Instruction Manual

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This manual is for the program that implements the image analysis method presented in our paper: Z. Huang, F. Senocak, A. Jayaraman, and J. Hahn. Integrated Modeling and Experimental Approach for Determining Transcription Factor Profiles from Fluorescent Reporter Data, BMC Systems Biology 2:64 (2008).

The original program was developed by Zuyi (Jacky) Huang. Based on this, the graphic user interface (GUI) was coded by Mark Deimund. This GUI program can be used to identify the objects at different intensity levels from images, e.g., fluorescent cell regions from fluorescent images. The image analysis method is briefly introduced in this manual, which is followed by a detailed description of how to use this program to get the fluorescence intensity profiles from fluorescent images. An example is presented for the purpose of instruction. Please contact Juergen Hahn (hahn@tamu.edu) for any question or suggestion.

1. Introduction of the image analysis method

The tristimulus theory states that any visual color can be represented by overlaying three color information channels. For television and computer graphics, the standard colors used are red, green and blue. An RGB image can be represent by a three-dimensional matrix

$$M = \begin{bmatrix} (r,g,b)_{11} & \cdots & (r,g,b)_{1j} \\ \vdots & \ddots & \vdots \\ (r,g,b)_{i1} & \cdots & (r,g,b)_{ij} \end{bmatrix} \tag{1}$$

where M is of size $i \times j \times 3$. $i \times j$ is the resolution of the image, which means that there are i rows of pixels in the images and each row has j columns of pixels. Each pixel of the image has three intensity values, i.e. one for red, green, and blue. M can be rewritten into a two-dimension matrix X with the size of $(i \times j) \times 3$ in (2) by listing the three intensity values of each pixel line by line so that each row of X represents the red, green and blue values of a pixel:

$$X = \begin{bmatrix} r_1 & g_1 & b_1 \\ r_2 & g_2 & b_2 \\ \vdots & \vdots & \vdots \\ r_{i \times j} & g_{i \times j} & b_{i \times j} \end{bmatrix} \tag{2}$$

The intensity for each pixel is defined as the sum of the red, green, and blue values.
$$I = r + g + b \tag{3}$$

The RGB image M can be transformed into a two-dimensional gray image by replacing each pixel by its intensity:

$$N = \begin{bmatrix} (r+g+b)_{11} & \cdots & (r+g+b)_{1j} \\ \vdots & \ddots & \vdots \\ (r+g+b)_{i1} & \cdots & (r+g+b)_{ij} \end{bmatrix} \tag{4}$$

where N is of dimension $i \times j$.

Image analysis extracts information from a time-series of images, represented by matrices recorded at different points in time. A common image analysis procedure is to (1) record images at different time points, (2) separately analyze each image, and (3) combine the image analysis results obtained at different time points to determine dynamics of the system.

The proposed image analysis is based on K-means clustering and Principal Component Analysis (PCA). This method distinguishes the regions of the image with similar brightness and then groups them into the same cluster. The pixels in the same cluster have the same fluorescence intensity level, and the clusters with higher fluorescence intensity levels are considered as the fluorescent area. Clusters are indexed in a decreasing order of fluorescence intensity, that is, Cluster 1 has the highest fluorescence intensity while Cluster 2 has the second highest fluorescence intensity. The image analysis program determines the fluorescent regions in the first step and then calculates the average fluorescence intensity over the fluorescent regions. Please refer to our paper for more detail on the image analysis algorithm. The following figures show an example for this image analysis method.
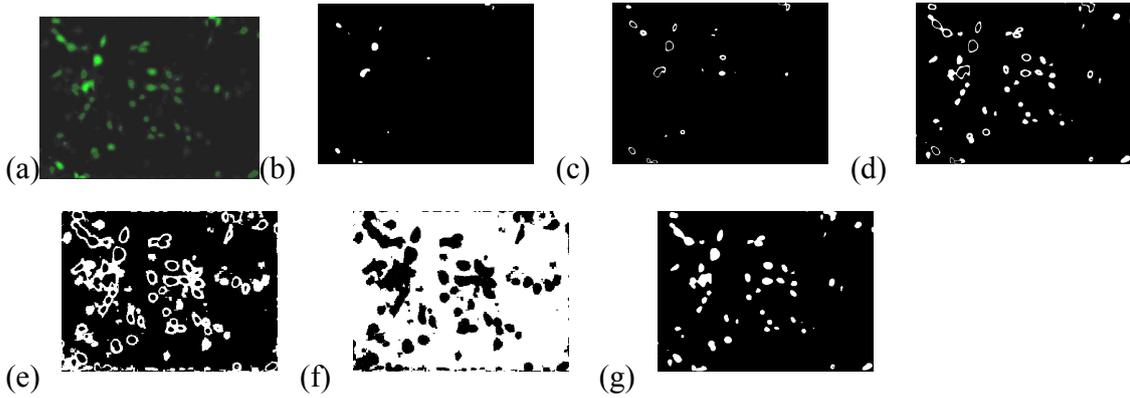
Figure 1: Fluorescent regions detected by the PCA-based image analysis procedure: (a) Fluorescent microscopy image; (b) cluster 1; (c) cluster 2; (d) cluster 3; (e) cluster 4; (f) cluster 5; (g) cumulative results of clusters 1, 2, and 3.

