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Exploring 3D Structured Illumination Microscopy Images
using the ImageJ Software

Faculty Sponsor
Dr. Chrysanthe Preza

Abstract

The use of ImageJ and fairSIM software to interpret and analyze 3D Structured Illumination Microscopy (3D SIM) images in the field of computational imaging is the main topic of this study. The study offers a thorough tutorial for brand-new users looking to master the fundamentals of utilizing ImageJ to work with 3D SIM images. The paper provides a platform for people to become familiar with the techniques and procedures required for assessing 3D SIM images by offering step-by-step instructions to help readers learn how to use ImageJ to manipulate 3D SIM images. The study's objective is to enable young researchers to develop new skills in using open-source programs, like ImageJ and Fiji, to explore and process 3D SIM data, thereby fostering the use of computational imaging science in microscopy.

Introduction

The motivation for this paper is to provide researchers with a helpful resource for analyzing and reconstructing 3D Structured Illumination Microscopy (3D SIM) images, as this technique is becoming increasingly popular for high-resolution imaging of biological samples [1]. By understanding how to use open-source software such as ImageJ [2] and fairSIM [3] to analyze these images, researchers can better extract information about the structure and function of cells and tissues, which can be used to further our understanding of various biological processes.

Super-resolution imaging methods like 3D SIM are used to see small features in biological material. In SIM specialized illumination and computational methods enable the reconstruction of high-resolution images of the underlying samples. Compared to conventional fluorescence microscopy, this method can increase lateral and axial resolution by a factor of 2, enabling imaging intracellular structures at high resolution [4]. Compared to wide-field fluorescence microscopy (light microscopy with uniform illumination), the advantages of 3D SIM include compatibility with common fluorophores, rapid recording times, high sensitivity, and good contrast. However, SIM requires longer data processing times, has a lesser improvement in lateral resolution than other super-resolution methods, and is susceptible to reconstruction artifacts due to insufficient system calibration and sample quality. For our study, we use Fiji, a variant of ImageJ2, a Java-based scientific image processing application that works with several image formats and has a wealth of documentation on manipulating images. The next two subsections in this introduction section briefly introduce the realm of 3D SIM and the ImageJ software.

3D Structured Illumination Microscopy (SIM)

Structured Illumination Microscopy (SIM) is a super-resolution imaging technique that visualizes fine details in biological samples that cannot be resolved using traditional light microscopy methods [4]. SIM uses a specific illumination pattern to excite specific regions of the sample selectively and then uses computational algorithms to reconstruct a high-resolution image from the resulting interference patterns. This technique was first developed in the early 2000s and has since become a popular tool for studying the structure and organization of cells and other biological samples at the sub-cellular level. 3D SIM is a wide-field imaging technology that illuminates the entire field of view with Moiré fringes, a striped structured pattern created by interfering light. Usually projected onto the specimen, the grid shadow effect is only visible in the focused plane. The out-of-focus light is removed

by capturing multiple images with the structured pattern placed in different orientations and using computational methods for data post-processing, and the specimen's higher-resolution information is revealed (Figure 1). Over traditional (widefield) fluorescence microscopy, 3D SIM increases lateral and axial resolution by 2 and is compatible with multicolor imaging [4].

The number of grid rotations and image resolution have a complicated relationship. The grid frequency, the number of rotation angles, geometric relationships between the grid lines, and the specimen characteristics significantly impact the outcome. Increasing the number of grid rotations can improve the final resolution but doing so inevitably results in slower data acquisition and more photobleaching.

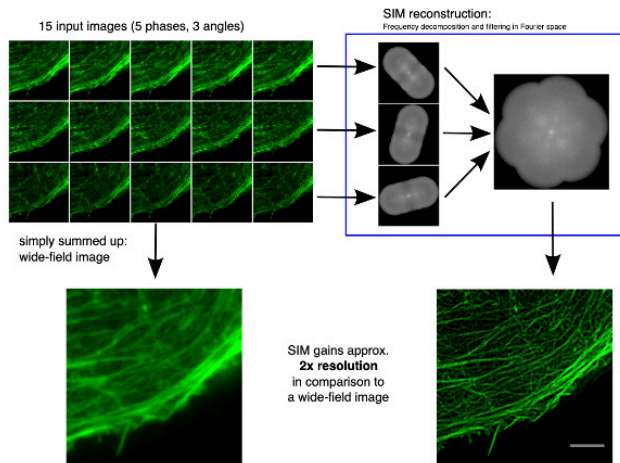


Figure 1. Overview of the SIM process, as seen on fairSIM's website [3]. A stack of 15 raw SIM images (top-left), is reconstructed (top-right) into a final SIM super-resolution image (bottom-right) [5]. The final SIM image has an improved resolution compared to the widefield microscopy image (bottom-left).

