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Irises

A practical tool for image-based analysis of cellular DNA content

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The DNA content of nuclei is a valuable measure of cell cycle status. Irises is a software tool to facilitate systematic in situ determination of DNA content for cell cycle analysis at single-nucleus resolution within complex tissues. We demonstrate the utility of the tool with analysis of DNA content in germline nuclei of *C. elegans*. Compared with results obtained by manual analysis, we find the tool greatly facilitates analysis by improving speed at least 5-fold while maintaining accuracy. The source code and instruction manual (including installation for both Mac and PC) are provided.

Introduction

In many areas of cell biological inquiry, knowing the cell cycle status of interphase nuclei in their native position within a tissue is critical. In contrast to fluorescence-activated cell sorting (FACS) where tissues are disrupted and individual cells isolated, in situ analysis is achieved by high-resolution imaging after fixation of the tissue and labeling of DNA with fluorescent dyes such as propidium iodide or 4',6-diamidino-2-phenylindole (DAPI). The relative distribution of fluorescence intensity is then correlated with ploidy (N) to determine which cells are in G1 (2N), S-phase, or G2 (4N) within interphase.[1] Despite its wide use in biology, data analysis in the image-based in situ analysis is not as highly automated. Manual analysis is often prohibitive for large numbers of cells.

The *C. elegans* germ line contains a population of stem/progenitor cells for which cell cycle is an important regulatory target of signaling by the insulin/IGF-like and TOR pathways, among others.[2-4] This tissue is not amenable to FACS analysis since each germ cell retains a small opening to a core of shared cytoplasm. In the past, the number, position, and DNA content of interphase nuclei were assessed by quantifying fluorescence from individual planes within a stack of images (pseudo3D by optical sectioning).[4-7] This technique requires manual identification and segmentation of each nucleus. While reliable, the process is extremely laborious, limiting the practicality of quantitative analysis.

Results

Here we present Irises, a software tool to facilitate the in situ analysis of cellular DNA content, using the *C. elegans* germ line as a test case. Irises uses an automated algorithm to segment nuclei from images and quantify DNA content. Each identified nucleus is modeled as a sphere and the DNA content is estimated by calculating the total pixel intensity of fluorescence within each sphere.[8] Meanwhile, it uses supervised data analysis, facilitating user interaction with the data to perform additional analysis such as normalized ploidy in biologically relevant regions.

The Irises Graphical User Interface (GUI) is shown in **Figure 1A**, with a summary of the workflow outlined in **Figure 1B**. First, given an input image stack, the GUI displays a maximum projection of the stack. Second, it enables the user to define a Region of Interest (ROI) in the input images using mouse clicks. Third, automated segmentation is performed in which the program identifies nuclei within the ROI. Fourth, Irises allows users to conveniently examine and edit the automatically identified nuclei for quality control. Editing is done in an additional GUI in which users can add, remove, or adjust the position and size of each individual nucleus. In addition, nuclei near the top and bottom of the image stack that may be unreliable due to optical aberrations can be removed in bulk by excluding all nuclei from specific image planes.[9] Fifth, Irises calculates the fluorescence value per nucleus. For each

