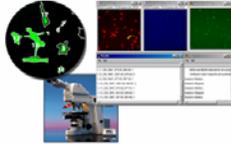




Quantitative Cell Biology



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Document: Indicator Cell Analyzer Program Installation and Operation

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I. Introduction

Indicator Cell Analyzer (ICA) is a java-based plugin written for the publicly available ImageJ image analysis and processing program. It performs a specific analysis

of corresponding three-color images of cells that have been collected using three-color fluorescence microscopy. One set of images is used to define each cell object, a second set of images is used to identify the number of nuclei in each cell object and the third set of images are used to quantify the presence of an indicator in each cell such as cytoplasmic green fluorescent protein or fluorescein isothiocyanate conjugated antibody (see Figure 1). It was specifically developed for evaluating cell stained with the high-contrast staining methodology developed in our laboratory and imaged with automated-fluorescence microscopy. In general, cells are cultured on polystyrene or other substrates, fixed, stained with Texas Red C₂ maleimide and DAPI. These general purpose stains provide high contrast images of the cell edge and nuclei, respectively. If the cells are also stained with a third probe with fluorescein like optical properties or express GFP, then the level of the third signal is quantified in a region defined by the Texas Red stained cells. In the absence of a third indicator signal, the ICA program will analyze images and provide high quality morphology measurements of the cells in culture. Cell objects can be classified as an individual cell or a cell cluster and morphological properties of each class can be determined. Only images of Texas Red C₂ maleimide and DAPI stained cells are required for morphological analysis.

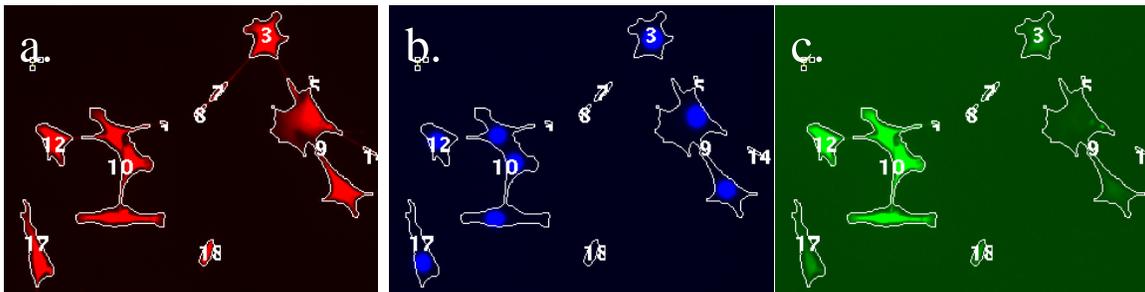


Figure 1. Corresponding images of fixed NIH-3T3 cells expressing cytoplasmic GFP collected with three-color fluorescent microscopy. White ROI lines were obtained with image analysis software. a. Tx-Red maleimide whole cell stain; b. DAPI stain; c. cytoplasmic GFP.

The initial ICA routine was written for the Innovision ISEE image analysis software package. Although these routines were completely functional, they could only be implemented by users of the ISEE software. Furthermore, low-level software access is not available with this program so user friendly aspects of the program interface could not be improved. To make the program available to any user, we decided to transfer the software routines to the ImageJ platform written by Wayne Rasband at the National Institutes of Health. This image analysis program is open source and is written in JAVA which allows it to be run on any operating system that has a virtual JAVA engine. This includes computers with Microsoft, Macintosh and Linux operating systems. Once the ICA program was written for ImageJ, we validated the results obtained from the ISEE and ImageJ versions using the same set of test images. This ensured that any unidentified bugs in the ICA software does not impact the quantitative results obtained from the program.

Operation of the ICA program is relatively self-explanatory. For any measurement of microscopy images, you only need two threshold numbers: one for cell edge definition and a second for nucleus detection. You can also set maximum and minimum size of object accepted as a cell. This latter setting can often limit the inclusion of cell objects that are very large cell clusters or unidentified debris. The

quantified properties for each cell object are listed in a results text box and the list is imported into a spreadsheet program for analysis. The ICA program can be used for only Morphology Analysis if an indicator directory is not selected. Below are instructions for installing and using the ICA program.

