HCA-Vision User Manual

CSIRO Quantitative Imaging

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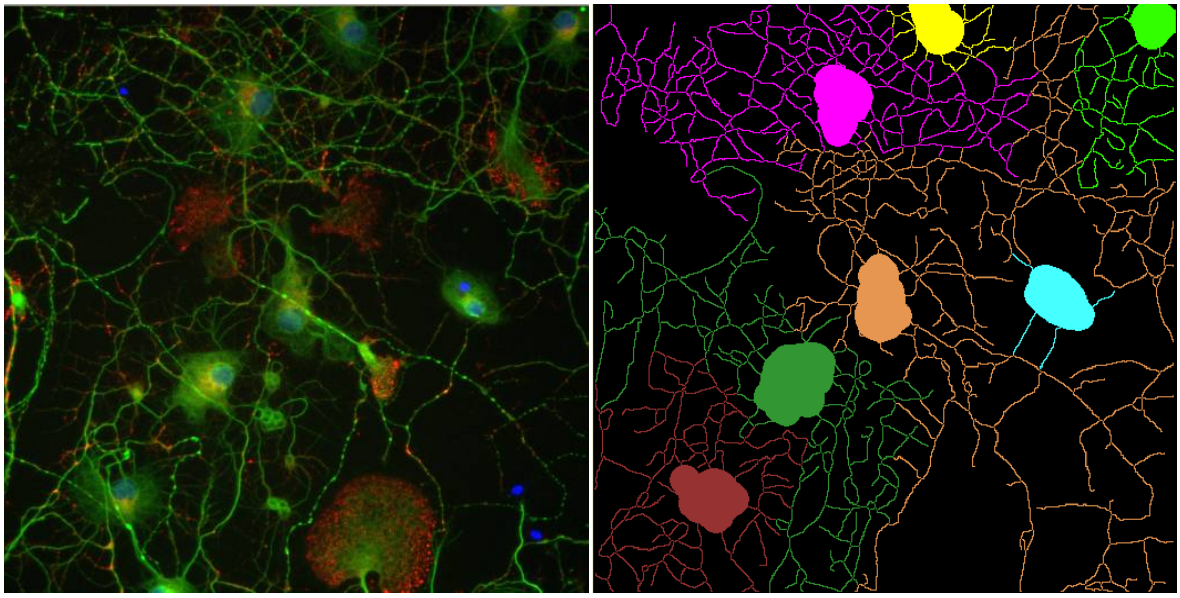

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Important Disclaimer

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1. WELCOME

Welcome to the Bio Image Analysis workshop.

This workshop provides case studies for representative High Content Analysis problems. These include neurite analysis of single and mixed cell populations, co-culture analysis to simultaneously analyse multiple cell-type populations, subcellular analysis to measure protein translocation and assess co-localisation of two different proteins, and cell scoring to count the number of cells that express two different proteins to a level above user defined values.

HCA-Vision will be used as the tool to solve these problems. It uses automated image analysis to rapidly produce measures of functional properties of cells.

2. HOW TO LOAD IMAGES & SWITCH TO A DIFFERENT MODULE

2.1 Launch HCA-Vision

First of all, launch HCA-Vision by double clicking HCA-Vision icon on the desktop of your computer, or from Start menu, click Start>All Programs>HCA-Vision>HCA-Vision.

2.2 Load an Image

After HCA-Vision is launched, you can load an image into HCA-Vision in one of the following two ways:

1) Using left hand side File Pane shown in the following figure. Navigate the directory tree to locate the image file you want to load, then click the image file name.

Or

2) Using File menu. Click File>Open pull-down menu and select the image file to load.

By default, the sample image shown in Figure 1 will be automatically loaded. Otherwise, it can be located at C:\HCA-Vision\SampleImages\Sample.tif.

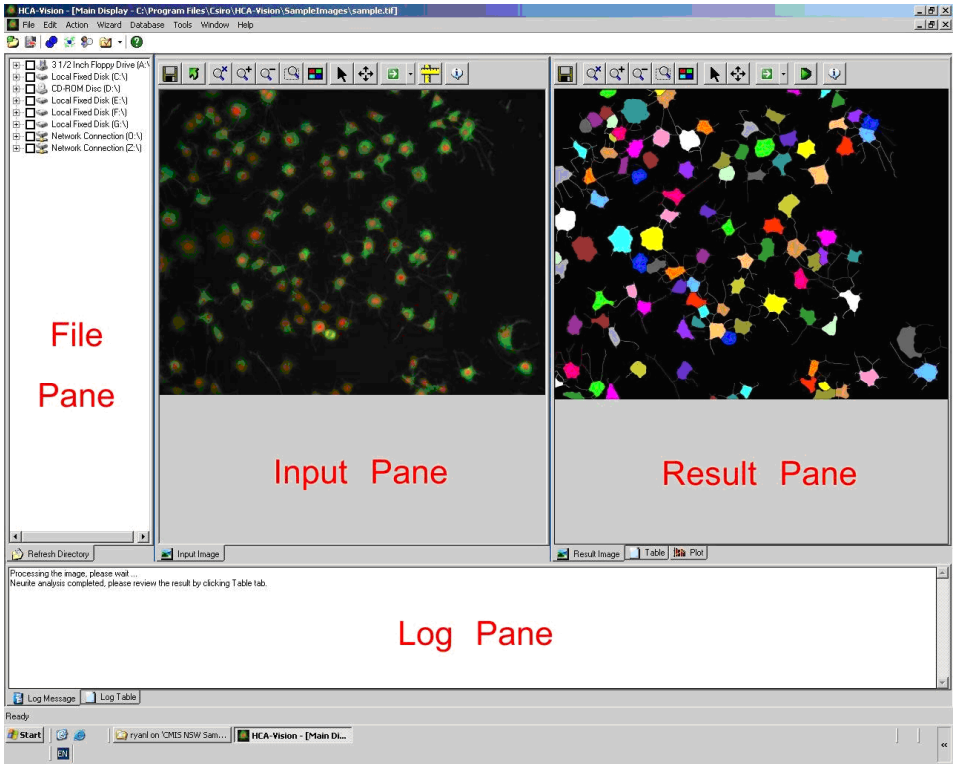


Figure 1 Layout of HCA-Vision

2.3 Switch to a Different Module

There are three modules currently available in HCA-Vision, including Neurite Analysis and Subcellular Analysis.

To switch from one module to another, just click Modules menu, then click the module you want to switch to.

3. NEURITE ANALYSIS

HCA-Vision's Neurite Outgrowth Module is a powerful tool for the analysis of neurite structure in fluorescence images. The software is designed to operate in both an exploratory mode (on single or small groups of images) or in batch mode (large batches of images for screening). The wizards provided in this module guide the user through the process of segmentation and analysis of neuron bodies and neurite outgrowth. Quantitative measures and statistics that are biologically relevant are delivered on both a cell-by-cell and image-by-image basis.

Some definitions of neurite segments are given in the following figure.

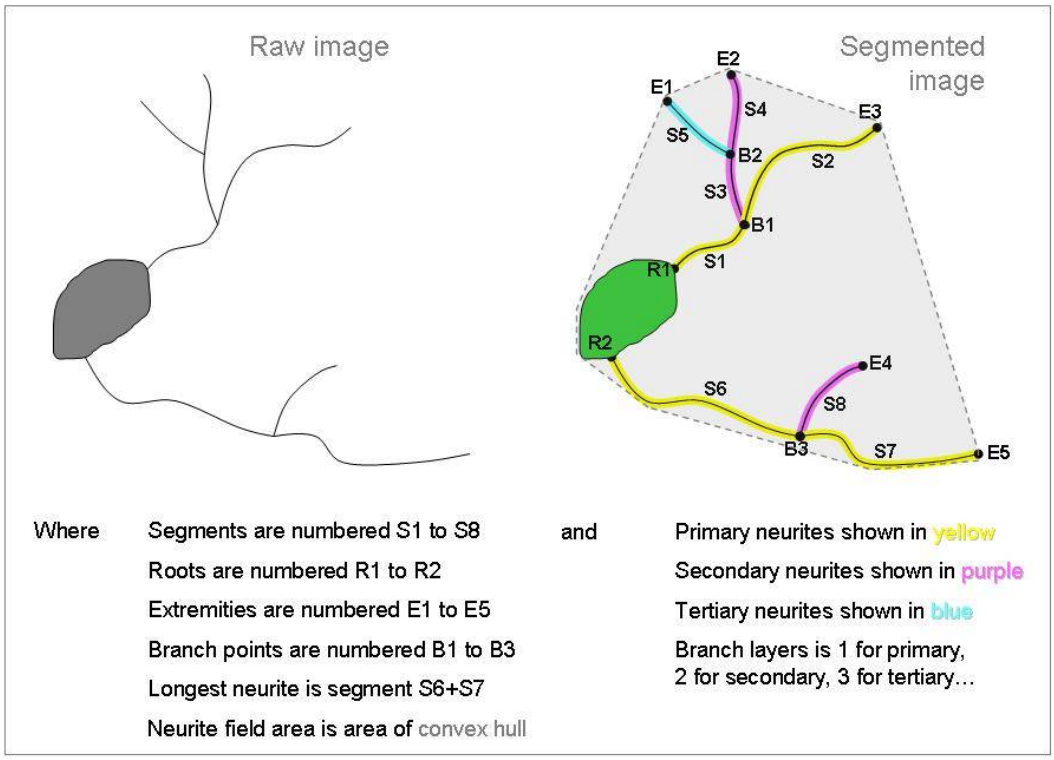


Figure 2 Neurite segment definitions

3.1 Neuron Body Detection

With HCA-Vision, neurite analysis is divided into three steps, including neuron body detection, neurite detection, and neurite analysis. After you load your image, the first thing to do is to tune the parameters for these steps by launching the wizard for each step. These wizards can be launched from Wizard menu. Click Neuron Body Detection Wizard to bring up the neuron body detection wizard. The first page shows there are nine steps for neuron body detection. These include:

3.1.1 Load an Input Image

The window will instruct you to choose an input image. Given you have loaded the image, we will assume this step has been performed. Click the Next button.

3.1.2 Select an Input Image

The Wizard now requires you to select the channel in the image which contains the neuron bodies. In our example, the neuron bodies are in the green channel, number 1. This is the default value loaded in the software. You can click on the up and down arrows to see the other channels. The numbering starts from 0, typically red, and continues to the maximum number of channels minus 1 in the image. Ensure that you are using the green channel and click Next.

3.1.3 Smoothing (Gaussian Filtering)

You are now required to set the pre-filtering parameter. The default parameter here is 6. Pre-filtering the image allows you to smooth your image to reduce the effect of noisy image capture. Moving the slider to the right increases the parameter. The result of pre-filtering will be shown in the Result pane of the software. If you set the slider to 0, no pre-filtering will occur. Return the slider to 6 and click Next.

3.1.4 Background Correction (Morphological Top Hat)

Background correction attempts to flatten the black part of the image. This is controlled by the "width" parameter. It should be set so the value is larger than the diameter of the largest neuron body. The default value here is 99. Moving the slider to the left makes the software treat the dark edges of the cytoplasm like background and flatten them. Return the slider to 99 and click Next.

3.1.5 Suppression of Neurites (Morphological Opening)

Neurite structure now needs to be removed from the image. This is done by setting the "width" parameter. The default parameter here is 7. You can see the result in the Result pane has removed the neurite outgrowth from the image. Moving the slider to the right removes more than just the outgrowth, but parts of neuron body as well. Move the slider to the left and the neurite outgrowth will re-emerge. Return the slider to the value 7 and click Next.

3.1.6 Intensity Thresholding

The image now needs to be separated into foreground (the neuron bodies) and background using a "threshold value". The wizard now presents the user with a slider for the value "threshold sensitivity". The default value here is 0.05 (se Figure 3). The resulting labelled image is automatically displayed in the Result pane. The cell bodies are now uniquely colour coded. Disregarding the "holes" where the nuclei in the red channel occur, we have managed to pick up most of the cell bodies. Moving the slider to the left will pick up some of the fainter cell bodies, but the trade off is the detected cell bodies for brighter objects may be too big. By increasing this parameter, you loose some of the fainter cell bodies, but get more correct results for bright objects. Return the slider to 0.05.

To have a comparison between your result image and the original image, you can click Overlay>Enable Flicker>Channel Image (see Figure 4) to have a flashing display in the Result pane showing the neuron channel image and the result image overlayed on the neuron channel image. By doing so, you can locate which neurons are missing from the result image and change the threshold value accordingly. You can also choose the original image or other image channels or inverted image channels from the menu shown in Figure 4 to compare the result image and the chosen image. You can apply the “Flicker” function on all labelled result images. Alternatively, you can apply the “Overlay” function by clicking Image Overlay menu shown in Figure 4, then choose a channel image or the original image to be overlaid.