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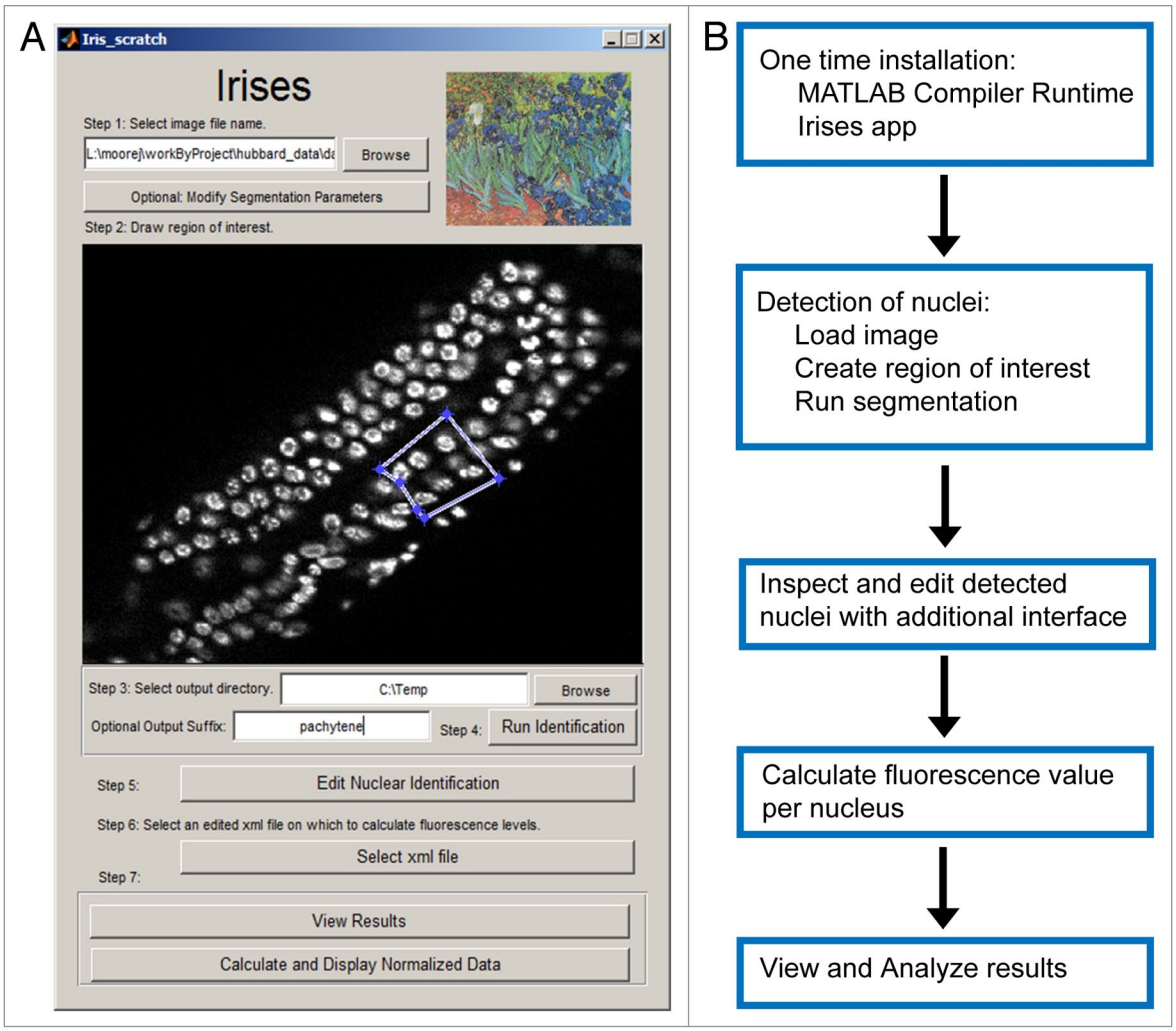
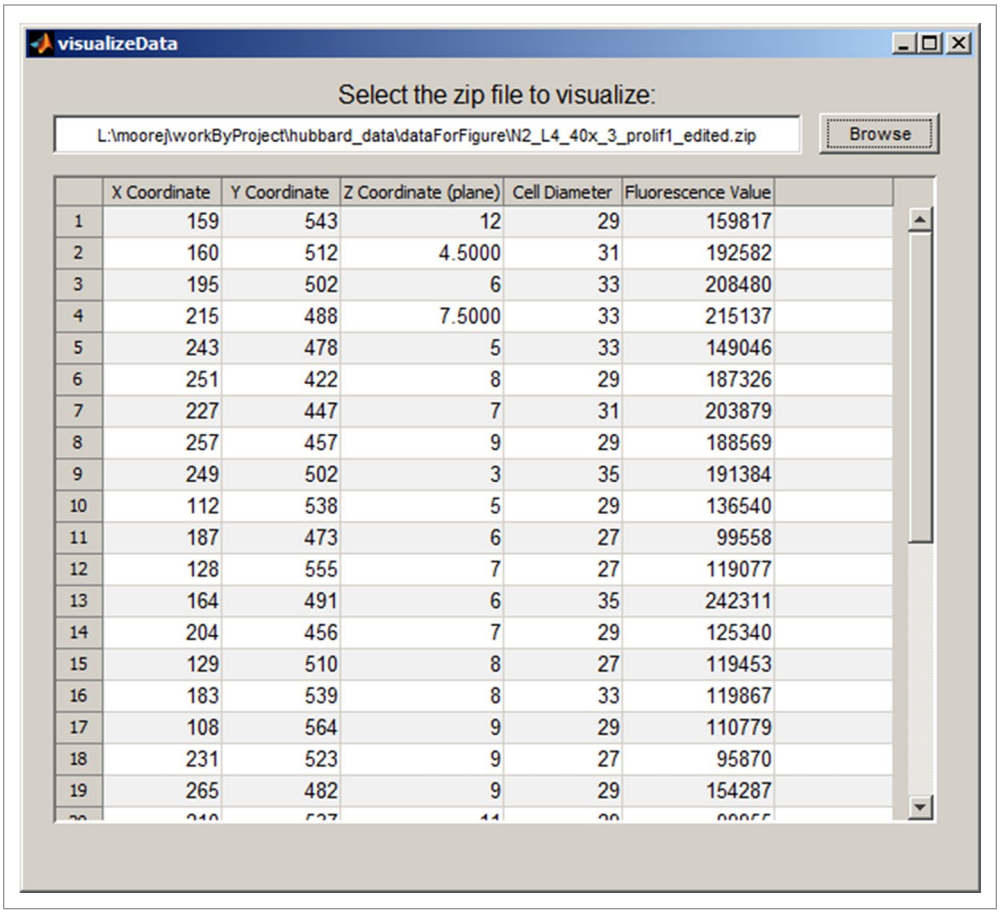


