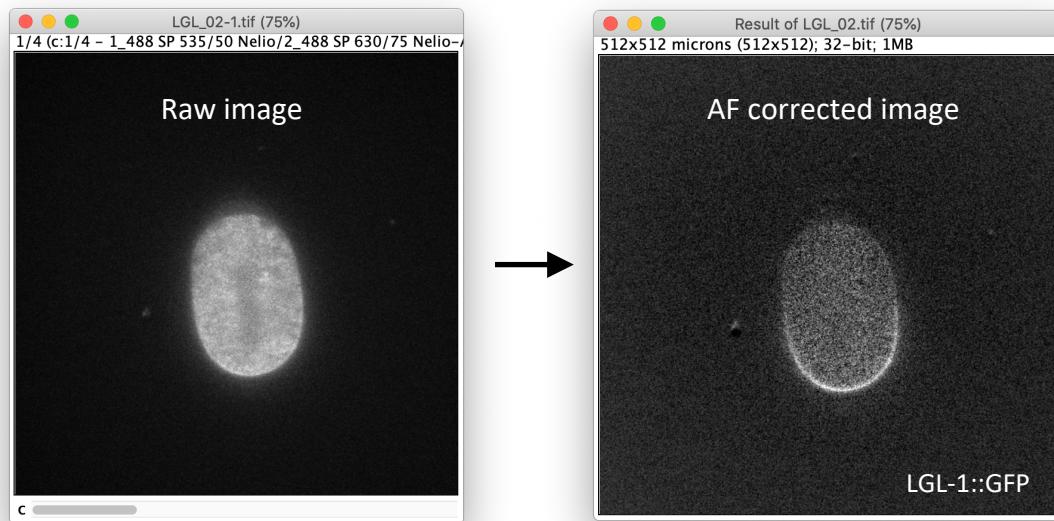


Autofluorescence correction FIJI plugin: User guide

Overview

This plugin provides an easy-to-use implementation of the autofluorescence (AF) correction method described in this paper: ???.

For a full description of the method, please see the paper. In brief, the method is designed to address the problem of autofluorescence which is commonly abundant in channels used to image green fluorophores, and can significantly obscure the true signal that you are interested in. The method takes advantage of the fact that autofluorescence typically has a much broader emission spectrum than GFP. By using a second channel with a red-shifted emission filter, we can obtain a clean readout of the level of autofluorescence in the sample, which can then be subtracted away on a pixel-by-pixel basis. A calibration step is required to obtain the necessary inter-channel correction factors for the autofluorescence calculations. In most cases, this will involve imaging unlabelled samples (e.g. wild-type N2 *C. elegans* samples) using the regular GFP channel (e.g. 488nm laser, 535/50nm filter) and a red-shifted autofluorescence reporter channel (e.g. 488nm laser, 630/75nm filter). If you wish to perform AF-correction on dual colour samples containing both green and red fluorophores, calibration should instead be performed using the relevant RFP-expressing single line (to account for ‘bleedthrough’ of red fluorophore signal). In this case, you also need to include your regular RFP channel (e.g. 561nm laser, 630/75nm filter) in this procedure.



Although developed with *C. elegans* in mind, the principles are general and thus should be applicable to any samples that exhibit similar broad-spectrum AF that can be captured in a red-shifted emission channel.

Installation

The plugin can be installed in one of two ways:

Method 1 (recommended): Linking FIJI to the update site (NOT YET AVAILABLE)

The recommended way to install is by linking FIJI to the update site for this plugin. This will ensure that any updates are installed automatically. To install in this way, follow the guide found at <https://imagej.net/update-sites/following>, and select the update site named ???

Method 2: Manual installation

To install manually, first download the repository from Github. Then in FIJI go to *Plugins -> Install...*, and select the .jar file found in the ‘target’ folder of the repository. Follow the onscreen instructions, restarting FIJI when it tells you to.

NOTE: If you use this installation method, you will have to manually install updates. To do this, you will have to first delete the existing version of the plugin from FIJI’s *plugins* folder (on a mac you can find this folder by right clicking on the FIJI icon in your Applications folder and clicking ‘Show package contents’).

