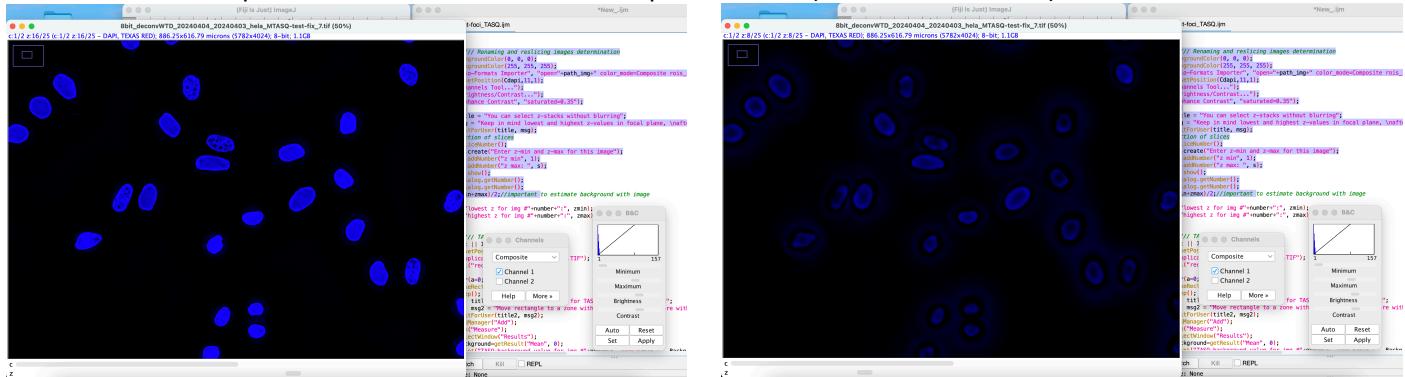


Features need to be optimized before quantification (developed in lines 19-120), we recommend setting on control image (untreated) and in positive control image (treated with PDS in our case). Macro is written as follows and we tried to address pitfalls that may occur.

1. Command macro starts at line 217 with opening images

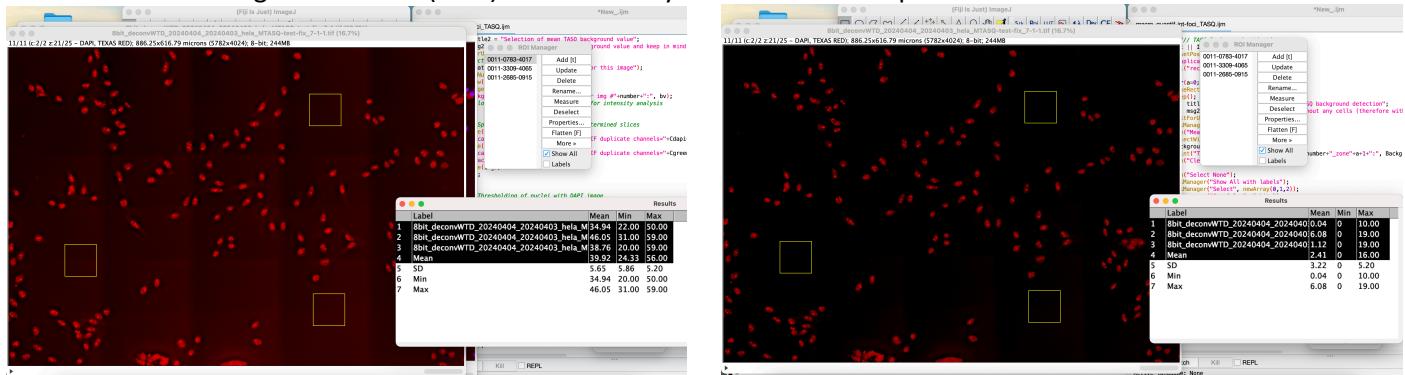
2. Renaming and reslicing images (lines 230-250):

- o Clear DAPI (left) vs blurred DAPI (right)
- o Keep same number of z-stacks for all quantification ("zmin" and "zmax")