The white color region in Figure 1(g) is chosen as the region representing cells with GFP while the white pixels shown in Figure 1(e) and 1(f) represent the background. Once the cell region has been determined it is possible to compute the average fluorescence intensity by the following formula:

$$I = \left(\frac{\sum_{k=1}^{N_f} I_{f,k}}{N_f} - \frac{\sum_{k=1}^{N_b} I_{b,k}}{N_b} \right)_{stimulation} \tag{5}$$

Where $I_{f,k}$ refers the fluorescent intensity of the k-th pixel in a fluorescent cell region, $I_{b,k}$ refers the fluorescent intensity of the k-th pixel belonging to the background, N_f is the total number of pixels in the fluorescent cell region, N_b is the total number of pixels in the background. The intensities for the images in different points in time construct the fluorescence intensity profile.

2. Procedure of image analysis

This section uses a simple example to illustrate the procedure of applying the proposed GUI program to get fluorescence intensity profiles from images. Assume that we have taken fluorescent images for different positions at different points in time and that different experimental conditions are applied at different positions. We try to get the fluorescent intensity for different positions or several positions that have been performed under the same experimental condition. The procedure for image analysis consists of the following steps: 1) Extract images in the format of jpg or tif from experiment data and then save them in the folders. The images for the same position are indexed according to the measurement sequence and saved in the same folder. The present version of GUI allows two image formats as these formats, i.e. jpg and tif, are the most extensively used. 2) the GUI program is used to analyze images and plot fluorescence intensity profiles for specific positions. Section 2.1 and 2.2 present the details of these steps while Section 2.3 shows how to run the program in MATLAB.

2.1 Extract images from experiment data

This section shows the procedure to extract fluorescent images from the Axiovert 200 M microscope system provided by Carl Zeiss Light Microscopy. The software AxioVision is used to extract fluorescent images from the experiment data. If you use a different imaging system, you might need to extract the images following the procedures instructed by the manual of the imaging system you use. Assume that we stimulate HepG2 cells with IL-1 of a concentration equal to 10 ng/ml in Well 2 and take fluorescent images every one hour for a period of 30 hours at each position in the well (i.e., Position 13~15). On the same time, images for Position 1~3 in Well 1 are used as the data set for the negative-control experiment condition, i.e. no stimulation is applied in Well 1.
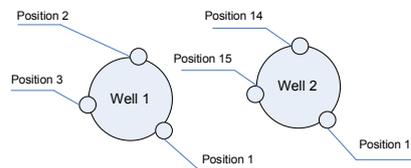

Figure 2: Positions and wells for experiment

The following steps are taken to extract the images that will be analyzed by the GUI image analysis program:

1) Run the software AxioVision Rel. 4.6 or higher version which is available for free on the website.
2) File->open image-> open your image. AxioVision Rel. 4.6 can open a time series of images for each position.
3) Each image has two channels of information: "Channel 1" is for the light microscopy image while "Channel 2" is for the fluorescent microscopy image. The buttons to access these two channels are located at the left-low corner of the user interface of the software (See Figure 3). The original fluorescent image is in gray color, not green color. The green color is added by the software, i.e., AxioVision Rel. 4.6. To get the original fluorescent microscopy image, the following operation should be taken:
Click the button "on" to "off"-> Click the button "2"

Figure 3: Operation to access the original fluorescent microscopy image

Click F8/F7 on the keyboard to zoom out/in the image to fit the full screen of the monitor.

4) To get the images with the best contrast, regulate image parameters, such as "Brightness", "Contrast", "Gamma value", and "Bits per pixel". These parameters should be kept the same for all sets of images during image analysis, as they may change the absolute value of the fluorescence intensity. If you have two sets of experiment data taken at different days, you need to make sure all the image parameters are kept the same when you extract images. To set up image parameters, the following operations should be taken:

 (1) Open the images at the positions stimulated with the highest concentration of the stimulus. Usually, these images have the highest fluorescent intensity, and image parameters chosen based upon these images are applicable to the images taken at other experiment conditions.

 (2) Click the following button shown in Figure 4 to display the image property window:

Figure 4: Button to access the image property window

The following window is opened:
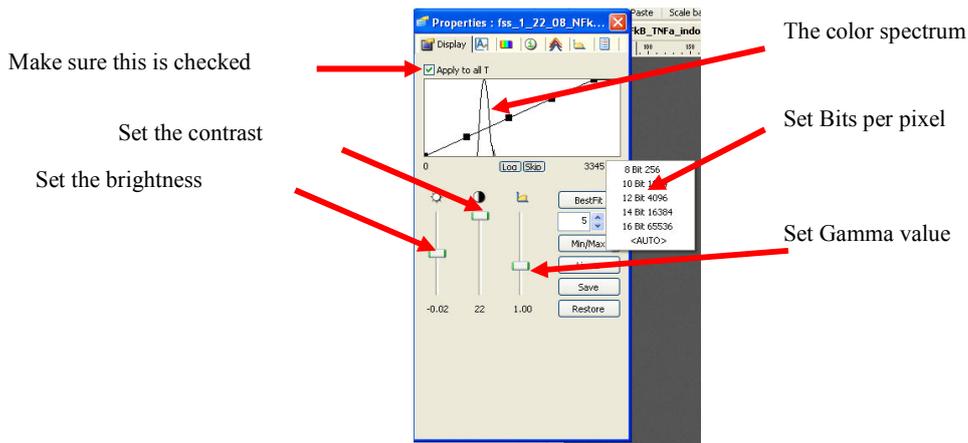
Figure 5: The window for setting values of image parameters

