

# AnalySIS® Tutorial part 2

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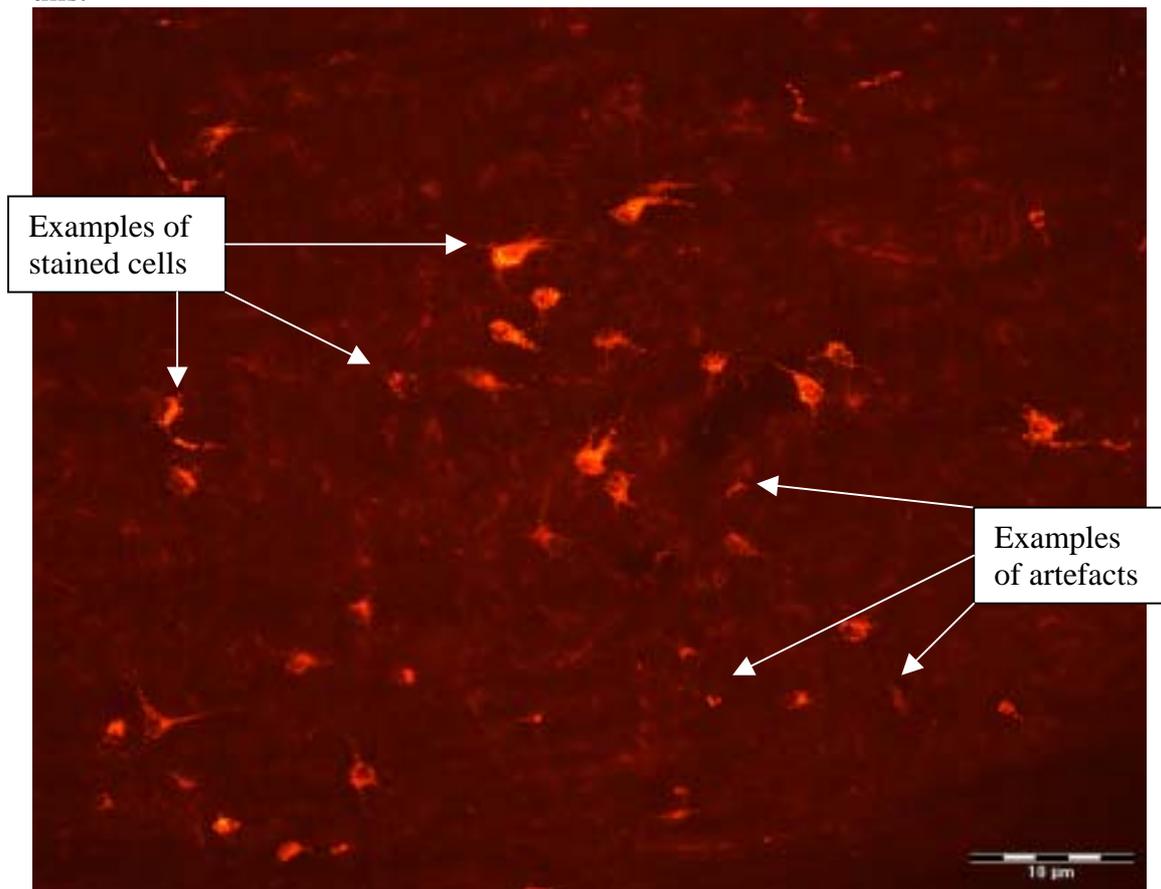
*Using AnalySIS® to automatically identify and record data from retrogradelly  
labelled cells in a fluororuby-stained section.*

This tutorial describes how to acquire a digital image of fluorescent cell profiles, separate labelled cell-profiles from background structures, filter the digital image to remove artefacts, detect cell profiles, and record x,y-coordinates for each profile for the purpose of 3-D reconstruction.

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Our task is to extract relevant data from a section that, in the microscope, looks like this:



### 1) Optimising image acquisition

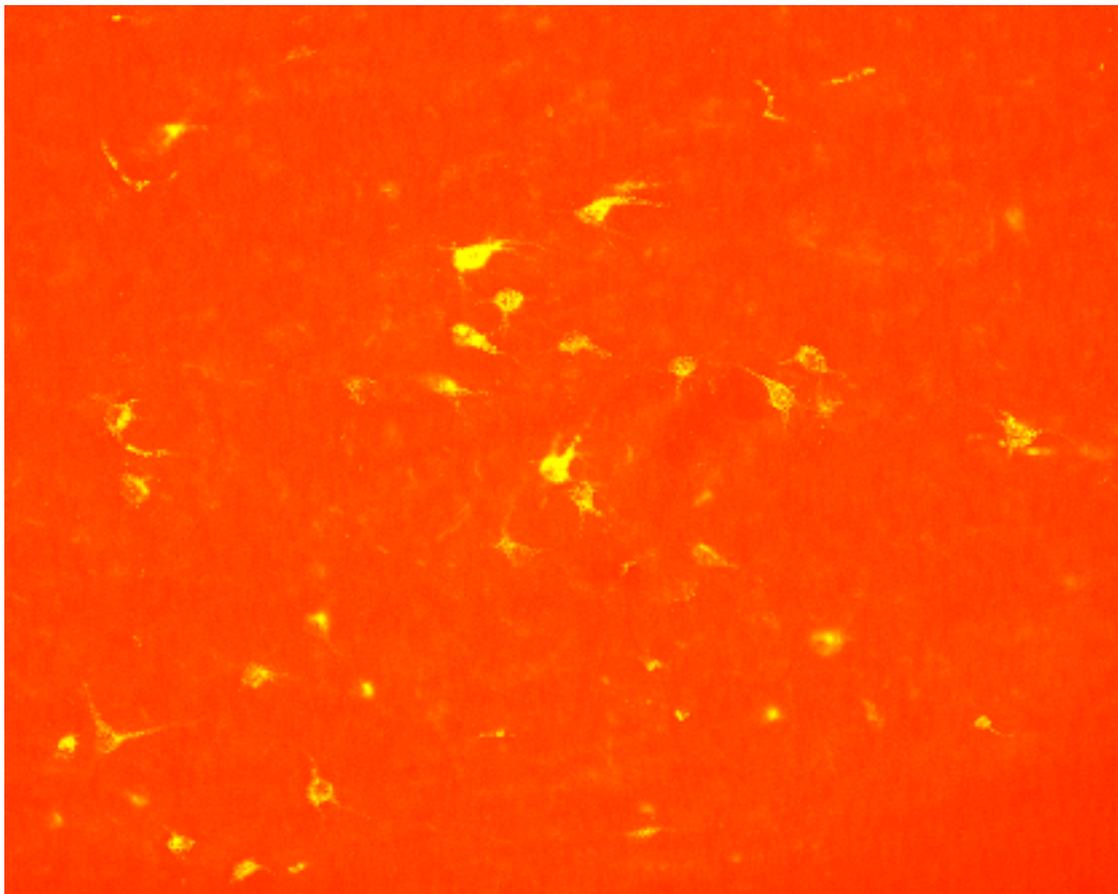
As you can see, the separation between stain and background is far from perfect in the original image. We found that increasing some colours and toning down others, increased contrast, thereby enhancing separation already at the stage of acquisition.

To do this you first have to make sure the live camera is active (press the acquire-button ). Choose *camera control* () from the toolbar, then *colour settings* () from the subsequent popup menu. You now have the opportunity to adjust five scale bars, all which will affect the live image.



Select and deselect which scale bar(s) you want to manipulate by checking/unchecking the checkboxes above each bar.

We found that using the set of values “RED:10, GREEN:5, BLUE:0, OFFSET:-100, GAMMA:1” suited us well. The image we acquired using these values looked like this:



Here the stained cells and, unfortunately, the stained artefacts are represented in tones of yellow, while the background is uniformly red.

## **2) Converting the image to greyscale format**

The next step will be to convert the image to an 8bit greyscale format. This is not strictly necessary, but it is usually easier to set thresholds (see later) for an image with only one channel (greyscale) than a 24bit full-colour image with three channels (RGB)

