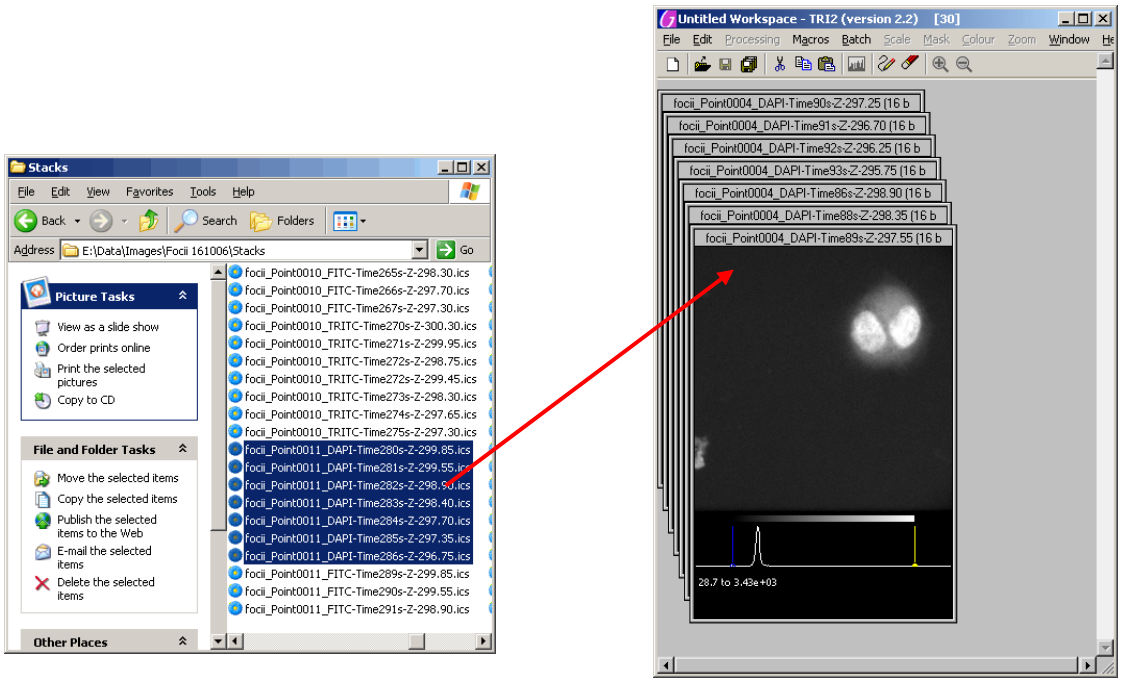
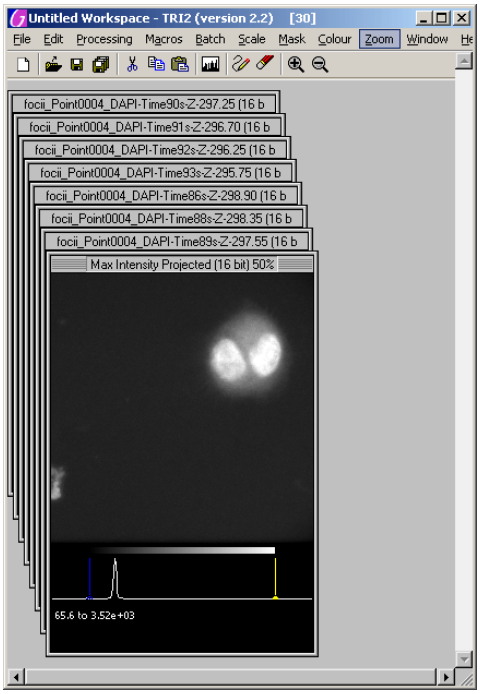
Batch Counting of Foci

Getting results from Z stacks of images.

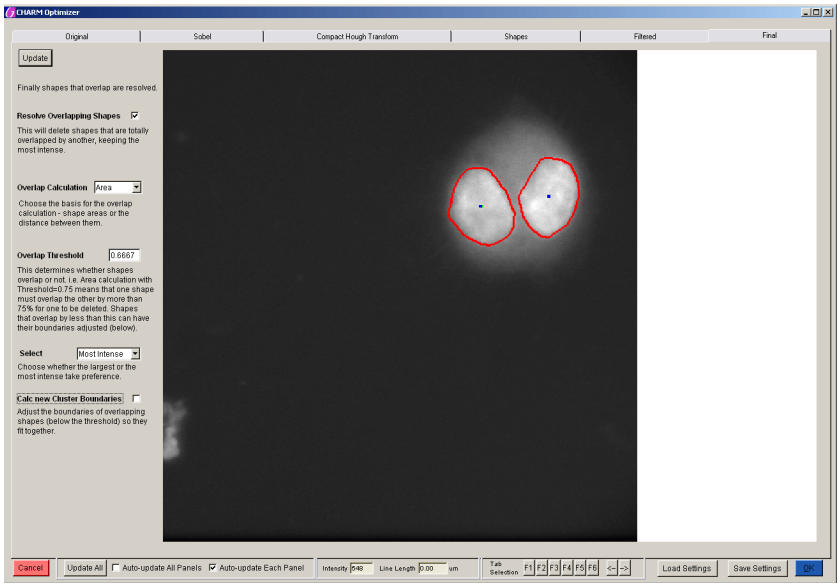
1. First it is necessary to determine suitable CHARM parameters to be used for batch counting. First drag a stack of images taken with the DAPI cube which will be used for region determination, (open TRI2, select the stack and drag them to the workspace) :-