 (3) To get the image parameters that make images have best quality, the following steps are taken: (A) change "Bits per pixel" so that the color spectrum has the largest spread. Usually, 8 bit, 10 bit or 12 bit works well. (B) Set the Gamma value as 1. (C)

Move the "slide rod" marked in Figure 6, observe how the color spectrum changes over time, and then set the value of brightness by moving the line across the color spectrum using the mouse.

Figure 6: Operation to observe the variation of the color spectrum versus time

(4) After setting the value of parameter "Brightness", move the line across of the color spectrum using the mouse to get the best value for parameter "Contrast". Make sure all the images in the position are not too dark and not too bright. The best values of image parameters make images have the best contrast. Write down the values of the image parameters, and check whether these parameter values are applicable to the images in other positions. Otherwise, regulate the values of image parameters until they are applicable to all images in the dataset.

5) Export the jpg/tif pictures

After applying image parameters to all images in the dataset, the jpg/tif pictures are exported from these images and then analyzed by the GUI program. The following steps are taken to export the jpg-format pictures from experiment data:

(1) Change the images in gay color back to green fluorescent images by clicking "off" to "on" and then clicking "1"

Figure 7: Operation to convert the image to GFP image

(2) File->export-> set up the options as shown in Figure 8:
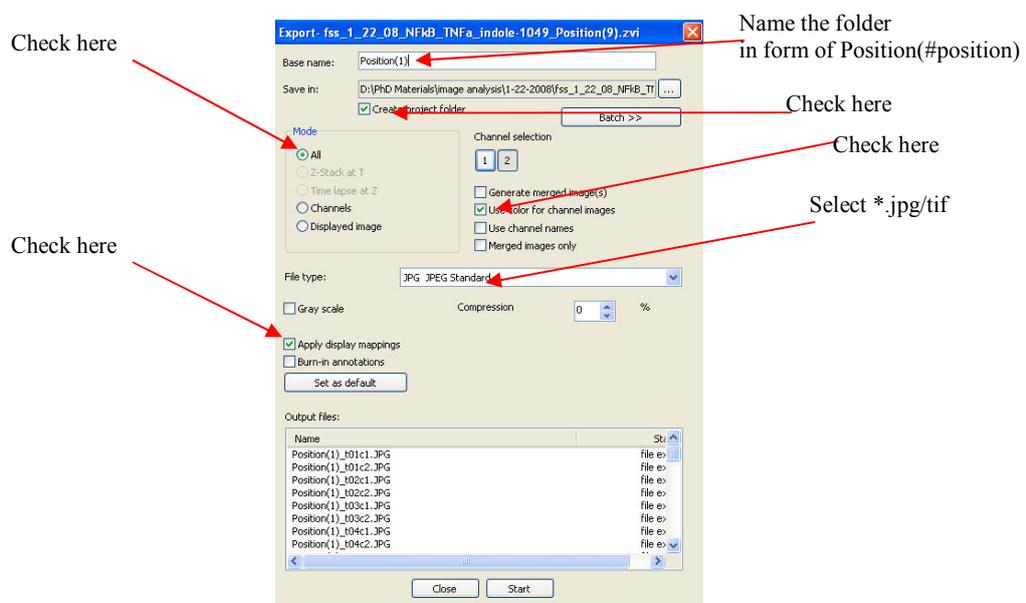
Figure 8: Interface to export the jpg-format images for each position

The folder to store all the images for one position should be named in the form of "Position(#position)". For example, the name of the folder for Position 1 should be "Position(1)" while the name of the folder for Position 13 is "Position(13)". By this way, images are guaranteed to be saved in the folder associated with the position where these images are taken. In addition, the sequence for measuring each image is coded in the name of the image. For example, "t01" in "Position(1)_t01c2.jpg" means that the image is taken at the first sampling time point, and "c2" means that these images are exported from channel 2. The indices of positions should be positive.

