Visualising 3D and Live Data

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Aim

Data that is captured on a confocal microscope or live imaging system can be represented in several ways that can best address the question being asked. For confocal data there are several ways of showing the 3D data that is captured and for live data the easiest way to represent it is to create a montage or a movie of the time series.

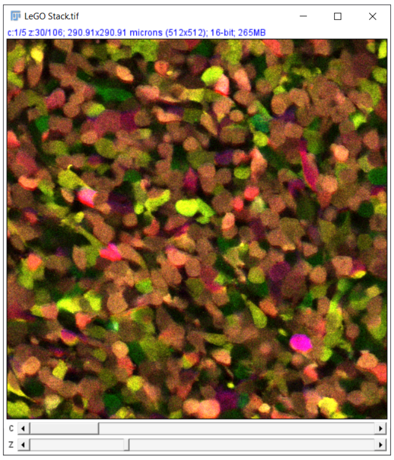
Other options are movies to play in presentations or kymograms to visualise movement of objects in a 2D image,

Visualising 3D Data

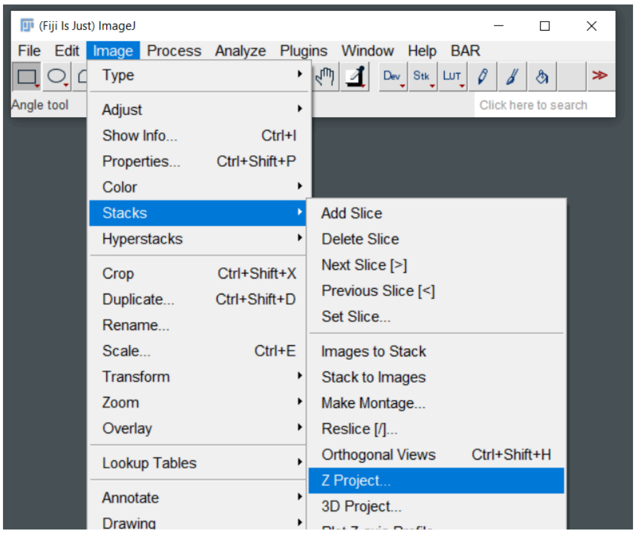
Z Projection

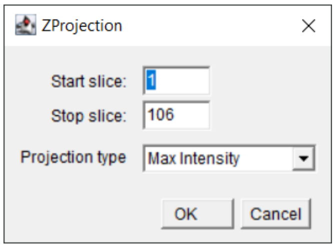
A Z projection is created by combining all the Z slices in a data set together to generate an image. The usual type of projection is a maximum intensity projection, this means that the highest intensity pixel from each slice is projected to the final image. Other projections are average, minimum, sum, standard deviation and median. Each has its uses, but a maximum projection is used most often in fluorescent imaging.

1. Open the **LeGO.tif** file found in the **Demo Images\Confocal Folder**

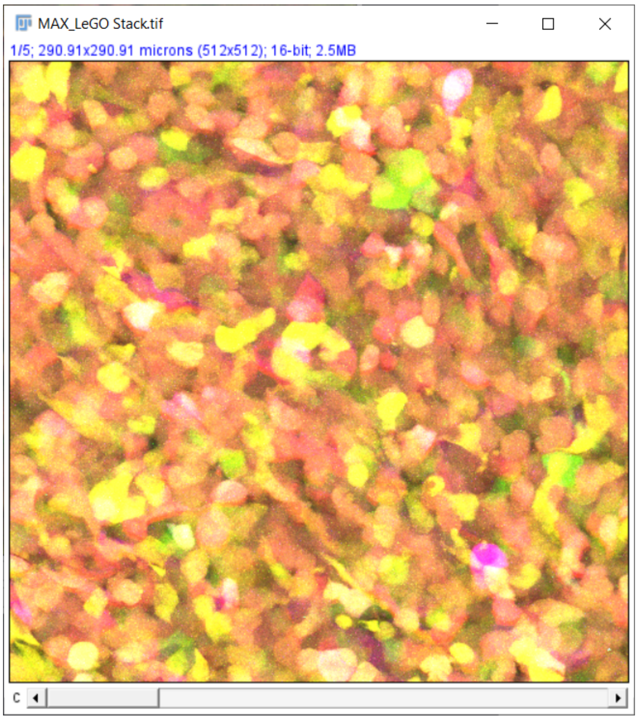


2. Go to **Image Stacks Z Project..** (or press the Stk button on the Fiji bar) and set the Projection Type to Max Intensity and press **OK**.

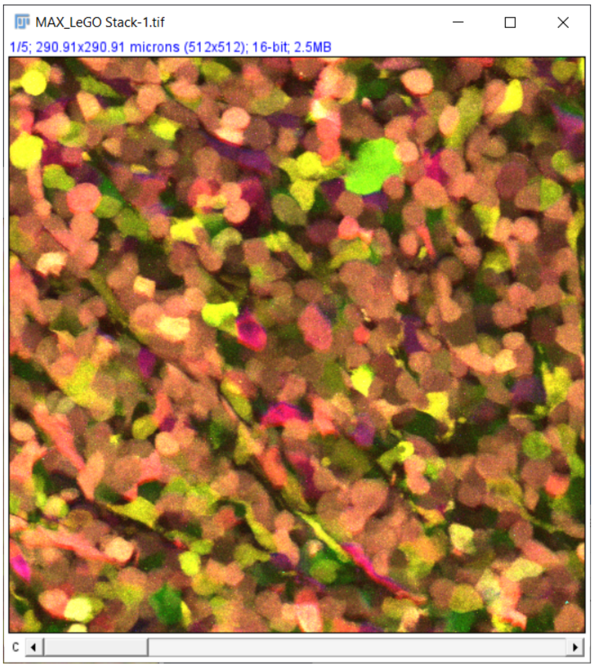


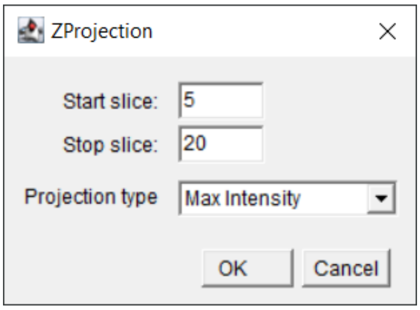


3. The resulting image will represent the brightest pixel (in Z) at each x,y position. For this image there is a lot of z slices that projected through leaving the image looking saturated and messy



4. Try again but this time set the start and end to cover less slices in the final projection, e.g. from 5 to 20. The resulting projection is cleaner and less messy.

