Application Note

Planimetry measurements of spheroids in a ClinoReactor using FIJI – A step-by-step guide

- Surface area measurements (planimetry) is a great way to normalise and follow growth of 3D constructs.
- Manual measurements of planimetry can be time-consuming, which is why this application note offers a description of an automatic method to measure the planimetry of 3D constructs.
- Contains a step-by-step guide using the free open-source software FIJI.

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Introduction

During standard cell culture, cell growth is monitored by confluence and cell count. With 3-dimensional cell culture, cell count is not possible to perform without destroying the construct. Instead, a non-invasive way to determine growth is by measuring the surface area (planimetry) of the spheroid, which can be performed directly in the ClinoReactor. However, manual planimetry determination can be time-consuming which is why this application note describes an automated way to measure the planimetry of several hundred spheroids in an automated way. Planimetry can, besides being a measure of growth, be used as a normalisation tool to for example normalise µM ATP per mm^2. This application note describes using a FIJI (open source) image analysing software to perform manual or automated planimetry determination.

Flow diagram

Method

1) Prepare FIJI

Install FIJI (FIJI Is Just ImageJ) on your computer through the following link:
https://imagej.net/software/fiji/downloads
Note: When installing FIJI, do not place it inside “program files” but instead place it in your user space.

When FIJI is installed make sure you have the “read and write excel” plugin. It is installed by the following steps in FIJI:

1) Help -> update... (figure 1A)
2) In the Updater window click “Manage update sites” (figure 1B)
3) Check the “ResultsToExcel” box (figure 1C)
4) Click “close” and then “apply changes” in the “ImageJ Updater” window
5) Restart FIJI, and everything is ready!

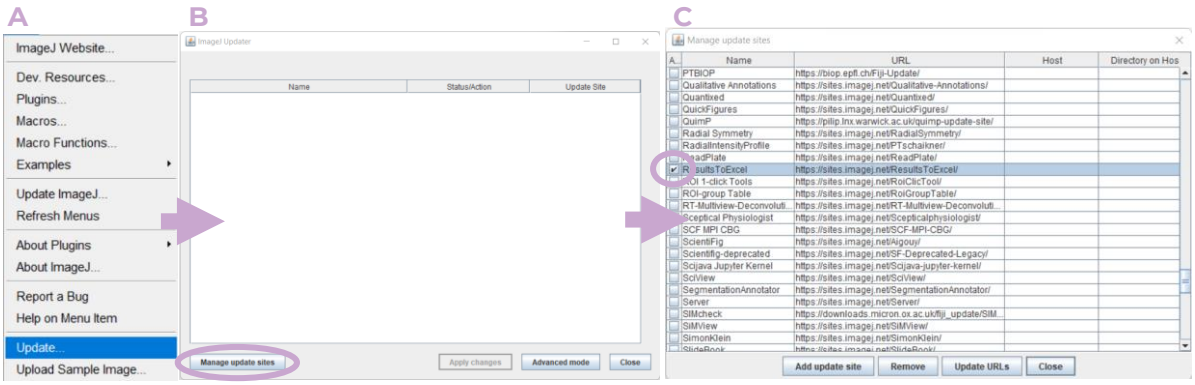


Figure 1: Flow of installing the plugin "read and write excel"

2) Capture microscopy pictures of the spheroids in the ClinoReactor

A good picture of the spheroids is important for accurate measurement. Therefore, the following parameters should be considered:

1) Large contrast between the spheroids and the background.
2) An equally illuminated background.
3) Avoid to the best of your abilities that the spheroids are touching each other.

Note: Remember to have at least one picture with a scalebar

3) Transfer the pictures of the spheroids to a folder on your computer

All the pictures you want to analyse simultaneously must be collected in the same folder. The pictures in the same folder must be taken with the same magnification. For convenience, rename the picture so you can identify it later in the process. Each ID must be unique.
E.g. date_name_number

Inside the folder with your microscopy pictures, make another folder and call it "output". It is extremely important that you spell it correctly and that all letters are in lowercase.

4) Open FIJI

5) Set a global scale for the pictures

To measure the area of the spheroids, FIJI must know how many pixels make a specific distance (the pixel/distance ratio). This can be set in two ways:

If you know the pixel/distance ratio, you can simply write it in FIJI by pressing Analyze -> Set Scale... and then write the number of pixels (figure 2A) necessary for a known distance (figure 2B). Remember to tick the "Global" box (figure 2C).
Note: You need to open a picture in FIJI to set the scale.

