FluxNorm User Manual

Software Installation:

Installation of Fiji

1. Follow the link below and click download for the system you are using.
   Fiji Site: https://imagej.net/Fiji/Downloads
2. Follow the installation instructions on the site. It should be as easy as opening the file for Fiji.

Installation of Python and Jupyter(Anaconda)

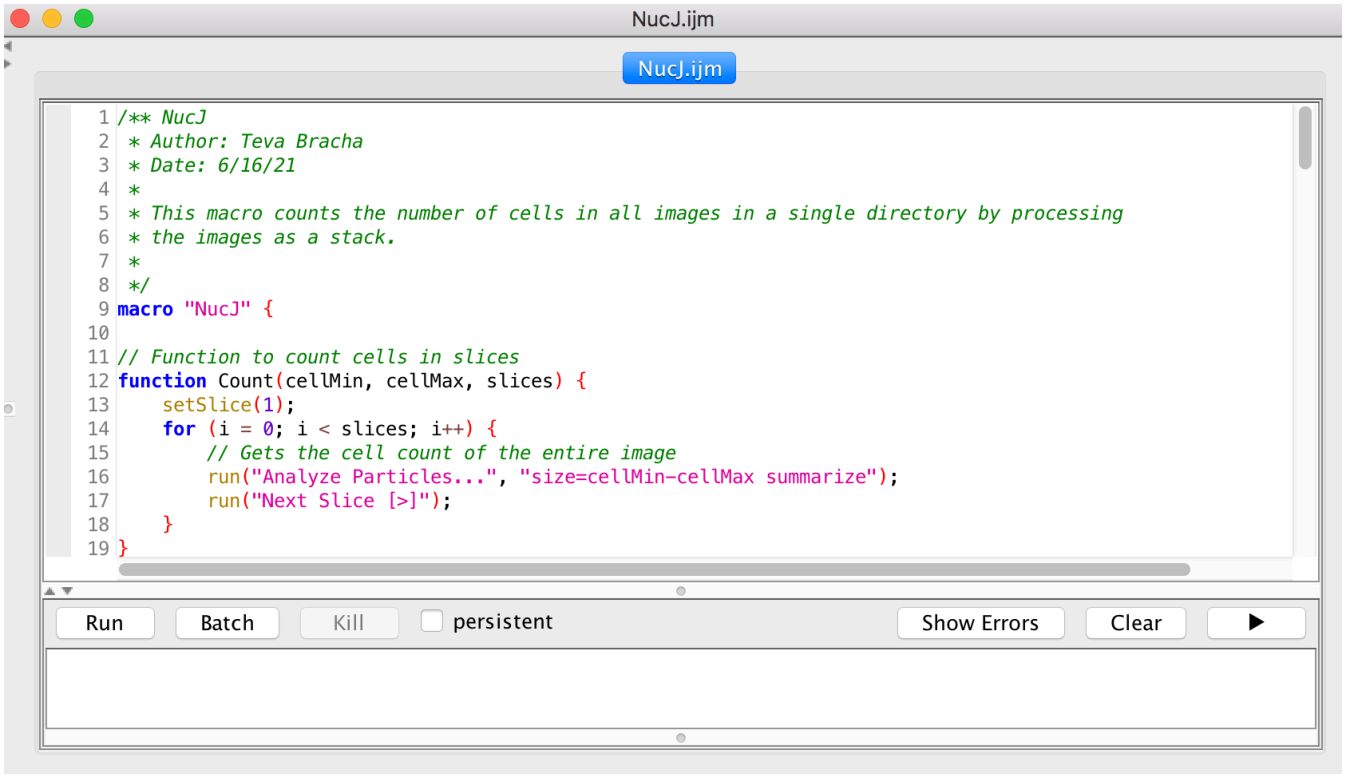
1. Use the anaconda installer to download both Python and Jupyter: https://www.anaconda.com/download/
2. As the python code is written in 3.6, choose to download the installer for Python 3.6.
3. If you are unfamiliar with the command line, install the anaconda Graphical Installer. You can do so simply by clicking the large green download button.
4. If you are somewhat familiar with the command line, you can download the command line version by clicking the smaller green Command Line installer link below the Graphical installer.
5. Follow the link: https://docs.anaconda.com/anaconda/install/, then choose the installation instructions for the Operating system you are using. Follow the instructions to install Anaconda.

