Journal of Visualized Experiments

High-Accuracy Correction of 3D Chromatic Shifts in the Age of Super-Resolution Biological Imaging Using Chromagnon

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60800R1
Full Title:	High-Accuracy Correction of 3D Chromatic Shifts in the Age of Super-Resolution Biological Imaging Using Chromagnon
Section/Category:	JoVE Biology
Keywords:	Fluorescence microscopy; Confocal microcopy; Fluorescence imaging; 3-D Imaging; Neuroimaging; time-lapse imaging; Image interpretation; Image processing; Colocalization analysis; Chromatic aberration
Corresponding Author:	Atsushi Matsuda Joho Tsushin Kenkyu Kiko Mirai ICT Kenkyu Center Kobe, JAPAN
Corresponding Author's Institution:	Joho Tsushin Kenkyu Kiko Mirai ICT Kenkyu Center
Corresponding Author E-Mail:	a.matsuda@nict.go.jp
Order of Authors:	Atsushi Matsuda
	Takako Koujin
	Lothar Schermelleh
	Tokuko Haraguchi
	Yasushi Hiraoka
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Kobe, Japan

National Institute of Information and Communications Technology

588-2, Iwaoka, Nishi-ku, Kobe,
Hyogo 651-2492, Japan
TEL: +81-78-969-2111

Dr. Xiaoyan Cao,
Review Editor *JoVE*

December 16th, 2019

Dear Dr. Cao, *JoVE* Editor,

We have revised a manuscript "High-accuracy correction of 3D chromatic shifts in the age of super-resolution biological imaging using *Chromagnon*" submitted to *JoVE*.

We appreciate your support during the revision process. We have thoroughly updated the manuscript following the comments made by the editor and reviewers. We have updated our software and its document. Finally, we made a set of image data available for download from the GitHub site as example images to test our software.

We believe the manuscript was improved greatly. I look forward to hearing back from you.

With my kindest regards,

Atsushi Matsuda, PhD
Senior Researcher,
Advanced ICT Research Institute,
National Institute of Information and Communications Technology
588-2, Iwaoka, Nishi-ku, Kobe 651-2492, Japan

TITLE:

High-Accuracy Correction of 3D Chromatic Shifts in the Age of Super-Resolution Biological Imaging Using *Chromagnon*

AUTHORS AND AFFILIATIONS:

Atsushi Matsuda[1,2], Takako Koujin[1], Lothar Schermelleh[3], Tokuko Haraguchi[1,2], Yasushi Hiraoka[1,2]

[1]Advanced ICT Research Institute Kobe, National Institute of Information and Communications Technology, Kobe, Japan

[2]Graduate School of Frontier Biosciences, Osaka University, Suita, Japan

[3]Micron Advanced Bioimaging Unit, Department of Biochemistry, University of Oxford, Oxford, UK

Corresponding Author:

Atsushi Matsuda (a.matsuda@nict.go.jp)

Email Addresses of Co-Authors:

Takako Koujin (koujin@nict.go.jp)

Lothar Schermelleh (lothar.schermelleh@bioch.ox.ac.uk)

Tokuko Haraguchi (tokuko@nict.go.jp)

Yasushi Hiraoka (hiraoka@fbs.osaka-u.ac.jp)

KEYWORDS:

chromatic aberration, fluorescence microscopy, super-resolution imaging, colocalization analysis, cell biology, image processing

SUMMARY:

Correction of chromatic shifts in three-dimensional multicolor fluorescence microscopy images is crucial for quantitative data analyses. This protocol is developed to measure and correct chromatic shifts in biological samples through acquisition of suitable reference images and processing with free software, *Chromagnon*.

ABSTRACT:

Quantitative multicolor fluorescence microscopy relies on the careful spatial matching of color channels acquired at different wavelengths. Due to chromatic aberration and the imperfect alignment of cameras, images acquired in each channel may be shifted, and magnified, as well as rotated relative to each other in any of the three dimensions. With the classical calibration method, chromatic shifts are measured by multicolor beads attached to the surface of a coverslip, and a number of software are available to measure the chromatic shifts from such calibration samples. However, chromatic aberration can, vary with depth, change with observation conditions and be induced by a biological sample itself, hindering determination of the true amount of chromatic shift in the sample of interest and across the volume. Correcting chromatic shifts at higher accuracy is particularly relevant for super-resolution microscopy where only slight chromatic shifts may alter the interpretation of multicolor images. We have developed methods

to measure and correct chromatic shifts in samples in 3D using our open-source, free software, *Chromagnon*. Here we provide a detailed protocol to correct the chromatic shifts using our approach. The protocol includes special requirements for sample preparation, data acquisition, and software processing to measure chromatic shifts in biological samples of interest.

INTRODUCTION:

Multicolor imaging is one of the fundamental aspects of biological fluorescence microscopy, in cases where the spatial relationship of different molecules or structures is of major interest. Chromatic aberration, an optical aberration of polychromatic light caused by dispersion, changes the apparent position of the colored objects of interest. Similarly, microscopes equipped with multiple cameras devoted to acquiring each color have more complex chromatic shifts due to differences in optical elements and imperfect alignment among the channels. Thus, such chromatic shifts may lead to a false conclusion unless explicitly corrected by the user. Although chromatic shifts have not been a major problem as long as the resolution of microscopy is limited by the classical resolution limit, recent development of super-resolution microscopy[1] has prompted the need for more accurate correction of chromatic shifts.

It has been a common practice to measure chromatic shifts of microscope systems using a multicolor bead calibration slide[2]. The bead-based calibration method is appropriate for measuring chromatic shifts from the entire optics of the microscope towards the surface of the coverslip[2]. This method, however, is unable to measure chromatic shifts in the biological samples of interest. It is important to note that many biological samples are three-dimensional (3D), and the chromatic shifts of such samples are different from those at the surface of the coverslip. Furthermore, chromatic shifts change with imaging conditions[2,3]. We have measured the chromatic shifts in 3D biological samples and found that the uncertainty of chromatic shifts was often as much as 350 nm by the classical multicolor-bead calibration method[3]. Therefore, chromatic shifts need to be measured in biological samples at the depth of interest and under the imaging conditions being used.

Here, we describe procedures to measure chromatic shifts in biological samples and correct these shifts using our software, *Chromagnon*[3]. To measure chromatic shifts in biological samples, our method uses two kinds of data sets, a “target” image and a “reference” image. The “target” image is a multicolor image of interest, for example, images stained for DNA, nuclear envelope, and microtubules. It is often impossible to measure chromatic shifts in such an image. Therefore, we need a “reference” image that is dedicated to measure the chromatic shifts in the sample. The only definition of a “reference” image is a multicolor image of the same object. In this sense, a multicolor beads image is also a type of reference image. Here, we describe three different types of reference image that are used to measure chromatic shifts in the biological samples: “crosstalk reference images”, “bright-field reference images” and “biological calibration reference images”. The type of reference image depends on the type of microscope being used or the correction accuracy required as summarized in **Table 1**.

[Place **Table 1** here]

"Crosstalk reference images" have the highest correction accuracy and are relatively simple to accomplish[3,4] (**Table 1**). The drawback is their limitation in microscopy applications due to their incapability of measuring chromatic shifts in excitation paths. Also, to obtain such images, the microscope should be equipped with multiband dichroic mirrors, and emission filters that are independently controlled from the excitation filters or light sources. Suitable microscopy includes conventional wide-field microscopy, single molecule localization microscopy (SMLM) such as photo-activated localization microscopy/stochastic optical reconstruction microscopy (PALM/STORM)[5,6] and expansion microscopy[7] observed with the wide-field mode. A crosstalk reference image is acquired from the target sample itself. It is an image of crosstalk (bleed-through) fluorescence of a dye obtained in all required channels. Fluorescence emission always expands towards the longer wavelengths, therefore dyes with the shortest emission wavelength are excited to obtain crosstalk fluorescence in channels of longer wavelengths. For example, when the sample is stained with blue, green, and red, only the blue dye is excited, and the emission light is obtained in the blue, green, and red channels. In this protocol, DNA stained with 4',6-diamidino-2-phenylindole (DAPI) was used to obtain crosstalk fluorescence.

"Bright-field reference images" are an easier and less phototoxic alternative to "crosstalk reference images" but are the least accurate[3] (**Table 1**). These are bright field images of the target sample, acquired in all the color channels used in the target image.

"Biological calibration reference images" have the advantage of being applicable to any type of microscopy due to their ability to measure the chromatic shifts both in the excitation and emission paths[3,8] (**Table 1**). Suitable microscopy includes wide-field microscopy, confocal microscopy, light sheet microscopy, stimulated emission depletion (STED)[9], structured illumination microscopy (SIM)[10], Airyscan/SORA[11,12], SMLM observed with the total internal reflection fluorescence (TIRF) mode, Olympus super resolution (OSR)[13], and so forth. A biological calibration reference image is acquired from a calibration sample similarly prepared as the target sample, but with staining of a single structure with multiple colors. The correction accuracy excels the resolution of most super-resolution microscopy and preparing a biological calibration sample can be relatively simple. Another advantage is the availability to "average" multiple reference images. Therefore, even though the individual images contain poor information for the measurement of chromatic shifts, the information content can be increased by averaging multiple images. The accuracy depends on how much the imaging conditions are kept constant. In this regard, the best performance is obtained when both target and reference samples are on the same slide, using, for example, 8-well chambered coverglasses (**Table 1**, right-most). In this protocol, actin stained with three colors of phalloidin was used as a biological calibration.

Once a reference image is obtained, then the chromatic shift is measured and corrected by our software *Chromagnon*. There is no limitation on the number of channels, Z sections and time frames that *Chromagnon* can measure and correct the chromatic shifts for. *Chromagnon* measures chromatic shifts in two steps. The first step acquires the "global" or "affine" alignment parameters of translation in the X, Y, Z axes, magnification along the X, Y, Z axes, and rotation around the Z axis. The calculation accuracy of the global alignment is ~16 nm in 3D and ~8 nm in 2D. The second step is an optional 2D iterative "local alignment" on projected images to obtain

a higher accuracy. In the local alignment process, the images are subdivided into multiple regions and chromatic shifts in these local regions are measured. Subsequently, the regions are further divided and chromatic shifts in the subregions are measured iteratively until the number of pixels in the region reaches the minimum number of pixels (usually 60 x 60 pixels). The resulting local alignment map is combined with the global alignment parameter and is applied to the target image by an elastic transformation. Following this step, the calculation accuracy is improved to ~14 nm in 3D and ~6 nm in 2D. The local alignment is not suitable for biological calibration reference images because biological structure in the reference is different from that in the target (**Table 1**). Therefore, only global alignment is used for biological calibration reference images.

The local chromatic shifts originate from two sources; microscope instrumental local distortion and biological structural inhomogeneity. Because microscope instrumental local distortion is constant, this can be measured from the multicolor beads reference sample and corrected as a fixed parameter. *Chromagnon* can combine the microscope instrumental local distortion map and the global alignment parameters from the biological calibrations (**Table 1**). Using this method, it is expected that the average accuracy of biological calibration will be improved by an additional 1−2 nm.

Here, we describe a protocol to correct the chromatic shifts of 3D fluorescence images using our software *Chromagnon*, from the easiest low end to the highest accuracy. We use immunostaining of HeLa cells as an example and observed them using 3D wide-field microscopy and 3D-SIM. In the first section, we describe how to prepare target samples and biological calibration samples. This part of the protocol should be optimized for the specific targets of the research. In the second section, we describe the acquisition methods for three kinds of reference images by microscopes. The assumption was to obtain blue, green, and red channels but channel composition should be modified by the specific targets of the research and by the setups of the microscope. It does not matter if the microscope is equipped with a single camera or multiple cameras. In the third section, we describe how one can use our software to measure and correct chromatic shifts of the target image by using reference images. Finally, in the fourth section, we describe a method to complement the biological calibration reference images by using a microscope's instrumental local calibration.