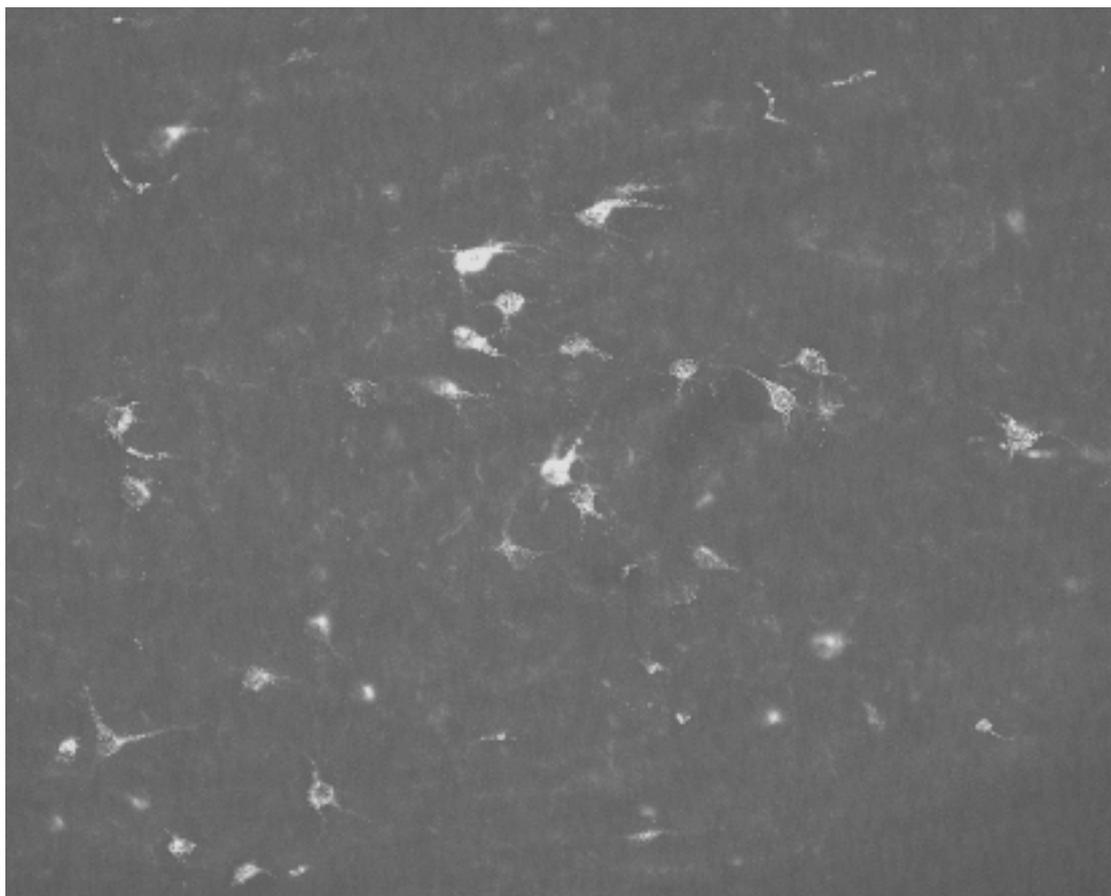
Remember: If you don't want to overwrite the original image, select an empty buffer as destination buffer. (This goes for all the subsequent image-transforming steps).

Choose *Oper menu* → *RGB Studio* → *Change Colour Depth*

From the popup window choose *Grey Scale* from the picklist next to *method*.

Click “OK”-button.

Your image should now look like this:



### 3) Binarizing the image

We now have an image where the stain is near-white and the background is dark grey. For analysSIS, however, this isn't quite enough. For particle detection purposes you need to convert the greyscale image to a 2bit (binarized) black&white image.

The main goal is to end up with a binary image where as much as possible of the stain and as little possible of the background is white.

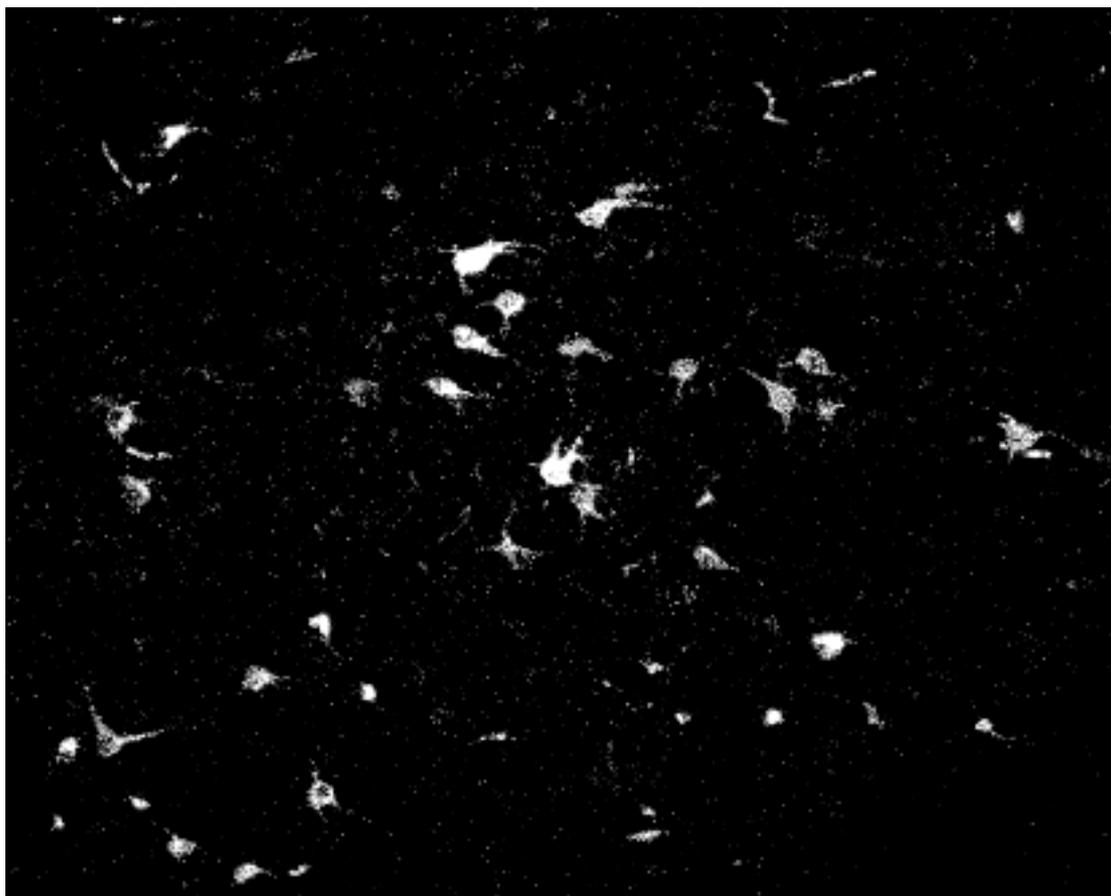
Note: Daniel Oyan describes the following tools in detail in his AnalysSIS® tutorial, available at [www.nesys.uio.no](http://www.nesys.uio.no)