Example datasets

A couple of example datasets are included in the Github repository which can be used to try out the workflow:

Example dataset 1: AF correction in single-colour GFP (LGL-1::GFP) zygotes

- Calibration images: 3x N2 zygotes
- Test image: 3x LGL-1::GFP zygotes
- Four channels in each image:
 - o Channel 1: 488nm laser, 535/50nm filter (i.e. the GFP channel)
 - o Channel 2: 488nm laser, 630/75nm filter (i.e. the AF channel)
 - o Channel 3: 561nm laser, 630/75nm filter (i.e. the RFP channel)
 - o Channel 4: DIC

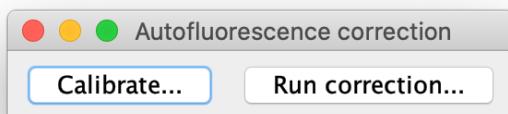
Example dataset 2: AF correction in dual-fluorophore (PAR-6::GFP, mCherry::MEX-5) zygotes

- Calibration images: 7x mCherry::MEX-5 zygotes
- Test images: 3x mCherry::MEX-5, PAR-6::GFP zygotes
- Four channels in each image:
 - o Channel 1: 488nm laser, 535/50nm filter (i.e. the GFP channel)
 - o Channel 2: 488nm laser, 630/75nm filter (i.e. the AF channel)
 - o Channel 3: 561nm laser, 630/75nm filter (i.e. the RFP channel)
 - o Channel 4: DIC
- 3x N2s are also included for comparison, but these shouldn't be used for calibration

The complete workflow is demonstrated below using example dataset 1.

Starting the plugin

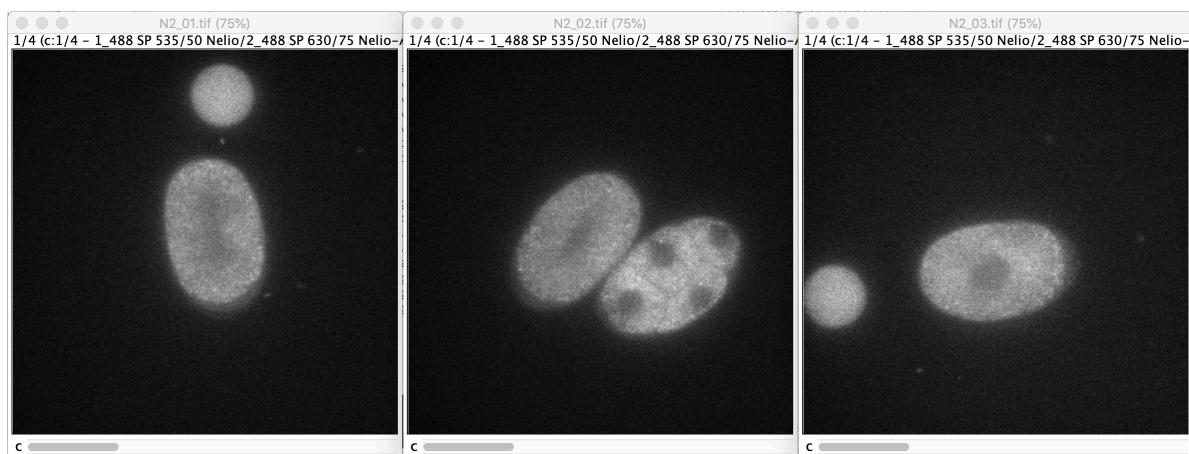
Open the plugin by going to *Plugins -> Autofluorescence correction*. The following window will appear:



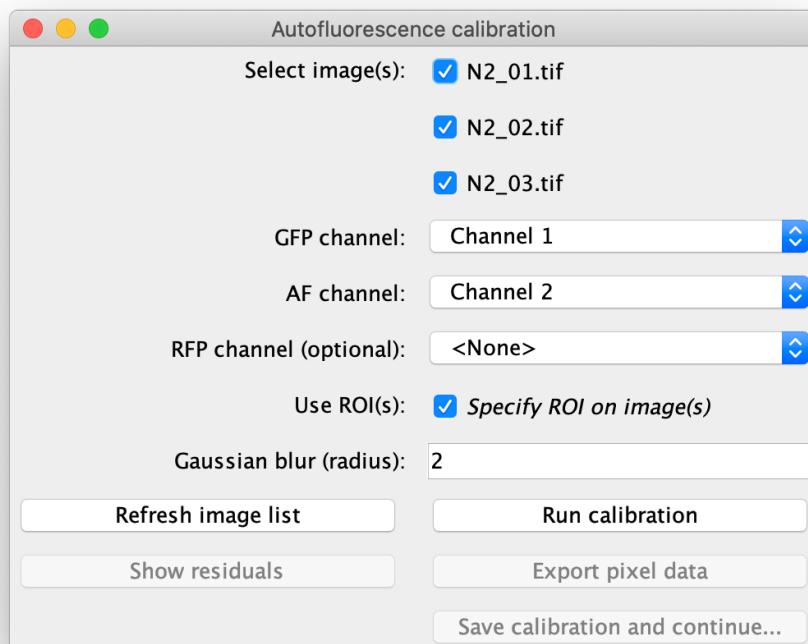
As a first step, you must calibrate the program using images of GFP-negative control samples. This can be done using the *Calibrate...* tool. Once you have calibrated the program, you can then perform autofluorescence correction on GFP-expressing samples, using the *Run correction...* tool. Both of these steps are outlined below.

