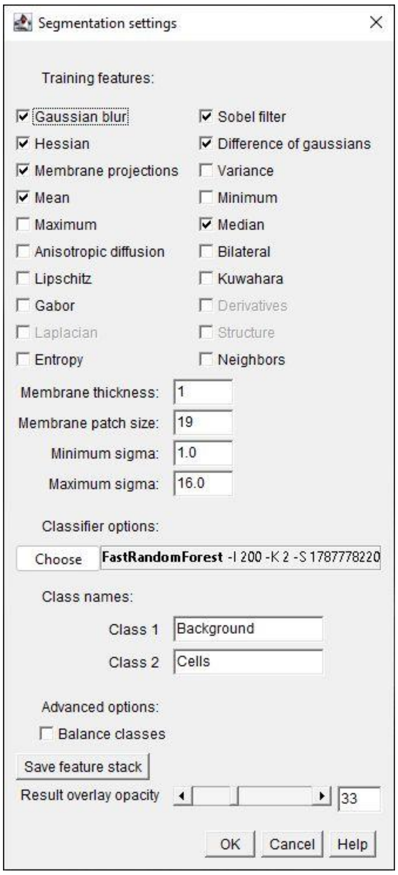
Fiji

1. Open Fiji and load one TIF file as input data
2. Click on *Plugins → Segmentation → Trainable Weka Segmentation*
3. Set *Settings* in the following way: add *Mean* and *Median* training features and change the classes' names as shown here:



Segmentation settings

Training features:

☑ Gaussian blur
☑ Hessian
☑ Membrane projections
☑ Mean
☐ Maximum
☐ Anisotropic diffusion
☐ Lipschitz
☐ Gabor
☐ Laplacian
☐ Entropy

☑ Sobel filter
☑ Difference of gaussians
☐ Variance
☐ Minimum
☑ Median
☐ Bilateral
☐ Kuwahara
☐ Derivatives
☐ Structure
☐ Neighbors

Membrane thickness: 1
Membrane patch size: 19
Minimum sigma: 1.0
Maximum sigma: 16.0

Classifier options:

Choose FastRandomForest -I 200 -K 2 -S 1787778220

Class names:

Class 1 Background
Class 2 Cells

Advanced options:

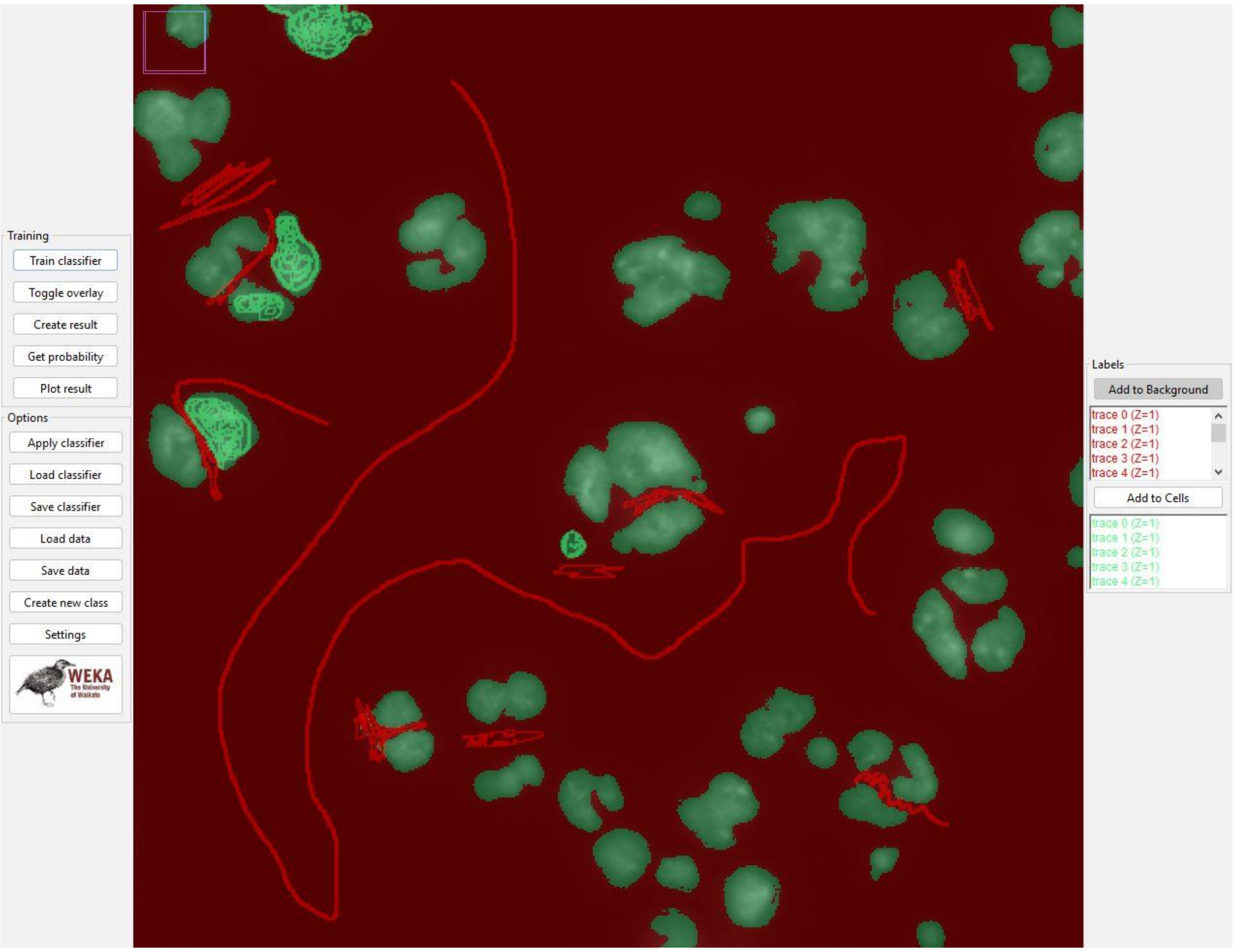
☐ Balance classes

Save feature stack

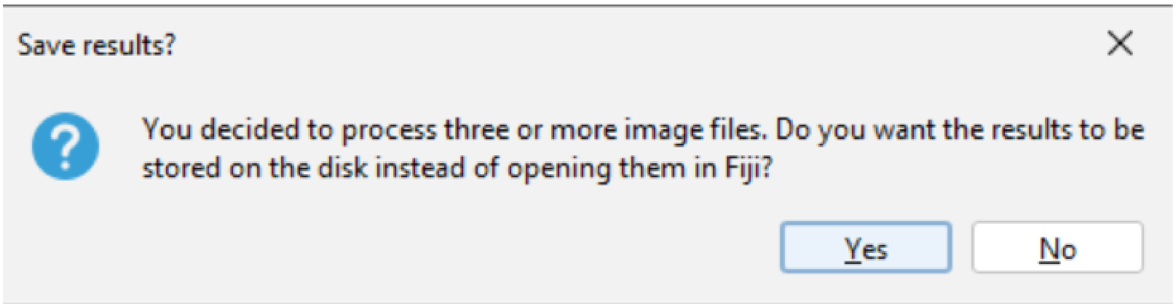
Result overlay opacity 33

OK Cancel Help

4. Mark parts of the image with the *Freehand Line* tool (set automatically in Fiji) and click on Add to Background/Add to Cells, depending on which part of the image you have marked
   a. After adding at least one entry to both labels, you can click on *Train Classifier*
   b. Train the classifier iteratively by drawing over pixels first, then choosing the label belonging to those pixels and clicking on *Train Classifier* to update it until you are satisfied with the result
   c. You can use *Toggle Overlay* to switch between the image as the classifier currently sees it and the original image
   d. 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:



5. Click on *Apply Classifier* and pick all images of the sample, including the one on which the classifier was trained
6. As there are more than three images, this window pops up:

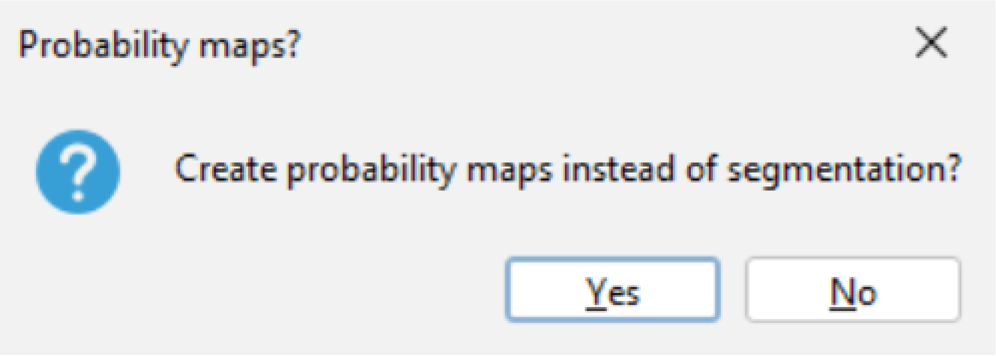


Save results?

You decided to process three or more image files. Do you want the results to be stored on the disk instead of opening them in Fiji?

   a. Click on *Yes* and pick the folder in which you want to save the segmented images

7. After picking the output folder, this window pops up:



Probability maps?

Create probability maps instead of segmentation?

   a. Click on *No*, as you want the segmentation results and not the probability maps

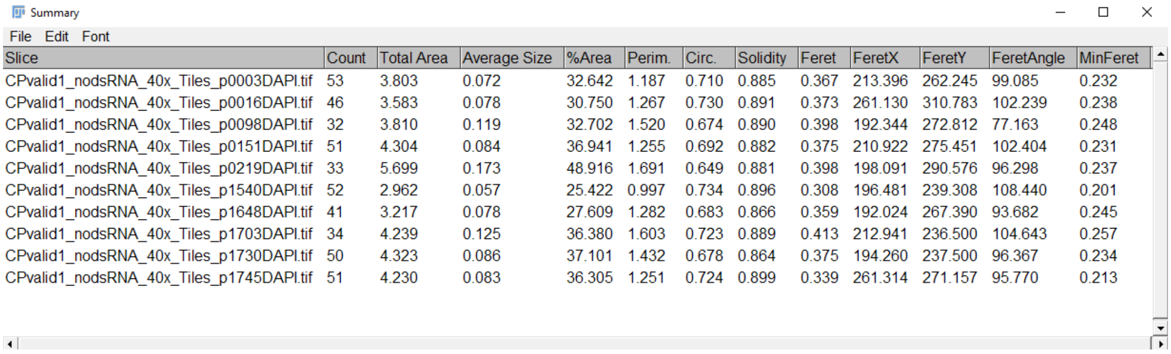
8. Now you should have all the segmented images in the specified output folder

9. Click on *Plugins* → *Macros* → *StartUp Macros* and open *ImageJ.ijim* macro supplied here to batch process all the files in the previously specified output folder

    a. Click on *Run* and specify the folder in which the segmented images are saved

    b. This macro loads the TIF file, 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:



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