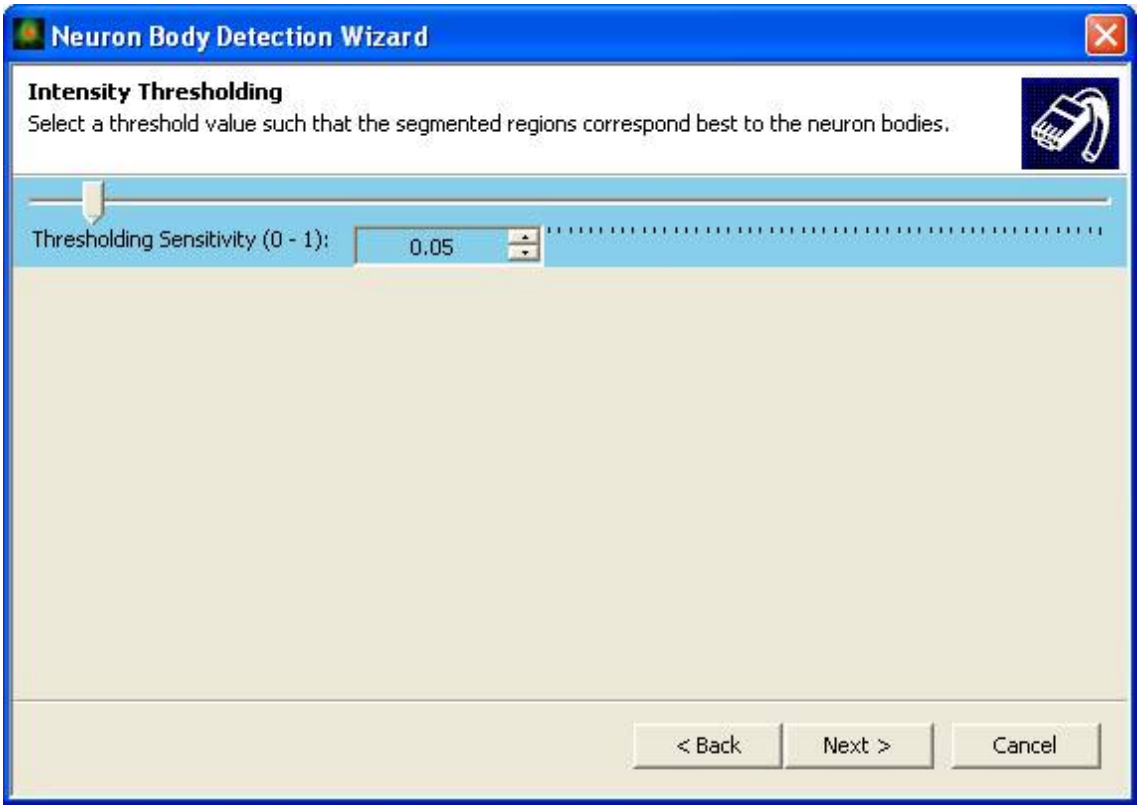


Figure 3 Intensity Thresholding wizard page

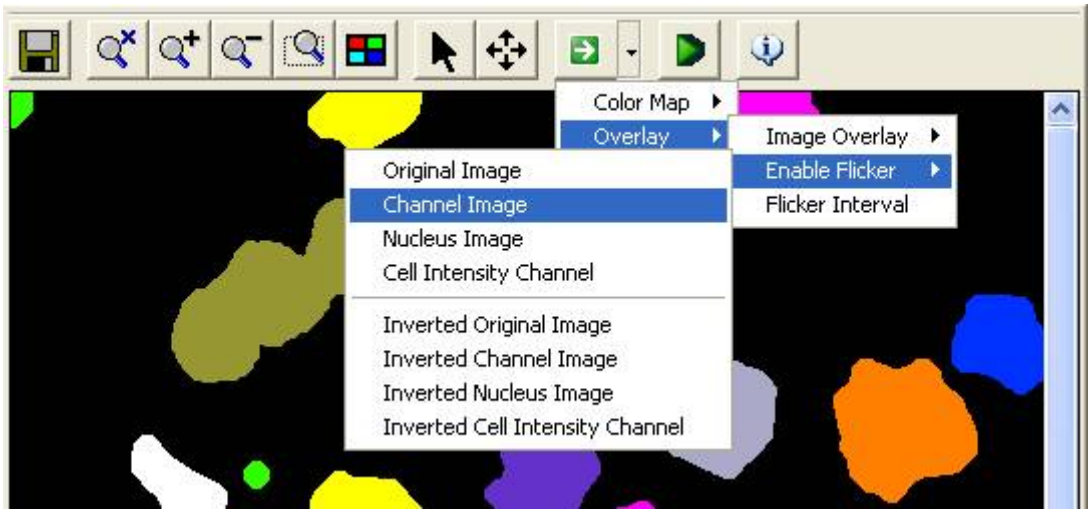


Figure 4 Menus to turn on “Flicker” feature

3.1.7 Nucleus Channel

To further improve the result of the above threshold, we can use nucleus channel information if it is available. In our example, a nucleus stain was captured in channel 0. Click the "Show" button in the wizard. In the Input pane you will see the nucleus stain and in the Result pane a result of thresholding the nucleus stain. If the nucleus stain is contained in a separate image you can load it by clicking on the "..." button. We need to threshold the nucleus image in much the same way as we did for the cell bodies. By adjusting the slider corresponding to "Nucleus Threshold Sensitivity" you can see the effect of setting this value closer to 1 (only detect bright objects) or closer to 0 (detect all objects, but results are too big and nuclei are merged). Return this slider to 0.34. The "Nucleus Area Threshold" is used to reject nuclei whose area falls below the threshold. If the “Declump” tick box is ticked, Maximum Nucleus Size can be used to specify the maximum nucleus area above which the nucleus will be declumped. However, the declumping function also depends on the average size of the nuclei in the image. You may find that some nuclei are not de-clumped even if their area is larger than the specified Maximum Nucleus Size. For the sample image, return the Maximum Nucleus Size slider to 300, untick the Declump box and click Next.

3.1.8 Nucleus Selection

Further manipulation of neuron body detection can be achieved by discarding nuclei that are too dark, or even too bright. The "Intensity Threshold" can be used to discard objects whose intensity, in a specified channel, is too low. The " Low Intensity Threshold" and " High Intensity Threshold" can be used to reject nuclei whose intensity fall outside a range of intensities in a specified channel. Return all the sliders to 0 and click Next for the sample image.

3.1.8 Object Selection

The next section allows you to remove some neuron bodies. You can remove neuron bodies touching the border or within a certain distance of the border by adjusting the "Border Width" slider. Neuron bodies less than a minimum area can also be removed by adjusting the "Minimum Neuron Body Area" slider. Return both sliders to 0 and click Next.

3.1.9 Optional Measurement

If the user is interested in intensity based information from another channel (eg. protein translocation studies), you can select the appropriate channel in this dialogue box. You can also choose various regions in which to measure intensity. The region choice is dependent on whether a nuclear mask was generated in the previous step. You can choose from cytoplasm, nucleus and cell membrane. If you don't wish to select any of these options, click "Next".

3.1.10 Save the Parameter Profile

We have completed the parameter tuning process for neuron body detection. Now you should save these parameters into a profile. Specify the profile name then click “Save” button to do so (refer to Figure 5) and then click Finish. If you do not wish to save the parameters, simply click Finish.

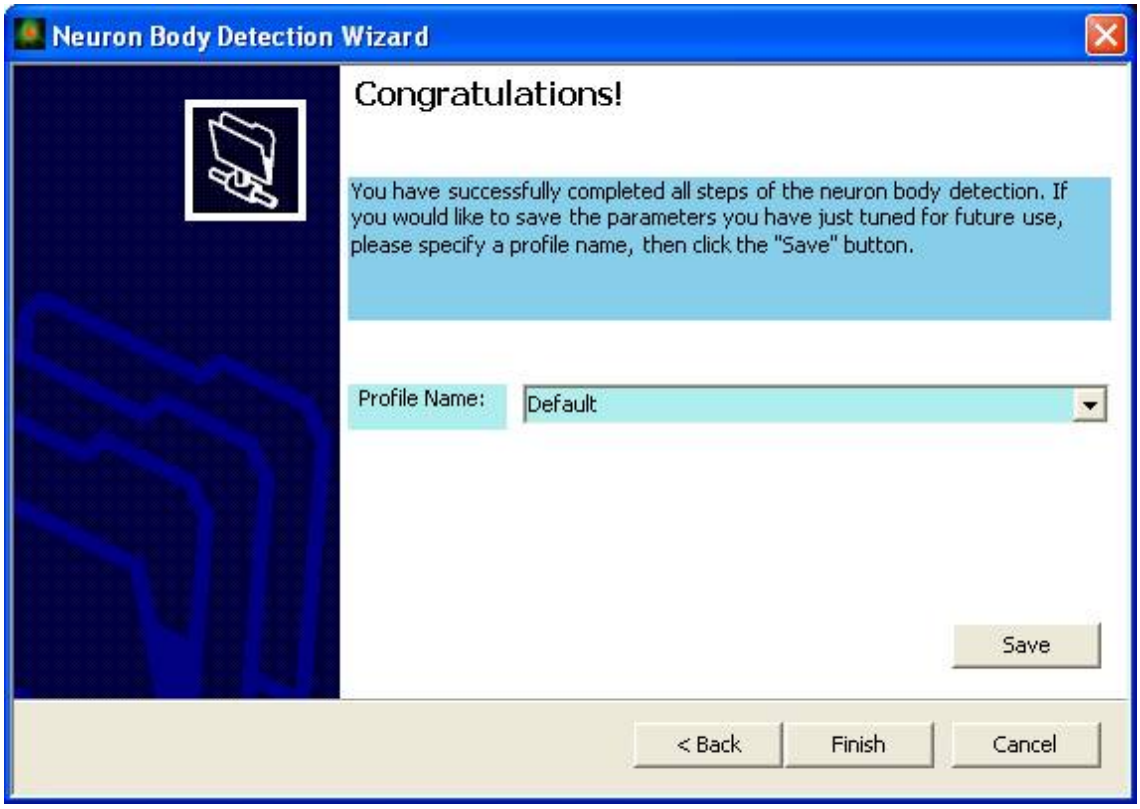


Figure 5 Save Neuron Body Detection parameter profile

3.2 Neurite Detection

The second stage of analysis is detecting the neurite structures. Go to the Wizard pull down menu and select "Neurite Detection Wizard". A window will pop up introducing you to the wizard facility and outlining the steps involved. The steps are addressed below:

3.2.1 Load an Input Image

You will be instructed to open an image file. If you have been following the Tutorial, you will already have the file "Sample.tif" open in the Input pane. If you are starting here, open the image "Sample.tif" as instructed in the Section 2 of this document. Click Next.

3.2.2 Select an Input Channel

You are now required to select the channel which contains the neurite outgrowth. In the current example, it is the green channel number 1. You can click on the up and down arrows to see the other channels. The numbering starts from 0, typically red, and continues to the maximum

number of channels minus 1 in the image. Ensure that you are using the green channel and click Next.

3.2.3 Preprocessing (Gaussian Smoothing)

You are now required to set the pre-filtering parameter. The default parameter here is 8. Pre-filtering the image allows you to smooth your image to reduce the effect of noisy image capture. Moving the slider to the right increases the parameter. The result of pre-filtering will be shown in the Result pane of the software. If you set the slider to 0, no pre-filtering will occur. Return the slider to 8 and click Next.

3.2.4 Linear Feature Detection

The Wizard now requires you to set 2 parameters, "Linear Window Size" and "Contrast". The parameter "Contrast" controls how different the brightness of a neurite can be compared to its background. By moving its slider to the left you can pick up more neurite structure. The opposite occurs as you move the slider to the right i.e. you start to miss structures. You will notice that the linear structures in some instances look broken. This is OK as you get a chance to join them, which is described in two paragraphs from here. Return the slider to the value 1.22. Adjusting the parameter "Linear Window Size" has a much more subtle effect. What it controls is the size of the window in which the background and foreground are determined for a particular pixel. The default value of 9 should be sufficient for most applications. You may try increasing it in images where neurite outgrowth is particularly wide. Click Next.

Please note, if you notice that some dots are detected instead of a line for a neurite no matter how you change the above parameters, please click Back to go back to Gaussian Smoothing step, try to increase the Gaussian Smoothing parameter, then click Next to come back to the Linear Feature Detection step and try again.

3.2.5 Remove Small Objects

Setting the value of "Diameter" for the major diameter of the best fitting ellipse of small objects is the next step. The default value of 7 removes all the small noisy objects we picked up in the last step while not removing any real neurite structure. Try moving the slider to the right and you will notice we remove too much neurite structure. Return the slider to 7 and click Next.

3.2.6 Gap Closing

Here we join broken neurite segments by adjusting the parameter "Distance". The default value of 9 works well for our example image. If you move the slider to the left you will see how setting this value too low will retain the broken appearance of our initial result. Return the slider to 9. The "Quality" parameter allows the user to discard links joining broken neurite segments that do not satisfy a brightness criteria. The mean brightness of the link must be greater than the average of the brightness at the endpoints it connects scaled by the "Quality" percentage. Moving the slider to the right will discard more of the links. Return the slider to 6 and click Next.

You now have completed the neurite detection. You can save the parameters you have tuned in a parameter profile. This parameter profile can have the same profile name as that used to save the Neuron Body Detection parameters or it can be saved with a different name. Click Finish.

3.3 Neurite Analysis

The final stage of analysis is neurite analysis. Here we associate neurite outgrowth with neuron bodies, construct the neurite trees and calculate various measurements described in the Results section. Go to the wizard pull down menu and select "Neurite Analysis Wizard". A window will pop up introducing you to the wizard facility and outlining the steps involved. Click the Next button.

3.3.1 Load an Input Image

You will be instructed to open an image file. If you have been following these notes, you will already have the file "Sample.tif" open in the Input pane. If you are starting here, open the image "Sample.tif" as instructed in the "Open and View an Image" section of the tutorial. It is also assumed that you have performed Neuron Body Detection and Neurite Detection, the parameters of which will be automatically loaded if you have followed the tutorial. These parameters can alternatively be loaded, if you have saved them, as described here. Click the Next button.

3.3.2 Debarb Small Neurite Branches

You are now required to set the parameter “Length”. Here, any branch in a neurite tree structure with length less than this parameter is pruned from the tree. The default value of 15 is usually sufficient for most applications. Click Next.

3.3.3 Thicken Neuron Bodies

The next step is to set the "Width" parameter. The neuron bodies are thickened to ensure that the neurites are touching the neuron bodies. The default value loaded into the program is 0. The effect of increasing this value too much is to connect neurite segments to cell bodies that are probably not actually related to the cell body. This in turn will inflate the number of primary neurites output (see Results section for description). Return the slider to 0 and click Next.

3.3.4 Remove Small Trees

The final parameter to set is “Length”. Small, spurious neurite trees can often occur around the edges of neuron bodies. Adjusting this parameter allows you to remove trees with length less than the value specified. If the value is set to 0, no trees will be removed. Return the slider to the default value of 4 and click Next.

You now have completed the neurite analysis. The results are all shown in the Result pane and are discussed in detail in the results section. You can save the parameters you have tuned in a parameter profile. This parameter profile can be saved with the same name as the previous steps or with a different name. Click Finish.