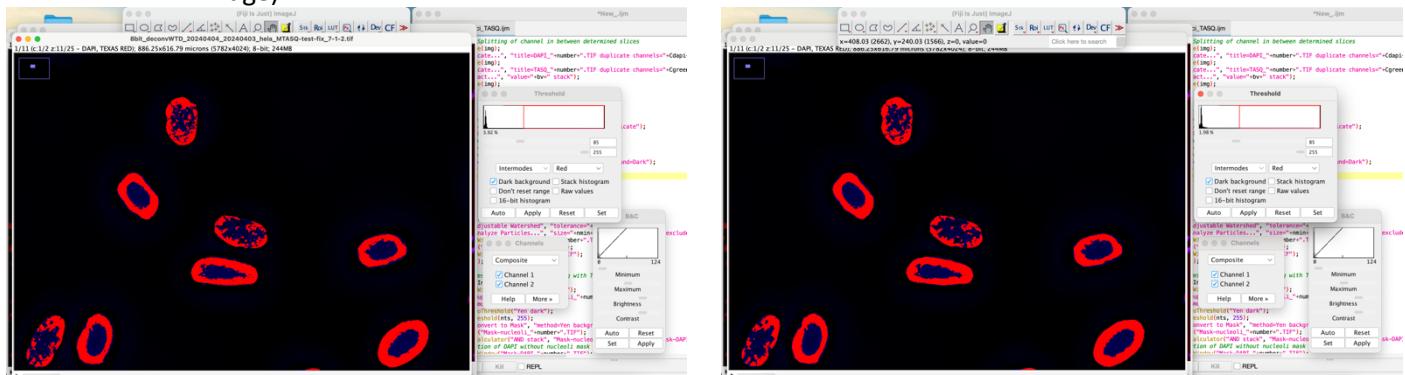
3. Removal of TASQ background (lines 256-308):

- o Before (left) and after (right; background value= 40 (nearest whole number)) background removal
- o Background values ("bv") are defined by mean measure of pixel value

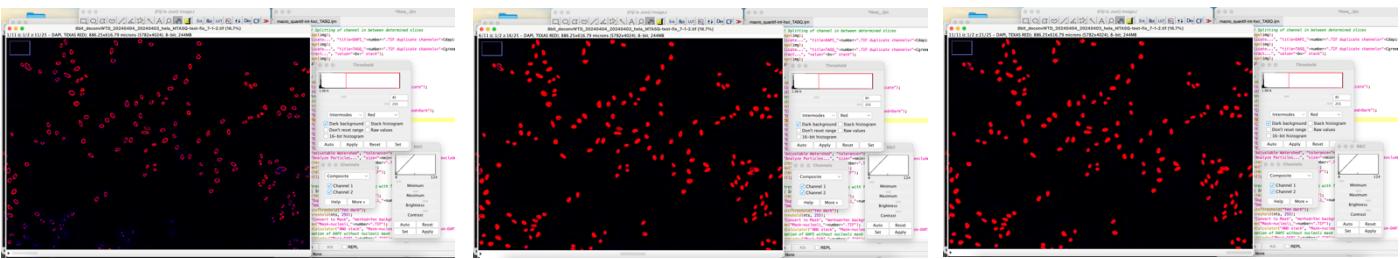


4. Thresholding of nucleus with DAPI staining (for each z-stack):

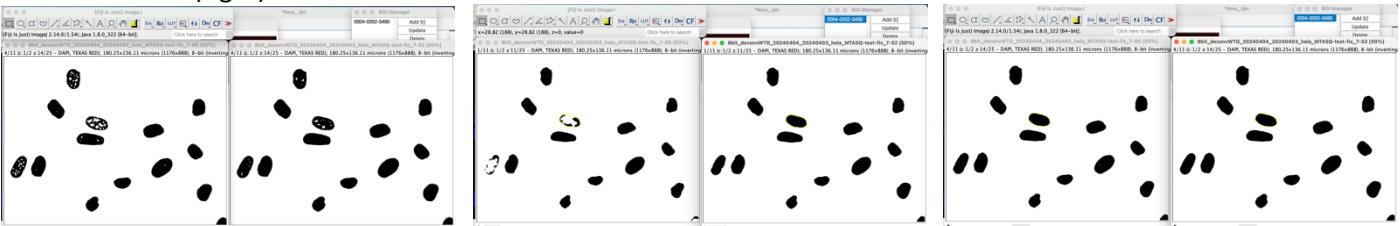
- o Before (left) and after (right) smoothing with same threshold value (in z-stack near in limit to blurred image)



