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TITLE:

Image Processing Protocol for the Analysis of the Diffusion and Cluster Size of Membrane Receptors by Fluorescence Microscopy

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SPT, chemokine receptor, tracking, clusters, diffusion, analysis software

SUMMARY:

Here, we present a protocol for single particle tracking image analysis that allows quantitative evaluation of diffusion coefficients, types of motion and cluster sizes of single particles detected by fluorescence microscopy.

ABSTRACT:

Particle tracking on a video sequence and the posterior analysis of their trajectories is nowadays a common operation in many biological studies. Using the analysis of cell membrane receptor clusters as a model, we present a detailed protocol for this image analysis task using Fiji (ImageJ) and Matlab routines to: 1) define regions of interest and design masks adapted to these regions; 2) track the particles in fluorescence microscopy videos; 3) analyze the diffusion and intensity

characteristics of selected tracks. The quantitative analysis of the diffusion coefficients, types of motion, and cluster size obtained by fluorescence microscopy and image processing provides a valuable tool to objectively determine particle dynamics and the consequences of modifying environmental conditions. In this article we present detailed protocols for the analysis of these features. The method described here not only allows single-molecule tracking detection, but also automates the estimation of lateral diffusion parameters at the cell membrane, classifies the type of trajectory and allows complete analysis thus overcoming the difficulties in quantifying spot size over its entire trajectory at the cell membrane.

INTRODUCTION:

Membrane proteins embedded in the lipid bilayer are in continuous movement due to thermal diffusion. Their dynamics are essential to regulate cell responses, as intermolecular interactions allow formation of complexes that vary in size from monomers to oligomers and influence the stability of signaling complexes. Elucidating the mechanisms controlling protein dynamics is thus a new challenge in cell biology, necessary to understand signal transduction pathways and to identify unanticipated cell functions.

Some optical methods have been developed to study these interactions in living cells¹. Among these, total internal reflection fluorescence (TIRF) microscopy, developed in the early 1980s, allows the study of molecular interactions at or very near the cell membrane². To study dynamic parameters of membrane protein trajectories obtained from TIRF data in living cells, a single particle tracking method (SPT) is required. Although several algorithms are available for this, we currently use those published by Jaqaman et al.³ that address particle motion heterogeneity in a dense particle field by linking particles between consecutive frames to connect the resulting track segments into complete trajectories (temporary particle disappearance). The software captures the particle merging and splitting that result from aggregation and dissociation events³. One of the output data of this software is detection of the particles along the entire trajectory by defining their X and Y positions in each frame.

Once particles are detected, we apply different algorithms to determine the short timelag diffusion coefficient (D_{1-4})^{4,5}. By applying the Moment Scaling Spectrum (MSS)⁶⁻⁸ analysis or by fitting the 'alpha' value by adjustment of the Mean Square Displacement (MSD) to the curve⁹, we also classify the particles according to the type of trajectory.

Analysis of spot intensity in fluorescence images is a shared objective for scientists in the field^{10,11}. The most common algorithm used is the so-called Number and Brightness. This method nonetheless does not allow correct frame-by-frame intensity detection in particles in the mobile fraction. We have, thus, generated a new algorithm to evaluate these particle intensities frame-by-frame and to determine their aggregation state. Once the coordinates of each particle are detected using U-Track2 software³, we define its intensity in each frame over the complete trajectory, also taking into account the cell background in each frame. This software offers different possibilities to determine the spot intensity and the cell background and, using known monomeric and dimeric proteins as references, calculates the approximate number of proteins in the particle detected (cluster size).

In this article, we describe a careful guide to perform these three steps: 1) detecting and tracking single particles along a video of fluorescence microscopy using U-track; 2) analyzing the instantaneous diffusion coefficient (D_{1-4}) of those particles and the type of movement (confined, free, or directed) of particles with long trajectories by MSS; 3) measuring the spot intensity along the video corrected by the estimated background fluorescence for each spot. This allows cluster size estimation and identification of the photobleaching steps.

The use of this protocol does not require specialized skills and can be performed in any laboratory with cell culture, flow cytometry and microscopy facilities. The protocol uses ImageJ or Fiji (a distribution of ImageJ¹²), U-track³, and some ad hoc made routines (<http://i2pc.es/coss/Programs/protocolScripts.zip>). U-track and ad hoc routines run over Matlab that can be installed in any compatible computer.

PROTOCOL:

1. Preparation of biological samples

1.1 Grow Jurkat cells in RPMI 1640 medium supplemented with 10% FCS, NaPyr and L-glutamine (complete RPMI). Electroporate Jurkat cells (20×10^6 cells/400 μ L of RPMI 1640 with 10% FCS) with a monomeric GFP-labelled chemokine receptor vector (CXCR4-AcGFP, 20 μ g) to allow its detection using fluorescence microscopy.

NOTE: It is possible to use other monomeric fluorescent proteins such as mCherry, mScarlet, etc.

1.2 24 hours after transfection analyze cells in a flow cytometer to determine both cell viability and CXCR4-AcGFP expression.

1.3 Select cells expressing low CXCR4-AcGFP levels by cell sorting of GFP^{low} positive cells (**Figure 1**), as low expression of transfected receptor is required in TIRFM experiments in order to ensure single particle tracking for tracing individual trajectories⁹.

1.4 Quantify the number of receptors in the cell surface.

NOTE: As an example¹³, $\sim 8,500 - 22,000$ AcGFP-labeled receptors/cell, correspond to $\sim 2 - 4.5$ particles/ μm^2 .

1.5 Resuspend sorted cells in complete RPMI and incubate for at least 2 h at 37 °C, 5% CO₂. Centrifuge cells (300 x g, 5 min), and resuspended them in TIRF buffer (HBSS, 25 mM HEPES, 2% FCS, pH 7.3).

1.5.1 Plate on 35 mm glass-bottomed microwell dishes ($2-3 \times 10^5$ cell/dish) coated with fibronectin (20 μ g/mL, 1 h, 37 °C) in the presence or absence of appropriate ligand (i.e., CXCL12, 100 nM, 1 h, 37 °C). Incubate cells (20 min at 37 °C, 5% CO₂) prior to image acquisition.

1.6 Perform experiments using a TIRF microscope, equipped with an EM-CCD camera, a 100x oil-immersion objective (HCX PL APO 100x/1.46 NA) and a 488-nm diode laser. The microscope allows temperature control and incubation with CO₂. Locate and focus cells with the coarse and fine focus knobs, using bright field to minimize photobleaching effects. For fine focus adjustment in TIRF mode use a low laser intensity, insufficient for single-particle detection or to induce photobleaching effects (5% laser power, 28 μ W).

1.7 Acquire movies (image sequences) of approximately 50 s minimizing the time interval between frames. Penetration of the evanescent field should be 70-90 nm of depth. Save the acquired movies for each experimental condition as ".lif" (video.lif).

NOTE: Movies in the described example were acquired at 49% at laser power (2 mW) with an exposure time of 90 ms, and a time interval of 98 ms, for 49 s (500 frames). Penetration of the selected evanescent wave was 90 nm.

2. Selection of images and creation of masks

2.1 For each experimental condition (video.lif), create a new folder (VideoName) that must contain different folders for every series. Each folder will contain a "videoSeq" folder for the video images and a "results" folder for the results of the analysis. Make sure that the file structure at this moment is the following:

VideoName/video.lif

VideoName/Series1/**videoSeq**

VideoName/Series1/**results**

NOTE: From the microscope different .lif files are obtained with several videos for every treatment condition (i.e. FN, FN+SDF). "video.lif" corresponds to the input.lif video file with all TIRF movies acquisitions (Series) performed at the microscope. "videoSeq" folder will contain the 500 frames of the movie that we are analyzing. The "results" folder will contain all files resulting from the analysis performed. Accurate nomenclature and localization of the different folders are essential for the correct function of the algorithms. Names in bold in the list above are fixed (i.e., they have to be called in this way because these are the names sought by the scripts). Names not in bold can change to reflect the experiment performed.

2.2 Open the TIRFM video (.lif file) with Fiji or ImageJ by dragging and dropping the file on the Fiji menu bar and click on **OK** to import the lif file using BioFormats (**Supplementary Figure 1**).

2.3 Select the series to process and click **OK (Supplemental Figure 2A)**. To design a mask for the analysis of this video, import also a multichannel image with the different chromophores (in the example, Series 1 is the multichannel image and Series 2 the corresponding video). The video (and the multichannel image) should open as an ImageJ stack. In the example (**Supplemental Figure 2B**), the image is on the left and the video is on the right.

NOTE: If creation of a mask for the video is not needed, go to Step 2.5.

2.4 Create a mask. Create a single image with the channels useful for the design of the mask. In this case, the interesting channels are the red, green and gray ones.

2.4.1 Split the channels from the multichannel image (**Supplementary Figure 3A**): select **Image** in the bar menu and click **Color | Split channels**. The different channels will show as separate images (**Supplementary Figure 3B**).

