Practical 7
Cell tracking

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Instrument: Imaging Workstation 2, next to room B501a

1 Purpose of the work

Your goal is to learn the practical side of tracking live cells and doing statistical analysis. Prepare yourself to present the results and answer the questions on the last page at the summary meeting.

2 Background

2.1 General

The motivation to track cells is to follow the change of properties for an individual cell over time. The property is often fluorescent brightness, but it may be the speed, roundness or other morphological attribute, etc

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 high gradients (quick changes) are selected as objects.

Watershedding is a basic technique to split objects touching each other. Since continuous regions are counted as one object, two objects joined even with single pixel width bridge are counted as one. Watershedding tries to detect positions where splitting should occur.

2.3 Counting

Counting means giving labels to thresholded areas (also called labeling), or counting how many continuous regions the thresholding returned. Thresholding is difficult with noisy images. Therefore it may be necessary to restrict the valid objects with filters. Filters can be based on object area, shape, intensity or any other calculable property. The selection procedure is also part of counting.

2.4 Tracking

Tracking is about finding out which counted objects are the same objects through a time series. The 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 the other track is lost

- Weak thresholding makes objects vanish at times. Tracking algorithm must remember a few time-steps backwards, which may increase confusion in objects moving close to each other, if the other is temporarily lost

3 Instruments/software used

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

4 Information on the data

The data is a time lapse video of cells. The cells are dish cultured transformed human bone cancer cells, which have been stressed by exposing to unhealthy environment. The blue staining shows the nucleus by coloring the DNA. The green color shows a type of a nuclear grain protein. 50 frames were taken every 20 minutes.

5 Your task

- View the statistical properties of the distributions of densities of the fluorochromes, nuclear areas and roundness

- Find out do any of the variables correlate significantly

6 Practical instructions

6.1 Starting up

Figure 1. Image-Pro Plus icon

- Start Image-Pro Plus

- Load the *senp_unflattened.seq* file from directory D:\MBI (load entire sequence)

6.2 Workflow of tracking objects

The basic workflow 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 restrictions 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.

6.3 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:

- Make a copy of the image sequence by selecting EDIT/DUPLICATE

- Go to PROCESS/FILTERS and preview a low pass filter in the Enhancement tab. Blur the image so that all objects are non-distinguishable (Figure 2 left image) by using a 7x7 kernel mask and increasing the amount of passes

- By clicking the sample image, the preview is refreshed with current settings

- Do not apply the low pass filter, but instead close the duplicate file and open a ready made image: *senp_blur.seq*

- Next, go to PROCESS/IMAGE OPERATIONS and subtract the blurred image from the original image (Figure 2 right image). The 1st operand is set to the original image sequence and the 2nd operand is the blurred image

- Close the original and low passed images to free up memory

- **Why is the result image darker?**

You may output the result in a new image and save the sequence to prevent losses in the 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.
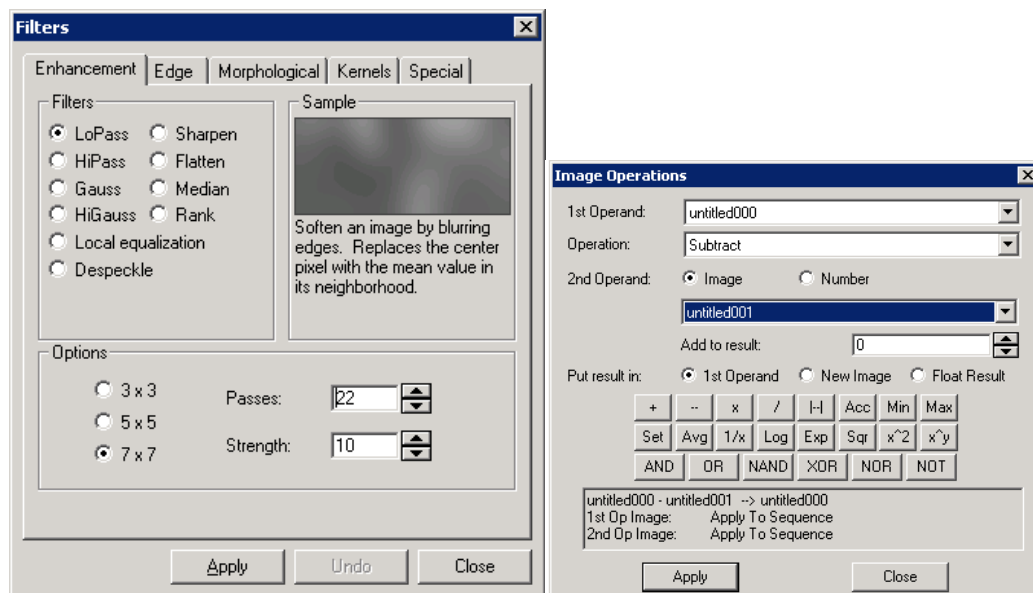