Choose *Image menu* → *Set Thresholds*

In the Set Thresholds window you can adjust the values until they suit your needs. In our example we found that one phase with values “LOW:135, HIGH:255” gave us an image we could use further. The thresholds are applied to the image through binarizing. In other words - the threshold values decide what is to be black and what is to be white when you convert to a 2bit image.

To create the binarized image, choose *Oper menu* → *Binarize*

Your image should now look like this:



We still got some artefacts to eliminate before any valuable results can be extracted. To do this we use morphological filters, some of the most intelligent features of AnalySIS® . Refer to AnalySIS® User's Guide page 339 for details on the different morphological filters.

#### 4) Preparing the image for analysis

- Choose *Oper menu* → *Morphological Filter* → *Morph.Close*

We use this filter to fill holes in particles<sup>1</sup> and smooth edges.

- Choose *Oper menu* → *Morphological Filter* → *Morph.Open*

This filter is used for object separation, reducing noise and to smooth particle edges.

Comment: Both filters are variants of standard erosion and dilation binary operators

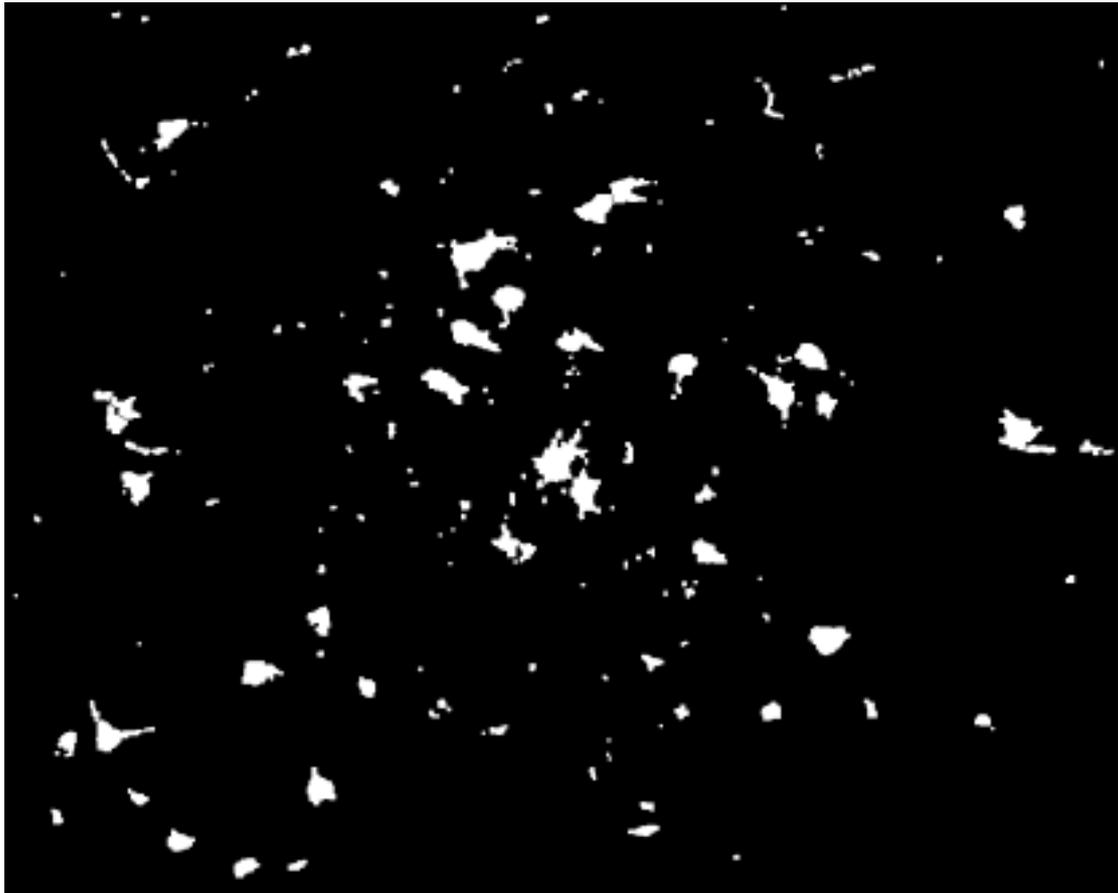
- Choose *Oper menu* → *Morphological Filter* → *Separate Particles*

The Separate Particles filter uses complex algorithms to separate connected particles according to shape criteria. The “thinner” the connection is, the better the filter works.