2.4.2 Merge again the three channels in a single image (**Supplemental Figure 4A**): select **Image** in the bar menu and select **Color | Merge channels**. Select the appropriate channels and press **OK (Supplemental Figure 4B)**. A new non-stacked image will be generated (**Supplemental Figure 4C**).

2.4.3 Synchronize the two windows by using the **Synchronize Windows** tool (**Supplemental Figure 5A**): select **Analyze** in the bar menu | **Tools | Sync Windows**. A new window with the synchronize image possibilities will be shown (**Supplemental Figure 5B**).

2.4.4 With the two windows synchronized (only the video if there is no multichannel image associated), the same region in both windows can be cropped. Draw the region of interest with the rectangular selection tool of ImageJ floating menu. Select **Image** in the bar menu and select **Crop (Supplemental Figure 6A)**. The two cropped images will show individually (**Supplemental Figure 6B**).

2.4.5 Unsynchronize both windows (**Supplemental Figure 6B**) by pressing the **Unsyncronize All** button in the **Sync Windows** manager.

2.5 Draw the region of interest with the rectangular selection tool of ImageJ floating menu if a mask has not been created as in 2.4. Select **Image** in the bar menu and select **Crop**.

2.6 Save the video as an **Image sequence** in the directory **videoSeq** under the corresponding video directory (**Supplemental Figure 7A**): select **File** in the bar menu and click **Save as | Image Sequence...**. Rename the labels for the video sequence as video0000.tif, video0001.tif, ..., video0499.tif (**Supplemental Figure 7B**): in the **Name** box, rename as **video** and click **OK**. The sequence must be alone in its directory to be successfully used by U-track.

NOTE: If not designing a mask for the video, go to Step 2.8.

2.7 Design a Mask. Select the multichannel image and open the Segmentation Editor plugin (**Supplemental Figure 8A**): select **Plugins** in the bar menu and select **Segmentation | Segmentation editor**. Add and rename the labels of the segmentation as necessary by right clicking on the labels of the Segmentation editor (**Supplemental Figure 8B,C**).

2.7.1 Choose the appropriate selection tool in ImageJ floating menu (here, use freehand), select a label (Green) and design first the outermost mask (**Supplemental Figure 9A**). Once designed, press the + button in **Selection** option of **Composite** window, and the selected mask will be displayed on the viewer (**Supplemental Figure 9A**). Repeat this step with next labels (Interior, in red) (**Supplemental Figure 9B**).

NOTE: After designing the mask for the Green and Interior labels, the Exterior mask will occupy the rest of the image.

2.7.2 Masks are coded in the image as regions 0, 1, 2, ... according to the order of the labels in the RGB labels window. When all masks for the different labels are designed, save the mask with the same filename as the video, with the name **mask.tif** (**Supplemental Figure 9C**): select **File** in the bar menu and select **Save as | Tiff....**

NOTE: The selected masks will be employed in the calculation of the diffusion coefficients and classification of the trajectories (see step 4.2).

2.8 Check that the file structure at this moment is the following:

```
VideoName/video.lif
VideoName/Series1/mask.tif
VideoName/Series1/videoSeq/video0000.tif
VideoName/Series1/videoSeq/video0001.tif
...
VideoName/Series1/videoSeq/video0499.tif
VideoName/Series1/results
```

NOTE: The mask.tif is an image with the mask as designed in Steps 2.4 and 2.7. The video*.tif is the video as saved in Step 2.6. As above, the names in bold in the list above are fixed, i.e., they have to be called in this way because these are the names sought by the scripts. Names not in bold can change to reflect the experiment performed.

3. Tracking the particles

3.1 Track all the particles seen in the selected videos using U-track.

3.2 Open Matlab and add U-track directory to the path by using **Set Path | Add with Subfolders** option in the menu. Save the path so that in future executions of Matlab U-track is in the path. This path setting needs to be done only once.

3.3 Change the working directory to the directory containing the Series to be analyzed. Invoke U-track by typing in the console (**Supplemental Figure 10**) **movieSelectorGUI** and press enter. The **Movie selection** window will be opened (**Supplemental Figure 11A**).

3.4 Press on the **New** movie button and the **Movie edition** window will appear (**Supplemental Figure 11B**).

3.5 Press on **Add channel** to choose the directory with the video (VideoName/Series1/video) and fill the movie information parameters. Set the output directory for the results of U-track to **Results** (videoName/Series1/results).

NOTE: The movie information parameters can be obtained from the microscope and the acquisition conditions.

3.6 Press on the **Advanced channel settings** and fill the parameters related to the acquisition. Look the values of the parameters in the example (**Supplemental Figure 11C**).

3.7 Press **Save** on the **Advanced channel settings** window and **Save** on the **Movie edition** window. The program will ask for confirmation of writing the file called **movieData.mat** on the **results** directory. Confirm.

3.8 After creating the movie, press on **Continue** in the movie selection window. U-track will ask about the type of object to be analyzed. Choose **Single-particles** (**Supplemental Figure 12**). The **Control panel** window will appear (**Supplemental Figure 13A**).

3.8.1 Select the first step **Step 1: Detection** and press on **Setting**. The **Setting Gaussian Mixture-Model Fitting** window will appear (**Supplemental Figure 13B**). In the example, “Alpha value for comparison with local background” is set to 0.001 and “Rolling-Window time averaging” to 3 (**Supplemental Figure 13B**).

3.8.2 Press on **Apply** in the **Settings Gaussian Mixture-Model Fitting** window and **Run** in the **Control panel**. With the configuration in **Supplemental Figure 13**, only the **Detection** step runs. This step takes a few (2-5) minutes. Check the results by pressing the **Result** button of the Step 1 (Detection, **Supplemental Figure 14**).

NOTE: As shown above, the movie shows red circles on the detected particles. If no red circle is shown, then this step has not worked correctly.

3.9 Perform the identification of tracks, that is, merging the particles detected in the previous step into tracks that span multiple frames. This is the **Step 2: Tracking** of U-track whose settings have to be defined as shown in **Supplemental Figure 15A-C**. The Step 2 cost function settings for **frame-to-frame linking** and **Gap closing, merging and splitting** in the example are shown in **Supplemental Figure 15B and C**, respectively.

3.10 After setting the parameters for Step 2, press **Run** in the **Control panel** and only Step 2 runs (**Supplemental Figure 16**).

3.11 **Perform track analysis, Step 3.** Define the settings as shown in **Supplemental Figure 17** (right panel). Then, press **Apply** in the panel of Setting-Motion Analysis, and **Run** in the Control Panel-U-Track. This step takes a few seconds.

3.12 **Verify with the Result button of Step 3 that the process has correctly identified all the tracks.** For doing so, click on **Show track number** of the **Movie options** window, and check frame by frame that each track has been correctly identified (**Supplemental Figure 18**). Manually annotate those particles that are not true particles.

NOTE: If this manual selection is not done, a weaker automatic selection can be performed later when the diffusion coefficient is calculated (see Step 4).

4. Calculation of the diffusion coefficients and classification of trajectories

4.1 Be sure that all the scripts are invoked from the directory of the video being analyzed (in the example, VideoName/Serie1).

4.2 Read all the trajectories to compute the diffusion coefficients by issuing in the Matlab console the command: **trajectories=readTrajectories(0.1)**, where 0.1 is the time in seconds between two consecutive frames (time interval, shown in panel **Movie Information**, Supplemental Figure 11B).

4.3 **Exclude trajectories corresponding to incorrectly identified spots/trajectories.** Give a list of the spots to exclude. For instance, to exclude spots 4, 5 and 28, type: **trajectories=readTrajectories(0.1, [4, 5, 28])**.

4.4 **Calculate the instantaneous diffusion coefficients for each one of the tracks of this cell. In this case, calculate the diffusion coefficient for a time lag = 4, called D_{1-4} .** For doing so, run in the Matlab console the command: **D=calculateDiffusion(trajectories, 113.88e-3, 0.0015, 'alpha', '', 20)** where trajectories are the trajectories obtained in Step 3, 113.88e-3 is the pixel size of the acquired images in microns, 0.0015 is an upper bound for the diffusion coefficients of immobile particles measured in $\mu\text{m}^2/\text{s}$, and 'alpha' is the fitted model as explained below.

NOTE: When using a faster camera and need more frames to calculate the diffusion parameter increase it, e.g. to 20, by **D=calculateDiffusion(trajectories, 113.88e-3, 0.0015, 'alpha', '', 20)**. The string parameter before 20, in the example above, '', is the suffix added to the output files. This suffix may be used to differentiate different analyses.

4.5 Fit the MSD with a different function by calling the calculateDiffusion function again with a different fitting mode ('confined', 'free', or 'directed'). In this example, 'confined': **D=calculateDiffusion(trajectories, 113.88e-3, 0.0015, 'confined')**.

4.6 Obtain the fitting results for the directed model, as shown in **Supplemental Figure 20**.

4.7 **Decompose the trajectories into short and long trajectories.** Use the command:
[shortTrajectories, longTrajectories]=separateTrajectoriesByLength(trajectories,50) where 50
is the minimum length in frames of a trajectory to be considered long (in the example shown).