- o Same threshold ("zts") in different z-stacks in low z-stack (left), in middle z-stacks (center) and in high z-stacks (right)



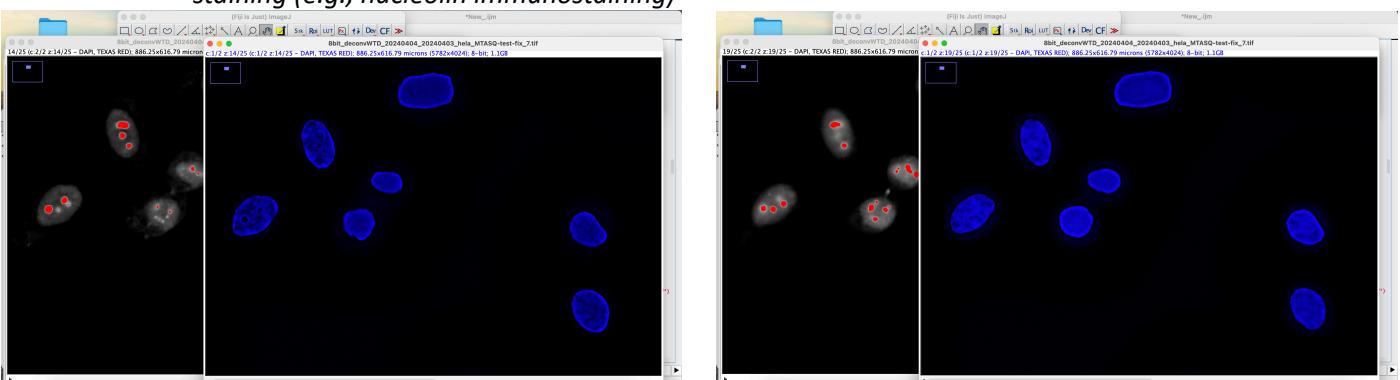
- Nucleoli may not be thresholded but holes will be filled by macro commands (lines 319-326)
- However, if threshold value is too high, holes could not be filled (intermodes threshold 85 (left-side of screenshot) vs 52 (right-side of screenshot)): in middle z-stack before “dilate-close-fill-erode” commands (left), after “dilate-close-fill-erode” commands in middle z-stack (center) and in low z-stack (right).



- Of note: if DAPI staining starts to be blurry, it is normal to have fragmented thresholded images as shown with threshold of 85; but it should happen with lower values of threshold.*
- Nuclei can be separated with watershed command (“ws2”)

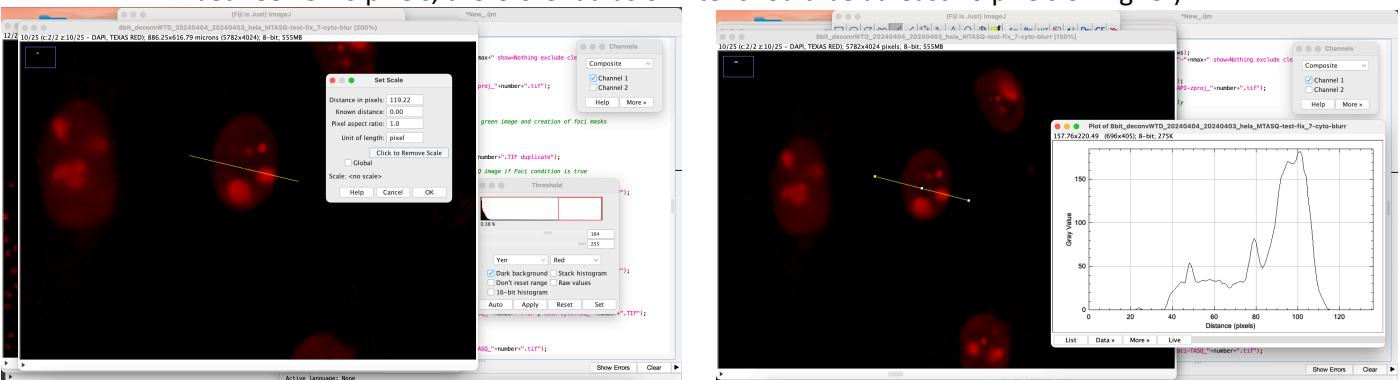
5. Thresholding nucleolus staining with TASQ image (LUT changed in grays) (lines 334-348):

