1. Open ImageJ.
2. If the image file is already a TIFF, drag and drop onto ImageJ. Alternatively, go to “File – Open” and select the appropriate folder. Jump to step 5.
3. If the data is still in the original format generated by Metafluor (one file per frame per channel), go to “File – Import – Image Sequence….”. Select the folder where the images are contained. Pick any file. In this step, if the folder selected contains multiple channels (ie, in the case of YC3.6 images, dual sensor, or similar experiments), these will need to be extracted separately. Use the “File name contains” box to define the channel (e.g. R-GECO1 for the Red Geco or R-GECO2 for the green Geco). Make sure the “Sort Names Numerically” box is ticked: this guarantees that the frames are correctly ordered in time, as each file is name like “xxxx.001”, and the last three digits define the frame number. Press “OK”, and a sequence file is generated.
4. Go to “File – Save As – Tiff”. This prevents the time costly process of having to import the image sequence again when next needed.
5. If analysing two channels simultaneously, it is important that all editing is done in the same way on both. This is particularly important when it comes to cropping, as spatial coordinates must match between both channels. To guarantee this, open both files as described above, and go to “Analyze – Tools – Synchronize Windows”. Once the windows are synchronized, any selection of regions of interest will be matched across windows.
6. If analysing two channels simultaneously it is essential to align the channels pixel by pixel, in a process known as registration. This process is not required if only one channel is being analysed. To register two channels, go to “Plugins – Registration – MultiStackReg”. Select one of the channels as Stack 1 and select “Use as reference”. Select the other channel as Stack 2 and select “Align to First Stack”. Select “Rigid Body” as the Transformation method.
7. To make the subsequent steps faster, this is the ideal step to crop images in space and time (as and/or if needed).
   1. To crop images in space, select the area to remain using the rectangular tool (under the button “File”). Go to “Image – Crop”. If cropping synchronized windows, simply select the second window and go to “Image – Crop” (the area of interest has already been selected for the first window.
   2. To crop images in time, go to “Image – Duplicate”. Define the range according to the time that will be analysed. Remember to tick “Duplicate stack”.~
8. To extract intensity data, open the plug-in Time Series Analyzer. Go to “Plugins – Stacks – Time Series Analyzer”. This will open the ROI manager and the Time Series Analyzer windows.
   1. Select the ROI or ROIs of interest using of the selection tools (square, circular, draw, etc…). On the ROI manager, click “Add [t]”. Several ROIs can be added sequentially and they will be numbered. Ticking the box “Show all” will show them simultaneously on the image.
   2. Once the ROI or ROIs are defined, press “GetAverage” on the Time Series Analyser window. This will yield a graph and a table with the intensity values over time of the selected ROI(s). These can be further analysed in Excel, R, Graphpad, etc….
   3. To get the intensity values of multiple channel (R-Geco/Green-Geco). Just click on the first window , get the value via average function, then click on the second window and get the average value of the second channel.

**Notes:**

* After every step, especially one that takes a significant amount of time to process, it is recommended to save the file and indicate on the file name the processing that took place (eg., “Plant 1 Registered”). This is because ImageJ does not have an “Undo” function and any mistake requires starting the process from the last saved file.
* Registration can also be used to keep a specific spatial feature (eg., the root tip) in the same place over the entire duration of the imaging. For this, go to “Plugins – Registration – MultiStackReg”, select the file to be registered in Stack 1 and select “Align from the drop-down menu. Do not select any other image as stack 2. Use Rigid body and press go. Bear in mind that the frame currently being visualized is the one used as a reference, and the alignment will create black space or eliminate parts of the image, to keep everything aligned with the reference.

Movie extraction:

* For YC3.6 – conversion to rainbow color – use YFP (frame 21)- Lookup tables – 16 colors
* Save as .avi

Control the speed of movie:

* Image – Stacks – Tools – Animation Options