Structured Illumination Microscopy is suitable for all common fluorophores, uses several quick recordings with a wide field of view, is incredibly sensitive when using the right camera, and provides a drastic improvement in image contrast compared to wide-field microscopy. SIM's thin sample 3D sectioning capability is suitable for applications that investigate live cells. However, compared to other super-resolution techniques, it provides a smaller improvement in lateral resolution; to obtain high-resolution images, large amounts of processing time are required; and the final result can be seriously harmed by errors in the grating position, system calibration, refractive index mismatch, and/or sample quality [4].

When working with images, it is important to display them for both qualitative and quantitative analysis and be able to manipulate them by processing them using computational methods. ImageJ is a Java-based public-domain scientific image processing program currently developed by the Laboratory for Optical and Computational Instrumentation [2]. Available in desktop and web-based formats, it can operate on 8-bit grayscale and 8-bit, 16-bit, 32-bit, and RGB color images saved in many formats, including raw uncompressed TIFF [2]. ImageJ supports scrolling, zooming, irregular area selections, geometric operations, arithmetic operations, color processing, and displaying multi-image stacks. It also has extensive documentation.

Several derivatives of ImageJ have been developed that add features and make it easier to use and maintain. ImageJ2 is an open-source rewrite of the original program focusing on advanced features and backward compatibility [6]. It has a built-in updater for the program and the plugins, support for larger images and more advanced file formats, and a more powerful script editor. For our study, we used Fiji, an easy-to-install distribution of ImageJ2 and several plugins [2].

FairSIM is a plugin for ImageJ that provides an open-source implementation of a two-dimensional restoration algorithm used in Structured Illumination Microscopy (SIM) [5]. FairSIM uses complex algorithms to process the blurry wide-field image stacks and reconstruct from the raw dataset a sharper super-resolution image as demonstrated in Figure 1 [3].

Methods

This section describes the tools and other resources used to conduct our study.

Installing Fiji

On its website, portable binaries of Fiji are provided for the three major desktop operating systems: Windows, macOS, and Linux. Once a Java 8 (or later) runtime is installed, the software can be used on any system. It has no installer – the necessary files and executables are included in the download, which means it can be run from an external drive and easily transferred to another system.

Installing fairSIM

The ready-to-use JAR file for the fairSIM plugin “fairSIM_plugin.jar”, can be downloaded from its website and loaded in Fiji, just like any other plugin, from “Plugins > Install...” (see Figure 2), and it can be chosen from the file browser.

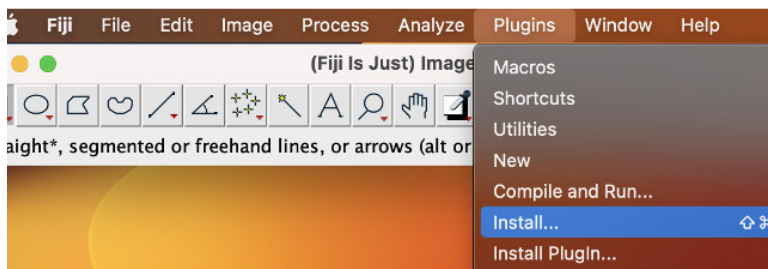


Figure 2. How to install the fairSIM plugin from Fiji’s menu.

Dataset

Opstad data

The dataset used in this paper was created by Opstad et al. [7] using 3D structured illumination microscopy to see how well 3D SIM could be used to examine mitochondria in living or fixed cells. The dataset includes images of lysosomes labeled with LysoTracker in both fixed and live cells. The data is particularly useful for SIM reconstruction because it includes raw 3D SIM data, optical parameters, and reconstructed 3D SIM images for validation.

The data in [7] was captured using a DeltaVision OMX V4 Blaze imaging system with sCMOS cameras and lasers for excitation. It uses sinusoidal illumination patterns to create images with resolution beyond the diffraction limit. The optical resolution of the system is 110-160 nm laterally and 340-380 nm axially. The system takes 120 images for every micrometer in thickness.

Image analysis and processing were performed by Opstad et al. [7] using Fiji. This included converting data format from DV to TIFF image files while maintaining bit-depth and metadata, creating AVI movies by using a maximum intensity z-projection and correcting for photobleaching through exponential fit, and visualizing the images using orthogonal views.