---

<sup>1</sup> Definition: A *particle* is a collection of interconnected white pixels surrounded by black pixels.

After applying the three filters, we should have this image:



The stained cells appear as solid, prominent white areas. We still have some artefacts to deal with, but these can be separated from the cells during the detection process. It's now time to begin analysing.

### **5) Defining detection**

We need to apply some criteria to make AnalySIS® search for the particles we want to detect. These must be as narrow as possible (to exclude artefacts), still wide enough to include all cell profiles. The criteria will have to be deduced from several samples to be functional through a given set of sections.

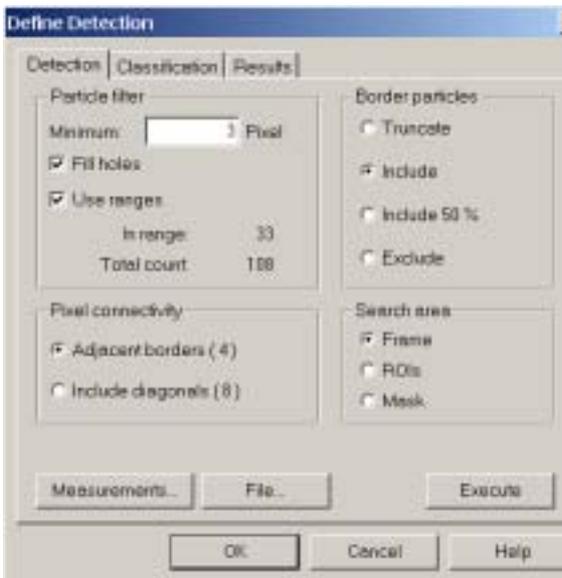
What we want to do in this example is to exclude the small particles (considered artefacts), exclude all “threadlike” structures (also considered artefacts) and include all particles larger than a given size.

Choose *Analysis menu* → *Define Detection*

Note: Since Daniel Oyan describes the following tools in detail in his AnalySIS® tutorial, I'll only give a brief overview.

Leave the classification- and result-tabs alone for now.

Explanation of our values:



**Particle filter:**

- Minimum 3 pixels: exclude all particles larger than 3pixels
- Fill holes: When measuring particle area, include the area of holes in the particle.
- Use ranges: Use the ranges applied under Measurements... (See later)

**Pixel connectivity:**

- Adjacent borders (4): particles with diagonal connections are counted as two different particles.

**Border particles:**

- Include: includes particles split by the border of the search area.

**Search area:**

- Frame: Search the full image.

Click the “Measurements...” button and you’ll get this window:

The relevant tab for us is *particles*.

By choosing measurements from this menu, you can include/ exclude particles to suit your purposes

The currently selected measurements

**Detection..:** Back to *Define Detection* menu  
**File...:** Save and load measurement settings

Insert max/ min values for each measurement. These will act like a “range”, and particles outside this range will be excluded from the search.

Detect particles in the selected image according to the measurements you’ve defined.

Description of the currently selected measurement

Selected Measurements	Range Min.	Range Max.
ID Particle	None	None
ID Class	None	None
Area	0.7	7.5

If you want an extensive explanation of how all the different measurements work, I advise you to consult AnalySIS® User's Guide page. 444.

To add a measurement to your list of selected measurements, simply check the checkbox next to the one you want to select. Click on a measurement under the "selected measurements"-heading to gain access to the Filter Range options. Enter Min and Max values to define a relevant range.

Note: "ID Class" and "ID particle" are default measurements, which cannot be removed.

To include/ exclude the particles we wanted, we found that two measurements were of value.