PROTOCOL:

1. Sample preparation

1.1. Preparation of a target sample

1.1.1. Seed 6 x 10^5 HeLa cells on a 35-mm glass-bottom dish and grow them in 2 mL of growth medium (Dulbecco's modified eagle medium with L-Gln and sodium pyruvate supplemented with 10% fetal bovine serum [FBS]) at 37 °C and a CO_2 concentration of 5%. Alternatively, seed 2.5 x 10^5 HeLa cells on an 8-well chambered coverglass and grow them in 0.5 mL of growth medium at 37 °C and a CO_2 concentration of 5%. Use #1.5 coverglass (with thickness of 0.17 µm) for high-resolution microscopy.

NOTE: Use 1/4 volumes for the 8-well chambered coverglass for further steps.

1.1.2. After about 24 h of incubation, replace the solution with 2 mL of 3.7% formaldehyde in phosphate-buffered saline (PBS). After gentle mixing, continue to fix cells for 15 min at room temperature (RT) on a rotation platform.

CAUTION: Work in a fume hood.

1.1.3. Wash cells with 2 mL of PBS, 3x each for 5−10 min on a rotation platform.

NOTE: Follow local regulations to dispose formaldehyde waste.

1.1.4. Permeabilize cells with 2 mL of 0.1% Triton X-100 in PBS for 5 min on a rotation platform, followed by three washes with 2 mL of PBS, each for 5−10 min on a rotation platform.

1.1.5. Incubate cells in 100 µL of 1% bovine serum albumin (BSA) in PBS for 1 h at RT on a rotation platform.

1.1.6. Replace the solution with 100 µL of a mixture of primary antibodies (anti-emerin polyclonal antibody[14] and anti-tubulin monoclonal antibody[15]) in 1% BSA in PBS at appropriate dilutions (1/500 for anti-emerin and 1/100 for anti-tubulin), and incubate overnight at 4 °C.

1.1.7. Wash cells with 2 mL of PBS, 3−5x each for 10 min on a rotation platform.

1.1.8. Replace the solution with 100 µL of a mixture of secondary antibodies (anti-rabbit IgG with Alexa Fluor 488 and anti-mouse IgG with Alexa Fluor 555) in 1% BSA in PBS at 1/500 dilution, and incubate for 3−4 h at RT.

1.1.9. Wash cells with 2 mL of PBS, 3−4x each for 5 min on a rotation platform.

1.1.10. Replace the solution with 2 mL of 0.5 µg/mL DAPI in PBS to stain DNA for 30 min at RT on a rotation platform.

NOTE: Instead of DAPI, Hoechst 33342 at the same concentration can also be used.

1.1.11. Replace the solution with ~100 µL of mounting medium (**Table of Materials**).

1.2. Preparation of a biological calibration sample

1.2.1. Prepare fixed cells as described in steps 1.1.1−1.1.4.

NOTE: Preferably, samples are prepared on a chambered coverglass to place both the target and reference samples in separate chambers on the same coverslip (**Table 1**, right-most). This

preparation is preferable when the experiment requires the highest correction accuracy. When the sample needs to be prepared on a regular coverslip, use precision coverslips with low thickness variation ("No. 1.5H") to insure reproducibility.

1.2.2. Prepare the stock solution of fluorescent dye-conjugated phalloidin in 1.5 mL of methanol and store at -20 °C. Mix phalloidin stock solution in PBS at the following dilution: 1/100 for phalloidin with Alexa Fluor 405, 1/1,000 for phalloidin with Alexa Fluor 488, and 1/200 for phalloidin with Alexa Fluor 594.

1.2.3. Replace the solution with 1 mL of the phalloidin mixture prepared in step 1.2.2 and incubate for 30 min at RT on a rotation platform.

1.2.4. Wash cells with 2 mL of PBS, 3−5x each for 10 min on a rotation platform.

1.2.5. Replace the solution with ~100 µL of mounting medium.

NOTE: The calibration samples can be stored at 4 °C or -20 °C for repeated use.

2. Acquisition of reference images

2.1. Crosstalk reference images

2.1.1. Place the target sample prepared in step 1.1 on a wide-field microscope.

2.1.2. Acquire a fluorescence image of the target in blue, green, and red channels.

NOTE: For 3D images, the Z step size in the reference image can be different from that in the target image as long as the image file contains information for the step size. The Z step size for reference and target images is preferably less than half of the optical resolution of the Z axis, which is calculated by $\frac{2\lambda}{NA^2}$, where λ is the wavelength in nanometers and *NA* is the numerical aperture of the objective lens. For example, if the axial resolution is 550 nm, then use a Z step smaller than 275 nm. No time series is required for the reference image.

2.1.3. Subsequently, to acquire a fluorescence image of the reference, select the excitation light only for DAPI, and choose to acquire in blue, green, and red channels. Acquire the reference image exactly at the same stage position and Z height, in case of a 3D stack.

NOTE: For longer emission wavelengths, increased illumination intensity and exposure time will be required.

2.2. Bright-field reference images

2.2.1. Place the target sample prepared in step 1.1 on a wide-field microscope.

2.2.2. Acquire a fluorescence image of the target in blue, green, and red channels.

NOTE: Z step size preferably fulfills the Nyquist criterion as described in step 2.1.2.

2.2.3. Acquire a bright field image of the target in blue, green, and red channels exactly at the same stage position as in 2.2.2 and the same Z height in case of a 3D stack.

2.3. Biological calibration reference images

2.3.1. Place the target sample prepared in step 1.1 on a 3D-SIM microscope.

2.3.2. Acquire a fluorescence image of the target in blue, green, and red channels by 3D-SIM.

NOTE: The type of microscope can be of any type such as 3D-SIM, confocal, STED, etc. Z step size preferably fulfills the Nyquist criterion as described in step 2.1.2.

2.3.3. Acquire multiple fluorescence images of the reference prepared in step 1.2 at different stage positions similar to the target image (step 2.3.2) by 3D-SIM.

NOTE: The total number of pixels in XY must be the same in all reference images. The step size in Z must be the same in all reference images. The total number of Z sections is preferred to be the same as that in the target image, but this is not an absolute requirement. The XY position on the stage for these reference images does not matter because the position or coverslip is already different from the position of the target sample and furthermore the difference in chromatic shift on a single coverslip is less than 15 nm^3. Imaging conditions including objective lens, observation temperature, immersion oil, pinhole size in confocal microscopy, and tilt angle in highly inclined illumination microscopy[16], should all match the reference for the best performance. If the microscope equips multiple cameras to acquire multiple channels simultaneously, the reference images should be acquired as often as weekly to correct the instrumental drift.

3. Correction of chromatic shift using *Chromagnon* software

3.1. Using a web browser, go to https://github.com/macronucleus/Chromagnon/releases, and download the newest binary release of *Chromagnon*.

NOTE: Binary releases are available for Windows, Mac, and some Linux versions.

3.2. Extract the program and put the executable file at a convenient location. On a Windows or Mac double-click the file to open it, or else execute the binary file from the command line on a Linux system.

NOTE: The graphical user interface as in **Figure 1** will open.

[Place **Figure 1** here]

3.3. Drag and drop the reference files in the "Reference box" (**Figure 1**) or click **Reference files** (**Figure 1A**) to open a file selector dialog. Depending on the file formats, if the program asks to install a Java Development Kit (JDK), press **yes** and the user will be navigated to the download page. Download and install the JDK for the operating system as instructed. *Chromagnon* should be able to read the image file format after restarting the program.

NOTE: *Chromagnon* can read most image file formats (multipage 'tif', 'czi', 'nd2', 'oib', 'lif', 'dv', etc.). The original microscope image format is preferred at this step because metadata can be lost when an image format is converted to a multipage tif file. If the channel name is shown as 0, 1, 2, etc. instead of wavelengths such as 528, 609 (**Figure 2A**, green boxes), then *Chromagnon* does not know the identity of the channels in the image. In this case, ensure that the order of the channels and the pixel size in the reference file match those in the target file. For example, if the order of channels in the reference is green and red, then the order of channels in the target must also be green and red, but not red and green.

[Place **Figure 2** here]

3.4. Drag and drop the target files in the "Target box" (**Figure 1**) or click **Target files** (**Figure 1B**) to open a file selector dialog.

NOTE: If there are multiple target images and each of them have corresponding reference images, the corresponding reference image and target image must be on the same row in the respective reference and target boxes (**Figure 2A**). A single reference image can also be used to align multiple target images (**Figure 2B**).

3.5. Check the **crop margins** checkbox if unchecked (**Figure 1C**).

NOTE: If this checkbox is checked, margins resulting from the alignment are cropped. Check this option for general use but uncheck if a pixel-level comparison is required between images before and after alignment.

3.6. Choose an output image format from the choice list (**Figure 1D**).

NOTE: The available formats are 'tif' (ImageJ[17] format), 'dv', and 'ome.tif'; the 'ome.tif' format is only available after installing JDK.

3.7. Specify the suffix for the output filename (**Figure 1E**, the default value is '_ALN'), which is added to the target filename.

3.8. For crosstalk reference images with high signal-to-noise ratio, use local alignment from the choice list of **Local align** (**Figure 1F**), choose **Projection** to use local alignment and **None** to disable it. Use a minimum window size of 60 (**Figure 1G**).

NOTE: When using local alignment, all target images must have corresponding reference images (**Figure 2A**). Local alignment is not recommended for biological calibration reference images as local chromatic shifts vary from one sample to the other (**Table 1**). For the same reason, single local alignment cannot be applied to many target images (**Figure 2B**). As an exception, it can be used when the sample of interest is only at the surface of the coverslip, and the field of view is filled with bright objects, just like the multicolor fluorescent bead samples.

3.9. For multiple biological calibration reference images, check the **average references** option (**Figure 1H**) to measure a single alignment parameter from the averaged image, and the single alignment parameter is then applied to all target images (**Figure 2C**).

3.10. Click **Run all** (**Figure 1I**) to start measurements; the alignment parameters are applied to the target images.

NOTE: After measuring chromatic shifts from the reference files, the program makes files with extension "chromagnon.csv" or "chromagnon.tif". The type of output file depends on whether local alignment is in effect (**Figure 1F**). If alignment is done without local alignment, the output is "chromagnon.csv", while if local alignment is being used, the output is "chromagnon.tif". The file names are the same as the reference file except for the specified suffix (**Figure 1J**, the default is without suffix) and the extension ("chromagnon.csv" or "chromagnon.tif"). A detailed description of the alignment process can be found in the "Chromagnon.log" file created in the same folder.

3.11. Wait until the corrected image shows up in the viewer (**Figure 3**).

NOTE: Dragging the image with the mouse moves the image and moving the mouse wheel changes the zoom. Move the slider (**Figure 3A**) to change Z (and/or T when applicable) section for display. If the viewer is too slow to refresh an image, click the **Load whole data into memory** button (**Figure 3B**) to stop it accessing the data on the hard disk. Dragging the left or right edge of color boxes (**Figure 3C**) can control the minimum or maximum values for display. Clicking the button for each channel (**Figure 3D**) toggles between showing or hiding the selected channel in the viewer. Right clicking on the color box (**Figure 3D**) enables users to choose the colors for display, or change the display options of the color bar, and it also allows users to specify the exact minimum and maximum values for display scaling by choosing "scale to …". Clicking the **Orthogonal view** button (**Figure 3E**) shows the images of ZY and XZ views. Moving the cross lines changes the position to show in the side views.

[Place **Figure 3** here]

3.12. To check if the measurement was performed correctly, drag and drop the reference images into the "Reference box" and "Target box". Run the program, and check if the images are perfectly overlapped.

NOTE: When available, “chromagnon.csv” or “chromagnon.tif” files can be used as references in order to skip measurements. If the chromatic shift in the image remains uncorrected, try solutions summarized in **Table 2**.

3.13. To access the alignment parameters in the sample, or when the alignment parameters need to be edited manually, open “chromagnon.csv” files directly with any text editor or spreadsheet software. When it is edited, save it as “csv”.

NOTE: The values are in pixels for “tz”, “ty”, “tx”, and in degrees for “r”, and in magnification factors for “mz”, “my”, and “mx”.

3.13.1. Alternatively, load a “chromagnon.csv” or “chromagnon.tif” file into *Chromagnon*’s Reference or Target box (**Figure 1**), then double-click it to open the alignment editor (**Figure 4**).