4.8 Study short trajectories using the same fitting procedure described in Step 4.3:
D=calculateDiffusion(shortTrajectories, 113.88e-3, 0.0015, 'directed', 'Short'). Analyze short
and anomalous trajectories with the command: **D=calculateDiffusion(shortTrajectories,**
113.88e-3, 0.0015, 'alpha', 'Short').

4.9 **Analyze long trajectories to classify the type of motion through their Moment Scaling**
Spectrum (MSS)⁷. The command:
trajectoriesClassification=classifyLongTrajectories(longTrajectories,113.88e-3,0.0015,'Long')
shows the analysis in screen and generates a file called **trajectoryClassification<Suffix>.txt** in the
directory **results\TrackingPackage\tracks**.

5. Calculation of cluster size through the particle density

NOTE: Be sure that all the scripts are invoked from the directory of the video being analyzed (in
the example shown, VideoName/Serie1).

5.1 **Analyze the intensity of each particle along their trajectory.** For doing so, invoke the script
by typing in the Matlab console: **analyzeSpotIntensities** that takes as input the trajectories
calculated by U-track in the first section. In its most basic form, simply call the script without any
argument from the directory of the video being analyzed (in the example shown,
VideoName/Serie1) **analyzeSpotIntensities()**. **Configure this basic behavior in many different**
ways by providing arguments to the script as in: analyzeSpotIntensities('Arg1', Value1, 'Arg2',
Value2, ...). Valid arguments with their corresponding variable values ('ArgN', ValueN) are listed.

5.1.1 ('spotRadius', 1)

Analyze the fluorescence intensity using the spotRadius of 1 pixel (by default) that corresponds
to a patch of size 3x3 centered at the spot ((2*spotRadius+1)x(2*spotRadius+1)).

NOTE: For a patch of 5x5 centered at the spot, choose a spotRadius of 2, etc.

5.1.2 ('onlyInitialTrajectories', 1)

If this argument is given (true, value of 1), analyze only the trajectories that start in the first frame
of the video. This is useful to analyze control images (by default 0, false).

5.1.3 ('trackTrajectory', 0)

If this argument is set to 0 (false), then keep the coordinate of the spot in its first frame for all
frames (this is useful for immobile spots). If the argument is set to 1 (by default 1, true), then the
spot is tracked along the video following the coordinates calculated by U-track.

5.1.4 ('excludeTrajectories', [4,5,28])

394 Include the trajectory number of those trajectories excluded in Step 4.3 (in the example 4, 5, 28).

395
396 5.1.5 ('extendTrajectory', 1)

397 If this argument is set to 1 (true), then analyze the intensity in the patch to the end of the video
398 (even if the trajectory stops earlier). The coordinate of the spot is either the last coordinate in
399 the trajectory (if trackTrajectory is true) or the first coordinate in the trajectory (if trackTrajectory
400 is false). This argument is false (0) by default.

401
402 5.1.6 ('subtractBackground', 1)

403 If this parameter is set, then correct the raw fluorescence measured at each spot by the estimate
404 of the background fluorescence for that spot (see below). This argument is true (1), by default.

405
406 5.1.7 ('meanLength', frame number)

407 If this parameter is set, then the mean intensity spot is measured at the length indicated. Set
408 'meanLength', 20 to measure the mean spot intensity at the first 20 frames. If the argument is
409 not set, then the spot intensity is calculated at the whole trajectory (by default, full length).

410
411 5.1.8 ('showIntensityProfiles', 1)

412 Set this parameter as 1 (by default 0, false), to plot the intensity profile along the different frames
413 as well as their background.

414
415 NOTE: These plots are very useful to identify photobleaching as shown in the **Supplemental**
416 **Figure 21**. For every path, the routine automatically analyzes if it is possible that there has been
417 photobleaching. This is done by comparing the intensity values with a Student's t in the first and
418 last N frames along the path. By default, N is 10, but this value can be modified through the
419 argument 'Nbleach'.

420
421 5.1.9 ('backgroundMethod', value)

422 Set this parameter to determine the background of each spot. This can be done in several ways,
423 and which one to use can be selected changing the "value":

424
425 5.1.9.1 ('backgroundMethod', 0)

426 Use this value to manually identify the background for the whole video. Allow to select 8 points
427 in the first frame of the video. A patch around these points is analyzed along the whole video,
428 and the 95% quantile of all these intensities is chosen as the background intensity for all spots.

429
430 5.1.9.2 ('backgroundMethod', 1)

431 Use this value to manually identify the background for each frame. Choose 8 points for every spot
432 and every frame. This is a time consuming task but it gives a lot of control to the user. The 95%
433 quantile of the intensities in these patches is chosen as the background intensity for this spot in
434 this frame.

435
436 5.1.9.3 ('backgroundMethod', 2)

Use this value to calculate the background of each spot estimated from 8 points located in a circle around the spot with a radius controlled by the argument `'backgroundRadius'` (by default, $4 \times \text{spotRadius}$).

5.1.9.4 (`'backgroundMethod', 3`)

Use this value to calculate the background for each frame by first locating the cell in the video and then analyzing the intensities of the cell in each frame (**Supplemental Figure 22**).

NOTE: The background is chosen as the gray value at a given quantile of this distribution (by default 0.5 (=50%), although this parameter can be controlled through the argument `'backgroundPercentile'`, this value can be set higher, for instance, 0.9 (=90%) if wanting most of the cell to be considered as background. To help in the identification of the cell, indicate which is the maximum background value expected along the frames using the argument `'maxBackground'` (for instance, in all the analyzed videos, the background value normally never goes beyond 6000)¹³. By default, this option is set to 0, meaning that this help is not used by default. See which is the cell detection and the area selected for the background estimation by setting the argument `'showImages'` to 1 (stop the execution at any time by pressing CTRL-C).

5.2 Gather the diffusion and intensity information for all the trajectories calculated in the Steps 4 and 5.1, respectively, using `gatherDiffusionAndIntensity()`. Gather only the diffusion and intensity information for short trajectories. For doing so, use the suffixes used in Step 4.7, and type: `gatherDiffusionAndIntensity('Short')` where `'Short'` is the suffix used in Step 4.7.

5.3 Gather the Moment Spectrum Scaling and the intensity information by typing: `gatherTrajectoryClassificationAndIntensity('Long')` where `'Long'` is the suffix used in Step 4.7. A summary of all the files generated using this protocol is shown in **Figure 2**.

REPRESENTATIVE RESULTS:

The use of this protocol allows the automated tracking of particles detected in fluorescence microscopy movies and the analysis of their dynamic characteristics. Initially, cells are transfected with the fluorescently-coupled protein to be tracked. The appropriate level of receptors presents on the cell surface that allows SPT is obtained by cell sorting (**Figure 1**). Selected cells are analyzed by TIRF microscopy that generates videos in a format that can be studied with the tools described in this protocol (**Supplemental Video 1**).

The videos generated cannot be directly analyzed, and independent frames of each video are required. The use of ImageJ generates files that can be interpreted using Matlab software such as video frames in .tiff format or masks. U-track tracks the particles seen in the selected video and saves the information in Matlab (.mat) files (*movieData.mat*, *Channel_1_detection_result.mat*, *channel_1.mat*, *Channel_1_tracking_result.mat*), see **Figure 2**.

As shown in **Figure 2**, the calculation of the diffusion coefficients and classification of trajectories commands, generates different files (*diffusionCoefficients.txt*, *diffusionCoefficientsMobile.txt*, *diffusionCoefficientsShort.txt*, *trajectoryClassificationLong.txt*, etc). The information contained in

these files are best managed using Excel and Prism software. Information that can be obtained from this analysis includes:

1. Percentage of immobile spots. You can use as reference of immobile particles: the 95% percentile of D_{1-4} values obtained (1) from single particles in fixed cells and/or (2) from purified fluorescent protein attached to the coverslips (**Figure 3A**).
2. Percentage of long trajectories (> 50 frames) (**Figure 3B**).
3. Type of movement of the long trajectories: directed, free, confined (**Figure 3C**).
4. Diffusion coefficient (D_{1-4}) of mobile particles in cells treated with different stimulus (**Figure 4A**).