3.4 Neurite Analysis Results

In this section the output of the neurite analysis is discussed. It is assumed you have been following the Tutorial up until this point. We refer to the output of the example image processed in the tutorial, but it also serves as the main reference point for describing the output.

3.4.1 Result Images

The neurite analysis produces two result images. The first of which is automatically displayed in the Result pane of the software or by clicking on the "Result Image" tab at the bottom of the right pane. This image is called the neurite parent image. It is colour coded so that neurite segments are given the same colour as the colour of their parent neuron body. The second image is called the branch layer image. You can view this image by first clicking on the "Result Image" tab in the Result pane and clicking the "Next Image" button, which is the arrow button just above the result image. This image shows the neuron bodies in green, primary neurites (those touching the neuron body) in yellow, secondary neurites (those touching primary neurites) in pink etc. Given the fairly simple input image, we only see a few examples of secondary and tertiary branch layers.

3.4.2 Table

HCA-Vision reports a range of quantitative measures of neurite outgrowth. You can view these by clicking on the Table tab at the bottom of the Result pane.

Firstly, 9 summary statistics are presented:

- number of cells
- total and average neurite outgrowth - the total length of all neurite structures (in pixels) and this value averaged by the number of neuron bodies
- total and average neurite area – the total area of all neurite structures (in pixels) and this value averaged by the number of neuron bodies
- total and average number of segments - a segment is a linear structure between branching points or a neuron body
- average branching layers and longest neurite from a cell - the average of the highest level of branching for a cell as shown in the branch layer image described above and the length (in pixels) of the longest path from a neuron body to an extreme segment
- total and average number of roots - the number of points where neurite structure touches a neuron body
- total and average number of extreme neurites - the number of terminating neurite segments
- total and average number of branch points - the number of points where a neurite structure splits into two or more branches
- total length of Orphan neuritis – neurites disconnected from a cell body

Secondly, for each neuron body we report:

- total neurite length - sum of the length of each neurite segment
- max neurite length - the length (in pixels) of the longest path from a neuron body to an extreme segment
- max branch layer - the highest level of branching for the cell as shown in the branch layer image described above
- mean branch layer - the mean level of branching for the cell as shown in the branch layer image described above
- number of branch points - the number of points where a neurite structure splits into two or more branches
- number of roots - the number of points where neurite structure touches a neuron body

- number of segments - a segment is a linear structure between branching points or a neuron body
- number of extremities - the number of terminating neurite segments
- neurite field area - the area of the convex hull containing the cells neurite structure
- max intensity neurite - the maximum intensity along the neurite structure
- mean intensity neurite - the mean intensity along the neurite structure
- integrated intensity neurite - the integrated intensity along the neurite structure
- std dev intensity neurite - the standard deviation of the intensity along the neurite structure
- branch layer statistics - for each branch layers 1 to 3, the number of branches, total neurite length and max neurite length are reported
- intensity statistics - for the neuron body and each region of interest and additional channel (these measurements are only available when you tick corresponding options in Section 3.1.9 “Optional Measurement”), the maximum intensity, mean intensity, integrated intensity and standard deviation of intensity are reported.

A diagram explaining the concept of each measure is shown in Figure 2.

The output table can be exported as a .csv file for loading into applications like Excel by clicking on the "Save the Table" icon, which is a disk icon above the result table, in the Result pane.

3.4.3 Cross Referencing

HCA-Vision allows you to interactively link per cell results between result images and the output table. There are three ways to do this:

If you have the input image open in the Input pane and the output table in the Result pane, you can click on the "row header" of a line in the output table and the corresponding cell will be highlighted in the input image. A "row header" is the tile at the very left of each row in the result table.

If you have the input image open in the Input pane and the output table in the Result pane, press the "select" button (this appears as an arrow) in the row of buttons at the top of the Input pane. You can now click on a cell and the corresponding row will be highlighted in the output table.

If you have the input image open in the Input pane and a result image in the Result pane, press the "select" button (this appears as an arrow) in the row of buttons at the top of the Result pane. You can now click on a cell in the result image and the corresponding cell in the input image will be highlighted.

3.4.4 Plot

By clicking on the "Plot" tab at the bottom of the Result pane you can view a plot of each of the quantitative measures per cell. The Y-axis corresponds to number of neurons (Counts). The X-axis corresponds to the measure for the neuron body, for example "Total Neurite Length". You can go through all the different plots by clicking the arrow icon at the top of the plot.

3.5 Load Parameters

The saved profile parameters can be used later for another image similar to the one you just analysed. You can load a saved parameter profile at any time by clicking Action>Load All Parameters menu to bring up the Load All Neurite Analysis Parameters dialog box shown in Figure 6. Just specify the parameter profile name, then click Load button to load the parameters when needed.

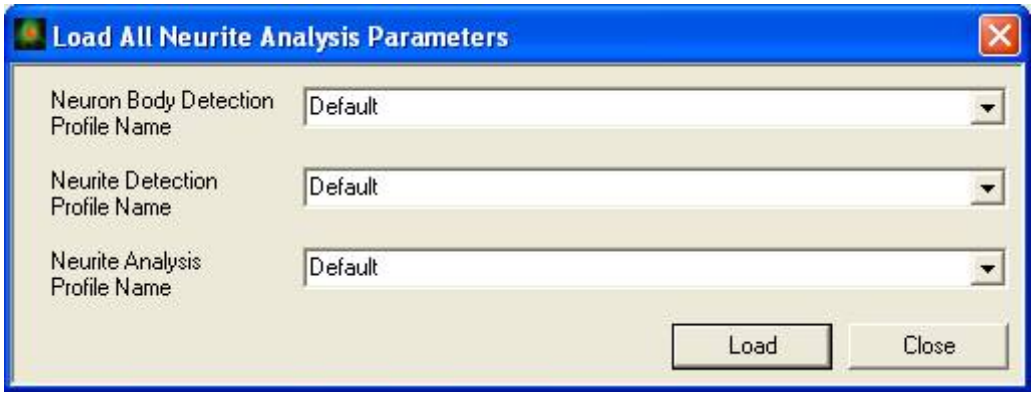


Figure 6 Load saved parameters

3.6 Run Neurite Analysis Directly

There is a toolbar (shown below) just below the menu strip. If you hover the mouse on individual buttons, you can see a tooltip for each button. You can simply load your saved parameters anytime, then click Neuron Body Detection, Neurite Detection or Neurite Analysis to see the corresponding results immediately. These functions can also be accessed from the Action Menu.

Figure 7 Toolbar for Neurite Analysis

3.7 Batch Processing

HCA-Vision allows you to process multiple images as a batch. You can perform a batch process for either Neuron Body Detection or Neurite Analysis. The use of the batch processing functionality is best described via example. We will perform the Neurite Analysis on two images.

Firstly, the user needs to select images for a batch. Navigate to C:\HCA-Vision\SampleImages, click on the black boxes next to the filenames "l1fccontrolpic7.tif" and "lifcpic5control.tif" in the directory viewer (left hand File pane). If your image files are all in one directory, you can alternatively just click on the box next to the directory name. Please note, the images to be processed in a batch may come from the same experiment or be similar “looking”. So when you tune the parameters, you just select one typical image, go through the parameter tuning wizard, then save the parameters. Then you are ready to process all images for the experiment by selecting the images you want to batch processing, then in Action menu click Batch

Processing>Neurite Analysis or click on the batch processing button, which is in the toolbar shown in Figure 7 (a directory icon with a tick inside). You will be presented with a dialogue box (shown in Figure 8) with the following options

- Batch ID - a unique label for your batch. If you do not enter a Batch ID, one will be automatically generated based on a time stamp for the process
- Comments - optional notes about the batch
- Preprocessing Option
- Profile Name(s) - parameter profile(s) the user has generated using the Wizards or from HCA-Vision's default sets. Select "lifcpic5controlr" for each of the 3 stages.
- Nuclei Image Suffix - during Neuron Body Detection the user can use a separate nuclei image to aid the detection (as opposed to a separate nuclei channel). If this is the case it will appear as part of the loaded parameter profile. This box allows you to enter a suffix that specifies the naming convention for the nuclei images in the batch. In our example, the parameter file associated with "Neuron Body Detection" makes no reference to a separate nuclear stain and hence this region is greyed out. If your image does not have nuclei or if the nucleus image is in a separate channel of your neurite image, ignore this box. This box only applies when you have separate nucleus image files for each neurite image to be batch processed.
- Save Output Images - an option to save output images and specify their file type. The program creates a Results sub-directory in the same directory as the files you have selected for the batch. Within the Results sub-directory, the output image files (with extension .jpg) are saved.
- Save Statistic Result in CSV file - optional to save the result statistics for each image into a separate exportable file for Excel etc. They are saved in the Results sub-directory described above. Click OK. Additionally, the results are saved to the HCA-Vision database by default.

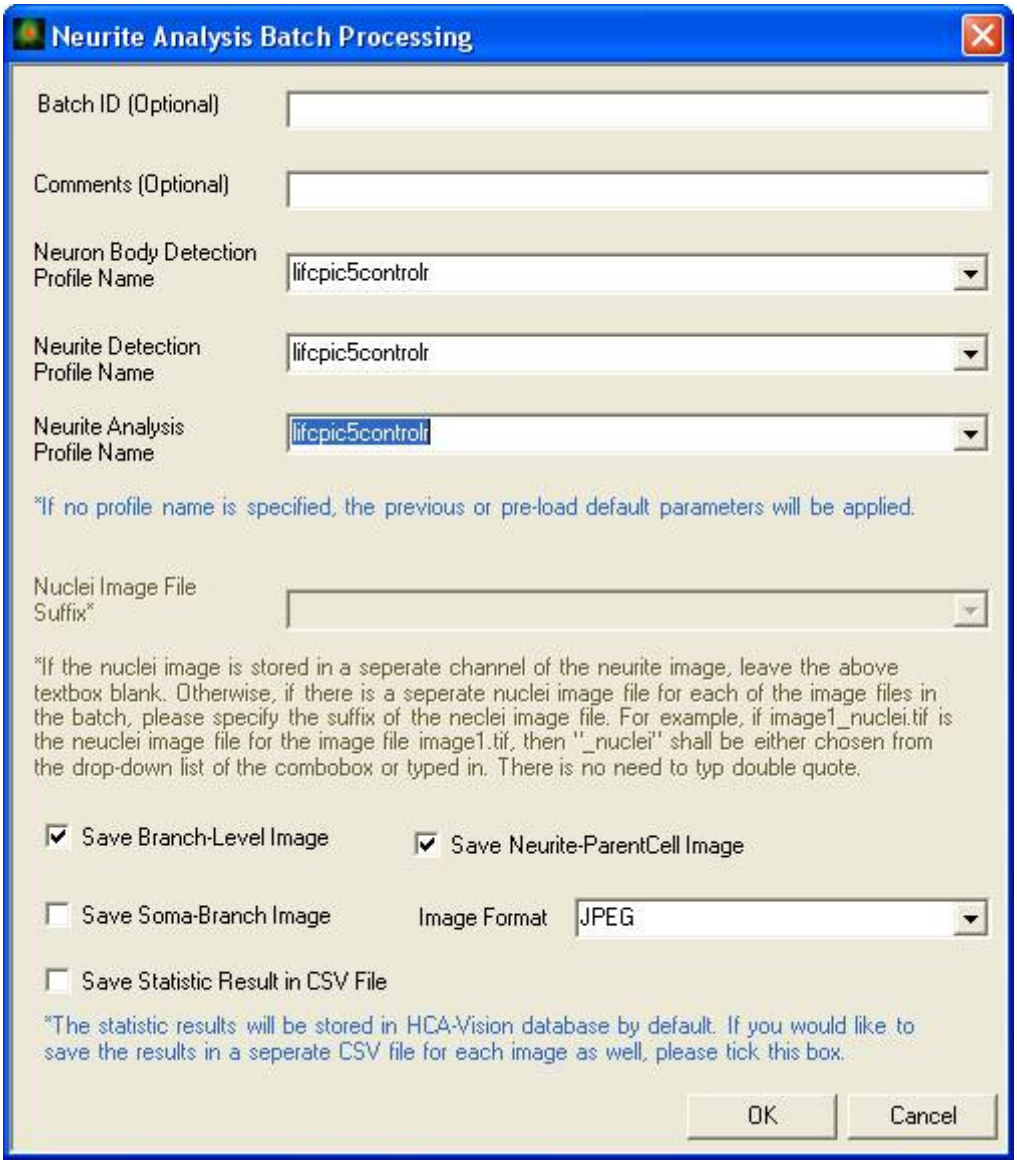


Figure 8 Batch processing dialog box

3.8 HCA-Vision Database

Upon completing a Batch Process, the results of either the Neuron Body Detection or Neurite Analysis are saved into a database. From the database menu you have access to the following functionality:

3.9 Database management

We define a database as a collection of results from various batch processes. You can set the current database which allows you to view the results from the database's various batches or append further results to it. The current available databases are listed in the Set Current database window with the current database highlighted as shown in the following figure.

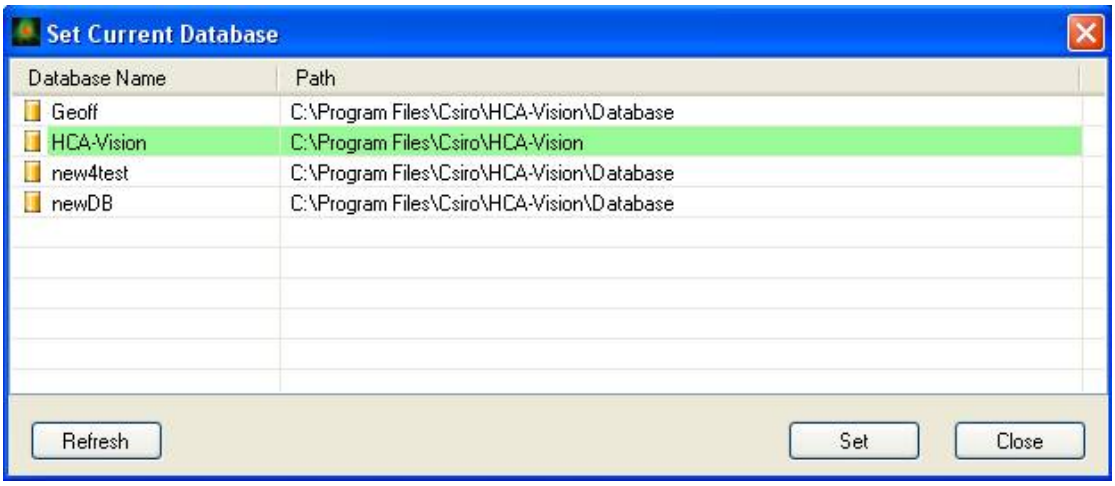


Figure 9 Set current database window

The user can also create a new database if you want to save the batch processing results for your images into a different database by clicking Database>Database Management>Create New Database menu. The create new database window is shown above.