2. From the “Processing” menu select “Maximum Intensity Projection”.

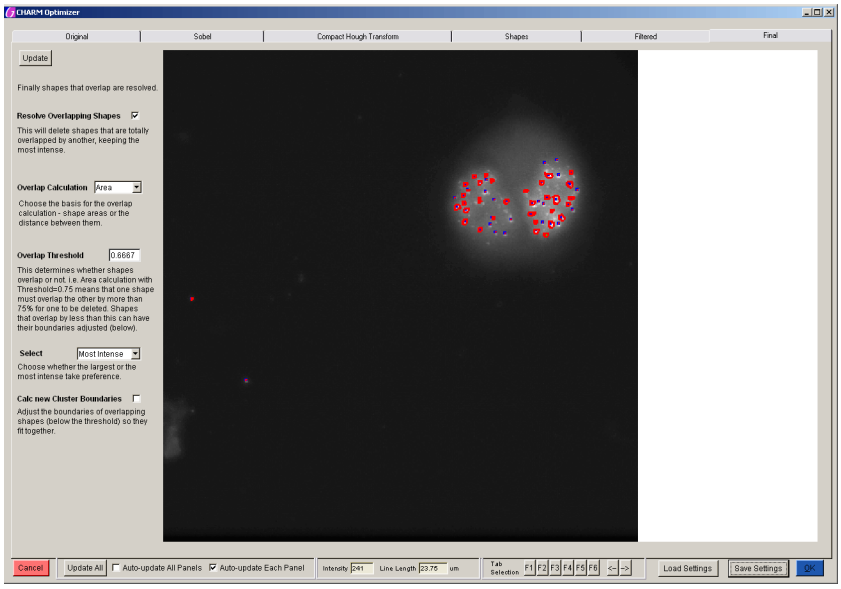


3. Run the CHARM Optimizer on the result to obtain good outlines of the cells. From the menu select “Processing->Counting->CHARM Counting->Optimizer”. See the document “Use of the CHARM Optimiser.doc” for guidance.

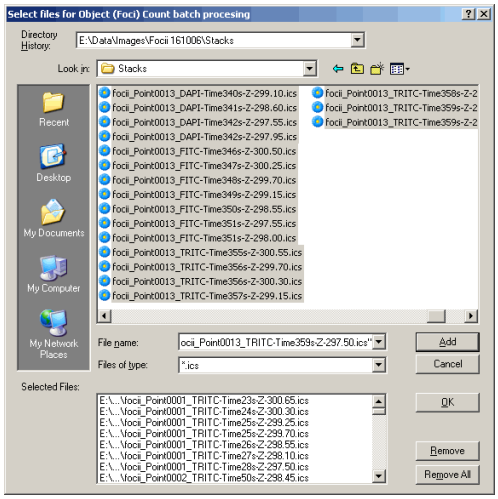


4. Use the “Save Settings” button to save the CHARM parameters. It is recommended to save the file in the same folder as the images to be analysed with a name like “region_params.xml”.

5. Clear the workspace, or open a new one and repeat to make “foci_params.xml”. This time use a stack of images acquired using the FITC or TRITC cube.

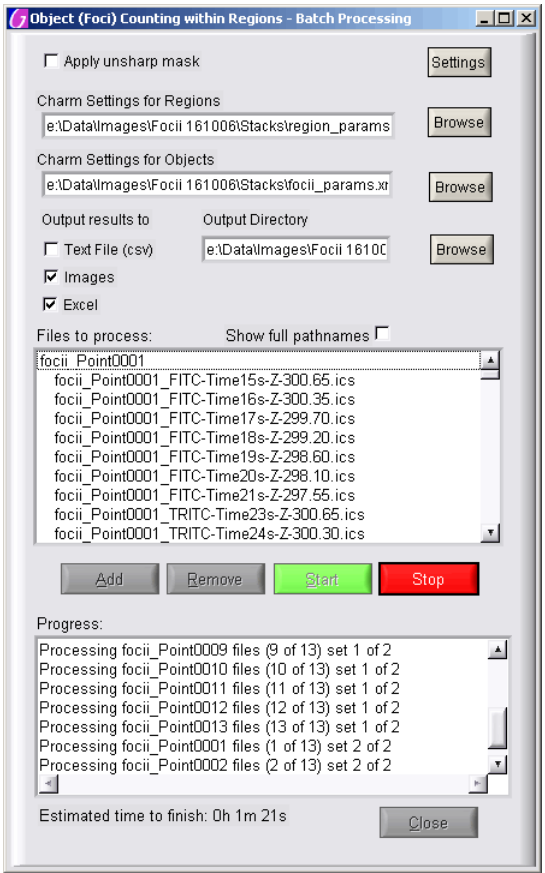


6. From the “Batch” menu select “Object (Foci) Count within Regions”. A file selection window appears. Navigate to the folder containing the stacks of images, select all those of interest and press the “Add” button. Then press “OK”.

