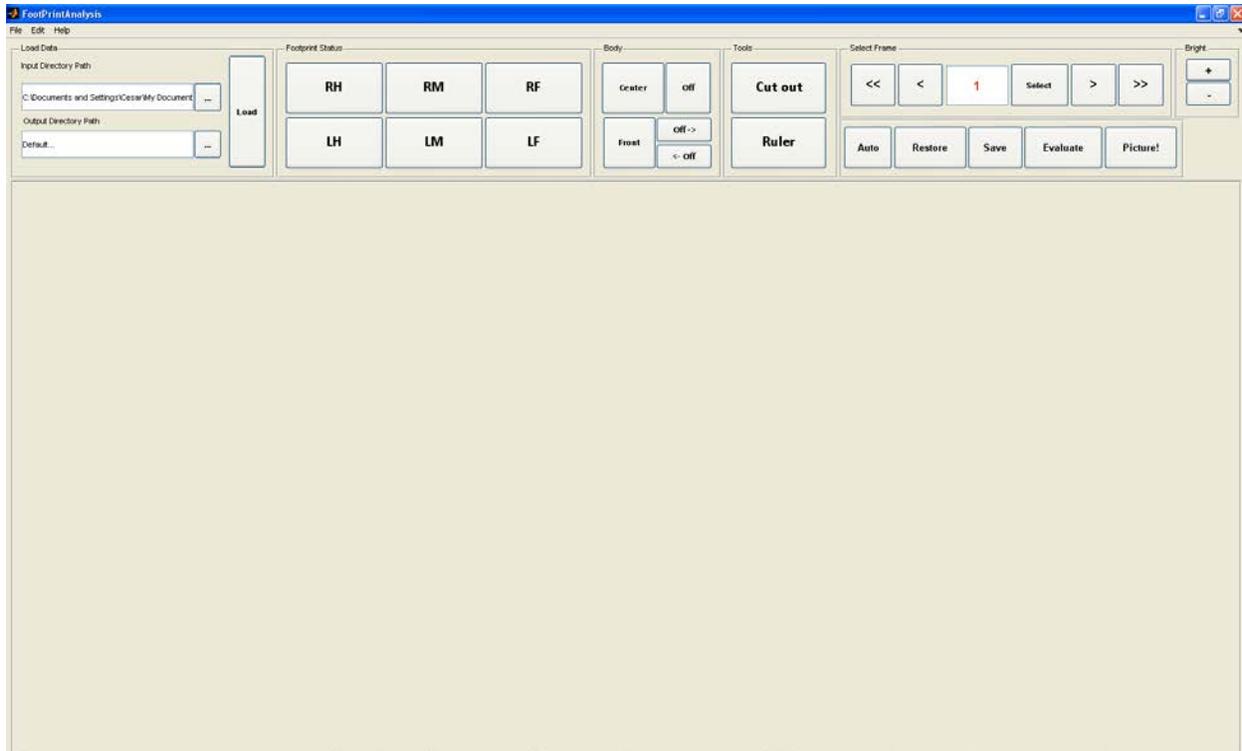
From acquiring the videos until quantification of the walking behavior, the following steps must be taken:

1. Set and verify the tracking settings. Importantly, scale calibration and animal size.
2. Complete auto tracking
3. Cure the tracked video.
4. Statistically evaluate the tracked video.

Below, **Sections II** and **III** describe the program's main window and setup window, respectively. **Sections IV** describes the list of shortcuts; **Section V** describes how to best setup the animal's size and **Section VI** discusses how to correct a tracked video.

## II. FlyWalker main window

Once FlyWalker is opened, the main window should open (**Figure 1**).



**Figure 1.** FlyWalker interface.

The main window is divided into several panels that allow the user to load the movies; scroll the movies; enable auto-tracking; cure the tracking and evaluate the tracked movie.