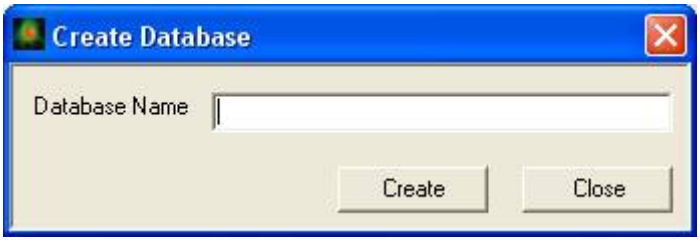


Figure 10 Create new database window

3.9.1 View batch processing results

After running the particular analysis in batch mode, you can view its results in database format. For example, if you have done the batch processing described in the example in the Batch Processing section, select Database>View Batch Processing Results>Neurite Analysis. This starts up a new window with 2 tabs, shown below.

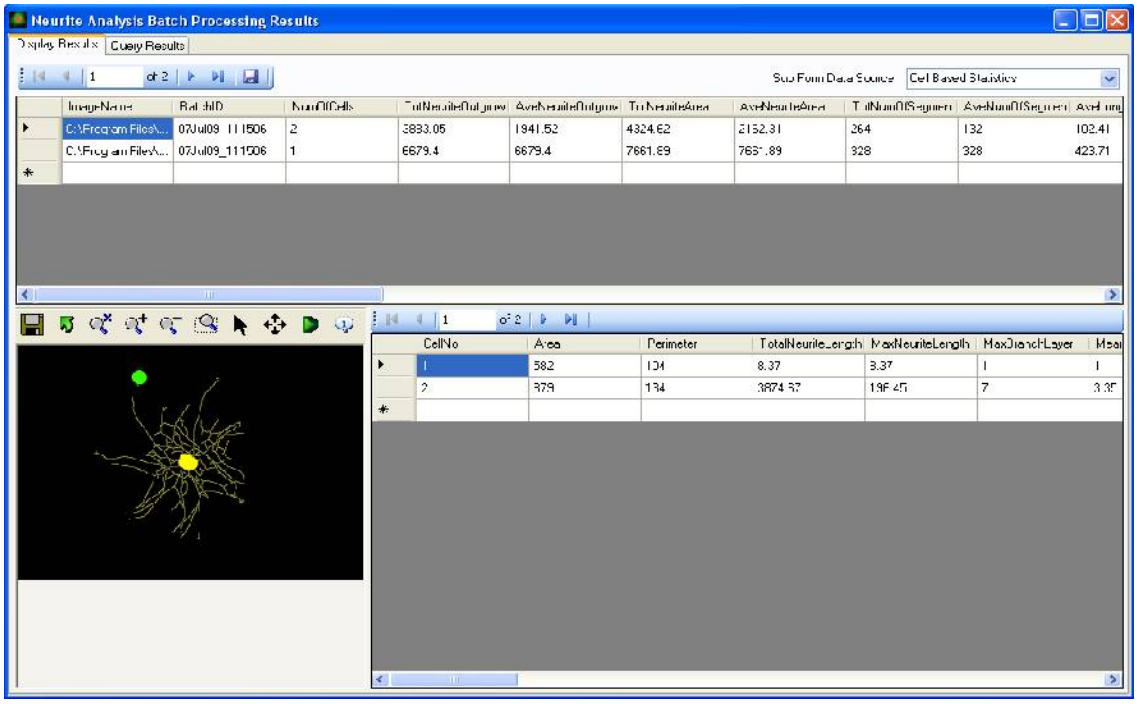


Figure 11 Neurite Analysis Batch processing Result display

The default here is the "Display Results" tab. The top pane shows the images analyzed along with their batch ID and the image-wide summary statistics. By clicking on the line corresponding to an image, the cell-by-cell results are returned in the lower panes, with the image shown on the bottom left and the results on the bottom right. You can cross reference from the image to the table in much the same way as described in the results section. The batch processing result for the highlighted image can also be exported by clicking the Save Data button (the disk icon in the top pane).

3.9.2 Query Database

Clicking on the “Query Results” tab brings you to the functionality for database querying. To build a query, first “Select a Table” of results, for example “CellBasedNeuriteStatistics”. You can then “Select Fields of Interest” such as “Image Name”, “CellNo”, “TotalNeuriteLength” and “NumBranchPoints” by holding down the Ctrl button while clicking on the appropriate field. You can now "Select a BatchID" for a particular batch, or select all batches (by not selecting any batches, you will query all the batches). Now to build a query, click on the "Where" button to bring up the Where Clause window shown in Figure 12, choose a field, a condition and a value, for example “TotalNeuriteLength” greater than 1000, then click “Add to Where Clause”. If you wish to add additional queries, you must select either the “And” or “Or” operators as the way to combine the queries before clicking “Add to Where Clause”. Continue adding queries in this fashion and then press “OK”. Now press “Create Query Statement”. The statement will appear in the “SQL Statement Box”. Finally, press “Execute” and the results will appear in the Query Result pane as shown in Figure 13. The selected results can be exported to Excel etc. using the Export icon (a disk icon above the query result). When finished querying the data, kill the Batch Processing Results window by clicking the Close button (the cross in the top right corner).

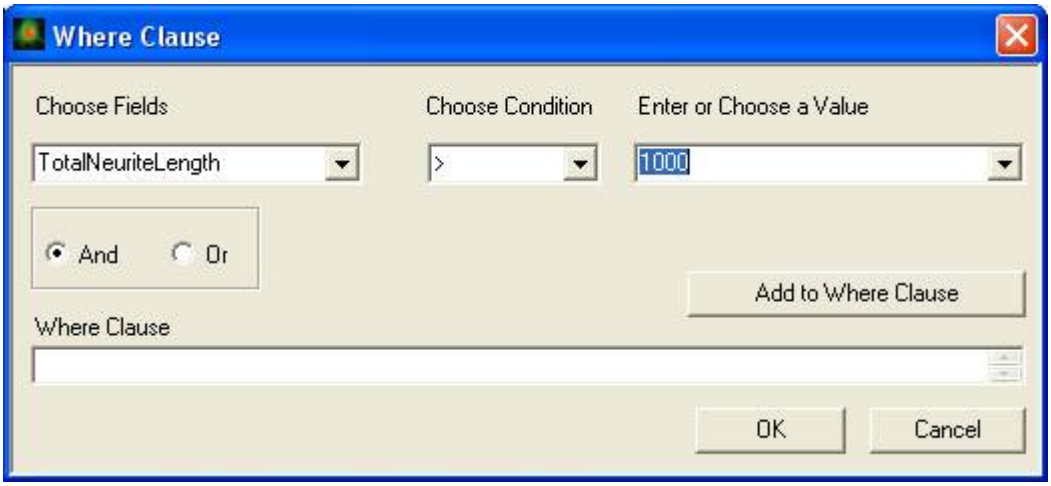


Figure 12 Where Clause window

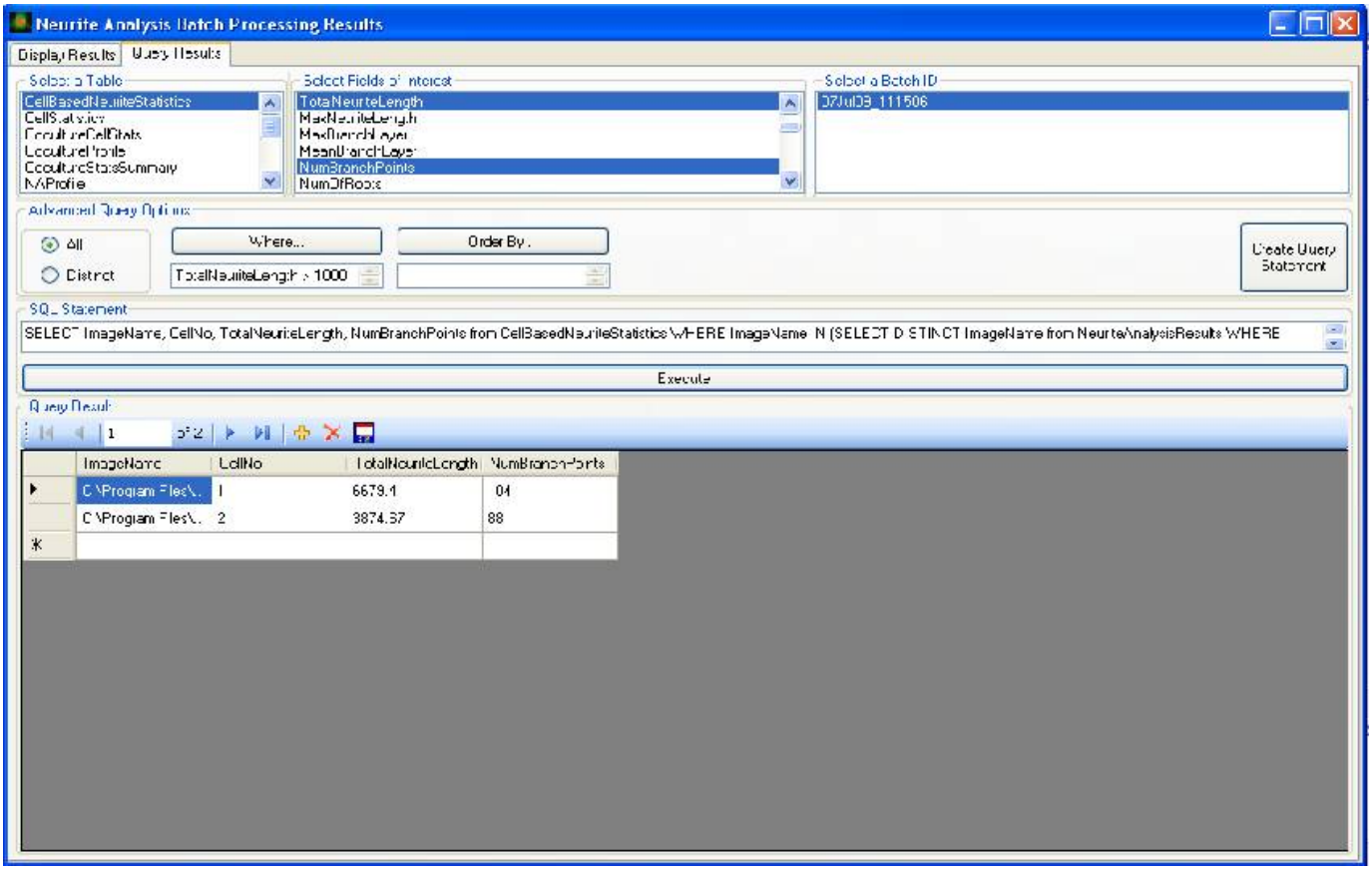


Figure 13 Database query window

3.10 Neurite Analysis with Mixed Cell Populations

In mixed cell populations, it is often necessary to filter and separate each cell type before cell counting and further analysis. For example, Figures 14 and 15 both contain a mixture of

neurons and other brain cell types. Neurite analysis was to be performed only on neurons. In Figure 14, the neurons (red) can be readily separated from the astrocytes (green) and other unstained glial cells based on high intensity in the red channel. In Figure 15 however, the non-neuron nuclei could only be filtered out by using a combination of their size, brightness and low intensity in the neuron (green) channel.

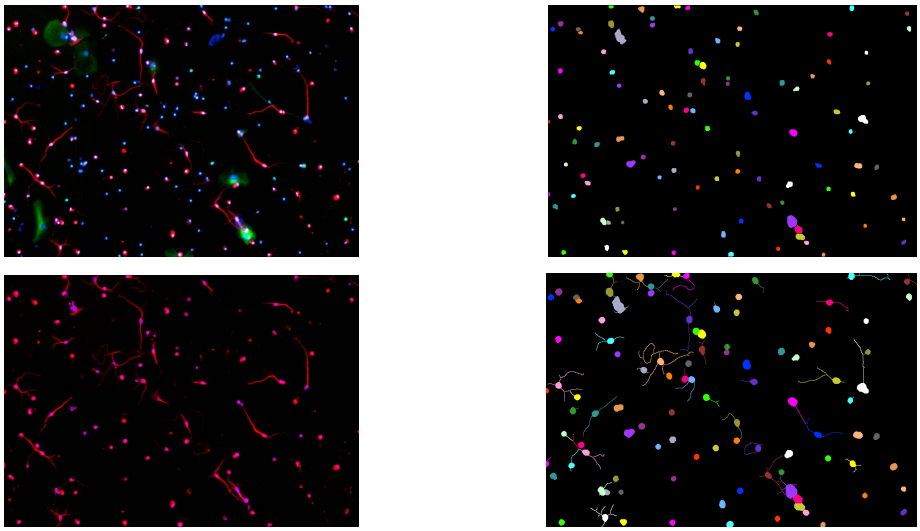


Figure 14 To screen for compounds which drive differentiation of mouse neural stem cells into neurons, filter the raw image (top left) containing neurons in red and astrocytes in green, to select just the neurons (bottom left). Nuclei of all cell types are shown in blue. Top right and bottom right images show the neuron body detection and neurite analysis results, respectively.