III. Installation:

1. Download ImageJ (v1.32j or greater) and install as described. Start the Image J program to ensure successful installation.
2. Download ICA_v2dot8.java text file (or latest version) and save it under the ImageJ plugin directory (i.e. c:/Program Files/Image J/plugin).
3. Download ICA_test_images.zip (optional). Extract the archive and save it under the ImageJ directory. This archive is large (300 Mbytes) and contains 3 directories of 50 microscopy images that can be used to test the ICA program. Under the ImageJ directory should be a “red_tiff”, “blue_tiff” and “green_tiff” directory with files in each directory. Also a test file of the expected results (test_results.txt) will be extracted from the archive.
4. In the ImageJ program, click Plugins->Edit->ICA_v2dot8.java. A new window named ICA_v2dot8 should open showing the JAVA source code for the plugin (see Figure 2).

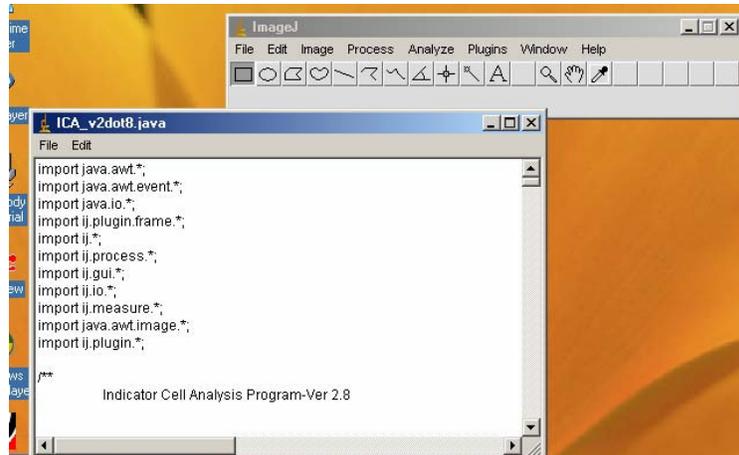


Figure 2. Opening the JAVA source code for the ICA program. The compile command is under the “File” menu of this window.

- In the ICA_v2dot8 window, click File->Compile and Run. The ICA_v2dot8 program should be compiled into executable code and a new window should appear. (see Figure 3) This indicates the program compiled correctly.

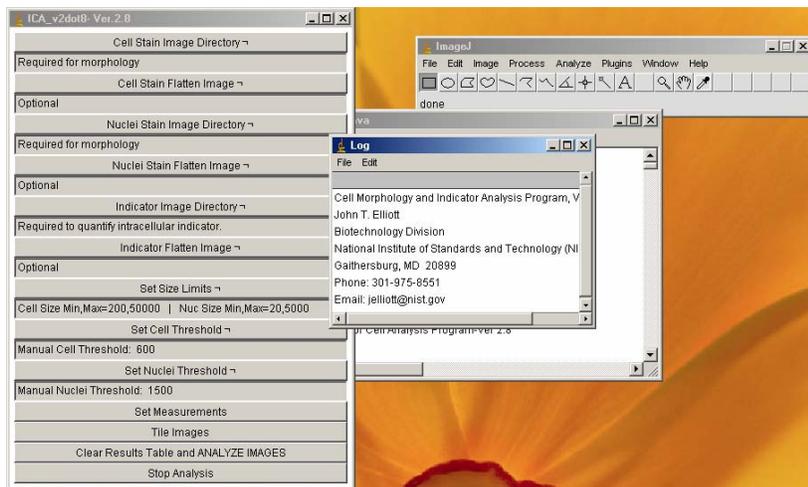


Figure 3. After compiling ICA program, the ICA control panel, Log and Results windows should open automatically. This indicates the program compiled successfully.

- Close Image J and restart Image J.
- To run ICA, click on Plugins->Edit->ICA_v2dot8 to start the ICA program.

After ICA has been compiled, only step 7 is required to run the program from ImageJ.