Calibration with GFP-negative control samples

To begin calibration, open a selection of control images (3-5 images is ideal, a maximum of 10 is supported). These must be opened as multi-channel images (one window per image, rather than separate windows for each channel). Images can be raw (i.e. direct from the microscope), as is the case with the example datasets, or can be pre-processed in any way of your choosing (e.g. background subtraction, flatness correction). This can sometimes improve performance, depending on your exact setup, but must be consistent for all images in your dataset.



Next, click the *Calibrate...* button on the plugin window. The following window will appear:



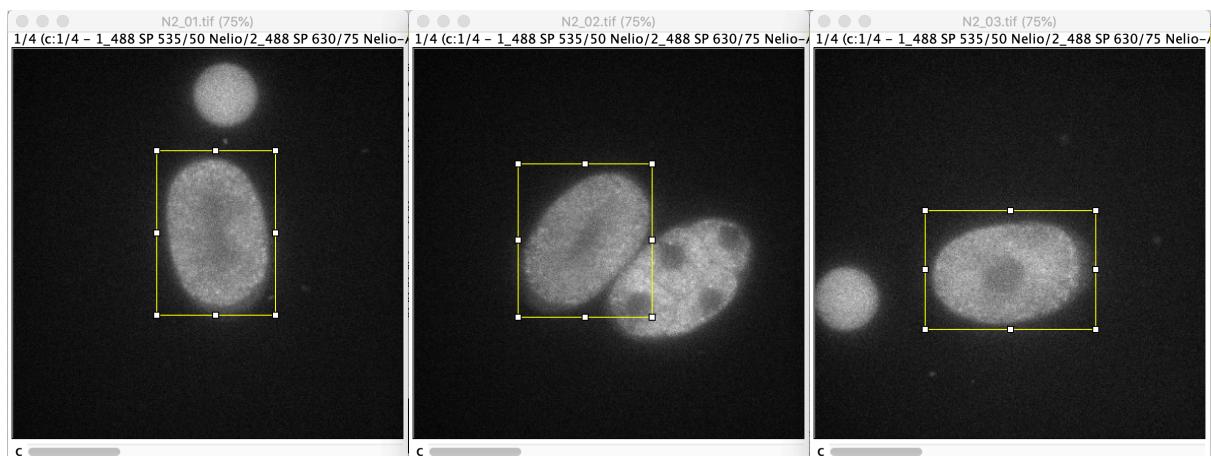
Select the images that you wish to use for calibration, and specify the following parameters:

GFP channel: The channel number corresponding to the GFP channel (typically taken with a 488nm laser and a 535/50nm filter)

AF channel: The channel number corresponding to the AF channel (typically taken with a 488nm laser and a 630/75nm filter)

RFP channel (optional): The channel number corresponding to the RFP channel (typically taken with a 561nm laser and a 630/75nm filter). This is not required in this case, but should be included in cases where a red fluorophore is present (e.g. example dataset 2, where the mCherry::MEX-5 single line should be used for calibration). It can also help to include this channel in some other exceptional cases, which are outlined in the main paper.

Use ROI(s): If selected, calibration will be confined to pixels within a user-selected ROI for each image. If unselected, the whole image will be used. You can select an ROI on each image using any of FIJI's ROI selection tools:



NOTE: It is often strongly advisable to use this feature. This is particularly useful if the sample only takes up a small part of the image, or in cases where other objects (e.g. beads) are visible in the frame, as these may throw off the calibration if included.