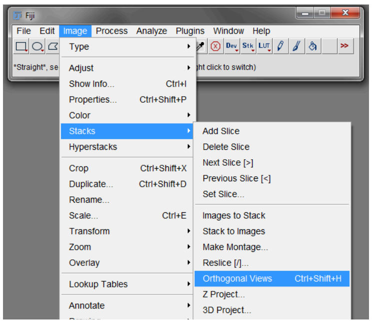




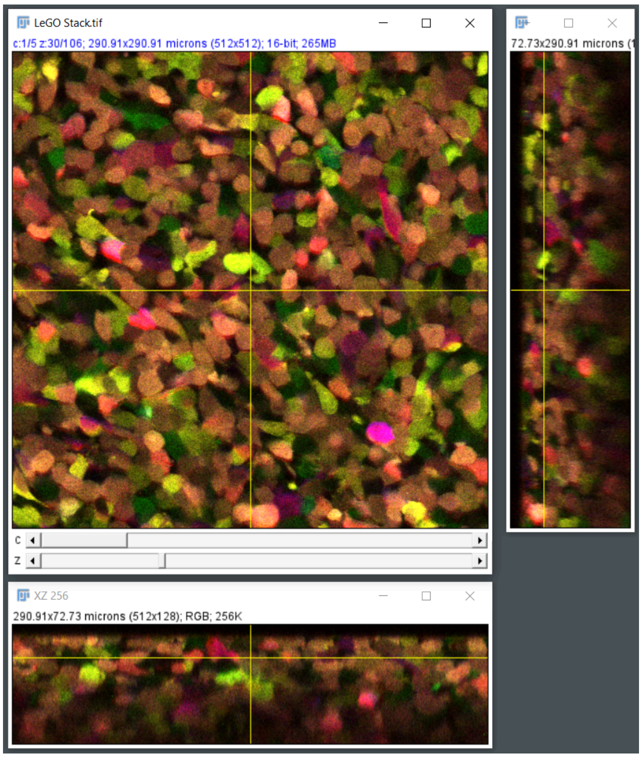
Orthogonal Projection

An orthogonal projection is a view created in the YZ or XZ dimension of an image stack. An orthogonal projection allows you to visualise depth information one slice at a time in your sample.

1. Open the **[LeGO.tif]** file found in the **[Demo Images\Confocal]** folder. Go to **Image Stacks Orthogonal Views.**



2. Two windows will open up, one to the right and one below the original stack. These windows show the orthogonal projections in XZ and YZ. To change the view seen in each window move the yellow cross hair in the original stack.

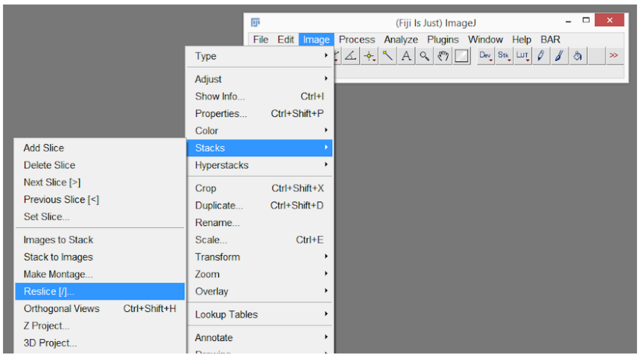


NOTE: Each of the individual XZ and YZ images can be saved for later use.

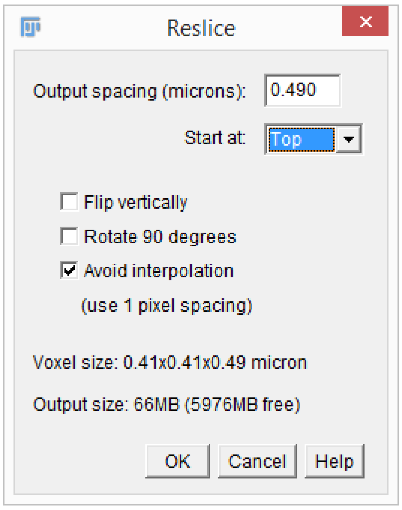
Orthogonal Stack/Reslice Stack

The orthogonal projection only lets you see one slice at a time and it has the overlay lines in the way. Sometimes it can be useful to generate a stack of orthogonal slices instead. This is easily achieved by reslicing the stack along a different axis.

1. Open the **LeGO.tif** found in the **Demo Images\Confocal** folder. Go to



2. The dialog that opens lets you set how the stack should be resliced (top, bottom, left, right) and to set the distance between slices. In this case the stack is already calibrated so there is no need to enter a value, if it is not calibrated and you know the calibration you can enter it here. NOTE: This is the z spacing calibration in this example, not the xy calibration.



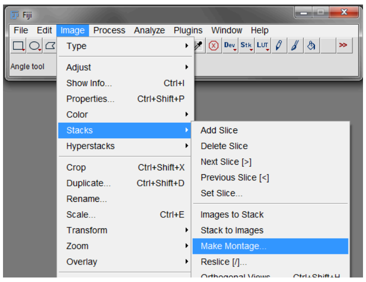
There is a tick box to select that says **Avoid Interpolation**. Ticking this will avoid adding information to your image but depending on slice spacing could end up giving you a very squashed or stretched stack.

3. A new stack is generated that represents the orthogonal planes of the image. This stack can be treated as any other stack and can be made into a movie or a montage.

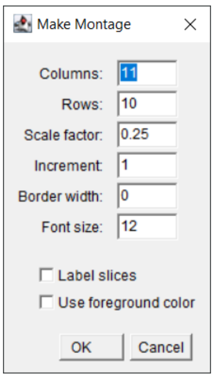
Slice Montage

Creating a montage allows you to show all (or a sub set) of the slices from a confocal series in one image. This technique can also be used for a time stack to show different time points in a data set.

