Work no 9: Cell tracking

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1 Purpose of the work

Your goal is to learn the effects of different parameters when tracking live cells.

2 Background

2.1 General

Tracking objects requires the objects to be identified, which requires the objects to be separated from background. This chain of processes can be roughly divided into three parts: segmentation, counting, and tracking.

2.2 Segmentation

Segmentation is the part where signal (objects) is separated from background. Easiest method is to select an intensity level and set all brighter pixels as part of an object, and darker pixels as background. This operation is called thresholding. The resulting binary image (mask) can then be modified with binary operators (dilation, erosion...). Also other means of thresholding exist, for example edge thresholding, in which steep gradients (quick changes) are selected as objects.

2.3 Counting

Counting means giving labels to thresholded areas, or counting how many continuous regions thresholding returned. Thresholding is difficult with noisy images and, thus, other criteria such as object area, shape, intensity, or any other calculable property may also be used for selecting valid objects. The selection procedure is part of counting.

2.4 Tracking

Tracking is about finding out which counted objects are the same objects throughout a time series. Main difficulties in tracking are:
- Sparse sampling frequency in time makes objects move a long distance between images. Tracks are lost.
- Objects moving close to each other or even overlapping are segmented as one object, and one of the tracks is lost.
- Weak thresholding makes objects vanish at times, which may increase confusion in objects moving close to each other. Thus, tracking algorithm must remember a few time-steps backwards.

2.5 Software

In this practice you will be using Image-Pro Plus 6.1 image analysis program and Microsoft Excel for numerical analysis.

2.6 Information on sample

In this practice you are using the data acquired earlier in live cell imaging practice (work 6). The image data should either be a single TIFF stack image, or series of separate TIFF images of moving chloroplasts.

3 Your task

- Find out the correlation between cell velocity and cell size (area).
- Find out the distributions of mean velocities and areas over all tracks.

4 Practical instructions

4.1 Starting up

- Start Image-Pro Plus.
- Select complete menu set.
- Click 'done' when Macro Player opens.
- Drag & drop your TIFF stack in the Image-Pro Plus window (load entire sequence), or go to menu *Sequence*/*merge files..* if you have a series of TIFF images.

4.2 Work flow of tracking objects

The basic work flow of tracking objects starts by separating the objects and background. The easiest way of doing this is thresholding each time frame into a binary image. The next phase is to set limits to the objects' sizes and forms, since thresholding usually returns some false results. Finally, the objects at different time points are linked to each other, producing tracks of identified objects over time. A selection of measurements is done on the objects, and the results analyzed.

Each of these steps contains various adjustable attributes. All of them must be considered each time a new set of data is segmented and tracked. Make sure to write down your parameter choices!

4.3 Display range

Often the images' intensity ranges are at the low end of the possible values (e.g. 12-bit data saved in 16-bit format), and the default mapping from these values to monitor brightness is linear. In practice it means that the acquired image is usually all black when opened in Image-Pro Plus. Therefore, you have to adjust the display range. Go to *Enhance* menu, and select *Display range* (Fig. 1).
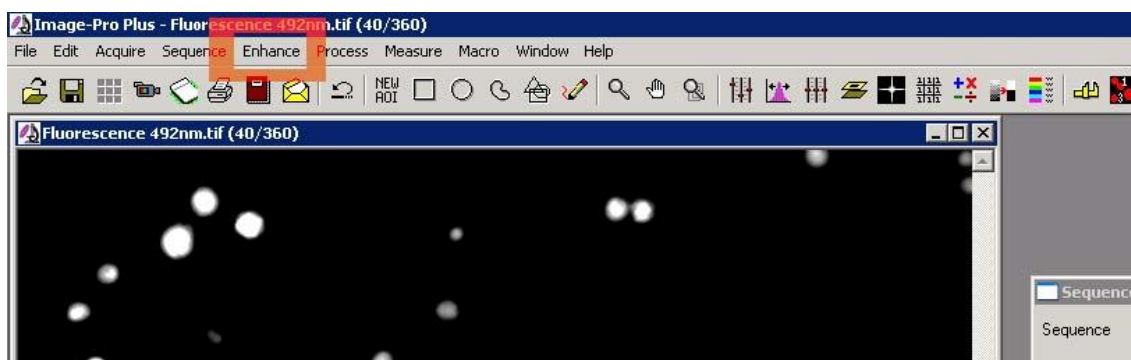

Figure 1: Enhance menu

Define the ends of the histogram so that the image data are well visible but the objects are not saturated (Fig. 2). Usually you only have to adjust the maximum value. Do NOT click apply, because that will alter the image data according to the selected intensity mapping. For scientific analysis, you should keep the manipulation of the original data to minimum. If any manipulation is done, it should be well documented with reasons why it was done. Although we may manipulate the data to acquire better thresholding, the

measurements can still be done on the original data by moving the resulting mask on top of the original image data.