3.13.2. To see how much a channel is shifted when the parameters are edited, drag and drop the reference image file onto the bottom area (**Figure 4A**) to open the image in the editor. Upon editing the values in the table (**Figure 4B**) and hitting the **enter/return** key, observe how the image of the corresponding channel moves as the values changed. After editing, click **save as…** (**Figure 4C**) to save the changes.

[Place **Figure 4** here]

3.14. To check the local alignment map, load the “chromagnon.tif” via *Chromagnon*’s Reference or Target box (**Figure 1**), and double-click it to open the alignment editor (**Figure 4**). Click **view** (**Figure 4D**) to view the local shift indicated by lines whose lengths are magnified by the factor specified in the **magnification** choice list (**Figure 4E**).

NOTE: By specifying the “original image file” (**Figure 4F**), the shifts are mapped along with the original image.

4. Generating a microscope-specific local alignment map

4.1. Dilute 2 µL of multicolor fluorescent beads of 200 nm diameter in 18 µL of ethanol.

4.2. Vortex the solution and place 10 µL of the bead solution at the center of a glass bottom dish. Make sure to use #1.5 coverglass (with thickness of 0.17 µm) for high-resolution microscopy.

4.3. Leave the dish at RT for 1 h to dry completely.

4.4. Place the dish on a microscope to be calibrated and focus on the fluorescent beads.

4.5. Obtain 2D or 3D images with the microscopy to be calibrated. Obtain multiple images by changing the stage position.

4.6. Open *Chromagnon* graphical user interface.

4.7. Drag and drop the bead image files on the "Reference box" (**Figure 1**) or click **Reference files** (**Figure 1A**) to open a file selector dialog.

4.8. Check the **average references** option (**Figure 1H**). From the choice list of **Local align** (**Figure 1F**), choose **Projection**. Use a minimum window size of 60 (**Figure 1G**). Click **Run all** (**Figure 1I**) to start measurements.

NOTE: A ".chromagnon.tif" file is created, in which the local alignment map of the microscope is stored.

4.9. After measurement, click **Extra parameters** (**Figure 1K**). From the **Local distortion of your microscope instrument** box, click the choice list and choose **New…** to open a dialog. Drag and drop the "chromagnon.tif" file generated in step 4.8 into the file name box or click **choose file** button to open a file selector dialog. Enter the name of the microscope and click **OK**.

4.10. When measuring chromatic shifts from crosstalk or biological calibration reference images, click **Extra parameters** (**Figure 1K**). From the **Local distortion of your microscope instrument** box, choose the name of the microscope specified in step 4.9. Click **OK**.

NOTE: The choice of the "Local distortion of your microscope instrument" is not saved after the program is shut down.

4.11. Proceed to chromatic correction without local alignment as in section 3.

NOTE: It is also possible to use local alignment in addition to microscope calibration. In this case, local alignment starts with microscope calibration.

REPRESENTATIVE RESULTS:

An example of chromatic shift correction using a crosstalk reference image is shown in **Figure 5**. The image was obtained with a wide-field microscope equipped with a single camera. Fluorescence emission from DAPI was used as a reference (**Figure 5A,B**) to correct the blue, green, and red channels. The image comprises 3 channels of 60 Z slices, each composed of 256 x 256 pixels. The images were deconvolved before measuring the chromatic shifts. Measuring the local chromatic shifts using *Chromagnon* took 51 s on a Mac with Intel Core i7 (quad core, 8-threads, 2.7 GHz), 16 GB RAM and 1 TB flash storage. The alignment parameter was applied to the target image (**Figure 5C,D**), which has exactly the same number of voxels as the reference image. Preparing the aligned file took 3 s. As a result of trimming the edge pixels during the alignment process (**crop margins** checkbox in **Figure 1C**), the number of voxels was reduced after alignment (**Figure 5B**, 51 Z slices, 252 x 251 pixels). DNA in the anaphase bridge (indicated by arrowheads) is seen incorrectly outside of the nuclear envelope before alignment (**Figure 5C**, obvious in the bottom panel showing the XZ view), but as expected inside the envelope after alignment (**Figure 5D**).

An example of chromatic shift correction using a biological calibration reference image is shown in **Figure 6**. The images were obtained with a SIM microscope equipped with three cameras. Three images of HeLa cells stained with phalloidins conjugated to blue, green, and red dyes were averaged (**Figure 6A**). The reference image comprises 3 channels of 76 Z slices, each composed of 1,024 x 1,024 pixels. Measuring chromatic shifts using *Chromagnon* without local alignment required 194 s on the Mac system described above. The parameter was applied to a target image consisting of 3 channels of 73 Z slices, each composed of 1,024 x 1,024 pixels. Generation of the aligned file took 25 s. The XZ view shows incorrect channel positions along the Z, and slightly along the X directions (**Figure 6A,C**) but this misregistration was corrected after alignment (**Figure 6B,D**).

FIGURE AND TABLE LEGENDS:

Figure 1: A screenshot of *Chromagnon* graphical user interface.

Figure 2: Example screenshot for loading multiple files. (**A**) A case in which all reference images have the corresponding target images. Channel names are correctly identified by wavelengths (indicated by a green box) in the image files used in this example. (**B**) A case in which a single reference image is used to correct multiple target images. (**C**) A case in which multiple reference images (indicated by a red box) are averaged, and the resulting reference image after averaging is used to correct multiple target images.

Figure 3: A screenshot of the image viewer.

Figure 4: A screenshot of an alignment parameter editor.

Figure 5: An example of alignment with a crosstalk reference image. HeLa cells were stained with DAPI for DNA (shown in magenta), Alexa Fluor 488 (shown in yellow) for nuclear envelope, and Alexa Fluor 555 (shown in blue) for microtubules. Images were acquired by 3D wide-field microscopy with a single camera and deconvolved. (**A,B**) A representative crosstalk reference image using DAPI emission, before and after alignment by *Chromagnon*. Three color channels are shown as overlaid. (**C,D**) An optical section of the 3D stack in three channels before and after alignment. Axial chromatic aberration is obvious at the anaphase bridge shown by arrowheads. Scale bar in panel A indicates 5 µm for all panels.

Figure 6: An example of alignment with a biological calibration reference image. Images were acquired with 3D-SIM equipped with three cameras. (**A,B**) A reference image averaged from three images before (**A**) and after (**B**) alignment. HeLa cells were stained with phalloidin conjugated with Alexa Fluor 405, 488 or 594. (**C,D**) The target image before (**C**) and after (**D**) alignment. HeLa cells were stained with DAPI for DNA (shown in magenta), Alexa Fluor 488 (shown in yellow) for nuclear envelope, and Alexa Fluor 594 (shown in blue) for microtubules. Scale bar in panel A indicates 5 µm for all panels.

Figure 7: Examples of reference images. Nuclear envelope in fission yeast cells labeled with GFP and mCherry. Images were acquired with conventional wide-field microscopy. Chromatic shifts were corrected using *Chromagnon* without local alignment using the images themselves as reference images. Images were then deconvolved to show the details. (**A**) A good example with many objects in the field of view. (**B**) A bad example with objects only at the top-left corner. Misalignment is obvious at a certain region of the image. (**C**) An undesirable example where one of the quadrisection (separated by dotted cross lines) is empty. Scale bar in panel A indicates 5 µm for the full field view and 1.25 µm for the enlarged view and is applicable to all panels.

Table 1: Parameters when choosing the type of reference images.

Table 2: Troubleshooting for chromatic correction.

DISCUSSION:

The procedure for chromatic correction is a tradeoff between accuracy and effort. To save needless efforts, it is better to know how much accuracy is required for your study. The highest accuracy may not be required for conventional wide-field live imaging, and thus, bright field reference images are often sufficient to correct the chromatic shift. Similarly, when the imaging condition and environment is constant, repeated use of a biological calibration will save energy. On the other hand, if a highly accurate registration is desired, high-quality crosstalk or biological calibration reference images are necessary. For the best performance, reference images should be obtained with as similar conditions and timings as the target images as possible. As long as both reference and target images are obtained by the same microscopy, higher spatial resolution will improve the correction accuracy. If deconvolution is available for both reference and target images, then implementing this before correction may improve the correction accuracy. Also, for the best performance, the sampling theorem for the optical (Z) axis should be fulfilled in both the reference and target file for precise subpixel interpolation (protocol step 2.1.3).

Failure to correct chromatic shift leads to incorrect conclusions. Furthermore, using the wrong calibration may even worsen the chromatic shifts rather than correcting them, and this therefore needs to be avoided. We have summarized the possible causes of failures, and their common solutions, in **Table 2**. To examine the cause of a failure, in the first place, it is necessary to visually check if the chromatic shift in the reference image is precisely corrected (protocol step 3.12). Most failures are due to the quality of the reference images and are easily remedied as per the descriptions in **Table 2**. Regarding to the quality of reference images, it is important to note that the accuracy of global alignment decreases if the entire field of view is not filled with the sample (**Figure 7**, **Table 2**). Compared to the good example in **Figure 7A**, the bad example in **Figure 7B** contains only three nuclear envelopes in the upper-left region, and *Chromagnon* failed to align a part of this image. This is because the global alignment method of *Chromagnon* splits the field of view into four regions (**Figure 7C**) in order to measure the differences in rotation and magnification with high accuracy[3]. This method, if correctly operated, is one order more accurate than other linear methods such as the log polar transformation and simplex methods[3]. If any of the four regions are unavailable, then *Chromagnon* will switch to less effective linear methods. Therefore, for the best performance, the examples in **Figure 7B** and **Figure 7C** are undesirable,

and the four regions should be filled with objects. Users can check if any quadratic region of the field of view is unavailable for measurement by looking at the log file ("Chromagnon.log"; see protocol step 3.10). Fortunately, this problem can be easily overcome by averaging multiple biological calibration images or using local alignment for crosstalk or bright-field reference images (**Table 2**). Contrary to the case of failure to correct reference images, failure to correct the target images is difficult to find. Because such failures arise due to differences in file formats, imaging conditions, imaging timings, imaging/alignment methods between the reference and target images (**Table 2**), users should always be careful when using reference images that are obtained in different conditions/timings from the target images. Some example images are available for testing (https://github.com/macronucleus/Chromagnon) to obtain concrete idea of the good and bad example images.

[Place **Table 2** and **Figure 7** here]

In this protocol, we described three different reference types (**Table 1**). Among them, crosstalk reference images and biological calibration reference images need further careful discussion. For crosstalk reference images, samples stained with DAPI or Hoechst 33342, and mounted in glycerol or commercial mounting media can be efficiently used to align the blue, green, and red channels. Similarly, Alexa Fluor 488 can be used to align the green and red channels. However, obtaining crosstalk fluorescence is often difficult since many blue dyes except DAPI and Hoechst are dimmer and decay faster than most green and red dyes. Furthermore, the emission spectra of modern dyes are narrower, which makes the alignment of more than three channels by this method challenging. Attention should also be paid to some common red dyes (e.g., Alexa Flour 568 and 594, but not Alexa Fluor 555) that can be excited by violet light, which prevent obtaining high-contrast crosstalk images from blue dyes. Another drawback is that this method cannot measure the chromatic aberration of excitation light paths in multicolor excitation, because only a single excitation wavelength is used for excitation (**Table 1**). As most advanced microscopy uses altered illumination optics, the application of this method is limited. Still, its higher correction accuracy is sufficiently advantageous for it to be described in this protocol. In general, a crosstalk image should be taken after a target image to prevent bleaching or phototoxic effects. For SMLM observed with the wide-field mode, a reference image should be acquired before acquiring a target image as fluorescence dyes can be bleached while imaging.

