

Access to segmented images and tutorial for segmentation

Requirements

Download and install:

Python 3 (<https://www.python.org/>) and scikit-image (scikit-image.org)

For software development, PyCharm (www.jetbrains.com/pycharm/) and anaconda (www.continuum.io) were used.

Program configuration and execution

Adjust the path where the images to be processed are located:

```
config/nuc_sec.ini > [Paths]  
RAW = [Path to images to be processed]
```

Program execution

The program can be started within ‘NucleoSegment’ folder with:

```
python nucleo_segment.py
```

Processing steps of the tutorial

The individual steps of the segmentation pipeline for the example in this tutorial can be accessed by using the following arguments:

Processing steps	TEST-process
Filter labels	TEST-merge
Filter nuclei	TEST-nuclei
Correct nuclei	TEST-correct

Segmented images

The segmented and corrected images from the report can be accessed with the following arguments:

4-days A	4-days-A
4-days B	4-days-B
5-days A	5-days-A
5-days B	5-days-B

Start a new segmentation

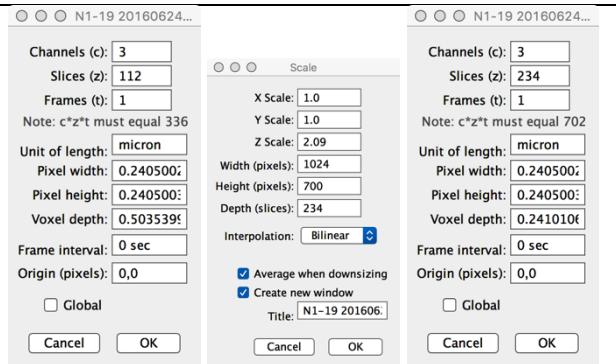
Before analysing an image, check that it has an isotropic resolution in Fiji with:

Image > Properties

In this case, the Z scale has to be adjusted to 2.09 (0.5035/0.2405) with:

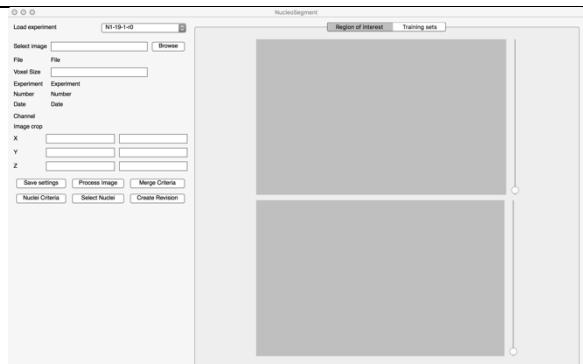
Image > Scale

Apply and save the stack for analysis.

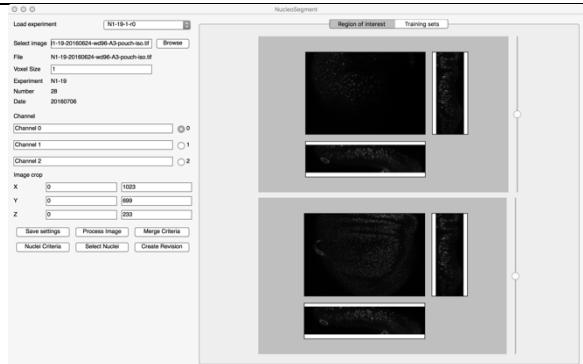


Start the program with:

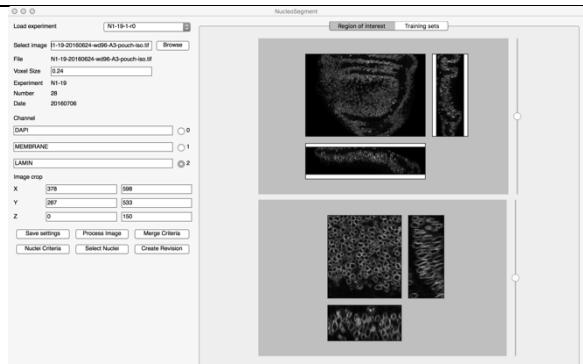
```
python nucleo_segment.py NEW -s
```



Select an image with 'Browse'.



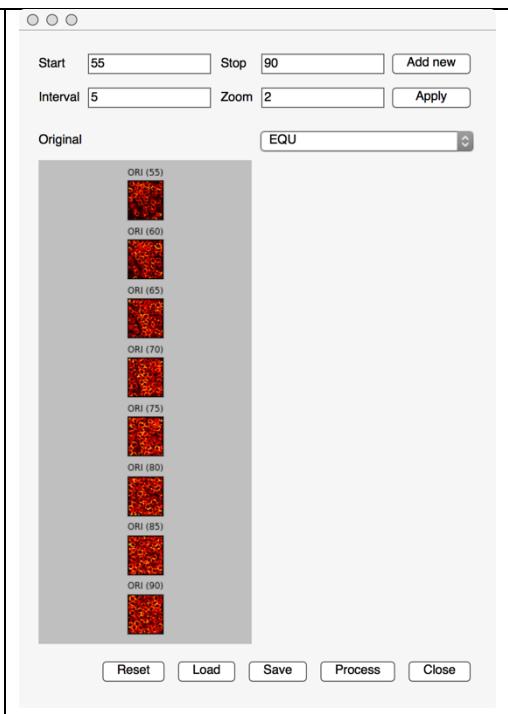
Define the voxel size as calculated in the first step (0.24). Define the membrane, DAPI and lamin channels. Define a region of interest on the image by dragging the mouse along the image while pressed. Modify X, Y and Z coordinates if needed. Click on 'Save settings' and close the window.



