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Irises: a practical tool for image-based analysis of cellular DNA content

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Irises

User Manual

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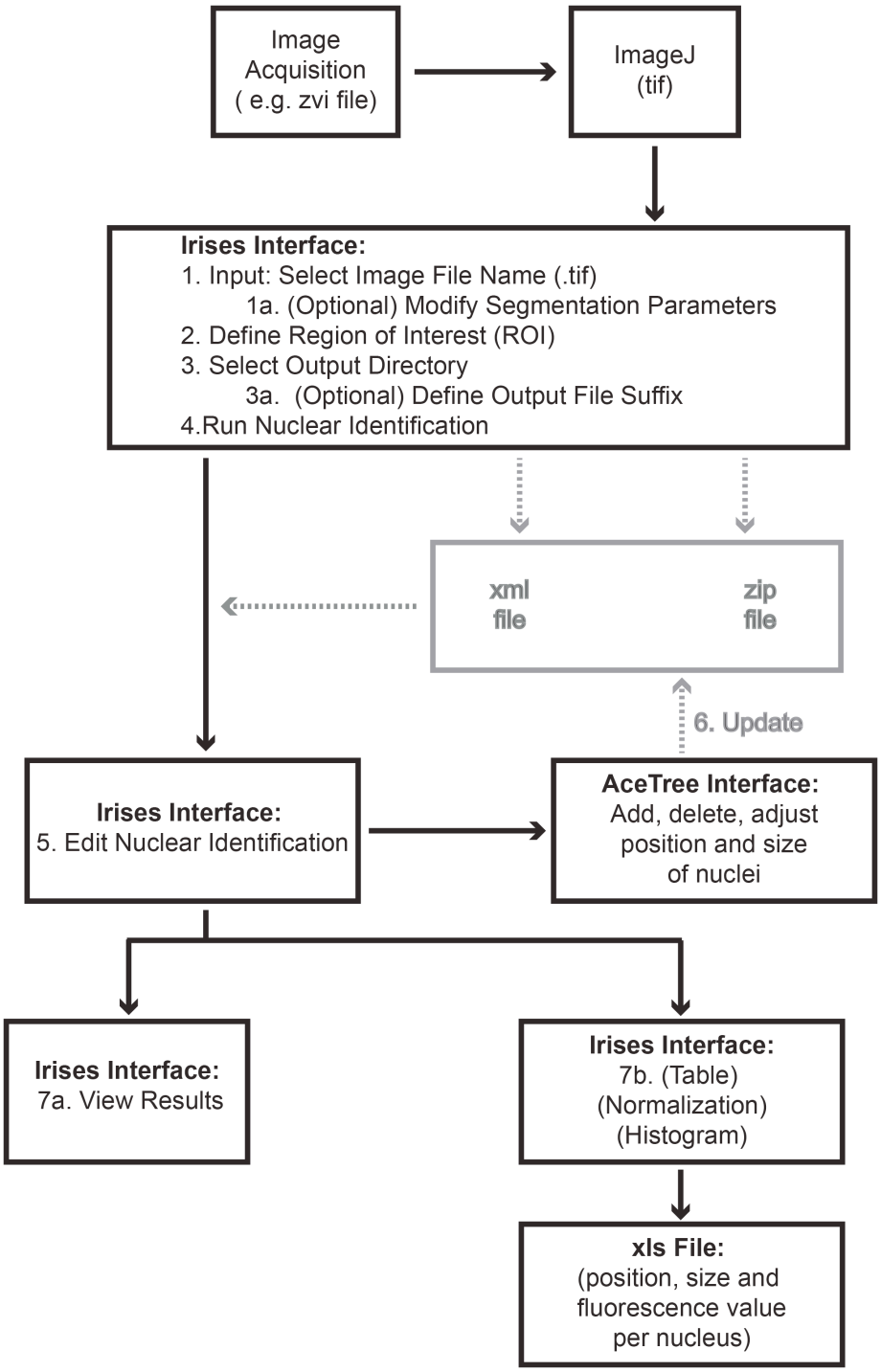
Workflow Diagram



To download and install Irises, see the **Download and Installation Instructions** at the end of this document.

The following steps prepare for image input:

To start an analysis, create a new folder. You will create various input files for your analysis as instructed below. Put all of these files in this folder. Irises will ask for this folder and will save the results into it.

