Exercises Session 5 – Automating measurements in Fiji

Exercise 13) Try some filters… (5 mins).

Run the macros:

- FiltersDemov001.ijm
- FiltersDemov201.ijm

Includes Anisotropic diffusion. (***Plugins / process / Anisotropic diffusion 2d***)

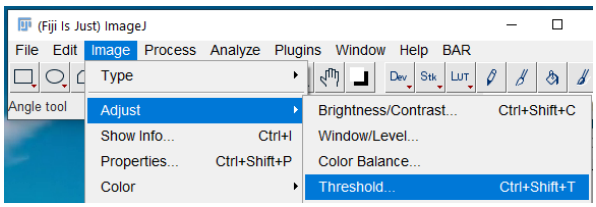
Removes noise with a Gaussian filter while also preserving edges.

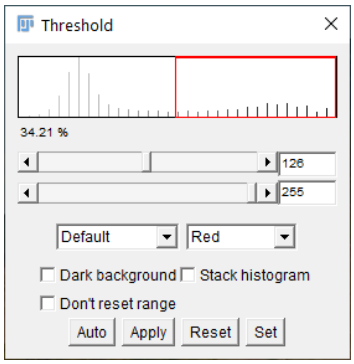
https://imagej.nih.gov/ij/plugins/anisotropic-diffusion-2d.html

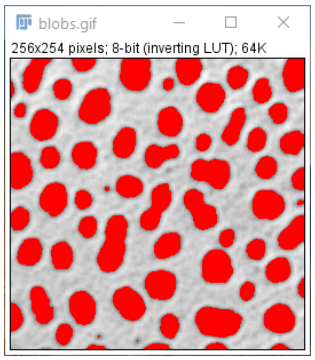
https://en.wikipedia.org/wiki/Anisotropic_diffusion

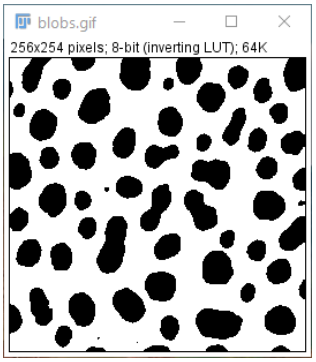
Exercise 14) Automatic counting Thresholding (20 mins)

- Open Blobs.gif
- Threshold (***Image / Adjust / Threshold***)
- Is your background Dark?
  Red shows what you are thresholding
- Press 'Apply' to generate a binary image.

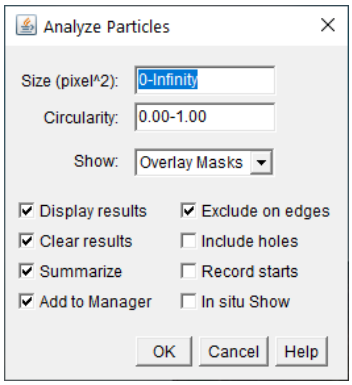


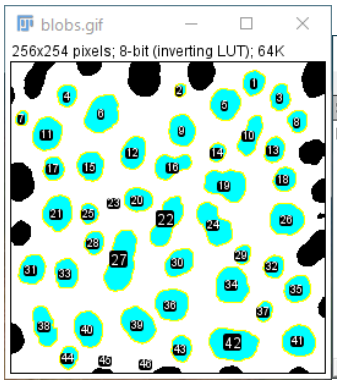


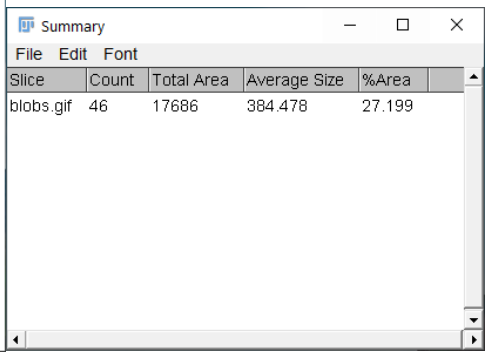




- Analyze particles (***Analyze / Analyze particles***) Set options, press 'OK'