Images of cells grown in two different media types—normal media containing glucose and glucose-free, galactose-adapted growth medium—are presented in [7]. Additionally, for both live-cell and fixed-cell imaging, two different imaging conditions are offered: for alive cells with or without LTDR

labeling and fixed cells chemically preserved with either paraformaldehyde (PFA) or PFA in conjunction with glutaraldehyde (GA). The dataset is hosted on DataVerseNO [7], a national, generic repository for open research data from researchers from Norwegian research institutions. Three-dimensional SIM data of mitochondria and lysosomes in cardiomyoblasts is present under normal and galactose-adapted conditions. Each file in the dataset consists of multiple images (“slices”) contained within a single file (a “stack”). Each two-dimensional slice was taken at different depths (z) and at about 1.1 seconds apart, showing the cell at different times and structured illumination angles [7]. The images from the dataset have a time-lapse interval dimension in addition to the three spatial dimensions (x , y , and z), hence making it 4D. To access the dataset, refer to Opstad et al. [7].

The “Experimental image”

For our study, we used the file “20210420_H9C2-dTag_GAL_37C_1520_sim-fasterer_016.tif” in the dataset’s RawSIM folder, and its corresponding ground truth “20210420_H9C2-dTag_GAL_37C_1520_sim-fasterer_016_SIR.tif” in the dataset’s 3D SIM folder of the Opstad data. At just under 900 MB, it is one of the smallest images in Opstad et al.’s data, which was the primary reason we chose this particular image. It is small enough to share with team members and open on lower-speed computers, yet enhancements applied to it were still evident.

Exploration

In this section, we show the interesting portions of our exploration of ImageJ as well as the results from this exploration.

ImageJ tools

Opening an image for display

We used Fiji to open the file used in our exploration (see Figure 3), and all team members could open it and display the image (Figure 4) on their personal computers of varying specs. However, it should be noted that several other images in the dataset are several times larger than the experimental image, so a desktop computer with the largest amounts of CPU and RAM may be required to work with these.

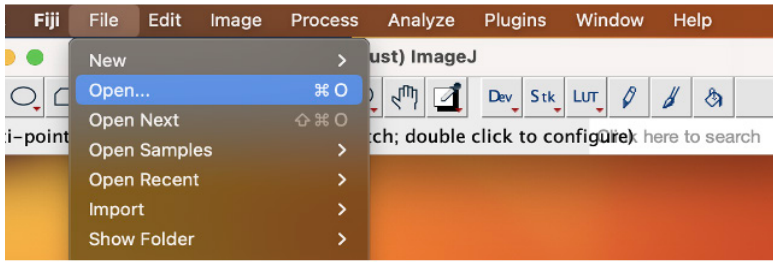


Figure 3. An image is opened using Fiji's menu.

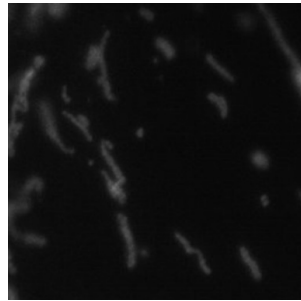


Figure 4. The experimental image, “20210420_H9C2-dTag_GAL_37C_1520_sim-fasterer_016.tif”. It has a resolution of 256x256x135 pixels and 50 time-lapse intervals (the first x-y-slice at $z = 1$ from the first-time interval is depicted).

Histogram

A histogram shows the distribution of pixel intensity values in an image. The "Histogram" feature in ImageJ's "Analyze" menu can be used to create and evaluate histograms. The histogram displays how frequently each pixel intensity value occurs in the image, which can reveal important details about the brightness and contrast of the image as a whole. One can better understand an image's overall brightness and contrast levels by studying its histogram, which can aid in making deft decisions about how to modify these values. The data from the histogram can be used to enhance the methods and algorithms for image processing that are employed in different image analysis applications. The pixel intensity distribution in an image is visually represented by histograms, which can be used to spot patterns and trends in the data. Individuals with little experience with image analysis can use ImageJ's "Histogram" tool because of its clear and straightforward user interface. Overall, using histograms in ImageJ can offer insightful information about an image's composition and aid in image improvement, processing, and analysis.

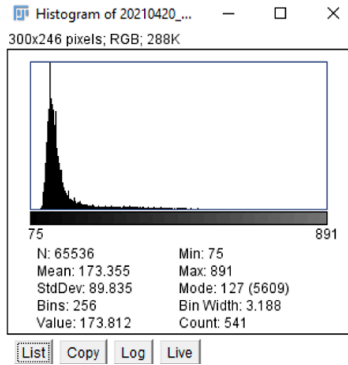


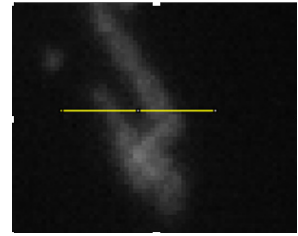
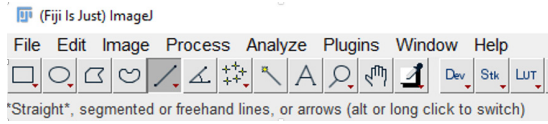
Figure 5. Histogram for the image shown in Figure 4