1. **Load Data:** this panel defines the directory where movie frames are loaded from (**Input Directory Path**), and the directory where results are saved to (**Output Directory Path**). The user can specify the output directory as "Default...", which will be "<Input Directory Path>/Results/". Data is loaded by pressing the **Load Data** button. Note that Load Data also loads the output that was generated previously for the given video.
2. **Footprint Status:** for a particular frame, each leg can be turned on or off manually (or of course, could be turned on/off by auto-tracking). The legs that are present on the given frame shown on the screen are indicated by the pressed state of the respective buttons (L,R,H,M and F correspond to left, right, hind, middle and fore, respectively). If a leg is not present and the user decides to place it, they can press the respective leg button, after which they can place the footprint on the screen using the mouse. In this case, the footprint will be placed for the current frame as well as the consecutive frames to the same location, constantly until two frames before the next appearance of the same footprint. For example, if the right foreleg (RF) does not appear on frames #40-49, but appears on frame #50, placing the footprint on frame #40 will

place it on frames #40-48. If the button was pressed erroneously and the user is prompted to place the footprint using the mouse, clicking outside the screen will cancel the action.

### 3. Body

- a. **Center:** lets the user place animal's body center manually using the mouse.
- b. **Front:** lets the user set manually the front of the animal. The front point, together with the center point (3.a), are used to calculate the direction and the length of the animal.
- c. **Off:** turns off labeled footprints and body features in the present frame.
- d. **Off->:** turns off labeled footprints and body features from the selected frame onwards. Useful to trim the tracking at the end of the video.
- e. **<-Off:** turns off labeled footprints and body features from the selected frame backwards. Useful to trim the tracking at the beginning of the video.

### 4. Tools

- a. **Cut out:** removes a user-defined rectangle from the whole movie. Once pressed, the user can specify the diagonal of a rectangle by consecutively placing the two end points of the diagonal using the mouse. The area of the rectangle will be cut out from the movie, i.e. it will be taken to have no information on the fly and will be turned dark. This feature is useful to remove parts of the movie that interfere with the tracking, e.g. dirt particles or defects in the glass. Note that the selected region will be removed during the entire duration of the movie. If a mistake was done, where for example, the region where a footprint will or was located was removed, the user can press **Restore** (see below). This feature can be used as many times as necessary.
  - b. **Ruler:** lets the user measure distances on the screen. Once pressed the user needs to indicate two points using the mouse. In return the program outputs the distance in pixels and micrometers on the upper left corner of the screen. Note that while the program can measure the distance in pixels, the output distance in micrometers uses the calibration parameter specified by the user (see below).
5. **Select Frame / <N> Frames:** allows the user to scroll the frames of the movie. The total number of frames present is indicated in the place of <N>.
- a. **<<:** jumps to the first frame.
  - b. **<:** moves one frame backwards.

- c. **Current frame:** the frame that is currently being visualized is indicated. Alternatively, the user can input the frame number manually and press **Select**.
  - d. **>:** moves one frame forward.
  - e. **>>:** jumps to the last frame.
6. **Auto:** once pressed, the program starts the auto-tracking of the frames starting from the current frame. This latter feature is important as the auto-tracking algorithm uses the leg positions on the previous frame for each frame for footprint-identification. For instance the user can specify the footprint locations on a given frame, and by starting auto-tracking on the next frame, the program will use the specified locations as initial condition.
  7. **Restore:** restores tracking information to the last saved status. I.e. restores footprint and body information, cut-out regions, etc.
  8. **Save:** saves all tracking information, as well as cut-out regions.
  9. **Evaluate:** statistically evaluates the collected tracking information, and creates a set of graphs and an excel spreadsheet. Quantification will only take into account tracked information (not untracked video). Note that the target file cannot be opened while the evaluation is ongoing, in case the same video is reevaluated. The files will be saved in a subdirectory where the original files were located (default directory: "Results"; thereafter Results folder). Incorrectly tracked videos will result in incorrect gait values.
  10. **Picture!:** creates a subfolder within the Results folder, called "Images", and starts saving images of the frames of the input video, with the addition of tracking information, as seen on the screen. Image saving starts on the current frame, and goes on continuously until the user presses the Picture! button again, that terminates the process. To save the complete video, the Picture! button has to be pressed on the first frame of the video. The features that are saved on the sequence files can be set on the Setup window (see below).
  11. **Bright.:** Increases (+) or decreases (-) the image brightness, as seen on the screen (i.e. it doesn't affect the tracking that uses the brightness thresholds set in Settings!). Useful to more easily identify the footprint onset/offset by the eye.

### III. Setup window

Before loading a movie (or starting the auto tracking), the settings must be properly set so that auto tracking and the measuring features are accurate. In order to open the Settings window go to Edit>Settings or press Control+S. Figure 2. displays the settings window.