1. Open the **LeGO.tif** found in the **Demo Images\Confocal**. Go to **Image ⍰ Stacks ⍰ Make Montage…**



2. The **Make Montage** widow will open. This can be configured to generate the montage you want. The default settings should be something like those below



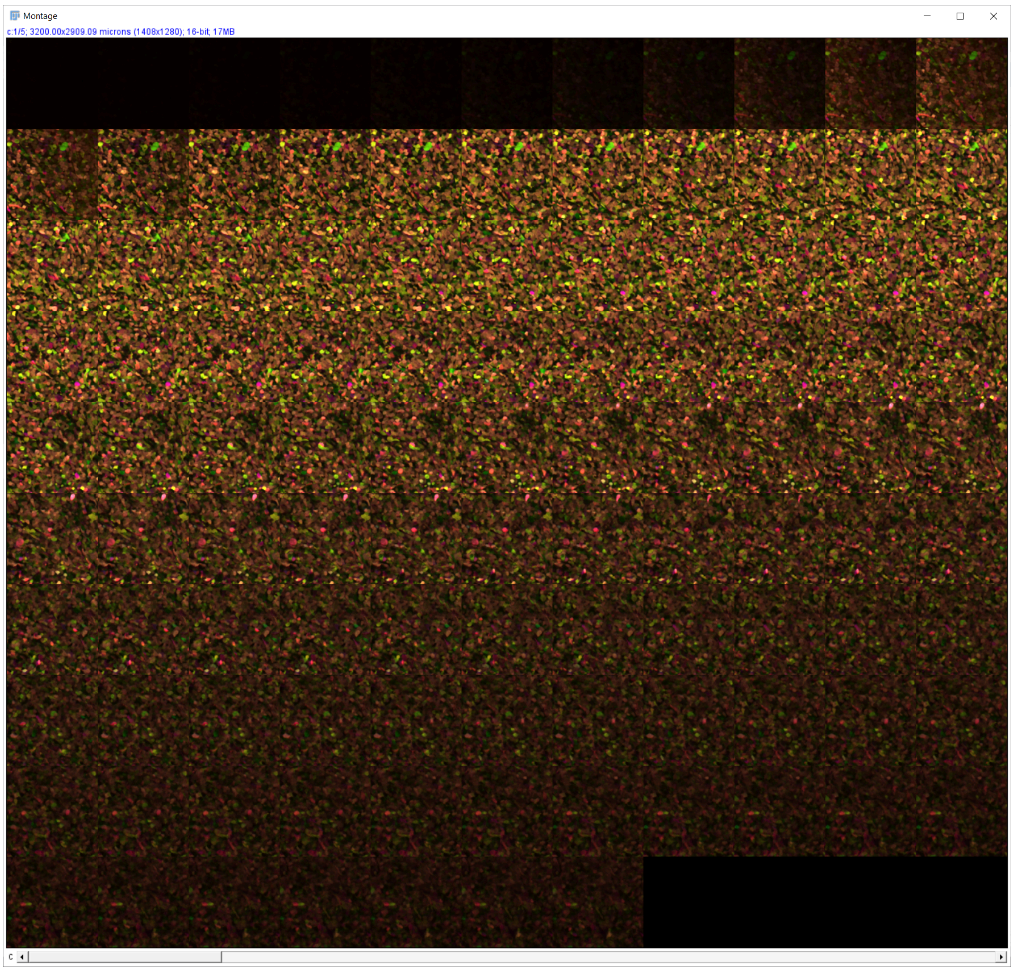
3. The **Columns** and **Rows** values can be changed to reflect the layout you want to achieve. **NOTE:** These values are not dynamically linked to the data so it is possible to change the values so that all your data will not fit in the montage.

   The **Scale Factor** is the amount each individual slice will be downsized to reduce the final size of the montage. If the montage is not going to be too big it is probably best to set this to 1 and resize the image later if required.

   The **Increment** value is the number of slices that will be used for the montage. A value of 1 means all slices will be used, a value of 2 means every second slice will be sued etc.

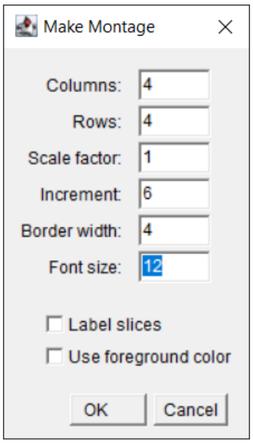
   The **Border Width** sets the thickness of the border line that will be drawn around each frame, a value of 0 gives no border.

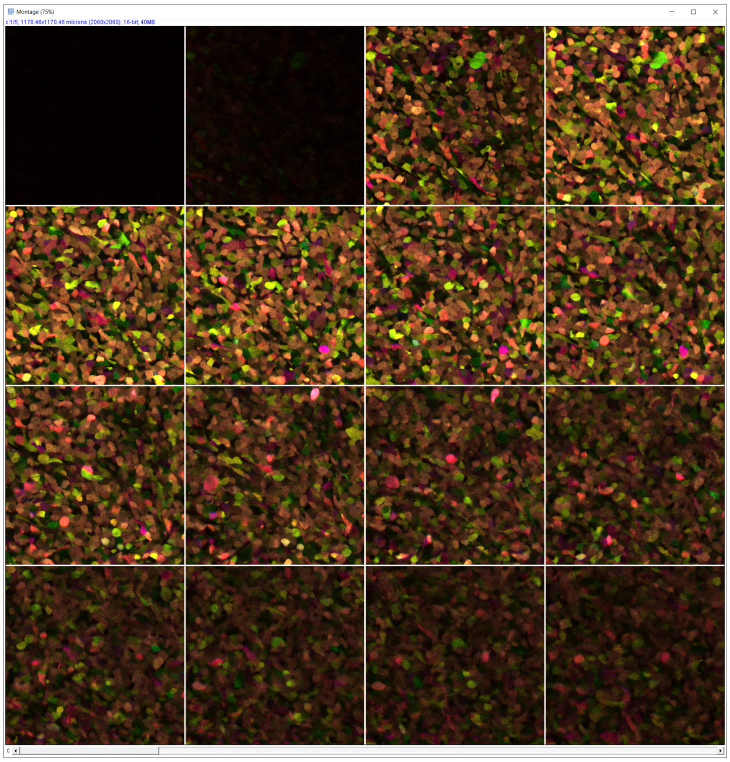
4. Leave the values at their default for now and press **OK.**



The resulting montage is a bit complicated looking. It is hard to tell where each slice is, the resolution is a bit low and there are way to many slices represented.