The step 5 of the protocol analyzes the intensities of each particle along the trajectory. The script **analyzeSpotIntensities** will print on screen all the information analyzed. For each spot and frame, the script shows the coordinate of the spot in that frame (x,y) in pixel units, the estimate of the background fluorescence (k_0), the raw spot intensity in the 3x3 patch, the corrected intensity (calculated as the raw intensity minus its background), the maximum value of intensity observed in the patch, and the region number within the mask where this spot is located at this frame. An example of the kind of output produced is

```
spot=43 frame=184
x=78.0397
y=72.5395
k0=1571
spotRawIntensity=5550.1111
spotCorrectedIntensity=3979.1111
maxCorrectedSpotIntensity=6243
maskRegion=2
```

All this information is stored in a log file (*results/TrackingPackage/tracks/log.txt*) and a table that can be read from Excel (*results/TrackingPackage/tracks/spotIntensitiesByFrame.txt*). After analyzing each spot, the script prints the average intensity along the trajectory and the majoritarian region within the mask

```
spot=43
meanCorrectedSpotIntensity along frames=4762.303
majoritarian region=3
```

This information is stored in the log file above and a table that can be read from a spreadsheet (*results/TrackingPackage/tracks/meanSpotIntensities.txt*). As an example, mean spot intensities (msi) for every particle along their trajectory in cells stimulated with different conditions is shown in **Figure 4B**. We may now use this information to roughly estimate the size of the fluorescent cluster. As a spot's mean corrected intensity is related with the number of fluorescent proteins present in this spot, and the fluorescence of a monomer can be measured through a similar but independent experiment, directly calculate the number of receptors per particle. The frequency distribution of receptor number per particle using as reference the intensity of the monomeric

protein expressed in the same cells is shown in **Figure 4C**.

The `gather diffusion and intensity` command create two files: one called *diffusionCoefficientsAndIntensitiesShort.txt* and another called *log_diffusionCoefficientsAndIntensitiesShort.txt* in the directory *results/TrackingPackage/tracks*. Both files contain 1) the spot index, 2) the diffusion coefficient, 3) the intensity, and 4) the region number within the mask. These files can be read from a spreadsheet.

Similarly, the `gather trajectory classification and intensity` command will create two files one called *trajectoryClassificationAndIntensitiesLong.txt* and another *log_trajectoryClassificationAndIntensitiesLong.txt* in the directory *results/TrackingPackage/tracks*. The first one contains 1) the spot index, 2) the movement type, 3) the spot first moment, 4) the intensity, 5) D_{1-4} and 6) the region number within the mask. This file can be read from Excel.

A summary of all the files generated using this protocol is shown in **Figure 2**. Other analysis that can be performed using this protocol includes the comparison of the dynamic parameters of small vs bigger spots, i.e. monomers vs oligomers, variations on these dynamic parameters induced by ligands, inhibitors, membrane composition, alteration of signaling pathways, etc. A complete analysis of CXCR4 behavior in response to its ligand CXCL12 under different experimental conditions has been performed using this protocol¹³.

FIGURE AND TABLE LEGENDS:

Figure 1. Cell sorting. Expression of GFP in Jurkat cells transfected with CXCR4-AcGFP before and after cell sorting. Cell expressing low levels of GFP (GFP^{low}) are selected and employed for TIRF experiments.

Figure 2. Summary of the files generated. The figure shows all the files generated using the Matlab routine described.

Figure 3. Classification of trajectories. Number of the different types of trajectories corresponding to cells treated with different stimuli. **(A)** Percentage of immobile spots, **(B)** percentage of long trajectories and **(C)** type of movement of the long trajectories.

Figure 4. Diffusion coefficient, mean spot intensities and number of receptors per particle. **(A)** Distribution of the short diffusion coefficients (D_{1-4}) values for each spot in response to different stimuli (I, II, III and IV). Red line represents the median value for D_{1-4} . **(B)** Mean spot intensities (msi) values for each spot along its first 20 frames in response to different stimuli (I, II, III and IV). Red line represents the mean intensity value (\pm SD). **(C)** Percentage of receptors/particle as extracted from intensity distribution of the individual particles, taking as bin width the monomeric protein intensity value.

Supplemental Figure 1. Opening a TIRFM file in Fiji or ImageJ. Options that appear on the Bio-formats window upon dragging and dropping a .lif video.

Supplemental Figure 2. Select series. Images present in the .lif video. (A) Window that permits selection of the series to be analyze, including multichannel images. (B) Result example of series selection, including multichannel image (left) and corresponding video (right).

Supplemental Figure 3. Split channels. (A) Window capture of the ImageJ commands needed for splitting channels of a multichannel image and (B) result example of the channel split.

Supplemental Figure 4. Merge Channels. (A) Merging different channels in a single image. (B) Channel selection for the merging. (C) Result example of the channel merge.

Supplemental Figure 5. Synchronize windows. (A) Localization in the ImageJ menu of the commands required for image synchronization and (B) resulting window.

Supplemental Figure 6. Select the region of interest. (A) Selection of the window of interest using the rectangular selection tool and (B) result of the image crop.

Supplemental Figure 7. Save the video. Save the region of interest as an image sequence and parameters for the Save as Image sequence window.

Supplemental Figure 8. Segmentation. (A) Open the Segmentation editor plugin in the ImageJ's plugins menu. (B) Add labels/materials for the segmentation.

Supplemental Figure 9. Mask design. (A) Selection of the appropriate labels and definition of the green mask. (B) Selection of the red mask. Example of two masks corresponding to two labels. (C) Save the masks as a .tiff file.

Supplemental Figure 10. Matlab working directory. Selection of the correct directory containing the series to be analyzed.

Supplemental Figure 11. U-track main menu. (A) Movie selection, (B) movie edition and (C) channel Settings menus.

Supplemental Figure 12. Select the type of object to track. Select tracking of single particles.

Supplemental Figure 13. Detection of particles. (A) U-track, control panel. (B) Example of settings for the detection of particles.

Supplemental Figure 14. Example of results for particle detection. Different windows that include a viewer menu, movie options menu and the movie.

Supplemental Figure 15. Tracking menu. Example of settings. (A) Tracking, (B) setting – frame to frame linking and (C) gap closing, merging and splitting menus.

Supplemental Figure 16. Example of results for particle tracking.

Supplemental Figure 17. Track analysis menu. Example of settings.

Supplemental Figure 18. Verification of track analysis. Screen displayed after track analysis, including a video window showing the particles detected and their corresponding tracks.

Supplemental Figure 19. Calculation of diffusion coefficients. Histograms of the diffusion coefficients calculated (left) and the mean squared displacement (MSD, right).

Supplemental Figure 20. Calculation of diffusion coefficients including the different fitting modes. Histograms of the diffusion coefficients calculated (left) and the mean squared displacement (MSD, right) for the confined model.

Supplemental Figure 21. Intensity Profiles. Example of spot intensity along its trajectory (blue line) and background (red line).

Supplemental Figure 22. Background for each frame. Left: Sample cell image. Middle: Automatically detected cell. Right: Area automatically selected as background.

Supplemental Video 1. Example of a typical TIRF video microscopy showing the presence of particles with different intensities and types of movements.

Supplemental Material 1. Files containing all the protocol scripts employed in the *ad hoc* routines employed for the classification of trajectories and analysis of the cluster size.

DISCUSSION:

The described method is easy to perform even without having any previous experience working with Matlab. However, Matlab routines require extremely accuracy with the nomenclature of the different commands and the localization of the different folders employed by the program. In the tracking analysis routine (step 3), multiple parameters can be modified. The “Setting Gaussian-Mixture Model Fitting” window (step 3.8) controls how U-track will detect single particles on the video. This is done by fitting a Gaussian mixture model as described in³. One of the key parameters for this fitting defines a filter to help identifying local maxima. The success of this operation depends on the image contrast and the noise present in the images. The first parameter (Alpha value for comparison with local background) controls the confidence of a maxima being a real spot, while the second helps to reduce noise during the identification of spots. Important parameters in the “Tracking” step (3.9) are the number of frames to close gaps (that is a track may span over frames in which the particle is not actually seen, but it is seen before these frames and after these frames; in the example, 0), and the number of frames that a track must span in order to be considered as a successful track (in our example, 20; this parameter is related to the camera acquisition speed and the number frames in the track must be such that it allows the calculation of the diffusion coefficient). It is also important to decide whether to merge and split segments (in the example these two possibilities were chosen). Then,

the parameters for the Step 1 in Cost functions (frame-to-frame linking) must be set. This function controls how the particles are tracked along the frames. The most important parameters at this point is the selection of the Brownian search radius, which control how far each spot is expected to be in the next frame. In our example we chose 0 and 5 as lower and upper bounds, respectively. Note that these parameters are very specific to the nature of the particles being tracked, and that they may have to be tuned in each specific case. Particularly important are the scaling power in the Brownian and Linear search radii. These scaling powers depend on the kind of movement of the particles (free or confined diffusion). In the example, the values (0.5, 0.01) were chosen for free diffusion. For the specific documentation of these parameters, see³.