Overview

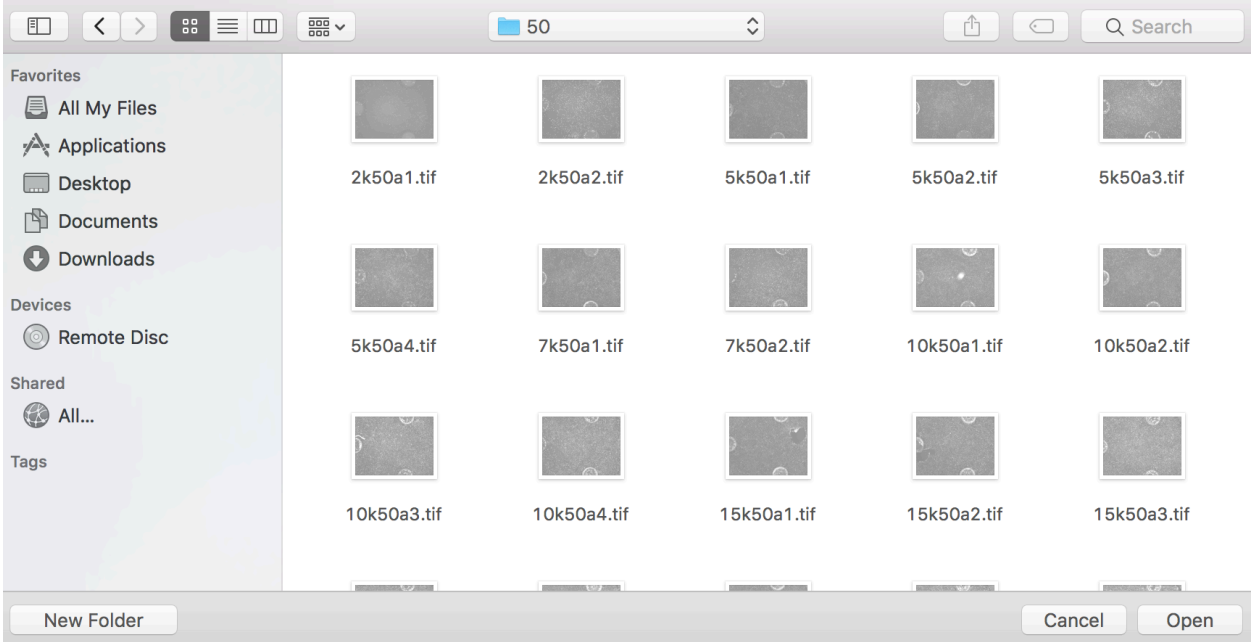
FluxNorm is a two step process using NucJ and ExtraPy to generate representative counts to normalize data from metabolic flux analysis. NucJ is semi-automated ImageJ macro which generates cell counts from nuclei stained images. ExtraPy is a python script which takes the counts obtained from NucJ and extrapolates them to represent the entire well. The first time a user runs through FluxNorm, it is recommended to perform an additional calibration step to ensure accurate counting for the cell type of interest. The end result should be quick, consistent, and accurate normalization of metabolic flux data.

NucJ Walkthrough

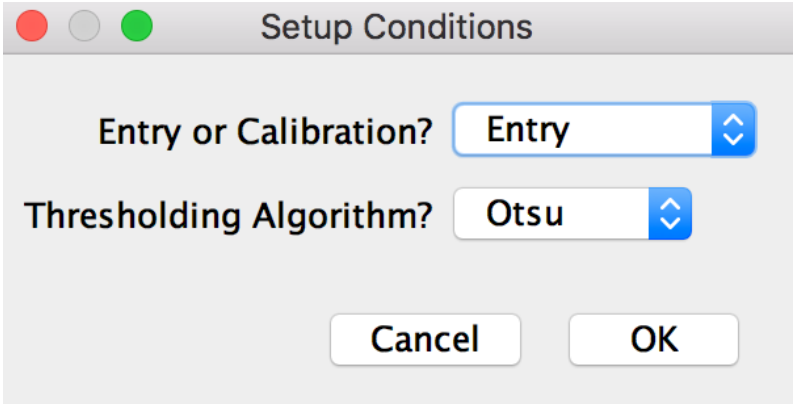
1. Make sure all the images you are counting are in a single directory with no other files in it.
2. Open “NucJ.ijm.” This can be done by dragging the program file into the Fiji bar.
3. Click the “Run” button in the macro window.



