

Autofluorescence correction FIJI plugin: User guide

Overview

This plugin provides an easy-to-use implementation of the autofluorescence 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 autofluorescence typically has a much broader emission spectrum than GFP. By using a second, red-shifted emission channel, 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 of unlabelled samples (e.g. N2 worm samples) using the regular GFP channel and a red-shifted autofluorescence reporter channel. If you wish to perform AF-correction on dual colour samples with green and red fluorophores, calibration should be performed using the relevant RFP-expressing single line instead (to account for ‘bleedthrough’ of red fluorophore signal).

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: Manual installation

Download the repository from Github. 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. After FIJI restarts, you should find “*Autofluorescence correction*” in the Plugins menu.

Method 2 (recommended): Linking FIJI to the update site (NOT YET AVAILABLE)

This will ensure that the plugin is updated every time a new version is released.

Example datasets

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

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

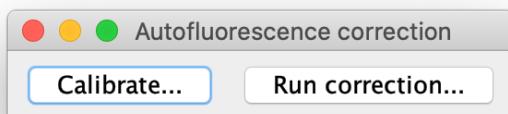
Example dataset 2: AF correction in dual-fluorophore (PAR-6::GFP, MEX-5::mCherry) zygotes

- Calibration images: 7x MEX-5::mCherry zygotes
- Test images: 3x MEX-5::mCherry, 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

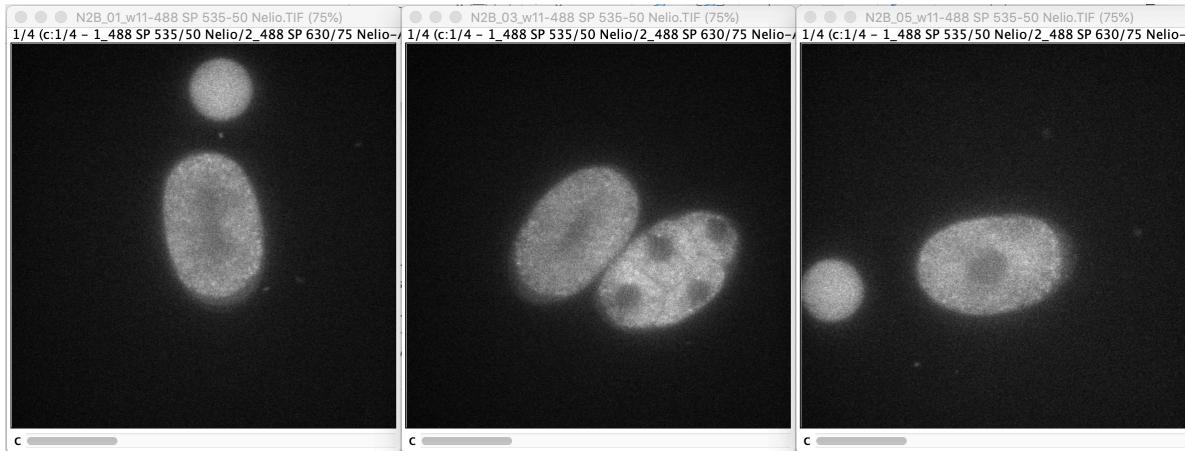
Open the plugin by going to *Plugins -> Autofluorescence correction*. The following window will appear:



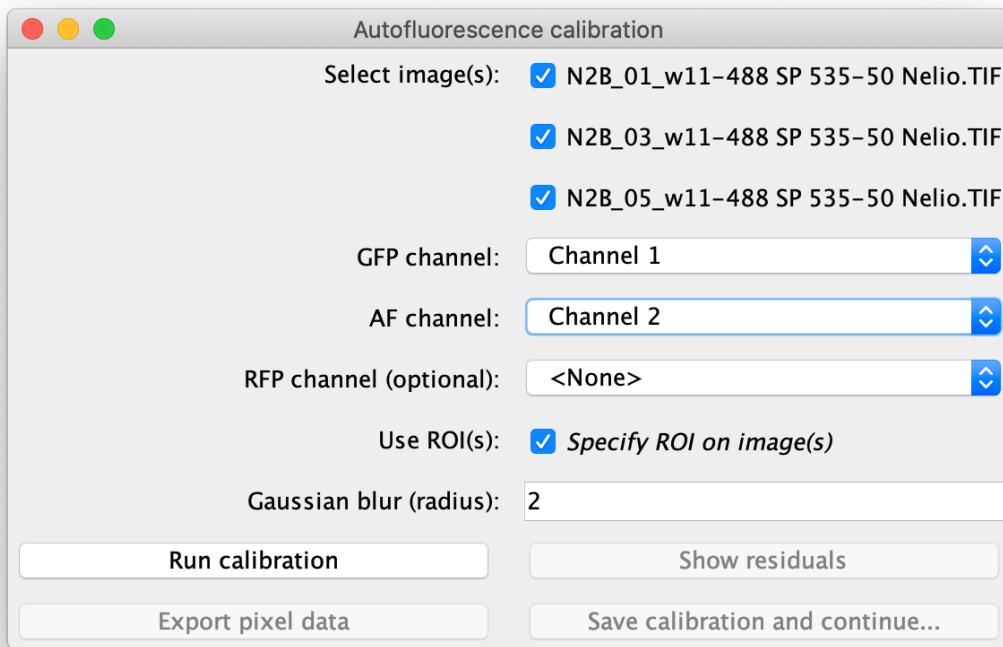
As a first step, you must calibrate the program using images of GFP-negative control embryos. This can be done using the *Calibrate...* tool. Once you have calibrated the program, you can perform autofluorescence correction on GFP-expressing embryos, using the *Run correction...* tool. Both of these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

Calibration with GFP-negative control samples

To begin calibration, open a selection of control images (3-5 images is ideal). These must be opened as multi-channel images (one window per image, rather than separate windows for each channel):



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



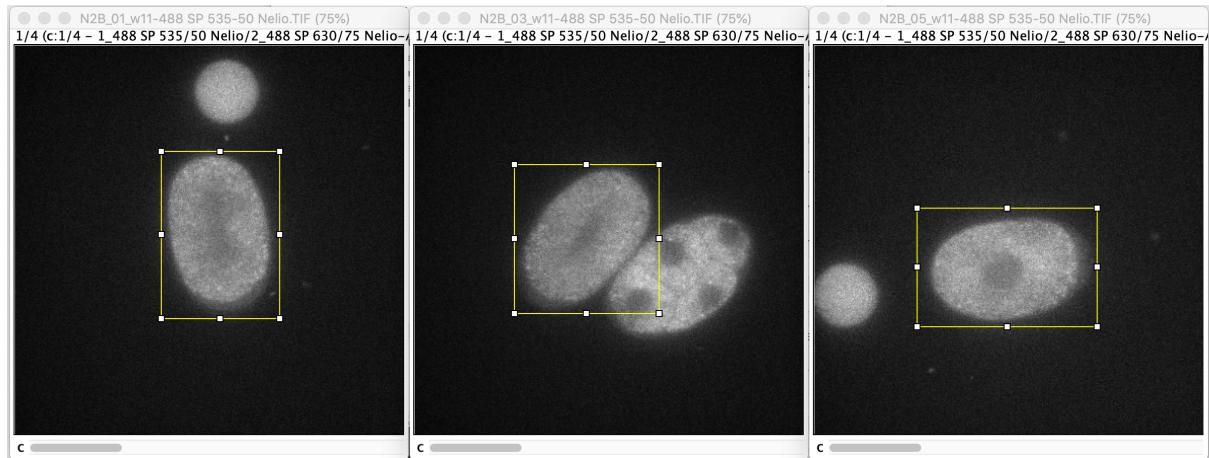
Select the images that you wish to use for calibration, and specify the following parameters:

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

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

RFP channel (optional): The channel number corresponding to the RFP channel (typically taken with a 561nm laser and a 630-75 filter). Typically, this is only required when a red fluorophore is present (calibration using an RFP single line), although there are some exceptions to this as outlined in the paper.