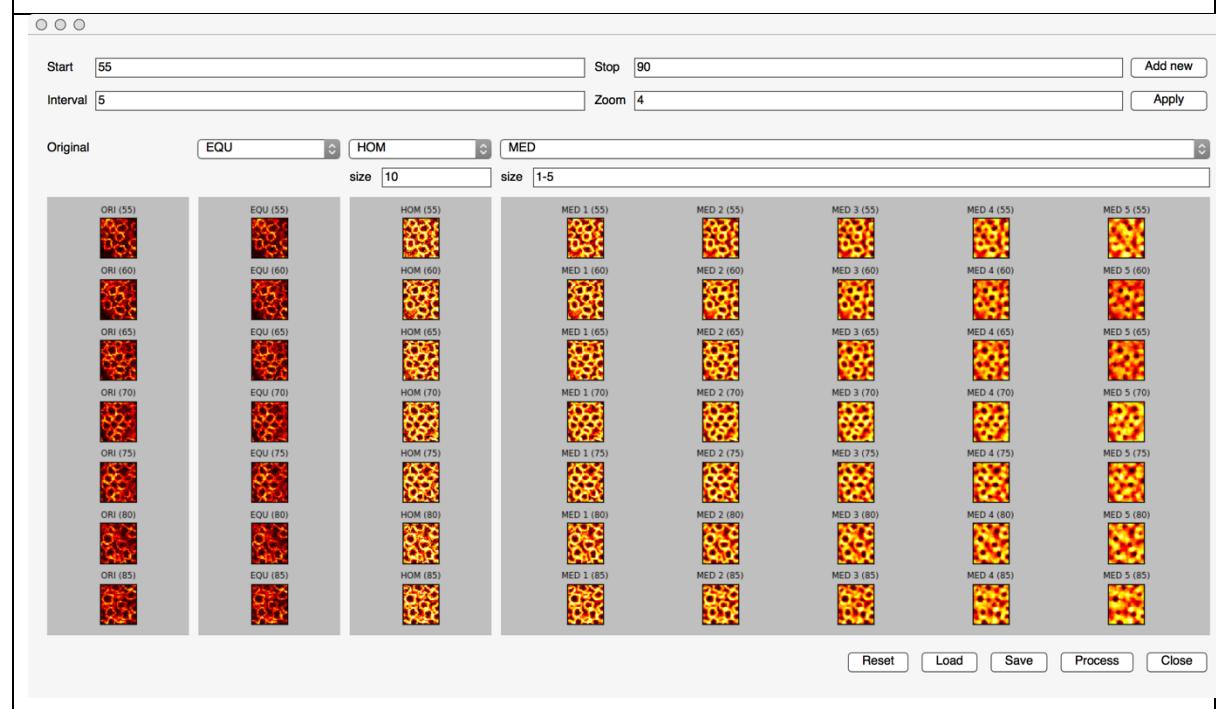
Select processing filters

Start program with the newly generated segmentation:

```
python nucleo_segment.py N1-19-28 -p1
```



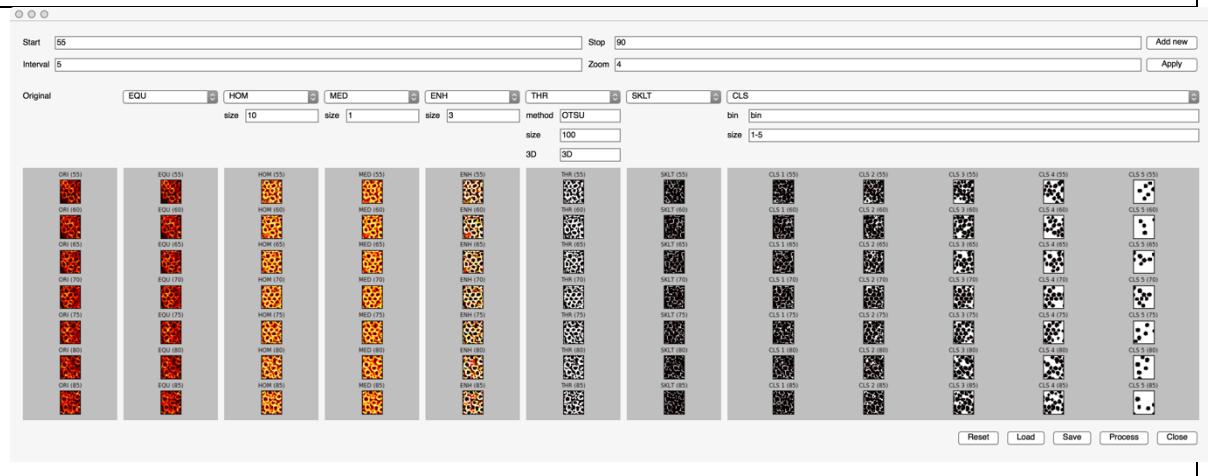
Increase the zoom to 4. Add filters by clicking ‘Add new’. Select ‘EQU’, ‘HOM’ and ‘MED’ from the lists. Set the parameters for ‘HOM’ as 10 and for ‘MED’ as 1-5. Click ‘Apply’.



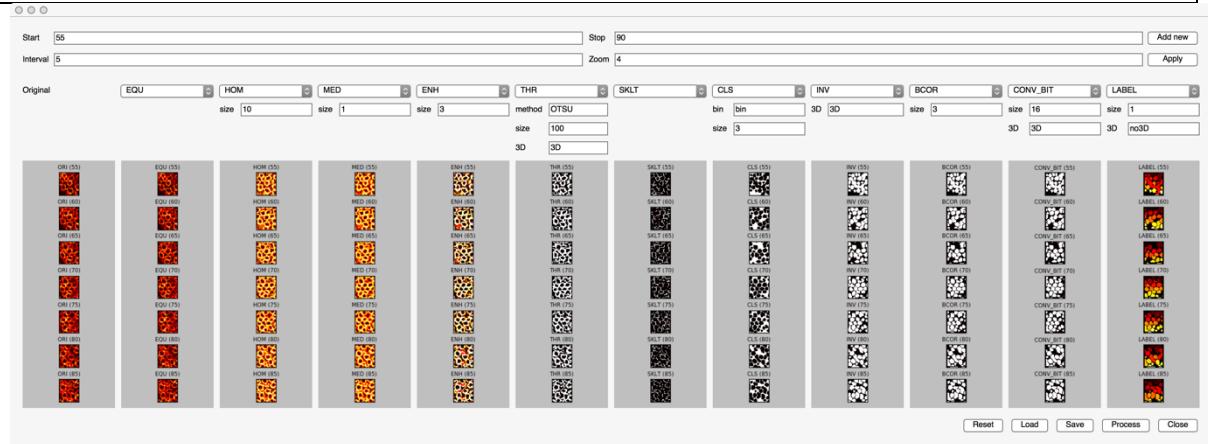
Set parameter for ‘MED’ as 1. Click ‘Apply’. Click ‘Add new’. Select ‘ENH’ and set parameters as 1-5. Click ‘Apply’.