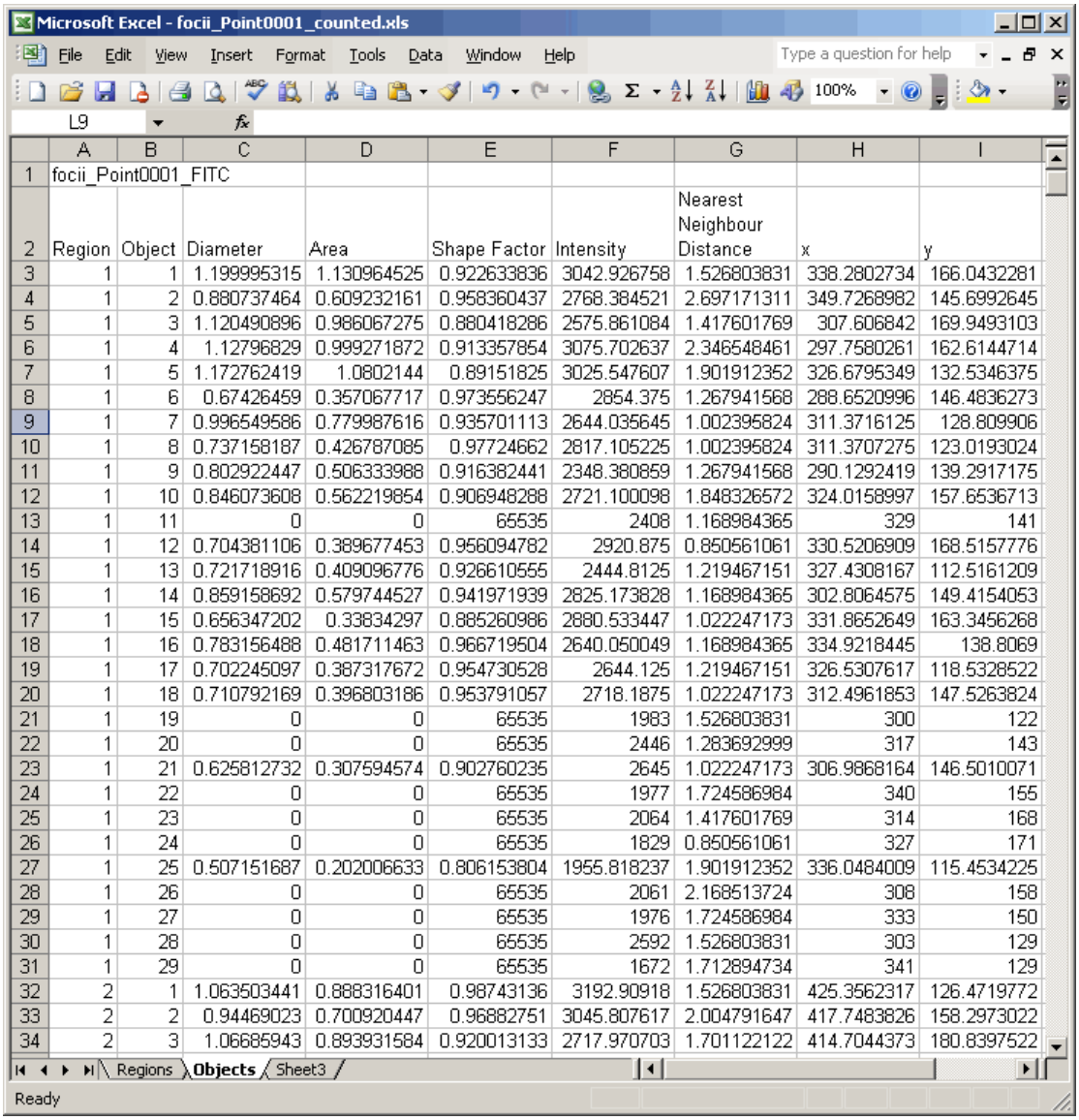


Images not captured on GCI systems may not have appropriate filenames, these files can be renamed to the following format:
Region/nucleus files: Any_name_region.ics
Object/foci files: Any_name_foci.ics
The important thing is that the text “region” or “foci” directly follows the last “_” character in the filename.
“Any_name” should be the same for region and foci files that match. A set of more that one foci file will be assumed as a through focus stack.

7. Use the “Browse” buttons to locate the CHARM settings files created earlier, and to select a folder where the results will be put.
Decide whether to output the results to text files, (one per stack), or Excel, (all results are put in one spreadsheet), or both.
Press “Start”.



8. When all the image stacks have been processed the spreadsheet is displayed.



Microsoft Excel - focii_Point0001_counted.xls

File Edit View Insert Format Tools Data Window Help

	A	B	C	D	E	F	G	H	I
1	focii_Point0001_FITC								
2	Region	Object	Diameter	Area	Shape Factor	Intensity	Nearest Neighbour Distance	x	y
3	1	1	1.199995315	1.130964525	0.922633836	3042.926758	1.526803831	338.2802734	166.0432281
4	1	2	0.880737464	0.609232161	0.958360437	2768.384521	2.697171311	349.7268982	145.6992645
5	1	3	1.120490896	0.986067275	0.880418286	2575.861084	1.417601769	307.606842	169.9493103
6	1	4	1.12796829	0.999271872	0.913357854	3075.702637	2.346548461	297.7580261	162.6144714
7	1	5	1.172762419	1.0802144	0.89151825	3025.547607	1.901912352	326.6795349	132.5346375
8	1	6	0.67426459	0.357067717	0.973556247	2854.375	1.267941568	288.6520996	146.4836273
9	1	7	0.996549586	0.779987616	0.935701113	2644.035645	1.002395824	311.3716125	128.809906
10	1	8	0.737158187	0.426787085	0.97724662	2817.105225	1.002395824	311.3707275	123.0193024
11	1	9	0.802922447	0.506333988	0.916382441	2348.380859	1.267941568	290.1292419	139.2917175
12	1	10	0.846073608	0.562219854	0.906948288	2721.100098	1.848326572	324.0158997	157.6536713
13	1	11	0	0	65535	2408	1.168984365	329	141
14	1	12	0.704381106	0.389677453	0.956094782	2920.875	0.850561061	330.5206909	168.5157776
15	1	13	0.721718916	0.409096776	0.926610555	2444.8125	1.219467151	327.4308167	112.5161209
16	1	14	0.859158692	0.579744527	0.941971939	2825.173828	1.168984365	302.8064575	149.4154053
17	1	15	0.656347202	0.33834297	0.885260986	2880.533447	1.022247173	331.8652649	163.3456268
18	1	16	0.783156488	0.481711463	0.966719504	2640.050049	1.168984365	334.9218445	138.8069
19	1	17	0.702245097	0.387317672	0.954730528	2644.125	1.219467151	326.5307617	118.5328522
20	1	18	0.710792169	0.396803186	0.953791057	2718.1875	1.022247173	312.4961853	147.5263824
21	1	19	0	0	65535	1983	1.526803831	300	122
22	1	20	0	0	65535	2446	1.283692999	317	143
23	1	21	0.625812732	0.307594574	0.902760235	2645	1.022247173	306.9868164	146.5010071
24	1	22	0	0	65535	1977	1.724586984	340	155
25	1	23	0	0	65535	2064	1.417601769	314	168
26	1	24	0	0	65535	1829	0.850561061	327	171
27	1	25	0.507151687	0.202006633	0.806153804	1955.818237	1.901912352	336.0484009	115.4534225
28	1	26	0	0	65535	2061	2.168513724	308	158
29	1	27	0	0	65535	1976	1.724586984	333	150
30	1	28	0	0	65535	2592	1.526803831	303	129
31	1	29	0	0	65535	1672	1.712894734	341	129
32	2	1	1.063503441	0.888316401	0.98743136	3192.90918	1.526803831	425.3562317	126.4719772
33	2	2	0.94469023	0.700920447	0.96882751	3045.807617	2.004791647	417.7483826	158.2973022
34	2	3	1.06685943	0.893931584	0.920013133	2717.970703	1.701122122	414.7044373	180.8397522