5. Try creating a montage with the following settings. This gives a better quality image that more clearly shows what is going on across the whole stack.

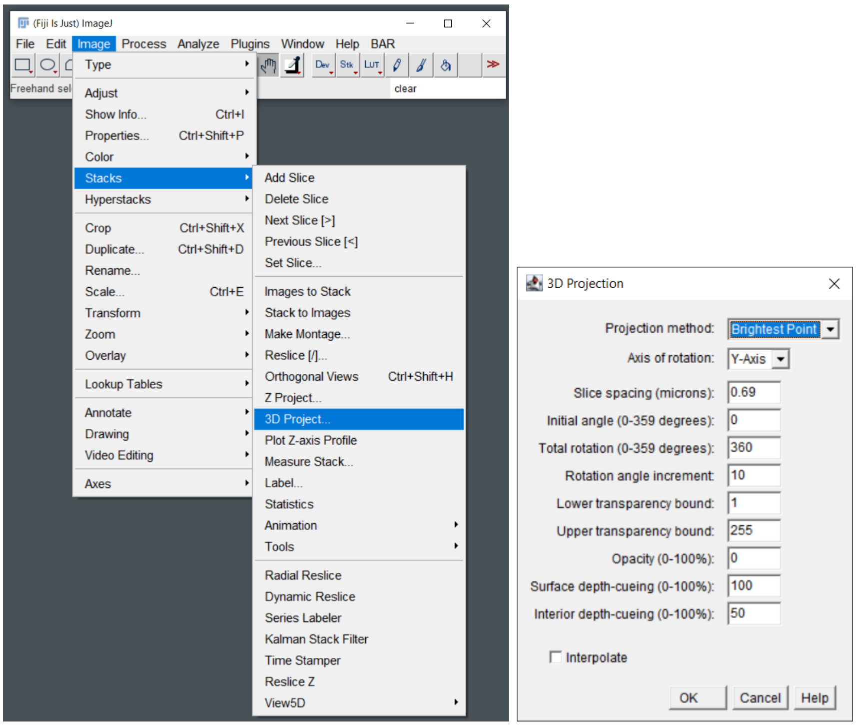




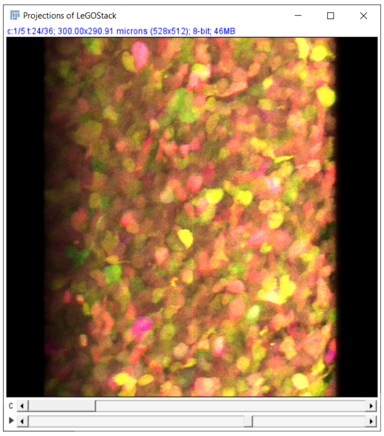
3D Volume – Simple

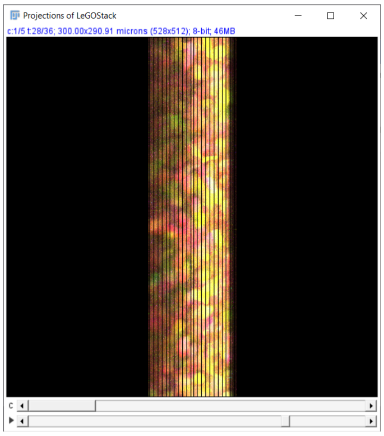
There are various ways to project 3D data in Fiji, some of which will be covered later in the course. The simplest method is a standard 3D projection.

1. Open [LeGO.tif]() found in the [Demo Images\Confocal]() folder
2. Now go to **Image Stacks 3D Project…** Leave the settings as default and press OK.



3. The resulting image will have a slider at the bottom like a normal stack but when you move it the image will rotate around the Y axis. You may notice when you look end on to the stack you can see lines between each of the slices. This is because the data has not been Interpolated (essentially had the missing information guessed at). You can fix this by building the volume again, but this time tick the Interpolate box.



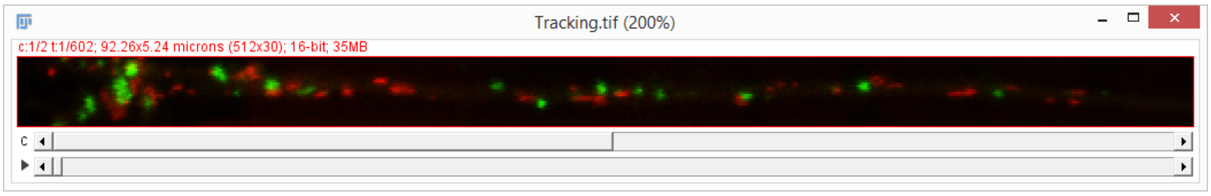


4. Try generating other models by changing the options in the 3D projection dialog box, try different rotation axis, angles or projection methods.

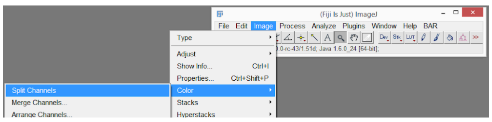
Kymograms

A kymogram is a way of representing live data in a kind of graphical way. Instead of showing live data as a movie it is represented as a 2D image with one dimension of the image representing a physical dimension (x, x or Z) with the other dimension representing time. They are a powerful way of easily representing what is going on in a live data set.

