Manual: Vector Field Analysis

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Contents

1 Features

- IgorPro (Wavemetrics)[1] Procedure.
- Optical Estimation by temporal local optimization.
- Plotting of vectors in the original video frame.
- Filtering of the vectors by image intensity, velocity and direction.
- Image masking.
- Histograms of velocity, directionality.
- Calculation of protein flow rates.

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2 Introduction

The vector field analysis program uses an algorithm that is called the optical flow estimation (Teklap, 1995). Optical flow is "the distribution of apparent velocities of movement of brightness patterns in an image" (Horn and Schunck, 1981). In video sequences the projection of temporal axis to the x-y plane results in an optical flow image. Since the optical flow is a result of the movement, it contains information on movement speed and direction. The optical flow estimation recovers these quantitative measures of the movement, which enables the statistical treatments of all movement that occurs in the sequence. A velocity vector field is the calculation result in which every movement, namely speed and direction, within the sequence is mapped. The largest difference to the other tracking technique is that this operation does not require the segmentation step.

Optical flow detection can be categorized into two types in terms of basic algorithm: the **matching method** and the **gradient method**. In the **matching method**, displacement is measured by searching for a particular region in the consecutive frame by matching the pattern of the previous frame. In the **gradient method**, optical flow is detected by assuming no changes in the signal intensity pattern at different time points and by using equations that correlate the spatial and temporal intensity gradient. This program uses the gradient method. Details are described elsewhere (Miura, 2005).

Briefly, a general assumption in the gradient method is that the total intensity of the image sequence is constant.

$$\frac{\partial I(x,y,t)}{\partial x}u + \frac{\partial I(x,y,t)}{\partial y}v + \frac{\partial I(x,y,t)}{\partial t} = 0 \tag{1}$$

$$\mathbf{v}(u,v) = \mathbf{v}(\frac{dx}{dt}, \frac{dy}{dt}) \tag{2}$$

[1] `http://www.wavemetrics.com`

Where $I(x, y, t)$ is the intensity distribution of the image and $\mathbf{v}$ is the optical flow vector. The equation (1) links the partial derivatives of the brightness pattern of the image sequence and the optical flow velocity. Since there are two unknowns, another constraint is required.

The temporal local optimization method (TLO) used in this program assumes that the optical flow field is constant temporally (Fig.6) (Nomura et al., 1991).

$$\frac{\partial \mathbf{v}}{\partial t} = 0 \tag{3}$$

The constant vector can be assumed for N frames. Then for a stack with frames , following error function can be generated:

$$E = \sum_{k} (I_x(i, j, k)u + I_y(i, j, k)v + I_t(i, j, k))^2 \tag{4}$$

By the least squared method, $\mathbf{v}(u, v)$ can be determined by two equations $\partial E/\partial u = 0$ and $\partial E/\partial v = 0$.

A series of tiff-format image frames is converted to a stack, which is then treated as a three-dimensional matrix $I(x, y, t)$. Partial derivatives of the image sequence must be first calculated as seen in equations 1 and 4. There are three popular ways for obtaining the first-order partial derivatives[2]; Sobel kernel, Roberts kernel and Prewitt kernel (also is called two-point central difference kernel). The Prewitt kernel was used as follows:

$$\frac{\partial I}{\partial t} = \left[\sum_{i=-1}^{1} \sum_{j=-1}^{1} \big\{ I(x+i, y+j, t+1) - I(x+i, y+j, t-1) \big\}/2 \right]/9 \tag{5a}$$

$$\frac{\partial I}{\partial x} = \left[\sum_{j=-1}^{1} \sum_{k=-1}^{1} \big\{ I(x+1, y+j, t+k) - I(x-1, y+j, t+k) \big\}/2 \right]/9 \tag{5b}$$

$$\frac{\partial I}{\partial y} = \left[\sum_{i=-1}^{1} \sum_{k=-1}^{1} \big\{ I(x+i, y+1, t+k) - I(x+i, y-1, t+k) \big\}/2 \right]/9 \tag{5c}$$

3 workflow

3.0.1 Preprocessing

For a successful analysis, following preprocessings are recommended:

1. Check average intensity fluctuation of the sequence (recommended: see Appendix 1).

2. Generate "Image Mask" using ImageJ (Fig. 1: see Appendix 5). You might not need this if there is no background area in the image.

