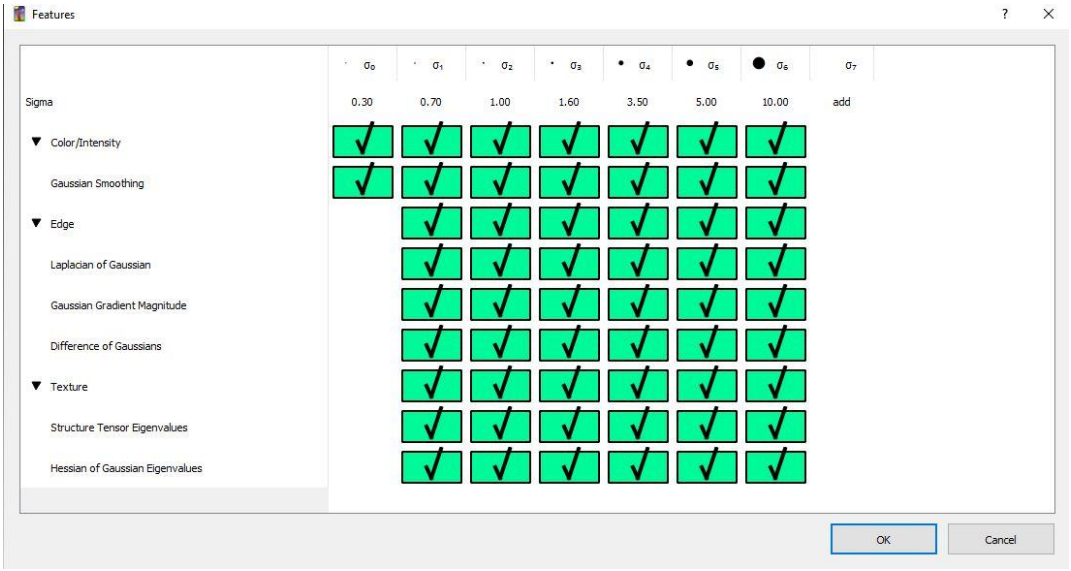
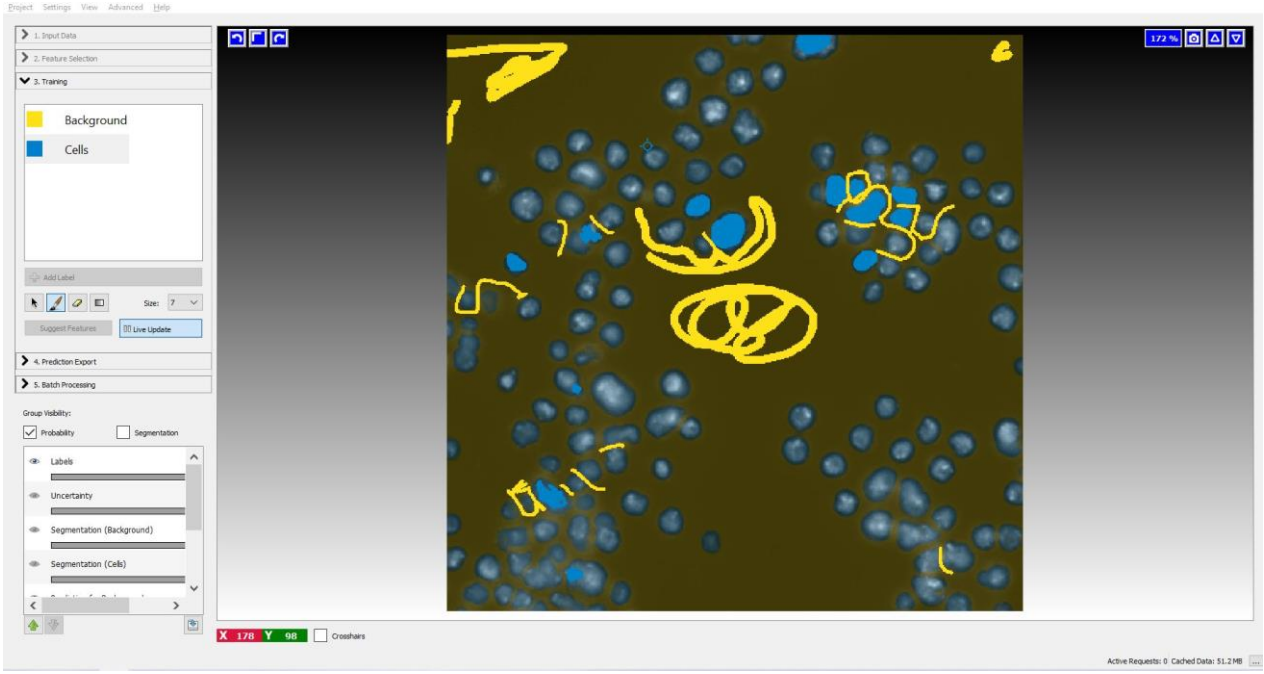
Ilastik