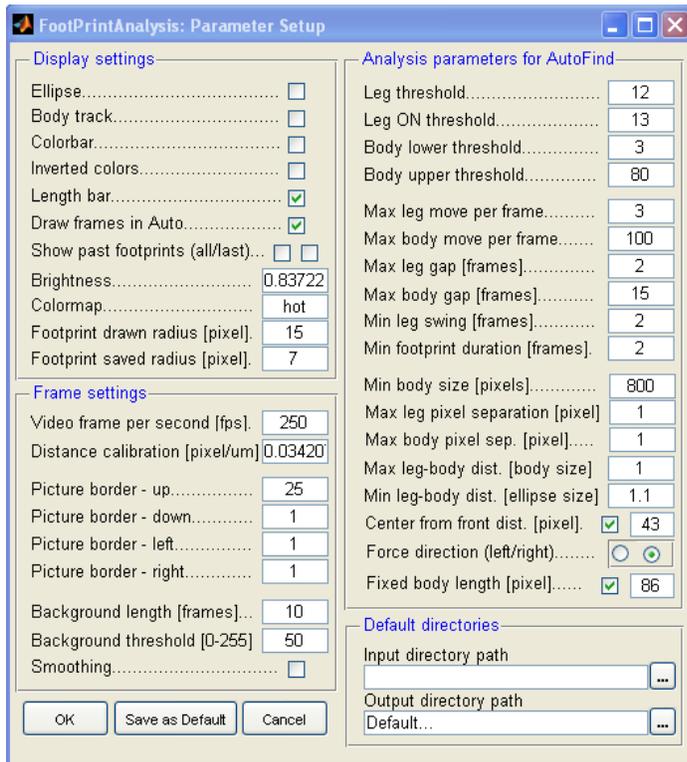
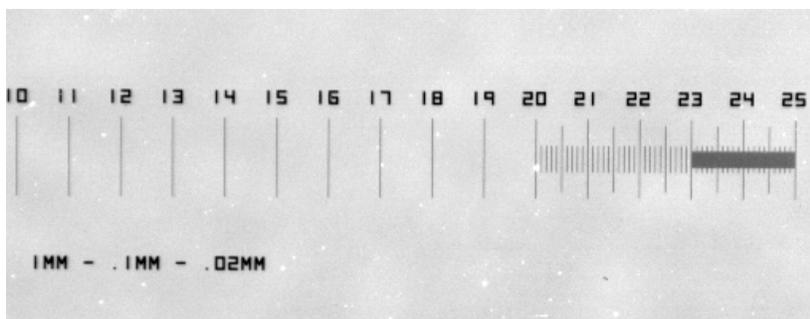


Figure 2. Settings window.

The Settings window is divided into four panels: Display settings; Frame setting; Analysis parameters for AutoFind; and Default directories. Settings can be set for a particular movie or set as default.

1. **Display settings:** addresses the features important for the visualization of the results on the screen (and which are saved in the output images).
  - a. **Ellipse:** if checked, an ellipse is drawn around the tracked animal, following its length and width.
  - b. **Body track:** if checked, shows the track of the body center for all frames prior the current frame.
  - c. **Color bar:** since pixel intensity is color-coded, the scale can be displayed on the right side of the screen.

- d. **Invert colors:** invert the colors on the frames, making bright dark and dark bright.
  - e. **Length bar:** if checked, shows a 1mm scale on the screen.
  - f. **Draw frames in Auto:** if checked, running Auto (i.e. auto-tracking) will redraw the frames with the newly tracked information on them so the user can visually follow how tracking is progressing. It allows the user to determine if the auto tracking is being done correctly.
  - g. **Show past footprint (all/last):** displays all past footprints in gray. If all is checked, past footprints are shown in all frames. If last is checked, past footprints are shown only in the last frame.
  - h. **Brightness:** sets the intensity of the loaded frames as shown on the screen (i.e. not for the tracking!).
  - i. **Color map:** sets the color scheme used to represent brightness for the frames. Options: 'Jet', 'HSV', 'Hot', 'Cool', 'Spring', 'Summer', 'Autumn', 'Winter', 'Gray', 'Bone', 'Copper', 'Pink' and 'Lines'. See <http://www.mathworks.com/help/techdoc/ref/colormap.html> for how these color scales look like.
  - j. **Footprint drawn radius [pixel]:** sets the radius of the circles that indicate each footprint, in pixels.
  - k. **Footprint saved radius [pixel]:** sets the radius of the circles that indicate each footprint, that is saved in the images (i.e. one can separately set the radius shown on the screen and the radius shown on the saved images!).
2. **Frame settings:** addresses the features of the video for calibration purposes, and to specify which part of the video should be used for the analysis.
- a. **Video frame per second [fps]:** specifies the frame rate of the loaded video. This is important for the program to be able to measure time in the video.
  - b. **Distance calibration [pixel/ $\mu\text{m}$ ]:** Specifies how many pixels correspond to a micrometer in the video. The FlyWalker can generate parameter values in millimeters or micrometers. In order for these values to be correct, each set of videos has to be calibrated with a reticle. For this, before or after a video session, take a snapshot (or small video) of a reticle on top of the glass (Figure 3). Afterwards on imageJ measure the amount of pixels per millimeter and calculate the pixel to  $\mu\text{m}$  ratio.