Biological calibration reference images allow users to easily align any desired number of channels at the cost of additional sample preparation. Another advantage of biological calibration reference images is the availability of "averaging" multiple references that helps fill all fields of view. This method may suffer from differences in imaging conditions if the calibration sample is prepared on a different slide. Most of this problem can be solved if both targets and references are prepared on the same slide by using commercial chambered coverglasses (**Table 1**), and other imaging conditions are kept constant as in protocol step 2.3.3. In this case, a correction accuracy similar to that of crosstalk reference images can be expected[3]. The protocol to use phalloidin as shown here is one of the easiest ways to stain a single cellular structure with multiple colors. There are numerous possible scenarios to prepare biological calibration samples. For immunostaining, a sample can be labeled with a single primary antibody followed by staining

with secondary antibodies of multiple colors. In this way, a single target structure can be labeled with multiple colors. Alternatively, 5-ethynyl-2'-deoxyuridine, detected by "click" chemistry labels newly synthesized DNA in multiple colors at high density, as described in detail previously[8]. For live cells, it is useful to prepare a transgenic strain harboring two copies of a gene that are fused to GFP or mCherry to label the same structure with two colors. If the copy number of the gene is critical as often observed for membrane proteins, a single copy of the gene can be tandemly fused to GFP and mCherry (**Figure 7**). Photoconvertible fluorescent proteins, such as mEOS2[18], can also be used by illuminating a moderate level of violet light to obtain both protein species with or without photoconversion. Under low oxygen conditions, GFP can also be used as a photoconvertible protein from green to red[19,20]. Choosing the right calibration sample will thus make the experiment more robust.

ACKNOWLEDGMENTS:

This study was supported by JSPS KAKENHI Grant Numbers JP19H03202 to A.M., JP18H05528 and JP17H03636 to T.H., and JP17H01444 and JP18H05533 to H.Y. L.S. acknowledges the support by the Welcome Trust Strategic Awards 091911 and 107457/Z/15/Z funding advanced imaging at Micron Oxford.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Schermelleh, L. et al. Super-resolution microscopy demystified. *Nature Cell Biology*. **21** (1), 72–84 (2019).
2. Manders, E.M.M. Chromatic shift in multicolour confocal microscopy. *Journal of Microscopy*. **185** (3), 321–328 (1997).
3. Matsuda, A., Schermelleh, L., Hirano, Y., Haraguchi, T., Hiraoka, Y. Accurate and fiducial-marker-free correction for three-dimensional chromatic shift in biological fluorescence microscopy. *Scientific Reports*. **8** (1), 7583 (2018).
4. Grünwald, D., Singer, R.H. In vivo imaging of labelled endogenous β-actin mRNA during nucleocytoplasmic transport. *Nature*. **467** (7315), 604–607 (2010).
5. Betzig, E. et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science*. **313** (5793), 1642–1645 (2006).
6. Rust, M.J., Bates, M., Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nature Methods*. **3** (10), 793–795 (2006).
7. Chen, F., Tillberg, P.W., Boyden, E.S. Expansion microscopy. *Science*. **347** (6221), 543–548 (2015).
8. Kraus, F. et al. Quantitative 3D structured illumination microscopy of nuclear structures. *Nature Protocols.* **2**, 1011–1028 (2017).
9. Hell, S.W. Far-Field Optical Nanoscopy. *Science*. **316** (5828), 1153–1158 (2007).
10. Gustafsson, M.G.L. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *Journal of Microscopy*. **198** (2), 82–87 (2000).

11. Schulz, O. et al. Resolution doubling in fluorescence microscopy with confocal spinning-disk image scanning microscopy. *Proceedings of the National Academy of Sciences of United States of America*. **110** (52), 21000–21005 (2013).
12. Müller, C.B., Enderlein, J. Image Scanning Microscopy. *Physical Review Letters*. **104** (19), 198101 (2010).
13. Hayashi, S., Okada, Y. Ultrafast superresolution fluorescence imaging with spinning disk confocal microscope optics. *Molecular Biology of the Cell*. **26** (9), 1743–1751 (2015).
14. Yorifuji, H. et al. Emerin, deficiency of which causes Emery-Dreifuss muscular dystrophy, is localized at the inner nuclear membrane. *Neurogenetics*. **1** (2), 135–140 (1997).
15. Woods, A., Sherwin, T., Sasse, R., MacRae, T.H., Baines, A.J., Gull, K. Definition of individual components within the cytoskeleton of Trypanosoma brucei by a library of monoclonal antibodies. *Journal of Cell Science*. **93** (3), 491–500 (1989).
16. Tokunaga, M., Imamoto, N., Sakata-Sogawa, K. Highly inclined thin illumination enables clear single-molecule imaging in cells. *Nature Methods*. **5** (2), 159–161 (2008).
17. Rueden, C.T. et al. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*. **18** (1), 529 (2017).
18. McKinney, S.A., Murphy, C.S., Hazelwood, K.L., Davidson, M.W., Looger, L.L. A bright and photostable photoconvertible fluorescent protein for fusion tags. *Nature Methods*. **6** (2), 131–133 (2009).
19. Sawin, K.E., Nurse, P. Photoactivation of green fluorescent protein. *Current Biology*. **7** (10), R606-607 (1997).
20. Elowitz, M.B., Surette, M.G., Wolf, P.-E., Stock, J., Leibler, S. Photoactivation turns green fluorescent protein red. *Current Biology*. **7** (10), 809–812 (1997).

Figure1

Click here to access/download;Figure;Fig.1.ai

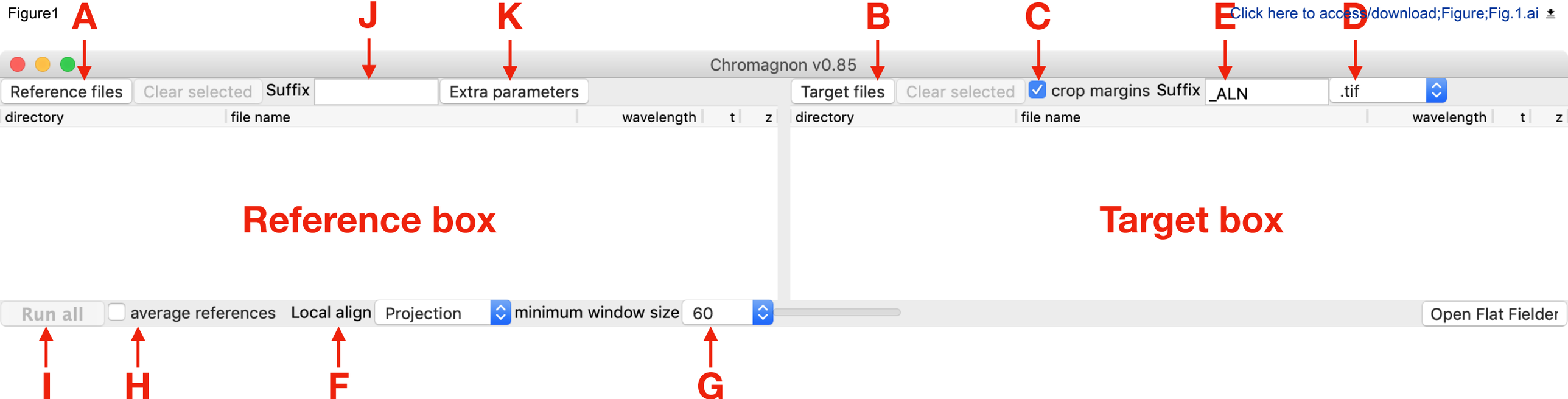
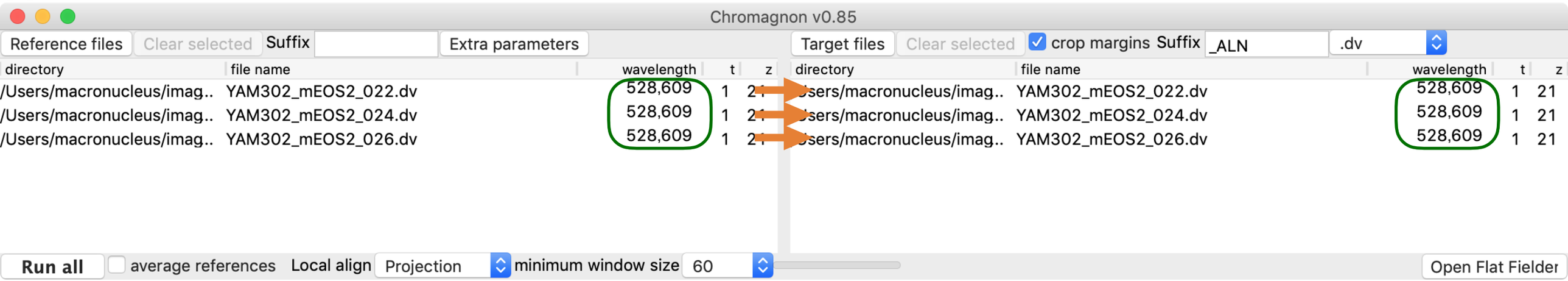