The Irises Interface:

The Irises Interface is designed with a top-to-bottom workflow in mind. Steps 1-4 represent the main input-to-output flow of the program. Steps 5-7 represent modifications and analyses of the data generated by steps 1-4. Step 5 allows the user to edit output files. Step 6 updates image intensity data stored in the zip file given edits made in step 5.

Running the analysis, starting with your image file

1) Open Z-stack file in ImageJ. Save as .tif file (File>Save as>Tiff…). Save the file to the folder created above. Other output files will be added to that folder below.

   STEPS 2-4 FOR MAC USERS ONLY. PC USERS DOUBLE CLICK ON Irises.exe AND SKIP TO STEP 5

2) Find the path to the file Irises.app on your computer.
   Example: To locate the path, select the file from the finder window and choose "File>Get Info". Copy the information under "Where" in the "Get Info" window after the first apostrophe up to the backslash before the file name.
3) Open a Terminal window by opening the Terminal application found under Applications>Utilities>Terminal.app. In the window that opens, type "cd[space]" and then paste or type the path to the folder that contains "Irises.app". Press enter.
   Example command: "cd /Users/username/Desktop/Irises/" (note that in some cases, the command line supplies home directory information by default, in which case the information after "username/" will suffice)
4) Type "./run_Irises.sh", press enter and wait for program to open (may take a minute; ignore Time Machine bypass error messages if they appear).).
   Note: Even while using the Irises Interface, the open Terminal window will give information on the progress of each step and will alert user if there are errors.
5) This will open a graphical user interface (GUI).
   Note: if you wish to scale the GUI window size it must be done proportionally to preserve the image appearance, but this does not affect the underlying calculation.
6) Irises Interface Step 1: Browse to find desired image (.tif file created in step (1) above), select it and wait for it to load. You will see a z-projection of the image.

7) Irises Interface Optional Step 1b: If you wish to edit any parameters from the parameter file for this image only (i.e. not a change you want to save), you can do this using the button labeled "Optional: Modify Segmentation Parameters."
8) Irises Interface Step 2: Define the Region Of Interest (ROI). (If necessary, return to the .tif file to find landmarks that may be less obvious in this rendering of the z-projection). Click once on image and move cursor slightly to activate cursor shape change (arrow to +). Click (but do not hold) once again to begin outlining ROI. A blue dot should appear marking the start point, and a line should appear from the start point as you define the ROI. Click to change the direction of the line and define the ROI polygon; each click will define a corner of a polygon. To finish the polygon, click on first point again to close – the cursor appears as a circle when you are over the starting point. Once a ROI is drawn, the next click will delete that ROI and you can start another one to analyze. Only one ROI can be analyzed at a time.

   Note: errors messages in the Terminal window referring to "finalpoints" may indicate aberrant ROI definition. In this case, quit Irises and return to the Terminal window "./run_Irises.sh" command.
9) Irises Interface Step 3: Use the browse button next to "Output directory:" to select the destination of your output.
10) Irises Interface Optional Step 3b: To individually distinguish files for each ROI, you can add a suffix at this step that will be added to all output files generated for that ROI (e.g., to match the region of the germ line you selected, i.e. proliferative, pachytene, etc.). This suffix will be added to the name of the output file. This makes it easier to keep track of output files later. **Otherwise successive ROIs for the same image output to the same location may overwrite previous results.**
11) Irises Interface Step 4: Click "Run Nuclear Identification". You can follow progress in the Terminal window. When it has completed the analysis it will return "All nuclei completed."
12) The segmentation results will be written to two ".zip" and two ".xml" files in a subfolder in your output directory. The file names will be related to your image file name and one of the two of each will include the suffix "_edited" in the filename. All later steps in both Irises and AceTree should be performed on the "edited" version of these files while the other remains clean for re-editing if necessary. The .zip files represent the numerical data generated by the program and the .xml contains links to the zip file and the image associated with that numerical data.

ALL SUBSEQUENT STEPS ALLOW EDITING AND ANALYSIS OF .zip AND .xml OUTPUT FILES GENERATED ABOVE

The user can open previously generated output files or work on newly generated files using the steps below.

13) Irises Interface Step 5: Click "Edit Nuclear Identification" to open the AceTree interface to modify the nuclei defined by the main program above. ***Manual editing nuclear identification is essential for accurate results and CANNOT be skipped.*** Instructions for this modification using AceTree are in a separate section below.