Regions | Objects | Sheet3

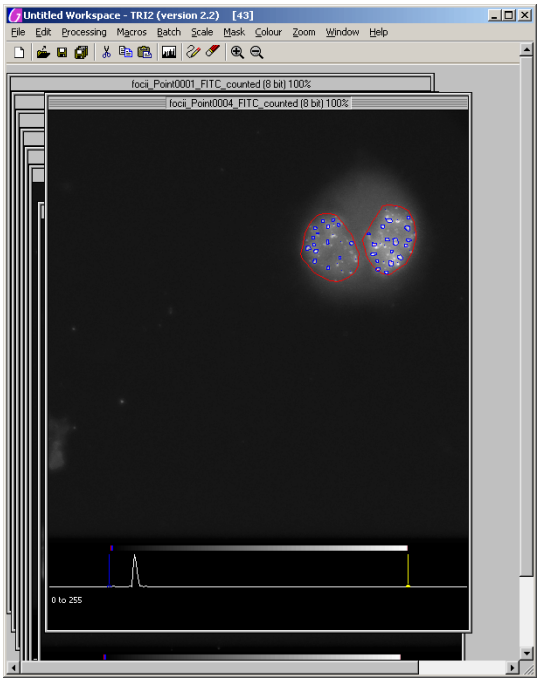
Ready

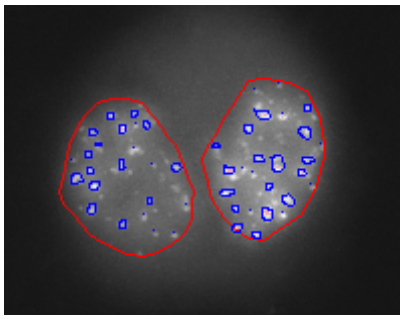
The following information is saved for each “Object”:-
Diameter and Area in microns
Shape Factor, (1.0 = circular).
Mean intensity.
Distance from its nearest neighbour.
The x and y position of its centre relative to the top left corner of the image.

In addition there is a sheet for the “Regions” with similar information and the “Object”, (Foci), count.

The Excel file is given the name of the first image stack. You will probably want to resave it with a more meaningful name and close Excel before analysing the next data set.

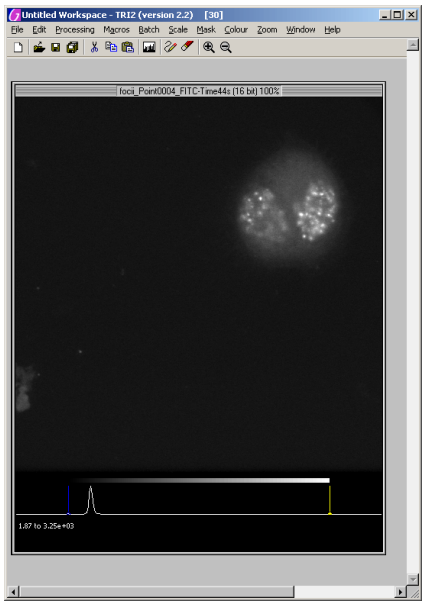
9. The images which indicate the results are saved in bmp format and these should be viewed to determine how well the algorithm has worked.
In the example below the region boundaries are good but clearly some foci have been missed. In the early stages it may be necessary to have several passes before arriving at suitable sets of CHARM parameters. Indeed for many data sets there will not be a “one size fits all” solution and there will have to be different parameters for each cube.



