**Reference:**

If you use this code, please cite the following reference to give proper credits.

Elaheh Alizadeh, Wenlong Xu, Jordan Castle, Jacqueline Foss, Ashok Prasad. A comprehensive suite of measures to quantify cell shape and actin organization. doi: https://doi.org/10.1101/372755

**Software requirements:**

1. Matlab installed with image processing toolbox
2. Optional: (an implementation using only Matlab is also provided)

Download the mij.jar and ij.jar from

<http://bigwww.epfl.ch/sage/soft/mij/>

Install it by putting them into the into the java directory of Matlab (e.g for Window Machine 'C:\Program Files\MATLAB\R2009b\java\jar').

1. Install the CellProfiler which can be downloaded from <http://cellprofiler.org/>

**Image Processing Protocol**

**Step 1 – Thresholding using MIJ (Matlab Interface with ImageJ)**

1. Before modifying and running the Matlab code, enter the following 3 lines into the Matlab command line window:

javaaddpath('C:\Program Files\MATLAB\R2016b\java\jar\mij.jar');

javaaddpath('C:\Program Files\MATLAB\R2016b\java\jar\ij.jar');

MIJ.start

Note: 1) The path to the java directory of Matlab may need to be modified accordingly.

2) After running these 3 lines, a ImageJ window should pop out.

1. Open the Matlab code named as “Auto\_ImageJ\_Threshold.m” in the folder “Step1” and make changes to the inputs as the first several lines in the code.
2. WorkingDir (working directory) as the path to the input folder. In the template files provided, it is “C:\Users\xuwl\Desktop\DLM8\_GDA\_TIFF\”. Make sure it is ended with “\”.
3. SpecStr (specification string) as the tag used for the images. In the template files provided, it is “DLM8\_GDA\_\*”. Make sure it is ended with “\*\_”.
4. OutputDir (Output directory) as the path to the output folder. In the template files provided, it is same as the WorkingDir (working directory). It can be specified to other path as well.
5. Output\_Folder into some name making sense.
6. Bitdepth as the bit depth of the input images. For gray-scale image, it is usually either 8-bit or 16-bit. You can find this out in the image properties. For the images used in this template, it is 8-bit.
7. Nuc\_affix as file affix for nuclear channel. This is the end of the corresponding image file name. For the images in the template, the image for nuclear channel is ended with “\_c1.TIF”.
8. Actin\_affix, same as the Nuc\_affix but it is for actin.
9. Now, you are ready to run the code. Note that every time after you finished tresholding, the program is paused (for better controlling purpose). You will need to go back to the Matlab main window and press any button to continue. Closing the ImageJ thresholding window is OK but not necessary.
10. After you are done with the thresholding using ImageJ, close the ImageJ in Matlab by typing

MIJ.exit

In the command line.

**Step 1 – Thresholding using only Matlab**

1. Open the Matlab code named as “Auto\_Matlab\_Threshold.m” in the folder “Step1” and make changes to the inputs as the first several lines in the code. Follow the same instructions as in the step 2 in the instructions for MIJ. The rest of steps should be self-instructive in the code.

**Step 2 – Declumping (separating the cells in clusters) using CellProfiler**

1. Open the CellProfiler by double click the “Template.cpproj” in the folder “Step2”.
2. In the leftmost panel in the CellProfiler main window, look for the Pipeline-Input modules-Images. Drag the folder(s) into the designated location.
3. In the Pipeline-Input modules-NamesAndTypes, type in the affix for Nuclei channel just above “OrigBlue”. The default is “NucInt.tif” and it should be fine for most cases. So is for OrigGreen and its corresponding affix “ActinInt.tif”. You can double check these two inputs by clicking “Update button”. If right, a list of files will appear in the list.
4. Click “Analyze Images” and wait…

**Step 3 – Visual check the declumping results from Step 2**

This is necessary because there are some cells with only nuclei left (dead cells) and if not removed by hand they will enter the final results. Also CellProfiler sometimes does funny stuffs.

1. Open the code named as “AfterCellProfiler2.m” in the folder “Step3”;
2. Change the WorkingDir and SpecStr accordingly. The WorkingDir here is different from the one in Step 1 and the WorkingDir here is actually the output folder in Step 1. SpecStr should be the same as in Step 1.
3. Follow the pop out windows to carry out the visual check.

Module 1: “Do you want to modify the cell masks?”

“Yes” – goes to Module 2 to carry out one of the operations;

“No” – Does no operation and goes to the next image;

Module 2: After selecting “Yes”, there are several operations can be done:

1. Remove/Combine
2. First click the cells as you wish to either remove from further analysis or combine into one cell.
3. After finish clicking, hit ENTER to choose these cells.
4. Then choose either Remove or Combine.
5. Adjust – allows fine-tune of the cell-cell boundary
6. First choose the TWO (only the case for two cells are coded now, may be extended to any number of cells in the future) cells you want to adjust the boundary by clicking.
7. Similarly, make the choice final by hitting ENTER.
8. Then, in a new popped out window showing only these two cells you want adjust, draw the new boundary by clicking around.
9. After done, hit ENTER.
10. A new window showing the newly cut cells with indexes will pop out. If the result is expected, you can choose “Yes” in the window asking “Do you have XX Cells?”. If not, you can choose either “No” to make changes based on current result, or “Start Over” to make the adjustment right from the beginning.
11. Cut – Remove the area you do not want from further analysis

Draw a shape by clicking around as you wish and hit ENTER to select. This will remove everything inside the shape in all channels. Then you will be asked “Are you satisfied with the cutting?” and three similar options as in “Adjust” which does similar things.

Module 3: “Are you satisfied with the operation?”

“Yes” – finalizes this operation and goes back to Module 1;

“No” – reverts this operation and do the operation again;

“Start over” – reverts all operations done so far and start working on the original image again.