2.2 Analyze images with the GUI program

Once the images for all positions are saved into the corresponding folders with the proper names, the GUI program can analyze all images automatically. The graphic user interface for the image analysis program is shown in Figure 9. The following steps are taken to calcuate the fluorescence intensity profiles from fluorescent images: (A) Use a representative image from the dataset to check how many clusters the pixels of the image should be divided into. (B) Select the folder where images and GUI program are stored, input the number of clusters hinted by the Step (A), set up the indices for all positions in the dataset, input the number of images sampled at each position, select the format of images (jpg or tif), and click the botton "Analzye" to analyze images. (C) Set up the indices of the positions for the negative control condition as well as for the stimulation, input the time interval between two samples, select the time unit and the type of intensity (i.e., absolute intensity or relative intensity), assign the number of clusters representing fluorescent region, and plot the fluorescence intensity profiles. (D) Fluorescence intensity profiles of all positions are saved into an Excel file (i.e. intensity.xls) and a file in the format of mat (i.e. intensity.mat). Operations for these steps are introduced in the following subsections.
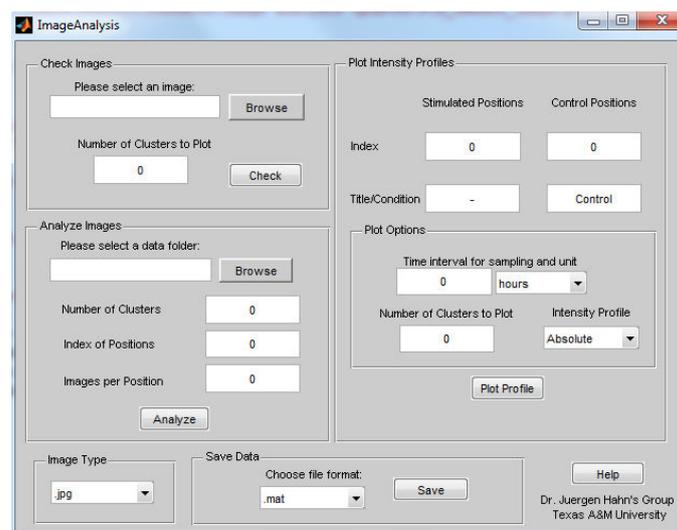
Figure 9: Graphic user interface for image analysis

2.2.1 Check images

An image named test.jpg is used as an example to illustrate the step for checking image. The purpose of this step is to use a representative image from the data set to check how many clusters the pixels of the image should be divided but also to check how many clusters should be used to represent the fluorescent cell region. The following operations are taken for this purpose:

1) Select an image and determine the number of clusters to be generated and plotted. By clicking on the "Browse" button at the top of the GUI (see it in Figure 10), a dialogue box will appear which allows users to select an image (see it in Figure 11).

2) Select the image (highlighted in red below) and click "Open" (highlighted in green below). In this example, "test.jpg" is used for checking. Refer to Figure 12 for this operation.
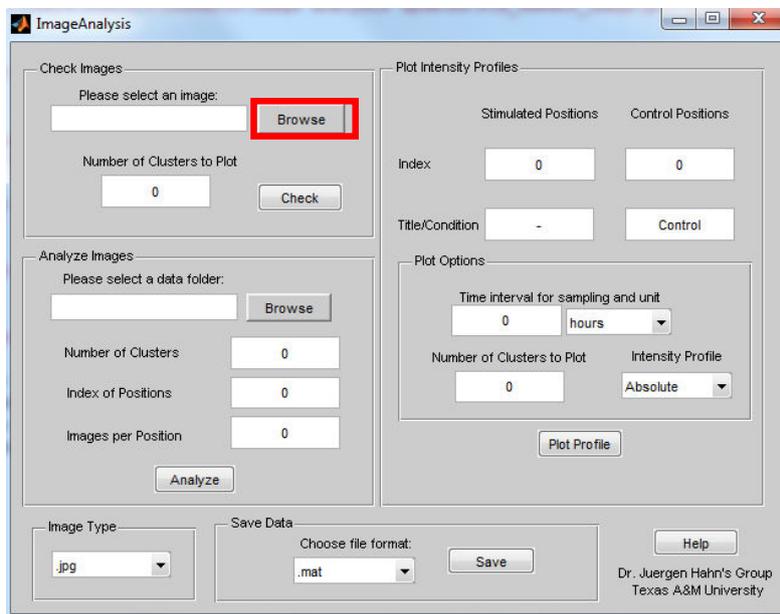
Figure 10: Step to browse the image for checking
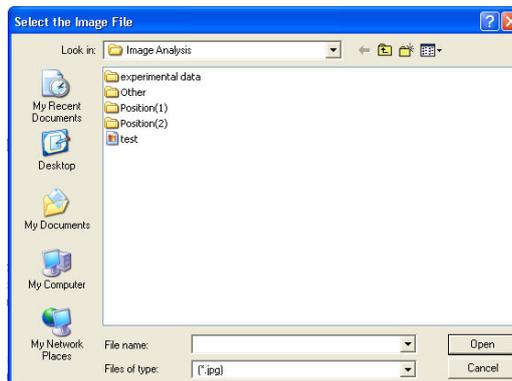
Figure 11: The dialogue box for choosing the image for checking

3) Enter the number of desired clusters to be generated in the box labeled "Number of Clusters". "5" is used in this example for the purpose of instruction. Once this value is input, click "Check" to generate plots of clusters for the image. The GFP image "test.jpg" is shown in Figure 13. The pixels for each cluster are shown in Figure 14 while the cumulative results of the first few clusters are shown in Figure 15. Figure 14 shows that five clusters can provide a good division of the pixels as the fluorescent region can be distinguished from the background region. It can be seen from Figure 15 that the pixels from the first 3 clusters are able to represent the fluorescent region of Figure 13.

