# Purpose:

Use STARDIST plugin for FIJI/ImageJ to segment and then count cells from microscopy. Commonly used for c-fos counting.

# Resources:

<https://imagej.net/plugins/stardist>

<https://github.com/stardist/stardist>

# Installation

Head to <https://imagej.net/plugins/stardist> and follow the directions for installation. This expects that you already have FIJI/ImageJ downloaded and updated. Use the following links if you need to [download](https://imagej.net/software/fiji/downloads) or [update](https://imagej.net/plugins/updater) FIJI/ImageJ.

# Workflow Overview

1. Convert your images to 8-bit grayscale .tif files and pre-process your images.
2. Hand score a semi-random sample of about 10% of your dataset.
   1. Get a lot of variety in this sample.
3. Calibrate STARDIST and Particle Analysis/post-processing to your signal using 4 or 5 images
   1. Use more if you have 200-300+ images
4. Run a test batch on the hand-scored sample then compare the results to your hand scoring
5. Check your correlation between hand and machine-scored and input/output images when there are discrepancies.
6. Recalibrate and repeat as needed until you have a pretty good correlation
   1. ~.97 is what I was able to relatively easily achieve with good signal.

# STARDIST Processing

## Step 1 - Image Conversion and Pre-processing

1. Your input images should be grayscale images in .tif format.
   1. When you open an image in FIJI, it may say “RGB” 
   2. If the image is already grayscale but showing RGB, the conversion step will be carried out automatically by running the “STARDIST Step1 - Preprocessing.ijm” macro in the following steps.
   3. If the image is NOT grayscale and shows RGB (i.e.: you have any color at all) you have additional steps. You must first split off your signal channel using the “STARDIST Step0 - Split Color Channels.ijm” macro
   4. Once you have run this macro, C1 (channel 1; the red channel) will go into a folder called RED, C2 into a folder called GREEN, and C3 into a folder called BLUE. These are your individual color channels. Choose the channel with the most obvious signal in it (e.g. RED for td-tomato) as your input folder.
2. Open FIJI and in the top bar go to File > Open. Navigate to the folder you unzipped the macros into and open “STARDIST Step1 - Preprocessing.ijm”
3. Hit the Run button
4. Fill out the prompts that appear in the dialog box. Leave Batch Process Mode checked.
   1. If you don’t want to downsample, set the downsample % to 100.
5. Downsampling is recommended for larger images (1,500+ x 1,500+) to increase speed
6. The images output from this step will be the input for the next step.

## Step 2 - STARDIST Model Application

### Calibration

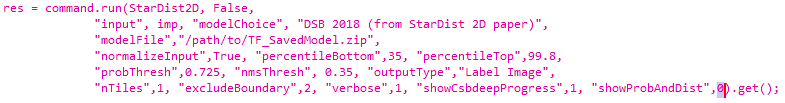
1. Take 4 or 5 of your preprocessed images and copy them into their own folder called “Calibration IMGs”
   1. Use a few more images for calibration if you have 200-300+ images
   2. Be sure to get some variety in there. Including images that are examples of your best and worst signal is advisable.
2. Open one image in FIJI (ensure no other image windows are open)
3. Go to the top menu Plugins > and scroll down until you see StarDist > StarDist 2D
4. Follow the prompts and use the directions found [here](https://imagej.net/plugins/stardist) to understand what they mean
5. Select “Label Image” for your output.
6. Hit OK and a few images will pop up. Wait until the label image comes up (should be the 3rd or 4th image generated.
7. Overlay your label image and your preprocessed image and see if it is parsing out the cell blobs or not and adjust from there.
8. Play with these until you get label images that do a pretty good job highlighting individual cells. The easiest way to understand these settings is to play with them using large jumps in value and then compare the resulting images.
   1. Note: STARDIST will highlight other things too that have high fluorescence like dust and edge fluorescence and weird stuff that is too big or too small to be a cell. Don’t worry. We will deal with these artifacts by area size in Step 3 later.
9. Record your calibration values. In the top bar go to Plugins > Macros > Record… and the Recorder window will pop up.
10. Run STARDIST again with the values that worked best for your images. These values will be recorded in the recorder and look something like this:

run("Command From Macro", "command=[de.csbdresden.stardist.StarDist2D], args=['input':'Preprocessed C1-SD 1 LS.tif', 'modelChoice':'DSB 2018 (from StarDist 2D paper)', 'normalizeInput':'true', 'percentileBottom':'35.0', 'percentileTop':'99.8', 'probThresh':'0.725', 'nmsThresh':'0.35', 'outputType':'Label Image', 'nTiles':'1', 'excludeBoundary':'2', 'roiPosition':'Automatic', 'verbose':'true', 'showCsbdeepProgress':'true', 'showProbAndDist':'false'], process=[false]");

1. The highlighted chunk of text above are your stardust settings. You will need these for the next step but you cannot just copy and paste the whole chunk of code.