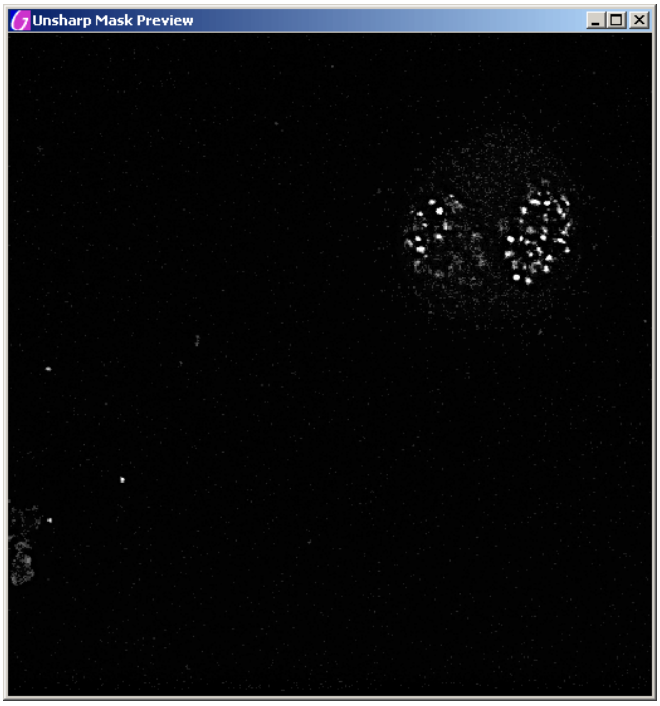


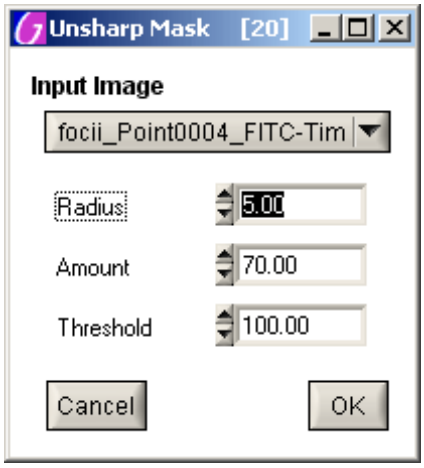
Getting results from extended focus images.

1. First it is necessary to determine suitable settings for the Unsharp Mask filter. Open an image acquired with the FITC cube. It will probably appear to be out of focus and overly bright, so drag the yellow cursor on the scale below the image to the left until an acceptable brightness is achieved.



2. From the "Processing" menu select "Unsharp Mask". An image preview window will pop up showing the results of applying an unsharp mask with the default set of parameters. The image will be updated as the parameters are changed.





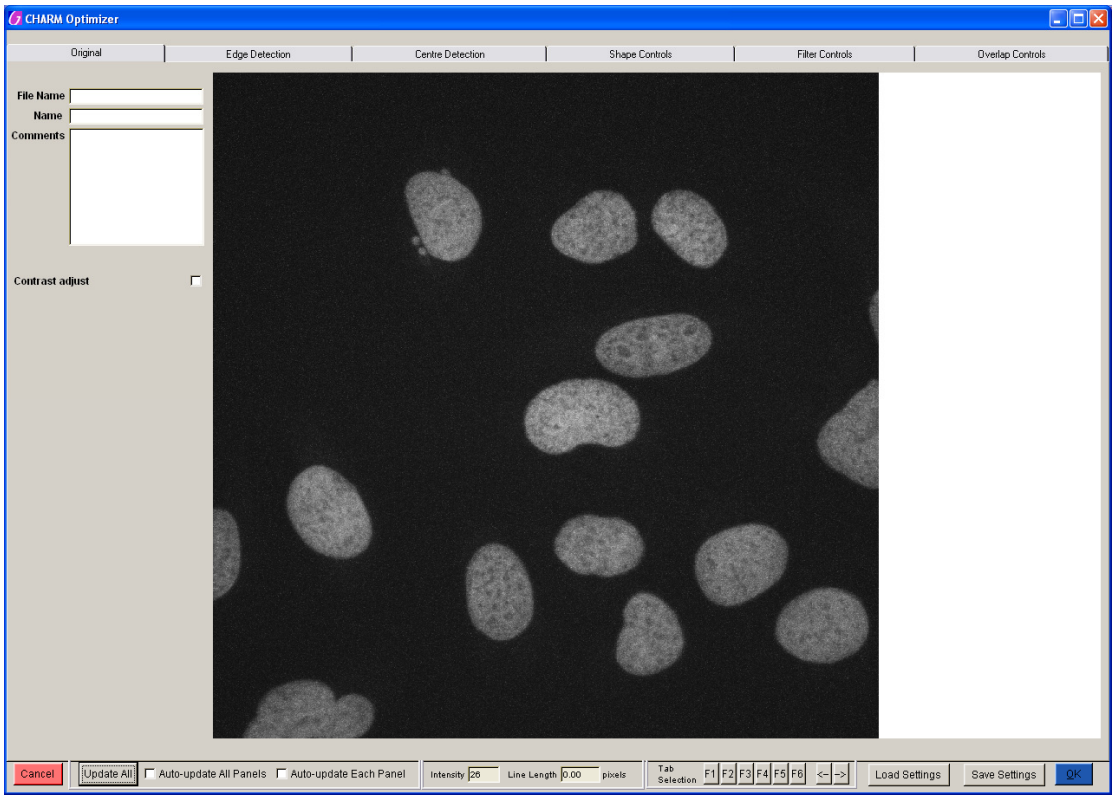
3. Check how these same parameters work for a few of the images and those taken with other cubes, (but not the DAPI cube – the unsharp mask will not be applied to the “Region” images).

4. Now it is necessary to determine CHARM parameters for the region images, (DAPI), and for the object images with the unsharp mask applied as described previously.

5. Now the batch object counting can proceed as before. The only difference is that the box labelled “Apply unsharp mask” needs to be checked.