The image for analysis
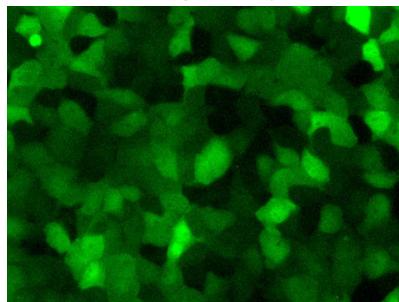
Figure 13: Image "test.jpg" is used as the image for checking

Cluster1

Cluster2

(a)   Pixels in Cluster 1
(b)   Pixels in Cluster 2
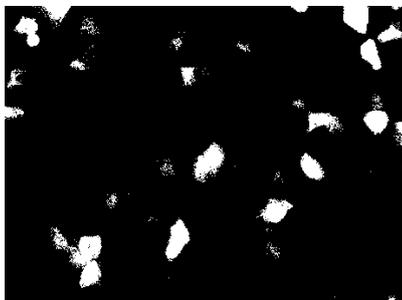
(c) Pixels in Cluster 3
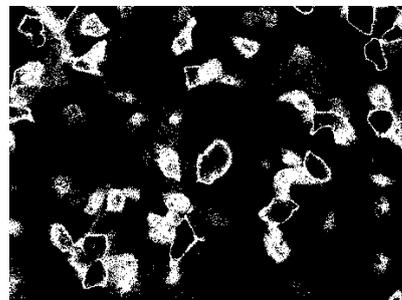
(d) Pixels in Cluster 4
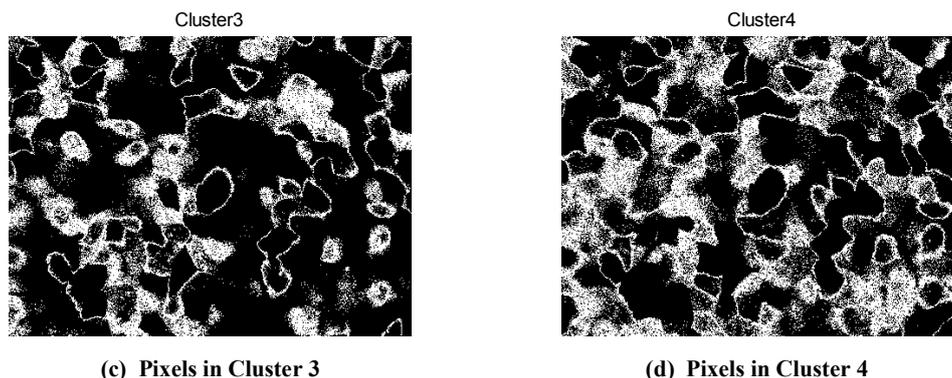
Figure 14: The pixels associated with Cluster 1 ~ 4

The summation of the first 1 clusters

(a) Pixels in the first cluster

The summation of the first 2 clusters


(b) Pixels in the first two clusters

The summation of the first 3 clusters

(c) Pixels in the first three clusters

The summation of the first 4 clusters

(d) Pixels in the first four Clusters
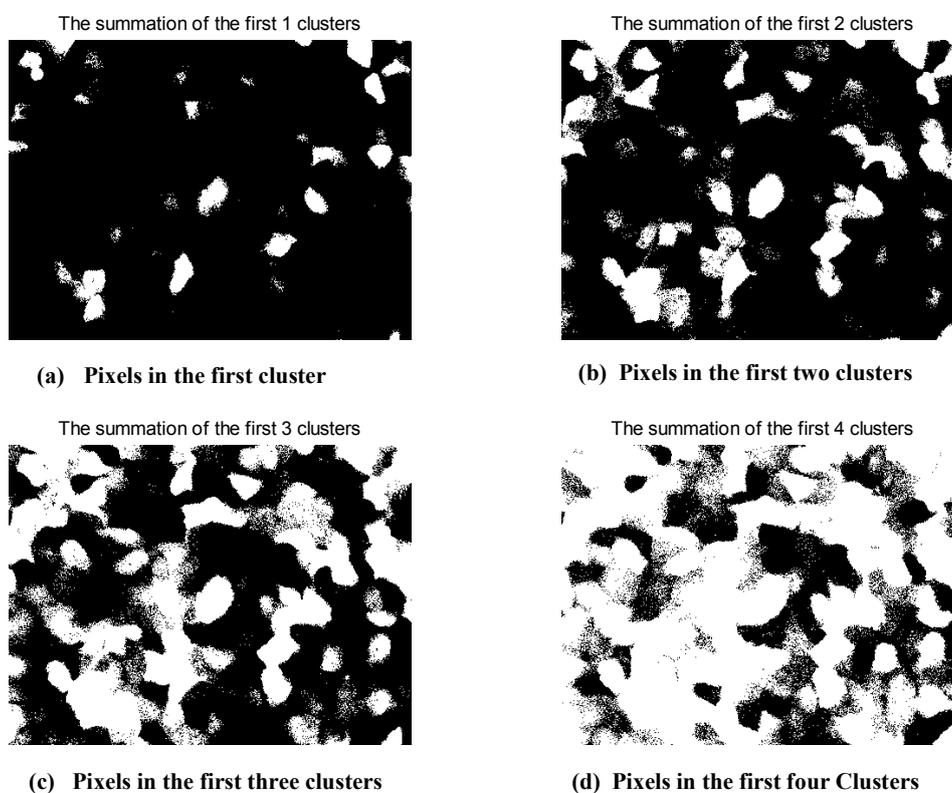
Figure 15: Cumulative results of the first *n* clusters, *n* = 1, 2, ⋯, 4.