### Batch Process

1. Open the “STARDIST Step2 - STARDIST Model Processing.py” script in FIJI.
2. Change the settings that make up the res variable starting with the second line so they match each of the settings you calibrated.
   1. For example, to match the above settings, it should look like this:



1. Scroll down to where the input directory (indir) and the output directory (ourdir) are defined (approx. lines 22 & 23). Change the input and output directories accordingly.
   1. Your input directory should be the folder containing your preprocessed files.
   2. Your output directory is where you will save your label images (images with each cell delineated as a single color/shade of gray).



* 1. NOTE: label images that output will appear completely black or white if you open them with a standard image viewer. Use FIJI to open them and you will see individual cells labeled.

1. Hit the Run button.
2. It may look like nothing is happening, esp. if you have larger images. Don’t worry, it is working (unless you get an error). If you want to track progress, open your output folder and see if label images are being saved there. If you dont see any after about 5-10 min, there may be a problem. But again, larger images = more processing time.
3. Overlay the preprocessed image and the label image to check to see that your labeled files have blobs that are primarily cells of interest. If this isn’t the case, perform additional calibration using some new images.
   1. If there are some small or large artifacts, don’t worry. We take care of that in the next step.

## Step 3 - Post-processing and Cell Counting

1. In the top bar go to File > Open. Navigate to the folder you unzipped the macros into and open “STARDIST Step3 - Postprocessing.ijm”
2. Hit the Run button
3. Your first time through, fill out the prompts that appear in the dialog box…
   1. **Initial Label Image Path:** The first time through,you are checking to see if your hand-counted counts match the machine-counted counts. **Copy** (do not cut and paste) the label images from the previous step for these files into a separate temporary folder. Use this as your input folder initially.
   2. **Label Image Path:** Once you have checked your settings and are happy with how well your counts align, your input folder should be the folder with all the label image files within. Once you have the data you need from it copied elsewhere, you can safely discard the temporary folder and its contents.
   3. **Output Path:** Your output folder is where you will save a .csv file with each image’s name and count included along with some other variables. As you are testing, you may want to use your input folder as your output as well. You will get rid of the temporary folder once you have confirmed that the images align and the output .csv will go with it.
   4. **Project Abbreviation:** for your first time through using just the images that correspond to your hand scored images, use “TEST” as the project abbreviation.
   5. **Batch Process Mode:** Checked
   6. **Minimum & Maximum Cell Area…:** These need to be adjusted for each project. You can measure the size of a few of your largest and smallest cells and get the area by using the magic wand selection tool , selecting a cell, and then hit ctrl + M to measure. It is better to allow the high end to be a bit too large. Make sure you leave some margin for variability in cell sizes that you might not see in this sample.
      1. NOTE: This also assumes all your images are the same size and zoom and the area of cells should fall within a range. If you want to count larger and smaller cells and the smaller cells look similar to noise that STARDIST picks up, you may not be able to effectively count the smaller cells without first dealing with that noise.
4. Copy your results from the .csv over to your datasheet, correlate, and compare.
5. Fiddle with the minimum and maximum area until you start to match pretty well with the hand counts by repeating from Step 3.2 above. This is especially true if you have lots of little artifacts that appear in the label image.
6. Once everything aligns, correlation data is recorded, and min/max settings are documented, discard your temporary folder.
7. Run the “STARDIST Step3 - Postprocessing.ijm” macro again.
   1. **Label Image Path:** Your input folder should be the folder with all the label image files within.
   2. **Output Path:** Your output folder is where you will save a .csv file with each image’s name and count included along with some other variables.
   3. **Project Abbreviation:** Whatever abbreviation you use for your project. This will be a prefix on the name of the output dataset. e.g. : “XX00”, “TW01”
   4. **Batch Process Mode:** Checked
   5. **Minimum & Maximum Cell Area…:** These need to be adjusted for each project. You can measure the size of a few of your largest and smallest cells and get the area by using the magic wand selection tool , selecting a cell, and then hit ctrl + M to measure. It is better to allow the high end to be a bit too large. Make sure you leave some margin for variability in cell sizes that you might not see in this sample.
      1. NOTE: This also assumes all your images are the same size and zoom and the area of cells should fall within a range. If you want to count larger and smaller cells and the smaller cells look similar to noise that STARDIST picks up, you may not be able to effectively count the smaller cells without first dealing with that noise.
8. As before, copy your results from the .csv over to your datasheet. You have your cell counts!