4. The program will open up a window. Navigate to the directory with the desired images in it and click “Open.”



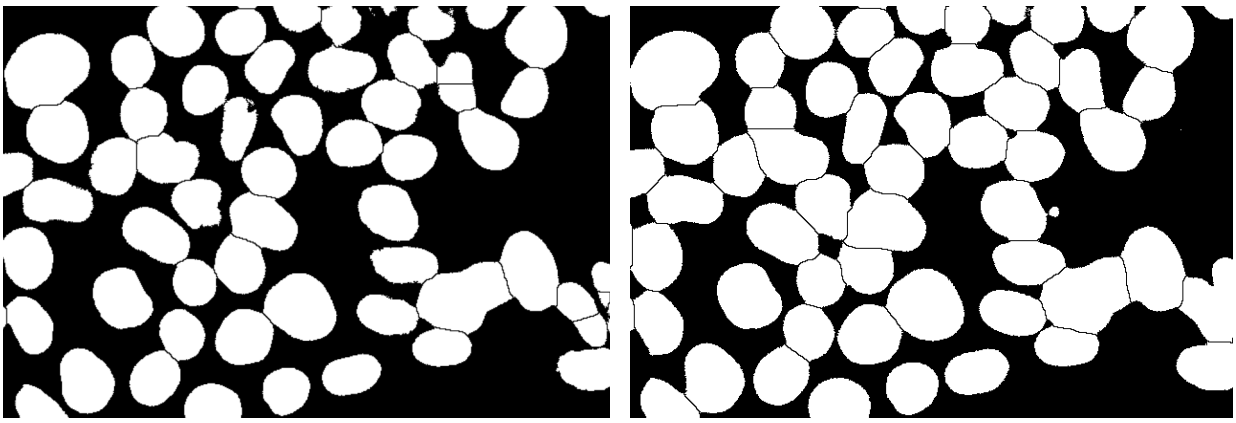
5. A dialog will appear that asks you to adjust the programs runtime parameters. Select the methods for thresholding, cell size cutoff determination, and ROI generation and whether or not to perform an alignment between all the images. We will go through each parameter individually.



Thresholding

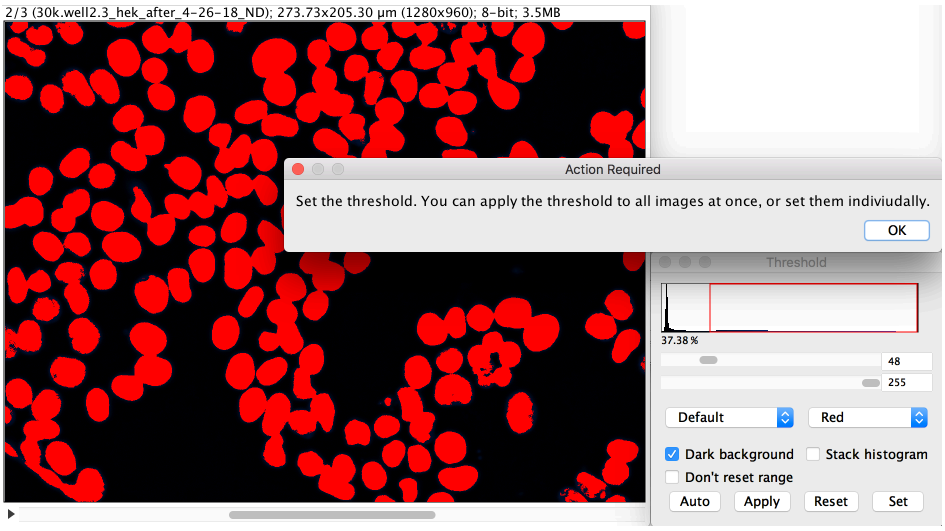
This parameter is to determine which automatic thresholding algorithm you would like to use for your images. Otsu and Huang are the two default thresholding algorithm, but you can also manually threshold your images.