Figure 1. Irises Interface and Workflow. (**A**) Screen capture of the Irises application. A region of interest containing nuclei in the pachytene stage is defined and is ready to be segmented. (**B**) Diagram of Irises workflow.

detected nucleus, the results include its position (the x, y, and z coordinates of its center), size (diameter in pixels), and the fluorescence value (**Fig. 2**). The results are saved in tabular form, which allows users to export the results and perform additional manipulations using other tools such as Excel.

Finally, Irises performs additional analysis to automatically create histograms of raw fluorescence values or normalized N-values (ploidy) by comparing with nuclei of known DNA content. Irises allows the user to sequentially define and analyze multiple ROIs from the same image stack. **Figure 3** provides an example analysis of two ROIs in an image stack of the *C. elegans* germ line. The ROI in **Figure 3A** (see also **Fig. 1A**) contains nuclei in the pachytene stage of prophase of meiosis I that are known to possess 4N content. The ROI defined in **Figure 3B** contains proliferative zone nuclei of unknown DNA content, which can be normalized to the data collected from the first ROI to infer the ploidy of each nucleus.

To test Irises, we compared its output with manual DNA quantification. We generated and analyzed images from 4th larval stage (L4) wild-type and *daf-2(e1370)* mutant animals. DAF-2 is the sole insulin/IGF-like receptor in *C. elegans*. Among many biological roles for this receptor, it is required in the larval germ line to promote the accumulation of proliferative germ cells.[3] The Irises-generated distribution of DNA content among nuclei in the proliferative zone (the distal-most 13 cell diameters, normalized to 4N pachytene nuclei in the same image) is shown from one individual gonad arm from each genotype (**Fig. 3C**). For the wild-type, we also determined that morphologically metaphase and prophase nuclei contained a G2 DNA content (≥ 4N) while each half of an anaphase figure yielded a G1 or early S phase DNA content (≤ 2.7N; data not shown). The Irises analysis indicates a shift in the *daf-2(e1370)* mutant to a greater proportion of nuclei in late-S and G2 (≥ 3.3N, **Fig. 3C**). These results are fully consistent with our previously published



	X Coordinate	Y Coordinate	Z Coordinate (plane)	Cell Diameter	Fluorescence Value
1	159	543	12	29	159817
2	160	512	4.5000	31	192582
3	195	502	6	33	208480
4	215	488	7.5000	33	215137
5	243	478	5	33	149046
6	251	422	8	29	187326
7	227	447	7	31	203879
8	257	457	9	29	188569
9	249	502	3	35	191384
10	112	538	5	29	136540
11	187	473	6	27	99558
12	128	555	7	27	119077
13	164	491	6	35	242311
14	204	456	7	29	125340
15	129	510	8	27	119453
16	183	539	8	33	119867
17	108	564	9	29	110779
18	231	523	9	27	95870
19	265	482	9	29	154287

Figure 2. The View Results window in Irises provides a tabular view of computed nuclear positions, sizes, and fluorescence for the region of interest.

manually collected data.[7] In addition, analysis with Irises is at least five times faster per sample, reducing the analysis from approximately 4 h to 45 min per L4 gonad arm.

The source code, a workflow diagram, and detailed instructions for Irises are provided in Supplementary Material, as well as methods for image collection used in this study.