4.4 Background removal

Before the actual thresholding, it is often useful to enhance the image set for easier thresholding. One possible enhancement is the removal of background. First, make a copy of the image sequence by selecting *Edit/Duplicate*. Go to *Process/Filter*s and apply a low pass filter in the Enhancement tab. Blur the image so that all objects are non-distinguishable (Fig. 3(a)) by using a 7x7 kernel mask and increasing the amount of passes. By clicking the sample image, the preview is refreshed with current settings.
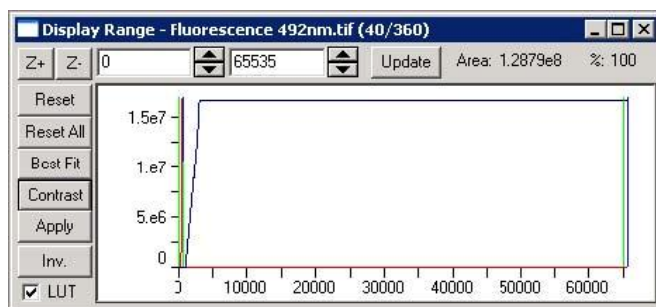

Figure 2: Adjusting display range

Apply the low pass filter. Next, go to *Process/Image Operation*s and subtract the blurred image from the original image (Fig. 3(b)). The 1st operand is set to the original image sequence and the 2nd operand is the blurred image. You may output the result in a new image and save the sequence to prevent losses in case of a crash. If the result image is dark, you have to adjust the display range as described earlier. Usually there is no need to adjust background further for better thresholding. You may still return to this phase for a tune-up later on.
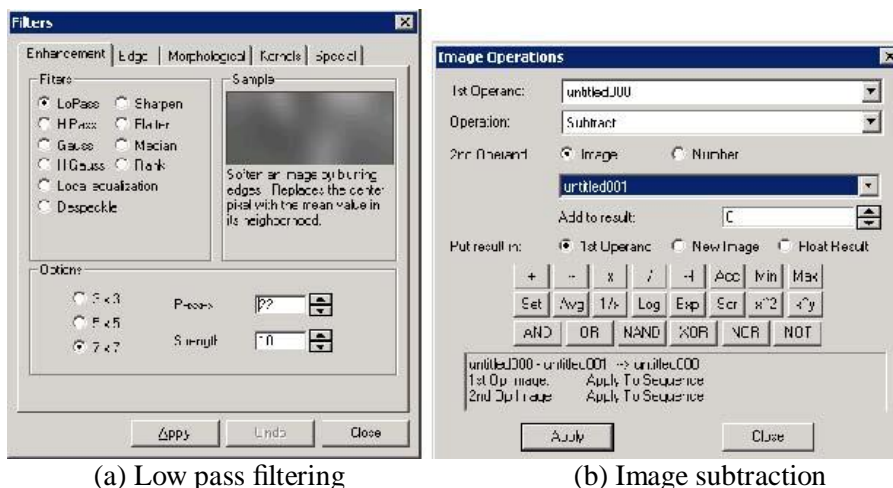