6. For most images we recommend thresholding through “Otsu.” However, if your images are poorly lit, and Otsu thresholding is unable to separate the cells from the background, you can try “Huang” for an alternative automatic thresholding method or adjust the thresholding by hand using “Manual.”



Otsu Huang

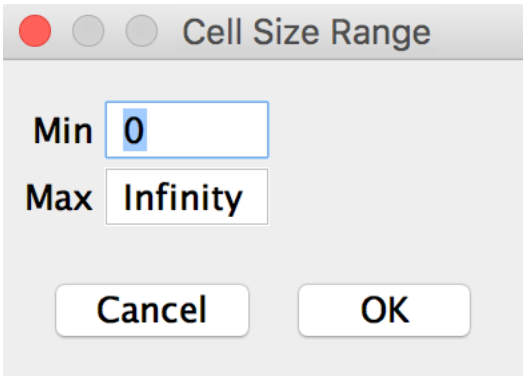
7. If you chose “Otsu" or “Huang” the program will automatically threshold the image using one of the given algorithms. If you chose “Manual,” a dialog will pop up telling you to threshold the images. Threshold the images manually and when finished press “OK”



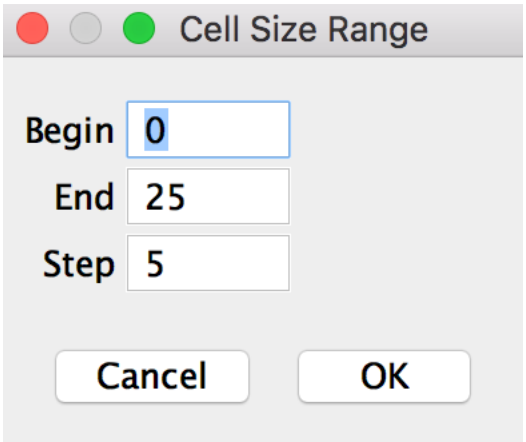
Cell Size Cutoff Determination

For NucJ to accurately count the cells, it must know at what size cutoff to consider a particle a cell. Determining this cutoff is crucial for generating accurate cell counts. When working with a new cell line or new imaging settings, it is important to perform a manual calibration to establish the cell size cutoff for these cells.

8. If you choose *"Entry"* the program will open a dialog with text boxes for the minimum and maximum cell size. Usually you will only need to enter the minimum, but if for some reason you wish to exclude particles greater than some size, that can be done as well.

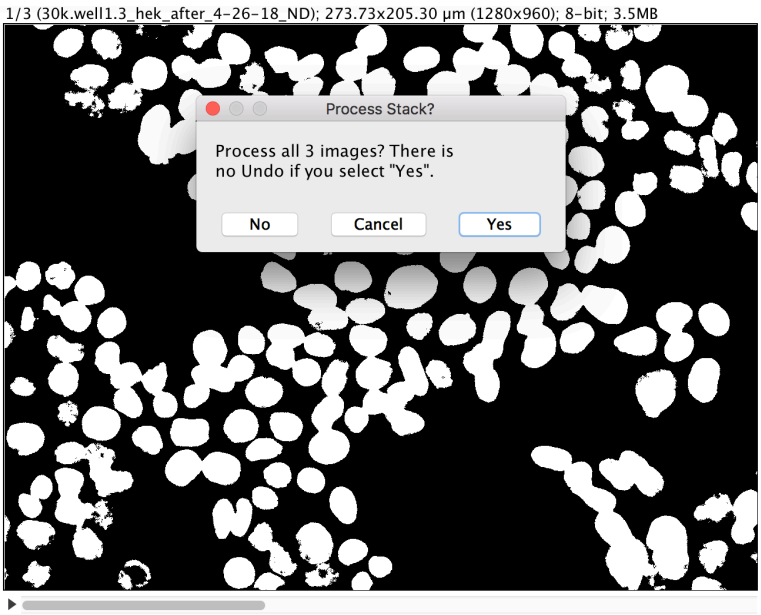


9. When determining the cell size cutoff for a new cell line, select *"Calibration."* Enter the range of cell size cutoffs you wish to test. A standard range for cell such as C2C12, Neurons, and HEK cells would be starting at 0, ending at 25, and having an increment of 5. These numbers will vary based on the resolution and magnification of your images. For a detailed walkthrough of the calibration process, reference *CaliPy Walkthrough* on page 9.