Set parameter for ‘ENH’ as 3. Click ‘Apply’. Click ‘Add new’ three times. Select ‘THR’, ‘SKLT’ and ‘CLS’. Set parameters for ‘THR’ as OTSU (method), 100 (size) and 3D (3D). Set parameter for ‘CLS’ as bin (bin) and 1-5 (size). Click ‘Apply’.



Set parameter for ‘CLS’ as 3. Click ‘Apply’. Click ‘Add new’ four times. Select ‘INV’, ‘BCOR’, ‘CONV_BIT’ and ‘LABEL’. Set parameters for ‘INV’ as 3D, ‘BCOR’ as 3, ‘CONV_BIT’ as 16 (size) and 3D (3D) and for ‘LABEL’ as 1 (size) and no3D (3D). Click ‘Apply’.



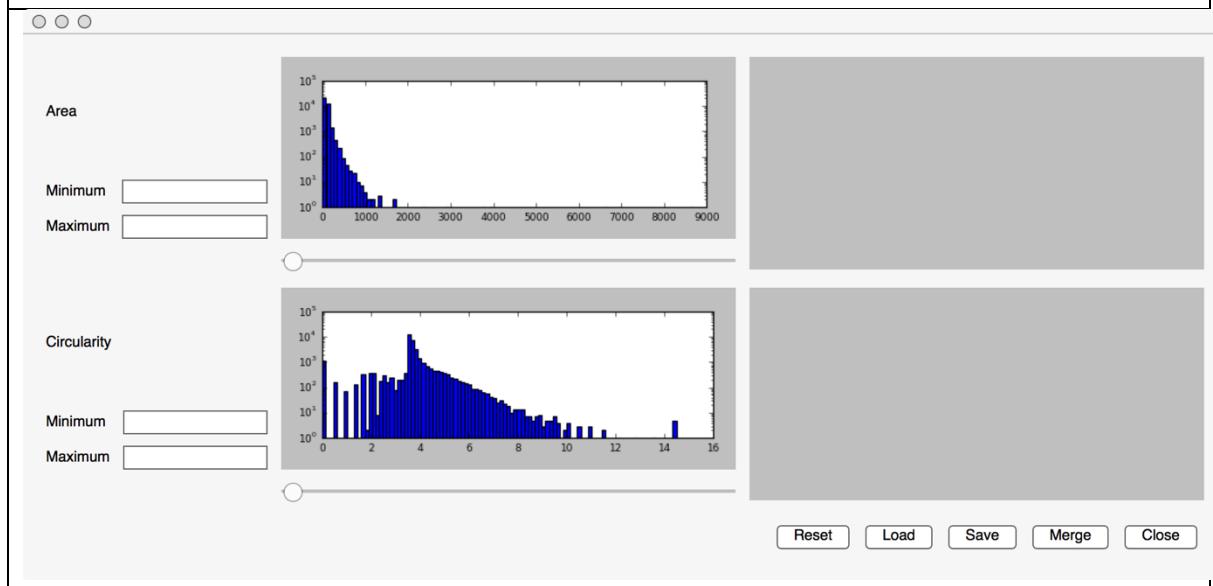
Click ‘Save’ and close the window.

Select filtering for labels to be merged

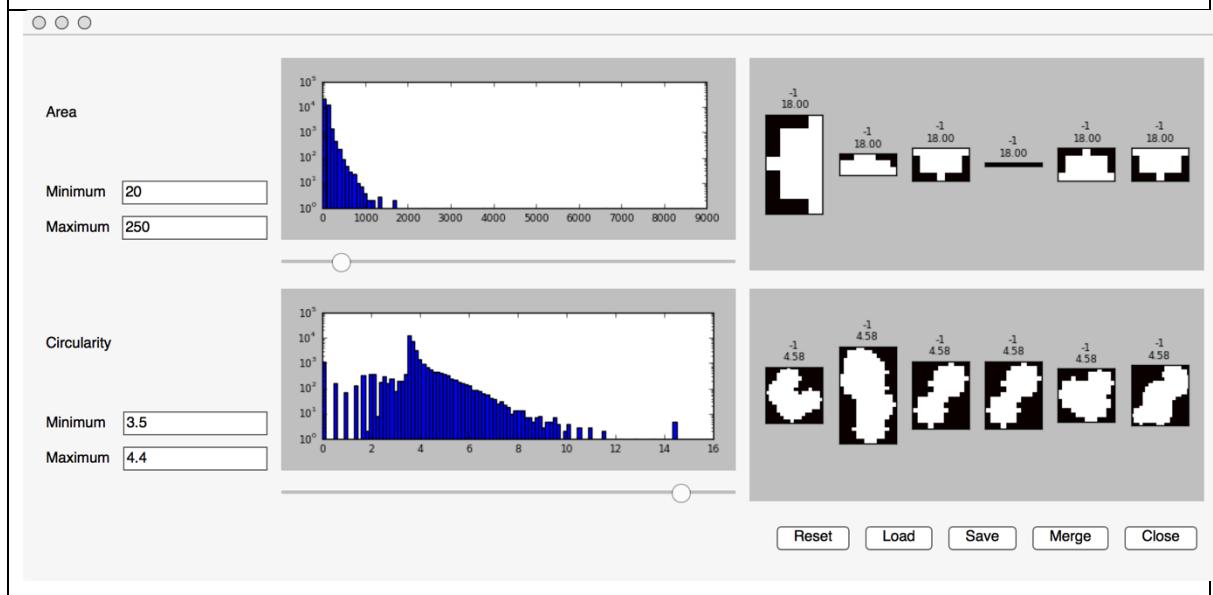
Process the image and filter labels for merging:

```
python nucleo_segment.py N1-19-28 -p2 -m1
```

The selected processing filters will be applied and the extracted labels will be presented.