(a) Low pass filtering   (b) Image subtraction

Figure 3: Background removal

4.5 Setting the track measurements and auto-tracking attributes

Next, open the object tracker from *Measure/Track Object*s (Fig. 4). Open the settings window by clicking the icon with a hammer and screwdriver. From measurements tab, select velocity and area measurements (Fig. 5(a)). Be sure to select the mean values over time to prevent too many values in the result window. In the View/Output tab (Fig. 5(b)), you can choose the coloring mode of the tracks (random or fixed). Sometimes it is more convenient to see all tracks in one color for finding continuous tracks that the algorithm has split into two.
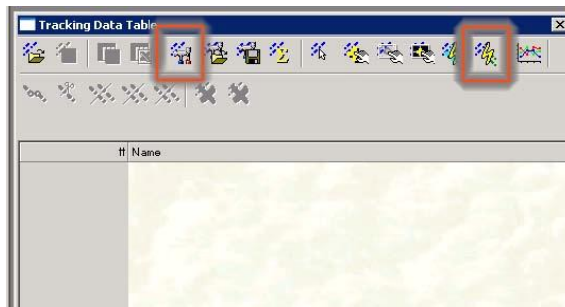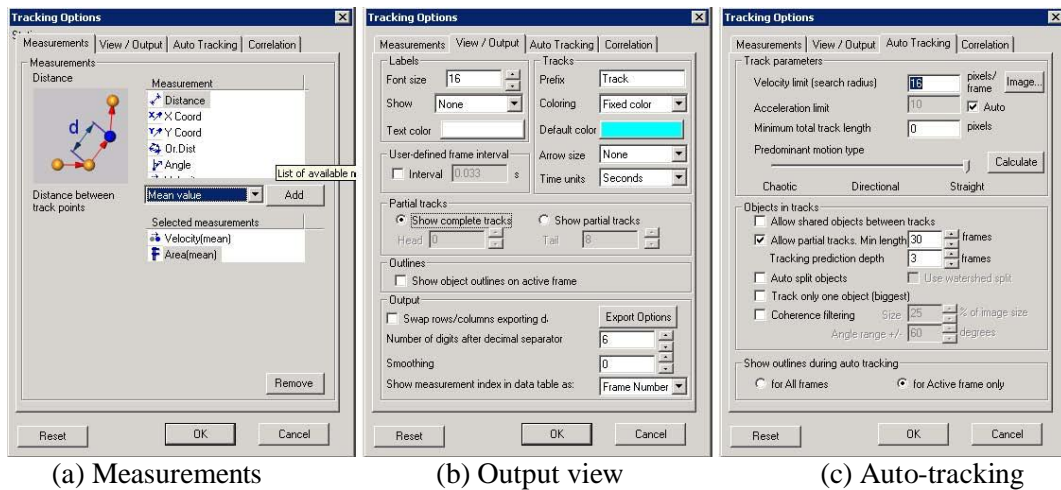
3

Figure 4: Object tracker window

(a) Measurements   (b) Output view   (c) Auto-tracking

Figure 5: Tracking options

The partial tracks setting determines whether the tracks are shown as complete in every time frame, or only the past part from the chosen time frame is shown. Try out the options to see the difference later on. Auto Tracking tab contains the parameters for the tracking itself (Fig. 5(c)). Velocity setting tells the algorithm how far it should look around when searching for the object in the next time frame. If there are many objects in the image sequence, you may want to check the *Allow partial tracks* and set its minimum length to at least one quarter of the sequence length. This operation only takes into account tracks that are very stable and well-segmented. If your thresholding has a fairly low value, which often results in objects clumped together, you may choose to *Auto split objects*. Auto-splitting is a CPU consuming procedure, which will slow down the tracking dramatically. Note that you do not know the amount of clumping until the next step in the process, so you may have to come back to the options later.

4.6 Choosing the threshold level

Close the options window and start auto-tracking from the rightmost lightning icon. The program will present a warning and open up the Count/Size window (Fig. 6). In the Count/Size window, click *Select ranges* to adjust thresholding level for segmentation.
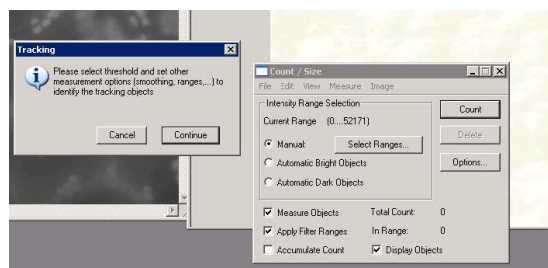

Figure 6: Starting up auto tracker

In the Segmentation window (Fig. 7), move the red selection area to find out the threshold level. Move both ends of the area until the cells are filled red, and the background is unfilled. Now you can see the amount of clumping, which determines whether you should select automatic object splitting in tracking options. Remember to browse through the whole image sequence with the Sequence Toolbar when adjusting the threshold level.