Figure 2. Low pass filtering and image subtraction

6.4 Setting the track measurements and auto-tracking attributes

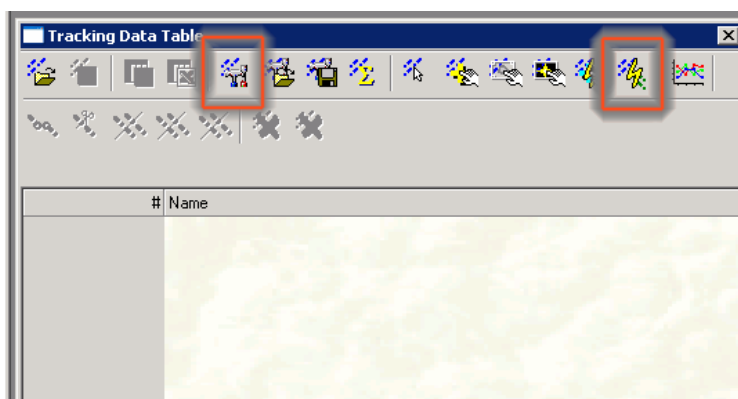- Next, open the object tracker from MEASURE/TRACK OBJECTS (Figure 3).


Figure 3. Object tracker window

- Open the settings window by clicking the icon with a hammer and screwdriver.

- From measurements tab, select Blue and Green Densities, Areas, Aspect, Center-X and Center-Y (Figure 4). Be sure to select the values (and not means) to get all the measurements in the result window. Aspect is the ratio of axes of an ellipse that is fitted on the object. Center-X and –Y are the coordinates of the object center, and they MUST be the 5th and 6th measurement in the list for automated speed measurements later on.
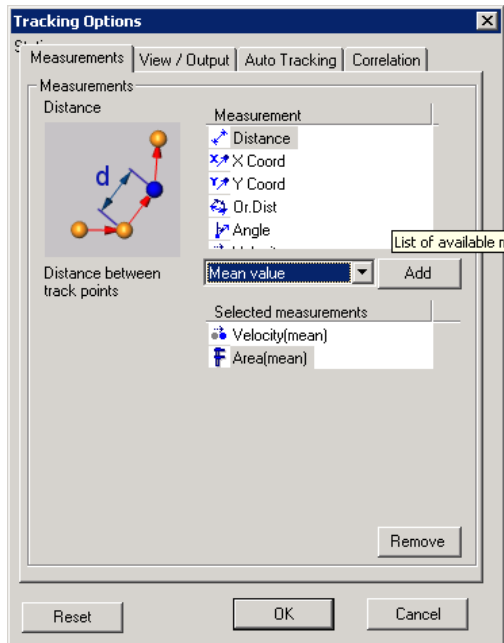
Figure 4. Measurements selection
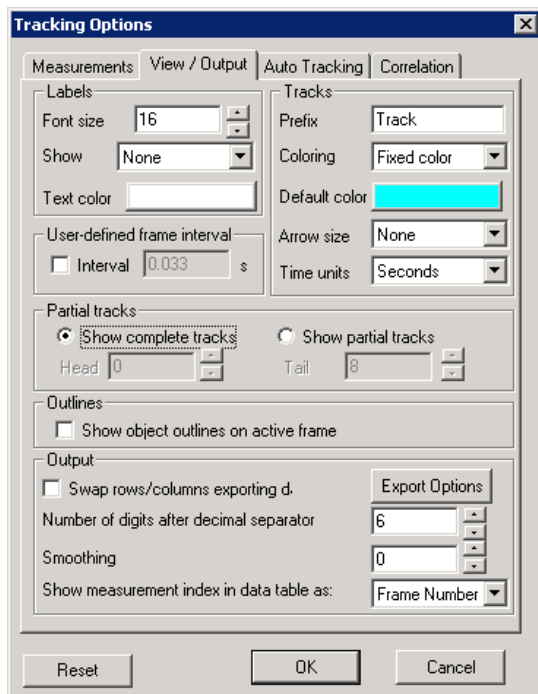
Figure 5. Track view selections
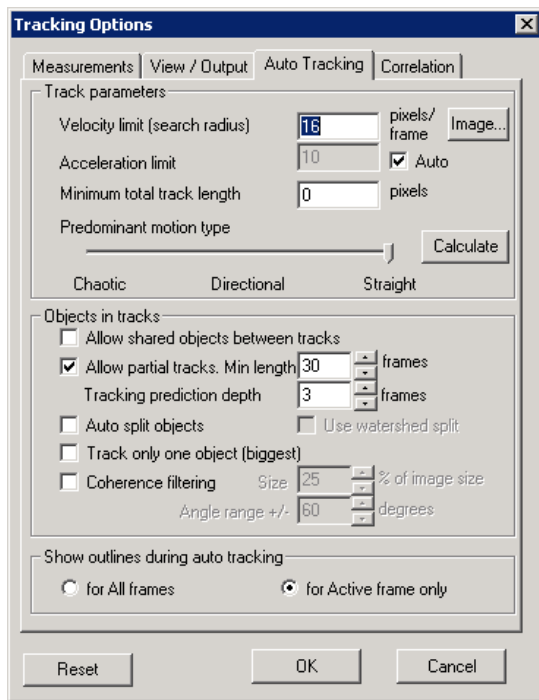
Figure 6. Auto tracking settings