- Should be done if **Intensity quantification** is done
- Thresholding (“nts”) should be done in parallel of DAPI staining to be sure nucleoli thresholding is accurate (some nucleoli are visible at z=14 (left) and not in z=19 (right))
- Of note: we would like to emphasize that nucleoli staining is more accurate if you use nucleoli specific staining (e.g., nucleolin immunostaining)*

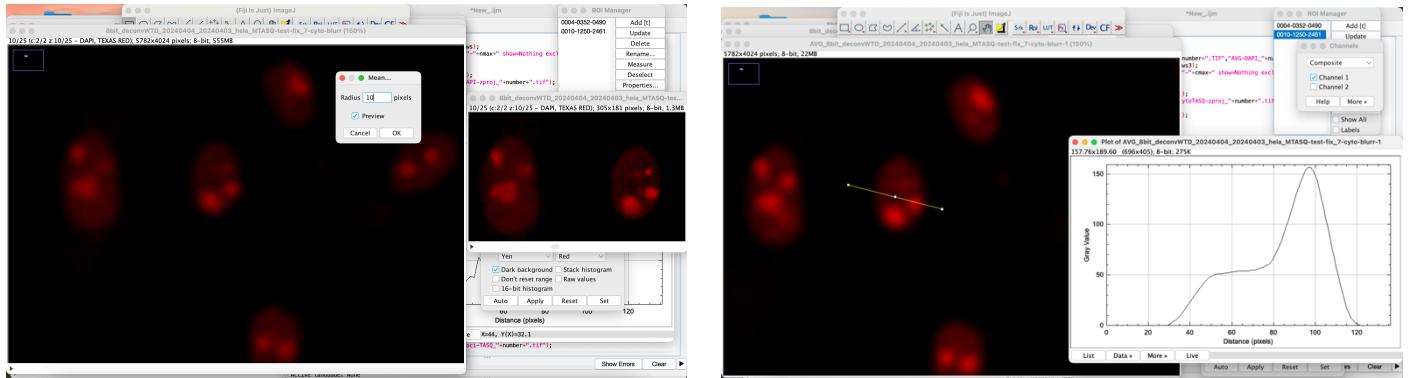


6. Thresholding cytoplasmic TASQ staining (for ROI delimitation: lines 351-365; for z-stack mask: lines 387-406):

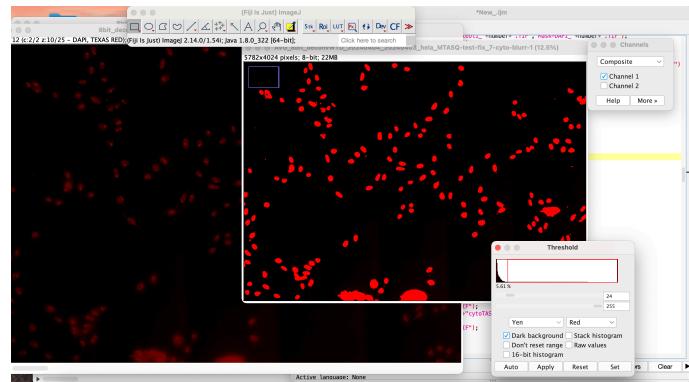
- Should be done if **Cytoplasmic (with Intensity and/or Foci) quantification** is done
- To create blur “mean” filter, you need to set radius (“r”, in pixel) bigger than objects of interest and scale need to be removed to know this size and gray values can be plotted (here small objects are between 5-10 pixels, therefore radius of filter should be at least 10 pixels or higher)