[2]Sobel filter

Figure 1: The "image filter" should look something like this. White area will be calculated for vector field. Such masking is required since noise in the background causes tiny vectors that contaminate the measurements.

3. Determine the range of frames to use for the Vector Field analysis by inspecting different time points of the stack. Check the shutter timing as well (App.3). A large fluctuation in the image intensity, avoid using those time points.

4. It is strongly recommended to do the walking-averaging of the sequence to decrease the noise (App. 4).

5. If required, adjust contrast (App.2).

3.0.2 Setting up the Vector Filed Program in IGORPro (Compiling)

In IgorPro, do [**File > Open File > Procedure...**] and select the file "**vec9.ipf**". Then click "Compile" at the left-bottom corner of the opened procedure window. In the menu bar, "Vector field" and "Directionality" appears.

Tip: If compiling does not work, it could be that other procedure files (.ipf files) are not in the reference path. Check "User > Procedures" folder, and see if the folder containing Vec9.ipf is linked or copied in that folder.

3.0.3 Vector Field Analysis

1. Import a image stack by [**VectorField > Load Tiff Stack**].

2. Import the image mask in the Vector Filed Analysis Program in the IGORPro. [**VectorField > Load Masking binary image...**]

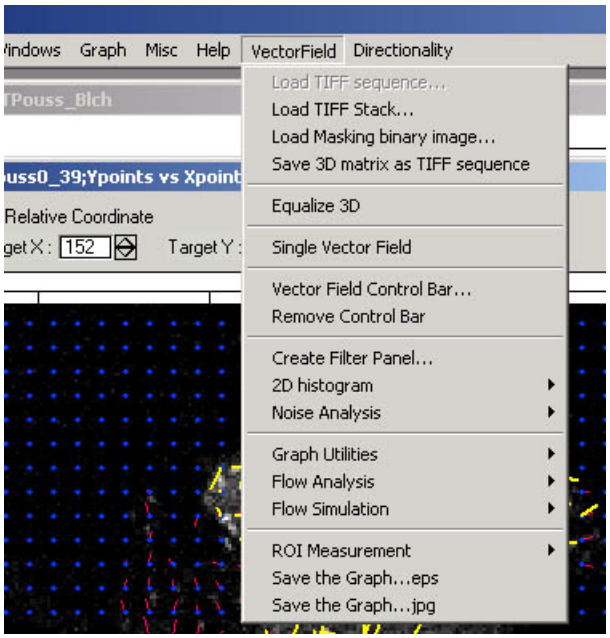


Figure 2: The menu tree of vector field analysis

- Image Filter can be inverted by [VectorField > 2D histogram > Invert Mask]. This function is sometimes convenient since one might prepare a mask which selects only the background of the sequence.

3. Calculate the vector field by **[VectorField > Single Vector Field]**. In a window that pops-up, select the tiff-stack, input the frame range (normally, I use 30 frames by experience that this is sufficient number of frames). Choose Bleaching Correction "**linear fit**", if there is a bleaching of the sequence. Other parameters do not have to be changed now. "Averaging" and "Scaling" could be adjusted later after the main calculation.

- It takes a while to calculate the vector field. While then there will be a rotating-disc icon indicating that the calculation is going on, at the right bottom corner of the IgorPro window (Fig. 4).
- If this calculation is first time in the current igor-experiment, then IgorPro asks you for a path to load noise reference data (Fig. 5).
- To set path, click 'Browse' button and select "NoiseParameter" folder within Vec9 folder.
- After the calculation, a new Vector Field window appears (fig. 6).
- There will be also two new windows, one for the speed histogram and another for the direction histogram (fig. 7).