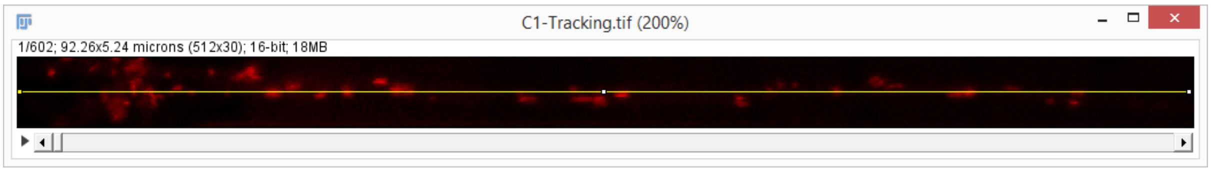
1. Open **Tracking.tif** from the **Demo Images\Confocal** folder



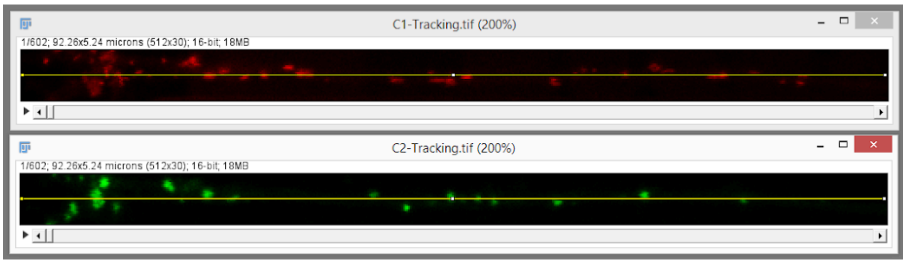
2. Split the channels by going to **Image → Colour → Split Channels**



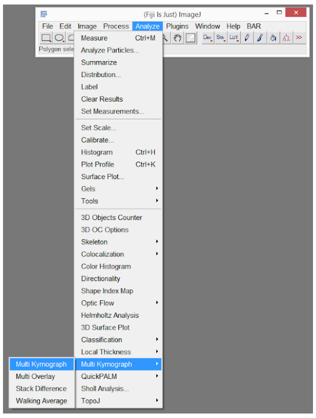
3. Use the line tool to draw a line down the middle of **channel 1** image. **TIP** hold down the **Shift** key to keep the line perfectly straight.



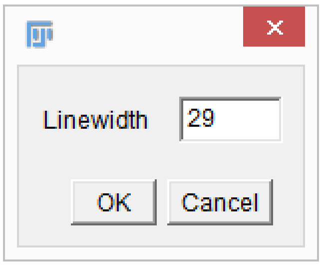
4. Select **Channel 2** and copy the line region to it by pressing **Ctrl + Shift + E**



5. Select **Channel 1**and go to **Analyze Multi Kymograph Multi Kymograph**



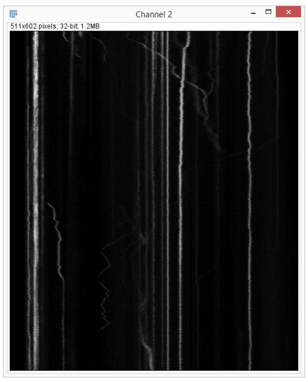
6. In the dialog that comes up you need to define a line width. This is the width of the line that will be used to average out the intensities to give you your kymograph. A higher line width will average out some noise in the resulting image, but too big a width will blur out real data too.



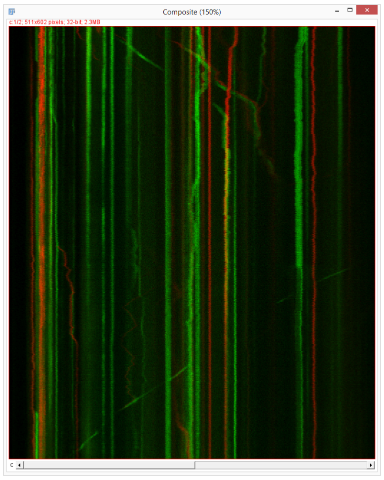
NOTE: Line width values have to be an odd number for this module to work

7. You will get an image like the following. This image show the x coordinates across the image and time, from start to end, down the image vertically. The angle of a track shows you its speed, a more horizontal line is from a particle traveling faster. The vertical lines represent particles that are not moving.

8. Rename the image to **Channel 1** by going to **Image → Rename**
9. Repeat for channel 2 so you end up with kymographs for each channel



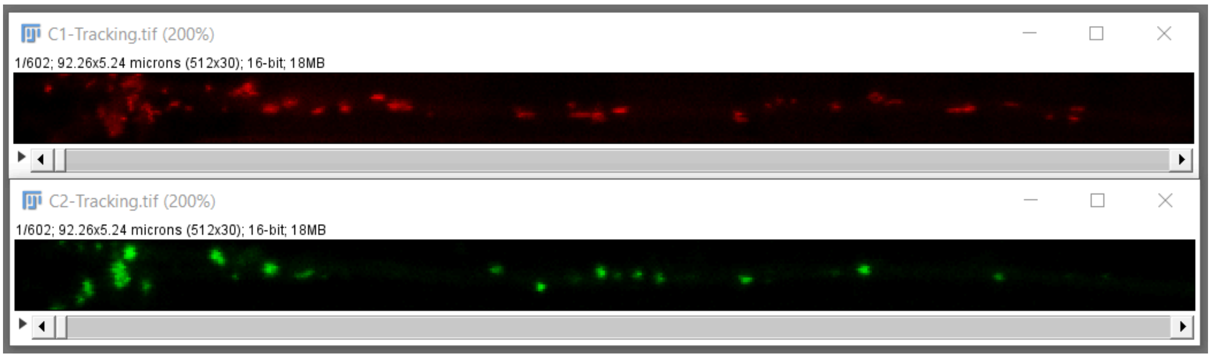
10. Merge the two channels together to see the difference in the tracks of the two markers (in this example channel 1 is green, channel 2 is red)



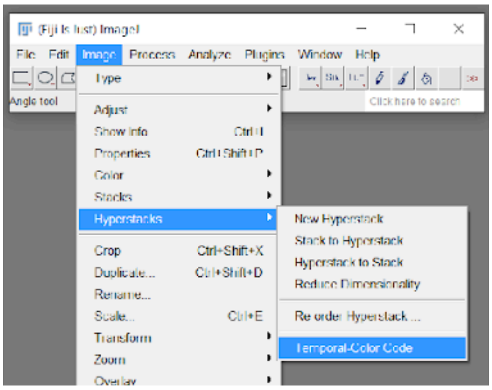
Temporal Colour Projections

Like a kymograph a temporal colour projection can convey live data information in a single image. A temporal colour projection takes all the different time points of an image and colours them differently (say from red to green to blue) and then projects them through into a final image. This allows where a given object or path occurs in time to be easily seen by its colour.