Brightness/Contrast

When working with any image, our first step will be manipulating the brightness and contrast. In ImageJ, the "Brightness/Contrast" tool, which can be found in the Image>Adjust submenu, is used to change an image's brightness and contrast. Select the desired image and click the Brightness/Contrast option, to use this tool. A dialog box allows the user to change the brightness and contrast settings. The settings can be changed by dragging the sliders or entering precise numbers in the text boxes. Alterations to the image can be applied by clicking "OK". Adjusting the brightness and contrast in ImageJ improves visibility and enables visual inspection of images.

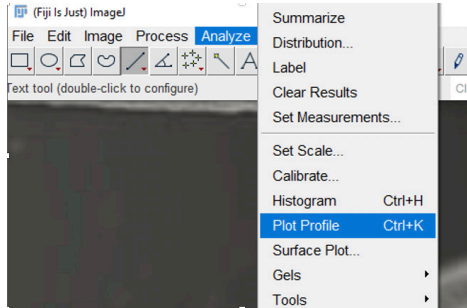
Intensity Plots

One can plot the intensity profiles of images on ImageJ. General information on image intensity plots can be found in [8]. We demonstrate here intensity plots taken from our experimental image. Figure 6 demonstrates how we use the intensity plot feature of ImageJ to analyze the SIM image. The intensity profile curve in Figure 6 (d) shows how this analytics visualization tool of intensity value versus distance helps us better understand the data quantitatively across a cross-section of our line of interest. This technique is useful for an image reconstruction algorithm researcher when comparing the resolution (which can be estimated from an intensity profile) of an unprocessed raw image with a reconstructed image.

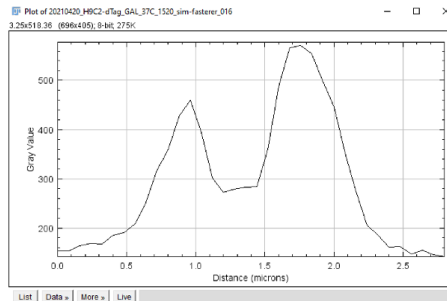


(a)

(b)



(c)



(d)

Figure 6. Creating an intensity profile. (a) Select the straight-line segment from Image’s main menu. (b) Drag a line over the RAW SIM image. (c) Go to Analyze in the main menu and select the Plot Profile option. (d) A new dialog appears depicting the intensity profile along the yellow line we set in (b).

Using fairSIM for image reconstruction.

This section provides the step-by-step process to create a reconstruction with fairSIM. FairSIM is largely an automated tool; it can approximate some input parameters, such as the Optical Transfer Function (OTF) and pixel size, without fine-tuning [9]. A new reconstruction can be started from the

“Plugins” menu (Figure 7). Once this process is initiated, dialogue windows prompt the user for input. In summary, the required steps are:

- Starting a new reconstruction (Figure 7)
- Selecting the image sequence, beams, angles, and phases (Figure 8)
- Importing an image slice (Figure 9, then Figure 10)
- OTF approximation (Figure 9, then Figure 11)
- Parameter estimation (Figure 9, then Figure 12)

We selected the OMX option (Figure 8) for the second step because the dataset was acquired using a DeltaVision OMX V4 Blaze imaging system. We chose 3 angles and 5 phases because of the specification on page 3 of [7]. The number of interference beams was not specified in [7].

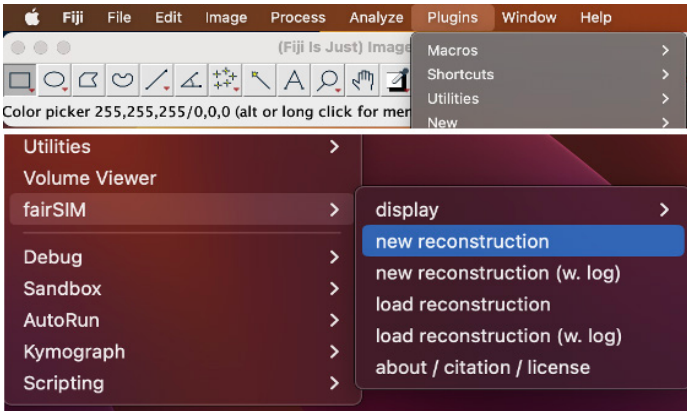


Figure 7. A new reconstruction is started using fairSIM, at “Plugins > fairSIM > new reconstruction”.