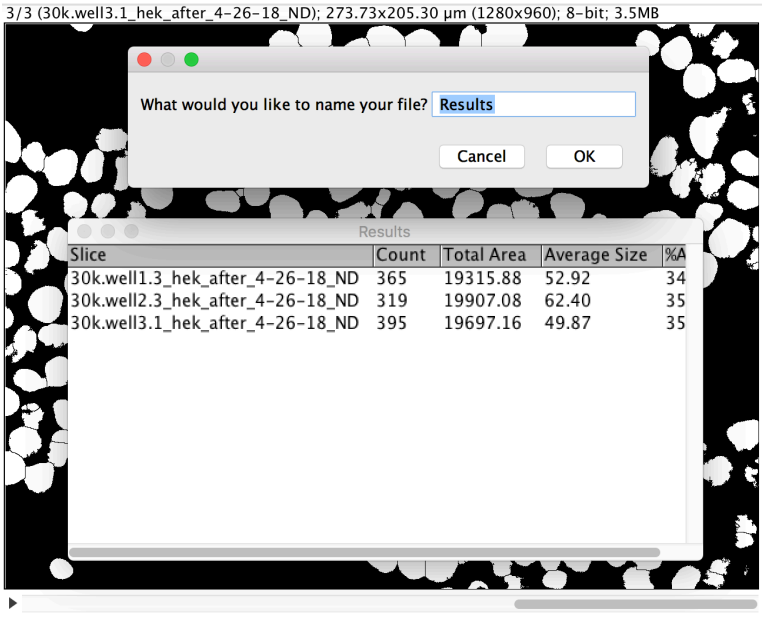


Finishing NucJ

10. Now that you are finished with the configuration, the program will run. You will have to click 'Yes' when prompted if you would like to apply this to all the images. This is NucJ performing the 'watershed' operation to all the images, which attempts to segment joined cells from each other. In the case of large clumps, there may be under sampling as the "watershed" operation cannot accurately split the cells.



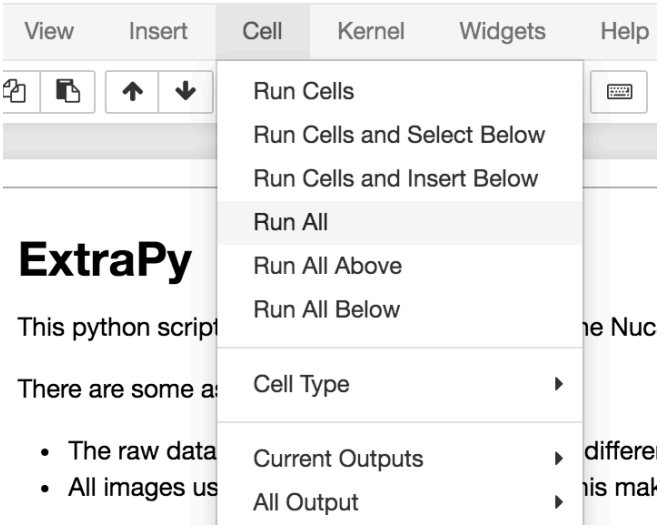
11. A results table should pop up with all your cell counts. The program will ask you what you would like to name your file and then save the file in the directory your images were in.



12. Keep track of where your raw output file is saved as you will likely need to move it for ExtraPy.

ExtraPy Walkthrough

13. Make sure your raw output from NucJ is in the same directory as ExtraPy.
14. Open up the Jupyter notebook file using either the command line or with Anaconda
15. Select “Cell” and then select “Run All”



16. The program will begin running, but will require some input from the user. The program will ask the user for the name of the raw data file. Type the name of the file, character for character, including the .csv extension, then hit enter.

```
Please input the name of your csvfile: Results.csv
```

17. The program will then ask whether or not you use to wish the default settings for the program. If this is the first time running the program, you should type ‘no’ and then enter in the following information as prompted: the name of the output file which will contain the cell counts, the area of the images you used, and the seeding area of the wells the cells were plated on.