1. Open **Tracking.tif** from the **Demo Images\Confocal** folder and split the channels



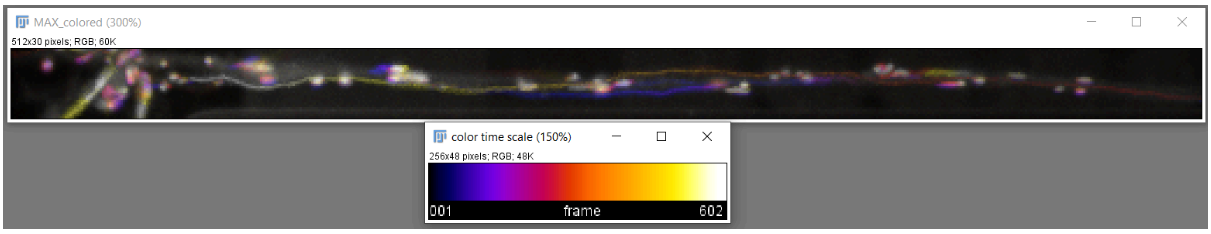
2. Select one of the channels, in this example channel 1 (red) and go to **Image → Hyperstacks → Temporal Colour Code**



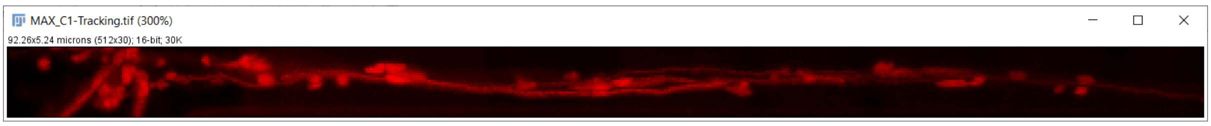
3. In the dialog that opens you can assign a **LUT** to the final image and chose which frames should be processed to generate the image. Spectral LUTs like Fire, Rainbow RGB etc work best for this. By default, the box should be ticked. This will generate an image showing what colours represent which t**Create Time Color Scale Bar**ime points.

   Press **OK**

4. The resulting image should be something like this showing the time points projected together with the calibration colour bar.



This shows much more useful information that a simple time projection/z projection

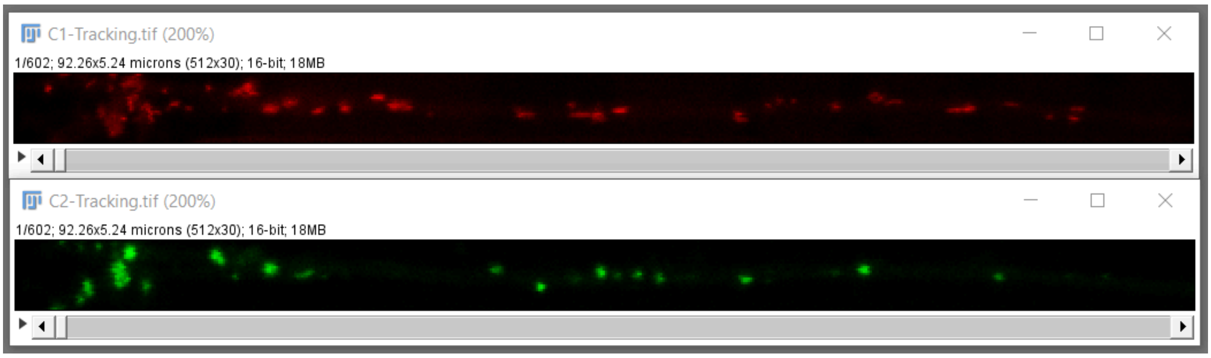


Cleaning up Time Data

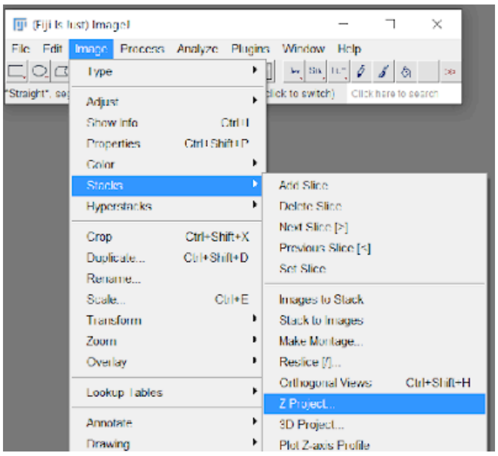
In both the kymogram and temporal projection data above there is noise contributed from objects that are not moving. These static objects result in vertical stripes in the kymograph and white blobs in the temporal projection.

These can be reduced or even removed by a simple process of subtracting an average image of the time series from the original data.