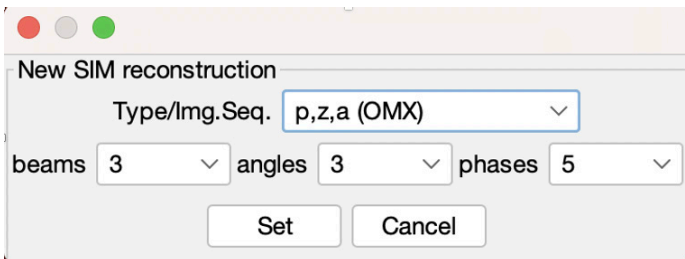


Figure 8. New SIM reconstruction dialog box for user input.

We use the default three beams for reconstruction as most 3D SIM datasets in the literature [1] use 3 beams. We clicked on the “Set” button in Figure 8 to display the fairSIM main window (Figure 9). For our study, we are only concerned with the “Load” tab. We chose an image slice from the stack containing multiple slices depicting the same subject at different depths and illumination angles over a time-lapse study.

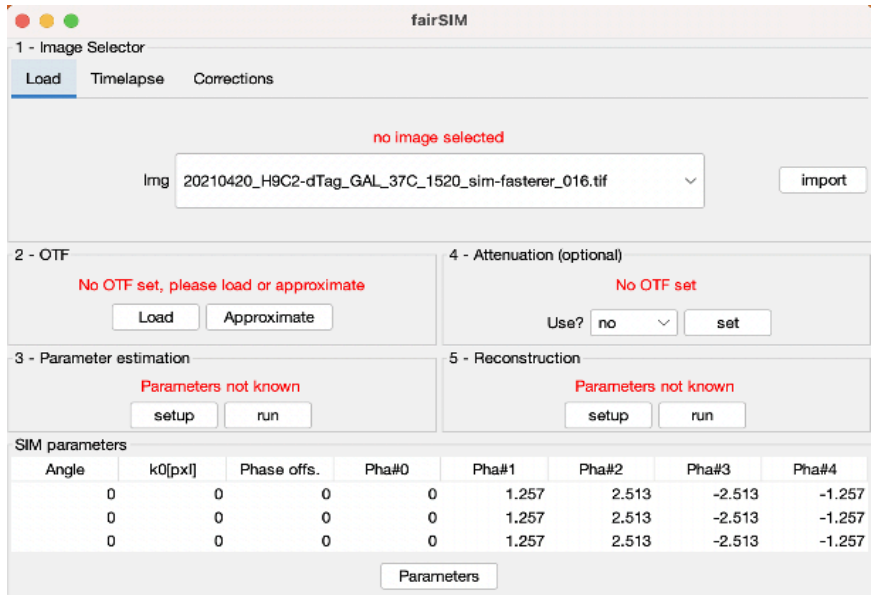


Figure 9. fairSIM main window.

We chose the first slice in the stack (Figure 10), but any slice will work. fairSIM automatically detects the pixel size, 80 nanometers (nm). “Background subtraction” will attempt to remove any background from the image, but this was not used for our study; the test images have a zero background.

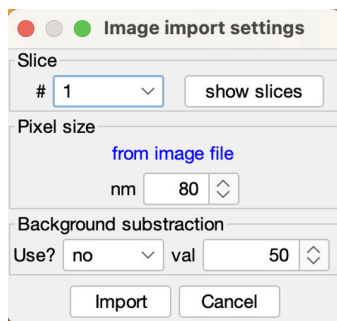


Figure 10. Image import settings.

The Optical Transfer Function (OTF) describes which frequencies are transferred through an imaging system, such as a microscope [10]. It is an indicator of image resolution. FairSIM can either use a given OTF file or (in this study) approximate the function. Attenuation also involves OTFs, but it is optional. The pictured parameters (Figure 11) are fairSIM's defaults, and they worked well on our study.

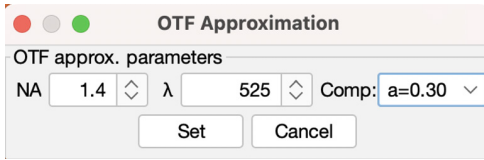


Figure 11. OTF Approximation.

Various parameters involve sub-pixel precision and the display of the fairSIM reconstruction [9]. Again, these default settings (Figure 12) did not require fine-tuning. Finally, the reconstruction was run, and the super-resolution image was generated and displayed (see Figure 13, (d) and Figure 17).

