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Quantifying Fluorescent Cells in Mammalian Cell Tissue Culture

Harley King[1]

[1]Institute for Bioscience and Biotechnology Research, University of Maryland

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Harley King
NIST Center for Neutron Research National Institute of Stand...

ABSTRACT

Quantifying the change in fluorescent status of cells over time can be an important data point in experimental analysis. Optimally, quantification can be accomplished with flow cytometers. However, these instruments are not available to every lab. Routine analysis and multi-sample preparation can be both time and cost prohibitive.

Fluorescent cell quantifcation in cell culture requires a fluorescent microscope and freely available imaging software like Image J.

Preparation and Transfection of Cells

1 A six-well plate is seeded with cells at a typical pre-transfection density.
The following day cells are transfected.

Nunc 6-Well Plate, Round
cell culture plate
Thermo Fisher Scientific 140675
Nunc 6-Well Plate, Round

Capture Plate Images

2 Images can be taken of the plate 24 twenty-four hours post transfection (hpt). Depending on the vector and the promoter, fluorescence may not be observed.

Images of the plate can also be taken at 48 and 72 hpt.

Save images in a folder renamed to include the hours after transfection, e.g. "6-w.plate.images.48hpt".

The LSM 710 confocal microscope requires a plate adapter. With this plate adapter, only the 10x objective can be used. Experimenters wishing for greater magnification may need to use chamber slides.

Universal mounting frame K-X
microscope stage plate adapter
Zeiss 451353-0000-000
LSM 710 culture plate adapter

Take a series of adjacent images in the plate's center to minimize shadows cast by plate walls. Images should not overlap. Fluorescence and brightfield should be overlayed. Additional excitatory channels may be added to verify fluorescent vs. autofluorescent emissions.

LSM 710
confocal laser microscope
Zeiss LSM 710

3 Quantifying fluorescent cells in images is complicated. The gold standard is eye-hand counting, or couting every fluorescent object in an image with a counter. However, multiple images from six wells at three different time points can result in hours of fluorescent cell counting.

Current software packages allow for algorithmic counting of fluorescent cells. CellProfiler allows for image-manipulation pipelines and batch-mode processing but can be difficult to setup. ImageJ (Fiji implementation) is easier to setup but is more limited in automation and cellular recognition.

This tutorial uses Fiji.

CellProfiler
by The CellProfiler project team is based in the Carpenter Lab at the Broad Institute of Harvard and MIT.

FIJI (Image J)
by NIH

4 Before importing the files into Image J, they must be adjusted so that the fluorescent layer is as bright as possible. Do this for all images systematically i.e. use the same values across all images to standardize them.

Use Image J or confocal-specific software to isolate the fluorescent channel. In this protocol, images are processed with Zen Lite (v 2.6) using GFP fluorescence.

Using Zen Lite, in "Camera" mode, each file is opened. The green channel histogram is set from default 255 to 50. The image is saved, then closed. Modified images were saved as in their binary format (.czi) in the same folder, different than the parent folder.

Next, "Processing"-->"Bacth" mode is selected. Select all .czi files in the folder and export using the following settings:
1. file type: "Tagged Image File Format (TIFF)"
2. checked "Convert to 8 bit"
3. Compression "None"
4. Resize: 100%
5. "Apply Display Settings and Channel Color"
6. Checked: "Individual Channels Image"
7. Selected: "Define Subset". Channels: "Ch2-T1 deselected, T PMT-T1 deselected"; "Ch1-T2 selected". Shows as "3".
8. The "Copy Parameters" button was selected. All files were selected and then "Paste Parameters". Added files were i) checked to ensure the same Tif export parameters were duplicated for each individual file.