Use of the CHARM Optimiser

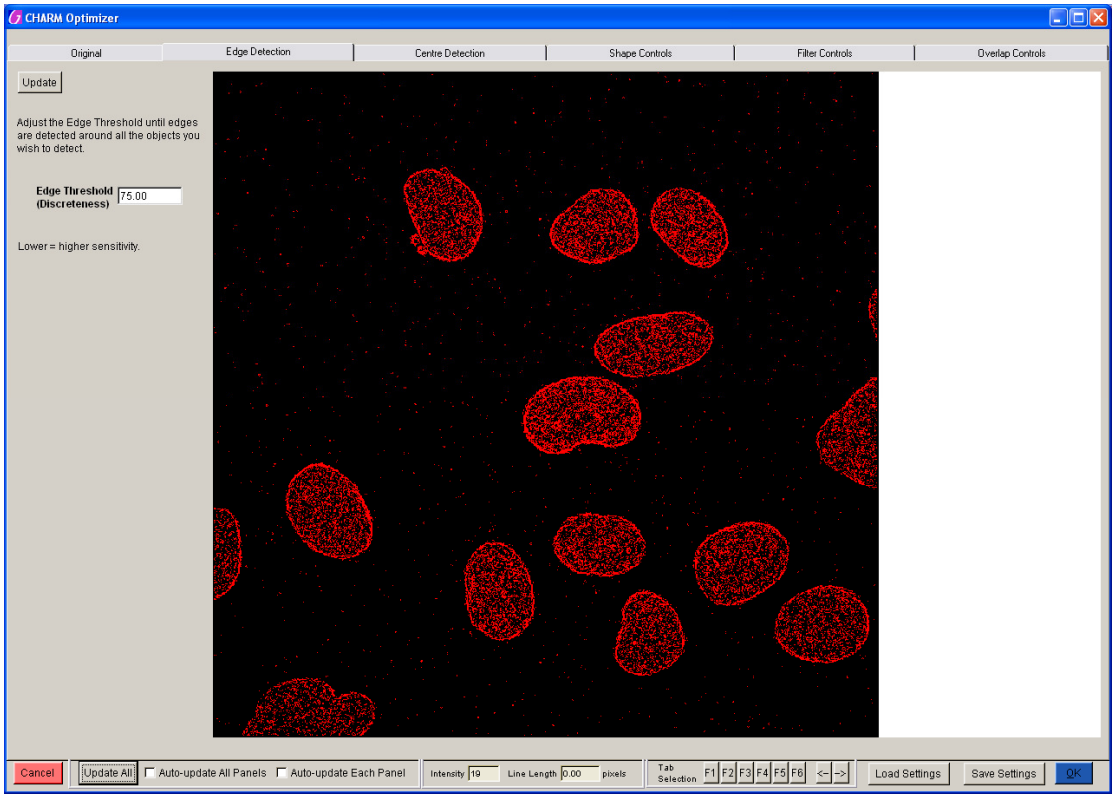
When the “CHARM Optimizer…” button is pressed the following screen appears:-



A name and some comments may be entered if desired. These will be saved with the parameters.
It is recommended to check the “Auto-update Each Panel” box, so that the effect of changing a parameter can be seen straight away.
If “Auto-update All Panels” is checked you will find the program becomes rather slow. This is only recommended for images containing very few objects.

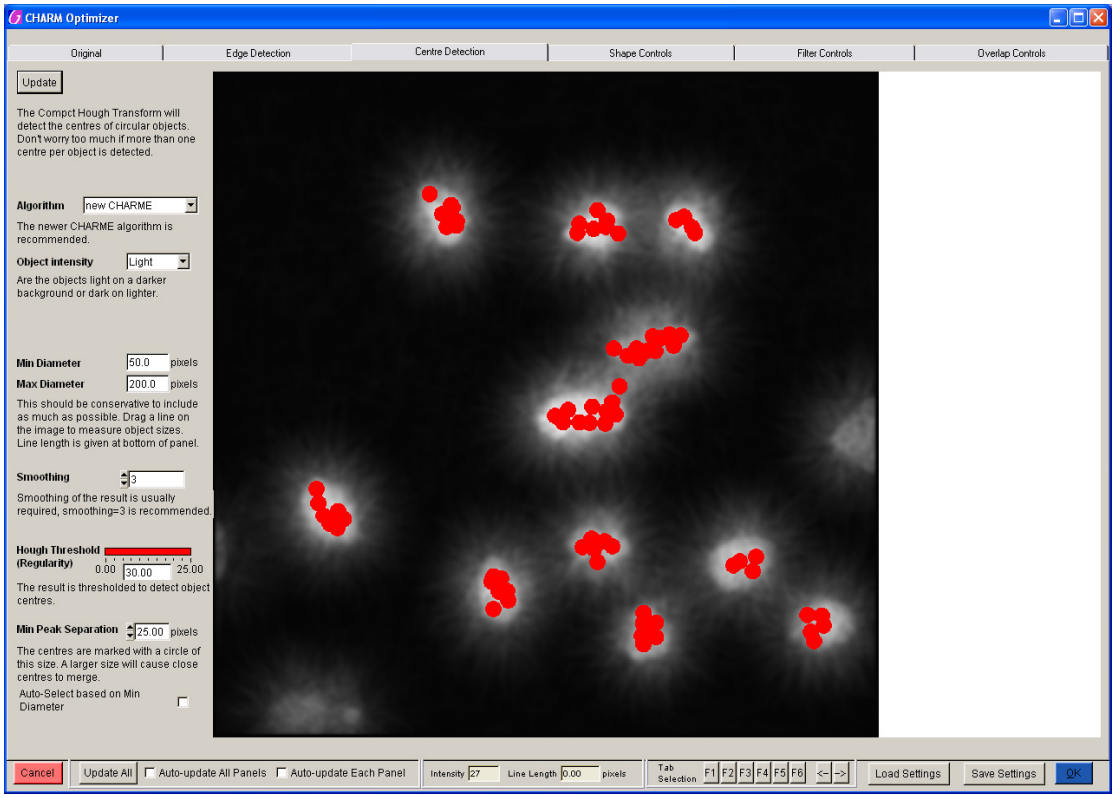
A useful feature here is the ability to measure cell diameters. If you draw a line on the image using the mouse, the length of the line in microns is displayed in the “Line Length” box. At this point it is a good idea to use this tool to estimate the diameters of the smallest and largest objects you wish to find, as this data will be needed later.