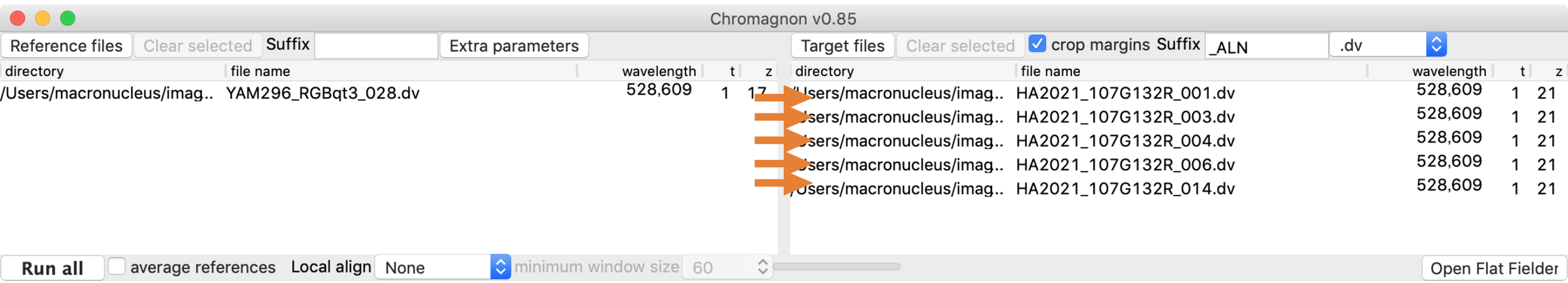
Figure2

Click here to access/download;Figure;Fig.2.ai

A



B



C

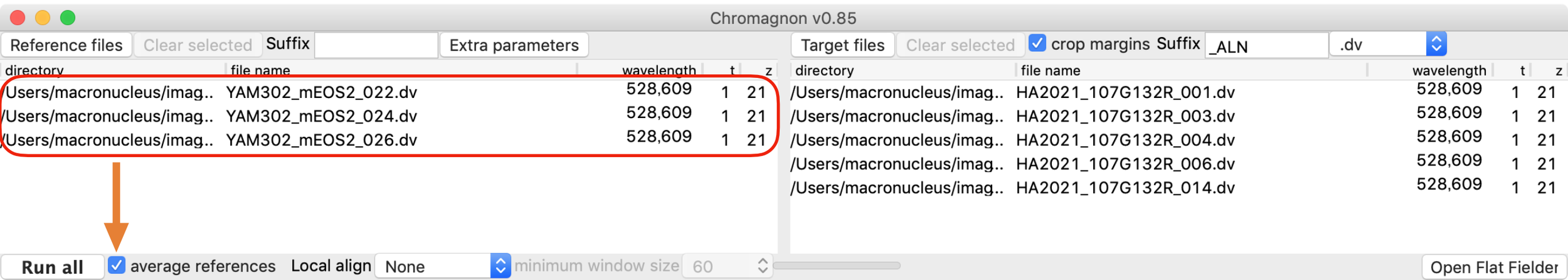
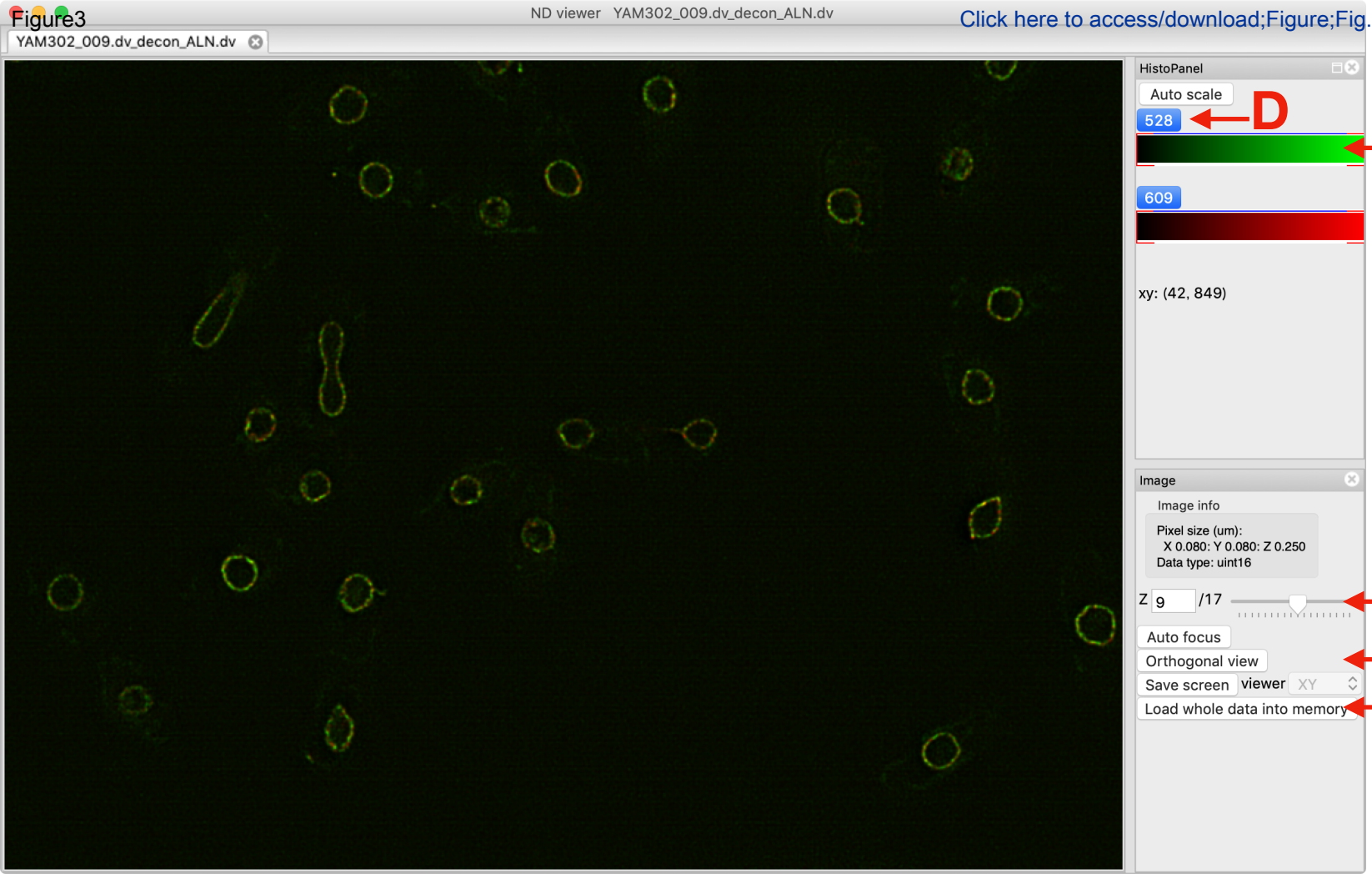
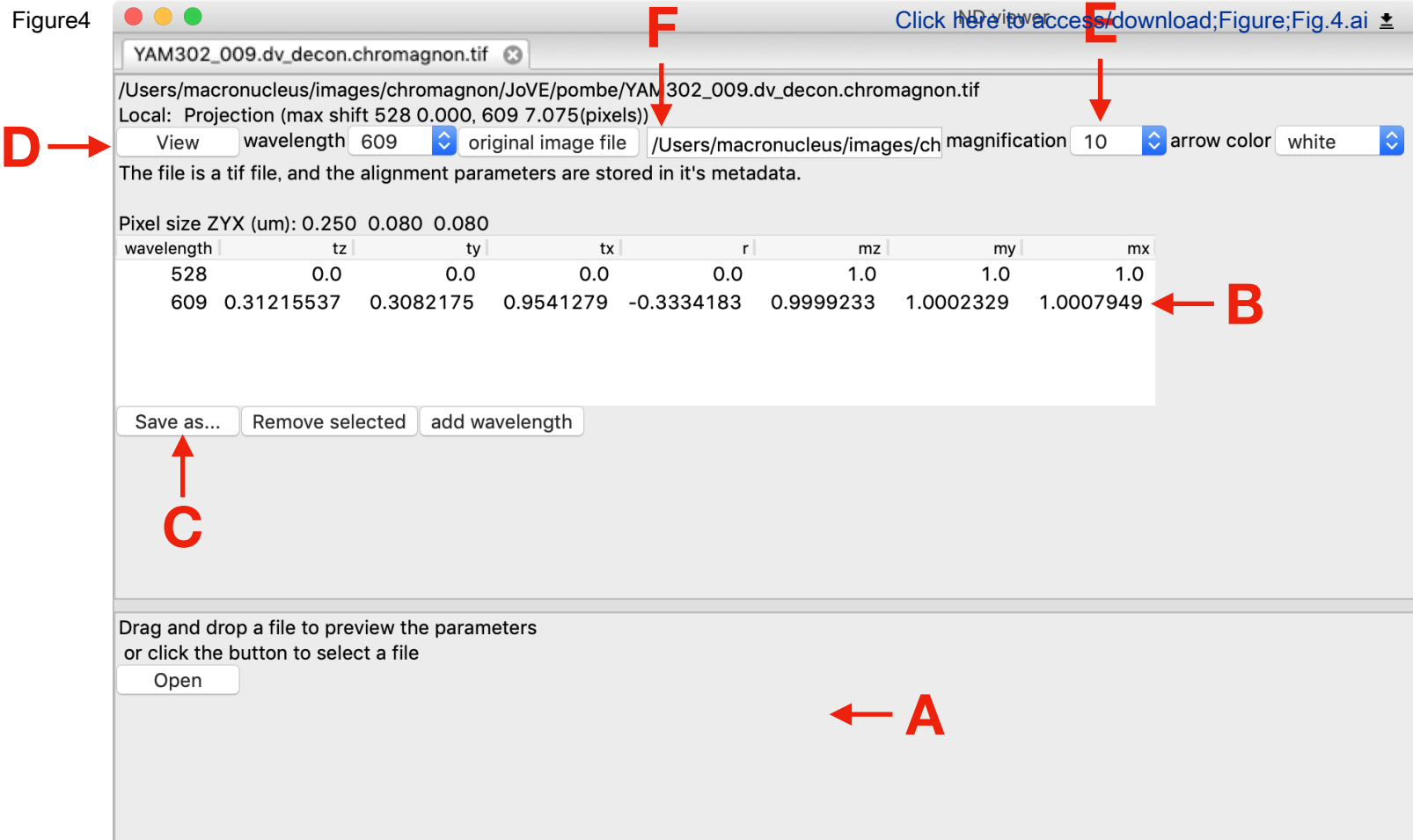