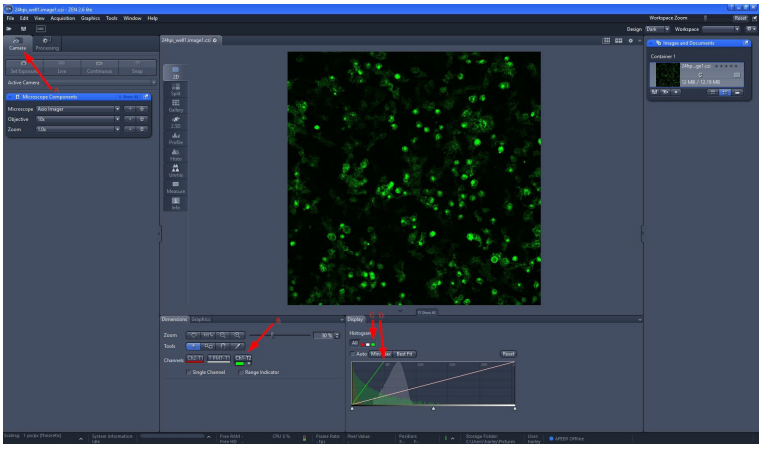

Figure 1: Screen capture of Zen Lite v2.6 software with image capturing cell fluorescence. Arrow (A) shows the software is in "Camera" mode. Arrow (B) shows the fluorescent layer is selected. Fluorescent layer in this image is GFP. With arrow (C) the histogram color to be modified is selected. Arrow (D) shows the histogram threshold moved from 255 (right side) to 50.

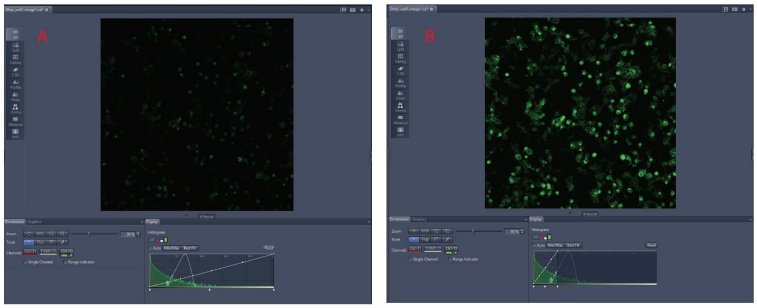

Figure 2: (A) Histogram color threshold set to 255. (B) Threshold set to 50. The enhanced GFP brightness is important for algorithmic recognition in subsequent programs like Image J.

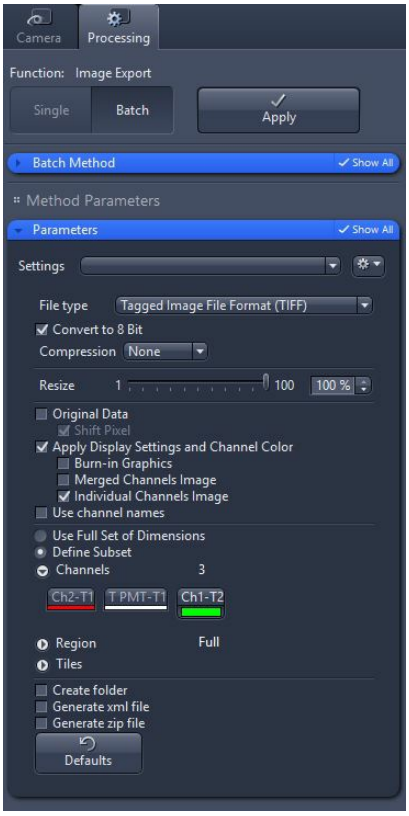

Figure 3: Batch mode export settings. These settings provide the GFP-channel to be exported as a .tiff file at full resolution with modified histogram parameter.

Zen Lite (Blue Edition) 2.6
by Zeiss

5 Exported .tiff images are opened in Adobe Photoshop to covert images to black and white and to invert the colors.
1. "Image"-->"Adjustments"-->"Black and White". Set green to "300%".
2. "Image"-->"Mode"-->"Grayscale". Select "Flatten" and then "Discard" to prompt requests for warnings about image flattening and layer loss.
3. "Image"-->"Adjustments"-->"Invert"
4. Export as a .tiff file.

Scripts or "Actions" can be written in Photoshop and then executed en batch.