In the View/Output tab (Figure 5) you can choose the coloring mode of the tracks (random or fixed). Sometimes it is more convenient to see all the tracks in one color to find continuous tracks that the algorithm has split into two. The partial tracks setting affects whether the tracks are shown as complete in every time frame, or only the past part from the chosen time frame.

Auto Tracking tab contains the parameters for the tracking itself (Figure 6). Velocity setting tells the algorithm how far it should look when searching for the object in the next time frame.

- Choose to AUTO SPLIT OBJECTS, but DO NOT USE WATERSHED SPLIT. Auto splitting is a CPU consuming procedure, which will slow down the tracking dramatically, but will increase the quality of tracking. Watershed will over segment this data set.

## 6.5 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 (Figure 7).
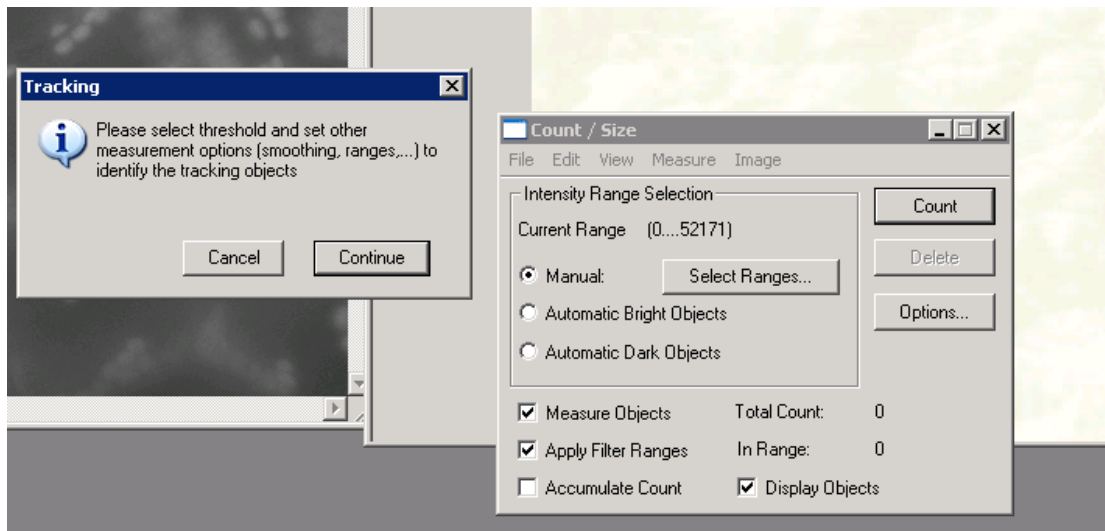
Figure 7. Starting up auto tracker