---------- Go to **Editing nuclear identification with AceTree** below----------

14) After you have performed the necessary edits in AceTree and saved the modified .zip file, quit AceTree Launcher and return to the Irises Interface.
15) Irises Interface Step 6: Click on the "Select xml file" to select the .xml file that you edited in AceTree. Once the file is selected, the fluorescence intensities for each nucleus will be calculated automatically for its corresponding .zip file. This updates the intensity values in the zip file to match the edits to nuclear positions already saved in the .zip file. If not done, edited nuclei will lack correct intensity values.
16) Irises Interface Step 7a: Click "View Results" to get a quick look at a data set. Another GUI will open. Use the Browse button to select the .zip file you saved in step (5) of the AceTree section below (intensity values will only be correct after you have done Step 6 above). In addition to displaying the data, the information will be automatically saved as an Excel file in the same folder. The Excel file is a comma separated value file; select the comma delimiter option under [Data>Text to Columns…] to display the X, Y, Z coordinates, cell diameter and fluorescence intensity for each nucleus.
17) Irises Interface Step 7b: To graph data and/or to use one ROI to normalize another ROI, click "Calculate and display normalized data" at the bottom of the results window. This will open another GUI where you can browse to select a .zip file to analyze. The user can graph raw fluorescence data (unnormalized) by clicking the "None" button. Browse to open a .zip file for the ROI to be analyzed. This is the only input file needed to graph unnormalized, raw fluorescence data. Or you can normalize one ROI to another ROI of known haploid equivalence (N-value). Choose either the 2N or 4N button depending on whether your ROI of known N-value is 2N or 4N. If the "2N" or "4N" buttons were clicked, browse to open a second .zip file for the ROI to use for normalization. If you would like the displayed histogram or an excel file of the normalized values to be saved, click on the appropriate "select to save" buttons and browse to an output directory to which it will be saved. Click "Calculate N values and plot in histogram" and your results will be displayed. As above (step 16), the Excel file is a comma separated value file.

Editing nuclear identification with AceTree

1) After clicking "Edit Nuclear Identification" (step 5 within the Irises interface), an AceTree window opens. Alternatively, you can double click on "AceTree.jar" file from the Finder window.
2) To open the .xml file related to your image, go to "File" in the AceTree window and click "Open config file" and choose the ".xml" file related to the series you are interested in (open the "_edited" version; it will be located in a subdirectory within the output directory you defined in step 3 on the Irises Interface, bearing the same folder name as the output directory).
When you open the file, the image will come up as a z-stack with circled nuclei that Irises has identified. These circles define the nuclei of interest. The AceTree interface lists each nucleus/circle defined within the entire stack. Clicking on the circle in the image will show/hide its identity. To select the z-center of the circle, control-click on the image or select in the list. Double-clicking on the list entry for a nucleus brings up the z-layer containing the center of the nucleus. Selected circles are white; all others are purple.

Example: "Nuc001_4_830_466" on the list defines nucleus in Z plane 4 and XY coordinates 830 and 466

3) Navigate through the image slices by clicking on the image window and using the "Up/Down" arrow keys on the keyboard. Start from the top of the stack (see image header – "p01" is the first plane) and navigate down.

   Note: Pressing the left/right arrow keys may produce a black image but you can navigate back to your image by return-navigating left/right.

   Notes on defining nuclei: The circles in each plane are a slice through the sphere defined by Irises. Every sphere defined by the circles should encompass the entire fluorescence of one nucleus without including extraneous fluorescence from neighboring nuclei.

   Each acceptable nucleus will therefore be defined as a small circle in one plane with increasing diameter in neighboring plane(s), and symmetrically smaller diameter until it is no longer associated with the nucleus.

4) To adjust, delete or add circles, go to the AceTree interface "Edit>edit tools". This will bring up two new windows, labeled "Adjust or Delete Cells" (used to adjust existing circles, delete circles, or delete all circles in an entire Z-plane) and "Edit Track" (used to add nuclei/circles).