Explore the extremes of the labels using the sliders. For ‘Area’, set minimum as 20 and maximum as 250. For ‘Circularity’, set minimum as 3.5 and maximum as 4.4. Click ‘Save’ and close the window.



Filter merged nuclei

Merge nuclei and filter the results:

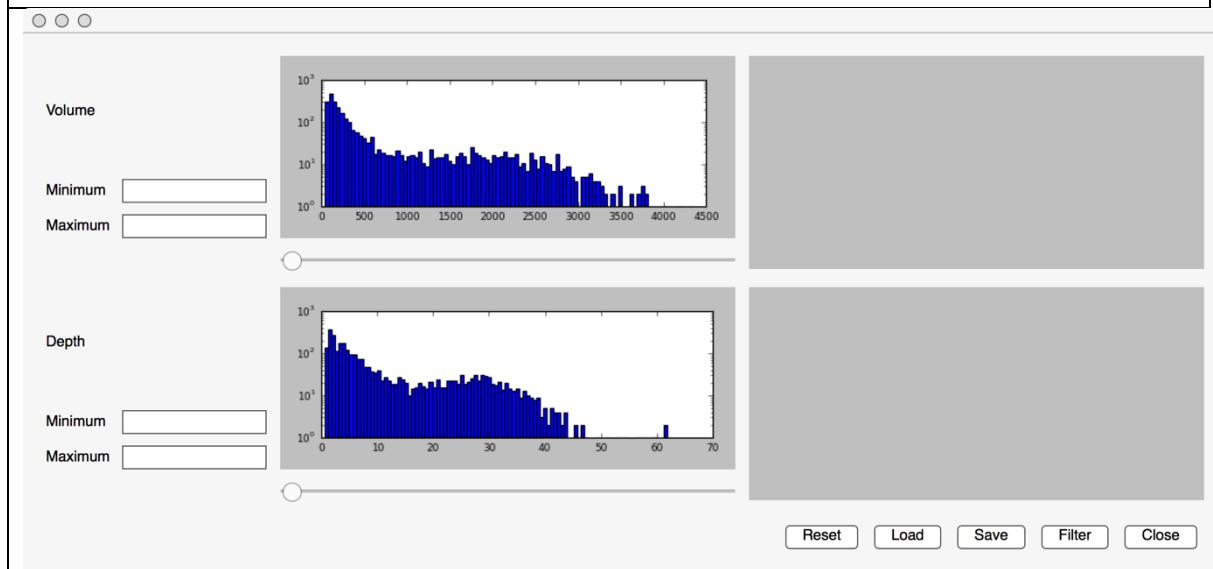
```
python nucleo_segment.py N1-19-28 -m2 -n1
```

The labels will be filtered and merged based on overlap defined in:

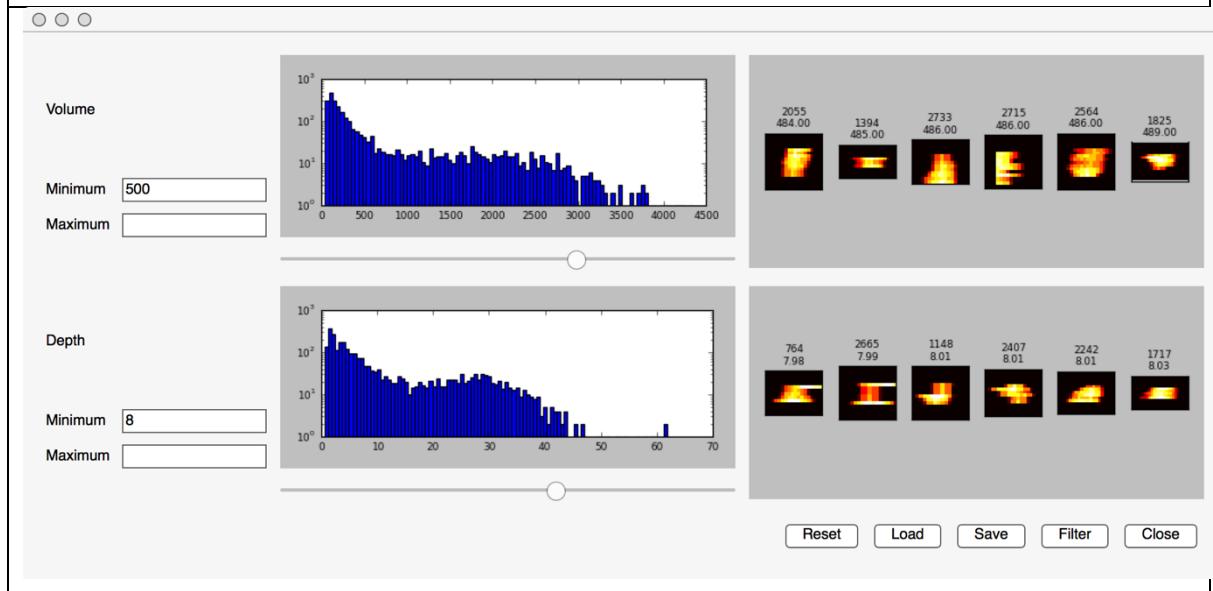
```
config/nuc_sec.ini > [NucleiMerge] > MIN_OVERLAP = 80
```

The properties of the merged nuclei will be calculated and their projections presented.

Depending on the amounts of nuclei, this step will take the longest.