- In the Count/Size window, click SELECT RANGES to adjust thresholding level for segmentation

- In the Segmentation window (Figure 8), select the HISTOGRAM BASED tab and move the red selection area to set the threshold level. Since we are setting the blue level, you must first set the red and green channels to accept any values (R and G buttons). Move the bars so that all colors are selected. For the blue channel 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 while adjusting the threshold level.
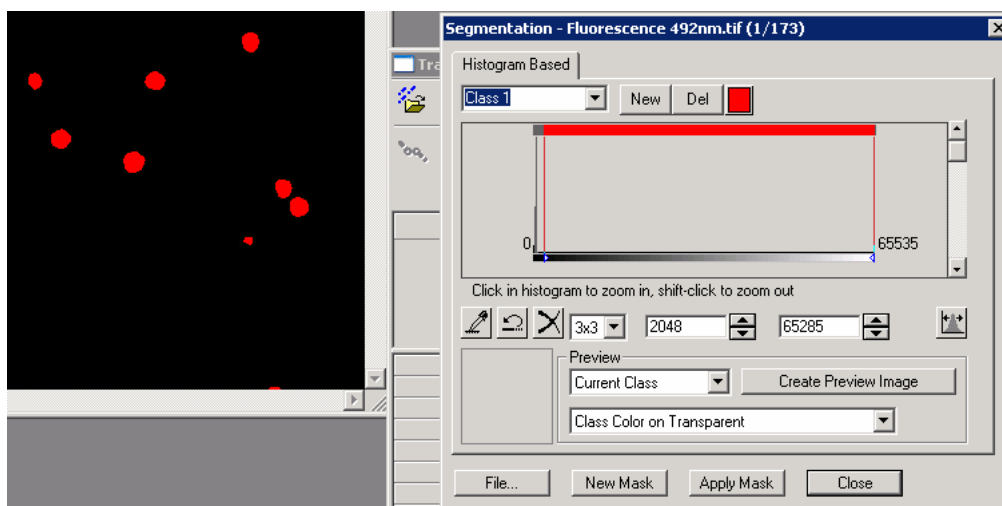
Figure 8. Segmentation threshold selection window

6.6 Object restrictions

- Click Close to get back to Count/Size window

- Open up MEASURE menu and SELECT MEASUREMENTS, which is used to restrict object segmentation (Figure 9)

If thresholding results in merged objects, they can be erased from the selection by setting a maximum value for accepted area of the object.

- Click AREA in Filter Ranges and then EDIT RANGE at the bottom

The new window (Figure 10) lets you graphically set the limitation values. You should also limit the minimum value to cut out noise. 10 pixels^2 is a good starting point.

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 restrictions.
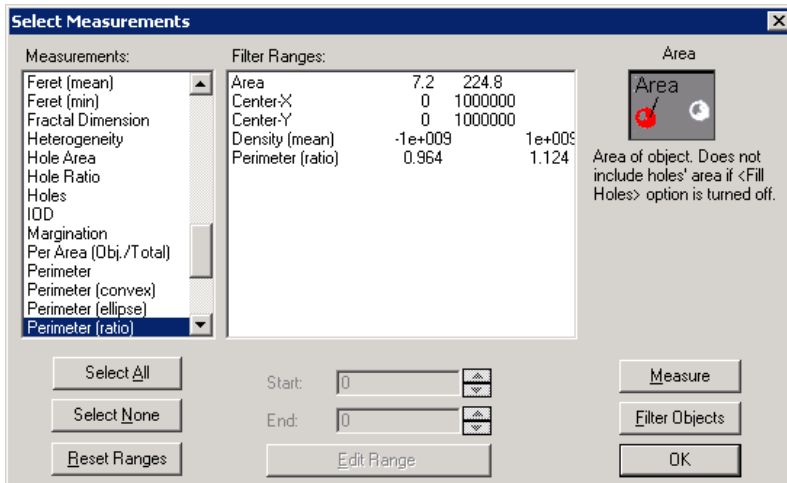