   Note: Only those layers that contain **whole nuclei** should be used in the analysis; ~15-20 layers are typically included in the analysis of *C. elegans* germ cells where the distance between each layer is .5 μm.

   a. To add a circle, click on the "Use ROOT Cell" button in the "Edit Track" panel. Check the box marked "Is early set correctly?" and click "Add Intermediate Cell". The next click on the image window will add a circle. Then click "Stop Adding." Repeat for further additions.
   b. To adjust a circle, click on its designation in the AceTree list and you can change its size or position using the "BIG", "SMALL", "LEFT", "RIGHT", "UP", "DOWN", "INCZ" and "DECZ" buttons. The last two change the plane that the nucleus is centered on; this is particularly useful to separate nuclei that otherwise overlap in the Z plane. If these adjustments do not resolve the problem, delete one or both circles. Size and position can also be changed by typing in the appropriate numbers in the spaces next to X, Y, Z, and D.
   c. To delete a circle, select the circle on the list and click "Kill cell." Click "Apply changes" to delete. Alternatively you can delete nuclei with the Delete Key on the keyboard.
   d. To delete cells above or below a given Z-plane click "Kill Group" and indicate *in the "Kill Group" window* (NOT on the image) from which layer number above or below which all circles should be deleted. The + and – buttons change the plane number in the "Kill Group" window. The "Above" and "Below" buttons may not be mutually exclusive so be sure that only one of them is checked. The "Estimate" button will provide the number of nuclei that will be deleted from the analysis. "Kill Cells" will perform the deletion.

      Note: If the image or AceTree interface is not responsive after this manipulation, close the "Kill group" window.

5) To save the changes made, go to AceTree interface "File" then "Save nuclei as zip". Click on the "_edited.zip" to overwrite the file originally designated with this name. This saving can be done at any time and it is recommended that you save often because there is no undo button. If

you made a mistake, you can reverse it by closing and reopening AceTree, but you will lose all unsaved changes.

6) Return to step (15) above (step 6 on the Irises Interface) to finalize these changes.

To return to previous files and analysis

The Irises interface can be used to view, edit and analyze results from any existing .zip or .xml file previously generated by Irises and AceTree by using the buttons in steps 5-7 of the Irises Interface.

Background Correction

Though not necessary in our images and analysis, in some circumstances background correction may be useful to remove intensity originating from out of focus light or from background staining. This is particularly the case when signal is weak relative to background intensity. If a uniform background correction is needed, during editing a dummy cell can be added in an empty region of the image. Follow the instructions above for adding a circle. Once results are generated the expression value for this 'cell' can be subtracted from all others in Excel. The dummy cell can then be deleted from the file and the results saved with an alternative name before plotting histograms or any other downstream analysis. A local background correction value specific to each cell is sometimes useful to avoid signal interference between tightly packed cells or in the case of spatially uneven background intensity. Local correction values per cell (see [4] for details) can be found in the raw nuclei .zip file output.

Image collection for *C. elegans* germ line studies

Images used in development of this tool were collected as follows:
L4 stage larvae were ethanol fixed, DAPI stained and mounted on 5% agar pads as previously described [1-3]. Sequential images at 0.5µm Z intervals were captured on a Zeiss Z1 Axioimager with Apotome using and AxioCamMRm digital camera and Zeiss AxioVision software, and were saved in the Zeiss zvi format. We found that images collected from either a 40x and 63x oil immersion objectives gave similar results without modification to the parameters used in Irises other than editing the number of slices setting in the parameter file to match the number of z-planes in the image stack.

References

1. Korta, D.Z., Tuck, S., and Hubbard, E.J. (2012). S6K links cell fate, cell cycle and nutrient response in C. elegans germline stem/progenitor cells. Development *139*, 859-870.
2. Michaelson, D., Korta, D.Z., Capua, Y., and Hubbard, E.J. (2010). Insulin signaling promotes germline proliferation in C. elegans. Development *137*, 671-680.
3. Pepper, A.S., Killian, D.J., and Hubbard, E.J. (2003). Genetic analysis of Caenorhabditis elegans glp-1 mutants suggests receptor interaction or competition. Genetics *163*, 115-132.
4. Murray, J.I., Bao, Z., Boyle, T.J., Boeck, M.E., Mericle, B., Nicholas, T.J., Zhao, Z., Sandel, M.J., Waterston, R.H. Automated analysis of embryonic gene expression with cellular resolution in C. elegans. Nature Methods. 2008; 5(8):703-9.

Download and Installation

You should **download** the following item available via SourceForge (http://sourceforge.net/projects/irises/files/)**:**

compiled_mac.zip or compiled_pc.zip

Unzip.