In the calculation of the diffusion coefficients command (step 4.4), the upper bound of the diffusion coefficient of immobile particles can be known from previous experiments or by running the calculateDiffusion function on a separate project with immobile particles (purified monomeric fluorescent protein) and seeing the diffusion coefficients reported. This function takes two extra parameters: 'outputSuffix', which is added to the output filenames and by default takes the empty value, and 'plotLength', which by default is 13 and is the number of time lag (seconds) in which the diffusion calculation is performed. The fitting mode 'alpha' implies an adjustment of the MSD to the curve

$$MSD = MSD_0 + 4Dt^\alpha$$

that is, an offset (MSD_0) and a power function of the time lag. The exponent of this power function, α , determines whether the movement is confined ($0 < \alpha < 0.6$), anomalous ($0.6 < \alpha < 0.9$), free ($0.9 < \alpha < 1.1$), or directed ($\alpha > 1.1$). For a review of this kind of analysis the reader is referred to Manzo et al.⁹

The calculation of the diffusion coefficients⁴ produces two output figures (see **Supplemental Figure 19**). The first one shows a histogram of the diffusion coefficients calculated (note that at this point, there is an independent diffusion coefficient for each trajectory). The mean and 95% percentile of these coefficients are shown in the Matlab console. The second plot shows the MSD (red curve) and the number of steps considered to calculate it as a function of the time lag for those trajectories considered to be mobile (those whose diffusion coefficient is above the threshold given in the command line). The average and standard deviation of the mobile trajectories is computed (these are the values for the trajectories in a single cell). In the example, the exponent is 0.59 meaning that the movement is confined. For this curve fitting, the program reports the uncertainty associated to each one of the parameters (shown as the standard deviation of each one) and the goodness of fit (a perfect fit would reach zero). At this point and after checking the value of alpha we may decide to fit the MSD with a different function:

$$MSD = MSD_0 + \langle r^2 \rangle \left(1 - Ae^{-\frac{4BDt}{\langle r^2 \rangle}} \right) \quad , \quad \text{if } 0 < \alpha < 0.6 \text{ (confined)}$$

$$MSD = MSD_0 + 4Dt \quad \text{if } 0.9 < \alpha < 1.1 \text{ (free)}$$

$$MSD = MSD_0 + 4Dt + (vt)^2 \quad \text{if } \alpha > 1.1 \text{ (directed)}$$

Anomalous trajectories cannot be fitted with a different function. In case of confined particles

you may calculate the confinement size (in microns) as in Destainville et al.¹⁴

$$L = \sqrt{3(\langle r^2 \rangle + MSD_0)}$$

A good indicator of a correct fitting is that the goodness-of-fit should decrease from the alpha fitting to the final fitting (in the example, the goodness fit falls from 0.30474 to 0.15749, **Supplemental Figure 19-20**). **calculateDiffusion** creates two files in the “results\TrackingPackage\tracks” inside the series directory called “*diffusionCoefficients.txt*” and “*diffusionCoefficientsMobile.txt*”. These files contain three columns: 1) the index of each input trajectory, 2) their corresponding diffusion coefficient, 3) the majoritarian region in the mask where this track belongs to (the regions are obtained from the file “mask.tif” that we generated in Steps 2.7; if this file does not exist, then this column is not present). You may use this file and the analogous files generated for other cells to analyze the distribution of the diffusion coefficient for a set of cells under similar experimental conditions.

In the calculation of the diffusion coefficient for short trajectories (steps 4.7-4.8), the last parameter ‘Short’ is a suffix added to the output filename so that you may analyze different subsets of trajectories without overwriting the *diffusionCoefficients.txt* files. The actual name of the output files is “diffusionCoefficients<Suffix>.txt” and “diffusionCoefficientsMobile<Suffix>.txt”. If no suffix is given, as in the general analysis performed above, then an empty suffix is assumed. The model parameters (D, v, and MSD₀) may differ from those fitted to all trajectories. Most remarkably, the standard deviation of the parameters as well as the Goodness fit normally increase. The reason is that short trajectories are more unstable and a reliable fitting is more difficult.

The analysis of long trajectories (step 4.9) classify them into confined (1), free (2), or directed (3) according to their first moment and its location with respect to the 2.5% and 97.5% percentiles of the first moment of 500 random paths with Brownian motion, the same diffusion coefficient and length as the trajectory being analyzed and simulated by Monte Carlo. If the first moment of the path being analyzed is below the 2.5% of the first moments observed in the simulations, the path being studied is classified as confined. If it is above the 97.5% of the simulated first moments, it is classified as directed; otherwise, the path is classified as Brownian. In the command employed, 113.88e-3 is the pixel size in μm, 0.0015 is the upper bound of the diffusion coefficient of immobile spots measured in μm²/s, and ‘Long’ is the suffix for the output filename. The first column of this file (“*trajectoryClassificationLong.txt*”) is the trajectory number, the second is its classification (1=confined, 2=free, 3=directed), and the third is the trajectory first moment.

The script for calculating the intensity of each particle (step 5.1) is highly flexible and allows tracking the fluorescence intensities in many different ways (each one well suited for different situations like tracking immobile spots of a control experiment or tracking highly movable spots over a cell with variable fluorescent background). The script will analyze all trajectories along all frames. For each spot at each frame it will measure the intensity of the pixels around the spot (it analyzes a square patch around the spot, by default of a size 3x3 pixels), estimate the spot

background and calculate the fluorescence difference between the spot and the background. The percentage of photobleaching particles and the number of fluorescent particles in a single cluster, seen as a single spot, can be estimated in this way.

This automated method (gatherTrajectoryClassificationAndIntensity) produces information on multiple parameters (spot intensity, lateral diffusion, type of motion), that can help to study the relation between spot size and its dynamic at basal conditions, and how different treatments can modify these parameters.

The main limitation of the method is that it requires cell transfection with the fluorescent protein to be tracked. Usually transfection leads to protein overexpression, a fact that hinders single protein tracking. A cell sorting step must be included to select cells expressing a number of receptors that allow detection and tracking of single particles by SPT-TIRF microscopy. This method could also be employed for tracking biomolecules labelled with quantum dots or Fab fragments. The described protocol requires a number of controls that must be previously analyzed in order to ensure that the conclusions driven from the analysis are correct. First of all, it is critical to determine the appropriate expression conditions that allow detection and tracking of single particles. Movies with densities of ~ 4.5 particles/ μm^2 , corresponding to 8,500-22,000 receptors/cell, were used to analyze the spatio-temporal organization of the cell membrane receptor¹³.

It is important to establish the minimum detectable diffusion coefficient by using purified monomeric AcGFP proteins or fixed cells. In both cases it is assumed that there is no diffusion and therefore we estimate that the diffusion values of particles analyzed in these conditions correspond to immobilized particles and used to discriminate between mobile and immobile trajectories¹³.

The analysis we have presented here is a general trajectory analysis tool, that can be applied to the analysis of diffusion of receptors by superresolution, to the analysis of diffraction limited images and to the analysis of cellular movement by standard microscopy. The main advantage of this method with others previously described, such as analysis of number and brightness¹¹, is that it allows the evaluation of the mean intensity values of each individual spot along its trajectory, taking into account the time of survival of the AcGFP monomeric protein before photobleaching. The survival time of a molecule before photobleaching will strongly depend on the excitation conditions.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

