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 We use this protocol and it's working

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Quantifying Fluorescent Cells in Mammalian Cell Tissue Culture

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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 quantification 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.

Equipment

Nunc 6-Well Plate, Round

NAME

cell culture plate

TYPE

Thermo Fisher Scientific

BRAND

140675

SKU

<https://www.thermofisher.com/order/catalog/product/140675>

LINK

Nunc 6-Well Plate, Round

SPECIFICATIONS

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".

Note

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.

Equipment

Universal mounting frame K-X

NAME

microscope stage plate adapter

TYPE

Zeiss

BRAND

451353-0000-000

SKU

<https://www.micro-shop.zeiss.com/en/us/system/accessories?pld=1005&gld=6016&cld=10255&pHld=400000000800&hld=400000000800700000&iCnt=21&pid=000000-1116-077,000000-1116-078,000000-1272-644,411860-9080-000,411860-9082-000,411860-9086-000,419195-9026-000,432>

LINK

LSM 710 culture plate adapter

SPECIFICATIONS

Note

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.

Equipment

LSM 710

NAME

confocal laser microscope

TYPE

Zeiss

BRAND

LSM 710

SKU

<https://www.zeiss.com/microscopy/us/products/confocal-microscopes.html>

LINK

Batch Process Images Using Zen Lite, Photoshop and Image.

- Quantifying fluorescent cells in images is complicated. The gold standard is eye-hand counting, or counting 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.

Software

CellProfiler

NAME

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

DEVELOPER

Software

FIJI (Image J)

NAME

NIH

DEVELOPER

- 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"-->"Batch" 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.

Software

Zen Lite (Blue Edition)

NAME

Zeiss

DEVELOPER

- 5** Exported .tiff images are opened in Adobe Photoshop to convert 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.

Note

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.

Software

Photoshop CC

NAME

Adobe

DEVELOPER

- 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.

Expected result

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.

Note

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	Well 1_1	Well 1_2	Well 1_3	Well 1_4	Well 2_1	Well 2_2	Well 2_3	Well 2_4	Well 3_1	Well 3_2	Well 3_3	Well 3_4	Well 4_1	Well 4_2	Well 4_3	Well 4_4	Well 5_1	Well 5_2	Well 5_3	Well 5_4	Well 6_1	Well 6_2	Well 6_3	Well 6_4
24 h pi		140	122	152	147	195	230	234	235	209	193	200	242	113	132	124	122	213	247	220	201	199	183	156	221
48 h pi		114	115	158	167	96	123	163	150	250	256	261	225	134	126	151	119	363	371	352	343	263	269	284	243

7 2 h pi	1 5 3	1 3 0	1 0 7	1 2 8	1 4	1 9	1 6	1 2	9 8	1 1 0	7 8	8 8	1 3 9	1 3 4	1 3 5	1 3 7	2 4 7	2 7 4	2 4 4	2 2 7	3 6 8	4 3 7	3 8 5	2 9 7
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Fluorescent Cell Counts vs Time

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.

Software	
Prism	NAME
GraphPad	DEVELOPER

Fluorescent Cell Counts by Well by Time

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.