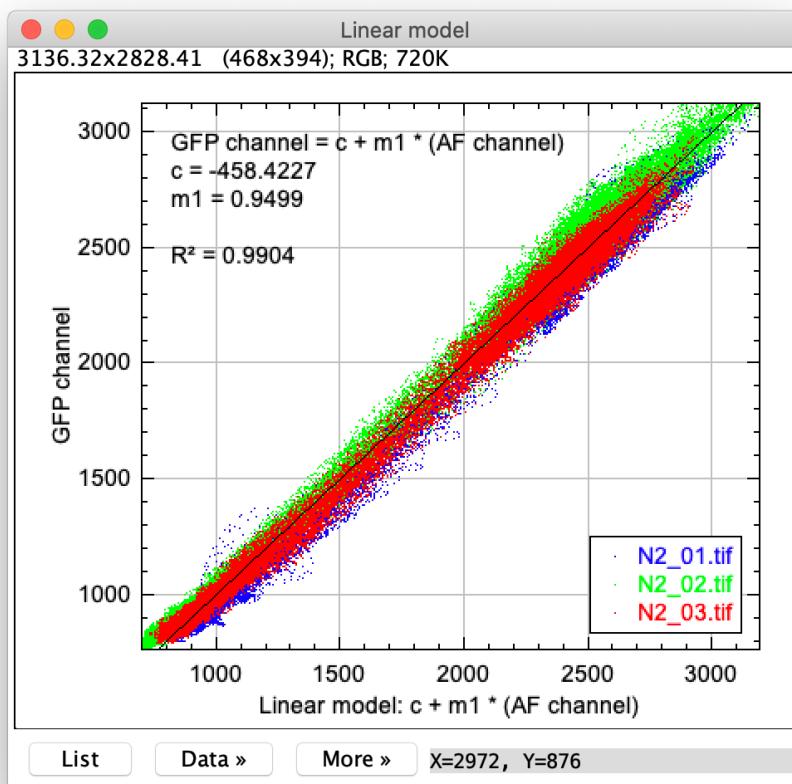
Gaussian blur (radius): The radius of the gaussian blur to apply to the images prior to pixel-by-pixel regression. The optimal Gaussian radius will depend on the resolution and signal to noise ratio of your images, and it may be useful to try a few different values.

After setting these parameters, click *Run calibration*. In brief, this will run the following algorithm:

- 1) A gaussian filter is applied to each channel of the selected images to reduce noise (note: this will not change the images on screen)
- 2) For each channel, all pixel values within the ROIs of the selected images are extracted and pooled

- 3) Linear regression (ordinary least squares) is performed on this pixel value data, capturing the relationship between pixel intensity in the AF channel (and RFP channel if applicable) and autofluorescence signal in the GFP channel

Once this has run, a window will appear showing the results of the linear regression:

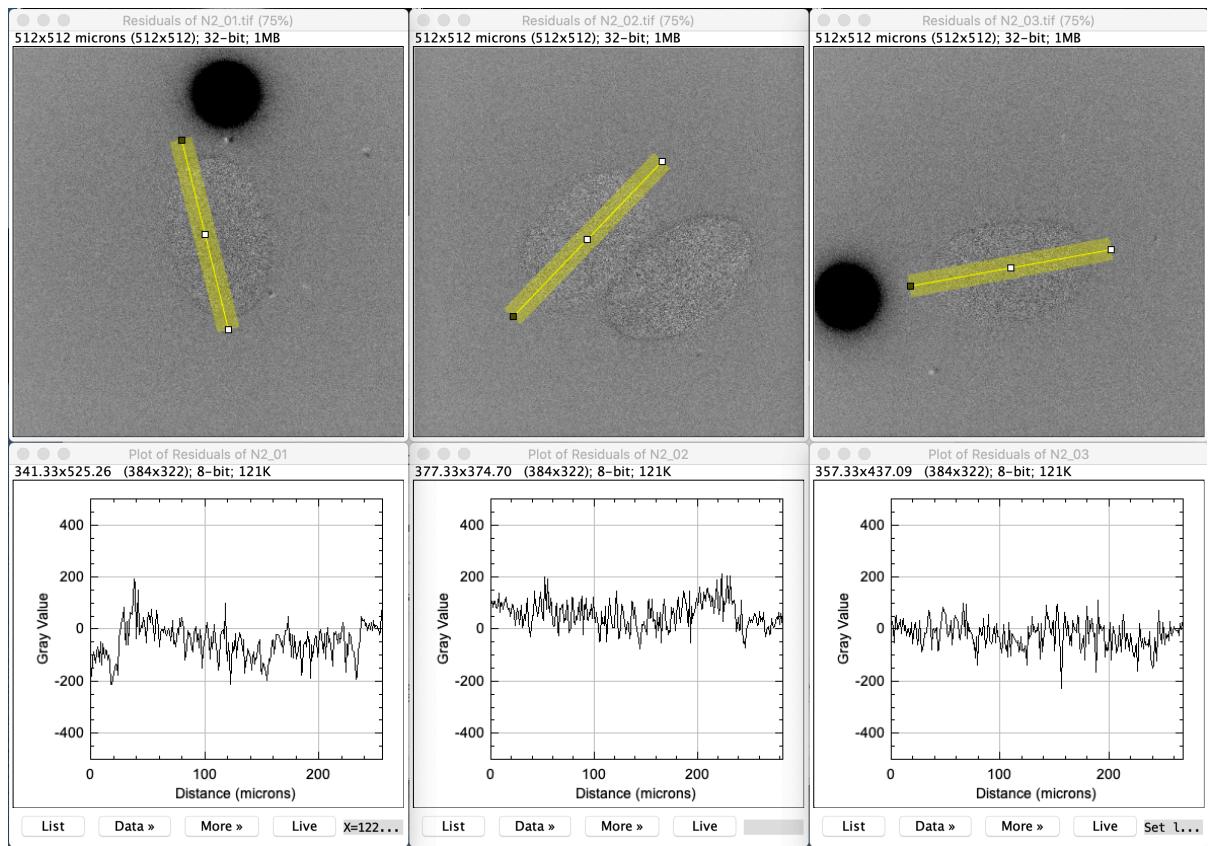


The scatter plot shows actual pixel values in the Gaussian-processed GFP channel (y-axis) vs values predicted by the optimised linear model (x-axis). It's important to study this plot carefully to make sure that it looks reasonable: a well-performing model should have a close 1:1 relationship across the entire range of pixel values for all images, and a high R-squared score (> 0.9). If the relationship looks particularly noisy, try increasing the Gaussian radius.

The parameters for the linear model (c and $m1$) are shown at the top-left of the figure. These will be saved by the program, but it's a good idea to note them down for your records.

Full data from the linear regression can be exported in tabular form by clicking *Export pixel data* on the main window. This may be useful should you wish to perform any further analysis.

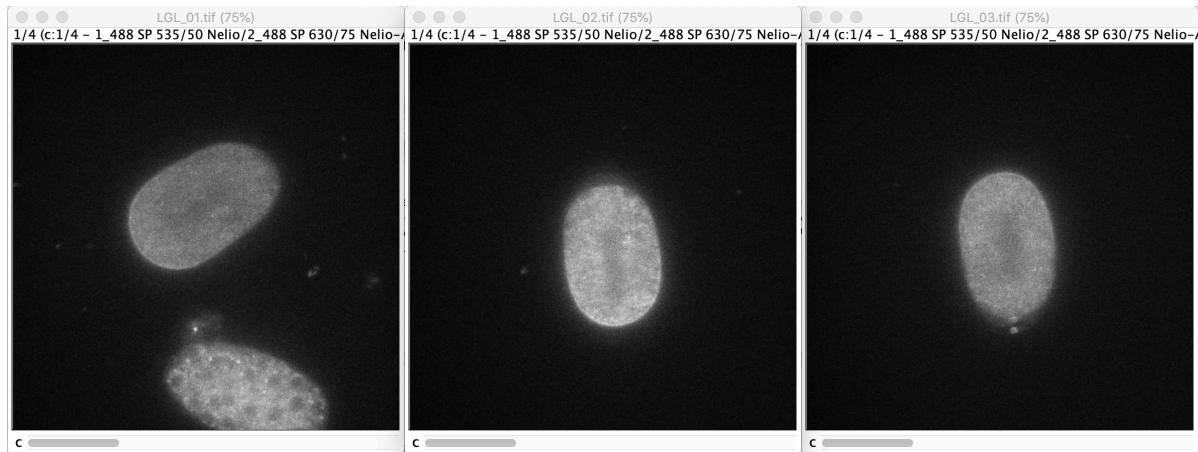
Finally, you can assess the calibration by looking at the residuals for each training image. Click the *Show residuals* button on the main window. The core region of each residuals image should be close to zero and free of any strong spatial features:



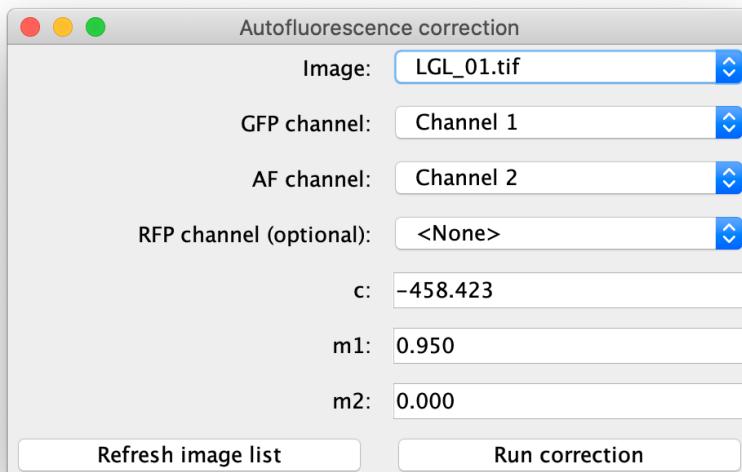
Once you are satisfied with the calibration, you can save the calibration and proceed with AF correction. Click *Save calibration and continue...* to return to the main menu.

Autofluorescence correction

First, open a selection of GFP-expressing images which you wish to correct:



Next, click *Run correction...* from the main menu. The following window will appear:



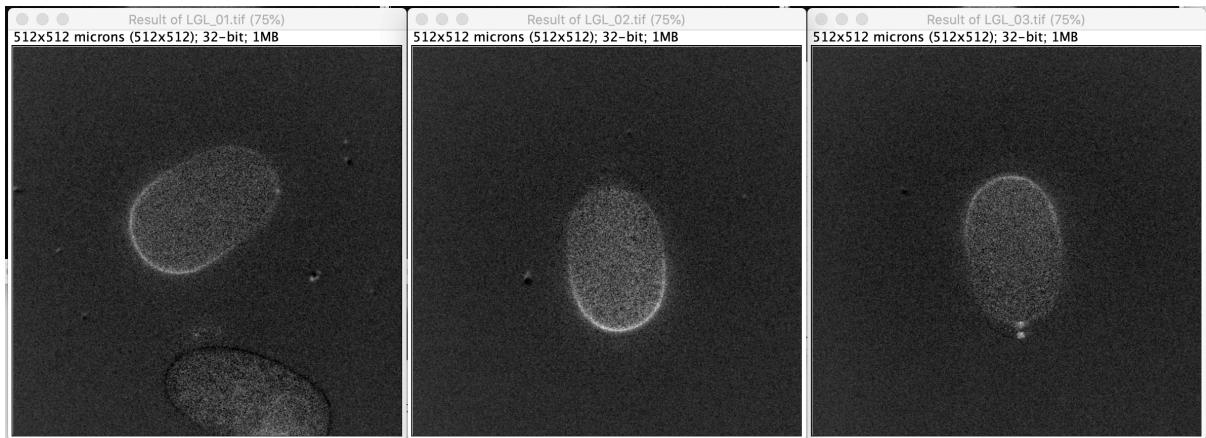
Specify an image at the top of the window. The other parameters will all be set automatically according to the calibration that has just been performed, but can also be set manually. For example, if you have quit and reopened the program since calibrating, these will have to be filled in manually (hence, it's a good idea to note down the parameters whenever you perform calibration!).

Click *Run correction*, which will perform the following operations:

- 1) The autofluorescence contribution in the GFP channel is predicted as a linear function of the AF (and RFP) channel(s)

- 2) This is subtracted from the GFP channel, giving a corrected image which represents the true (autofluorescence-free) GFP signal.

The resulting autofluorescence-corrected image will appear onscreen. This step can then be repeated for all of the images in the dataset:



NOTE: the new images will be 32-bit, with an appropriate scaling factor to ensure that pixel values are in the same units as the original images. Please DO NOT convert images back to 16-bit in FIJI as this will change the scaling of the pixel values.

Citation

This program is made available under a CC BY 4.0 licence. If you use this program as part of a publication, please cite the following reference: ???