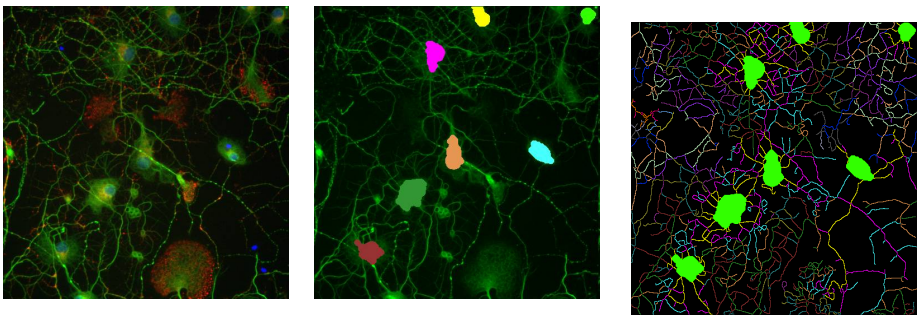


Figure 15 left – raw image with all nuclei labelled in blue (Hoechst), neurites in green (Tuj1) and synapses in red (Synaptophysin); middle – detected neuron bodies overlaid on the neurite channel, with non-neuron nuclei filtered out; right – neurite analysis result showing different levels of branching for neurons only.

The filtering by nucleus size can be done by specifying the nucleus area in the Neuron Body Detection wizard page shown below:

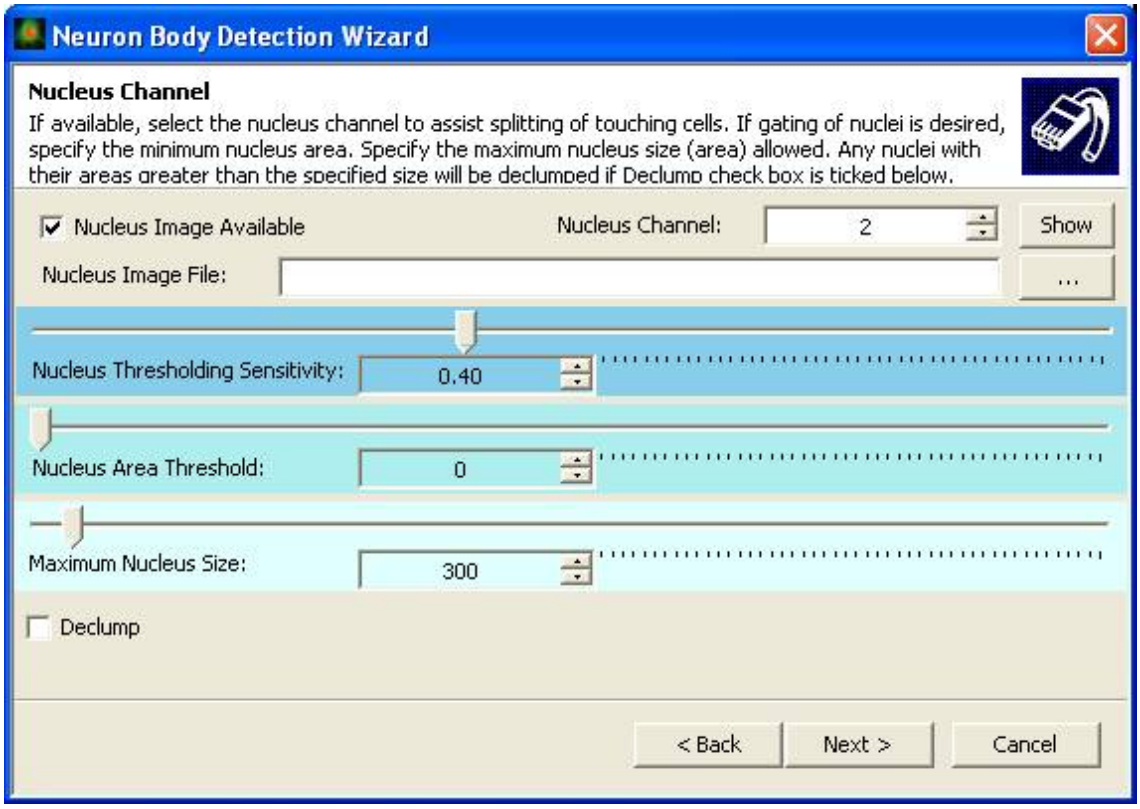


Figure 16 Nucleus filtering using area criterion

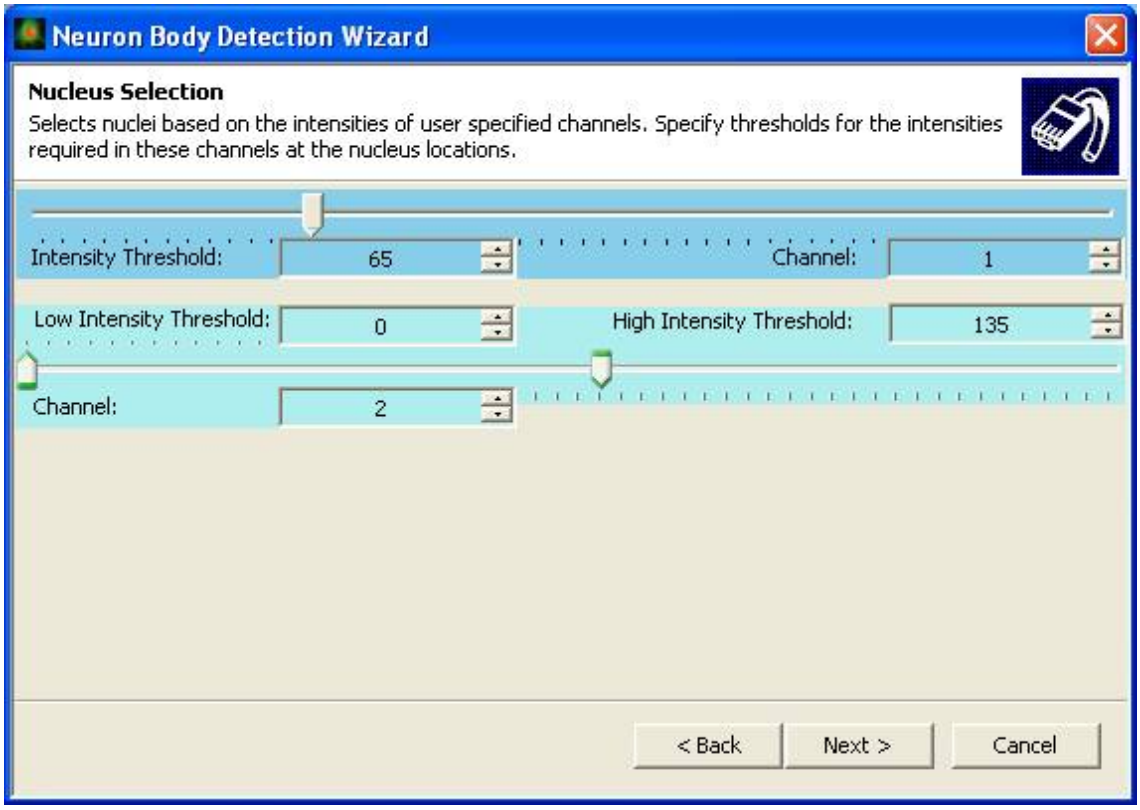


Figure 17 Nucleus filtering using intensity criteria in different channels

4. SUBCELLULAR ANALYSIS

Switch to the Subcellular Module by clicking Modules>Subcellular Analysis.

The Subcellular Analysis module allows measuring the translocation of proteins between virtually any two cell compartments. The functions include creating a mask or a region of interest, then applying the mask to capture different kinds of information or to measure intensities in different channels and regions.

This module includes Nucleus Detection, Cell Body Detection, Membrane Detection, Cell Scoring, Colocalisation Analysis.

4.1 Nucleus Detection

It should be noted that if a nucleus image is available, it will be used as a mask to detect cell bodies. So if this is the case, please run the Nucleus Detection function before running the Cell Body Detection function. However, if you do not have nucleus image and you still want to detect cells, this can be done by just running the Cell Body Detection function.

4.2 Cell Body Detection

The Cell Body Detection function is almost identical to the Neuron Body Detection Function in the Neurite Analysis Module, except that it is made generic for any type of cell instead of just neurons.

4.3 Cell Scoring

Before running the Cell Scoring function, Cell Body Detection needs to be run to get the cell body masks to be used for Cell Scoring.

This section describes how to do a multi-channel cell analysis using this option in the Wizard pull-down menu. This analysis is best explained by the example below.

4.3.1 Cell Body Detection

Choose the file "Colocalisation.tif" from the SampleImages directory in the installation if it is not loaded. This image is the default for this module. We would like to do some intensity measurements to determine the number of positive cells with respect to the green and red channels. Firstly we need to detect the cells. Simply click on the Cell Body Detection button in the tool bar (hover your mouse on the double cell icon in the toolbar, you will see the tooltip “Cell Detection”), or go through the wizard to produce the cell body detection result.

4.3.2 Cell Scoring

From the Wizard menu, select Cell Scoring. A dialogue box will pop up describing the steps involved - setting the primary intensity threshold, setting the secondary intensity threshold and generating the report. A screenshot of the Cell Scoring module is shown in Figure 18 with the details for each step given below:

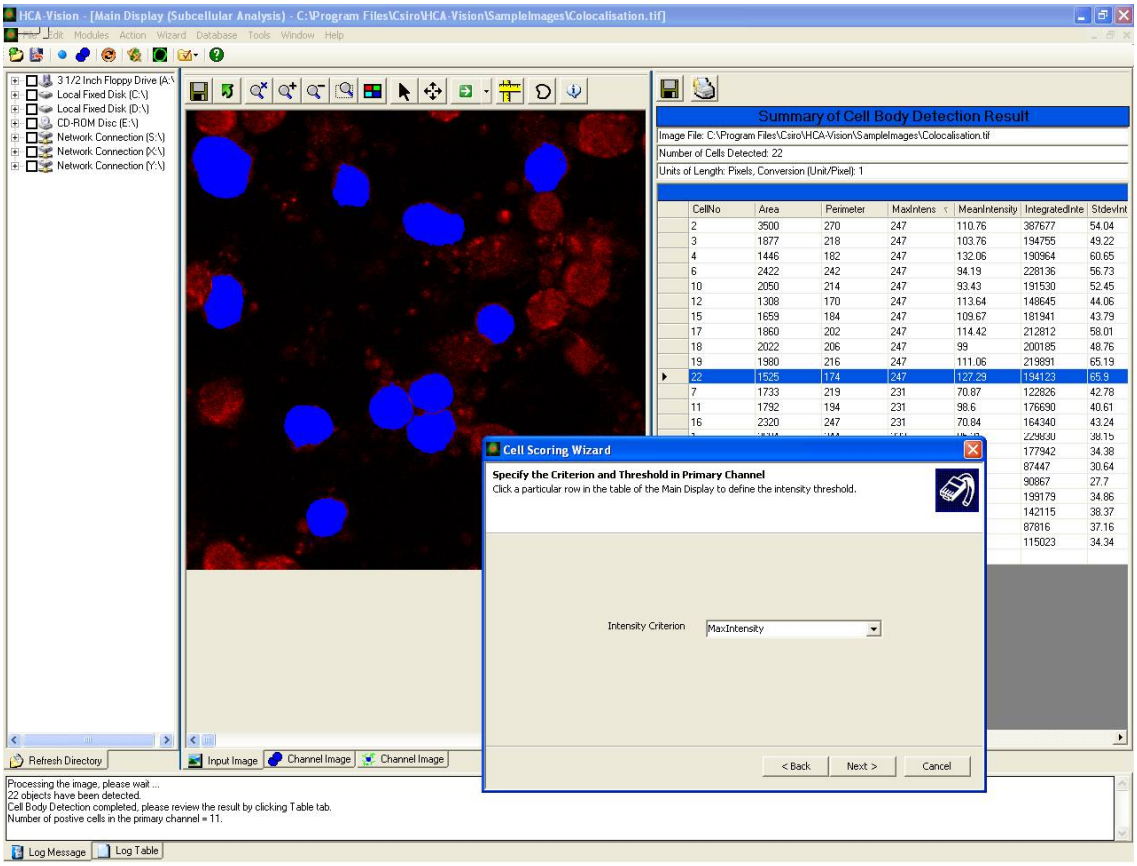


Figure 18 Cell Scoring screenshot

- Setting the primary intensity threshold - choose from the four different intensity measures in the combo box for the primary channel. (the primary channel is specified as the channel used to do the cell detection, in this case the green channel). This orders the table based on this measure. You can now click on a "row header" of the row corresponding to a threshold for a positive cell with respect to the primary channel. The Result pane will show which cells are now considered positive due to this threshold. Click Next.
- Setting the secondary intensity threshold - choose from the four different intensity measures in the combo box for the secondary channel (the secondary channel is chosen during the cell detection above as the "Additional Intensity Channel", in this case the red channel). This orders the table based on this measure. You can now click on a "row header" of a row corresponding to a threshold for a positive cell with respect to the secondary channel. The Result pane will show which cells are now considered positive due to this threshold. Click Next.
- Create report - click on the create report button and the results will appear in the Log pane of HCA-Vision. You will see the total number of cells, the number of positive primary channel (green) cells, the number of positive secondary channel (red) cells and the number of cells which are positive in both the primary and secondary channel. Click Finish.

4.4 Co-localisation Analysis

The Co-localisation function reports both image-wide and cell-based features, such as Number of Cells detected, Pearson's Colocalization coefficient, overlap, Manders' coefficients (K1, K2, M1, M2), Costes' co-localisation coefficient (M1/M2), cell area, perimeter, intensities in nucleus and reporter channels etc.

Before you run this module, you need to run the Cell Body Detection function to get cell body masks. The cell body mask will be used to calculate cell based co-localisations.