2.2.2 Analyze images

A dataset from a C/EBPβ GFP reporter system for HepG2 cells is used as an example in this section to illustrate the procedure of using the GUI program to analyze images extracted in Section 2.1. The format of jpg is used in this example. Images are taken for the negative control condition as well as for the situation where HepG2 is stimulated by IL-1 with a concentration of 10ng/ml. Two wells are used for these two experimental conditions, and each well has three positions for taking fluorescence images. Figure 3 shows a schematic illustration of the wells and

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positions. Image analysis using the GUI program consists of two portions: (A) assign the directory for the folder containing the image data; (B) Input information about the image data and then run the program. Steps for image analysis are described as follows:

1) Click on the "Browse" button on the GUI (see it in Figure 16). A smaller dialogue box that is shown in Figure 17 opens up.
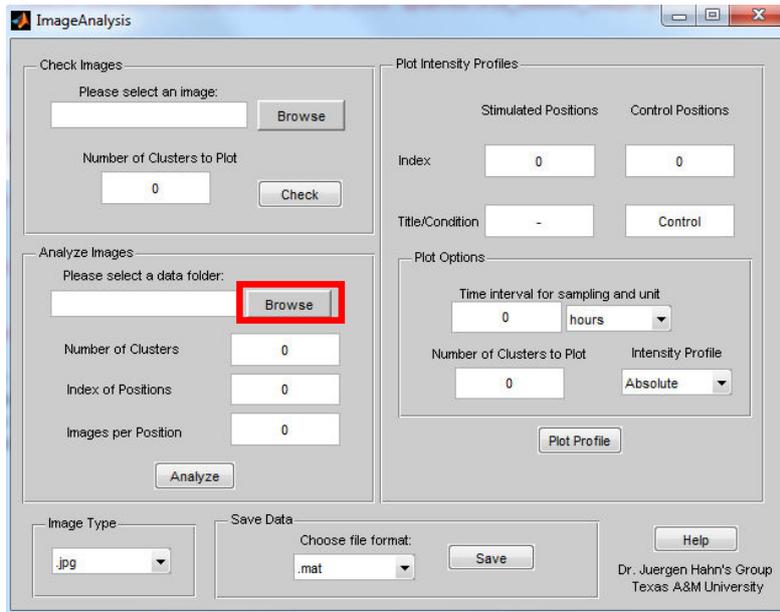
Figure 16: The "Browse" button that is used for setting the path for the folder containing image data

2) Find the folder containing the image data and ensure that it is shown in the "Folder:" blank (highlighted in Figure 17) of the dialogue box, and click "OK" (highlighted in green below). In this example, the image data folder is "E:\Zuyi Huang\Undergraduate student\Mark Deimund\Image analysis".
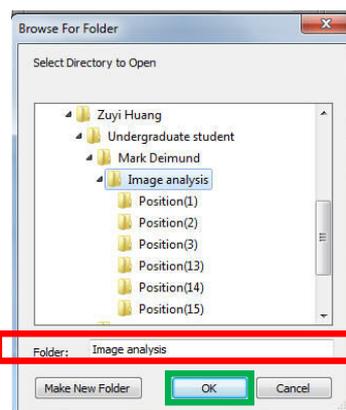
Figure 17: Assign the path in the dialogue box that pops up when the "Browse" button in Figure 16 is clicked
Now, the blank below "Please select a data folder:" in GUI should now read as "E:\Zuyi Huang\Undergraduate student\Mark Deimund\Image analysis"

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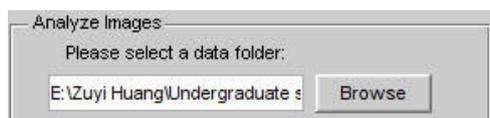
Figure 18: The blank showing the path for image analysis

3) Enter the following information for image analysis:

(1) Number of Clusters: This is the number of clusters into which the pixels of each image will be divided. The numbers 6 and 7 are commonly used for images from GFP reporter systems. To enter the number of clusters, simply click on the blank to the right of "Number of Clusters," and type in "7" (or any other desired number).

(2) Index of Positions: This index vector represents the positions. For example, if one wants to analyze the images taken at Positions 1, 2, 3, 13, 14, and 15, the index would be entered as "1:3,13:15" or "1, 2, 3, 13, 14, 15".