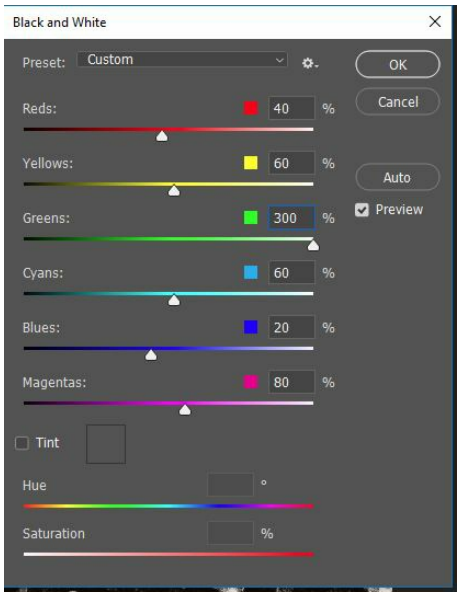

Figure 4: Convert image to black and white with "Greens" equal to 300%.

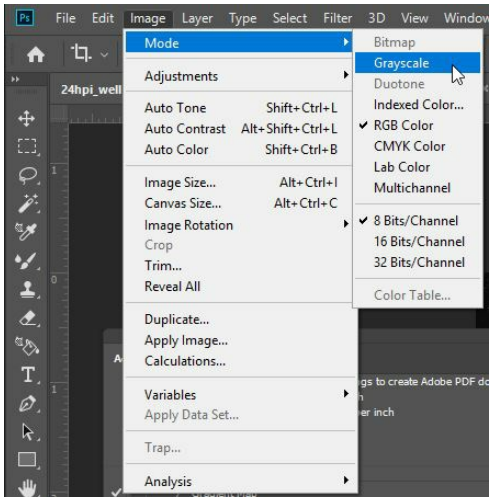

Figure 5: Convert to Grayscale.

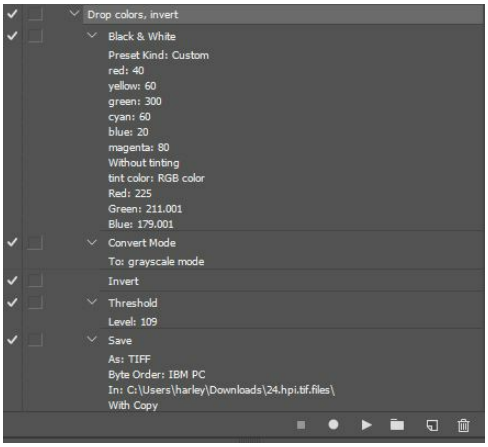

Figure 6: These are the Photoshop "Actions" set up to automatically run the conversion to black and white, grayscale and inversion.

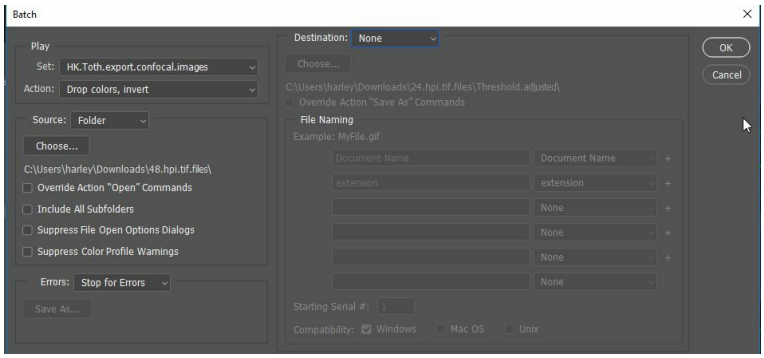

Figure 7: Photoshop batch options.

Photoshop CC 20.0.0
by Adobe

6 Get Fluorescent Cell Counts in Image J by importing the image. Select "Analyze"-->"Analyze Particles". Adjust the size and circularity settings so that a majority of the fluorescent cells are properly labeled after selecting "OK".