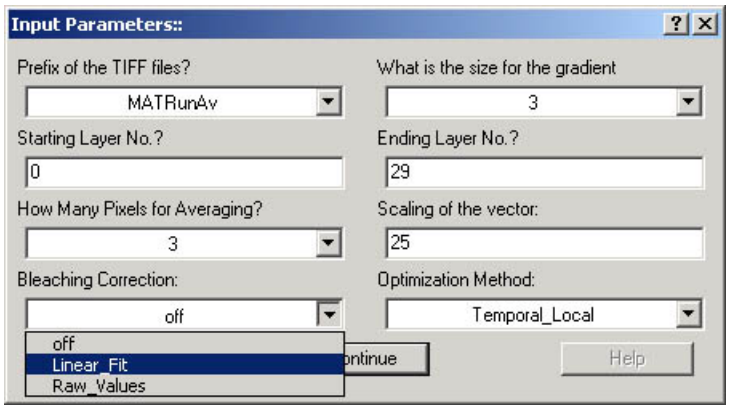


Figure 3: Interface of Vector Field Analysis

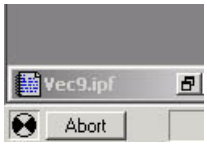


Figure 4: The icon indicating the process of computation.

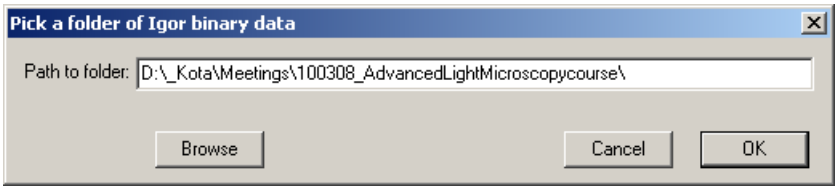


Figure 5: The interface for setting path to noise data.

4. To decrease the visualized vectors, adjust the vector scaling and averaging higher. To do so, activate the vector field window and then do [**Vector-Field >Vector Field Control Bar...**]. This will append a header bar at the top of the vector field plot, and averaging and scaling of vectors could be adjusted interactively.

3.0.4 OPTIONAL: Compute the angle distribution against a reference point.

Within the Vector Field Control Bar, select "Statistics" from the pull-down menu. Check "relative", then input the coordinates for the reference points. The coordinates can be measured easily by making a target ROI in the vector

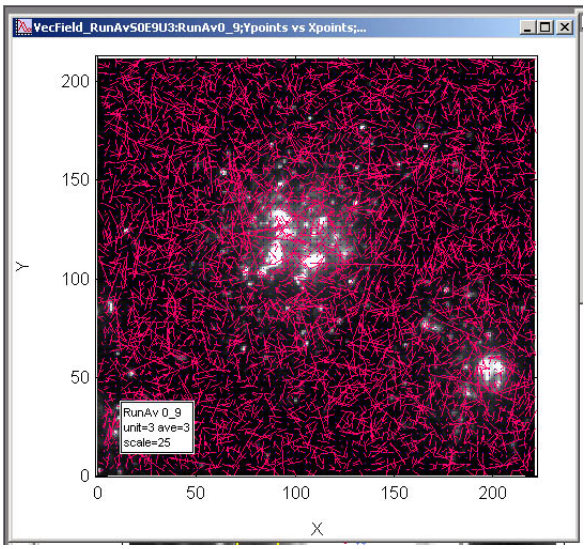
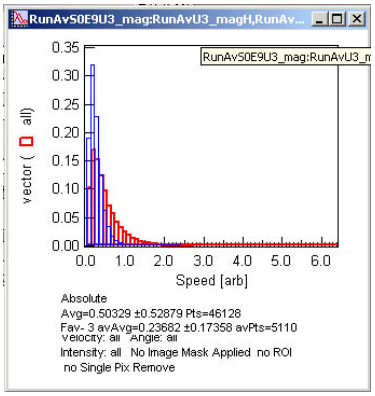
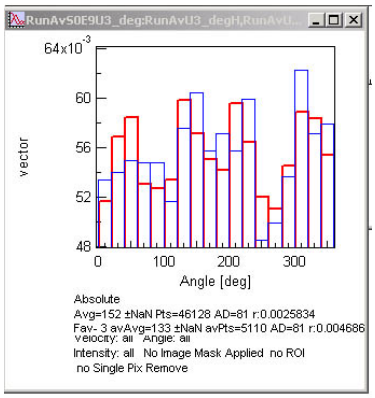


Figure 6: The initial vector field. In general, it needs scaling and filtering for a better view of vectors.



(a) Histogram of speeds.

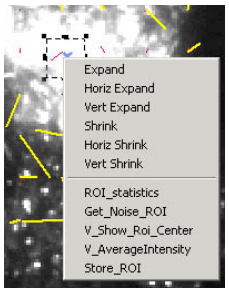


(b) Histogram of angles.