4.4.1 Cell Body Detection

Click the Cell Detection icon in the toolbar or click the menu Action>Cell Body Detection

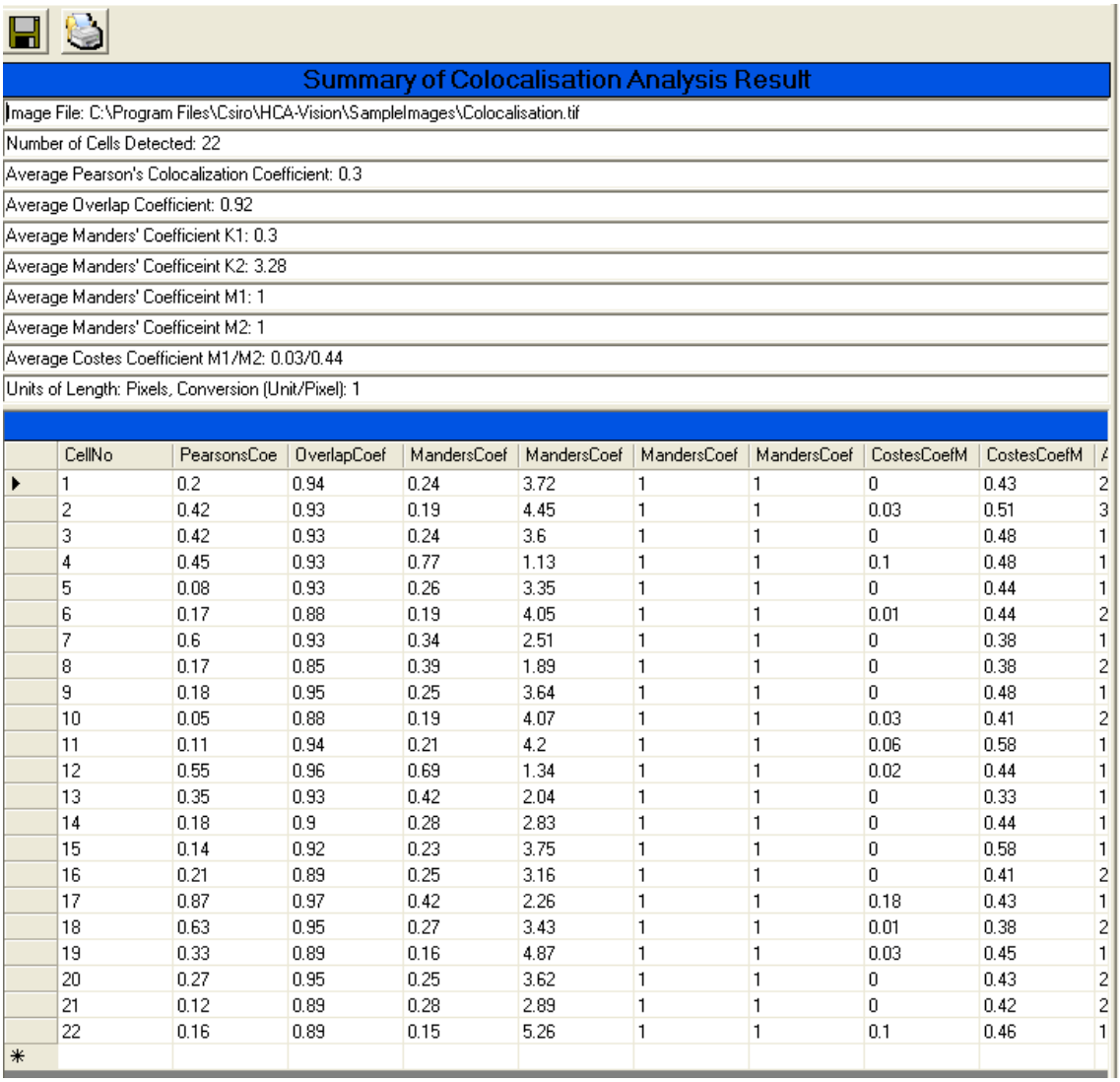
4.4.2 Colocalisation Analysis

Launch the Colocalisation wizard by clicking Wizard>Colocalisation Analysis Wizard.

In the wizard, you need to specify the red and green channels for the colocalisation analysis. To Specify the Background Values for Red and Green Channels, leave both intensity values 0 unless you know the background intensity value. In a future version, the average background will be calculated automatically.

The colocalisation parameters reported in the Table of Results are displayed in

Figure 19. For more details of these parameters, please refer to the publications listed in the reference section of this document.



Summary of Colocalisation Analysis Result

Image File: C:\Program Files\Csiro\HCA-Vision\SampleImages\Colocalisation.tif

Number of Cells Detected: 22

Average Pearson's Colocalization Coefficient: 0.3

Average Overlap Coefficient: 0.92

Average Manders' Coefficient K1: 0.3

Average Manders' Coefficeint K2: 3.28

Average Manders' Coefficeint M1: 1

Average Manders' Coefficeint M2: 1

Average Costes Coefficient M1/M2: 0.03/0.44

Units of Length: Pixels, Conversion (Unit/Pixel): 1

CellNo	PearsonsCoe	OverlapCoef	MandersCoef	MandersCoef	MandersCoef	MandersCoef	CostesCoefM	CostesCoefM	A
1	0.2	0.94	0.24	3.72	1	1	0	0.43	2
2	0.42	0.93	0.19	4.45	1	1	0.03	0.51	3
3	0.42	0.93	0.24	3.6	1	1	0	0.48	1
4	0.45	0.93	0.77	1.13	1	1	0.1	0.48	1
5	0.08	0.93	0.26	3.35	1	1	0	0.44	1
6	0.17	0.88	0.19	4.05	1	1	0.01	0.44	2
7	0.6	0.93	0.34	2.51	1	1	0	0.38	1
8	0.17	0.85	0.39	1.89	1	1	0	0.38	2
9	0.18	0.95	0.25	3.64	1	1	0	0.48	1
10	0.05	0.88	0.19	4.07	1	1	0.03	0.41	2
11	0.11	0.94	0.21	4.2	1	1	0.06	0.58	1
12	0.55	0.96	0.69	1.34	1	1	0.02	0.44	1
13	0.35	0.93	0.42	2.04	1	1	0	0.33	1
14	0.18	0.9	0.28	2.83	1	1	0	0.44	1
15	0.14	0.92	0.23	3.75	1	1	0	0.58	1
16	0.21	0.89	0.25	3.16	1	1	0	0.41	2
17	0.87	0.97	0.42	2.26	1	1	0.18	0.43	1
18	0.63	0.95	0.27	3.43	1	1	0.01	0.38	2
19	0.33	0.89	0.16	4.87	1	1	0.03	0.45	1
20	0.27	0.95	0.25	3.62	1	1	0	0.43	2
21	0.12	0.89	0.28	2.89	1	1	0	0.42	2
22	0.16	0.89	0.15	5.26	1	1	0.1	0.46	1

Figure 19 Colocalisation Analysis result

4.5 Membrane Detection

The Membrane Detection module can be used to quantitatively measure protein expression and translocation to the cell surface. It automatically traces the cell surface based on the calculation of a minimum cost path around a point inside the cell.

To launch the Membrane Detection Module, click Wizard>Membrane Detection Module. Please load the image “MembraneDetection.tif” in the SampleImages directory. The image is shown in the following figure. It shows results obtained on images of 3T3-L1 adipocytes retrovirally expressing a HA-GLUT4 reporter and immunostained. The Membrane Detection module was used to measure the relative amounts of GLUT4 on the plasma membrane under different conditions.

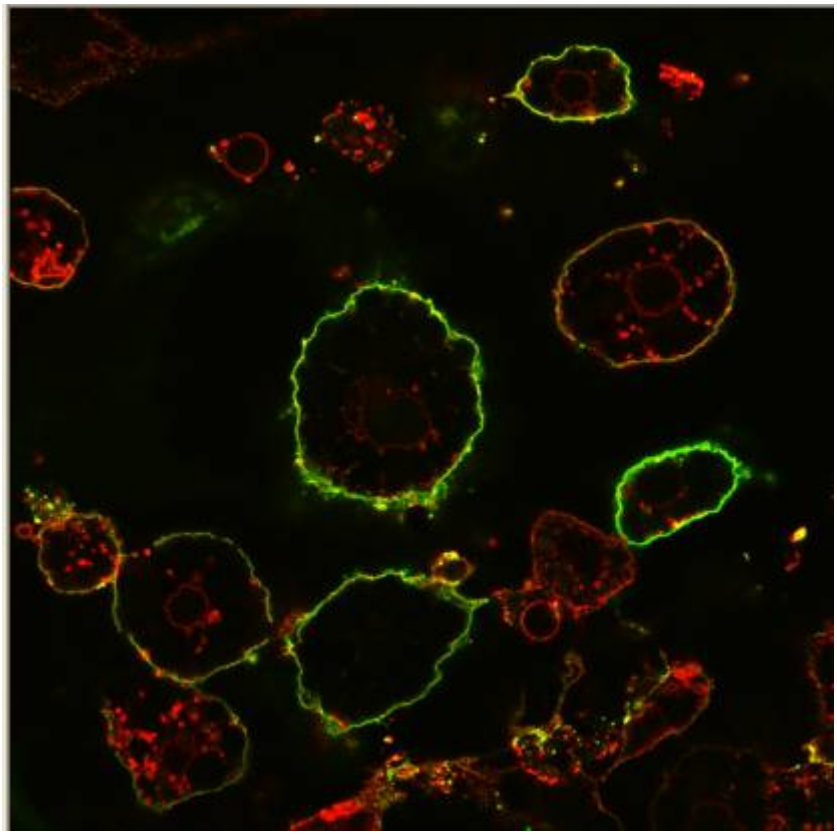


Figure 20 Membrane detection input image

The most important wizard page is the Specify Membrane Centre/Guiding Points page. When you bring this page up, the Specify Centre Point is ticked by default. Just choose one of the cells shown on the screen and click the centre of the cell (anywhere inside the membrane), an overlaid image will be shown on the Input Image pane and also the membrane will be shown in the Result Image pane. If the detection result is correct, just click “Save Result” to save the detection result for the current membrane. If you wish to add more cells, click “Next Membrane” button and click the centre of another membrane to detect. If a cell’s membrane has not stained uniformly, the detected membrane may not be perfect as for the cell in Figure 21. However, you can improve it by:

- Click Specify Guiding Points
- Click a few guiding points in the area where the membrane was not detected correctly as shown in Figure 25.
- Click “Apply Guiding Point” to update the detected membrane. You can always add more guiding points to improve the membrane detection results if needed.

When sufficient guiding points are added, the membrane is detected properly as shown in Figure 26. Click “Save Result”.

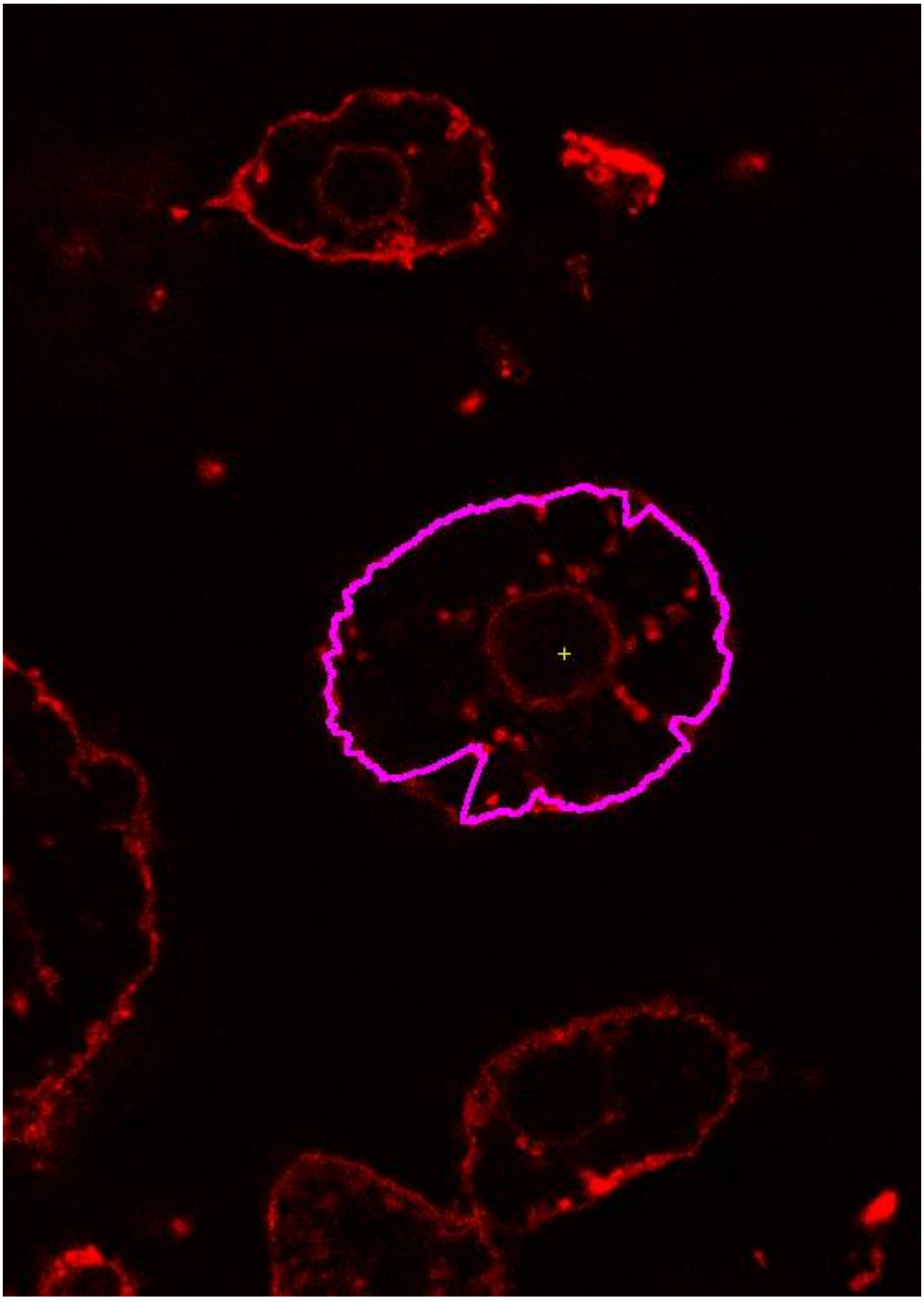


Figure 21 Initial membrane detection result by clicking membrane centre point only