4.7 Object restrictions

Click Close to get back to Count/Size window. Open up *Measure* menu and *Select Measurements*, which is used to limit object segmentation (Fig. 8(a)). If thresholding results in merged objects, they can be rejected for the selection by setting a maximum value for the accepted area of the object. Click *Area* in Filter Ranges and then *Edit Range* at the bottom. The new window (Fig. 8(b)) lets you graphically set the limitation values. You should also limit the minumum value to cut out noise.
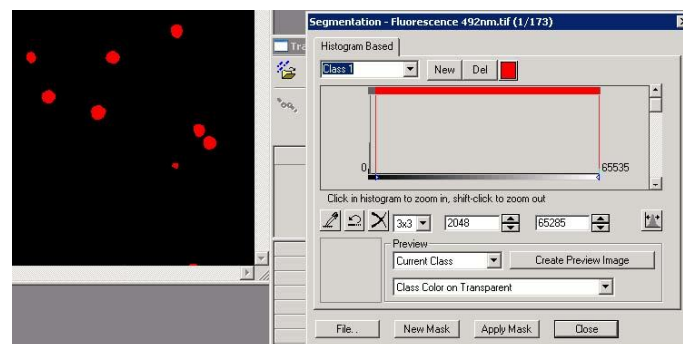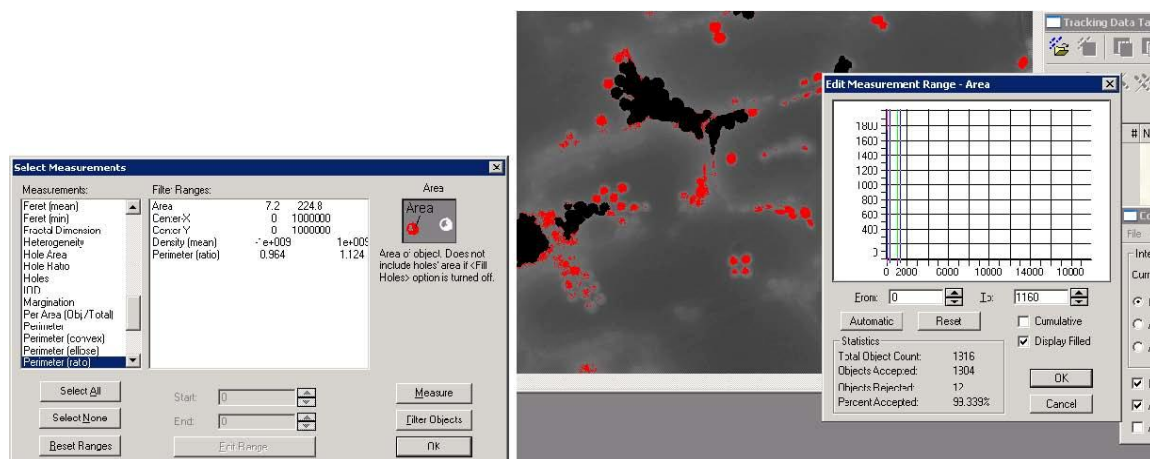

Figure 7: Segmentation threshold selection window

10 pixels^2 is a good starting point. Add a *Perimeter (ratio)* restriction so that only close-to-circular objects are accepted. When clicking *Count* in Count/Size window, the program shows how many objects were found in each time frame and how many of them were discarded due to the ranges set.

(a) Selecting object limits   (b) Graphical range editor

Figure 8: Segmentation attributes

4.8 Initiating auto-tracking

By clicking *Continue* in the Tracking window, the auto-tracker starts. If there are too many tracks you will be notified. Scroll around the tracking data table to see the results. When a row is clicked, the track in question is highlighted in the image. You may try to improve the tracking outcome by changing any of the parameters chosen earlier. The parameters affecting the outcome most are: threshold level (if segmentation is

not good), tracking search area (if tracks are often lost), and object ranges. Try to track at least 20 objects for more reliabile statistics.

4.9 Saving the results

At the top of the data table, there is an *Export to Excel* button. Press it to export the data. Save the data for later use.

4.10 Analysis

4.10.1 Analysis questions

After measuring the mean velocities and areas of the objects over time, at least two kinds of analyses can be done:
On average, does cell area correlate with velocity?
What are the distributions of the mean area and velocity?

4.10.2 Correlation analysis

Here is a reminder of how to produce a linear correlation analysis in Excel:
- Mark the two columns containing *area* and *velocity* values.
- Sort the left column (menu: Data/Sort -select the left column character).
- Click Chart Wizard.
- Select an XY scatter plot with lines and press finish.
- Select the plot (by pressing a left mouse button on the plotted line).
- Right-click -Add Trend line, in options tab select *Display equation* and *Display R-squared.*

What is the R^2-value? Are the measurements correlated?

4.10.3 Distribution analysis

To see how the measurements are distributed, make a histogram of each measurement.
- First create 10 bin centers for the histogram.
- For example, if your values are from 10 to 100, simply create 10 cells with values 10,20,30 ... 90,100. Scale to fit your data in the bins.
- Go to menu Tools/Data Analysis and select Histogram.
- In the histogram window, select the *cell area* values for Input Range and bin values for Bin Range.
- Select output range at an empty spot in the worksheet.
- When bins and frequencies have been printed in the worksheet, select the frequency values and press Chart Wizard.
- Select a clustered column chart (the default type) and press next.
- In the new window, choose Series tab and use the bin values as Category (X) labels.
- Then click finish.
- Repeat the same for *velocities*.

Are the distributions similar? Do they represent any theoretical distribution (e.g. normal, Poisson, χ^2 ...)?