One time setup for computer to be able to run analysis:

Install MATLAB Compiler Runtime:

For MAC --

1) Go to http://www.mathworks.com/products/compiler/mcr/index.html and click the link under row R2013b and column Mac to download "MCR_R2013b_maci64_installer".
2) Double click "InstallForMacOSX".
3) Click "Next" to being installation. Accept the terms of the license agreement and hit "Next".
4) You will be asked where to select an installation folder. The default should be "/Applications/MATLAB/MATLAB_Compiler_Runtime". If it is not, choose this folder. Click "Next" and create the folder. **Note: that if the compiler runtime is already installed in a different location it will be necessary to edit the contents of run_irises.sh to point to this alternate location.**
5) Click "Install" and wait for installation to complete. Click "Next" when told "Your installation may require additional configuration steps." This is already taken into account.

For PC --

1) Go to http://www.mathworks.com/products/compiler/mcr/index.html and download the R2012b Windows version that is appropriate for your machine.
2) Double click "MCR_R2012b_win32_installer".
3) Click "Run".
4) After a brief wait, it will bring up a screen asking you to install "MATLAB Compiler Runtime 7.16". Click "Next", accept the license agreement then click "Install".
5) When the installation is done, click "finish".
6) Typically it is necessary to reboot the PC even if not prompted to.

Initial Setup:

Each lab will have to set up the program optimized for their own purposes. There is a single text file, named "example_parameter_file.txt" which is used to specify the parameters Irises requires to operate, including the paths to necessary files, calibration of xy and z microns per pixel, etc. The default file is a starting point, but several parameters may need to be optimized for each application. It is recommended that the user save a copy of the example_parameter_file.txt before editing. The one

intended for use with the program should maintain the name “example_parameter_file.txt.” The annotation in the parameter file explains what each parameter tells the program and how to edit for better results. The most commonly altered parameters can be changed within the Irises Interface for that particular run of the program (optional step 1b in the flow chart), but these changes will not be made within the saved parameter file.

The parameters that often need to be edited are listed below. **It is recommended that you do not change the other parameters.**

Note: only change parameters to the right of equal signs and only change them when you need to.

1) *firsttimestepdiam*: specifies the approximate diameter of nuclei in pixels. This should only need to be changed if nuclei are grossly abnormal in size.

   Note: For *C. elegans* germline nuclei, although the actual size of nuclei in pixels changes slightly relative to the magnification, we found that an estimated diameter of 30 works well for images captured by either 40x or 63x immersion lenses. The *boundary_percent* parameter (below) compensates.

2) *xyres* and *zres*: tells you the size of each pixel in microns. XY is within the image plane and Z is between them. If the size of each pixel in microns is not known, open the image file (.zvi or .tiff, see below) in ImageJ. The information can be found in Image>Properties or Image>show info under “scale factor for X…for Y…for Z”.

3) *slices*: number of planes in the image.

   NOTE: the default is for Irises to analyze all planes. This parameter can be set to fewer planes than are in the stack within the GUI to exclude unwanted planes for a particular analysis.

4) *parameters.intensitythreshold*: specifies the intensity threshold to identify nuclei. Lower this number if nuclei with weaker fluorescence need to be identified.

   Note: For *C. elegans* germline nuclei, an intensity threshold of about 6 is optimal under the conditions used to collect example images associated with this paper.

5) *parameters.boundary_percent*: this specifies the ratio between the brightness outside a nucleus and inside a nucleus. A lower value indicates a larger difference between the two areas. To improve accuracy and reduce manual corrections (see below), increase this number if background is high or lower this number if background is low.

   Note: For *C. elegans* germline nuclei, a combination of *firsttimestepdiam*=30 and *parameters.boundary_percent*=.14 is optimal for performance under the conditions used to collect example images associated with this paper.

As long as all program files, including the parameter file, are kept in the same folder and are not renamed, Irises will be able to find and use them. This does not include input and output files which are specified using browse buttons within the Irises Interface. If a file is moved or renamed, the parameter file will need to be edited to reflect these changes. To avoid this situation, it is recommended that the files downloaded as part of the Irises package are not moved or renamed.

You should also have

An image stack to analyze. Any format can be used, provided it can be opened in ImageJ and saved as a tif stack (see instructions on using Irises).