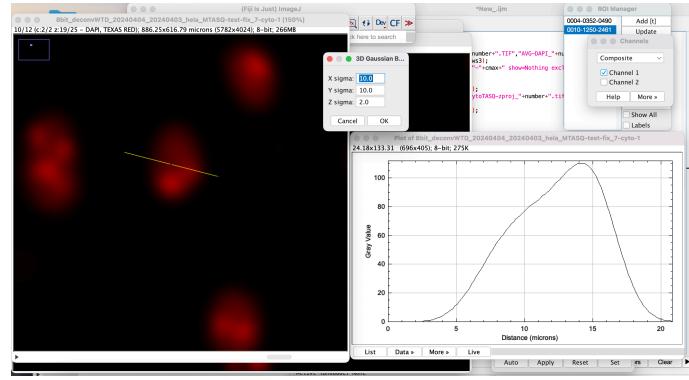
- Mean filter should create a “mask” of TASQ staining that can be also used to remove background and thus highlighting some small foci (see 8. Background removal). Average Z-projection of blurry mean TASQ is used to create ROI.



- But cytoplasmic thresholding to create ROI (“yts”) is not adapted to all staining; here staining is limited to nuclei.

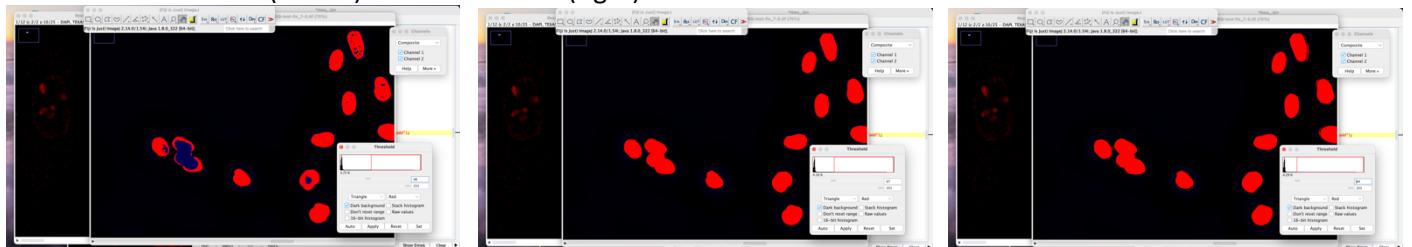


- If cytoplasmic quantification has to be done, same values of filter can be used for ROI determination and z-stack determination of cytoplasm with 3D gaussian blur (“xGB”) command (“r” value = “xGB” value) (lines 387-406). As for ROI determination, blur filters are used only to create a mask to delineate areas for quantification.