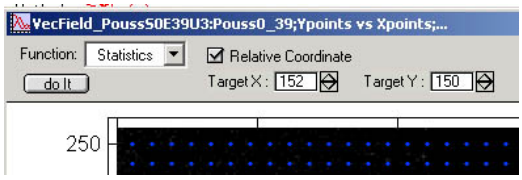
Figure 7: Vector Field analysis results.

field, right click the mouse button within that ROI. A menu will appear and choose "V_show_ROI_center" (fig 8a). The centroid coordinates of the selected ROI will be printed in the History window. Input those values in "TargetX" and "TargetY" (fig. 8b). Clicking "Do it" button will change the measurement of direction of the vectors against the reference point.

The direction histogram changes its range from 0 - 360 to -180 - +180. In the latter case, 0 is directed towards the reference point, and 180 is directed



(a) Selecting reference point.



(b) The interface for setting reference coordinates.

Figure 8: Optional: computing vector angles against a reference point.

away from the reference point (fig. 9).

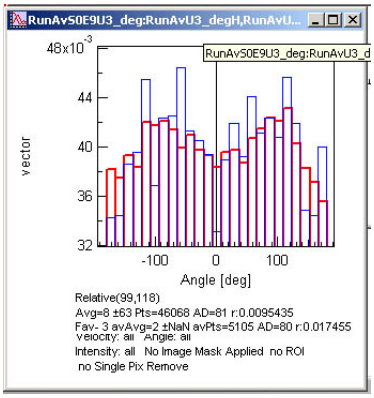


Figure 9: Histogram of vector angles measured against a reference point

3.0.5 OPTIONAL: Vector Filtering

If vector field is influenced by noise largely, these noise derived information must be removed from the measurement. To do so, do [**VectorField > Create Filter Panel...**]. This then pops up a window titled "Filter Panel" (fig. 10). There are several types of filters and you can set their parameter to remove noise derived vectors.

1. Find the optimum lower and higher limit for the intensity filter (compare the original movie and the vector field). Input the values within the Filter Panel. For selecting intensity range, threshold function in ImageJ is useful [**Image > adjust > threshold**].

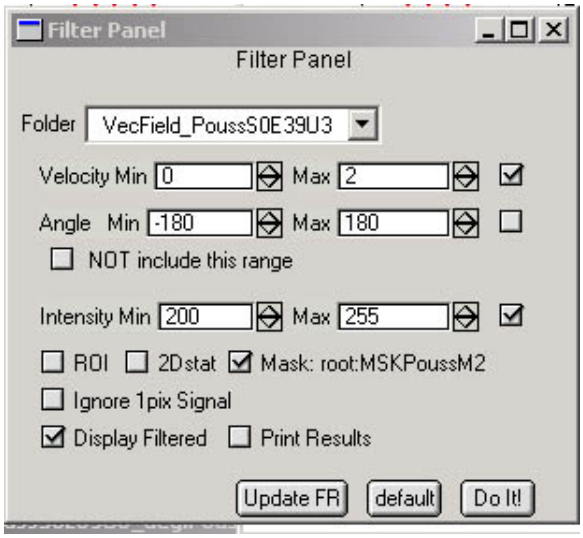


Figure 10: Vector filtering panel.

2. Find the "speed of noise" from the back ground and set the lower limit for the speed detection. Input the value in the Filter Panel.

3. Set the image-filter by checking the "Mask" check box. A dialogue window will appear. Select the Image Mask from the pull down menu.

4. Don't forget to check the "Display Filtered" check box.

5. Click "Do it" to execute the filtering. After the filtering is done, yellow vectors will appear, which are the vectors after the filtering. Statistics will also change, showing only the results from those yellow vectors.

6. Filtering parameters can be changed and re-calculated.

3.0.6 OPTIONAL: Flow Rate calculation

1. Check the background intensity. Move the curser to the background region of the vector field, click right mouse button and select "V_AverageIntensity" form the menu. The average background intensity will appear in the command window.

2. Set background intensity by VectorField Flow Analysis Set Background Intensity.

3. Do the analysis by VectorField Flow Analysis Calculate Flow. New graphs will appear.

4 Appendix

4.1 Appendix 1: Checking the average intensity fluctuation.

Dealing with Image Sequences with Blinking (Fluctuation of the fl. Intensity)

One could measure the temporal changes in the average intensity of frames in the following way using ImageJ. First, open the stack that you want to measure. Then a macro program should be installed.