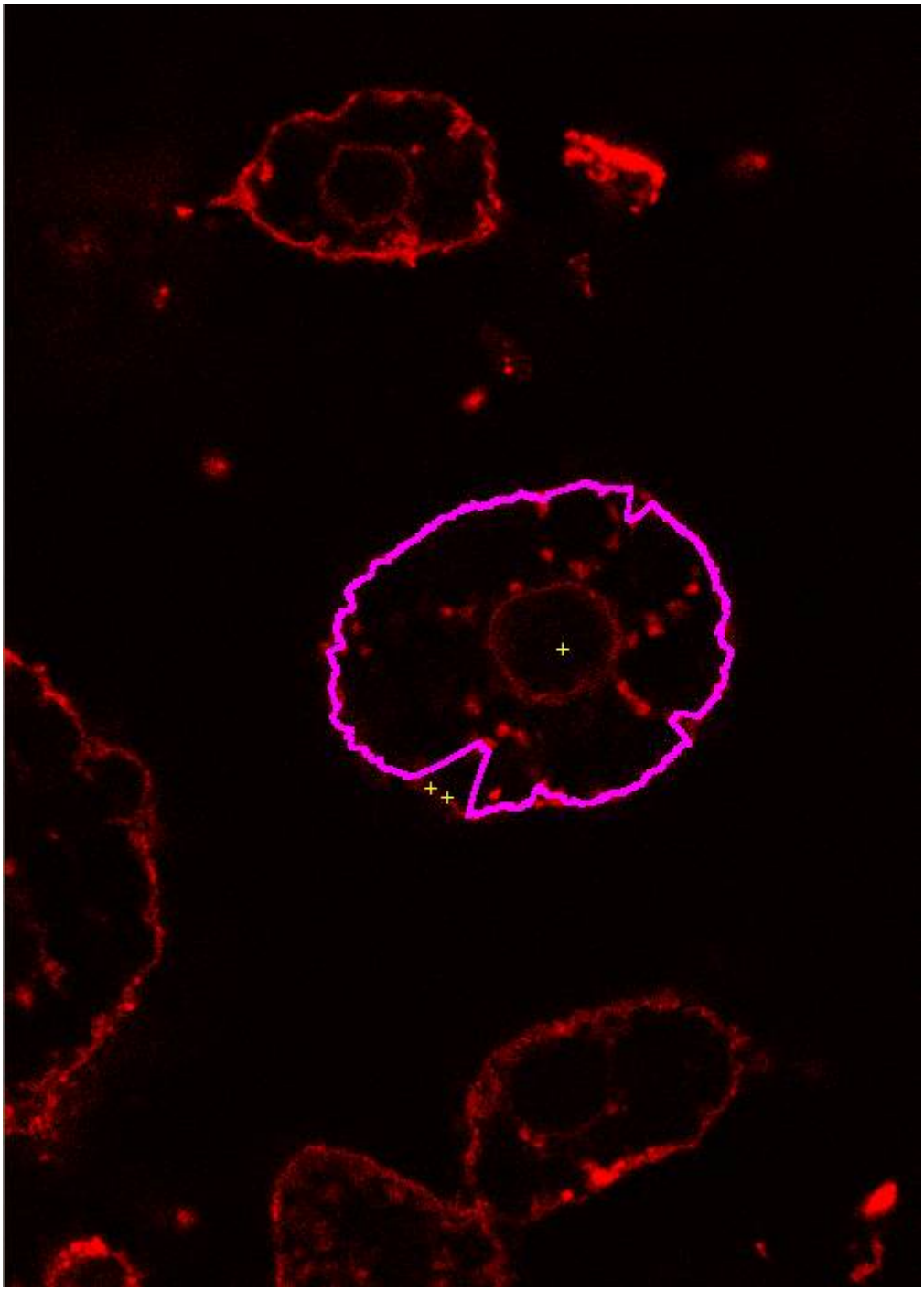


Figure 22 Overlaid image after clicking adding guiding points

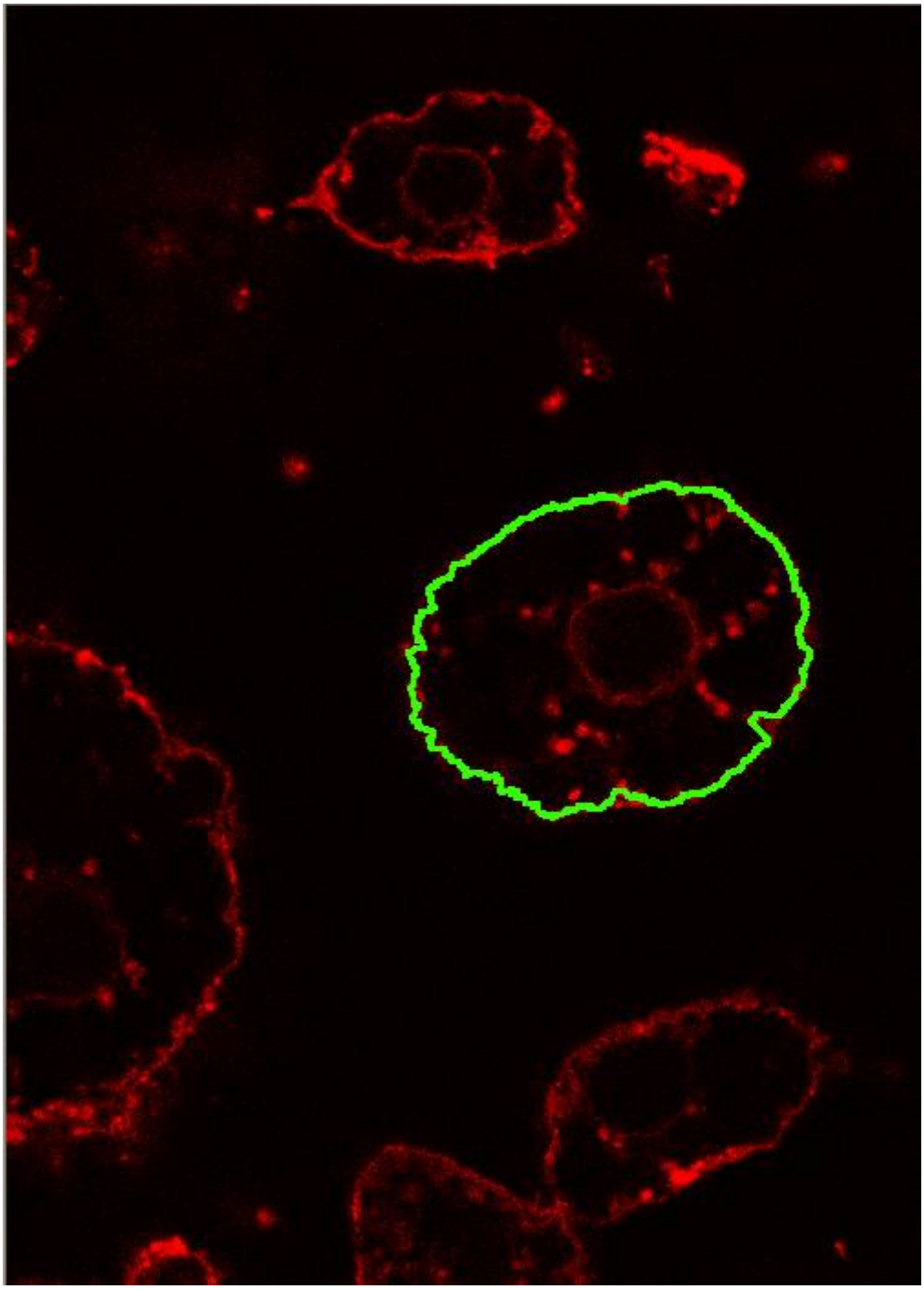


Figure 23 Membraen detection result after adding guiding points

Having defined the masks for the membranes you wish to measure, you have two options in the Specify Membrane Centre Point/Guiding Points page to modify those masks. You can thicken the membrane mask by changing the Dilate parameter. To exclude the parts of the membrane

which stained faintly, you can threshold the membrane mask by changing the Intensity Threshold parameter. When you are satisfied with the masks, click Next to display all the membrane masks. Click Finish.

After you have defined the membrane masks using the Membrane Detection Wizard, the intensity measurement of membrane and other channel images will be displayed in the Result pane by clicking Table tab. The Result Image will show all membranes you selected. An example is shown below:

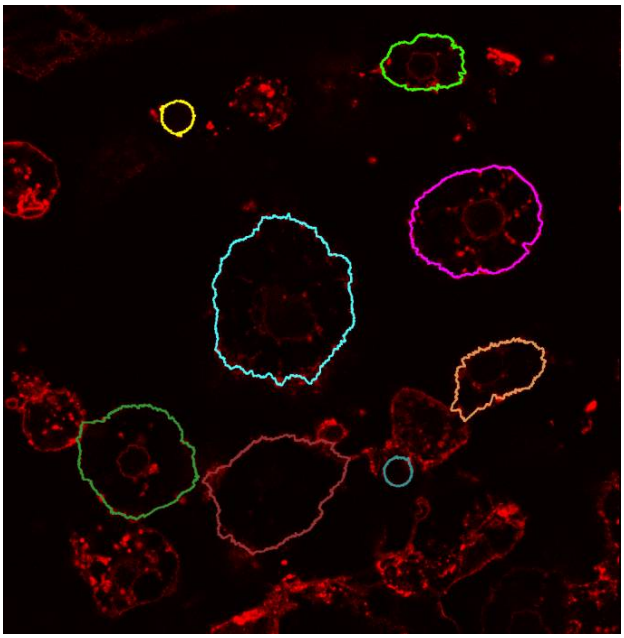


Figure 24 Membrane detection result image of chosen membranes

4.6 Region of Interest

Whenever you load an image, you can choose to perform all subsequent operations on only a portion of the image, termed a “Region of Interest” (ROI), rather than the whole image. Simply click the Region of Interest button located above the input image, the second from right. It looks like an irregular ellipse. Then you will be able to draw any irregular shape, as shown in the following figure, by holding down the left mouse button, dragging it around the required region and releasing it to close the loop.

Once you have created a region of interest, you can do nucleus detection, cell body and cell membrane detection, colocalisation analysis, intensity measurement etc. on that region following the same procedure as you do for the whole image. As the region may have a different intensity distribution compared to the whole image, you may need to go through individual wizards to tune the parameters instead of simply using the parameters previously tuned for the whole image.

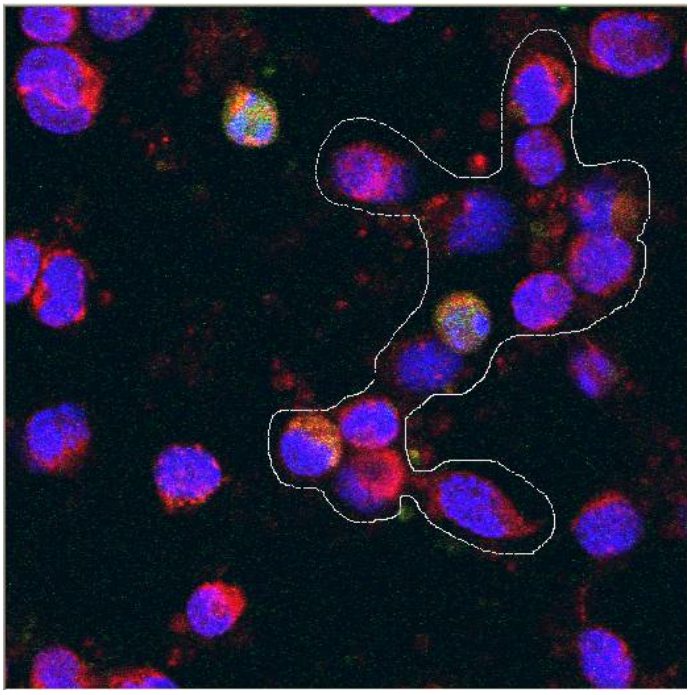


Figure 25 Region of interests created using HCA-Vision ROI tool

4.7 Intensity Measurement

You can measure the intensity of each channel in an entire image by loading the image then clicking the Action>Intensity Measurement menu. Then you will see the intensity measurement result in the Table of Results pane. Intensities will be reported for all channels of the image.

Alternatively, you can create a Region of Interest, then click Action>Intensity Measurement menu to measure the intensities for just that region.

5. TOOLS

From the Tools menu you can run the Create RGB Image function and also configure the default environment parameters of the HCA-Vision.

5.1 Create a RGB Image

If you happen to save your red, green and blue images into different files, you can combine them using the Create RGB Image function. Simply specify where your R, G, B images are stored, then specify the output image file location and name as shown below. If you just have an R and G channel image, you can ignore the B channel by leaving the B Channel Image File textbox blank.