If you do not know the pixel/distance ratio, it can be measured in FIJI.

a) Open a picture with a scale bar with the magnification you want to measure.
b) Select the line tool (figure 3).
c) Draw over the scale bar, so the line is as long as the scale bar.
d) Open Analyze -> Set scale.
e) Write the known distance (figure 2B) (the length of the line is automatically entered in the "Distance in pixels" (figure 2A)).
f) Make sure the tick is in "Global" (figure 2C).
g) Press "OK".

Now the scale is set for the picture you want to analyse!

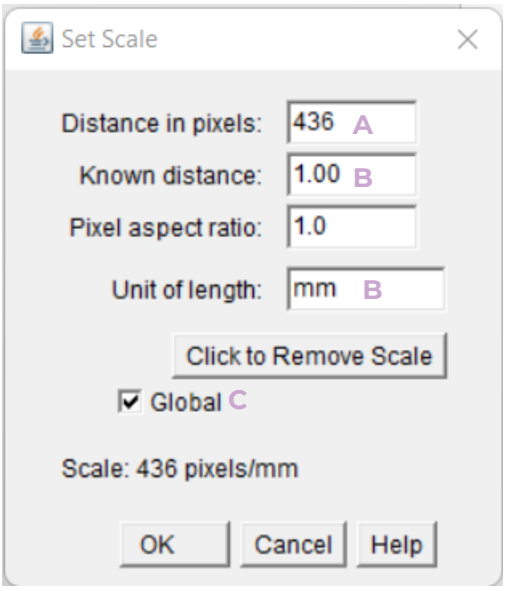


Figure 2: This figure shows the "Set Scale" window. A) a given distance in pixels. B) The distance that A corresponds to all well as the unit of measurement. C) When this box is ticked, the scale will be used on all pictures.

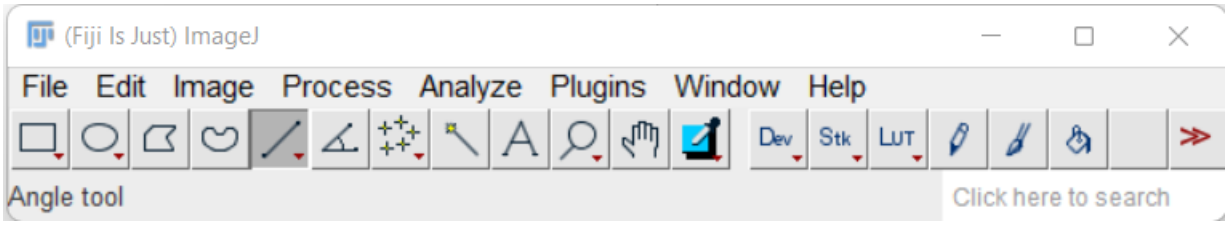


Figure 3: Selection of the line tool

6) Run the macro

a) The macro, which measures the area of the spheroids, is run by pressing Process -> Batch -> Macro...
b) Choose the folder that contains your pictures as "Input..." (figure 4A)
c) Choose the "output" folder you created in step 3 as "Output..." (figure 4B)
d) Copy macro from appendix 1 and paste the macro inside the square (figure 4C).
e) Press "Process"

All pictures are now processed, and the output is stored in the "output" folder. All the data is stored in one tab in the excel file. Check appendix 2 for more information about the macro.