**Figure 3.** Reticle for distance calibration.

- c. **Picture border (up; down; left and right):** defines the part of the images that should be used for the analysis. Sets the borders (in pixels) which are ignored by the tracker. Useful if a part of the video has a moving element, such as a time stamp.
  - d. **Background length [frames]:** Any FTIR signal present in frames prior to the appearance of the fly (due for example a dust particle or a defect in the glass) can be subtracted from all subsequent frames in order to minimize false positives. These frames are the last ones in the movie sequence. This parameter sets the number of frames at the end of the series of frames in the frame folder that will be used for calculating the background masks. Note that the background images are used from the end of the video as the fly might carry dirt with it that will appear in the video at an arbitrary time, i.e. they might not be present right at the beginning.
  - e. **Background threshold [0-255]:** pixels below this threshold will be considered to be background, and will not be used in obtaining the background that will be subtracted from every image. I.e. small fluctuations of the brightness due to the “flickering” of the illumination will not be taken into account in the background calculation, only real defects present on the screen with no fly on. Image information is stored using a 0-255 brightness scale (0 being completely dark and 255 being completely bright).
  - f. **Smoothing:** Defines level of smoothing of the frame pictures. Smoothing is a low-pass spatial filter used to remove non-physical small scale variability that may affect the results (the brightness of the fly may only change on a longer scale, while background brightness fluctuation due to noise may be significant on the pixel-scale). 0 if there should be no smoothing. 'n' if there should be smoothing over a distance of n from a given pixel. E.g. if smoothing is 2 then the pixels value will be the average of the pixels over a  $2*n+1$  rectangle centered around the pixel.
3. **Analysis parameters for AutoFind:** settings used by the auto tracking algorithm. These settings determine if the body and footprints are detected and the quality of the tracking. The User may want to iteratively tune these parameters upon seeing how the auto-tracker performs with some given parameters.

- a. **Leg threshold:** minimum pixel brightness over which a pixel is considered to be potentially part of a footprint, given that the footprint was already present in the previous frame. A footprint will only be identified if its brightness is above this threshold (see also 3.b).
- b. **Leg ON threshold:** minimum pixel brightness for which a pixel will be considered to be the part of a footprint that has not been present on the previous frame (i.e. that just turned “on”). This parameter is specified separately from “Leg threshold” as if a footprint is already identified, we have increased confidence that something that resembles a footprint in the vicinity of a previously identified footprint is likely a footprint. In the same time, if nothing was present somewhere, we need to be more certain that it is not just flickering background. Therefore “Leg ON threshold” should be greater than “Leg threshold”.
- c. **Body lower threshold:** minimum pixel brightness for a pixel to be considered to be the part of the body.
- d. **Body upper threshold:** maximum pixel brightness for a pixel to be considered to be the part of the body. We define an upper threshold for the body as brighter pixels may be footprints and not body parts.
- e. **Max leg move per frame:** maximum displacement allowed for a footprint once it is detected. We will consider a footprint to be the continuation of a previous one only if they are within this distance from each other. Greater distances indicate that the footprints are by a different foot.
- f. **Max body move per frame:** maximum allowed displacement of the body once it is detected. The fly can’t “jump” more than this per frame.
- g. **Max leg gap [frames]:** even if a footprint is not identified in a frame, it may be only due to noise. If a leg is detected, disappears, and then shortly appears again, the program will assume that the disappearance is only because of misreconstruction, will fill in the gap, and will consider the newly appeared footprint to be the continuation of the previous one. The number of frames for which the footprint can disappear without the program considering it a new footprint is specified by this variable. Of course, the new footprint has to be in the vicinity of the old one in order to be considered the continuation, as specified by 3.f.
- h. **Max body gap [frames].** same as “Max leg gap”, but for the body.
- i. **Min leg swing [frames]:** a given leg has to be swinging for at least this number of frames before it can reappear again. This means that a footprint will not be identified as a given leg if the given leg was down in less than “min leg swing” frames ago. For example if a leg typically swings for 10 frames, and a footprint appears 3 frames after the RF footprint disappeared, it is more likely that it is RM.
- j. **Min footprint duration [frames]:** minimum number of frames for which a footprint is considered a footprint. If the number of frames for which the footprint is continuously