- Area: (Filter Range: Min:0,7 Max:10)

This will exclude all particles less than 0,7 $\mu\text{m}$  square. The maximum value is set high enough to include all particles

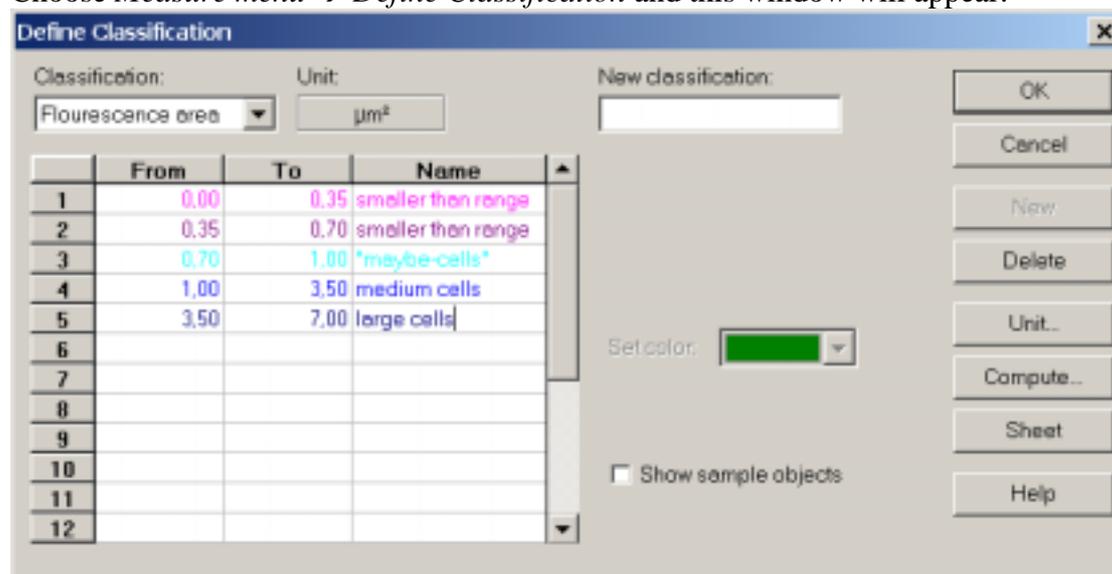
- Elongation: (Filter Range: Min:0 Max:3,5)

This filter excludes particles based on their shape. The value "0" represents a perfect circle. The higher the Max-value is, the more oval shapes are allowed to be within the search range. In other words, we use this measurement to exclude "threadlike" particles (like some of the artefacts in our example).

After applying the measurements, click "OK"-button to finish.

## 6) Defining Classification and Detecting Particles

Choose *Measure menu*  $\rightarrow$  *Define Classification* and this window will appear:



Define Classification provides AnalySIS® with a table used to divide particles into groups. This can be useful for various reasons, e.g. comparing groups, counting particles of different area and visualizing the various particles (thereby giving you

further ideas on how to configure measurements, divide groups, exclude/ include particles etc.)

We used it to define a group of “fragment-particles”. We discovered particles with an area between 0,70 and 1,0 $\mu\text{m}^2$  square mainly represented cell-fragments and wanted to keep them as a separate category.

To define a new classification, enter a name in the “New Classification”-box.

Click the “New”-button.