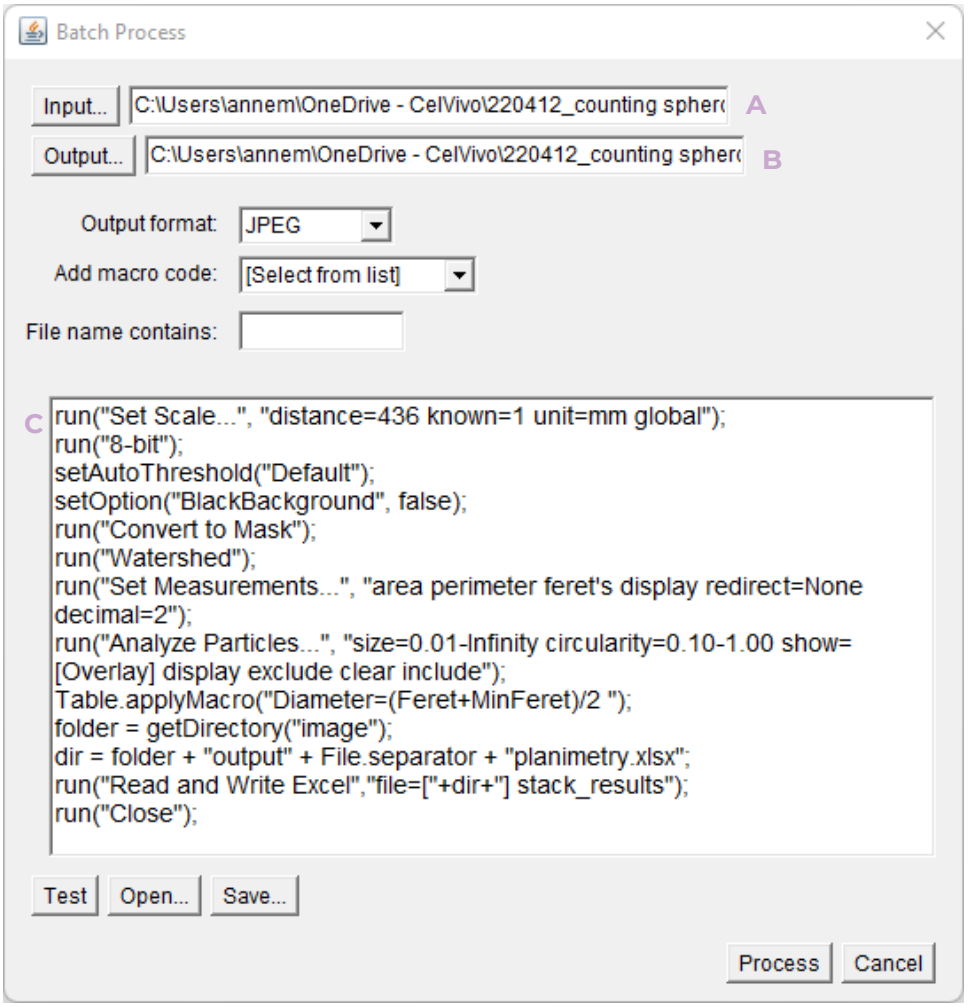


Figure 4: The "Batch Process" window in FIJI that show a) the directory where the microscopy pictures are stored, b) the directory where the output is stored and c) the script that is used.

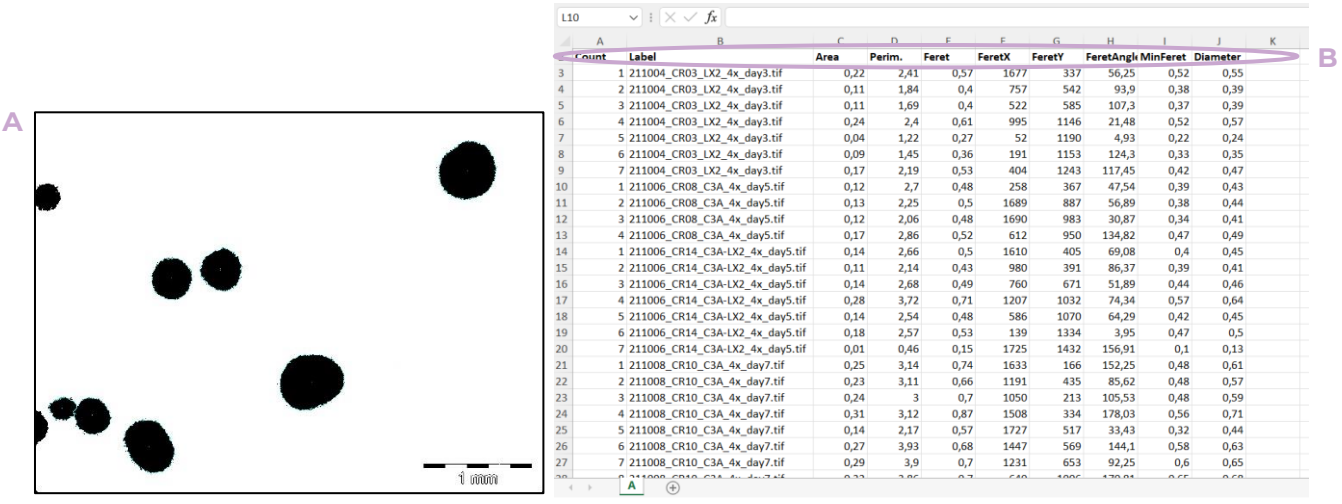


Figure 5: Output from the macro. A) Shows each spheroid, that was measured on a picture, as well as their unique ID number. B) Shows the results with counts (the ID for the counted spheroid), label (name of the picture that was analysed), area, perimeter, Feret, and diameter.