present is shorter than this value, the frame is cut out OF THE ANALYSIS, although it will still appear in the video.

- k. **Min body size [pixels]:** Define the minimum size of the cluster of pixels which is identified as the body. Below this value no body (and footprints) will be identified.
  - l. **Max leg pixel separation [pixel]:** maximum empty space (gap) allowed between two pixels that are the parts of the same leg. If there are two bright pixels and they are less than this value apart, they will be considered to be the parts of the same footprint, even if the pixels between them are dark.
  - m. **Max body pixel sep. [pixel]:** maximum separation allowed (in pixels) between two pixels being part of the same body. Similar to “Max leg gap”.
  - n. **Max leg-body dist. [body size]:** maximum distance between a footprint and the body center in units of body length. The leg has to be closer than this parameter to the center of the body to be considered a leg.
  - o. **Min leg-body dist. [ellipse size]:** minimum distance of legs from the center of the body, defined as the size of the ellipse that circles the body (i.e. the allowed distance depends on the direction of the footprint from the body). In the simple case of this value being 1, footprints will only be considered to be footprints if they are outside of the ellipse. This value only applies for the onset of the footprint, therefore it can “flow in” within this distance!
  - p. **Center from front dist. [pixel]:** if checked, the center of the body will be placed along the automatically reconstructed body direction, at the specified distance from the foremost point of the body, as identified by the tracking algorithm.
  - q. **Force direction (left/right):** Forces the body axis to move to the left or to the right, i.e. allows auto-tracking to only consider the fly going in the specified direction.
  - r. **Fixed body length [pixel]:** chooses between using automatic length detection (check mark), or a defined fixed distance.
4. **Default directories:** sets the default directory where the movie sequence can be found and where the files generated by the program are saved. These values will be given to every newly analyzed video, or will appear every time one starts FlyWalker.

## IV. Shortcuts

Leg/Body Shortcuts	
Right Front	e
Left Front	d
Right Middle	w
Left Middle	s
Right Hind	q
Left Hind	a
Center	r
Front	f
Frame shortcuts	
<	z / left arrow
>	c / right arrow
Select	x / down arrow

## V. Animal size settings

In order to set the proper size settings (body length and distance to centroid) go to the first set of frames where the fly shows up and use the ruler tool to measure the Anterior/Posterior size of the fly in pixels. Run the Auto button to confirm that the line matches the length of the fly. Also check that the centroid is between the midlegs. If not, change the Fixed body length and Center from front dist. parameters accordingly. Test the Auto mode for a few frames in order to confirm the settings.

## VI. Footprint editing

Once the auto tracing feature was completed, the user needs most of the times to correct mislabeled footprints. Footprints can be deleted or inserted by pressing the according button. If a footprint is deleted, then all successive (and continuous) footprints will also be deleted. Similarly, if a footprint is added it will stay in the subsequent frames until the same label reappears elsewhere (See section II.2.).

If a footprint is absent in a particular frame but present in the precisely subsequent one, then the user can press the corresponding footprint key plus the Shift key. In this case the footprint will be placed precisely on the same location as the following frame. On the other hand, if the footprint is absent in a particular frame but present in the precisely preceding one, then the user can press the corresponding footprint key plus the Shift and Alt keys. In this case the footprint will be placed precisely on the same location as the preceding frame.

Pressing "Evaluate" will generate a large set of plots. By quickly inspecting these graphs, the user will realize if the tracking was correctly done, in particular if "lost" footprints were overlooked. In these situations correct the problem and re-evaluate the video.