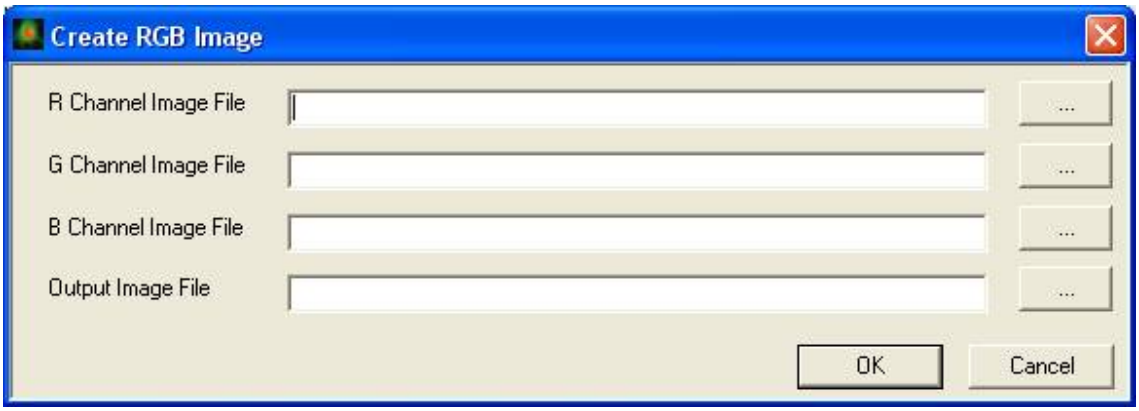


Figure 26 Create RGB Image window

5.2 Options

You can modify some general options via Tools>Options menu. The Options window is shown below. Click General tab to display some default environment parameters used in HCA-Vision. These are described below:

- Measurement Units: set the measurement unit to either pixels, micrometers, millimetres or centimetres.
- Conversion (Unit/Pixel): set the conversion rate between pixels and your selected unit.
- Max Number of Cells: set the maximum number of cells per image, default value is 3000.
- SQL Command Timeout: set the timeout for an SQL command, default value is 300 seconds.
- Display Accumulated Membrane Result – when ticked, membranes detected from different images will be displayed in the same result table for further analysis.

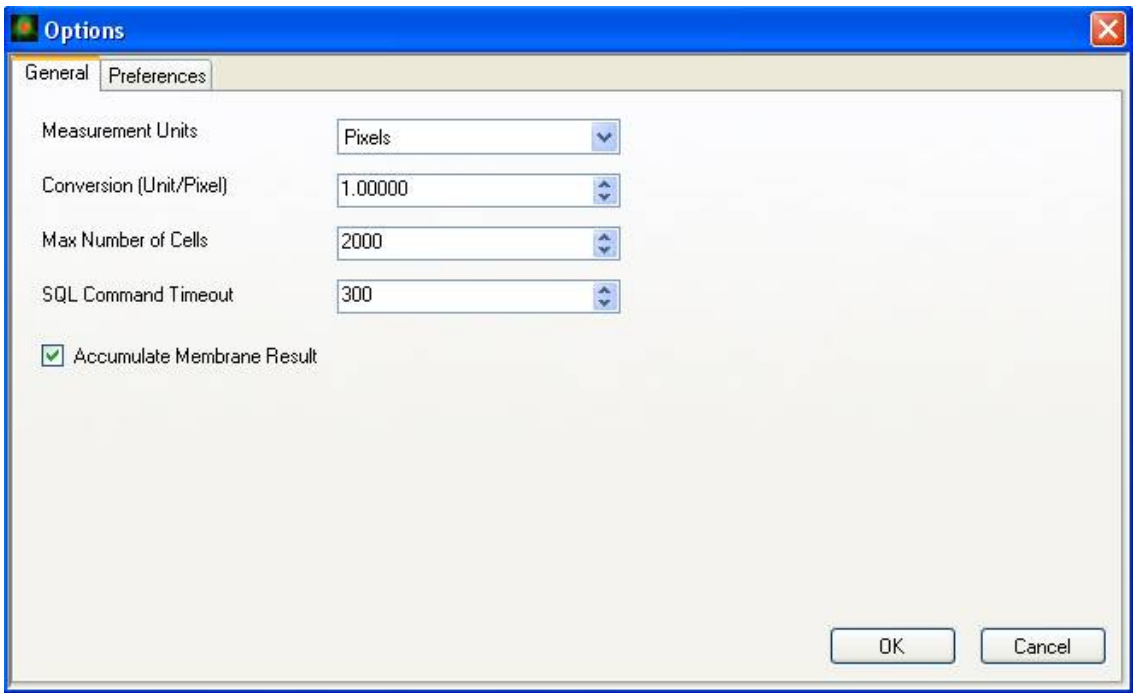


Figure 27 Option window to specify HCA-Vision global environment variables

Click the Preferences tab to display the following parameters:

- Default Nuclei Image Directory: when browsing for separate nuclei images, this is the default location
- Sample Image File: default image when no images are selected
- Regions File: a file for colour mapping output images
- Cell Detection Parameter Files: files which store all the user and default parameter settings for the current analysis module:

Neurite Analysis	**Subcellular Analysis**
Neuron Body Detection	Nucleus Detection
Neurite Detection	Cell Detection
Neurite Analysis	Membrane Detection

- Help File: the location of the help file
- Licence Key File: licence file location

6. HANDS ON PRACTICE – A STEP-BY-STEP GUIDE ON NEURITE ANALYSIS

In this session, we are going to reproduce the results presented in the following paper:

Vallotton et al. Automated Analysis of Neurite Branching in Cultured Cortical Neurons Using HCA-Vision. Cytometry, Part A, 71A(10):889--895, October 2007.

Sample images and tracing results reported in the paper can be downloaded from the following website:

http://www.hca-vision.com/CytometryA_files/

Download image files in “Min” and “Pos” directories.

Our purpose is to assess in a systematic manner the phenotype of Sez-6 null neurons (Sez-6 is a protein involved in brain development).

6.1 Detecting Neuron Bodies

- Launch HCA-Vision.
- Set Module to “Neurite Analysis”
- Open the image **Min\mNM1.tif**. This image is the first of about 50 images corresponding to the null mutant (the one lacking the protein).
- Go to the menu **wizard – neuron body detection wizard**. As the name suggests, the purpose of this assistant is to segment the cell bodies (i.e. the neurons without their neurites). Measurements are to be obtained on a per-cell basis.

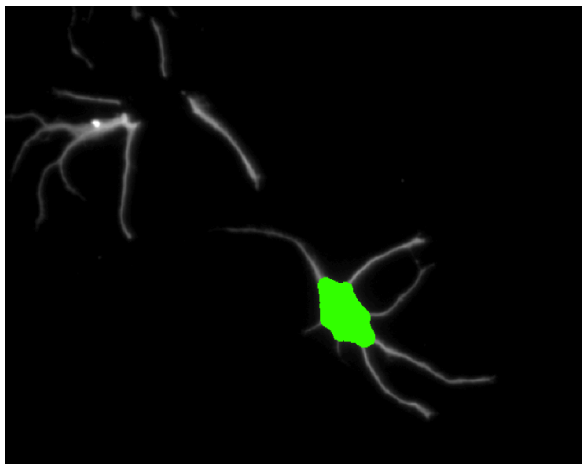


Figure 28 The detected neuron body is shown in green

- Read the outline of the different steps for detecting the neuron bodies. Press **Next**, and **Next** again, because you have already selected your image.
- Press **Next** again, because the image features only a single channel (no color).

- Gaussian filter the image using a filter width of 5 pixels. Press **Next**.

- Remove background bias using a value for the size of the structuring element equal to 99 pixels. Press **Next**.
- Suppress neurites using a size of the structuring element equal to 10 pixels. Observe that the neuron body maintains its approximate shape because it is much larger than that threshold. In contrast neurites have mostly disappeared. Press **Next**.
- **Threshold** the image using a normalized value equal to 0.6. Activate the flickering mode by clicking on white arrow on a green background (above the result image). This develops a set of menus. Activate **overlay – enable flicker – original image**. Convince yourself that the suggested value is reasonable. Try other settings and revert to the value of 0.6. Press **Next**.
- Observe that you do not have a **nucleus image** available. Therefore, untick the checkbox having that name. Do not worry about the image appearance and press **Next**, and **Next** again.
- Tick the **Segmentation** box to ensure that touching neuron bodies will be split
- Specify a minimum **neuron body area** of 1600 pixels, which is just enough to suppress cell debris but not enough to remove genuine neuron bodies. Press **Next**, and **Next** again because we do not have an additional channel to measure signals from. Press **Next**.
- Save the **profile name** under your own name, and **Finish**.

6.2 Detecting Neurites

- Proceed to the menu **wizard – neurite detection wizard**, and read the different steps. Press **Next**, and **Next** again, because we wish to act upon the currently selected image.
- Press **Next** as there are three channels available, select channel ‘0’. Press **Next**.

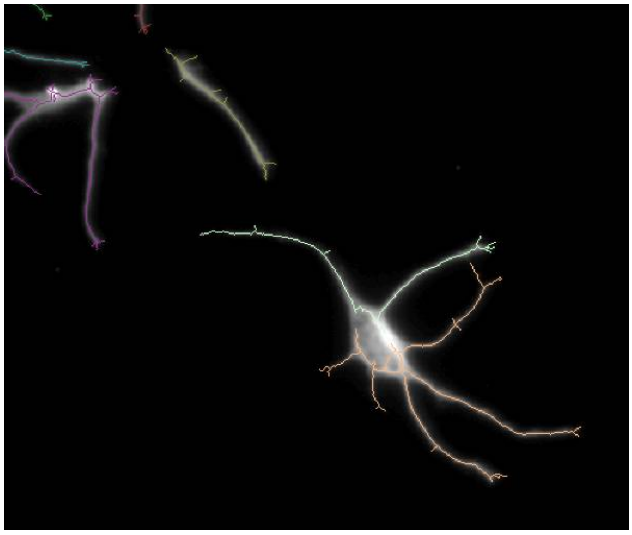


Figure 29 All linear features are overlaid onto the original image

- Filter the image using a Gaussian filter of a width equal to 7 pixels. Experiment with this setting. Too large a width will suppress the contrast of neurites. Too small a width does not improve the continuity of neurites along their length. Press **Next**.
- Use a **window size** of 9 pixels, which is the length of the line along which the algorithm assesses whether a pixel represents a local intensity maximum.
- Specify a **contrast** of 1.5, which (roughly) demands that the intensity of the center pixel along the line be at least 1.5 times the average intensity along that line. Notice how you can miss all neurites by specifying a very large value. Experiment with a value of 0.1. Revert back to a value of 1.5. Activate the flickering mode. Convince yourself that the suggested value is reasonable. You may want to zoom on the cell to see details. Press **Next**.
- **Remove objects** smaller than 10 pixels in diameter. Press **Next**.
- Close gaps of a maximum **span** of 9 pixels and demand that the **quality** of the bridging path be at least 30%. Press **Next**.
- **Save** the profile under your name and finish. Observe that the cell body boundaries are detected as linear features by mistake. This should not worry you because the neuron body masks will be covering these linear features.

6.3 Analysing Neurites on a Per Cell Basis

- Start the **neurite analysis wizard**. The purpose of this wizard is to combine results obtained in the **neuron body detection wizard** with those of the **neurite detection wizard**. Read the different steps and press **Next**, and **Next** again.
- **Debarbing** is the process of removing small lateral branches from the neurite traces that may have been created as a result of noise in the image. Select a length threshold of 11 pixels. Press **Next**.

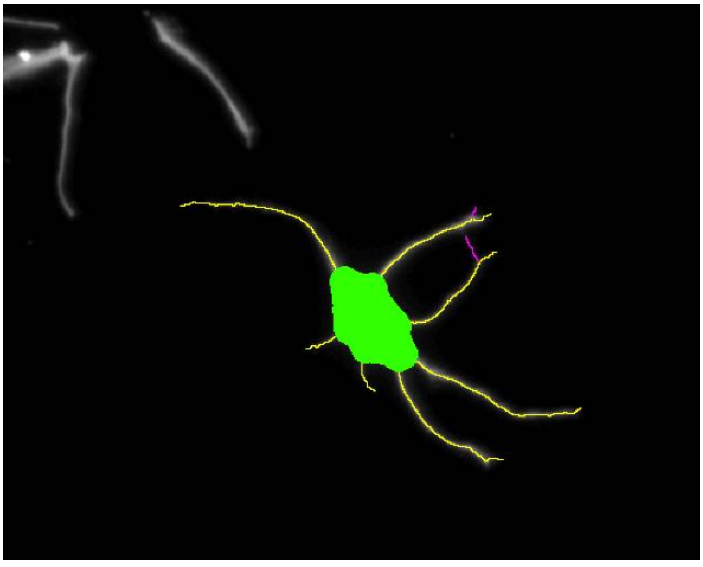


Figure 30 The neuron body is shown in green. Primary neurites are traced in yellow, and secondary neurites are in purple. The neurite analysis wizard relies on the parameters of both the neuron body wizard, and the neurite tracing wizard to produce these results.

- **Thicken** the neuron body by 4 pixels. This will ensure that neurites are connected to their respective neuron bodies, and therefore not removed from the analysis.
- **Remove** trees smaller than 8 pixels. Activate again the flickering mode and convince yourself that you've done a decent job of analyzing this image.

- Save the profile under your name and **finish** the wizard.

6.4 Batch Processing

- Select another image of the sequence, such as mNM10.tif and directly click on the icon corresponding **to neurite analysis**, which is the one representing a man with a magnifying glass in the toolbar. Evaluate the quality of the results using the **flickering** mode and the **zoom**.
- Do the same with a few images randomly selected in the same directory. This is the stage whereby, if you find that one particular image is analyzed sub-optimally, you need to modify the analysis parameter without affecting the quality of the results obtained on the other images. This may be relatively time consuming for a dedicated user.
- Use the batch processing capabilities to analyze all images in the directory. To that effect, in the directory tree check the box next the "Min" directory and click on the **batch processing** icon in the toolbar, selecting **neurite analysis**, and specifying your newly created parameter profiles if needed. Launch the batch analysis using the **OK** button and be patient…
- Let us examine the results by launching the menu **database – view batch processing results – neurite analysis**.
- Observe that each batch run is characterized by its own batch ID, in order to avoid mixing results from one experiment with those of another experiment.
- The averaged results in the top table are not always useful because the information should be analyzed on a per-cell basis, rather than on a per image basis. The table on the right of the result image contains information for every cell. Click at the very beginning of one row to highlight in the result image the cell for which that row pertains. Copy-paste the various statistics for cells that are entirely in the image and that do not touch other cells in ExcelTM.
- Now, using the same parameter profiles, repeat the batch processing for the dataset in the directory "Pos" (corresponding to the wild type cells) and assess whether the phenotypic statistics are significantly different for the wild type cells compared to the Sez-6 null cells.

6.5 Database Query

Using the **query results** function of the database window, isolate all cells that possess more than 3 roots segments, and that feature a neuron body area over 2000 pixels. Measure the average neurite field for only these cells.

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