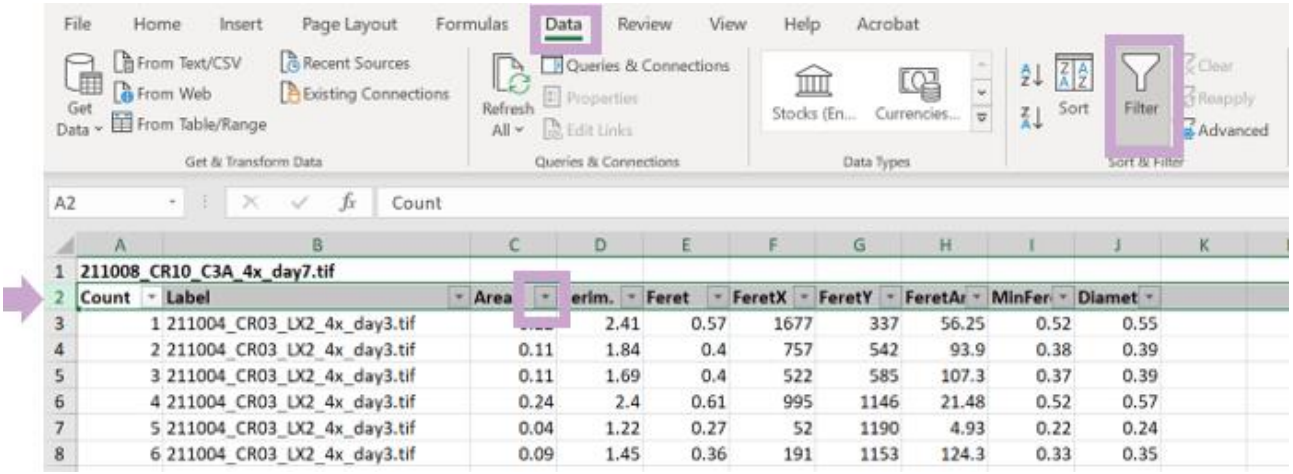
Quality assessment

a) Open output folder.
b) Check if spheroid area is measured correctly by comparing the original image to the area of spheroids measured (figure 5A). (Macro excludes spheroids on the edge where only a part of the spheroid can be measured).
c) Check the planimetry.xlxs file to see if results are as desired.
d) If they are, proceed with data analysis.
e) If smaller cell debris or other unwanted shapes are counted as well proceed to filtering and troubleshooting.

Filtering and troubleshooting

A way to circumvent a suboptimal planimetry determination is to filter out cell debris that are measured. Filtering can be performed in excel.

a) Determine the area of the smallest spheroid by manually measuring it as described below.
b) Open excel file from output folder.



	A	B	C	D	E	F	G	H	I	J	K
1	211008_CR10_C3A_4x_day7.tif										
2	Count	Label	Area	erim.	Feret	FeretX	FeretY	FeretAr	MinFer	Diamet	
3	1	211004_CR03_LX2_4x_day3.tif		2.41	0.57	1677	337	56.25	0.52	0.55	
4	2	211004_CR03_LX2_4x_day3.tif	0.11	1.84	0.4	757	542	93.9	0.38	0.39	
5	3	211004_CR03_LX2_4x_day3.tif	0.11	1.69	0.4	522	585	107.3	0.37	0.39	
6	4	211004_CR03_LX2_4x_day3.tif	0.24	2.4	0.61	995	1146	21.48	0.52	0.57	
7	5	211004_CR03_LX2_4x_day3.tif	0.04	1.22	0.27	52	1190	4.93	0.22	0.24	
8	6	211004_CR03_LX2_4x_day3.tif	0.09	1.45	0.36	191	1153	124.3	0.33	0.35	