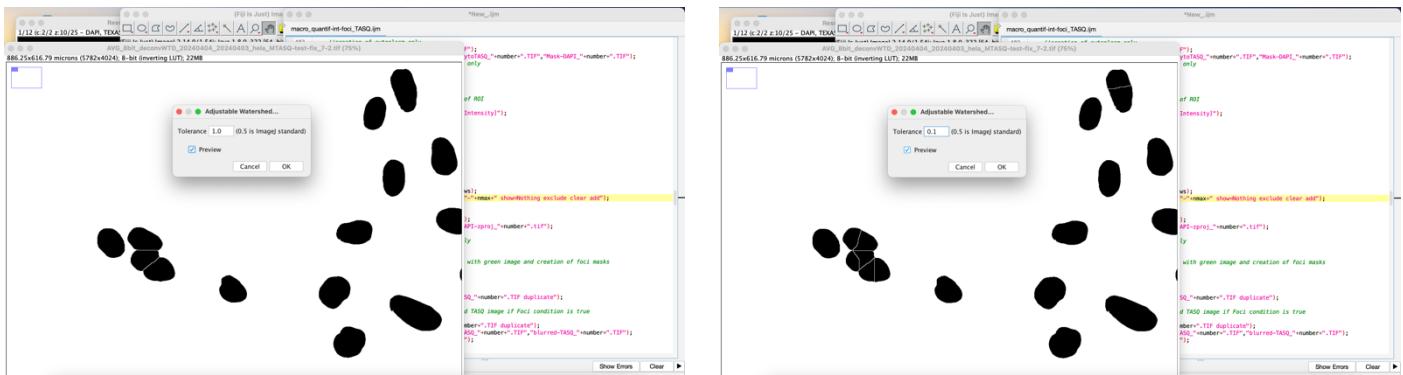


7. Thresholding nuclear DAPI staining (lines 365-378 or 409-431):

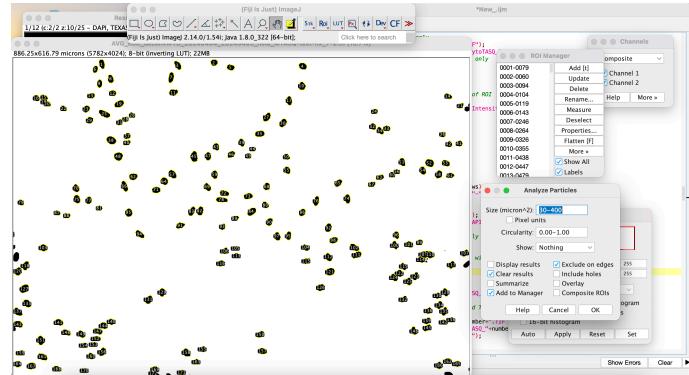
- Should be done if **Cytoplasmic (with Intensity and/or Foci) quantification** is done (lines 365-378) and if **Nuclear (with Intensity and/or Foci) quantification** is done (lines 409-431)
- After average projection, triangle threshold has to be well set (“dts”): too high value (left) vs correct value (center) vs too low value (right)



- After “close-fill-erode-dilate” commands, separation of nuclei could be needed with adjustable watershed (“ws”) with correct value (left) vs with incorrect value (right) depending on DAPI staining

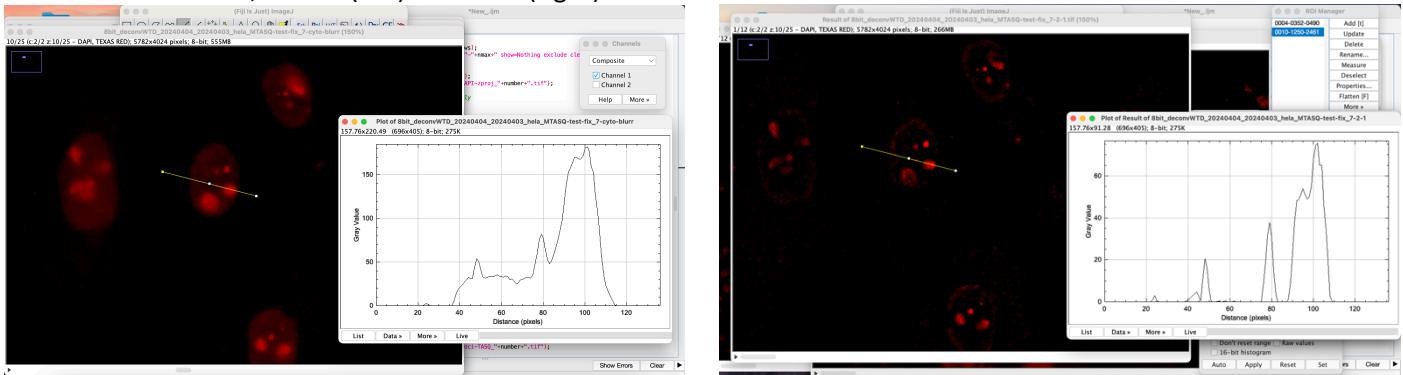


- Size of particles must be selected ("nmin" and "nmax") to obtain list of ROI in ROI manager