III. Operating ICA for Cell Morphology

The ICA_v8 interface is shown in Figure x. Using the test images as an example, here are the steps to operate the program without an indicator image.

- Press “Cell Stain Image Directory”. Browse to ICA_test_images/red_tiff. Select a file in the directory and press “OK”.
- Press “Cell Nuclei Image Directory”. Browse to ICA_test_images/blue_tiff. Select a file in the directory and press “OK”.
- Press “Set Size Limits”. The default values are adequate for most cell types.
- Press “Set Cell Threshold”.
 - Use the Brightness control until cell edge can be visualized.
 - Use the Threshold control until the blue boarder touches the cell edge.
 - Use Next Image to test the threshold on the next image. Adjust appropriately.
 - When an appropriate Threshold value has been determined, press OK.

5. Press “Set Nuclei Threshold”. Follow a-d in step 4.
6. Press “Set Measurements”. Select the measurement parameters to collect.
7. Press “Tile Images”. Adjust the windows so the images and text files can be easily observed.
8. Press “Clear Results Table and ANALYZE IMAGES” to start analysis.

The computer generated ROI outlines of each object should be briefly visible as the program runs. Data should be printed in the results table.

Transferring Data into a Spread Sheet.

The data in the results file can be saved and opened with Spread Sheets like MS Excel or it can be directly copy and pasted into the spread sheet. The data is delimited by commas. Below are the instructions for MS Excel.

1. Start MS Excel.
2. Select the ImageJ results window, click Edit->Select All, then click Edit->Copy.
3. Select the MS Workpage, highlight box A1, and click Edit->Paste. All of the data should be pasted in column 1.
4. In MS Excel, highlight column 1, click Data->Text to Columns. Click the “Delimited” button, click “Next”, click the “Comma” check box, then click “Finish”.

The data should be separated into columns and can be analyzed. Only the basic measurements are provided. Parameters such as roundness, axial ratio, etc. can be calculated from these values.

ICA operation (Morphology and Indicator):

Using the test images as an example, here are the steps to operate the program with indicator image.

9. Press “Cell Stain Image Directory”. Browse to ICA_test_images/red_tiff. Select a file in the directory and press “OK”.
10. Press “Cell Nuclei Image Directory”. Browse to ICA_test_images/blue_tiff. Select a file in the directory and press “OK”.
11. Press “Cell Indicator Image Directory”. Browse to ICA_test_images/green_tiff. Select a file in the directory and press “OK”.
12. Press “Set Size Limits”. The default values are adequate for most cell types.
13. Press “Set Cell Threshold”.
 - a. Use the Brightness control until cell edge can be visualized.
 - b. Use the Threshold control until the blue boarder touches the cell edge.
 - c. Use Next Image to test the threshold on the next image. Adjust appropriately.
 - d. When an appropriate Threshold value has been determined, press OK.
14. Press “Set Nuclei Threshold”. Follow a-d in step 4.

15. Press “Set Measurements”. Select the measurement parameters to collect. The measurements specifically for the indicator cells are arranged in a separate section of the window..
16. Press “Tile Images”. Adjust the windows so the images and text files can be easily observed.

Press “Clear Results Table and ANALYZE IMAGES” to start analysis.

Additional notes:

1. All images must be 16 bit TIFF files. It has only been tested with 12-bit images (0-4096). Modifications may have to be made for other image depths.
2. The images for each color must be in a separate directory and must have an index “.001”, “.002” following it. The indexes between the images must correspond to each other. The program relies on the automatic numerical ordering of the files in the folder to keep all files in sync.
3. Flattening images can be used for each directory. This may improve thresholding or indicator quantification during analysis.
4. For indicator quantification, a local background is calculated by eroding the cell object 3 times and calculating the average background in the area immediately outside of and surrounding the cell.
5. Although this program was developed to perform image analysis on images collected by automated microscopes, it can also be used to analyze images collected manually. The only requirement is that each specific image in the directory (cell edge, nuclei, indicator) must correspond to the same cells.
- 6.
7. Questions? Email me at jelliott@nist.gov.

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