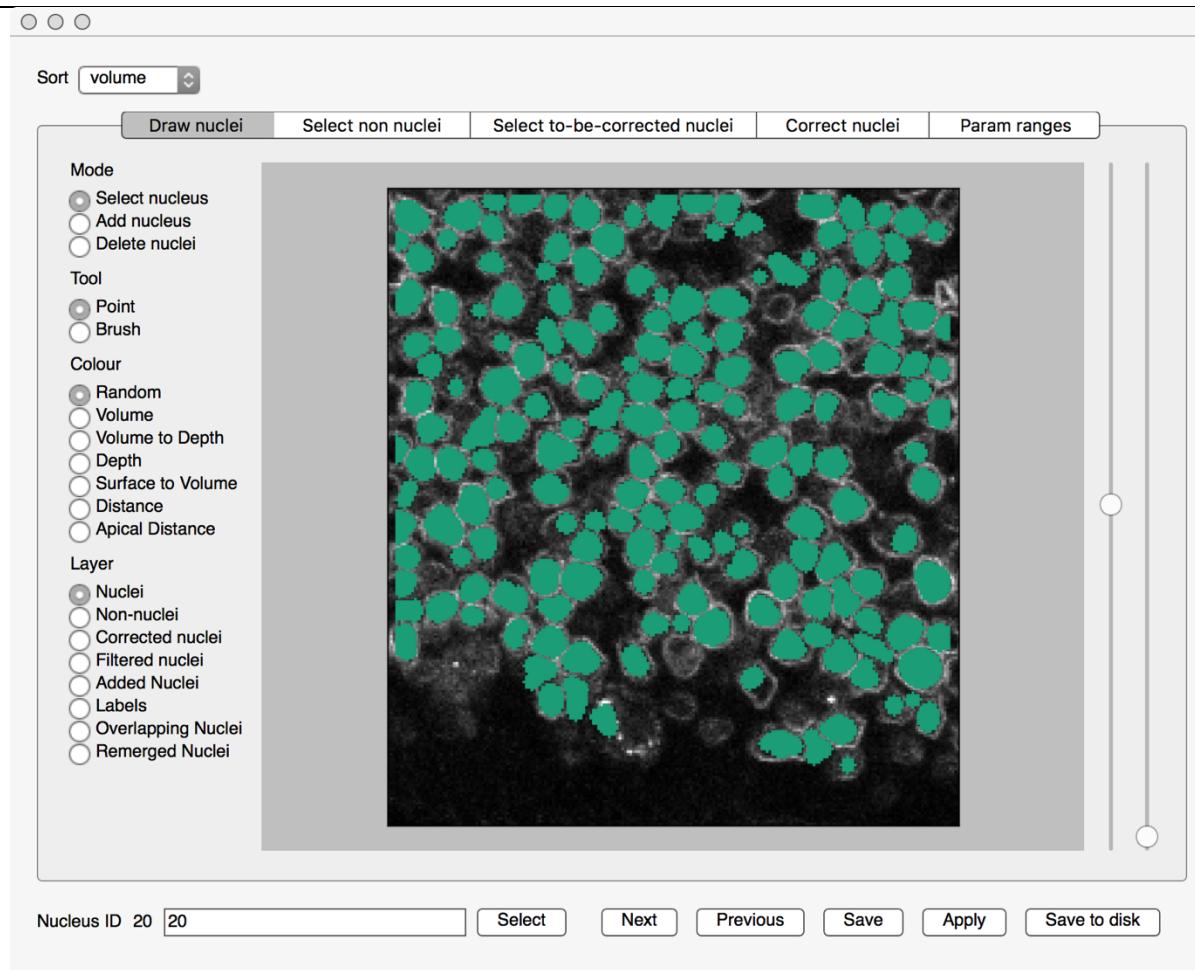
Explore the extremes of the nuclei using the sliders. For ‘Volume’ set minimum as 500 and for ‘Depth’ set minimum as 8. Click ‘Save’ and close the window.



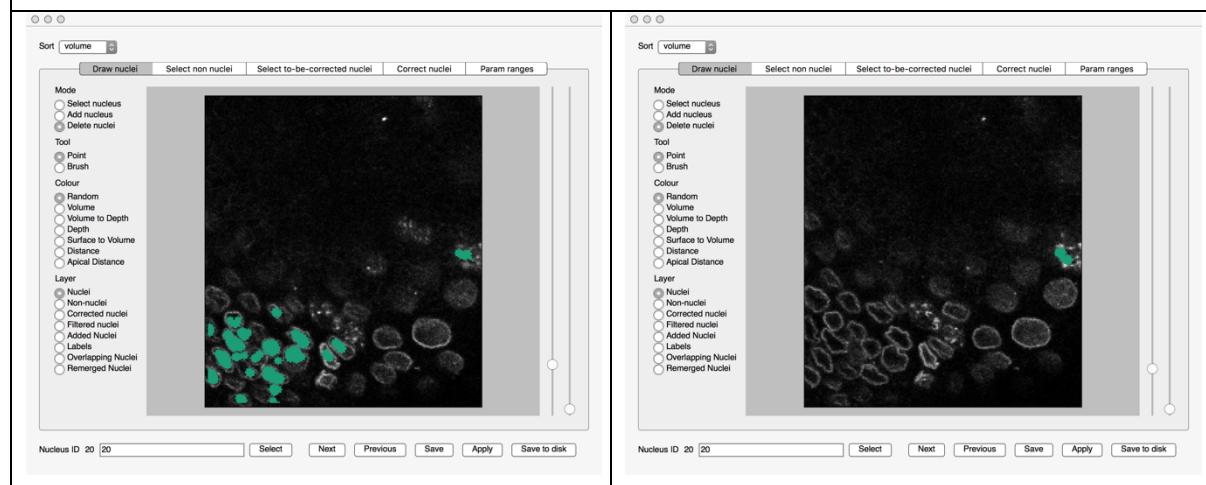
Correct segmented nuclei

Filter nuclei and show correction interface:

```
python nucleo_segment.py N1-19-28 -n2 -c1
```



Select ‘Delete nuclei’ in ‘Mode’ and click on the nuclei not in the region of interest. The mouse can also be dragged along while pressed. Select ‘Add nucleus’ and click on a deleted nucleus if a nucleus needs to be recovered. Start from the top as many peripodial cells are also labelled. Once all nuclei are deleted, click ‘Save’ and then ‘Apply’.