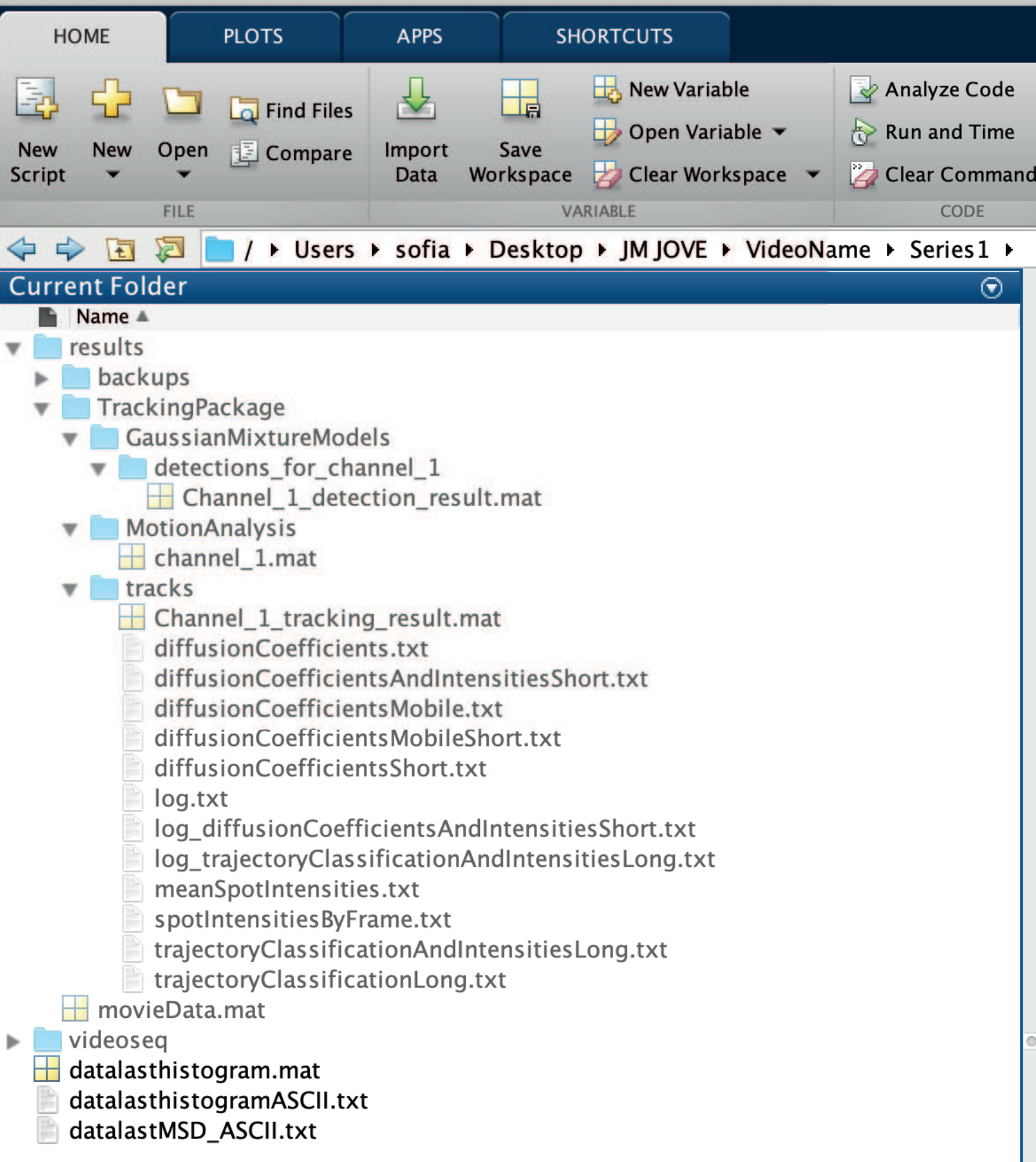


Figure 2

Figure 3

[Click here to access/download;Figure;Figure 3.eps](#)

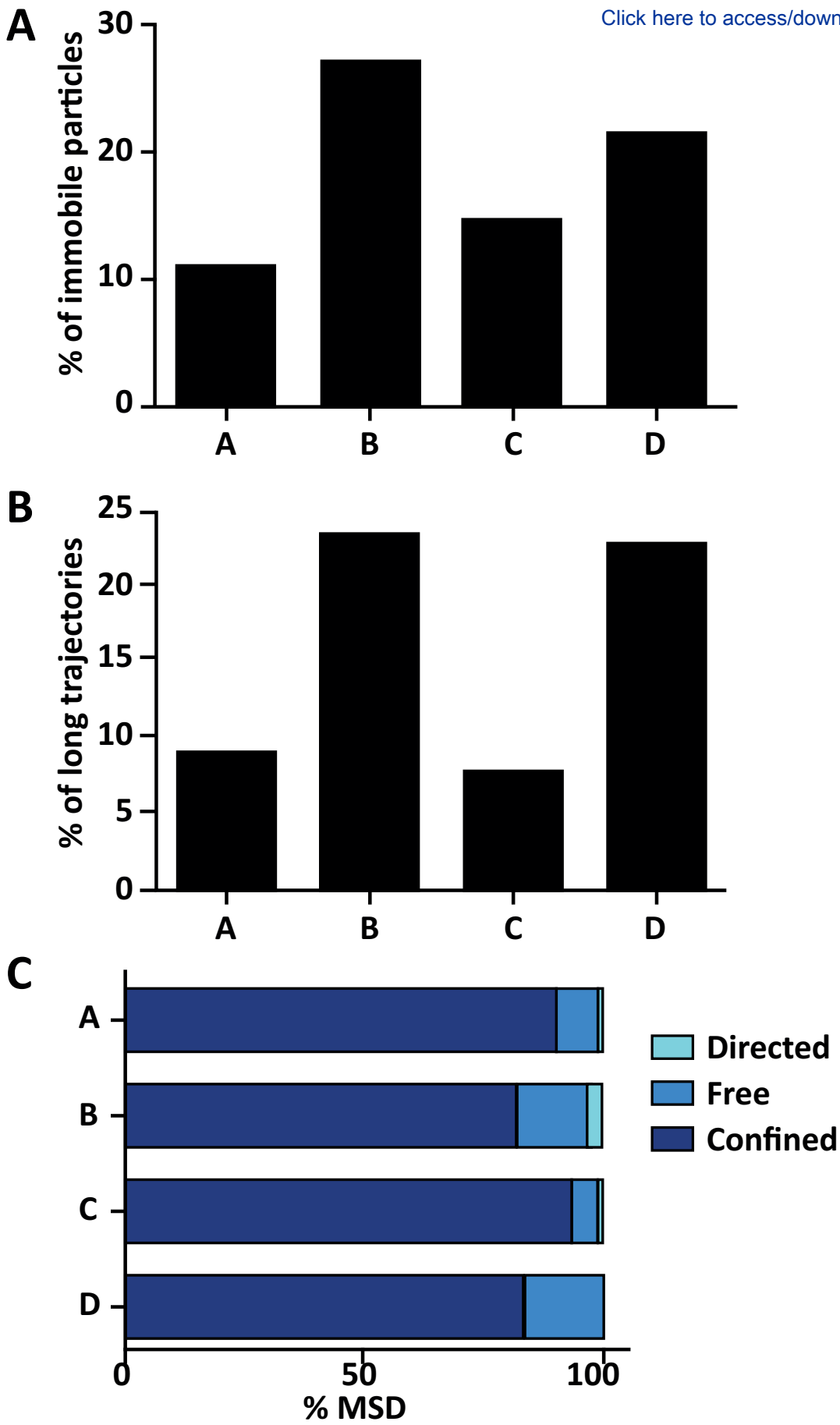


Figure 3

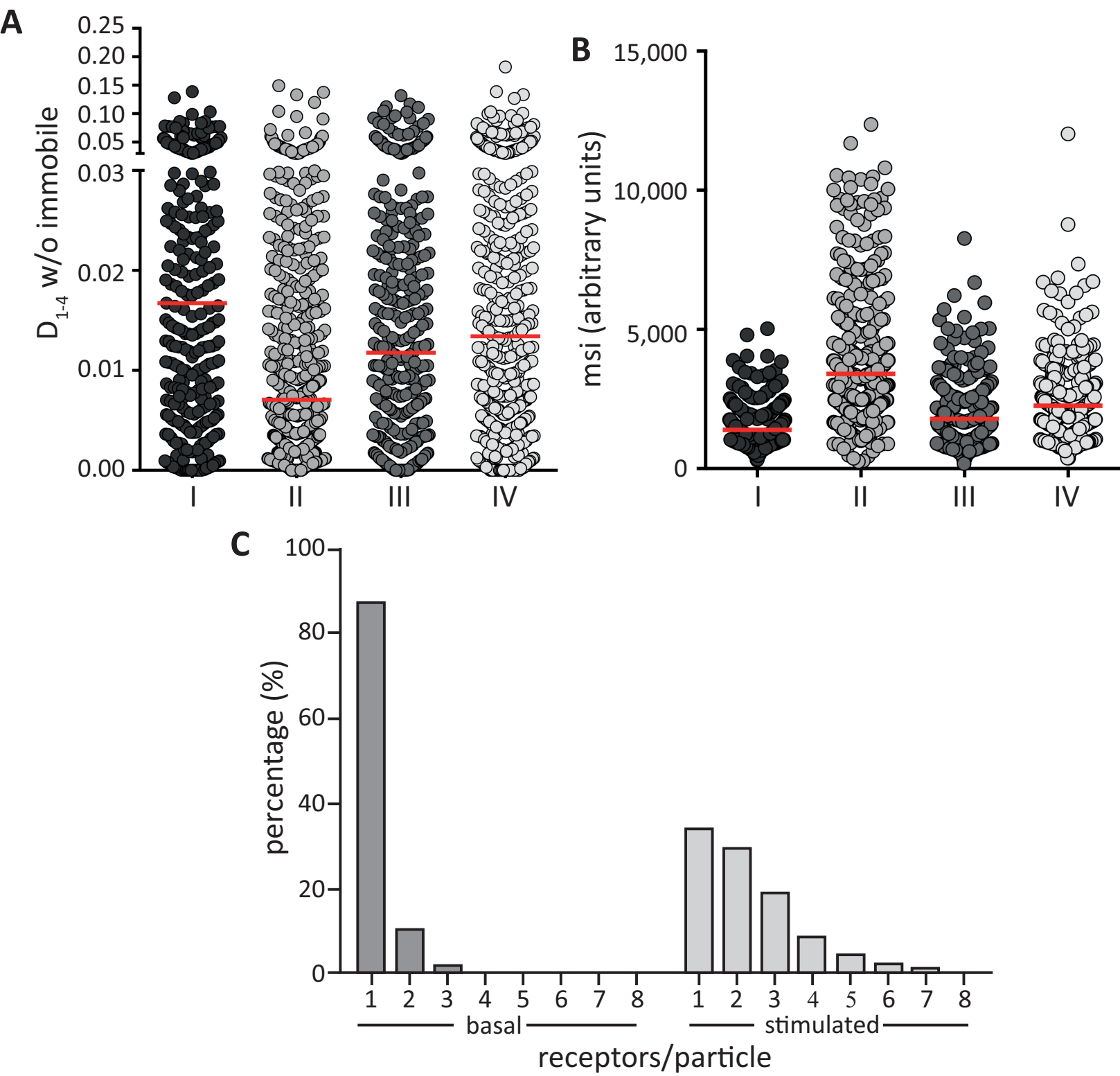
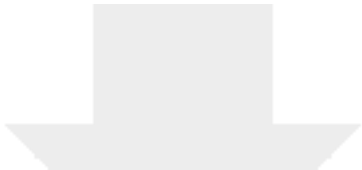
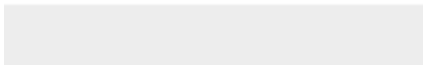