Enter relevant values in the table

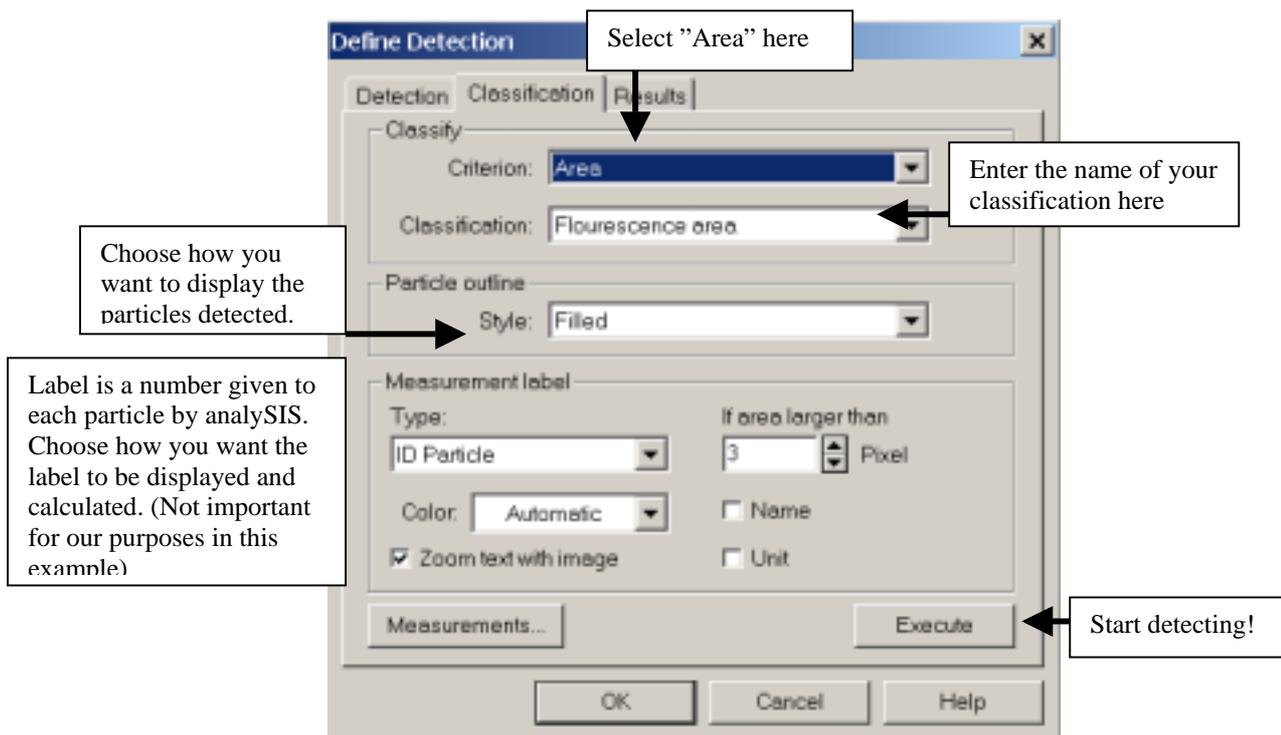
Optionally give each group a name

Click the “OK”-button

Note: You can use the “Compute...”-button to calculate a new classification. Enter number of groups under “bins”, enter Min and Max values, choose measurement under “criterion” and choose between a linear or a logarithmical calculation of group ranges under “mode”.

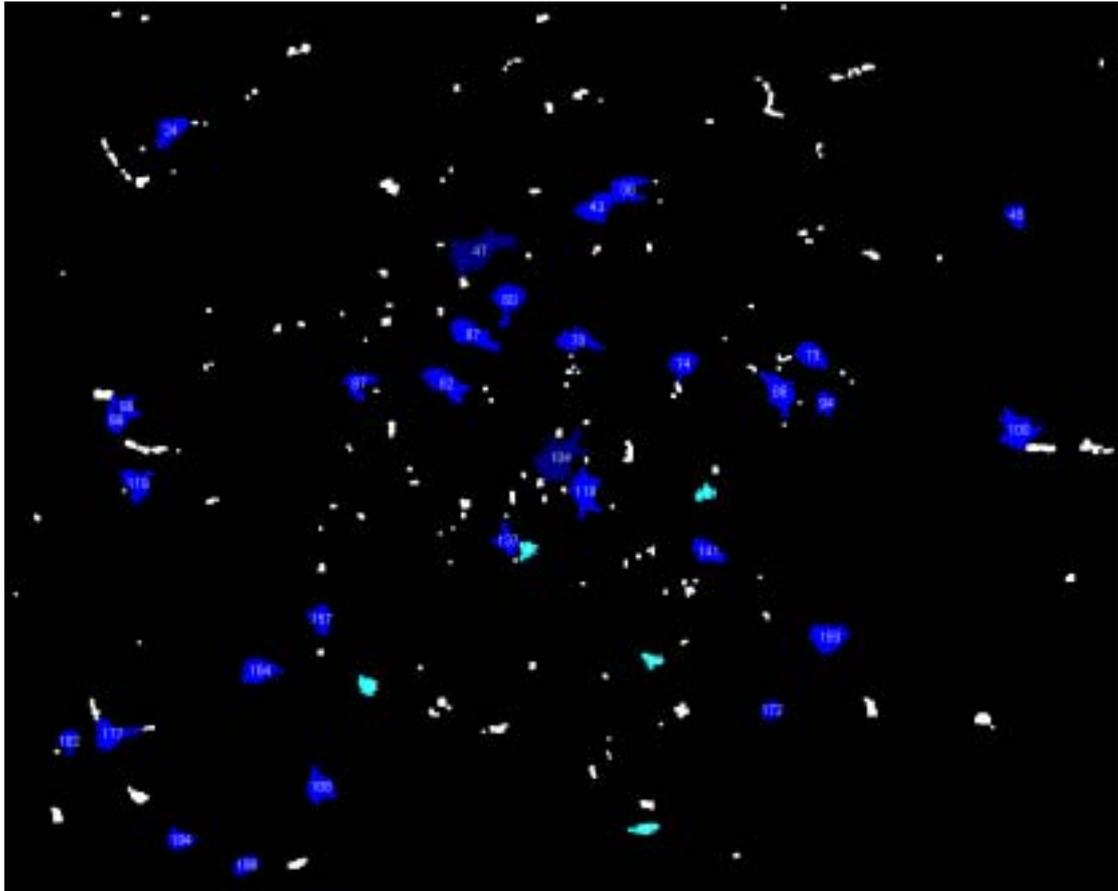
Choose *Analysis menu* → *Define Detection*

Click the *classification*-tab and this window pops up



Now click the “Execute”-button, and if you have chosen to use our values and colours all along, your image should look like this:

Note: You can also execute detection by choosing *Analysis menu* → *Detect*



"fragment-cells"
     
  medium sized cells
     
  large cells

You've detected the cells, but may want to display the result in a more orderly way. To do this, choose:

*Analysis menu* → *Particle results*

AnalySIS® then opens this sheet:

ID Particle	ID Class	Area	Elongation
		µm <sup>2</sup>	
1	34	4	1.76
2	36	4	1.21
3	40	4	1.60
4	45	4	1.26
5	41	5	1.22
6	80	4	1.34
7	80	4	1.30
8	80	4	1.30
9	80	4	1.30
10	80	4	1.30
11	72	4	1.89
12	72	4	1.81
13	81	4	1.81
14	85	4	1.34
15	85	4	1.25
16	85	4	1.35
17	85	4	1.34
18	85	4	1.45
19	125	5	1.34
20	111	4	1.70
21	116	4	1.24
22	134	4	1.81
23	134	4	1.81
24	134	4	1.81
25	180	4	1.38
26	181	4	1.80
27	182	4	1.41
28	182	4	1.30
29	170	4	1.80
30	175	4	1.25
31	180	4	1.71
32	180	4	1.71
33	180	4	1.34
34	111	4	1.34
35	182	4	1.40
36	184	4	1.21

As you can see, the table displays for each particle the measurements you've defined earlier (page 6).

If you want additional information displayed in the sheet, add parameters to your list of selected measurements under *Analysis menu* → *Define Measurements*. Remember not to define Max/ Min values for filter range unless you want them to be used as exclusion criteria during particle detection.

We wanted to get xy-coordinates for each particle for later being able to import the position of the cells into Micro3D.

Add "Centre of Gravity X" and "Centre of Gravity Y" to your list of selected measurements. Press the "Apply"-button, then press "OK"-button. Choose *Analysis menu* → *Particle results* again, and the mission should be completed.

## 7) Comments and tips

- Streamlining the process – creating a macro

The methods described in this tutorial may seem extremely time consuming, but it is not necessarily so. Many of the steps can be done only once (e.g. setting thresholds, defining detection and defining classification) and most of the steps can be integrated in a macro, reducing your workload considerably.

In our example we made a macro containing all the steps from grayscaling the image to getting particle results. In other words - the only manual procedures were to acquire the image and press a button. The finished Particle Results would then appear on screen.

Our macro (comments displayed in red):

```
int disp;  
introduces the variable "disp".  
disp=0;  
sets the value for "disp" to 0. (regular programming procedure)  
disp=Op.Display;  
sets "disp" equals Op.Display (the number of your currently selected buffer) We now  
have the opportunity to change the destination buffer for later stages in the macro by  
setting Op.Dest (destination buffer) = disp (our variable) + x (any number)  
Op.Dest=disp+1;  
sets destination buffer to your current buffer +1 (the next buffer)  
ImgxChangeColorDepth(ColorMode:=0, DitherMode:=0,  
    ColorCount:=256);  
grayscales the image  
Op.Display=disp+1;  
Op.Dest=disp+2;  
sets destination buffer to start buffer +2 (the third buffer from your starting buffer)  
{  
    HTHRESHOLD hThr=thrLoad("p2");  
    thrSave(hThr, NULL);  
    thrDestroy(&hThr);  
}  
loads the file "p2" containing threshold values. You can create threshold-files by  
choosing Image menu → set thresholds. Save your values as a file and change the  
name in the macro so that it corresponds with the name of your file.  
Note: whenever a macro is changed, it's a good custom to save it as a new file.  
BinarizeGrayScaleImage(Thresholds:=NULL, Phase:=-1);  
binarizes the image  
Op.Dest=disp+2;  
MorphClose();  
Op.Dest=disp+2;  
MorphOpen();  
Op.Dest=disp+2;  
SeparateParticles();  
applies the three filters described previously in the tutorial.
```

```
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,  
Inclusions:=FALSE, MinPixel:=3, Min:=0, Max:=DBL_MAX,  
Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,  
UseRanges:=TRUE);
```

defines your detection criteria. Your current values from Analysis menu → Define Measurements and Measure menu → Define Classification will be used.

```
Op.Display=disp+2;
```

```
Detect();
```

detects particles

```
ParticleResults();
```

displays the sheet containing your particle results

To use this macro, choose *Special menu* → *Define macros*. Enter a name in the “Macros”-box and press the “Add”-button. Copy the macro code (without the comments) and paste it into the “Macro text:”-field. Press “OK”-button.

You can apply the macro to a new button in your toolbar by choosing *Special menu* → *Edit Button Bars*. Consult AnalySIS® User’s Guide page 509 for detailed information.

## 8) Acknowledgments:

I thank Daniel Oeyan and Jan G. Bjaalie for critical comments to this tutorial. The histological material was kindly provided by Marius Svanevik and Trygve B. Leergaard.