Use ROI(s): If selected, calibration will be confined to pixels within a user-selected ROI for each image. If unselected, all pixels 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 embryo only takes up a small part of the image (i.e. low magnification images), or in cases where other objects (e.g. beads, worm remnants) are visible in the frame, as these may throw off the calibration if included.

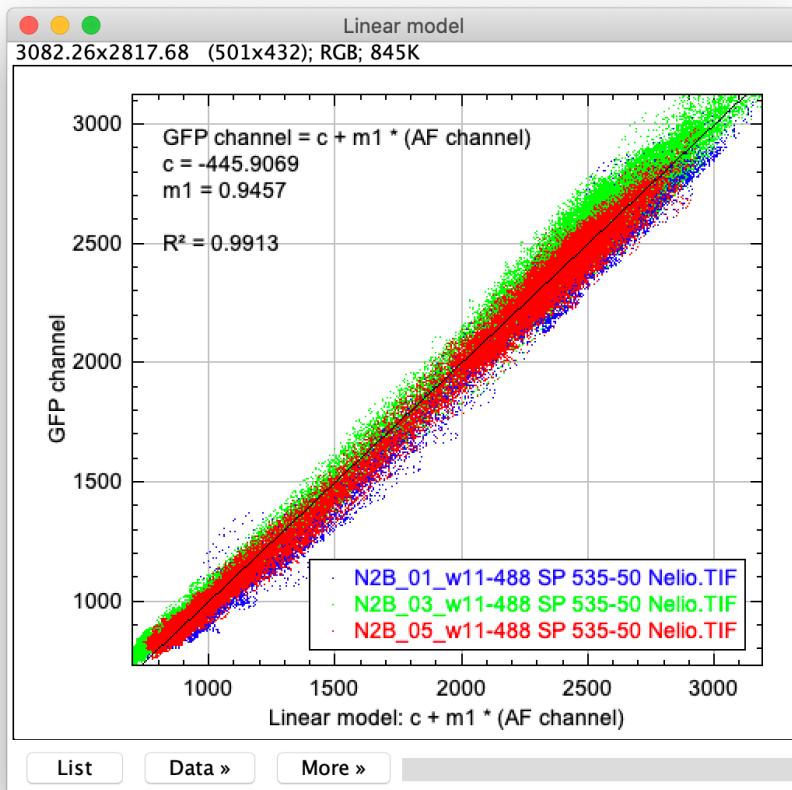
Gaussian blur (radius): The radius of the gaussian blur to apply to the images prior to pixel-by-pixel regression. The optimal Gaussian radius will depend on the resolution and signal to noise ratio of your images, and it may be useful to try a few different values.

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 these images are extracted and pooled

- 3) Linear regression (ordinary least squares) is performed on these pixel values, 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:

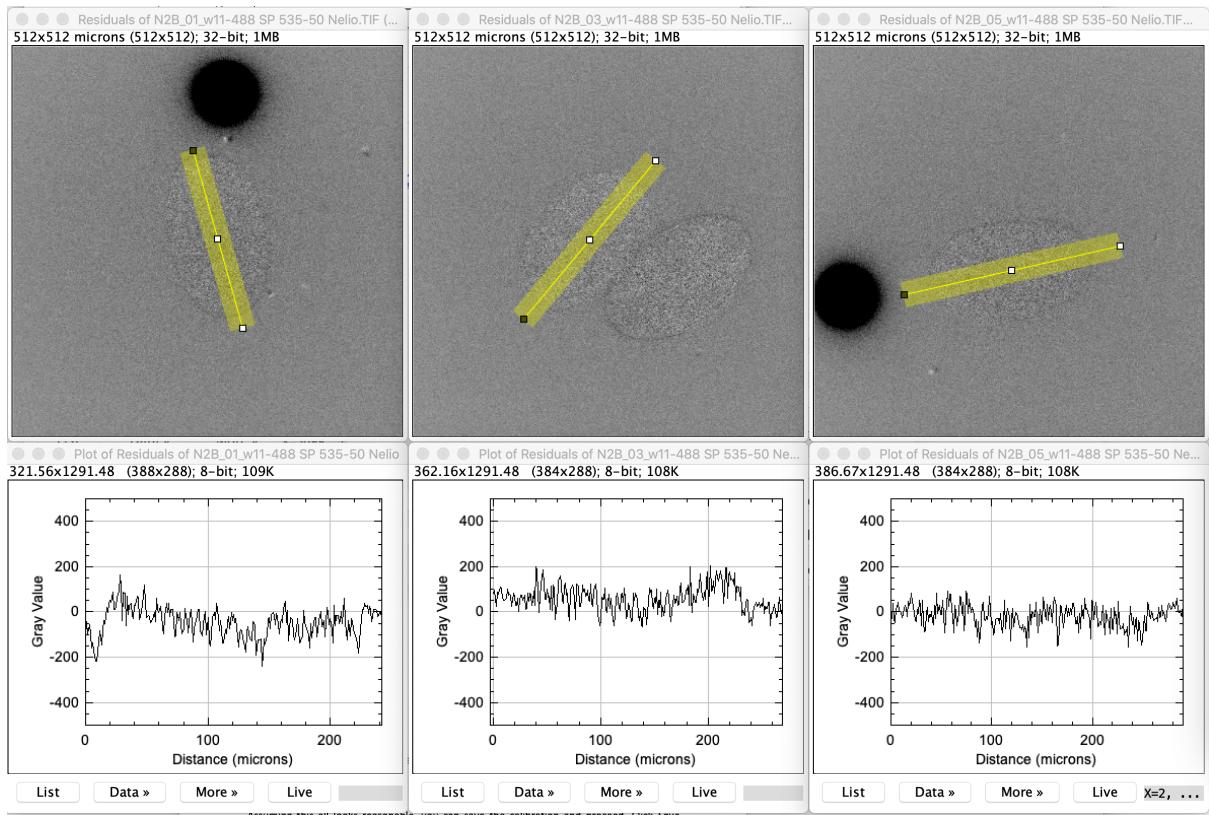


The scatter plot shows actual pixel values in the Gaussian-processed GFP channel (y-axis) vs values predicted by the optimised linear model (x-axis). It's important to study this plot carefully to make sure that it looks reasonable: a well-performing model should have a close 1:1 relationship across the entire range of pixel values for all embryos, 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, for further analysis if desired.

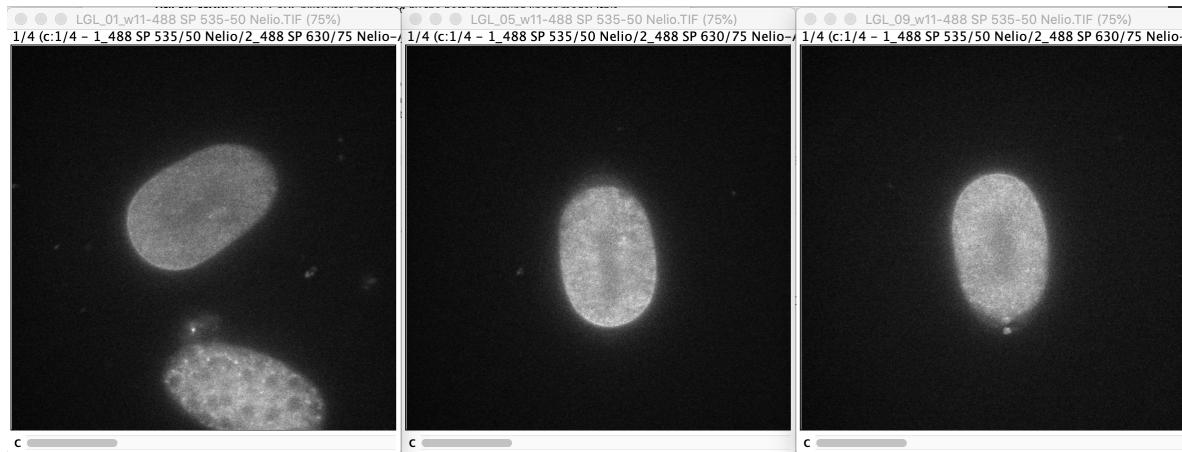
Finally, you can assess the model by looking at the residuals for each training image. Click the *Show residuals* button on the main window. The core region of each residuals image should be close to zero and free of any strong spatial features:



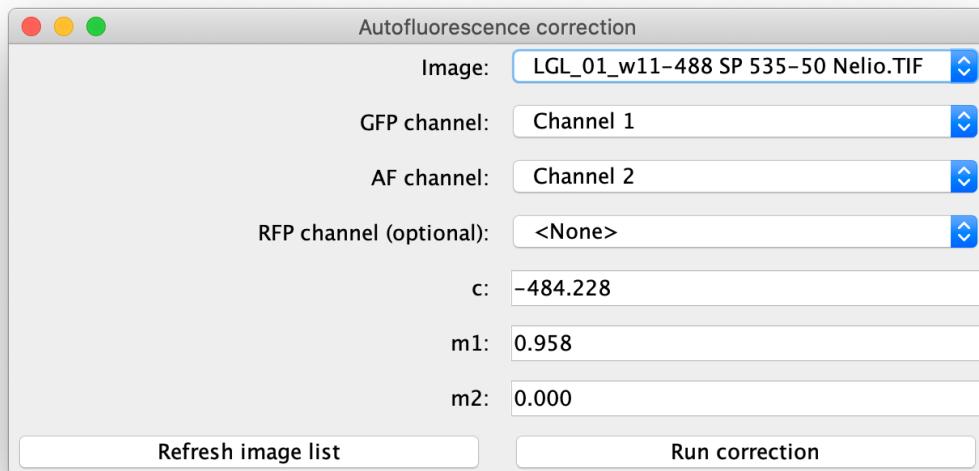
Once you are satisfied with the calibration, you can save the calibrated model and proceed with AF correction. Click *Save calibration and continue...* to return to the main menu.

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:



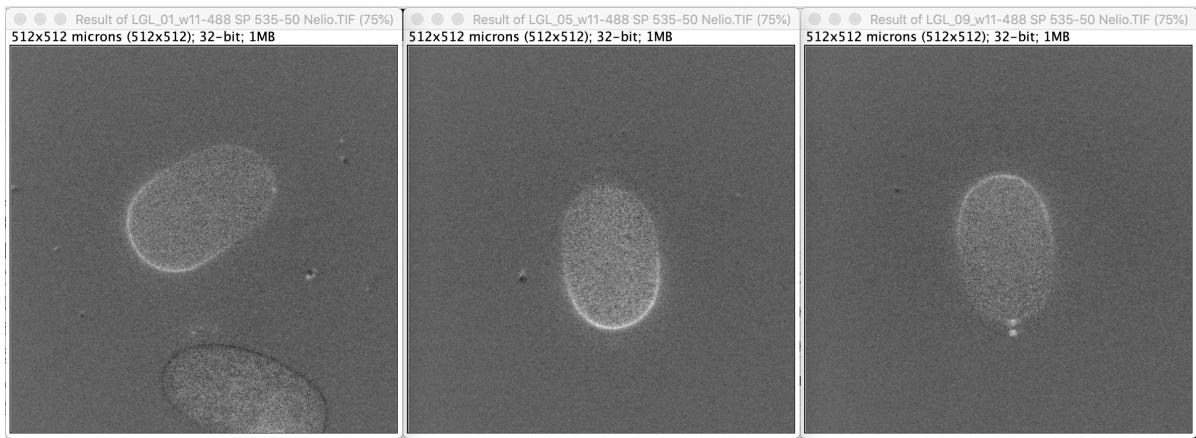
Specify the image that you wish to correct at the top of the window. The other parameters will all be set automatically according to the calibration that has just been performed, but can also be set manually. For example, if you have quit and reopened the program since calibrating, these will have to 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 to 16-bit in FIJI as this may change the scaling of the pixel values.

Citation

If you use this program as part of a publication, please cite the following reference: ???