Discussion

Irises is a useful software tool for image analysis to measure DNA content from 3D fluorescence images in a systematic and objective fashion. It is worth noting that proper setup for image acquisition is a vital step to obtain the appropriate data for informative measurements. In particular, one should consider the appropriate spacing between z planes given different types of microscopy. Detailed considerations and guidelines can be found in a recent review.[10] Two features in Irises help users to evaluate potential over- or under-sampling. First, the tabular output (**Fig. 2**) contains the diameter of each detected nucleus. This allows the users to estimate the number of z planes that are sampled in individual nuclei. More importantly, it allows users to examine potential shape and size changes of nuclei in mutants, which could affect the number of z planes sampled per nucleus and the apparent total fluorescent signal due to changed sampling rate. Another common issue in 3D imaging is the reduction of the apparent fluorescent intensity at the deep end of a specimen due to optical aberrations. The tabular output contains the z position and total fluorescent signal per nucleus. A simple plot in Excel using the tabular output would allow users to examine the correlation between the two variables and determine the meaningful z range to be used. As mentioned, Irises allows users to remove nuclei in bulk by excluding all nuclei from specific image planes. Finally, when imaging a complex tissue, it would not be practical to obtain the ideal image of each nucleus. Therefore, it is critical to sample a sufficient number of nuclei to achieve meaningful statistics. In this regard, the automated and objective measurements provided by Irises is particularly valuable.

Irises is useful for studies in which the relative position of nuclei together with their DNA content is of interest and/or for situations where FACS analysis is not feasible. In principle, any tissue where nuclei can be imaged with consistent fluorescence levels between planes can be analyzed using Irises and simple DNA stains. The underlying algorithm sums up the per pixel fluorescence intensity within an identified nucleus, regardless of the fluorophore used for labeling. More importantly, Irises allows specific cells to

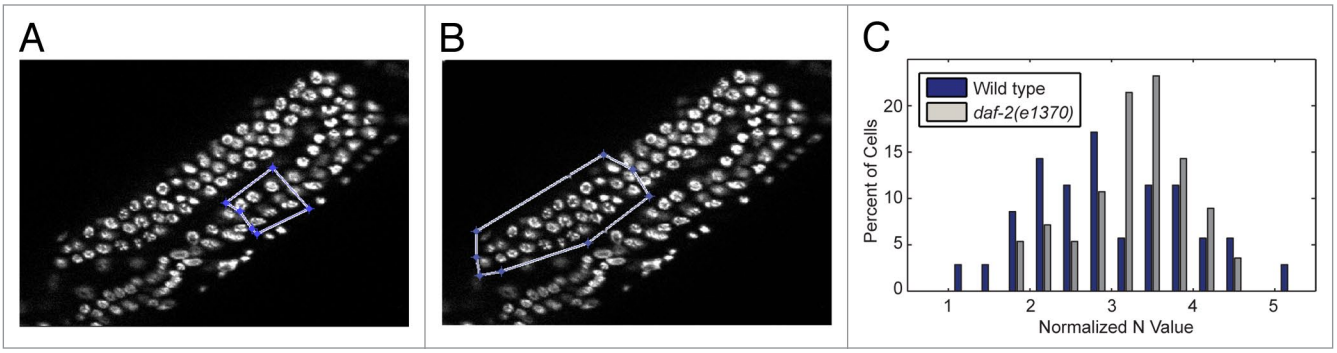


Figure 3. Ploidy analysis in the *C. elegans* germ line (**A and B**) Regions of interests specifying nuclei in the pachytene (**A**) and proliferative (**B**) zone. The average nuclear intensity at the pachytene stage, which has known 4N content can be used normalize florescence values at the proliferative stage. (**C**) Normalized histograms. Normalized florescence values can be used to compare the distribution of DNA content within the proliferative region in WT and mutant worms. The rightward shift in the *daf-2* distribution indicates an increased proportion of nuclei in late-S and G2.

be included or excluded from the analysis based on position or other information (e.g., additional markers). For example, in the developing brain and retina, stereotypical movements are associated with cell cycle under normal conditions.[11,12] The cell cycle status may be more easily tracked under a variety of conditions and in conjunction with other markers using this approach. In addition, the Irises approach could potentially be useful in analysis of samples in which changes in ploidy beyond 4N are linked to development or pathology.[13] In some experimental systems this is currently achieved with methods such as fluorescence in situ hybridization FISH.[14] Finally, we envisage that this tool could also be used to track subtle changes in other fluorescent markers such as label dilution experiments.[15]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Material

Supplemental material may be found here:
www.landesbioscience.com/journals/worm/article/29041/.

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