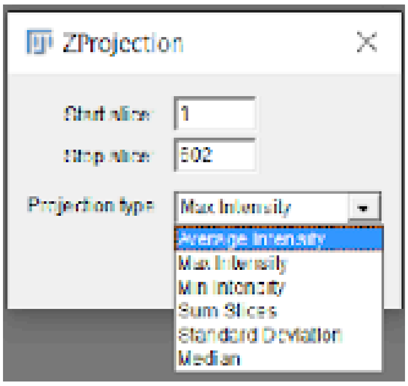
1. Open **[Tracking.tif]** from **[Demo Images\Confocal]** and split the channels



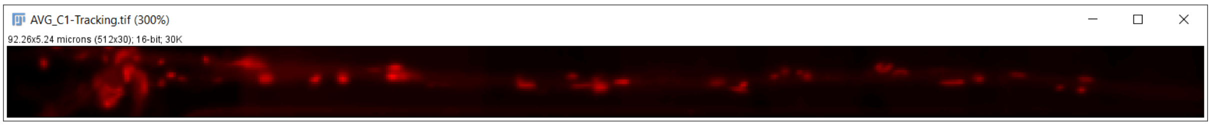
2. Select one of the channels, in this example channel 1, and go to **Image → Stacks → Z Project…**



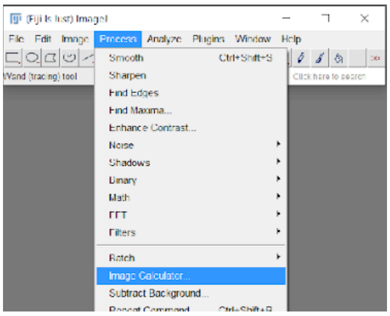
3. In the dialog that opens change the **Projection Type** to **Average Intensity** and press **OK**



4. The resulting image may look a bit fuzzy in some places but that is ok, it is what we are going for. The image is an average of all the intensities across the time series at each pixel. Pixels that change during the time series, for example from objects moving, will have an average that will be low. Pixels that are part of objects that stay still will have an average value very similar to those in the original image.

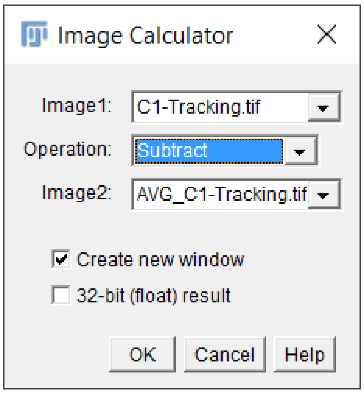


5. Go to **Process ⍰ Image Calculator**

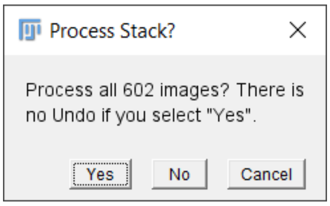


6. They dialog that opens allows various image arithmetic and Boolean logic functions to be performed. For this example, we need to subtract the average intensity image from the original time series. Because the average intensity image is only one plane it will be subtracted from each of the frames of the time series.

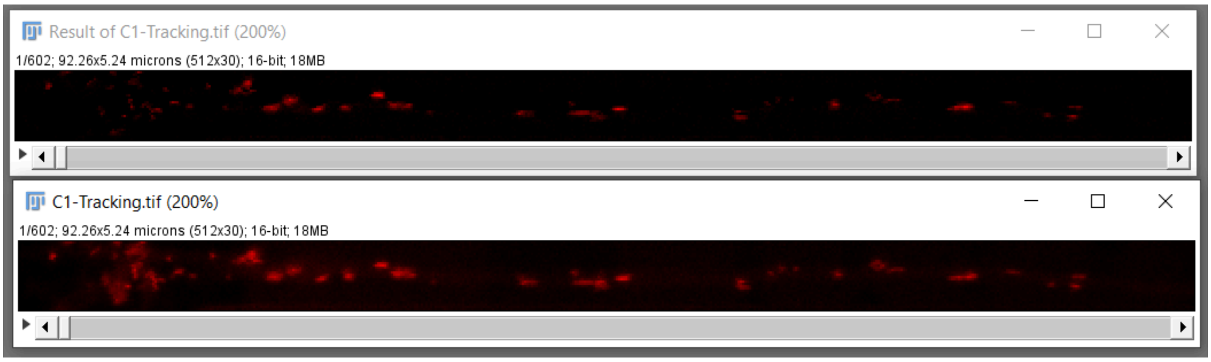
   Make sure **Create new window** box is ticked and press **OK**



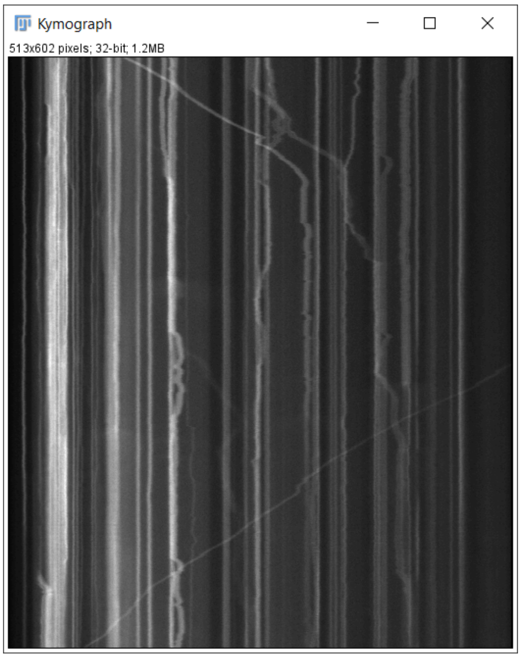
7. Click **Yes** in the dialog that comes up

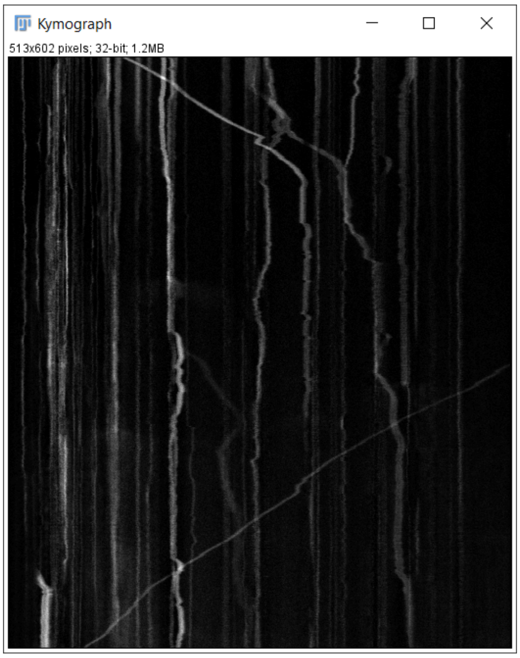


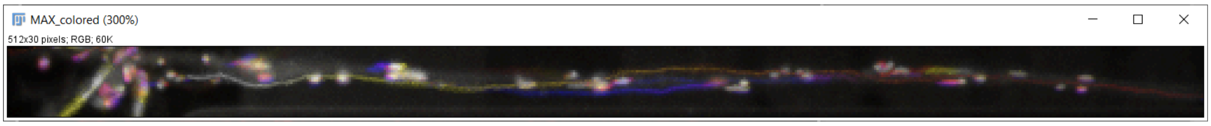
8. The resulting stack should be a cleaner version of the original. In this example there are still some stationary blobs that remain. This is due to the fact that they do move slightly over the time course so are not averaged out.