(3) Images per Position: This quantity is the number of images taken at each position, which might range from only a few images to 30 or more. In this example, 31 images are taken for each position, i.e. one image taken per hour for a period of 30 hours.

(4) Image Type: there are two options for this category, the jpg or tif. Jpg is used in this example. After the above information has been entered, the module for image analysis should look as shown in Figure 19.

4) If all of the necessary parameters are input, the image data is ready to analyze. After confirming all information is correct, click the "Analyze" button highlighted in red below.
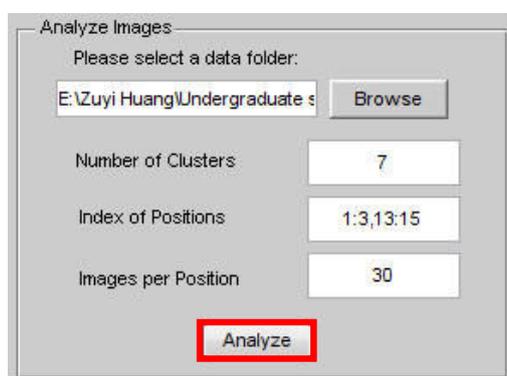
Figure 19: Images are ready to analyze after corresponding information has been entered

Once the "Analyze" button is clicked, the GUI automatically goes through each image at each position and saves an intensity file for each position. This process can take several hours, depending upon the number of positions, the number of images per position, the size of the images, and the computing capacity of the computer. For example, it takes about 30 seconds to process an image with a resolution of 1388×1042 in a computer of dual-core 2.2G CPU and 3G memory.

2.2.3 Plot intensity profiles

The intensity profiles for different experiment conditions can be plotted after all the images have been analyzed. The intensity profiles for the positions where cells are stimulated can be plotted with absolute intensity values or the values relative to the intensity values for negative control condition. For each experiment condition, stimulation or control, multiple fluorescence intensity profiles are plotted as images are taken at multiple positions. Error-bar profiles where the bar indicates one standard deviation of uncertainty are then drawn for each experiment condition.

The following information needs to enter before clicking the "Plot Profile" to plot the fluorescence intensity profiles.

1) Stimulated Positions: These are the indices of positions in the experiment that have been stimulated with stimuli.
2) Control Positions: These are the indices of positions in the experiment that have not been stimulated.
3) Index: The blanks below the "Stimulated Positions" and "Control Positions" are where the indices for the respective positions should be input. For this example, the control positions should be Positions 1~3, and the stimulated positions should be Positions 13~15.
4) Title/Condition: The title or condition of each experimental position can be input in this blank. By default, the "Control Positions" title is set to "Control", although the user can change this title. The "Stimulated Positions" title should describe the experiment conditions. For this example, the stimulated positions title is set to "IL-1 10 ng/ml".
5) Time Interval for Sampling and Unit (x-axis): Time interval is period between two sampling. In this example, images are taken every one hour. Therefore, one hour is the time interval. The pop-up menu allows the user to select the time scale for the x-axis. This scale should be selected based on the time between subsequent images at each position. In this example, this parameter will be set to "hours".
6) Number of Clusters to Plot: This parameter is the number of clusters that represent fluorescent regions. This number can be found in Subsection 2.2.1 (i.e. Check image). For example, if pixels in the first 5 clusters represent fluorescent cell regions, this parameter should be set to 5. Generally, this number will be 3, 4 or 5 if pixels of fluorescent images are divided into 7 clusters. Usually, a larger number is assigned for the images with high contrast. User can test this parameter by plotting the corresponding pixels and inspecting the plotted fluorescent regions using the approach presented in Subsection 2.2.1. For our example, 5 clusters will be plotted.
7) Intensity: This pop-up menu allows the user to choose between absolute and relative intensity profiles to be plotted. The absolute intensity profile plots for both the control condition and the simulated condition. For each condition, absolute intensity profiles of all individual positions are plotted in one figure while the average intensity profile with error bars is plotted on another. If the relative intensity profile is selected, the average intensity profile for the control condition is subtracted from absolute intensity profiles of the simulated positions. The obtained relative intensity profiles are then plotted, accompanied by the corresponding average value profile with error bars.

   Figure 20 shows how the parameters would be input into the GUI for our example. Figure 21 and Figure 22 show the corresponding absolute and relative intensity profiles respectively.