Next view the “Edge Detection” tab:-



Modify the “Edge Threshold” value until the outlines of all cells are as complete as possible without too many of the background pixels being red as shown above.
(If “Auto-update Each Panel” has not been checked it will be necessary to click the “Update” button after changing the value.)

Next view the “Centre Detection” tab:-



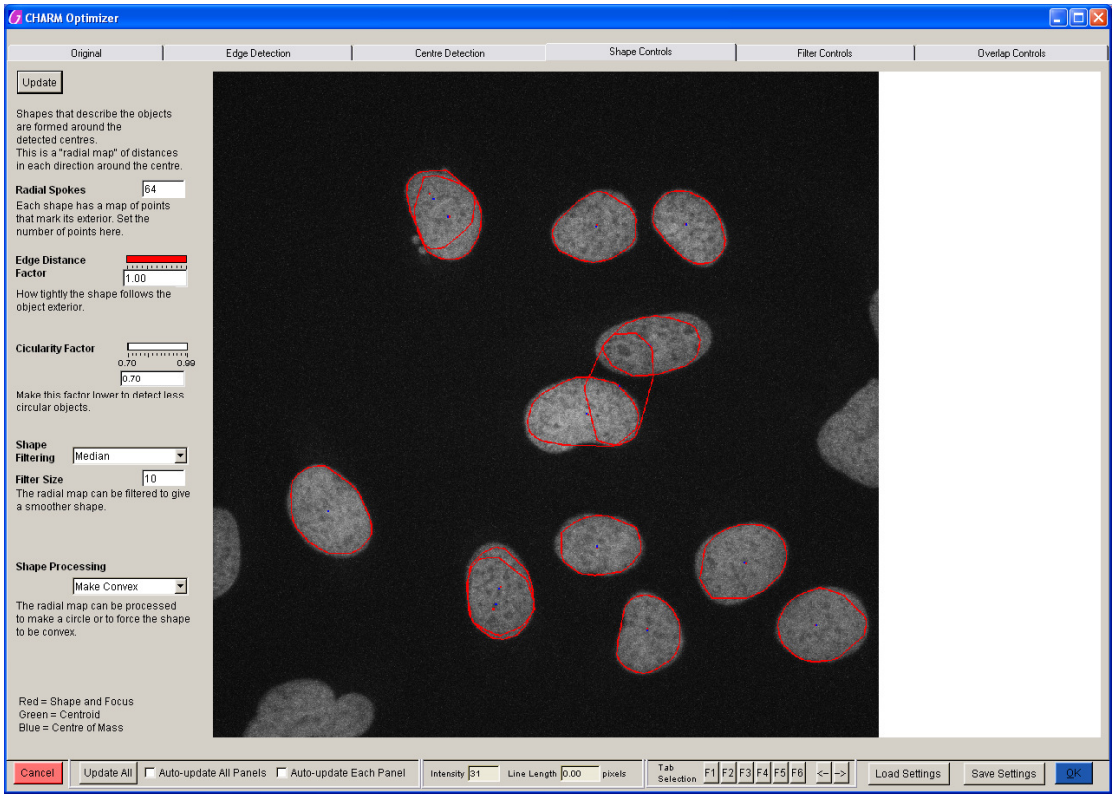
The instructions under each control give a guide as to how to set suitable values.

The “Min Diameter” and “Max Diameter” values should be changed to reflect the size of object.

“Smoothing” should be probably left at 3 and “Hough Threshold” should be adjusted until red dots appear showing the centres detected.

By comparing the “Hough Transform” image with the “Sobel” image it can be seen that many cells contain more than one red dot. The “Min Peak Separation” should be increased such that such dots join together. However, if it is increased too much then adjacent cells will also be joined and treated as a single object. Do not worry that you cannot get exactly one red object for each cell as co-incident objects will be filtered out later. Therefore it is better if this number is too small rather than too big.