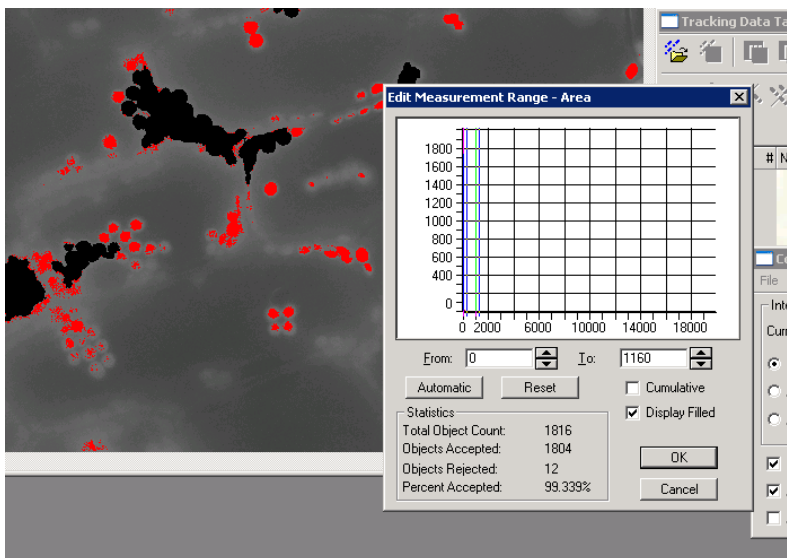**Figure 9. Selecting restrictions**

Figure 10. Restriction adjustment from a histogram

6.7 Initiating auto-tracking

- By clicking CONTINUE in the Tracking window, the auto tracker starts
- Scroll around the tracking data table to see the results. When a row is clicked, the track in question is highlighted in the image

6.8 Saving the results

- At the top of the data table, there is a SAVE TABLE button (icon with a table and disk). Export the data using the Comma Separated Values format. Save to D:\MBI\.

7 Data analysis

7.1 Analysis questions

After measuring the densities, areas and roundness of the objects over time, at least the following analyses can be done:

- How do the distributions of the variables look like?
- How do the values change over time?
- Which variables correlate with each other?

The analysis is done with ready made scripts for Matlab. You can see the contents of the script in question by typing *type [scriptname]*.

7.2 Loading the data

To load the data in Matlab,

- Start matlab (you may close image pro plus)
- Move to correct working directory by typing in the command window: *cd d:\mbi*
- Write the command: *parseipp('filename'),* where *filename* is the file you saved

7.3 Distribution analysis

To see how the measurements are distributed, make a histogram of each measurement.

- Run the function *dhist*
- **Are the distributions similar?** Do they look like any known distributions (e.g. Normal, Poisson,....)?

7.4 Change over time

- Run the command *timeplot*
  - o The command will show each track, their mean and standard deviation over time
  - o Mean change of speeds can be seen with commands: *figure; plot(nanmean(spds)')*
- **How do the values change over time?**

7.5 Correlation analysis

To see the correlation coefficient matrix of each variable, not taking the time change into account:

- Type in the command *dcorrmtx*
- The resulting output shows a matrix of correlations i.e. how a row variable correlates with a column variable
- What is the correlation coefficient? **Are any of the measurements correlated**? The X and Y coordinates are left out from the analysis, since the correlation would not be biologically meaningful
- Pick out the most correlating variables and type *cftool(dpoint(n1), dpoint(n2))*
- The *n1* is the number of the first variable (e.g. 1) and the *n2* is the other

- In the Curve Fitting Tool click FITTING…, NEW FIT and APPLY

- In the Results window you see the R-squared value. **What is the R-squared value? What does it mean?**

- Close the Curve Fitting tool

- Next, type in *tcorrmtx*. Now you see the correlations when the tracks have been averaged over time. **What are the most correlating variables?** Can you see this in the time plot?

- Try the cftool with the averaged data: *cftool(tpoint(n1), tpoint(n2))*

- **What is the R-squared value this time?**

8 Questions for summary

Prepare to answer these questions at the summary meeting:

- **What issues does sampling rate (how often images are acquired) bring to tracking and data analysis? Describe the possible effects of different sampling rates on the fluorochromes and the sample itself?**

- **What do you think was the most meaningful parameter in successful tracking?**

- **Are the speed measurements reliable?** (hint: images are only 2D)

- **How reliable is the area measurement? Can you be sure of the shape of the objects?**

- **How would you compare time series analysis and fixed samples? Is there a single time point that represents the cells in general?**