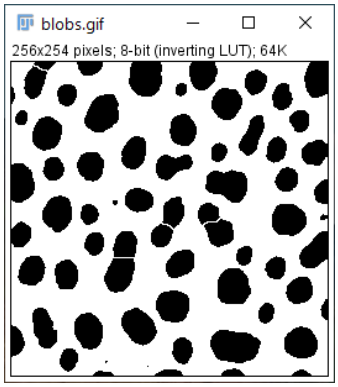


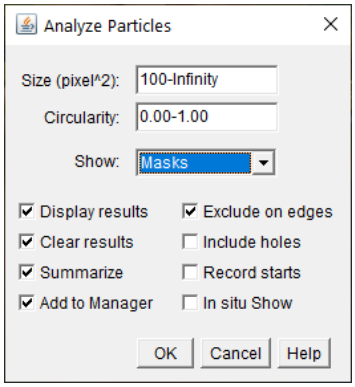


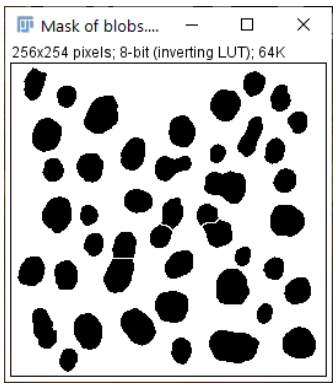
Some are clearly overlapping, should be counted twice.

Some are too small… Start again…

- After the threholding: ***Process / Binary / Watershed***
- In 'Analyze particles' set a minimum particle size.
- Show: **Masks** (And tick boxes as shown below)





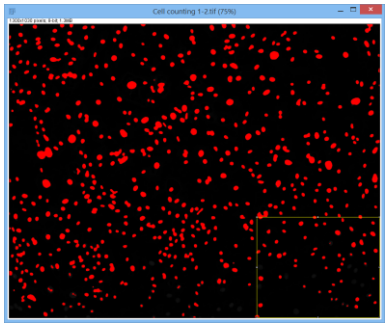


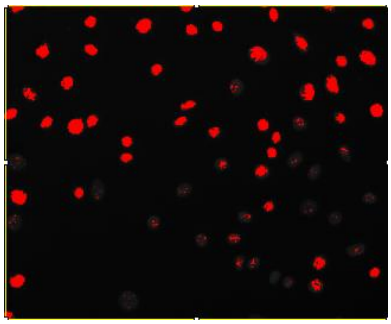
- Use the ROI manager to 'Show All' the ROIs on the original image.

Exercise 15) Counting real samples (thresholding, local threshold, find maxima) (15 mins)

Open image 'Cell Counting 1' (In folder '15-Counting & Segmentation). Duplicate it (good practice, so you aren't working on the original file).

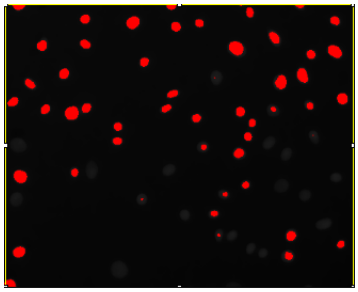
- Press 'Shift + T' (Threshold). (Don't press 'Apply', press 'Reset' to see the original image)





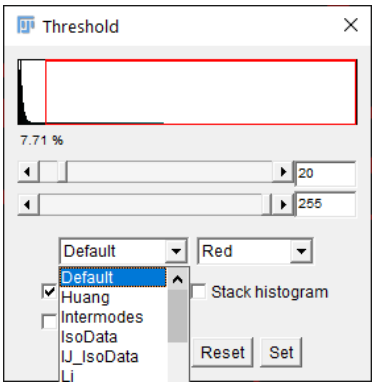
The image isn't evenly bright, so bottom right corner cells are missed.