[**Plugins Macros Install...**]

Then a popup window appears and a file called "**StackManager.txt**" must be chosen from wherever it is saved. This file could be downloaded from

`http://cmci.embl.de/dls/StackManager.ijm`

as well (right click and "save link as").

You will see new commands in the Macros menu [Plugins > Macros > ...] (fig. 11).

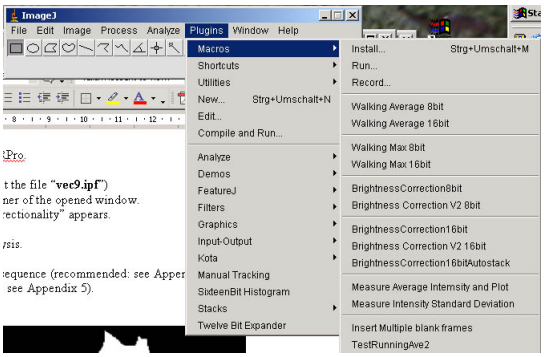


Figure 11: Installed macro appears in the ImageJ menu.

Activate the Stack window by clicking the title bar, and then select "**Measure Average Intensity and Plot**" form the macro menu. The program goes through the sequence once and then there will be a new window (fig. 12).

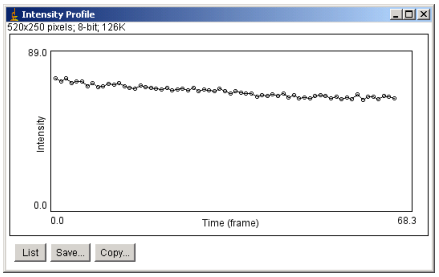


Figure 12: Intensity changes over time.

This plot shows how the average intensity of your sequence changes from frame to frame. The average intensity is decreasing, because there is a slight acquisition-photobleaching of the fluorescence.

In some cases, the intensity fluctuates largely. This could be caused by several reasons such as unstable light source (this happens a lot with old lamps) or changes in the focus plane. In any case, avoid using such a sequence, or use only a part of the sequence with a constant intensity (or with a constant photo-bleaching rate)

4.2 Appendix 2: Enhancing contrast and down-scaling from 16 bit to 8bit

This section explains how to convert 16-bit image sequences to 8-bit for faster computation of vector field. Note that this processing changes pixel intensity, so if you need to treat pixel intensity for measurement of protein density (such as for flow rate estimation), avoid this processing.

1. Open 16bit Tiff Sequence as a Stack in ImageJ.
2. Select a frame at a position about $2/3^{\text{rd}}$ of the whole sequence.
3. Check that the selected frame is not extraordinarily bright or dark compared to other frames.

   [Analyze > Histogram]

   This command displays an intensity histogram of the image. In most of images from fluorescence microscopy, histogram has a narrow peak towards left-side with a long tail towards the right side of the histogram.

   Since this is a 16bit image, Grayscale has 65536 ($=2^{16}$) steps between black(0) and white(65535) You need to down scale this and set a full rage in 8 bit, which consists of 256 ($=2^8$) steps.

   For example, if histogram of a frame distributes in a range of pixel values between 82 and 1009 ("min" and "max"), it means that there are ca. 900 gray scale in the image. Such a wide scale might not be necessary for your analysis as the range of the target signal might be narrower. To determine this meaningful range, the histogram must be examined carefully. You could use two different ways.

   - **Interactive pixel value reading**: Hover the mouse cursor over the histogram. As you move your cursor across the histogram, you will notice that "Value" (= pixel value) indicated below the histogram changes dynamically e.g. if your cursor is at "Value" 328 and "Count" is 26, it means that the number of pixels with a pixel value 328 in the image is 26 pixels. As you move your cursor closer to the peak, "Count" starts to increase. e.g. the "Value" is 165, "Count" is 435, and so on. Compare the value readout and actual pixels values in

the image to figure out the pixel value range that you are interested in.

In an example case fig. 13, the right side was determined to be "Value"=144 and the left side at "Value"=86.