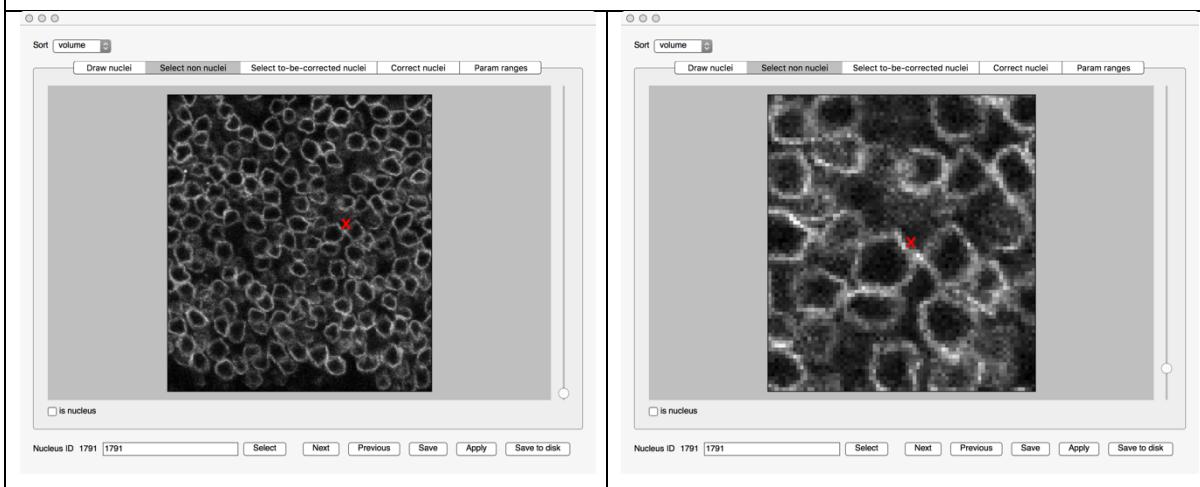


Select ‘Select non nuclei’ tab. Navigate through all nuclei and decide if it is a nucleus or not. The following keyboard shortcuts should be used:

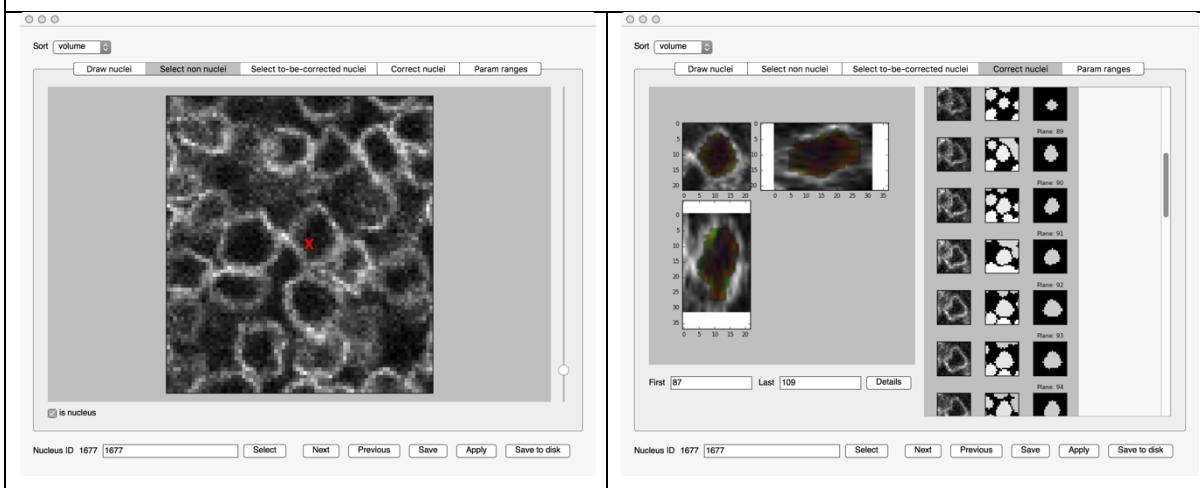
- > Forward
- < Backward
- x Toggle ‘is nucleus’

Many non-nuclei are easily identifiable because their centre (red X) is not on a nucleus.