- ***Process / Subtract Background*** (rolling ball radius 50), then
- ***Process / Filters / Median…*** (4 pixels – to remove the bright spots in the nuclei)
- How is the threshold now? (Press 'Auto' in the threshold window)

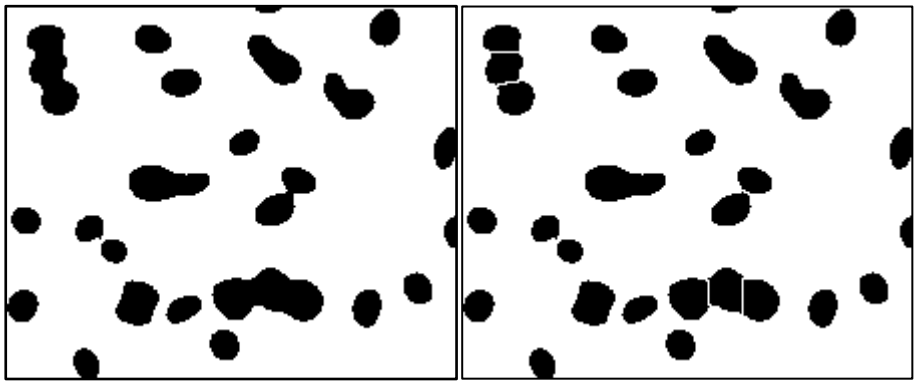


Fixed the spots but still missing some nuclei.

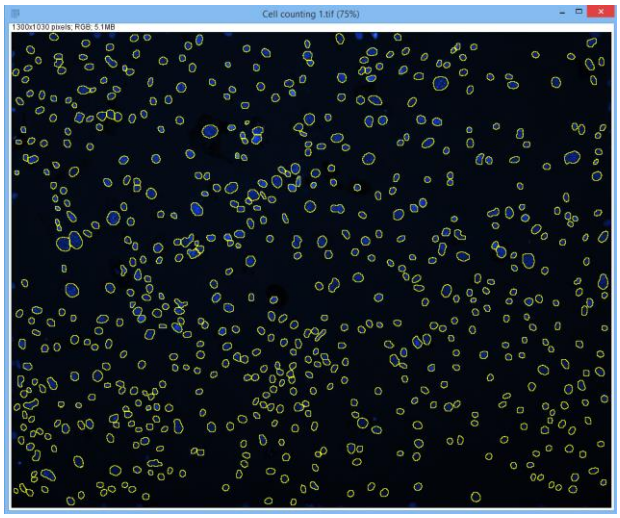
Try all the different threshold algorithms…

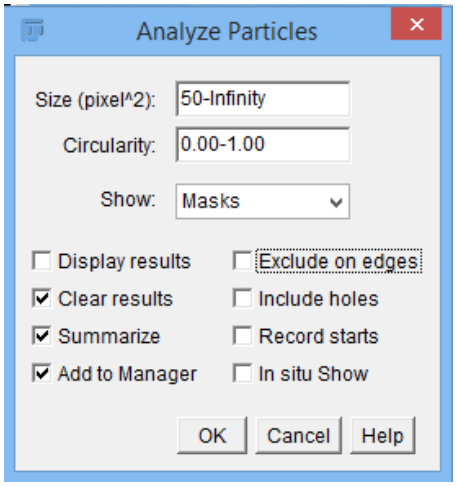


- Try different algorithms, I decided Triangle was best
- You can click the drop down menu, then use arrow keys on your keyboard.
- Watershed to split overlaps: ***Process / Binary / Watershed***



- finally, Analyze particles (choose to exclude edges or not)



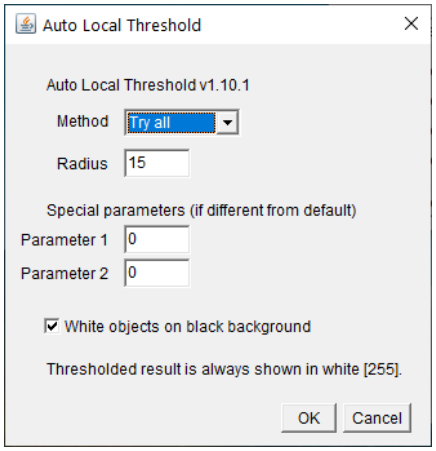


Counting with Find Maxima…

- On a duplicate of the original image, subtract background (20), Gaussian Filter (3) (WHY??).
- ***Process / Find Maxima…*** (try different values using preview)

Optional:

- You could also try Image / Adjust / AutoLocal Threshold…



I prefer not to use a local threshold.