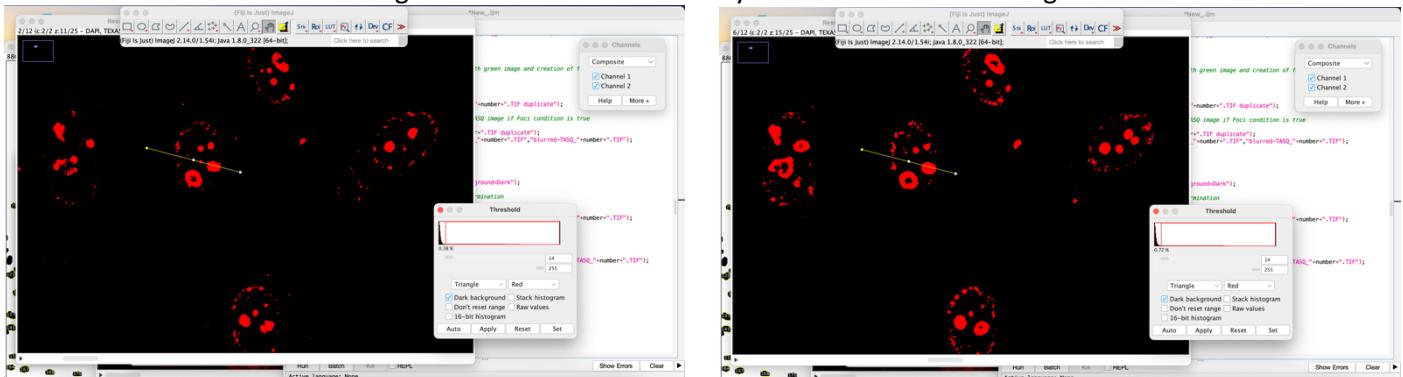


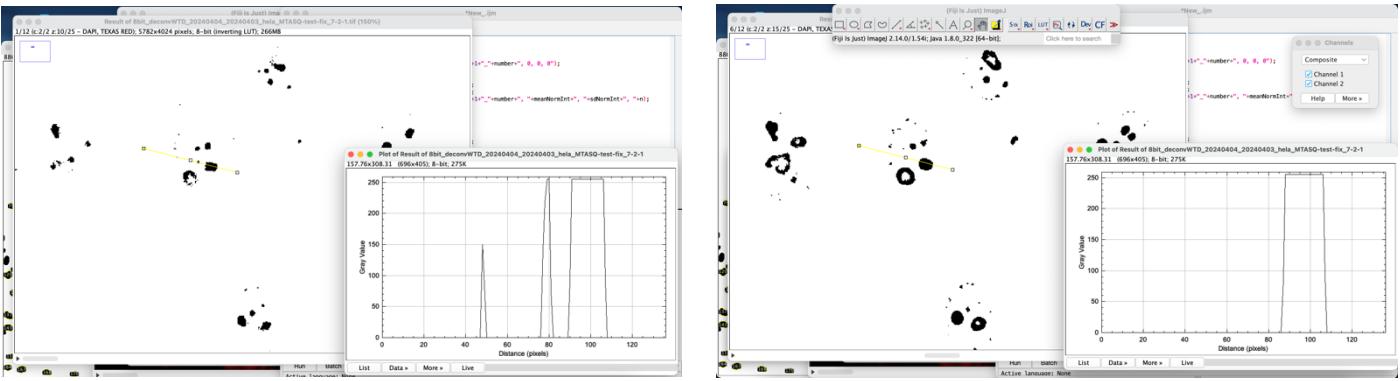
8. Background removal of TASQ staining (lines 434-468):

- Should be done if **Foci quantification** is done
- Used with mean filter mask (with image calculator; operation: subtract) increasing signal/background ratio, before (left) and after (right) subtraction



- Thresholding ("gts") must be carefully done throughout z-stack: low value of threshold (top images) can lead to poor segmentation of different foci; correct value of threshold (low images, in binary) allows correct segmentation of intense nearby foci but should be too high to avoid loss of faint foci.

