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Procedure for segmenting grayscale images:

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1. Open ImageJ
2. Once in ImageJ with the images to be segmented open, press “cntrl o” and select the frame to be manipulated. One can also press: File, then Open…
3. Click on “Image” then roll over “Type” then click on “RGB Color”. The convert
4. Double click on Color Picker to open the Color Pallet.
5. Select either black (0,0,0) or yellow (255,255,0) or any other color as long as the same color is used to outline each cell.
6. Click on “Image” then click on “Adjust” and click on “Brightness and Contrast”.
7. Adjust the brightness of the frame so that a fair amount of grayscale is visible. Then in the B&C box click “Apply”.
8. Select Paintbrush Tool by double clicking it. This will not only switch to paint brush but it will allow you to change the thickness of the brush as well. Adjust brush thickness to 1 pixel.
9. Use zoom in and zoom out to manipulate the frame. This can be accomplished by pressing the magnifying glass and then by left clicking to zoom in and right clicking to zoom out.
10. In order to trace the cells well, zoom in really far in on the edge of the cell. Trace the cell by having the brush edge paint over the very edge of the cell.
11. Save the frame by clicking “File”, “Save as”, “.tif” then choose where to save it.
12. To make a mask, you must first duplicate your images. To do this, click on “Image”, then click “Duplicate”, and check the box that says “Duplicate stack”.
13. Go to “Image”, then “Type”, and select “8- Bit”.
14. Next, click on “Image”, then click “Adjust”, then click “Threshold”.
15. In the first dropdown menu, make sure that “Default” is selected and in the second dropdown menu, make sure that “Red” is selected.
16. Adjust the two scrollbars until you see a red outline around each cell with as little red within the cell as possible. Click on apply.
17. Next click on “Process”, then click “Binary”, then click “Fill holes”. (NOTE: Real interior holes will be lost)
18. Finally, if necessary, click on “Analyze”, then on “Analyze particles” to remove any small objects that are not cells. You will have to change the setting to 100-infinity and you will need to select “Show Masks” in the dropdown menu. Save these images.

    Since steps 16 through 18 result in a mask where the interior holes are removeds follow the steps below to create a masks with interior holes intact (after performing step 16 above).

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