Local threshold looks at the histogram of an image in small sections. So rather than setting one threshold for the entire image, it uses different thresholds in different regions. This may then correct for uneven background across an image.

However, I'd prefer to normalise the background, or enhance object contrast then apply a single threshold over an entire image.

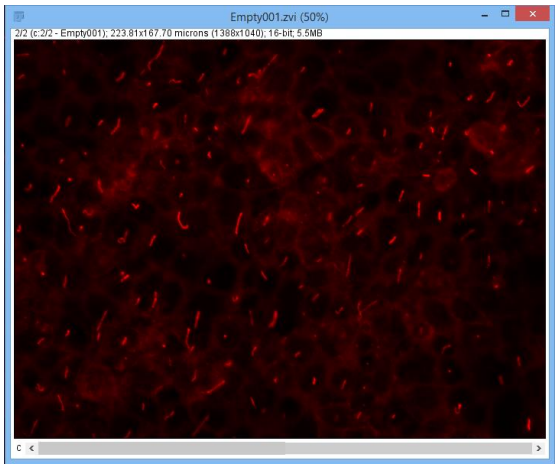
Exercise 16) Count cilia and measure lengths (20 minutes)

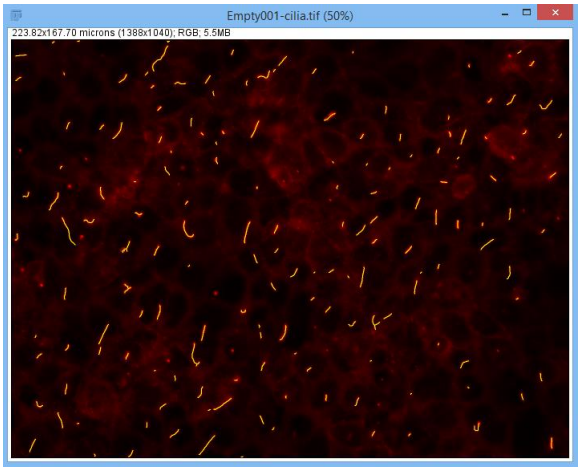
Cilia. Need to know how many and how long they are.

- Find maxima is no good (only counts them).
- Therefore, segment to a binary image then measure the lengths.

How??

- Image: Exercise images/ Cilia lengths. Choose one image.





Hints:

- Background correction.
- Filter (keeping edges).
- Threshold to get a binary (black and white) image.
- Binary Dilate to join up cilia pieces that may have split in two.
- Binary – Skelotonize, makes the cilia 1 pixel wide.
- Measure the skeletons. Length isn't an option, so perimeter divided by 2 will be the length.
- Add results to ROI manager. Use the ROI manager to overlay the identified cilia on the original image.

Once you've done this try the macro: Cilia2Dlengths.ijm (drag to ImageJ, press RUN).

Alternative Approaches

As we are trying to segment objects with distinctive properties, we could use the shape of the objects rather than just the intensity to threshold them. You could try the following plugins:

- Tubeness (***Plugins / Analyse / Tubeness***) https://imagej.net/Tubeness based on the original version: https://www.longair.net/edinburgh/imagej/tubeness/

- Ridge detection (Plugins / Ridge detection) https://imagej.net/Ridge_Detection Requires the Biomedgroup update site to be selected, the plugin is then automatically installed.