Figure 4



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Video or Animated Figure
Supplemental Video 1.mov



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Human Jurkat cells	ATCC	CRL-10915	Human T cell line. Any other cell type
pAcGFPm-N1 (PT3719-5)DNA3GFP	Clontech	632469	Different fluorescent proteins can be
Gene Pulse X Cell electroporator	BioRad		We use 280V, 975 μ F, for Jurkat cells.
Cytomics FC 500 flow cytometer	Beckman Coulter		
MoFlo Astrios Cell Sorter	Beckman Coulter		Depending on the level of transfection
Dako Qifikit	DakoCytomation	K0078	Used for quantification the number o
Glass bottom microwell dishes	MatTek corporation	P35G-1.5-10-C	
Human Fibronectin from plasma	Sigma-Aldrich	F0895	
Recombinant human CXCL12	PeproTech	300928A	
Inverted Leica AM TIRF	Leica		
EM-CCD camera	Andor	DU 885-CSO-#10-VP	
MATLAB	The MathWorks, Natick, MA		
U-Track2 software	Danuser Laboratory		
ImageJ	NIH		https://imagej.nih.gov/ij/
Fiji	Fiji		https://imagej.net/Fiji/
u-Track2 software			Matlab tool. For installing, download
GraphPad Prism	GraphPad software		

can be analyzed with this software

followed and analyzed with this routine

Use the transfection method best working in your hands.

n, cell sorting may not be required. You can also employ cells with stable expression of adequate levels of the receptor of interest.
f receptors in the cell surface.

.zip file from the web page (<http://lccb.hms.harvard.edu/software.html>) and uncompress the file in a directory of your choice

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Author(s):

C.O.S. Sorzano, L. Martínez-Muñoz, G. Cascio, E.M. García-Cuesta, J. Vargas, M. Mellado, J.M. Rodríguez-Frade

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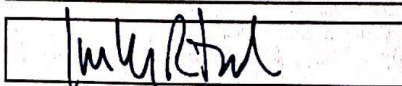
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Dear Editor,

Following your instructions, we have carefully revised the updated manuscript, 59314_R1.docx "Image Processing Protocol for the Analysis of the diffusion and cluster size of membrane receptors by fluorescence microscopy," to address all editorial comments.. All changes have been tracked in the revised version of the manuscript.

We hope that this revised version of the manuscript is appropriate for publication in JoVE
All the best

José Miguel Rodríguez Frade

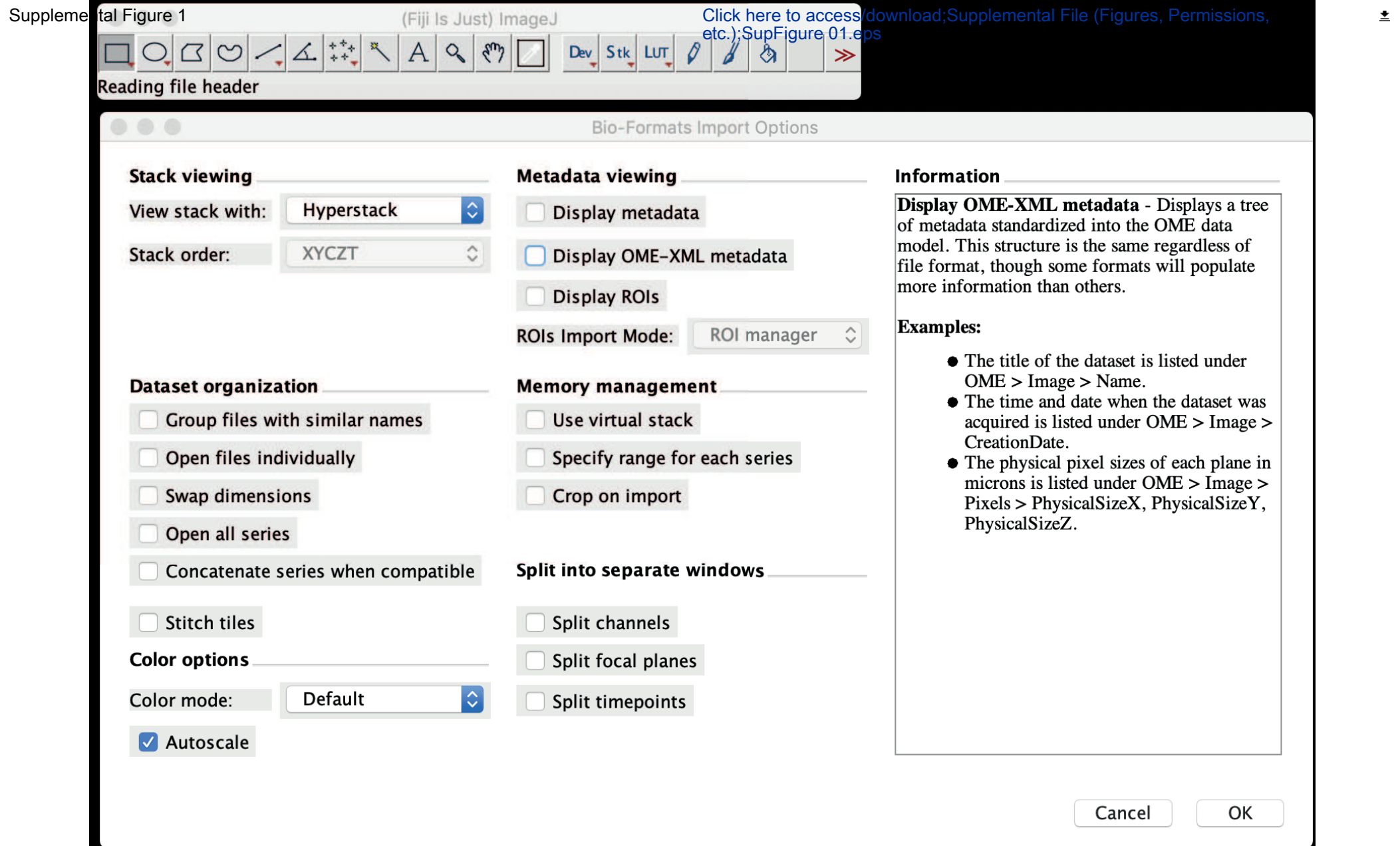
Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