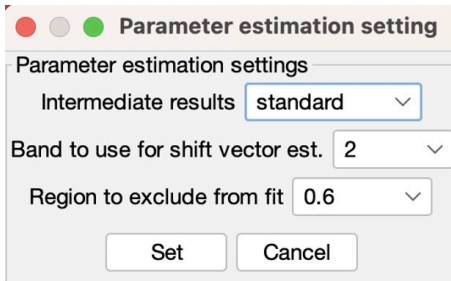


Figure 12. Parameter estimation

Resolution Enhancement

The wide-field image (Figure 13b) has a digital resolution of 1024 x 1024 pixels, while fairSIM's reconstructed image has 2048x2048 pixels (Figure 13d) – it is twice as large, because the optical resolution is doubled through the reconstruction process [1]. Many blurry and grainy details became much sharper after processing with fairSIM; the shapes of the cells are more apparent in the reconstruction (Figure 13d). We see a lot of artifacts in this fairSIM reconstruction because it was performed using default reconstruction parameters estimated by the fairSIM software. These are known to be

residual artifacts due to inaccurate knowledge of the structured illumination pattern parameters. Trying to optimize the input parameters could possibly result in a better reconstruction than what is shown in Figure 13d. It is not quite as sharp as the ground truth image (Figure 13c) provided in the Opstad data, because the two reconstruction methods used to generate these images are different. FairSIM's reconstruction methods are based on two-dimensional processing (instead of three-dimensional processing), but they may be useful as an alternative for researchers who lack more advanced commercial software. Figure 13 qualitatively demonstrates the resolution enhancement we achieved by using the fairSIM plugin in ImageJ. The fairSIM result is a sharp, super-resolution image, twice as large as the experimental raw data image.

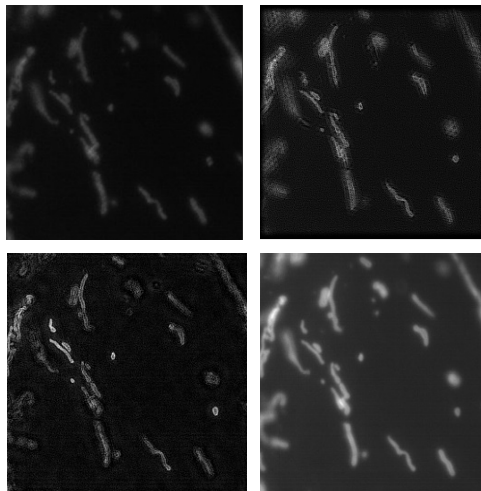


Figure 13. Resolution enhancement achieved using the fairSIM plugin in ImageJ. (a) Top left - Raw SIM image (1 out of 135). (b) Top right - wide-field image for depth 1/9. (c) Bottom left - ground truth image for depth 1/9. (d) Bottom right - fairSIM reconstructed image for depth 1/9.

Adding pseudo-color with a lookup table

Pseudo-color or false-color is widely employed to display images in many fields of science, including biology. Most scientific-grade cameras used in microscopes record grayscale images. To make it easier for researchers to see details in an image, scientists digitally add colors corresponding to different densities or fluorophores used to stain the sample. The human eye

is more sensitive to color gradations and variations than it is to variations in the tones of black and white [9].

In ImageJ, pseudo-color can be applied to a grayscale image using one of the many built-in lookup tables. Lookup tables (LUTs) are functions that convert shades of gray to shades of another color or color palette. For this study, several different LUTs were applied chosen from those available in the ImageJ menu (Figure 14).

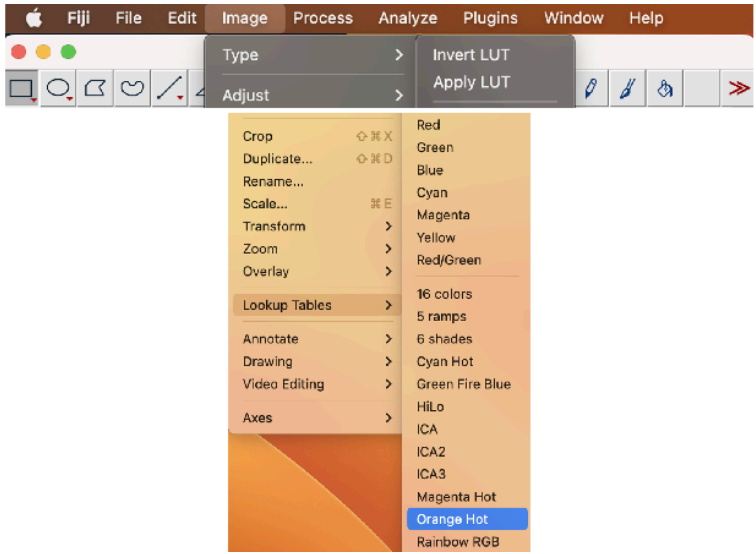


Figure 14. ImageJ’s lookup tables menu, with various color options displayed.