18. If you often use the same parameters for every experiment, you can change the following lines of code:

```
# Default value for Image Area in microns. RECOMMENDED: change this to a common value used by your lab.
ImageArea = 56196.769 # Change this value if you wish to have a different default Image Area

# Default Seeding Area for a 96 well seahorse plate
SeedArea = 10600000 # Change this value if you wish to have a different default Seeding Area
```

Simply change the values for ImageArea or SeedArea by changing the numbers in green. The units are by default in micrometers squared. The ImageArea will depend on the magnification of the microscope and can be calculated from the dimensions of your image. The SeedArea will depend on the number of wells on the plate and can generally be found from the product specifications.

19. ExtraPy will generate an output file with the chosen name in the directory you were working in. The output file contains the cell counts for all of your images.

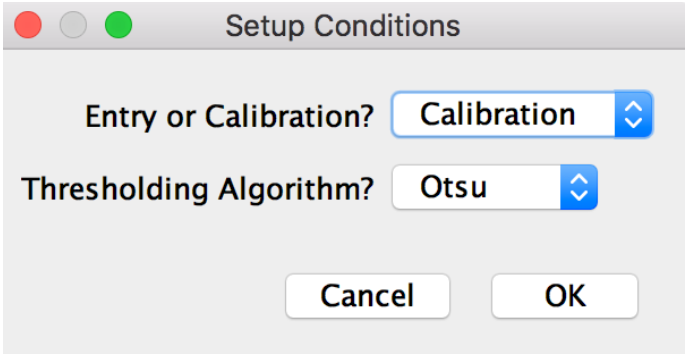
Slice	Count
30k.well1.3_hek_after_4-26-18_ND	68847.37
30k.well2.3_hek_after_4-26-18_ND	60170.72
30k.well3.1_hek_after_4-26-18_ND	74506.06

Final output of cell counts in .csv format. Image slice names remain to identify which count belongs to which image.

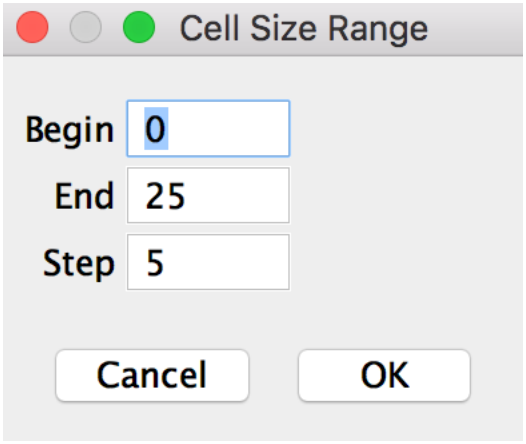
CaliPy Walkthrough

This section will go step-by-step through the manual calibration process. This is recommended anytime you are working with a novel cell line or changing your imaging conditions.

1. Collect a representative test set of images. If going through your first experiment, you can simply use those images.
2. Run NucJ normally and select "Calibration" mode



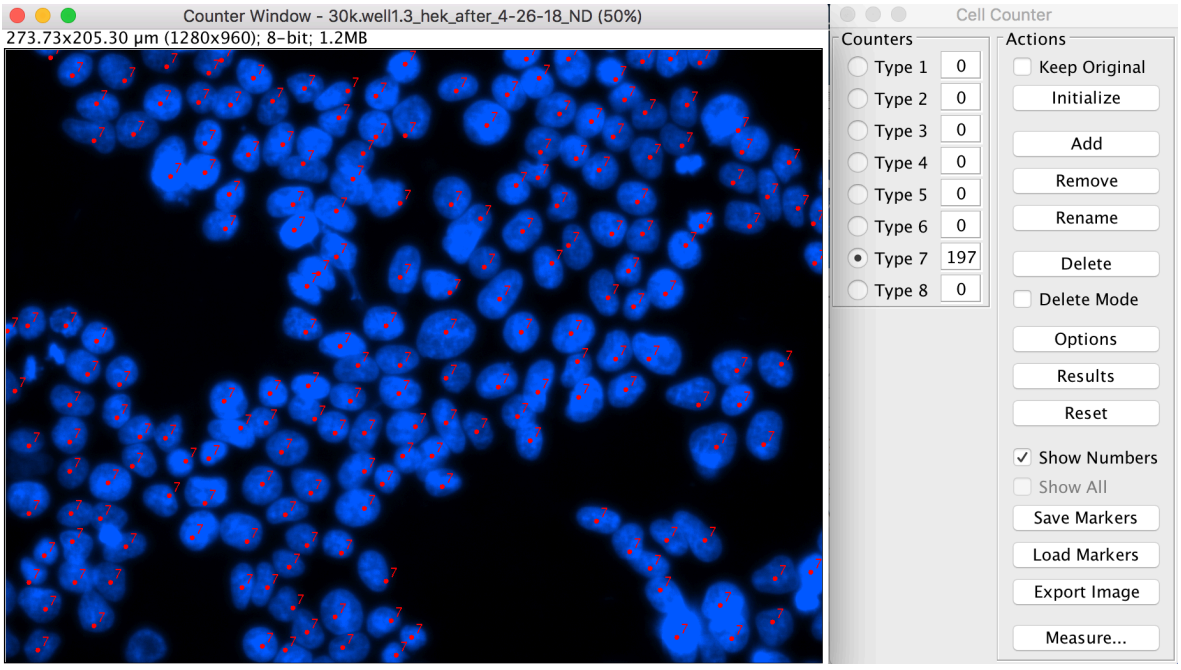
3. Enter your desired range for Cell Size Cutoffs (CSCs) and step size. In the example below, we would test the minimum CSCs of 0, 5, 10, 15, 20, and 25 um^2. Depending on the size of your cell, your desired range will vary.