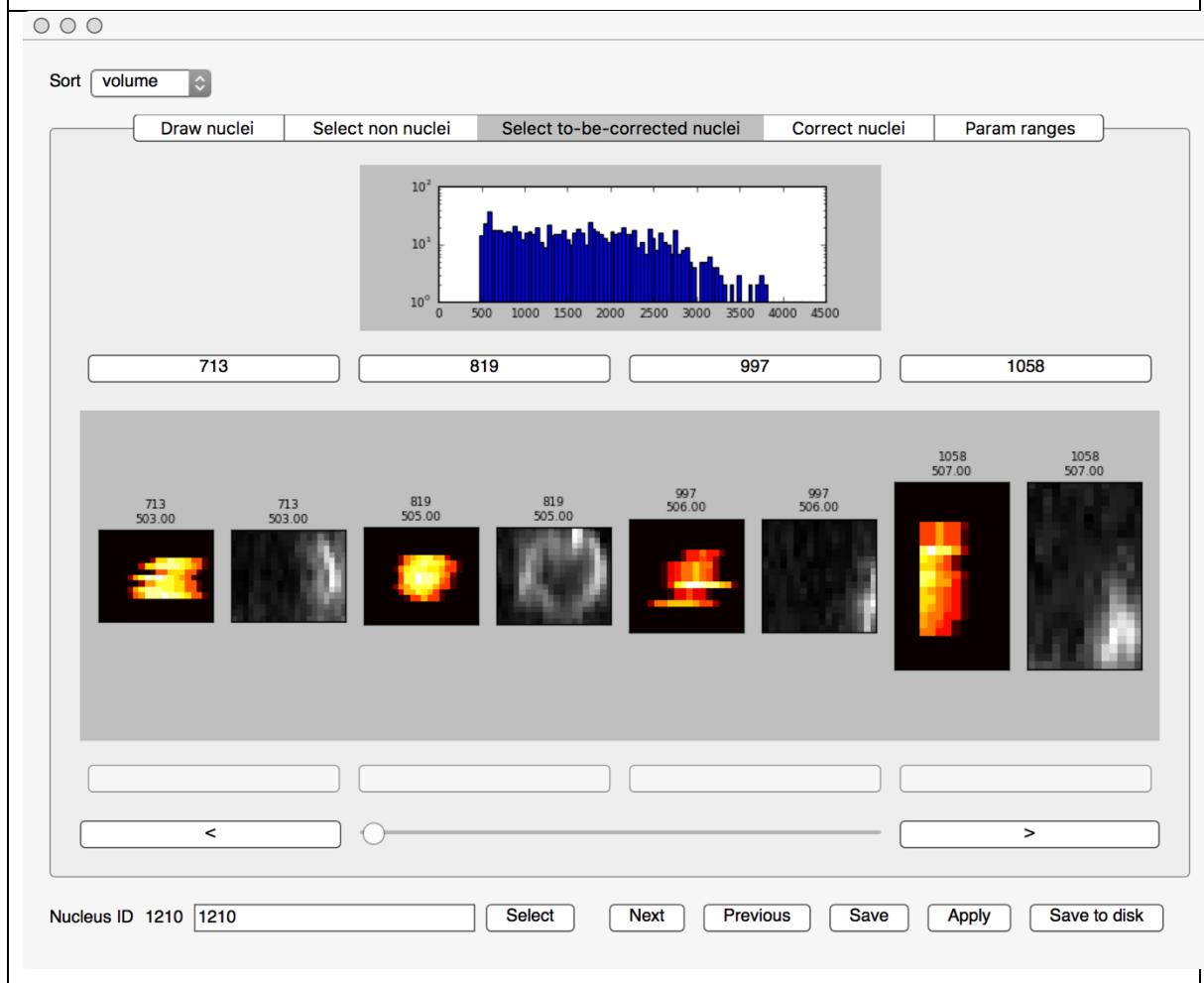
The first nuclei are mostly non-nuclei as the list is ordered according to volume. Therefore, the first nuclei can be removed by using ‘>’ and ‘x’ until the red marker is in the middle of a nucleus. Use the slider on the side to zoom into the image.



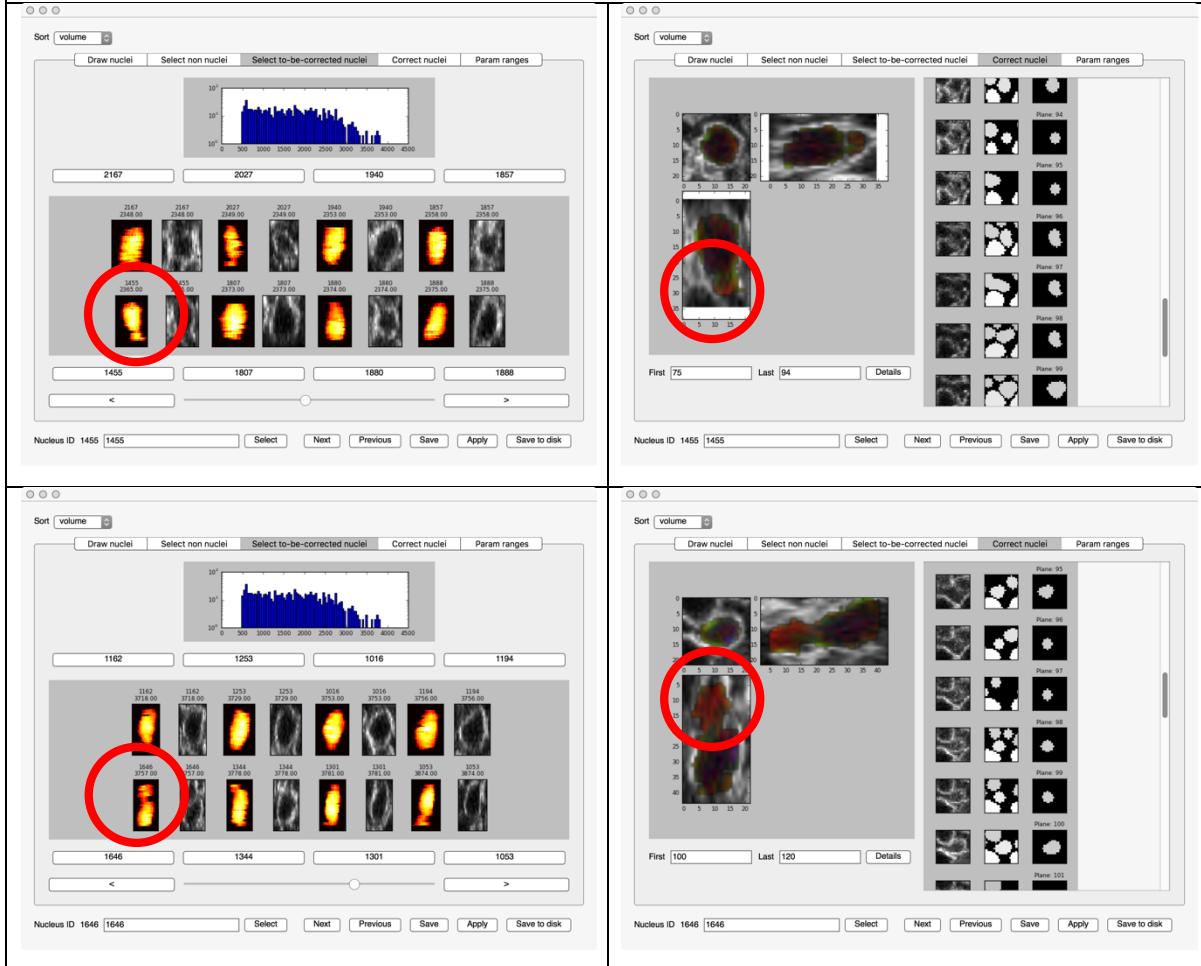
Once the red marker marks the middle of nuclei, it can be difficult to decide whether the nucleus is a true nucleus or not. Use ‘;’ to switch to the ‘Correct nuclei’ Tab and click ‘Details’ to show the individual planes (left: lamin; middle: labels; right: nucleus labels). Once decided if the nucleus is true or not, switch back to the selection tab with ‘k’. Focus at this point only at removing nuclei, not at correcting. Once at the end of the list, click ‘Apply’.