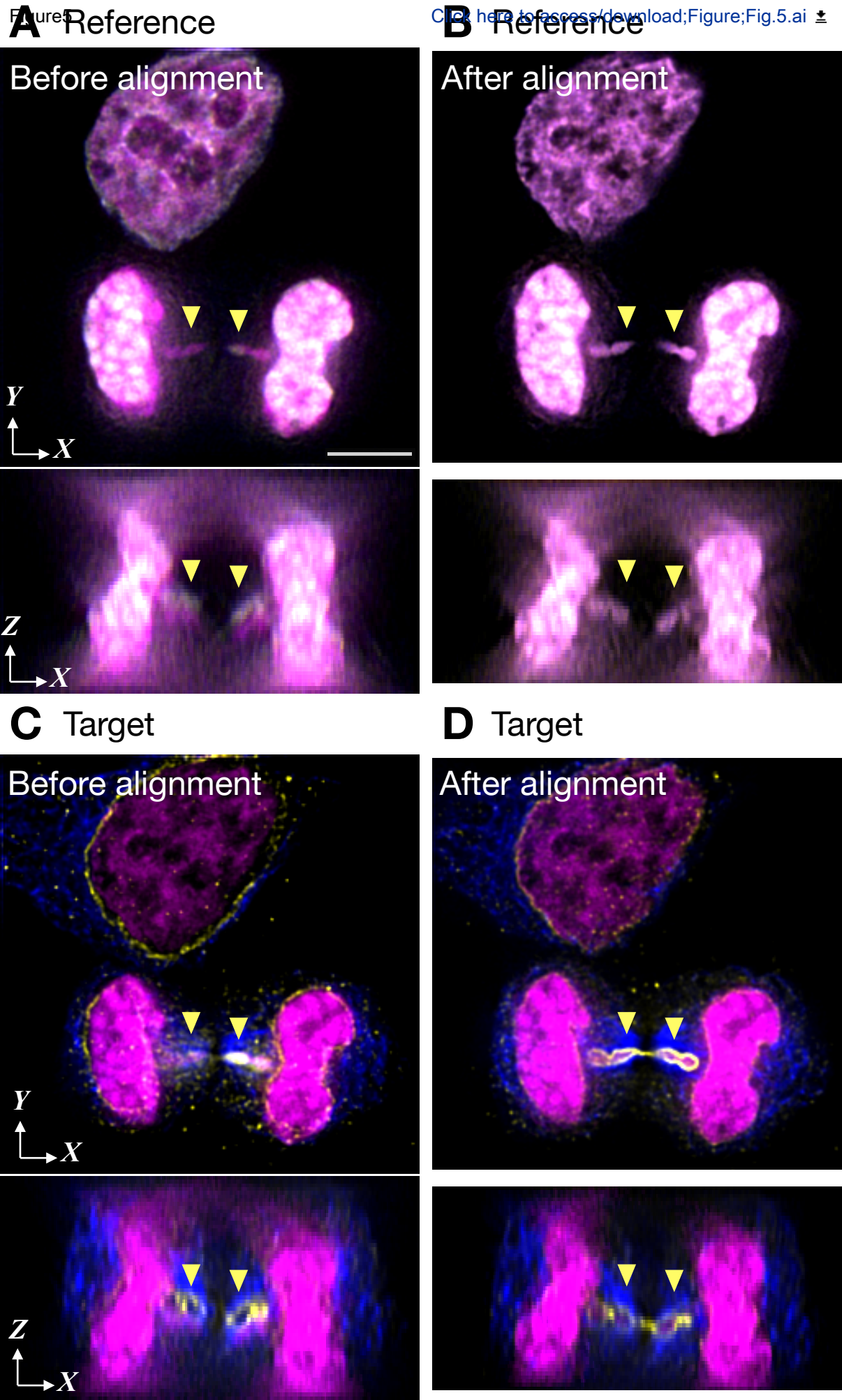
Figure3

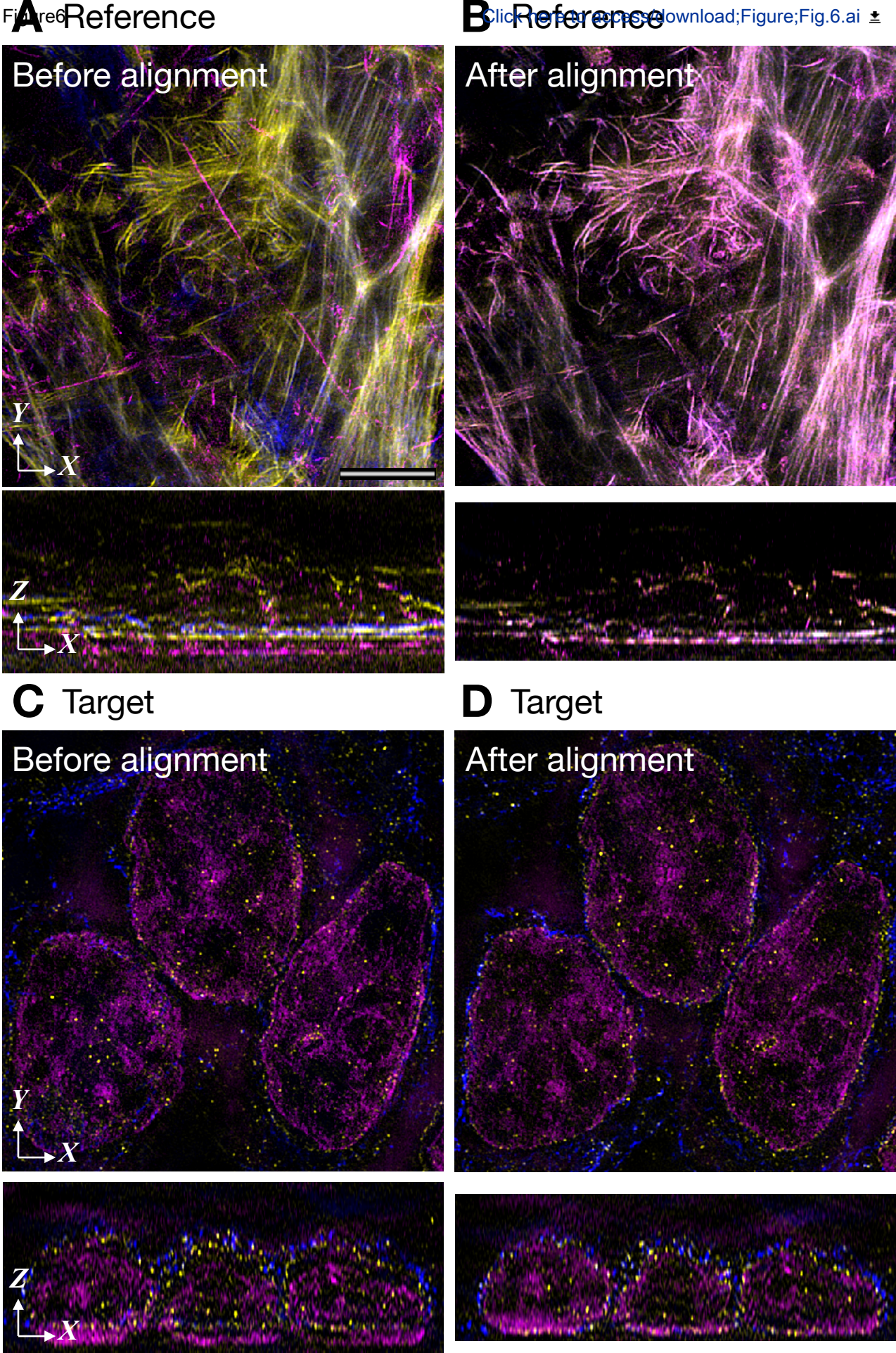


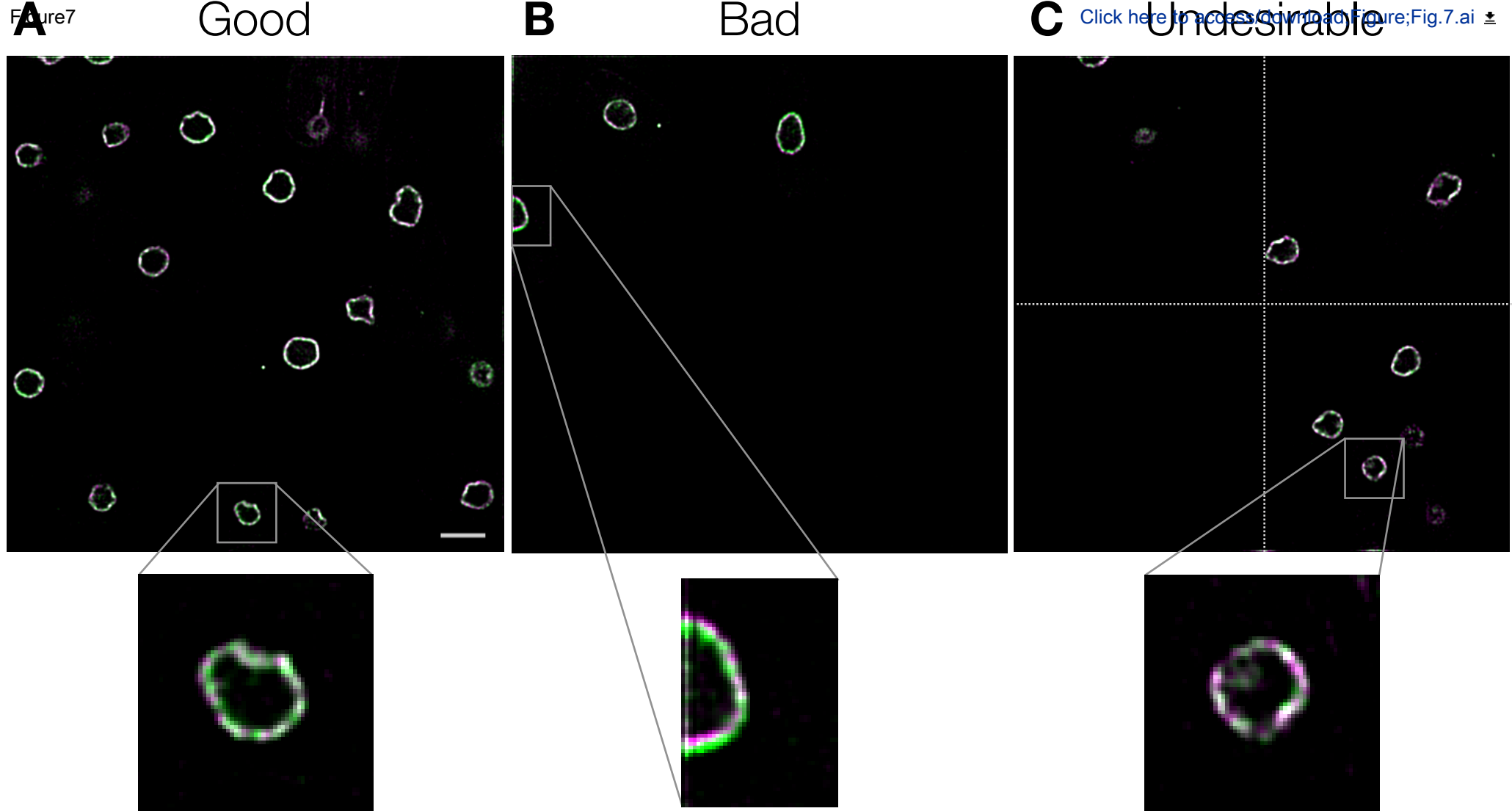
Click here to access/download;Figure;Fig.3.ai

Figure4


Click here to access/download;Figure;Fig.4.ai
NDviewer
YAM302_009.dv_decon.chromagnon.tif
/Users/macronucleus/images/chromagnon/JoVE/pombe/YAM302_009.dv_decon.chromagnon.tif
Local: Projection (max shift 528 0.000, 609 7.075(pixels))
View
wavelength
609
original image file
/Users/macronucleus/images/ch
magnification
10
arrow color
white
The file is a tif file, and the alignment parameters are stored in it's metadata.
Pixel size ZYX (um): 0.250 0.080 0.080
wavelength tz ty tx r mz my mx
528 0.0 0.0 0.0 0.0 1.0 1.0 1.0
609 0.31215537 0.3082175 0.9541279 -0.3334183 0.9999233 1.0002329 1.0007949
Save as...
Remove selected
add wavelength
Drag and drop a file to preview the parameters
or click the button to select a file
Open
A
B
C
D
E
F







	Crosstalk	Bright-field	Biological calibration (on a different slide)	Biological calibration (on the same slide)
Accuracy[a]	+++	+	++[b]	+++
Simplicity	++	+++	++	++
Applicable microscopy	Wide-field	Wide-field	All	All
Availability of local alignment	+	+	-[c]	-[c]

[a]: Number of "+" indicates increasing rating. Single plus is about 50 nm and three plus is about 15 nm in 3D.

[b]: The accuracy depends on how much the variable imaging conditions are kept constant.

[c]: Local calibration measured by multicolor bead samples can be combined as described in protocol section 4.

Problem	Cause	Solution
Failed to correct the reference image	Low contrast	Acquire a higher contrast image if possible. If a bright-field reference image is used, reacquire the image in a water-based solution to obtain higher contrast of the cell. Alternatively, try applying computational noise reduction (e.g. Gaussian filtering). Turn off local alignment, which is more sensitive to noise.
	Contamination of unrelated images	Remove the source of the unrelated images in the sample if possible. For crosstalk reference images, check the excitation spectra of the dyes used for the target images. If the dyes are excited during acquisition of a crosstalk image (e.g. Alexa Fluor 568 or 594), consider other dyes (e.g. Alexa Fluor 555). If dusts on the camera chip creates an obvious channel difference, clean the camera chip or use a computational flat-fielding method.
	An extremely bright spot made by a cosmic ray	Acquire the image again if possible. Alternatively, try applying computational noise reduction (e.g. Median or Gaussian filtering).
	Deconvolution artifacts (artificial signals at the axial and lateral edges)	Trim the edge pixels or Z sections after deconvolution. If one side is trimmed, the other side should also be trimmed to maintain the image center.
	The Z step size too sparse	A Z stack should be acquired to fulfill the Nyquist criterion as written in protocol 2.1.3.
	Optical aberration	Spherical aberration is the major aberration caused by users. Choose the right objective lens for the sample and use a coverslip thickness of 170 µm. If the objective lens is equipped with a correction ring, adjust it to find the position where the highest fluorescence count is obtained from the focus. In the case of an oil-immersion objective without a correction ring, adjust the refractive index of the immersion oil that increases the fluorescence count at the focus.
	Field of view is unfilled (Fig. 7)	In the case of biological calibration reference images, average many images. In the case of crosstalk or bright-field reference images, use local alignment.
	An unidentified software bug	Report the issue through GitHub (https://github.com/macronucleus/Chromagnon/issues)

Failed to correct the target image	Metadata of the image file is lost	Use the original microscope file format which contains complete metadata, and avoid converting to a multipage tiff file before processing. Use the same ordering of channels as written in protocol 3.3.
	Wrong alignment methods for the given microscopy	Do not apply the local alignment method when measuring from biological calibration reference images to target images. Do not use crosstalk reference images other than wide-field microscopy.
	Differences in imaging conditions	Keep the imaging conditions constant between the reference and the target images as written in protocol 2.3.3.
	Differences in sample (including coverslip)	Always use the same mounting medium, coverslip (e.g. No. 1.5H) and a similar depth of
	Microscope drift since the calibration was last made	Make a calibration as often as every two weeks. Keep the temperature constant, and use a floating table to avoid hardware drift of the microscope.

Name of Material/Equipment	Company
16% formaldehyde solution	Polyscience
35 mm glass-bottom dish	MatTek
Alexa Fluor 405 phalloidin	Thermo Fisher Scientific
Alexa Fluor 488 phalloidin	Thermo Fisher Scientific
Alexa Fluor 594 phalloidin	Thermo Fisher Scientific
Bovine Serum	Thermo Fisher Scientific
Coverslip	Matsunami
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Thermo Fisher Scientific
Dulbecco's Modified Eagle Medium with L-Gln and sodium pyruvate	Nacalai Tesque
Mounting medium (VECTASHIELD)	Vector Laboratories
Mouse anti-tubulin monoclonal antibody (TAT1)	
Nunc Lab-Tek II chambered coverglass (8 well)	Thermo Fisher Scientific
Rabbit anti-emerin polyclonal antibody (ED1)	
Secondary antibody with Alexa Fluor 488	Thermo Fisher Scientific
Secondary antibody with Alexa Fluor 555	Thermo Fisher Scientific
Secondary antibody with Alexa Fluor 594	Thermo Fisher Scientific
TetraSpeck Microspheres, 0.2 µm	Thermo Fisher Scientific

Catalog Number	Comments/Description
18814-10	
P35G-1.5-10-C	
A30104	
A12379	
A12381	
16170078	
No. 1S HT	
D1306	
08458-16	
H-1000	
	Described in Ref 15.
155409	
	A gift from Hiroshi Yorifuji, Gunma University, Gunma, Japan and Kiichi Arahata, National Center of Neurology and Psychiatry, Tokyo, Japan; deceased.
A-11034	
A-21424	
A-11032	
T7280	

Dear Editor

We appreciate the editor giving us enough time to revise this manuscript, and thank both reviewers for their important comments, which helped us make a lot of improvements to the manuscript.