Ensure that counts from the images are properly recorded in a text file or spreadsheet. Image J will prompt for saving.

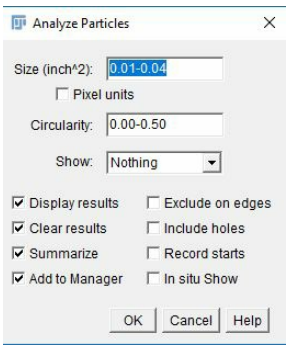

Figure 8: Every image and cell type will require settings to be tested empirically for precise coverage using Image J "Analyze Particles."

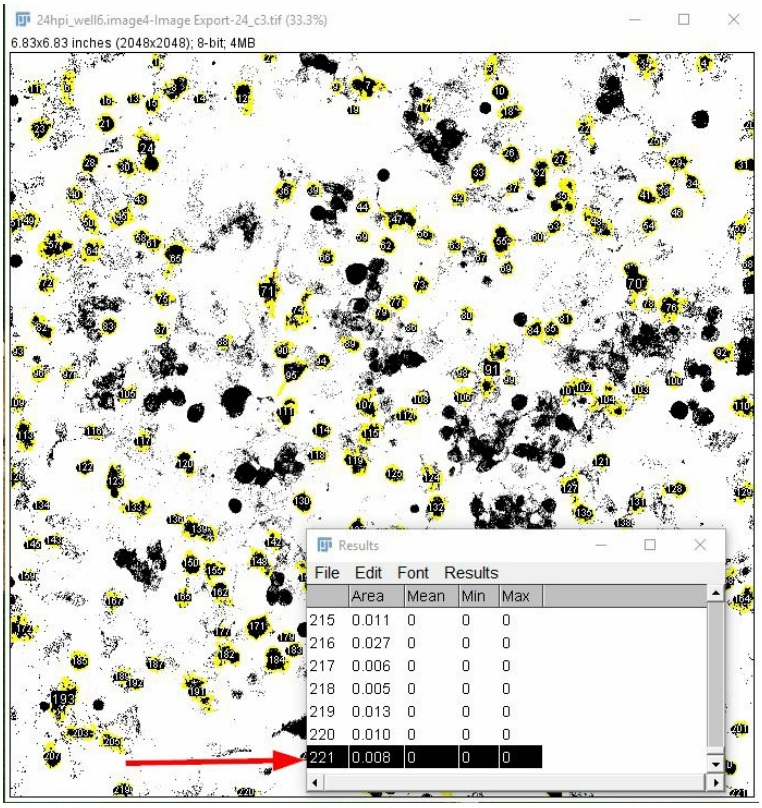

Figure 9: Cell counts for the previous settings and image count 221 cells (arrow). Not all cells will be captured by the analysis. For relative counts, the same settings must be used across all images and times.

Image J settings can be saved to a "Macro" which can also be used for a batch process.

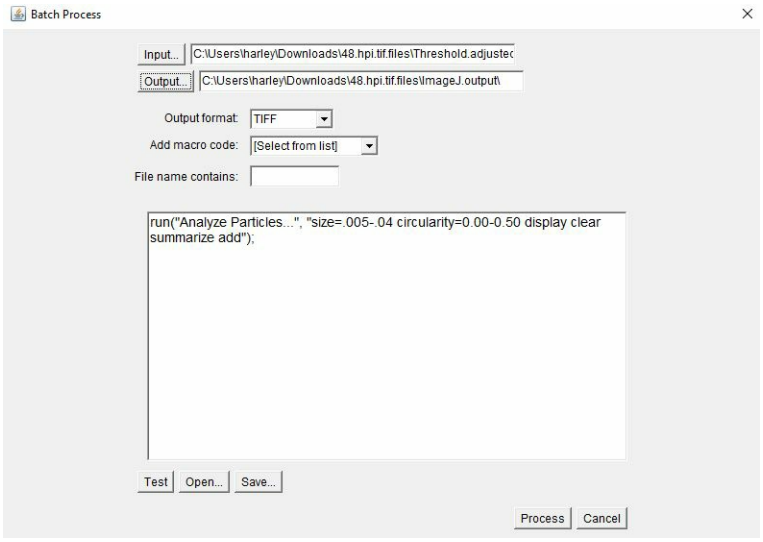

Figure 10: Batch process a macro in Image J.