Pseudo-color

In this section we display the images from file “20210420_H9C2-dTag_GLU_37C_1520_sim-fast_005.tif,” of the Opstad dataset using pseudo color. Applying pseudo-color to the raw SIM image was somewhat effective in increasing contrast (Figure 15). Portions of the image that were practically invisible in Figure 15a (mainly the corners) are now much more apparent. Still, the effect of the lookup table is neutered by the low resolution; it cannot make a blurry image sharp. The exact outline of the cell is still difficult to interpret as seen in the cropped images in Figure 15(c & d).

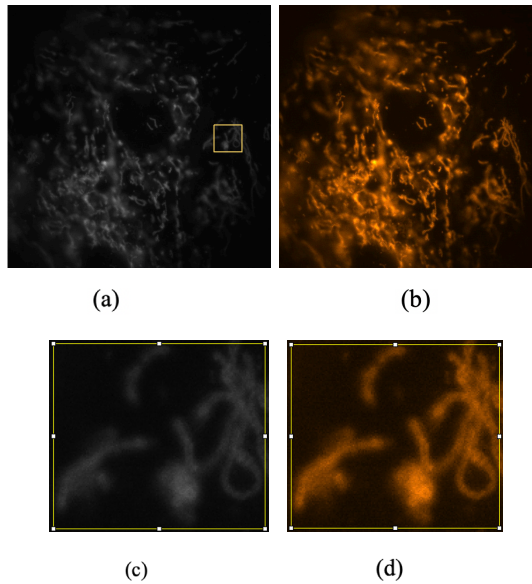


Figure 15. Impact of color in visual inspection of an image. (a) Input grayscale wide-field image from file “20210420_H9C2-dTag_GLU_37C_1520_sim-fast_005.tif” in the Opstad data. (b) The same image displayed using the “Orange Hot” LUT. (c) & (d) Cropped zoomed in images for better visualization taken from the yellow box shown in (a) and corresponding area in (b).

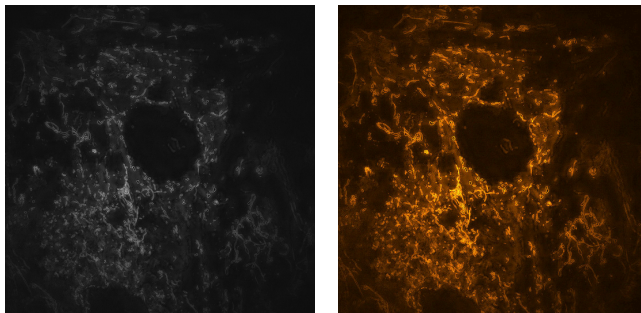


Figure 16. Impact of color in visual inspection of an image. Left: The ground truth image corresponding to the image shown in Figure 15. [7]. Right: The same image, displayed using the “Orange Hot” LUT applied.

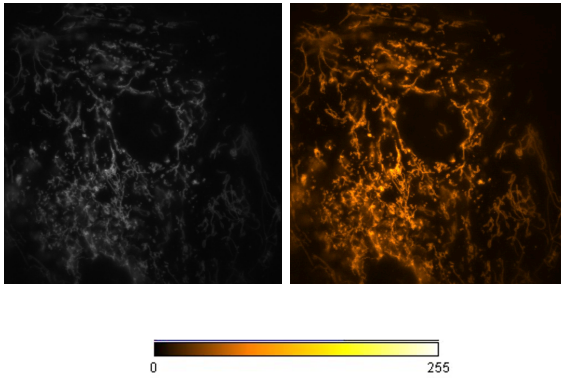


Figure 17. Impact of color in visual inspection of an image. Left: The output reconstruction from FairSIM. Right: The same image, with the “Orange Hot” LUT applied.

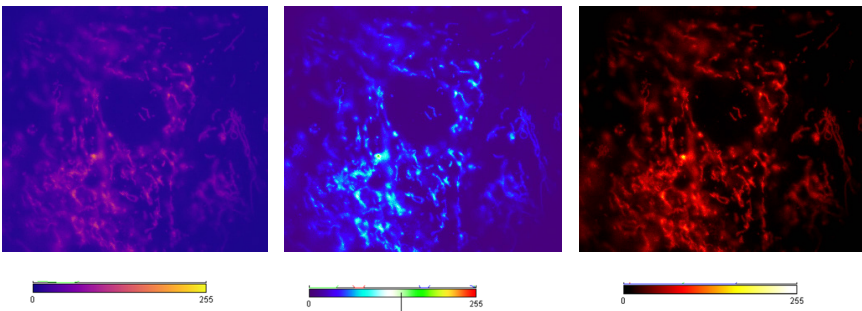


Figure 18. Impact of different LUTs in the visual inspection of the same image. The name of the LUT in each case is: (a) “mpl-plasma” (b) “Thermal” (c) “Red Hot.”