Switch to the ‘Select to-be-corrected nuclei’ tab and move the slider to the left. This is a gallery of the nuclei projections and helps to identify which nuclei need correction for their planes. Remove nuclei that were missed in the previous step. The numbers on the eight buttons around the gallery correspond to the identifiers on the gallery. Click on the identifier button to select a nucleus. The view will switch to ‘Correct nuclei’ where the planes can be corrected and the nucleus also deleted (by pressing ‘x’). The last selected nucleus always appears at the bottom left of the gallery. Use the arrow buttons to navigate back and forth.



Once the nuclei look reasonable, start correcting the planes if they look not correct. Commonly the top or bottom of a nucleus is merged with background. To correct the planes, modify the last or first plane by judging the individual planes by clicking ‘Details’. Click ‘Save’ and switch back to the ‘Select to-be-corrected nuclei’ tab.



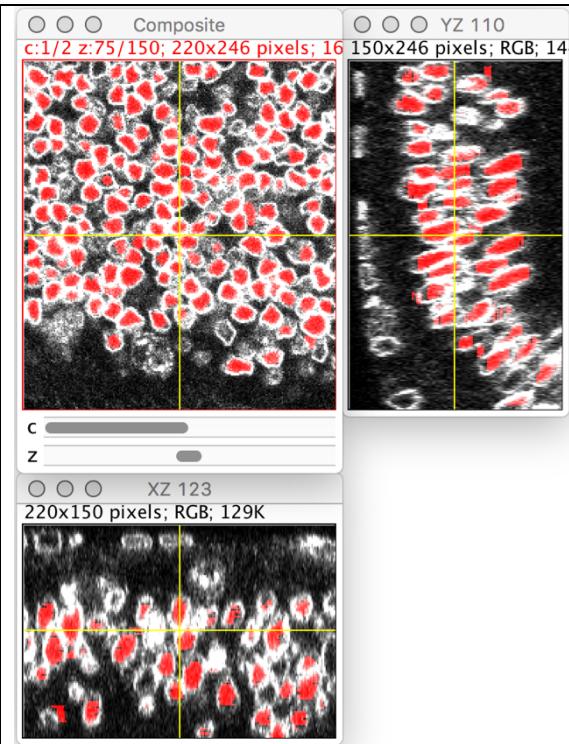
Once all nuclei are corrected, click ‘Save to disk’. Close the window. Restart the correction interface with:

```
python nucleo_segment.py N1-19-28 -c1
```

The volume distribution should now look more normally distributed.



The saved corrections will be applied on the resulting stacks will be saved in ‘results/[Experiment]/stacks_corr’. The main stacks are ‘lamin.tif’ and ‘nuclei.tif’. Load both into Fiji and merge the channels with ‘Image > Color > Merge Channels’. Select red channel for ‘nuclei.tif’ and gray channel for ‘lamin.tif’. Click ‘OK’ and show orthogonal view with ‘Image > Stacks > Orthogonal Views’.



Quantification of segmented nuclei

Adjust the tiling parameters in:

```
config/nuc_sec.ini > [Quantification]
TILING_DIM = 50
TILING_COUNT = 5
```

All values relating to volume will be converted by multiplying the number of pixels with the voxel size defined at the very beginning.

To quantify the corrected segmentations, use the following command:

```
python nucleo_quant.py 4-days-A 4-days-B 5-days-A 5-days-B
```

or, use the specific experiment identifiers:

```
python nucleo_quant.py N1-19-9 N1-19-22 N1-19-23 N1-19-24
```

To generate the heatmaps, load all TIF images in ‘analysis/maps_d50’ into FIJI (the d50 refers to the chosen tiling dimension). Load the macro ‘apply_LUTs.ijm’ into Fiji. Define the segmentations to be converted in:

```
images = newArray('N1-19-9', 'N1-19-22', 'N1-19-23', 'N1-19-24');
```

Adjust the tiling dimension in:

```
dim = 50
```

Define the result path in (omit the dimension in the path, i.e. ‘maps_d50’ > ‘maps_d’):

```
results_path =  
"/Users/schiend/PycharmProjects/NucleoSegment/analysis/maps_d"
```

Click run. The resulting images will be stored in the ‘maps_d50’ folder under ‘hot’.

The individual values for each averaged tile are stored in lists under ‘analysis/lists_d50’ in CSV format.

```
Name;N1-19-9;N1-19-22;N1-19-23;N1-19-24  
;2424.10526316;nan;nan;nan  
;2424.10526316;nan;nan;nan  
;2424.10526316;nan;nan;nan  
;2424.10526316;nan;nan;nan  
;2424.10526316;nan;nan;nan  
[...]
```

These can be loaded into the statistical analysis tool of your choice. As example, GraphPad Prism is used. Create a new chart with grouped samples. Import the data with ‘Insert > Import Data’. Select a list, e.g. hist_volume.csv. Click on the filter tab and set ‘Missing values are denoted by’ as ‘nan, -1’ and set the start row as 2. Click ‘Import’. You can now analyse the data using histograms, cumulative graphs or bar-charts.