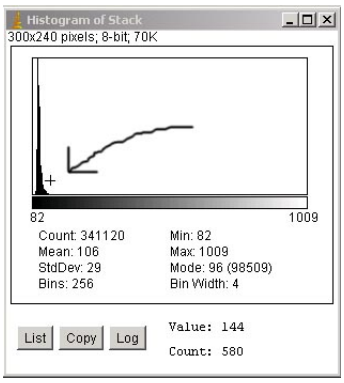


Figure 13: Checking pixel values using histogram window.

- **Numerical readout**: Click the "List" button in the histogram window. A list of number appears. This is the actual values of the histogram. The first column is the "Value" and the second column is the corresponding "Count" By examining the histogram values, the approximate steps that contain the peak position can be determined.

4. [**Image > Adjust >Brightness/Contrast**]

   This command displays Brightness and Contrast adjustment GUI. Click "Set" button. A small window pops-up. Set the "Minimum Displayed Value" to 86 and the "maximum displayed value" to 144. Now you see that the Image is contrast enhanced. Note that this only changes the LUT, not the pixel values of the image.

5. To apply this LUT and convert the image to 8-bit, choose the following command.

   [**Image > Type > 8bit**]

6. Check that pixel values are changed.

   [**Analyze > Histogram**]

   The histogram should be nicely distributed between the value 0 and 255.

4.3 Appendix 3: Checking the shutter timings.

Some sequences contain "blinking" of the frames. This could be due to

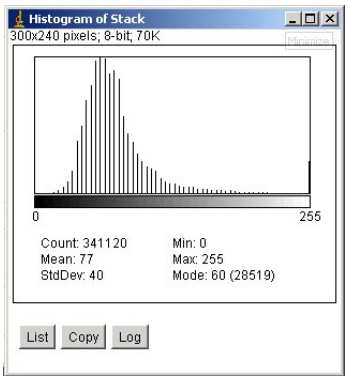


Figure 14: The histogram after conversion to 8-bit.

1. Light-source shutter is unstable.
2. Camera shutter is unstable.
3. Light source itself is blinking.
4. Focusing position changed.

To evaluate the stability of camera shutter timings, one can go back to the shutter log ("time stamps") and check if the sequence was taken with a constant shutter timings (fig. 15).

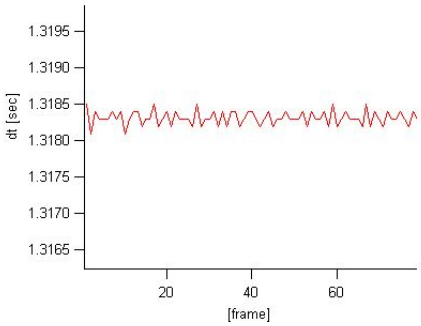


Figure 15: *dt* plot generated from the".tim" file.

4.4 Appendix 4: Walking average.

Walking averaging of a image sequence converts each frame of the sequence to an average of successive frames.

This file could be downloaded from

`http://cmci.embl.de/dls/StackManager.ijm`

After installing the macro, ImageJ macro menu looks like figure 16.

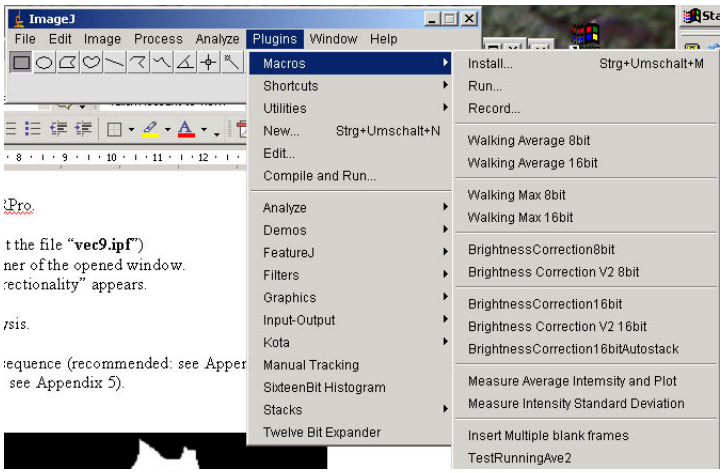


Figure 16: Stackmanager in ImageJ menu.