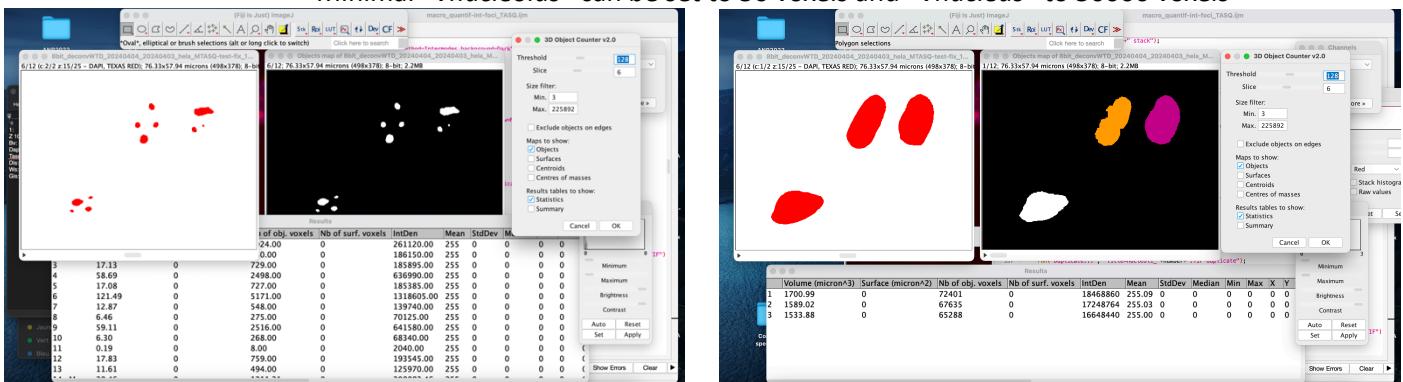




9. Loops to quantify TASQ staining (lines 475-645):

- **With fluorescence intensity quantification**
- To determine volume of macrostructures (nucleoli, nucleus, whole cell (in case of cytoplasmic quantification)), 3D objects counter plugin can be used with set of measurements “Nb of Obj. voxels” ticked.
- Results are presented as follows, on a restricted number of cells (3 cells analyzed as an example) : a/ on left, minimal value for nucleoli volume (with threshold “nts” previously determined) can be set: most of values vary between 268 and 5171 voxels (except one object with a volume of 8 voxels that can be considered as an outliers); b/ on right, minimal value for nuclei volume (with threshold “zts” previously determined) can be set: most of values vary between 65288 and 72401 voxels.

- Minimal “Vnucleolus” can be set to 50 voxels and “Vnucleus” to 50000 voxels



- **With foci quantification**

- Cut-off (“cot”) to discriminate small foci of TASQ and large foci of TASQ often correspond to value of minimal volume of nucleolus as nucleolus deciphering is based on intense and large TASQ staining.

10. Data analysis:

- Data are represented as follows for nucleus analysis:

Name: 8bit_deconvWTD_20240404_20240403_hela_MTASQ-test_fix_

Extension: .tif

Number of images analyzed: 2

Channels of cells: Channel DAPI: 1 Channel TASQ: 2

Size of nucleus: 30-400

z-DAPI Intermodes threshold: 52

z-DAPI adjustable watershed: 1

z-TASQ nucleoli Yen threshold: 149

AVG-projected DAPI Triangle threshold: 67

AVG-DAPI watershed #2: 1

Min. Voxel volume of nuclei: 5000

Min. Voxel volume of nucleoli: 150

radius of mean filter for TASQ background subtraction: 10

TASQ Triangle threshold for foci: 21

TASQ foci cut-off size: 150

Results for intensity analysis as follow: image analysed (nuclear, nucleolar or nuclear w/o nucleoli) #cell #img, TASQ-MeanInt, sdMeanInt, nb ROI analyzed

Results for foci analysis as follow: image analysed (small nuclear or large nuclear) #cell #img, foci-MeanVolume (in μm^3), sdMeanVol, nb of foci

lowest z for img #1: 10

highest z for img #1: 21

TASQ-background value for img #1_zone1: 27.2571

TASQ-background value for img #1_zone2: 34.3554

TASQ-background value for img #1_zone3: 46.7061

background value to be subtracted for img #1: 36

nuclear-TASQ-int_cell#1_1, 89.7809, 35.9863, 1
nucleolar-TASQ-int_cell#1_1, 188.439, 7.4755, 4
nuclear-wo-nucleolar-TASQ-int_cell#1_1, 81.9692, 22.0505, 1
small(\leq 150 voxels)-nuclear-foci-TASQ_cell#1_1, 0.4683, 0.7471, 15
large(>150 voxels)-nuclear-foci-TASQ_cell#1_1, 26.2382, 20.546, 5
(...)