On the other hand, applying pseudo-color to the super-resolution image helps us see the contrast that is there differently. The differences between the lighter and darker portions of the image are much more visible to the human eye – thin parts of the cells easily missed against the black background stand out more in the color image (Figure 16). The use of this LUT was also effective in visualizing our fairSIM reconstruction (Figure 17), with some portions (bottom middle) being sharper than in the grayscale image. We also compared various LUTs to observe differences in the image due to the different colors used (Figure 18).

Discussion

ImageJ and fairSIM are useful tools when studying 3D SIM images. Data from super-resolution fluorescence microscopy can be analyzed and visualized using fairSIM and ImageJ as shown in our study. By combining the two software programs, researchers can benefit from the tools offered by fairSIM for SIM data processing and analysis and the extensive capabilities of ImageJ for image analysis and visualization. ImageJ has a user-friendly interface that makes it accessible to researchers with varied degrees of technical skill. While fairSIM offers sophisticated capabilities for SIM image reconstruction, a versatile tool for researchers, fairSIM is compatible with various SIM data types and techniques, while ImageJ has a wide variety of picture file formats. However, the learning curve for ImageJ can be difficult for certain users, and some fairSIM features may be too sophisticated for researchers with little technical competence or lack of knowledge about the SIM imaging technique. For certain users, integrating fairSIM with ImageJ may be difficult, time-consuming, requiring some technical skills. In conclusion, combining fairSIM with ImageJ can result in a complete set of tools for the study and display of super-resolution fluorescence microscopy data. Organizations and researchers must, however, carefully assess potential complexity issues to avoid restoration artifacts and misinterpretation of images.

Conclusion

In this study, we demonstrate the effective use of ImageJ and fairSIM with a 3D SIM dataset and detailed step-by-step procedures. ImageJ is a free, open-source image processing program for multidimensional scientific images, and fairSIM is an open-source framework for image reconstruction in super-resolution microscopy. Both programs are widely used in scientific communities for image analysis in biology, medicine, and material science. ImageJ can be used to display, edit, analyze, process, and measure images and allows the creation, integration and modification of image macros, while fairSIM enables reconstruction of super-resolution images from SIM raw data. Both programs are written in Java and run on various platforms, including Windows, macOS, and Linux.

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References

- [1] M. G. L. Gustafsson et al., “Three-Dimensional Resolution Doubling in Wide-Field Fluorescence Microscopy by Structured Illumination,” *Biophys. J.*, vol. 94, no. 12, pp. 4957–4970, Jun. 2008, doi: 10.1529/biophysj.107.120345.
- [2] “Fiji,” ImageJ Wiki. <https://imagej.github.io/software/fiji/index> (accessed Jan. 27, 2023).
- [3] “The fairSIM project by fairSIM.” <https://www.fairsim.org/> (accessed Feb. 01, 2023).
- [4] M. G. L. Gustafsson et al., “Three-Dimensional Resolution Doubling in Wide-Field Fluorescence Microscopy by Structured Illumination,” *Biophys. J.*, vol. 94, no. 12, pp. 4957–4970, Jun. 2008, doi: 10.1529/biophysj.107.120345.
- [5] M. Müller, V. Mönkemöller, S. Hennig, W. Hübner, and T. Huser, “Open-source image reconstruction of super-resolution structured illumination microscopy data in ImageJ,” *Nat. Commun.*, vol. 7, no. 1, p. 10980, Mar. 2016, doi: 10.1038/ncomms10980.
- [6] “ImageJ2,” ImageJ Wiki. <https://imagej.github.io/software/imagej2/index> (accessed Jan. 27, 2023).
- [7] I. S. Opstad et al., “Three-dimensional structured illumination microscopy data of mitochondria and lysosomes in cardiomyoblasts under normal and galactose-adapted conditions,” *Sci. Data*, vol. 9, no. 1, Art. no. 1, Mar. 2022, doi: 10.1038/s41597-022-01207-7.
- [8] “Intensity Profile.” <https://imagej.nih.gov/nih-image/more-docs/Tutorial/Profile.html> (accessed Feb. 06, 2023).
- [9] M. Müller, “fairSIM quickstart guide”.
- [10] “18 EELE582_S15_OTFMTE.pdf” Accessed: Jan. 29, 2023. [Online]. Available: https://www.montana.edu/jshaw/documents/18%20EELE582_S15_OTFMTE.pdf