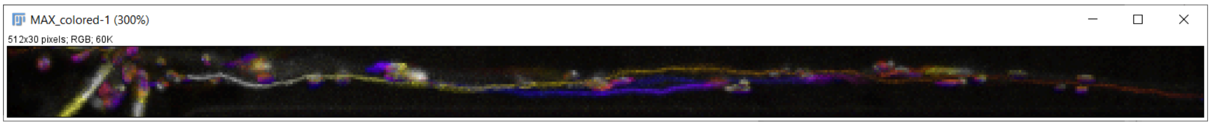


9. Kymographs or temporal projections of the cleaned-up time series should look cleaner that the original series





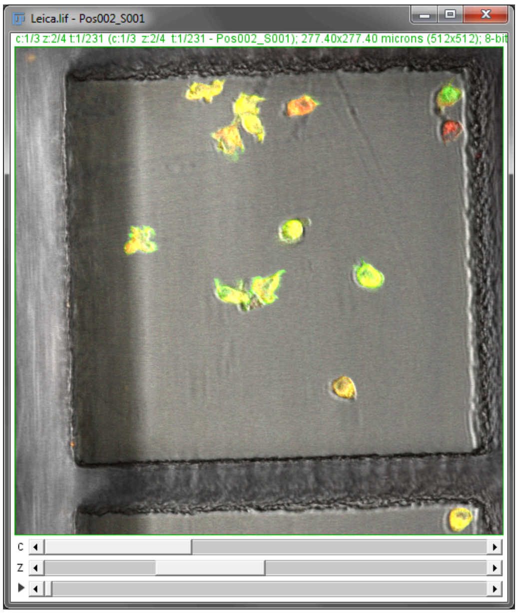




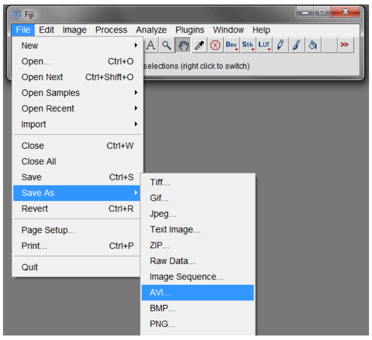
Making Movies

Any stack (Z series, time series, 3D rotation etc.) can be easily exported as a movie to show or play in PowerPoint.

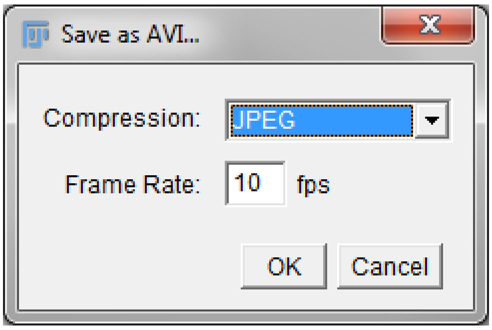
1. Open one of the data sets from the **Leica.lif** file from the **Demo Images\Confocal** folder. Set it to be a composite image and select one of the middle z slices.



2. Go to **File → Save As → AVI..**

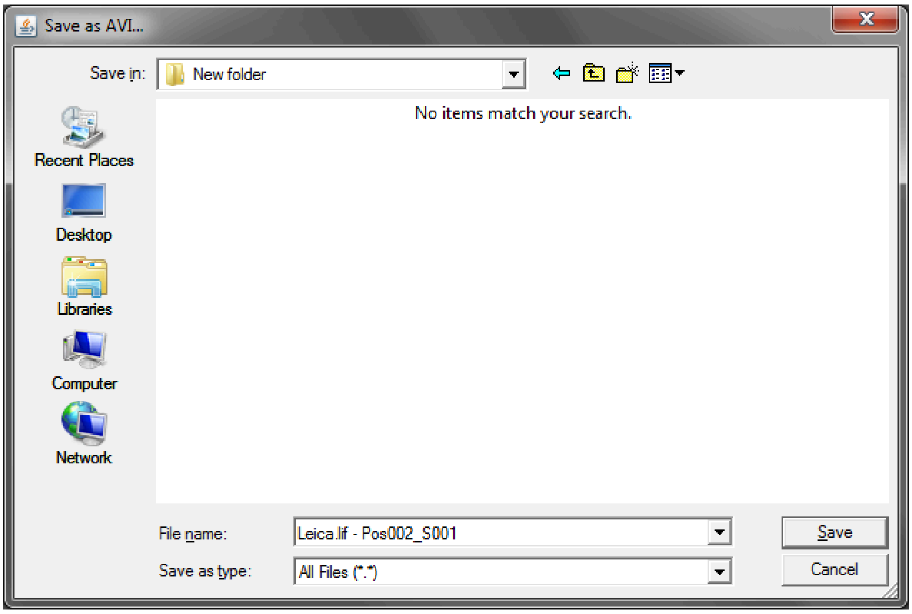


3. In the **Save as AVI** window select **JPEG** as the Compression: type and set the **Frame Rate** to **10**. Press **OK**.



The setting for the frame rate will depend on several factors: the length you want your movie to be, the number of frames/slices etc.

4. In the next window choose a name (or leave it as default) and a location to save your AVI file.



5. Open the file you just saved to see the movie in the default media player. **NOTE**: if you wish to save the file in a different format (e.g. MOV, mp4 etc.), save it as an AVI with no compression and then use third party software to convert it.