Before going into details, we would like to draw one's attention to major change in terminology that we have made in this manuscript. We have changed the term "bleed-through reference images" to "crosstalk reference images" because the latter is shorter, and consistent with our previous paper (Sci Rep 2018) where we used "crosstalk-on-demand methods".

Below, we describe our responses in black, and all quoted texts in the "times" font with changes indicated in red. The line numbers refer to lines in the revised version.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We used an English proof-reading service to ensure there are no spelling or grammar issues.

2. As some authors are affiliated with UK institutions, please check whether open access is required by your funding agencies.

We are in the process of changing our license to open access.

3. Keywords: Please provide at least 6 keywords or phrases.

Six keywords were supplied.

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
5. 1.1.1: Please specify composition of the growth medium and growth conditions.
6. 1.1.2: When is the growth medium replaced?
7. 1.1.5: Please specify incubation temperature.
8. 1.1.8: What are the secondary antibodies used in this step? Please specify.
9. 1.1.11, 1.2.5: Please specify the mounting medium used in this step.
10. 2.2.3: Please specify the reference sample use in this step.
11. Section 3: Please revise to avoid the use of personal pronouns (e.g., I, you, your, we, our). Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "NOTE".

12. Table of Materials: Please sort the materials alphabetically by material name.

Comments 4 to 12 were addressed in the revised manuscript to clarify some of the uncertain protocols.

Reviewer #1:

Major Concerns:

The concept of bleed through images needs more description in the introduction.

More description has now been given in the INTRODUCTION (line 91-104) for the concept of bleed-through (now renamed "crosstalk") reference images.

The problem of potential artifacts needs to be discussed.

We devoted the new second paragraph in DISCUSSION to the discussion of potential artifacts (line 536). Furthermore, we made a new table, Table 2, where the possible causes of failure and their solutions are summarized.

Minor Concerns:

A few additional items are worth to discuss.
Inhomogeneity in the field of view could be problematic for the protocol and should be checked independently with other means.

We assume the reviewer's concern is about Fig. 7. We described how to check for this inhomogeneity and solve it in DISCUSSION (line 552-556).

When applying a fixed calibration on live-cell imaging, several problems may arise; here fixation plays a role, but also temperature. It would be worth to discuss this problematic.
An additional problem that haunts some SIM devices is temp/draft dependent misalignment. Here an upfront recorded image bears the danger to worsen or overcompensate the correction. This problematic is also worth to mention.

These points are surely problems and they are dealt with in the new second paragraph in DISCUSSION (line 536-538 line 556-560) and in Table 2.

Reviewer #2:

Manuscript Summary:

The manuscript on "High-accuracy correction of 3D chromatic shifts in the age of super-resolution biological imaging using Chromagnon" was reviewed with the understanding that it provides a protocol to a publication by these authors on the topic of chromatic corrections in fluorescence images acquired with diffractive optics. The original work is well known to me and any suggestions made in this

review are meant solely as help to users of the software package and not as critique of the original work. My believe is that clear explanation of features of the approach or software are essential to the community and pointing out possible limits, even those likely based on the interpretation by non-deep-expert users, is a strength rather than a weakness to the authors work!

That being said, I am struggling somewhat with the format of this article as my understanding is that the final product is a video documentation of this protocol and the written text might not be accessed frequently. For this reason, some comments are made towards points that would need some explicit mentioning in the video if it is used as the sole resource for using the software - although they might be covered in the original publication.

First of all, we would like to express our gratitude to the reviewer. We cannot thank the reviewer enough for testing the protocol to this depth and giving valuable feedback. We believe both the manuscript and software have now been improved a lot.

We are changing our license to open access, so that everybody can read the text version of this article. We will also put a link to this article on the GitHub site so that those users who wish to learn more can easily navigate to the article.

As a preamble: addressing chromatic shifts is of vital importance to any meaningful data analysis and I do fully support the notion that a best-case alignment based on bead images is utterly useless in the context of biological imaging. As such this protocol is well worth publishing and the work of the authors is much appreciated. One limitation that would help downstream users if discussed in more detail is strategies to verify alignment performance in the user's hand. Assessing the precision of alignment for Chromagnon is difficult. It is hence appreciated that Chromagnon is not presenting an artificial measure for alignment success that would lure a user in safety - good job! It would help though to suggest the use of good and bad bead data (e.g. add noise to good data) for users to develop an intuitive understanding of alignment precision and to check if they do use the software within reasonable bounds.

We have made the test images available for download from GitHub. In addition to the “good” data, we also added “bad” data in which the signal to noise ratio was less than 3. However, because *Chromagnon* is very tolerant to noise, users will notice only slight mis-registration.

Although the images are biological objects, not fluorescent beads, we believe users will benefit from them, because they teach users what kind of data is required in reality and how the registration really works in both good and bad scenarios.

The test data set is mentioned at the end of the second paragraph in DISCUSSION (line 560).

Major Concerns:

On the critical side of this review my lab members and I find the protocol to be lacking structure and clarity. For example, the sample preparation starts with "immunostaining…" but does not reference the imaging type(s) that this sample would be used to collect. Parallel structure between sections will improve reader understanding as well as increase the ease of a reader to go between sections and reference the information relevant to the particular imaging method they are testing the software with. Our recommendation would be to outline what should be said for each type of imaging/reference used and rebuild the entire manuscript using that outline. The discussion suffers from the same issues as the introduction but magnified. The sentence structure for the discussion of how individual images are used for comparison and how the images connect to the method of alignment is very confusing. We also find the software we tested not to function in the way described and besides error messages are losing frames from z-stacks during the processing. It would be hugely beneficial if this kind of problems could be eliminated prior to publishing, possibly using more extensive testing by third parties?Below are specific examples we found unclear:

We have added the following new descriptions:

1. We described the types of microscopy used in the protocol in INTRODUCTION (line 156-157) and in PROTOCOL (protocol 2.3.1-2.3.3).
2. We described an outline of the protocol at the end of INTRODUCTION (from line 157).
3. DISCUSSION was also reorganized with respect to the contents of individual paragraphs, and some introductory sentences were also added to help the logical flow.
4. We have elaborated on the use of the software in the protocol. We have also fixed the problems in the software (see below). We have collected a lot of feedback on the software through GitHub and we will continue to make improvements to the software.

Abstract:

What is meant by information is magnified in lateral and axial dimensions? Does this refer to an increase in intensity?

The sentence was changed as follows:

Due to chromatic aberration and the imperfect alignment of cameras, images acquired in each channel may be shifted, and magnified, as well as rotated relative to each other in any of the three dimensions.

Introduction:

What is meant by optical misalignment is not clear, nor are there any citations regarding optical misalignment and the causes thereof. Is this point referring to a poorly constructed microscope or is there minute misalignment inherent to even adequately aligned components? The article suggests that it is impossible for a multiple camera system to be perfectly aligned. As a result, chromatic shift as a concept has no context, which leaves the reader ill prepared to continue reading.

We like to note that even sequential imaging on the same camera suffers from chromatic aberrations and likely more so than a well aligned multiple camera system.

The sentence was changed as follows (line 57):

Similarly, microscopes equipped with multiple cameras devoted to acquiring each color have more complex chromatic shifts due to differences in optical elements and imperfect alignment among the channels.

Also, a similar sentence in ABSTRACT was changed (see above).

The practice of using multicolored beads is discussed negatively here but is the recommended method for at least one situation in the protocol. When the use of beads is appropriate should be indicated here.

The explanation was changed (line 66-68):

Next, what constitutes a target versus a reference image is not well defined, even in basic terms. Also missing are generalizations regarding properties the two images should have. The necessary parameters for reference images, ideal and/or minimum to meet for each imaging scenario (as delineated in Table 1), are not clearly outlined, though some mentions are made in the paragraphs following the one beginning, "Here we describe…".

The explanation for the “reference” image has been added (line 83-84).

With respect to fluorescence, as discussed in the next paragraph, the explanation, "Since… blue, green and red channels," is not clear. Which specific channels are compatible and how many channels does the software support? The set-up described is not the only type found used in fluorescence microscopy, in other words, although immediately clear to the expert it is not clearly stated that the ideal alignment data cannot be obtained with a filter cube-based microscope. It would be helpful to clearly state what is desirable (e.g. cross talk signal) and what is technically needed to record such data.

The explanation of the requirement for bleed-through (now called “crosstalk”) reference images was expanded (line 91-95).

In addition, we added information regarding how many channels the software supports (line 129).

For potential users that process images using deconvolution, it is not specified if the chromatic shift correction should be done before or after the deconvolution, or if Chromagnon would outperform the chromatic shift estimation done by most deconvolution software. Again, this is pretty clear to the expert, but does confuse learners and non-experts.

The application of deconvolution has been commented on in DISCUSSION (line 530-532).

Furthermore, use of deconvolution was stated in PRESENTATIVE RESULTS (line 462) and the legend for Fig. 5 (line 497).

Protocol:

With respect to the protocol, having the set-up of the references under the section title "Data acquisition" is confusing to the reader, consider renaming.

The section title has been changed to "**Acquisition of reference images**"

Using test data from our lab we were only able to test the least preferred reference image, limiting the following comments to this scenario:

2.2.3 the article instructs you to use a sample. It can be assumed that the target and reference are one and the same for the bright field correction, but it is far from clear.

We made changes to the protocol as follows:

2.2.1. Place the target sample prepared in step 1.1 on a wide-field microscope.

2.2.2. Acquire a fluorescence image of the target in blue, green, and red channels.

2.2.3. Acquire a bright field image of the target in blue, green, and red channels exactly at the same stage position as in 2.2.2 and the same Z height in case of a 3D stack.

2.3.3. "The XY position on the stage does not matter because the amount of chromatic shift is mostly constant on a single coverslip."
Mostly constant? For the sake of consistency shouldn't an attempt be made to keep the x,y position consistent?

This sentence has been explained more carefully (line 281-284).

What about the use of a camera that does not take all the channels at the same time?
Preference either way is not stated here, nor is it in the Introduction.

This is now mentioned in INTRODUCTION (line 162-163).

What is meant by local alignment? Is there a defined volume of pixels which undergo alignment independently from the whole image? Is this done iteratively through the whole image volume?

The description of "local alignment" has now been moved from DISCUSSION to INTRODUCTION and is explained more carefully (line 134- 141).

The so called "local alignment" is performed on projected images; aren't projections inherently less accurate?

Yes, they are. However, most cell biologists collect only a few tens of pixels in the Z axis. Such a small number of pixels is not suitable for local alignment in 3D. In fact, for projected images, we set the minimum number of pixels at 60, which is often already reaching along the Z axis before subdivision.

4.8. "average references" - what is this?

The explanation has been added to INTRODUCTION (line 119-122).

"It is better to know how much accuracy is required for your study." - generally as best as one can get? This statement needs clarification

The explanation has been added at the beginning of DISCUSSION (line 521).

Why is accuracy of global alignment dependent on how much of the field of view is used?

A citation was inserted rather than an explanation of the theory (line 548).

The manuscript states four regions are used for accuracy with respect to rotation OR switches to "other methods". As neither method is well described, it does not make sense why the four regions are preferable over one.

This is briefly explained at line 549.

It is unclear why the region with the objects can't be divided into four regions and adjust the algorithm to account for difference not being about z at the origin.

There should be recommendations to center the objects imaged, which may be mentioned in the documentation under "alignment of time frames". However, the wording is not clear and seems specific to time frames. The inability to adjust might be a weakness of the method that needs to be addressed.

Yes, it is only for time series, this is now clearly stated in the documentation.

Magnification difference is a common feature in chromatic aberration. Images should be magnified from the optical axis which is assumed to be the center of the image. Therefore, if the center is artificially moved to a position that is the wrong place in regards to the optical axis, then the true alignment parameter cannot be found.