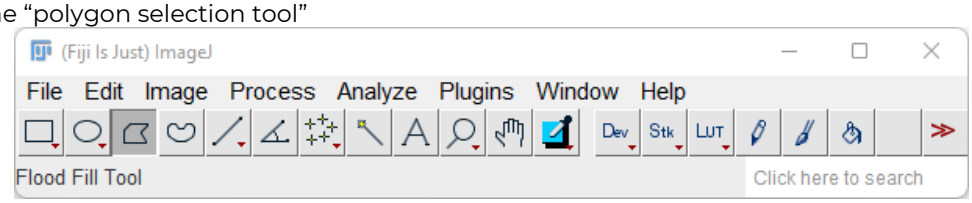
c) Go to data tab.
d) Select row 2 containing headers and press the filter botton.
e) Choose the drop-down menu with Area.
f) Select number filters ->Greater than..
g) Put in the area of your smallest spheroid manually measured and press OK.
h) Now you have your filtered list of spheroids within size range.

If this is not working try deleting the line of watershed from the macro and run the macro again with a new output directory.

Manual planimetry measurement

If the spheroids are too small or translucent, the measurement might be inaccurate. In such cases, it is necessary to do planimetry manually. This is done by following these steps:

a) Set scale as described above.
b) Select the “polygon selection tool”



c) Draw around the periphery of the spheroid by left-clicking with your mouse. Finish by right-clicking, or by clicking the yellow square, which marks where your drawing started.

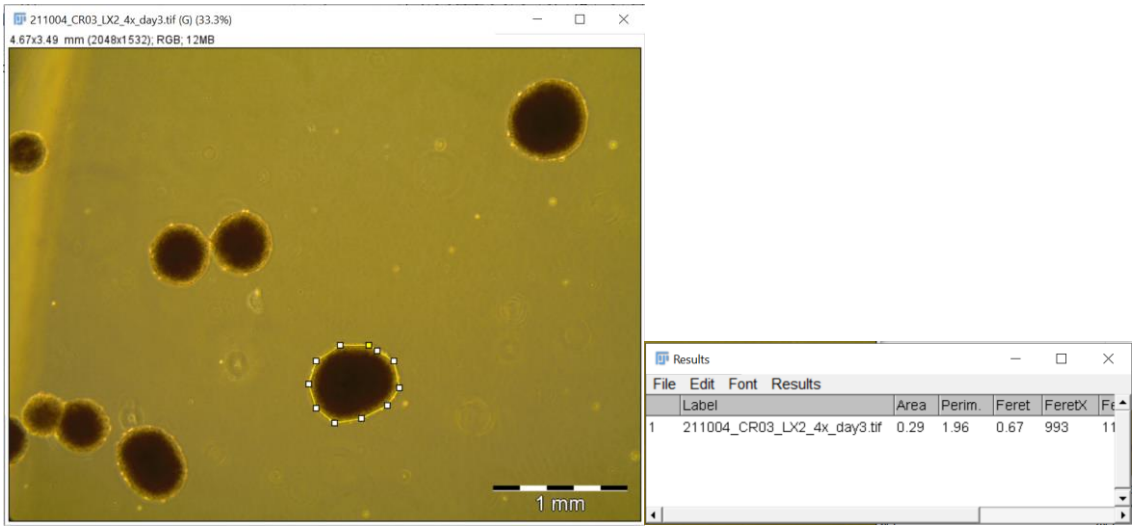


Figure 6: Peripheral drawing around spheroid using polygonal selection tool (left) and results from manual selection measurement (right).

d) Press ctrl + m
e) Repeat point c and d until you have drawn around all spheroids of interest.
f) Copy results by selecting them and paste the results to an excel file and save the results.

Figures or Data

Below is an example of data generated from one image and the output it generates in form of mask and table containing area results.