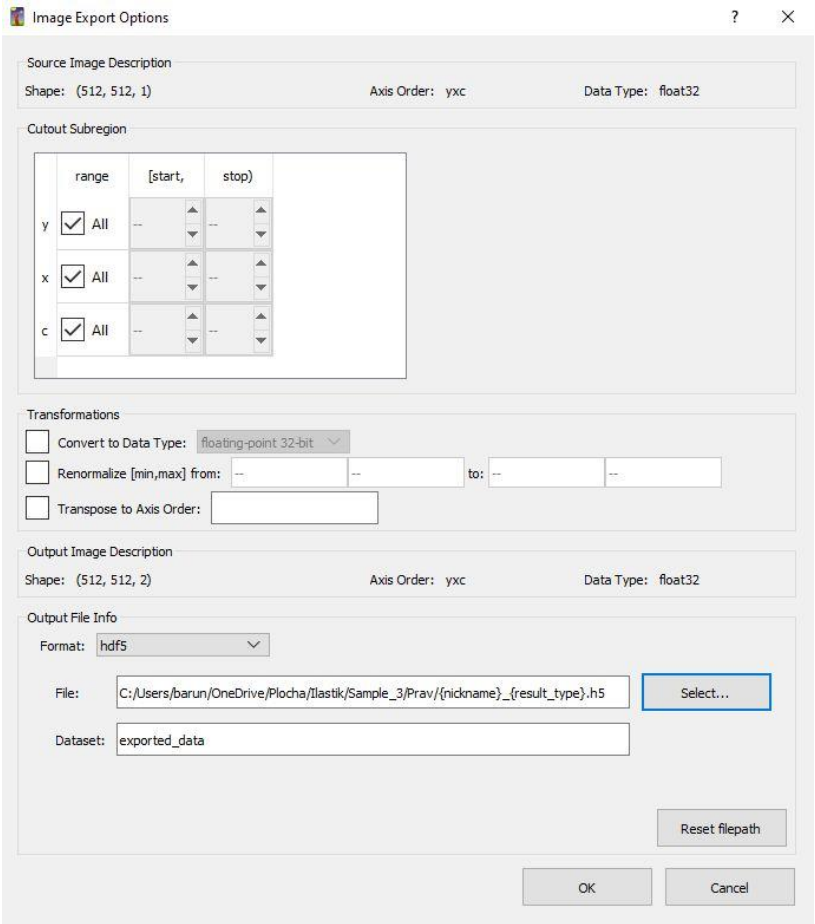
1. To be able to load the images in TIF format into Ilastik, save the images with a greyscale look-up table using Fiji, as importing images with the default colour palette is impossible
    a. Open Fiji and click on *Process → Batch → Macro*
    b. Specify the input folder in which all images are saved and the output folder in which you want to save the modified images
    c. Insert this code into the white box: *run("Grays"); run("Save");*
    d. Click on *Process* and check that all your images are saved in the specified output folder
2. Open Ilastik and create a project with *Pixel Classification* segmentation workflow
3. Load one TIF image file as input data
4. In *Feature Selection*, tick all options so the result looks like this:



5. In *Training*, train the classifier to recognise cells and background
    a. Rename *Label 1* (yellow) to *Background* and *Label 2* (blue) to *Cells*
    b. Click on the *Live Update* button, so it is possible to evaluate the classifier in real-time
    c. Train the classifier iteratively by choosing the label first and then drawing over pixels belonging to that label until you are satisfied with the result
    d. In the *Size* option, you can change the thickness of the brush to achieve greater detail
    e. The result can look like this, with the rich colours showing your interaction with the classifier and the muted colours showing the predictions of the classifier:



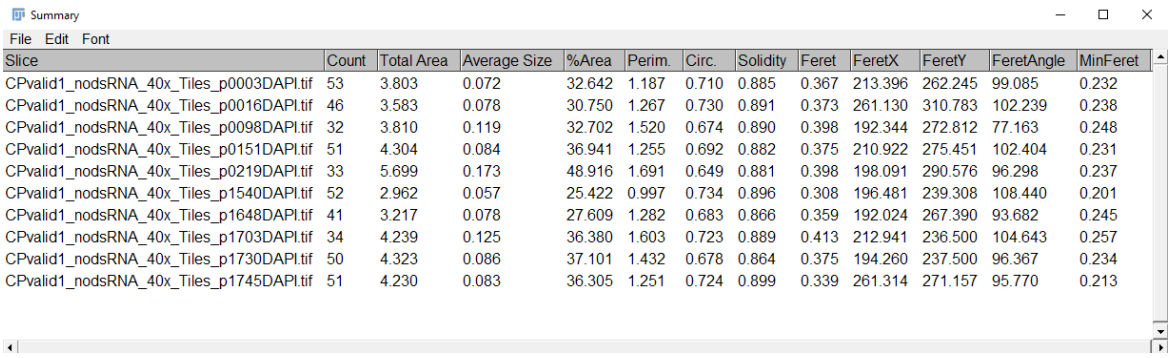
6. In *Prediction Export*, export the probabilities of pixels being background/cell
    a. In *Export Image Settings*, specify the HDF5 file format and the output folder (must be without spaces and diacritics) in which you want to save the results
    b. Leave the other settings in the default state, as shown here:



    c. Click on *Export All* to run the export
7. In *Batch Processing*, export the probabilities of pixels being background/cell for all other images of the sample
    a. Click on *Select Raw Data Files* and choose all corresponding TIF files

b. Click on *Process all files* to run the export
c. The export settings remain the same as those specified in *Prediction Export,* so the results are saved to the same output folder

8. Open Fiji and, if you do not have it already installed, install the Ilastik Fiji plugin as described here
9. Click on *Plugins → Macros → StartUp Macros* and open *Ilastik.ijim* macro supplied here to batch process all HDF5 files saved in the Ilastik output folder
    a. Click on *Run* and specify the Ilastik output folder
    b. This macro loads the HDF5 file using the Ilastik Fiji plugin, uses filtering and smoothing to reduce the noise in the data and counts the cells in the image
    c. The cell counts of all images are shown in *Summary* window, with an example here:


Summary

File Edit Font

Slice	Count	Total Area	Average Size	%Area	Perim.	Circ.	Solidity	Feret	FeretX	FeretY	FeretAngle	MinFeret
CPvalid1_nodsRNA_40x_Tiles_p0003DAPI.tif	53	3.803	0.072	32.642	1.187	0.710	0.885	0.367	213.396	262.245	99.085	0.232
CPvalid1_nodsRNA_40x_Tiles_p0016DAPI.tif	46	3.583	0.078	30.750	1.267	0.730	0.891	0.373	261.130	310.783	102.239	0.238
CPvalid1_nodsRNA_40x_Tiles_p0098DAPI.tif	32	3.810	0.119	32.702	1.520	0.674	0.890	0.398	192.344	272.812	77.163	0.248
CPvalid1_nodsRNA_40x_Tiles_p0151DAPI.tif	51	4.304	0.084	36.941	1.255	0.692	0.882	0.375	210.922	275.451	102.404	0.231
CPvalid1_nodsRNA_40x_Tiles_p0219DAPI.tif	33	5.699	0.173	48.916	1.691	0.649	0.881	0.398	198.091	290.576	96.298	0.237
CPvalid1_nodsRNA_40x_Tiles_p1540DAPI.tif	52	2.962	0.057	25.422	0.997	0.734	0.896	0.308	196.481	239.308	108.440	0.201
CPvalid1_nodsRNA_40x_Tiles_p1648DAPI.tif	41	3.217	0.078	27.609	1.282	0.683	0.866	0.359	192.024	267.390	93.682	0.245
CPvalid1_nodsRNA_40x_Tiles_p1703DAPI.tif	34	4.239	0.125	36.380	1.603	0.723	0.889	0.413	212.941	236.500	104.643	0.257
CPvalid1_nodsRNA_40x_Tiles_p1730DAPI.tif	50	4.323	0.086	37.101	1.432	0.678	0.864	0.375	194.260	237.500	96.367	0.234
CPvalid1_nodsRNA_40x_Tiles_p1745DAPI.tif	51	4.230	0.083	36.305	1.251	0.724	0.899	0.339	261.314	271.157	95.770	0.213

    d. Save the *Summary* window with the cell counts as a CSV file
    e. Your results are in the *Count* column

10. Repeat this procedure five times, once for every sample, with the classifier being trained on one image of the sample and then used without further modifications on the remaining nine images of the sample