Regarding the potential weakness of the method, we added the solution for this problem in new Table 2 and in line 554-556.

It is likely that most users would like to understand how the chromatic aberration is calculated and have access to the aberration as found between each pair/combination of channels. No such readout was available in the GUI, although some detection was mentioned during the loading of the file. The final corrections are available if one digs through the log file, but how these corrections are determined, that is what the shifts were that resulted in the corrects, is not. It also seems unclear to us if there is an explicit statement of the alignment used, e.g. if it is always an elastic, or ridged or mixed model that is used?

The text for protocol 3.13 was modified.

Also, the presence of a log file was mentioned at line 366-367. Furthermore, the contents of the log file were fully reorganized so as to easily follow the alignment process.

The kind of alignment method that is used (rigid or elastic) can be checked by looking at the output file type (chromagnon.csv or chromagnon.tif). The explanation has been modified (line 361-364).

Table 1:

This rating system seems to be ordinal; it uses a meaningless scoring system for which we do not know the maximum score nor is it clear if the scores are based on a ratio, in which case we need the reference value indicated by "+", or set difference, in which case we need the base and difference associated with each "+".

We removed the category “Simplicity” because they are only “++” and “+++”. In addition, rough estimates for the accuracy expected for “+” and “+++” were added in the legend.

The difference between biological calibration on the same versus different slides should be flushed out in the discussion, preferably following a quantitative results section.

Sentences have been added in INTRODUCTION (line 122-125) and in DISCUSSION (line 587-589).

Is there a difference between the two biological calibrations if the local calibration is measured by multicolored bead samples?

The expected difference was mentioned at line 150-152.

Documentation Edits:

* The disadvantage for bleed-through images is worded in a confusing way, instead of bleed-through maybe use 'cross talk' or 'bleed-through - also known as cross talk - …'

We have changed “bleed-through reference image” to “crosstalk reference image”. The document for the software has also been changed.

* Why are the only rotations looked at in the z-direction?

The current *Chromagnon* measures rotations around the Z-axis (from the XY plane) and X-axis (from the ZY plane), but the X-axis rotation is not used because it only has a minor effect and its robustness in the measurement process has not been tested enough. We are not ready to correct this rotation, but it is one of our future plans.

Testing the software:

We were only able to create the brightfield reference because we used a cube based microscope and also did not have three colors of phalloidin, and we were only able to obtain diffraction limited images. We used a Leica DMi8 widefield microscope with a 63x oil 1.4 NA Leica Plan Apoachromat, Hamamatsu ORCA flash 4 sCMOS camera, and mercury halide lamp. Our sample was of formaldehyde fixed Jurkat T cells stained for lamin B and nuclear pore complex. The Brightfield reference and fluorescent target image were acquired at the exact same position and z stack. The target image was deconvolved with ten blind iterations.

When the non-deconvolved image was corrected in chromagnon, it resulted in a highly pixelated and granular appearance. The pixel number was conserved, but the image quality was not. Also, the correction took nearly ten times longer than the estimate provided by the article despite being processed on a dual core liquid cooled imaging computer with 64GB RAM.

We are sorry that the result of the non-deconvolved image was not good. Presumably, the software failed to find the true magnification factor. The problem might be that the contrast of the brightfield image was too low. Solutions written in Table 2 may help in obtaining better results ("Cause" … "Low contrast").

As to the processing time, we did not mention the number of CPU core and threads in our system. The number of threads is important for correctly estimating the processing time because *Chromagnon* uses parallel processing. Numbers of cores and threads and a type of storage on our Mac were written in the revised manuscript at line 463-464.

Assuming the power of the computer is similar for simplicity's sake, the processing time is not extremely different from expectation. As always, the calculation time depends on the number of pixels. The data for Fig. 5 was 60 Z slices, 256 x 256 pixels, 3 channels, and it measured local alignment. If the reviewer's image was 21 Z slices, 2k x 2k pixels, 4 channels, then number of pixels to be calculated is about 30 times greater. Therefore, if local alignment was measured, the simplest calculation suggests that it can take even 30 times longer. If we compare the reviewer's image with the data in Fig. 6, 76 Z slices, 1k x 1k pixels, 3 channels, then the reviewer's data is 1.7 times larger in terms of number of pixels to be calculated. However, the experiment in Fig. 6 was measured *without* local alignment, so it should take at least twice the amount of time to measure the local alignment of the same number of pixels, and this gives an expected time of about 3 times longer for the local alignment of the reviewer's data. Other factors such as different storage types (SSD vs HDD) may also affect the processing time, as data are read several times during processing. From these considerations, we think the processing time was within reasonable bounds.

The deconvolved image was then corrected. This time, the image quality was preserved completely, but the number of z slices was reduced from 21 to 18. It is

not clear what happened to the other three slices, or which three slices were removed. Is the software limited to 18 z slices? This made it exceedingly difficult to compare the corrected image to the original as we could not determine which z slices corresponded.

We added explanations to the "crop margins" option of *Chromagnon* as protocol 3.5 and we also added these to the REPRESENTATIVE RESULTS (line 466-468).

We have never deconvolved bright-field images. But if such deconvolution was successful, then we would also consider using it.

The original deconvolved image had slight chromatic aberration as seen below. In the upper left of the nucleus, the red lamin ring extends past the yellow nuclear pore complex ring which is not physiologically accurate. As expected, the opposite is seen on the lower right side of the nucleus. The effective pixel size is 103nm, and as such, the chromatic aberration can be estimated to be approximately 206nm or two pixels on a linear-lateral vector.

The corrected deconvolved image largely shows the same chromatic aberration as the original. Subjectively, there is a slight shift of the lamin ring on the bottom right toward the NPC ring, so there may be a marginal correction. Overall, using a brightfield reference did not cause sufficient chromatic correction.

///IMAGE to be added here/// submission system unfortunately did not allow for upload of documents.

When I tested the software with the provided example images, the brightfield, bleed-through, and biological calibrations all adequately corrected the chromatic aberration between two channels. The images that were provided had higher lateral resolutions than the image we acquired and that may be why the correction was easier to qualify.

If the correction of chromatic shift in the image was only marginal, one possibility for this is low contrast of the bright-field reference image. The solution for this problem is mentioned in Table 2 ("Cause" … "Low contrast"). Alternatively, the camera chip may have acquired dust, which can compromise the correction accuracy. This problem was also mentioned in Table 2 ("Cause" … "Contamination of unrelated images"). Yet another possibility that the reviewer raised is the lateral resolution. If lower lateral resolution is a result of spherical aberration, it also affects the correction accuracy of the axial (Z) direction. This is mentioned in Table 2 ("Cause" … "Optical aberration"). Finally, Z step size may have not fulfilled the Nyquist criterion. This important explanation was lacking in the previous version of the manuscript. We added it to Protocol 2.1.2, 2.2.2 and 2.3.2 and Table 2 ("Cause" … "The Z step size is too sparse").

Whilst having such uncertainty, it is still possible that the reviewer's image is well-corrected. Although red fluorescence from lamin was sometimes inside and sometimes overlapped with green nuclear pores before alignment, it was slightly but always inside the nuclear pores after alignment. If the target NPC protein was

at the cytoplasmic side or at the plane of the nuclear envelope, then the localization patterns of two proteins are biologically correct. Even though the lateral resolution of the microscopy is, say, 250-300 nm, we can distinguish slight but invariable differences in the localization of two proteins that are separated by only 20 nm by visual inspection, if chromatic shifts are well-corrected. Therefore, slight differences in localization between red and green may be an expected result.

To find out if it is true, the reviewer can use the image of lamin and NPC as both reference and target images for chromatic correction by *Chromagnon* without local alignment. If multiple nuclei are present in the image, then the image should work as a good reference for correcting itself by global (linear) alignment. If the red fluorescence is slightly inside of the green fluorescence in all nuclei as in the image provided, then that is likely a truth.

The provided images also calculated faster than the one we tested, although we tested an image with twice as many channels. The calculation time may be proportional to the number of channels? Again, the corrected images were missing z slices. When the image was corrected with different reference types, the resulting images all had different numbers of z slices between 32 and 37.

The calculation time and reduction in number of z slices was answered above. The calculation time should be proportional to the number of channels (excluding one reference channel).

Finally, when more than one corrected image was displayed in the build-in image viewer, changing the z stack position caused the image to suddenly and irreversibly become highly skewed and obfuscated, and as such became completely unviewable. The image file itself was fine when I inspected it in FIJI.

We believe that opening multiple images in the viewer is not a big problem because only 2D images are loaded into memory each time. We assume that the problem you encountered is due to handling error of a large image file (2k x 2k). We added a new function in the viewer, namely “Load whole data into memory”. The new function is explained in protocol 3.11 (line 373-374).

Minor Concerns:

Copy Edits:
* Line 38-39. Change to "lateral (x,y) and axial (z) dimensions"…
* Line 78. Change envelop to envelope
* Line 91. Change to: "blue, green, and red"…
* Line 351. The files must have equal x, y, and z dimensions, so "pixels" should be replaced with "voxels".
* Line 418. Envelops to envelopes

These points have been corrected as suggested.

* Line 304. There are a lot of glass bottom dishes with different thicknesses. Does this refer to a #1.5 - 0.17mm glass bottom?

Yes, an explanation was added to protocol 4.2.

* Line 441. What is meant by more than three channels? 4-6? Infinite? NIR?

The corresponding phrase (line 583) was changed to any desired number of channels.

Program Bugs:

Unable to get to the screen in Figure 4.

The explanation at Protocol 3.13 (line 396) was changed to:

load a ".chromagnon.csv" or ".chromagnon.tif" file into *Chromagnon*'s Reference or Target box (Fig. 1), then double click it to open the alignment editor (Fig. 4)

Significantly more time to load and process than indicated in the article on a more powerful computer. The time given for running the program does not hold true; it took us much longer with 2048x2048 images with 21 slices in the z-stack and lambda = 4.

The problem is discussed above.

Crashed often when adjusting element "B" in Figure 3.

We assume that this is again due to handling error of a large image (2k x 2k). We added a new function in the viewer, namely "load whole data into memory". The new function is explained in protocol 3.11 (line 373).

The color/channel assignment was ordered in a randomly and changed between test images.

The problem is discussed in the NOTE of protocol 3.3, and Table 2 ("Cause" … "Metadata of the image file is lost"). We are currently working on a way to solve this important problem.

The result of our test use had 18 slices from 21 in the original z-stack. It is not clear which ones were removed as the "margins." This made comparison between the original and the processed images subjective.

We added an explanation for the "Crop margins" option at protocol 3.5. Please uncheck this check box if comparison is required.

Unclear what all the options that appear when one R-clicks on the color bar mean.

They are briefly explained in protocol 3.11 (line 377-380). In addition, we removed a few menus that are not used anymore.

Orthogonal view… did not seem to work, cannot move through image this way. What it was showing and the purpose was unclear.

This is again likely due to handling error of a large image (2k x 2k). We modified the program so that once the “orthogonal view” button is pressed, the whole image is loaded into the memory. The problem should now be resolved.

Log File Edits:

"geuss" is this guess or Gauss? Word is not found in protocol text
"affine" - Word is not found in protocol text

These words were corrected or changed to other words found in the text.

GitHub Edits:

Documentation spelled incorrectly in the initial markdown file.

The misspelling was corrected.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article:	High-accuracy correction of 3D chromatic shifts in the age of super-resolution biological imaging using Chromagnon
Author(s):	A. Matsuda, T. Koujin, L. Schermelleh, T. Haraguchi, Y. Hiraoka

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode ; "**CRC NonCommercial License**" means the Creative Commons Attribution-NonCommercial 3.0 Agreement (also known as CC-BY-NC), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License. If the "Standard Access" box

has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC NonCommercial License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video - Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video - Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties**. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

discretion andwithout giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contaminationdue to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Atsushi Matsuda
Department:	Advanced ICT Research Institute Kobe
Institution:	National Institute of Information and Communications Technology
Title:	Senior researcher
Signature:	
Date:	12/16/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140