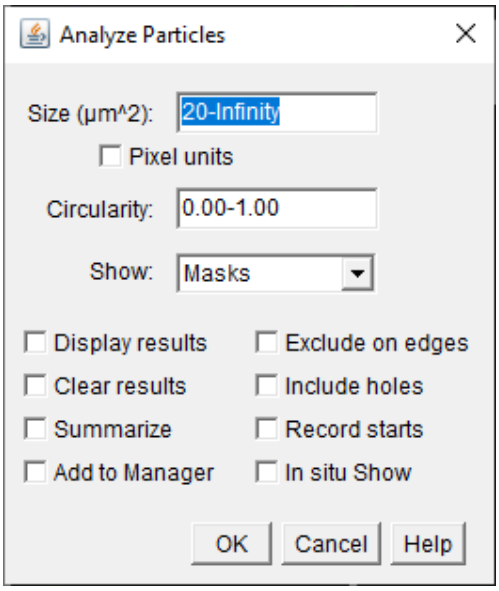
Exercise 17) Identifying double stained cells (20 minutes).

Often you need to measure cells that are positive for a more than one stain.

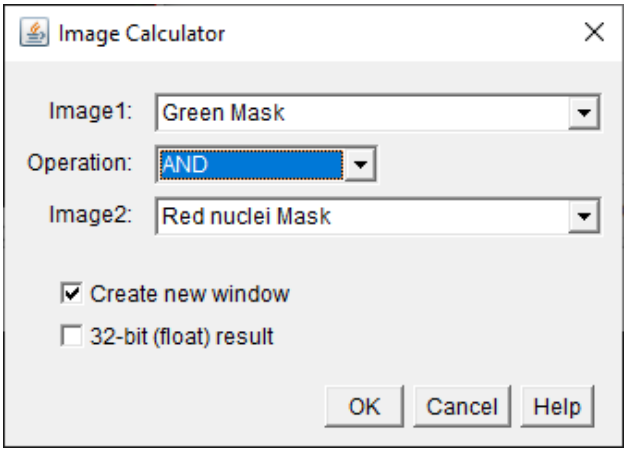
Use thresholding to make a binary image of each channel, then use 'Image Calculator' to add or subtract images from each other to identify overlaps.

File: Exercise Images / 17 Double Stained cells/ Dapi_GreenCytoplasm_RedNuclei.lsm

Procedure:



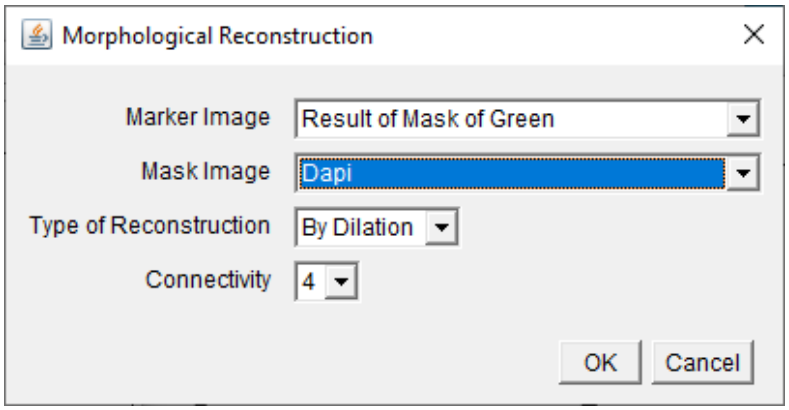
- ***Image / Color / Split channels***, and threshold each channel:
- C1-Dapi: Median filter (3). Threshold Triangle. Watershed. Analyze particles (20-Infinity – remove small objects) Show Masks. (Untick all other boxes)
- Green: Median(2). Threshold Default. Analyze particles (50-Infinity) Show Masks.
- Red: As for Dapi.
- Image Calculator: Combine 2 binary images, using AND



Optional extras:

How to make the output overlaps the size of the nuclei:

- You need to ***Help /update…*** and 'Manage Update sites'
- Select IJPB-Plugins & restart Fiji.
- ***Plugins/ MorpholibJ / Morphological reconstruction***:



Marker Image – starting points

Mask Image – the image that the markers grow into

You could also try and make a final image that has 3 original channels, and each mask as a new channel. (Split channels, then merge channels, choose all the relevant images).