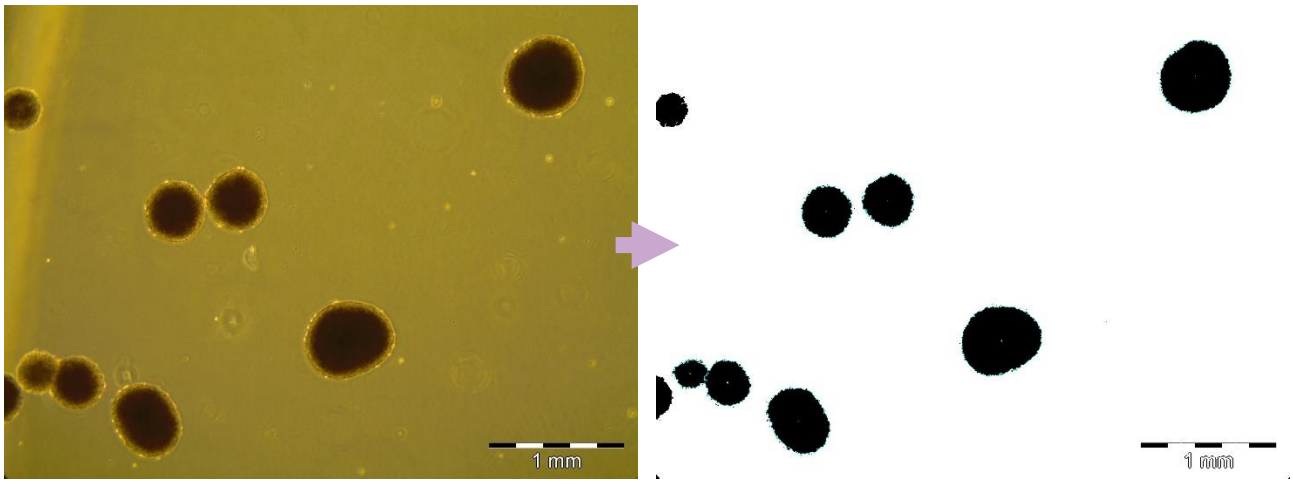


Figure 7: The figure shows how a microscopy picture looks after it has been converted and measured using FIJI.

Table 1: Results from above image in excel file.

211008_CR10_C3A_4x_day7.tif									
Count	**Label**	**Area**	**Perim.**	**Feret**	**FeretX**	**FeretY**	**FeretAngle**	**MinFeret**	**Diameter**
1	211004_CR03_LX2_4x_day3.tif	0.22	2.41	0.57	1677	337	56.25	0.52	0.55
2	211004_CR03_LX2_4x_day3.tif	0.11	1.84	0.4	757	542	93.9	0.38	0.39
3	211004_CR03_LX2_4x_day3.tif	0.11	1.69	0.4	522	585	107.3	0.37	0.39
4	211004_CR03_LX2_4x_day3.tif	0.24	2.4	0.61	995	1146	21.48	0.52	0.57
5	211004_CR03_LX2_4x_day3.tif	0.04	1.22	0.27	52	1190	4.93	0.22	0.24
6	211004_CR03_LX2_4x_day3.tif	0.09	1.45	0.36	191	1153	124.3	0.33	0.35
7	211004_CR03_LX2_4x_day3.tif	0.17	2.19	0.53	404	1243	117.45	0.42	0.47

Citation

Cite this as: A.P. Thulesen et. al, CelVivo application note 10x, 220505

For additional product or technical information visit www.celvivo.com or consult CelVivo Aps at info@celvivo.com or +45 70 228 228.

Appendix 1

```
run("8-bit");
setAutoThreshold("Default");
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Set Measurements...", "area perimeter feret's display redirect=None decimal=2");
run("Analyze Particles...", "size=0.01-Infinity circularity=0.10-1.00 show=[Overlay] display exclude clear
include");
Table.applyMacro("Diameter=(Feret+MinFeret)/2 ");
folder = getDirectory("image");
dir = folder + "output" + File.separator + "planimetry.xlsx";
run("Read and Write Excel","file=["+dir+"] stack_results");
run("Close");
```

Appendix 2 - Description of the macro

How does the macro work?

First, the macro prepares the picture for analysis. This is done by:

1) Converting the picture to an 8-bit (grayscale)
2) Converting the picture to black and white, where the spheroids (and everything else that has the same value as the spheroids = noise) become black.
3) Using the watershed plugin to separate any spheroids that are so close together that they would be counted as one.

Then the macro analyses the picture by measuring the black areas. The output is gathered in a file called “planimetry.xlsx” in the output folder.

Output from the macro

In the “output” folder two types of files can be found. The first type of output is a picture of the spheroids that were measured. It is possible to see the spheroids, as well as their unique ID number (figure 5A).

The second type of file is an excel file called “planimetry”. This file contains the measured area, perimeter, and diameter of each spheroid (figure 5B). There are furthermore some measurements called “Feret”. These are used to calculate the diameter. Feret is the longest possible diameter while minFeret is the shortest. The diameter is the average of these two. FeretX and FeretY give the coordinates to the start and stop of the line used to measure the diameter and are not important for further analysis.