Figure 20: Interface to input parameters for plotting fluorescence intensity profiles


(a)

(b)

(c)

(d)
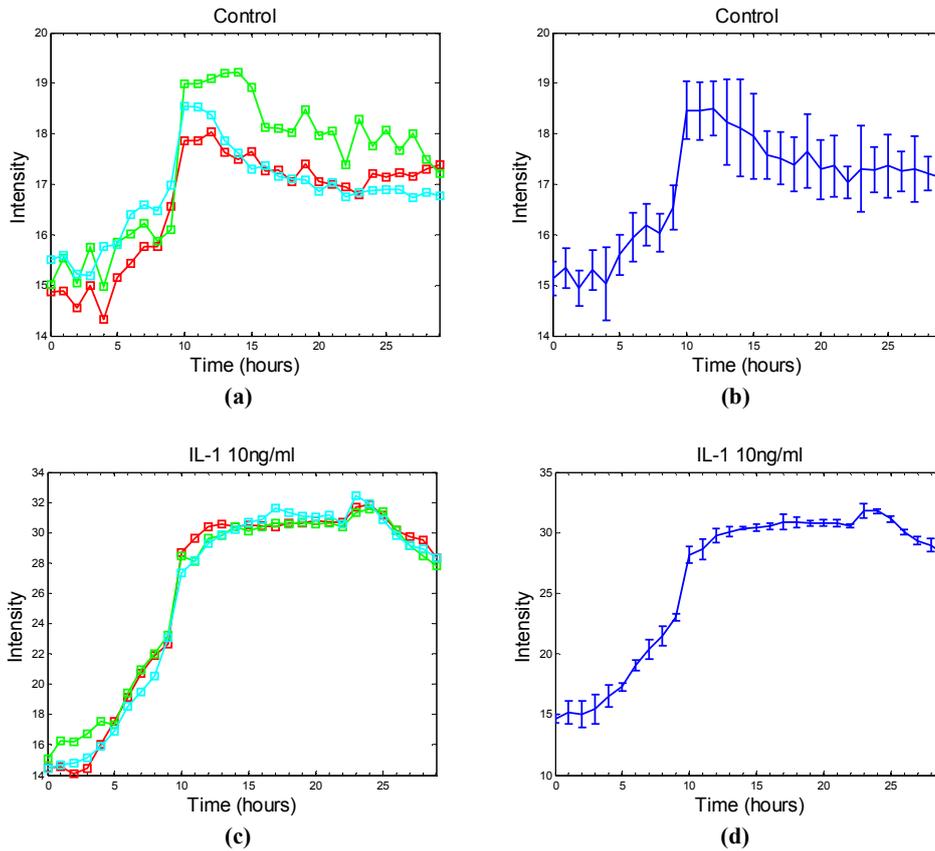
Figure 21: Absolute intensity profiles: (a) absolute intensity profiles for control positions; (b) the Error-bar profile for control positions; (c) absolute intensity profiles for stimulated positions; (d) the Error-bar profile for stimulated positions
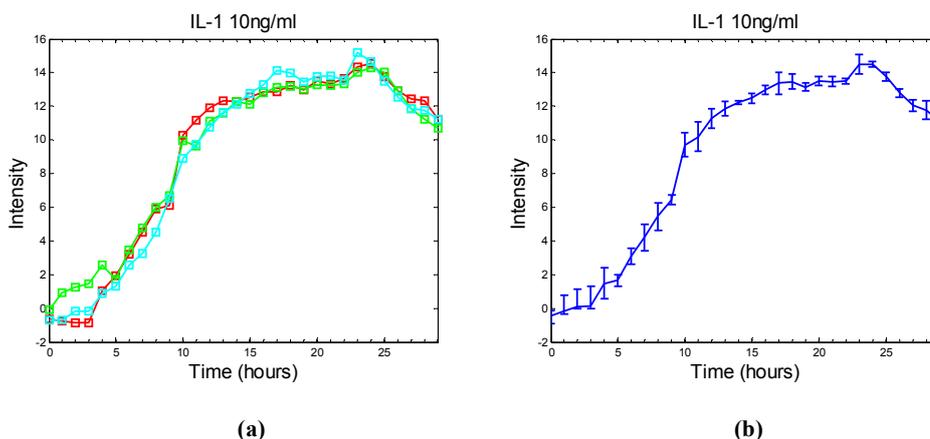
Figure 22: Relative intensity profiles: (a) relative intensity profiles; (b) Error-bar profile

2.2.4 Save data

Once all necessary profiles have been plotted, the data can be exported and saved by the following steps:

1) Select the file format in which to save the data from the pop-up menu under the "Choose file format:" (highlighted in green in Figure 23). The fluorescence intensity profile data can be exported to a mat file (i.e. intensity.mat) and an Excel file (i.e. intensity.xls).
2) Click the "Save" button (highlighted in red below), and the data will be saved in the same folder as the original image data.
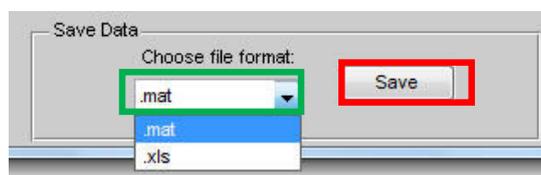

Figure 23: Interface to save fluorescence intensity profile data

2.3 Run the program

The following procedure is implemented to run the program:

1) Put all the MATLAB programs in the same folder where the folders of the images for different positions locate.
2) Make sure the path of the MATLAB command window is set to the folder containing all MATLAB programs.
3) Type ImageAnalysis in the command window of MATLAB. After this, the graphic user interface shown in Figure 9 pops up.
4) Follow all the instruction described in Section 2.2 to analyze images.

Acknowledgment