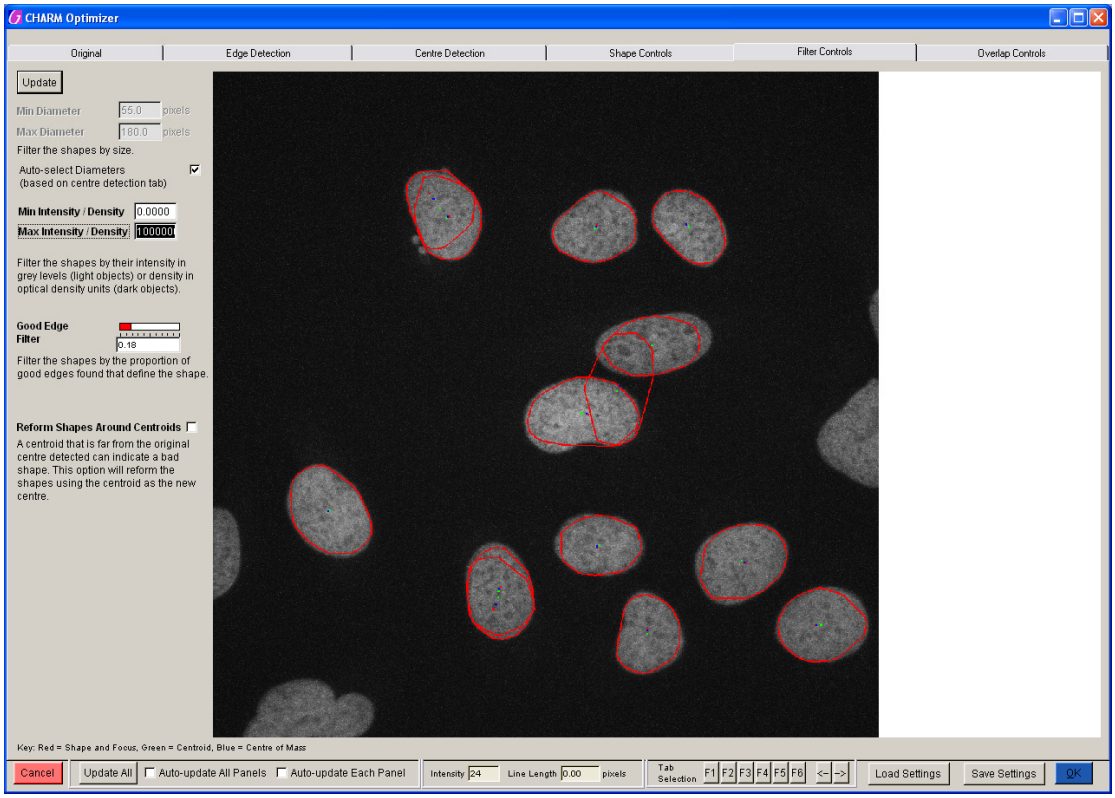
Next view the “Shape Controls” tab:-



By default 64 “Radial Spokes” are used to define the shape around the points found in the previous step. This may be reduced to 32 to speed up the cell-finding with little degradation of the shapes.

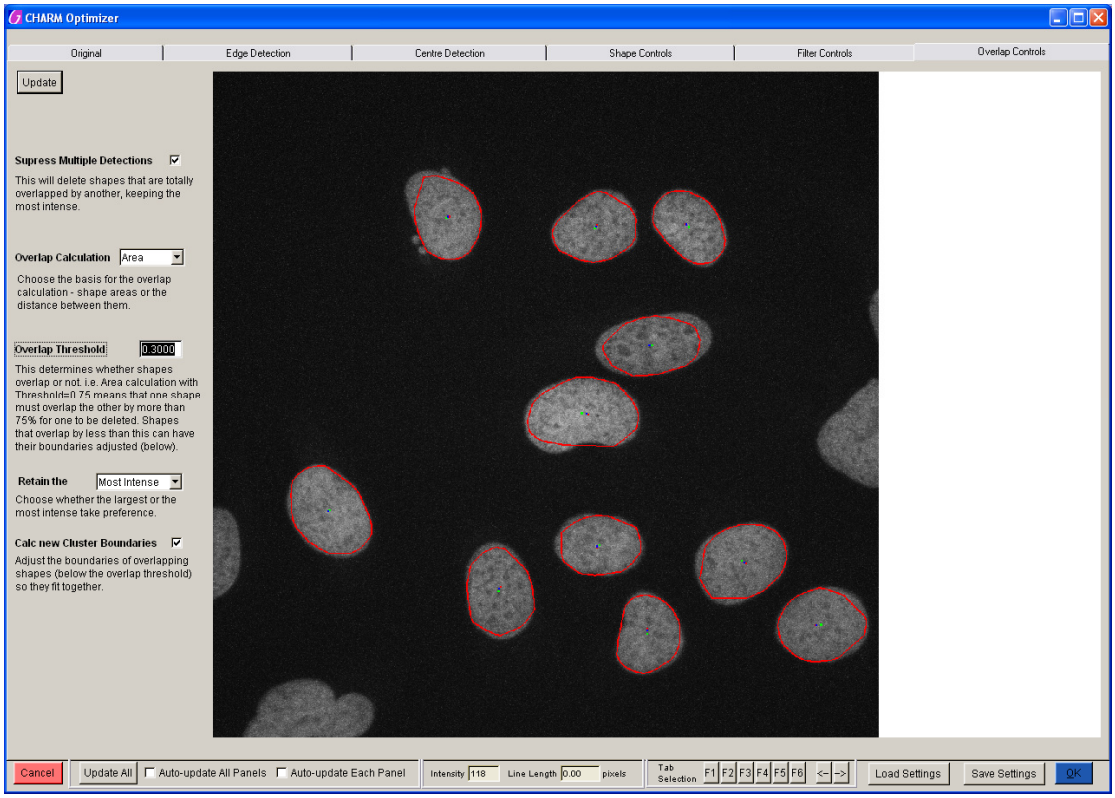
The other values control how well the shapes match the objects.

Next view the “Filter Controls” tab:-



The parameters are used to reject shapes on the basis of size and/or intensity and the proportion of good edge points found.

Next view the “Overlap Controls” tab:-



These parameters are used to process over-lapping objects. In the example above if more than one third of the area of a cell overlaps another object the least bright of the two objects is rejected.

If they overlap by less than one third neither is rejected but their boundaries are modified.

When you happy with the results, the parameters can be saved with a filename of your choice.

If “OK” is pressed these parameters become the ones which will be used from now on, whether they have been saved or not.

The idea is that parameter files can be saved for different objects, and then “Load Settings” can be used to retrieve the appropriate one for each experiment.