Analyze Results

7 Analysis software like GraphPad Prism or Excel can be used to view the results.

TIME	Well1_1	Well1_2	Well1_3	Well1_4	Well2_1	Well2_2	Well2_3	Well2_4	Well3_1	Well3_2	Well3_3	Well3_4	Well4_1	Well4_2	Well4_3	Well4_4	Well5_1	Well5_2	Well5_3	Well5_4	Well6_1	Well6_2	Well6_3	Well6_4
24hpi	140	122	152	147	195	230	234	235	209	193	200	242	113	132	124	122	213	247	220	201	199	183	156	221
48hpi	114	115	158	167	96	123	163	150	250	256	261	225	134	126	151	119	363	371	352	343	263	269	284	243
72hpi	153	130	107	128	14	19	16	12	98	110	78	88	139	134	135	137	247	274	244	227	368	437	385	297

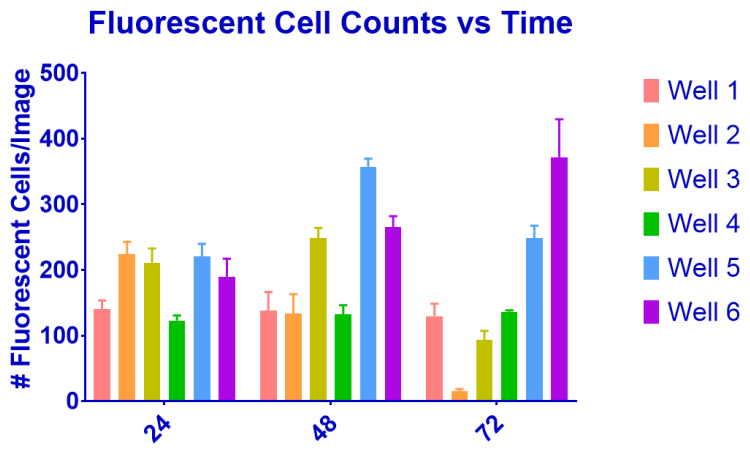

Figure 11: Example result graph. The average count for each well is plotted. The error bars represent standard deviation. Over time, fluorescent cell counts expected to increase as cells undergo mitosis. The reduction in fluorescence compared to a control can be observed in well 2 vs well 1, respectively.

Prism 8.0
by GraphPad

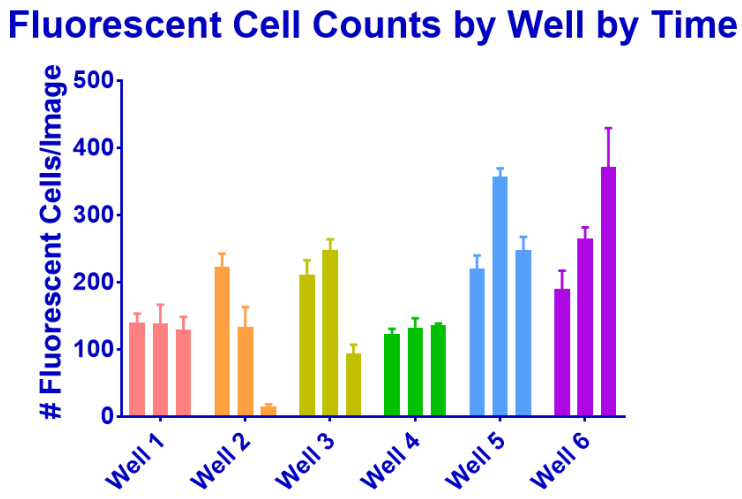

Figure 12: Example result graph displays data as shown in figure 11 but grouped and arranged by time. The decrease or increase in fluorescence compared to a control can be more easily observed.