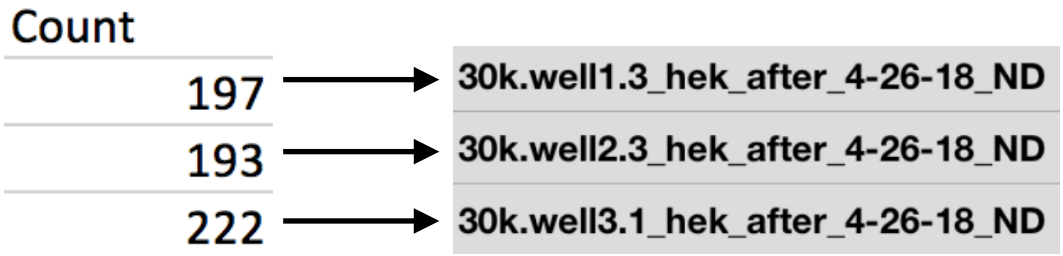
	Slice	Count
CSC = 0	30k.well1.3_hek_after_4-26-18_ND	365
	30k.well2.3_hek_after_4-26-18_ND	319
	30k.well3.1_hek_after_4-26-18_ND	395
CSC = 5	30k.well1.3_hek_after_4-26-18_ND	233
	30k.well2.3_hek_after_4-26-18_ND	216
	30k.well3.1_hek_after_4-26-18_ND	243
CSC = 10	30k.well1.3_hek_after_4-26-18_ND	221
	30k.well2.3_hek_after_4-26-18_ND	206
	30k.well3.1_hek_after_4-26-18_ND	230

4. NucJ will repeatedly count the images at the CSCs specified. Save the results as a csv.

5. Manually count the images. These will function as “Ground Truth” from which we can determine the accuracy of NucJ. We recommend using the CellCounter plugin built into Fiji.



6. Once you've obtained the counts for your test set of images. Enter them into any spreadsheet software (e.g. Excel, Numbers, Google Sheets) with the following format requirements:
    1. Enter them in a single column, matching the order they were counted by NucJ
    2. Title the column "Count"



7. Save/Export your spreadsheet as a csv. The original spreadsheet file is not necessary, but can be saved if desired.
8. Ensure the counts obtained with NucJ and the manually obtained counts are in the same directory as CaliPy.
9. Run CaliPy via terminal commands or using Anaconda
10. Enter the filenames as directed, including the ".csv" extension. Also enter the parameters entered in step 3 as directed.

```
Please input the name of the csv file containing manual counts: manual_counts.csv
Please input the name the csv file containing calibration counts: calibration_counts.csv
What was your minimum CSC? 0
What was your maximum CSC? 25
What was your step size? 5
```

11. CaliPy should return the best CSC of the ones tested. If none of the CSCs accurately captured the data, CaliPy will reflect this. In this case consider improving the imaging conditions or testing other CSCs.

```
The Best Cell Size Cutoff is 25um^2 with a p-value of 0.959477955076.

All Cell Size Cutoffs tested were statistically significantly different from Ground Truth.
```