If the bit-depth of your image stack is 8-bit, choose "Walking average 8 bit" If 16bit, choose "Walking average 16 bit". Then a pop-up window appears (fig 17)

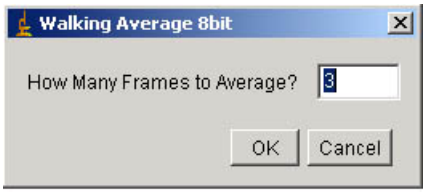


Figure 17: Stackmanager in ImageJ menu.

Input number of frames that you want to average, then click "OK". A new stack appears that is walking averaged.

4.5 Appendix 5: Generating a Image Mask for Vector field.

To make image mask, you need to use ImageJ. First, you must make two copies of a frame in the sequence you want to analyze. This can be done by

[Image > Duplicate...]

This will create a copy of the frame in the stack. If image is not 8-bit, then convert the image to 8 bit by

[Image > Type > 8bit]

Using this duplicated frame, you need to trace the edge of the cell. This is done by selecting freehand tool. Click the freehand ROI icon in the ImageJ menu bar (fig. 18).

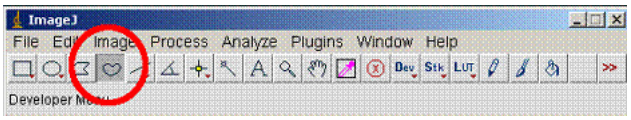
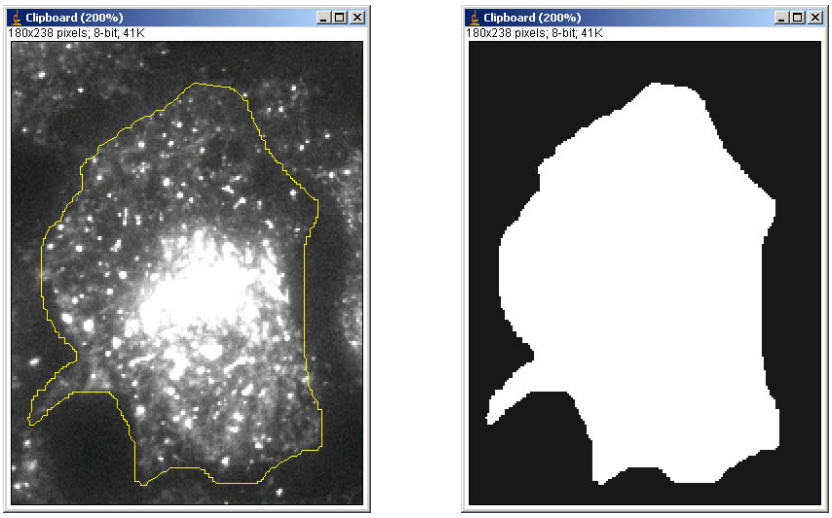


Figure 18: Selecting freehand ROI tool.

Then trace the cell edge like figure 19a.



(a) Cell edge traced using freehand ROI. (b) Generated mask from the trace.

Figure 19: Preparing cell mask.

Then after tracing, do

[Edit > Fill]

Tip: If inside area of the ROI do not become white, then check color option by [Edit > Options > Color...]. Check that the color assignment shown in the window appeared is like the one shown in figure 20.

Then

[Edit > Clear outside]

This will fill black in the outside area of selected ROI. By these last two steps, image should have become black and white (fig. reffig:celledgeMask).

OPTIONAL: masking area inside cell.

Sometimes signal is too high inside cell and this interferes with yourvector analysis. In such a case, another mask could be prepared to mask that high-intensity area, and combine it with the cell edge mask prepared above. You first make another duplicate of a frame by **[image > duplicate]**. Then Trace the high intensity area, and convert them to black ad white just like the first one.

Since we do not want to measure the white area, we invert the image so that the selected area becomes black (fig. 22).

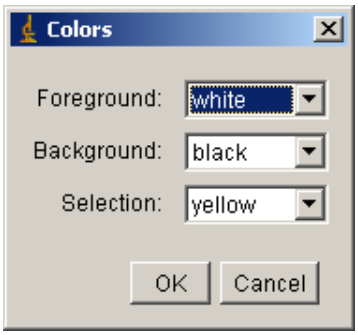
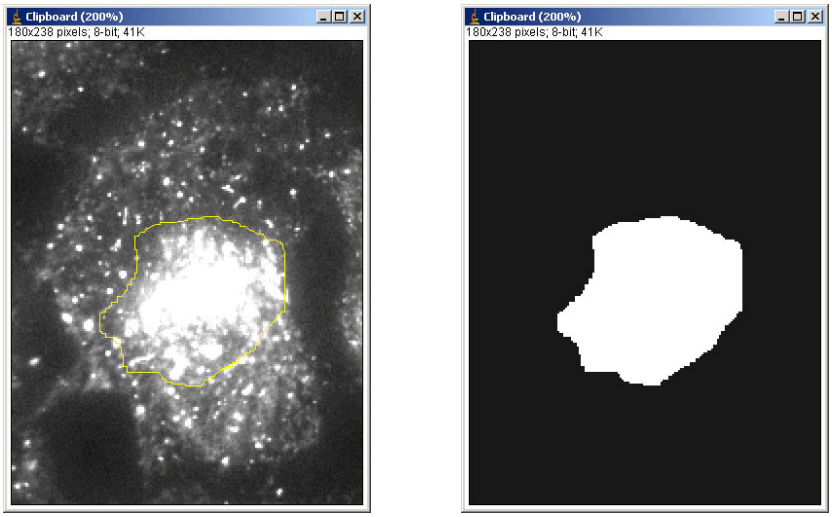


Figure 20: Color settings



(a) Cell nucleus traced using freehand ROI.(b) Generated nucleus mask from the trace.

Figure 21: Preparing nucleus mask.

[Edit > Invert]
Finally, use Image calculator to combine two masks (fig. 23).
[Process > Image Calculator...]
Select the first clipboard as image 1 and the second clipboard as the image 2. Operation is "Min". Then the combined image mask is created (fig 24) and can be imported from IgorPro.

5 References

Horn, B. K. P. and Schunck, B. G. (1981). Determining Optical Flow. *Artificial Intelligence* **17**, 185-203.

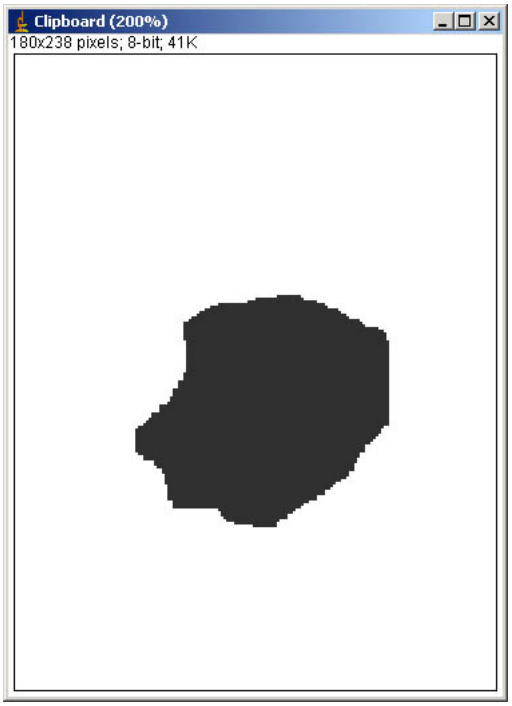


Figure 22: Inverted mask

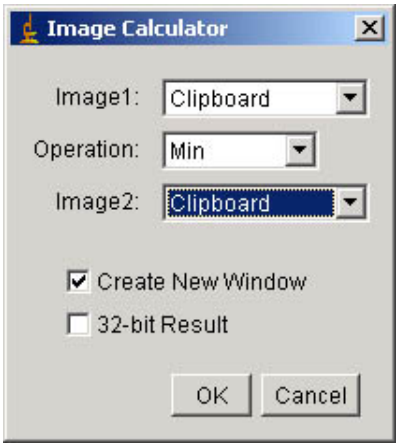


Figure 23: Image Calculator.

Figure 24: Combined mask.

Miura, K. (2005). Tracking Movement in Cell Biology. In *Advances in Biochemical Engineering/Biotechnology*, vol. 95 (ed. J. Rietdorf), pp. 267. Heidelberg: Springer Verlag.

Nomura, A., Miike, H. and Koga, K. (1991). Field theory approach for determining optical flow. *Pattern Recog. Lett.* **12**, 183-190.

Teklap, M. (1995). Digital Video Processing. Englewood Cliffs, N.J.: Prentice Hall.