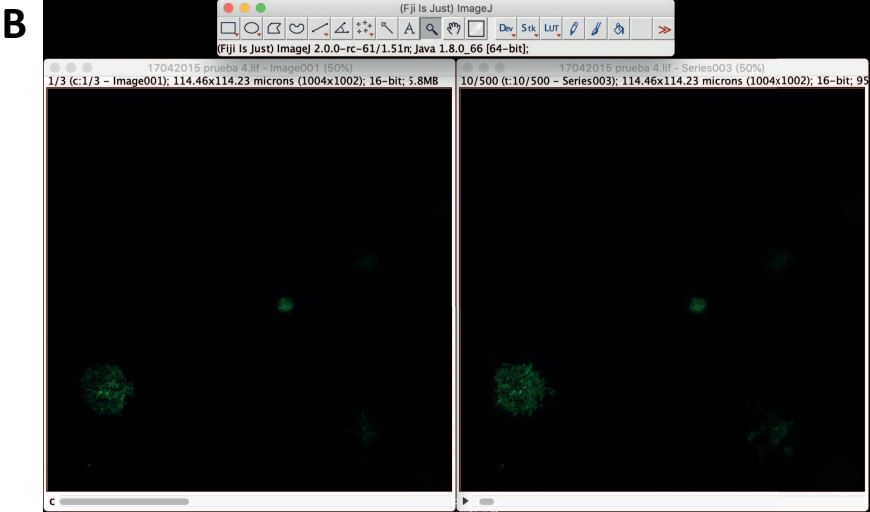
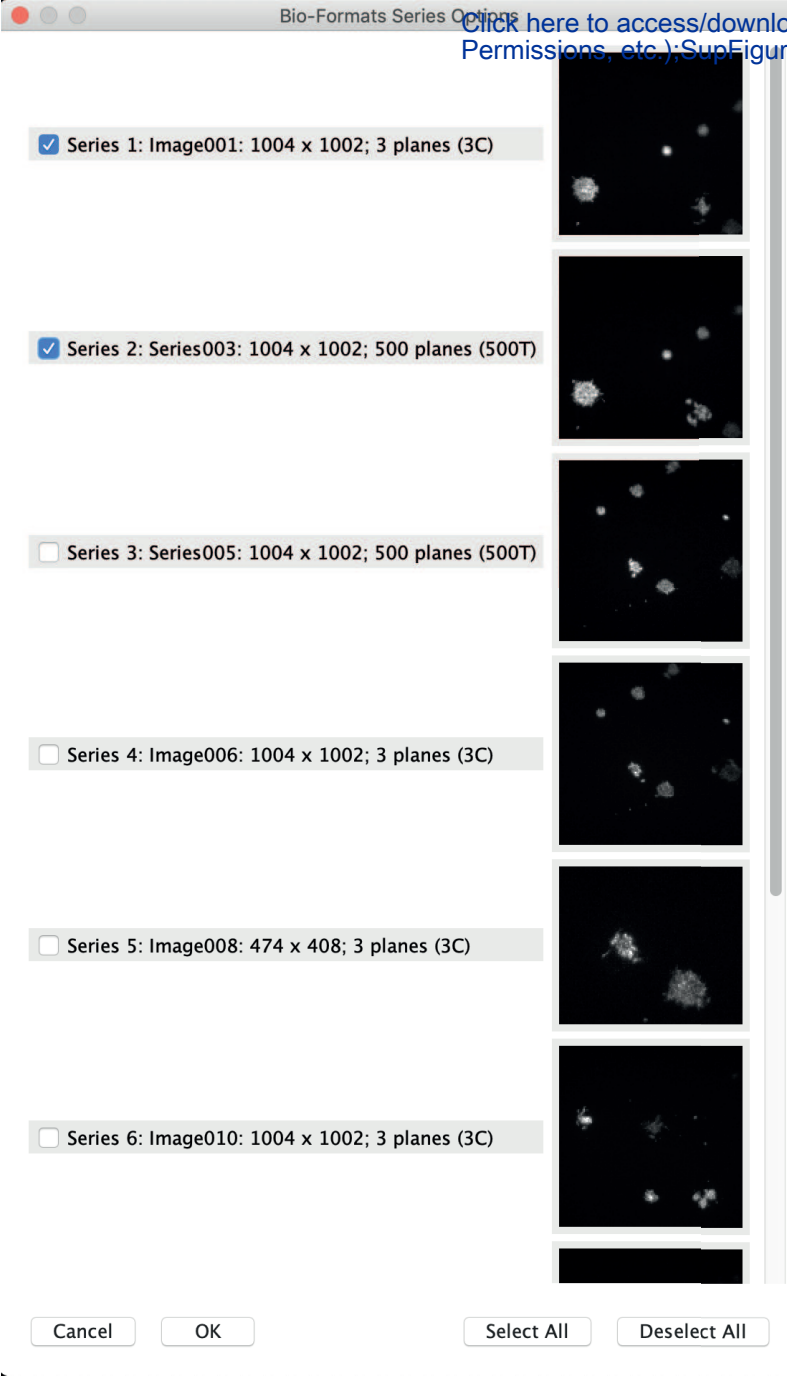
We have thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The spelling or grammar issues that may remain in the manuscript correspond to Matlab scripts and arguments that have to be written exactly as included in the manuscript

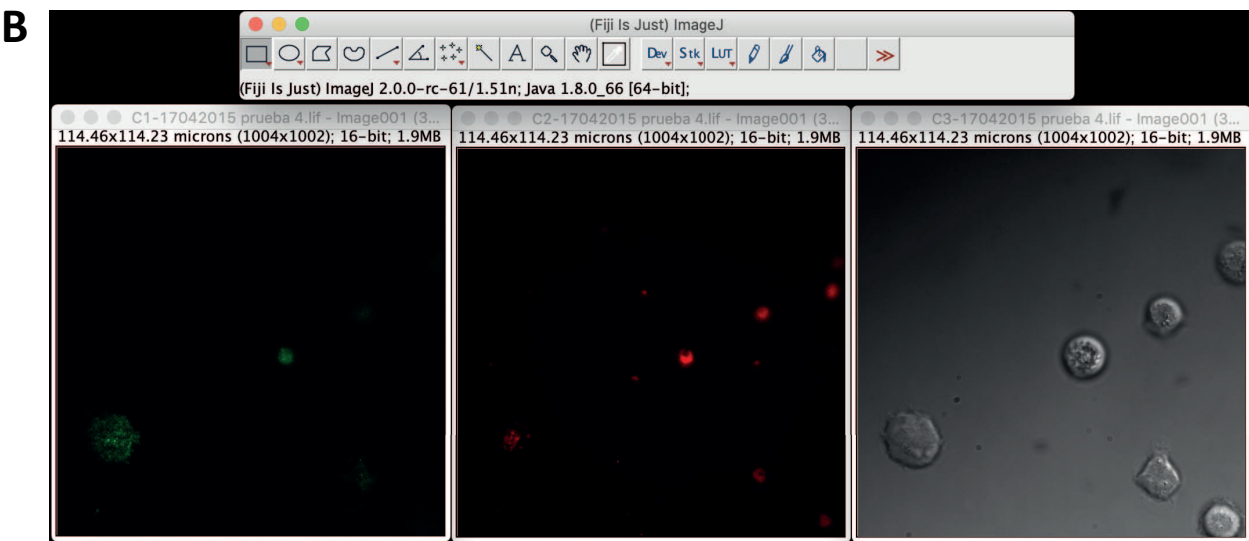
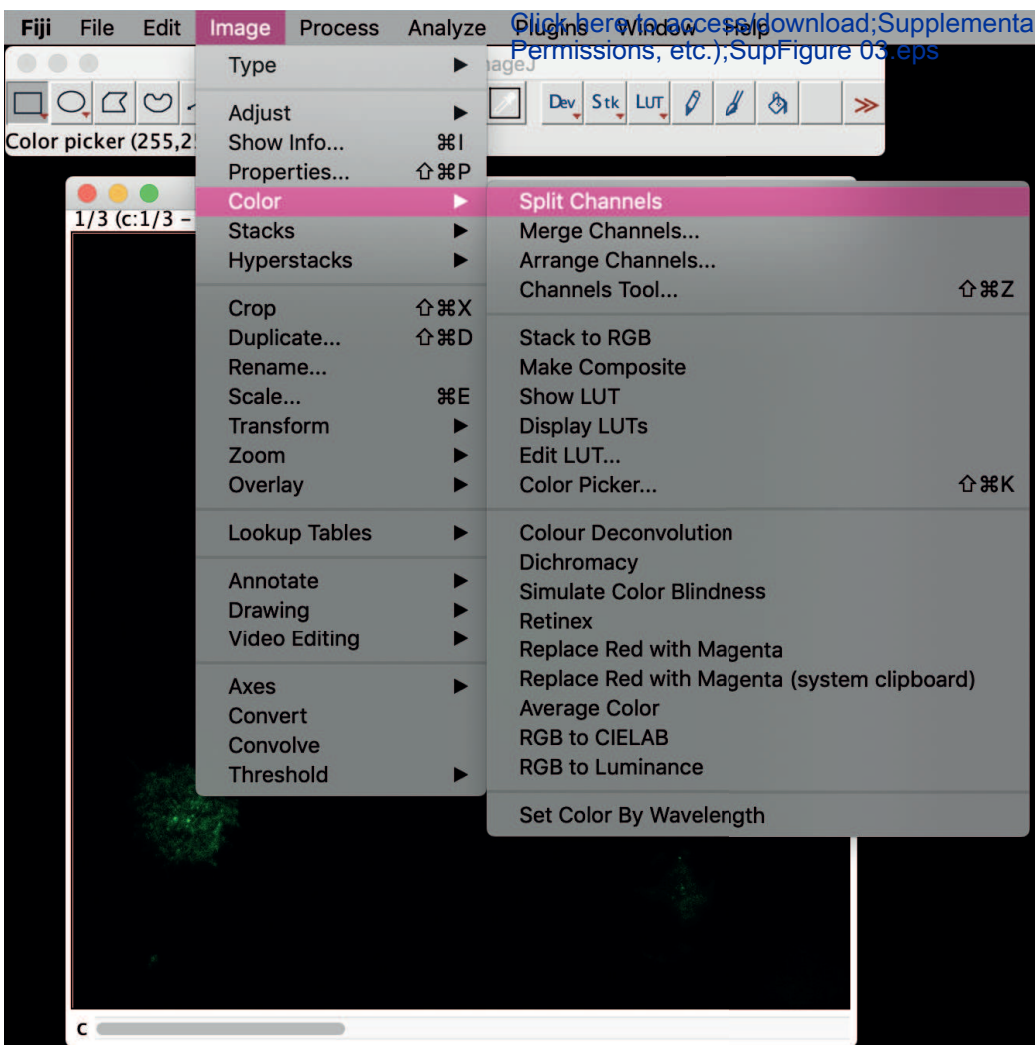
2. Not only the highlighted protocol steps must be written in the imperative tense, but also all other protocol steps. So, please rewrite steps 5.1.1-5.1.8

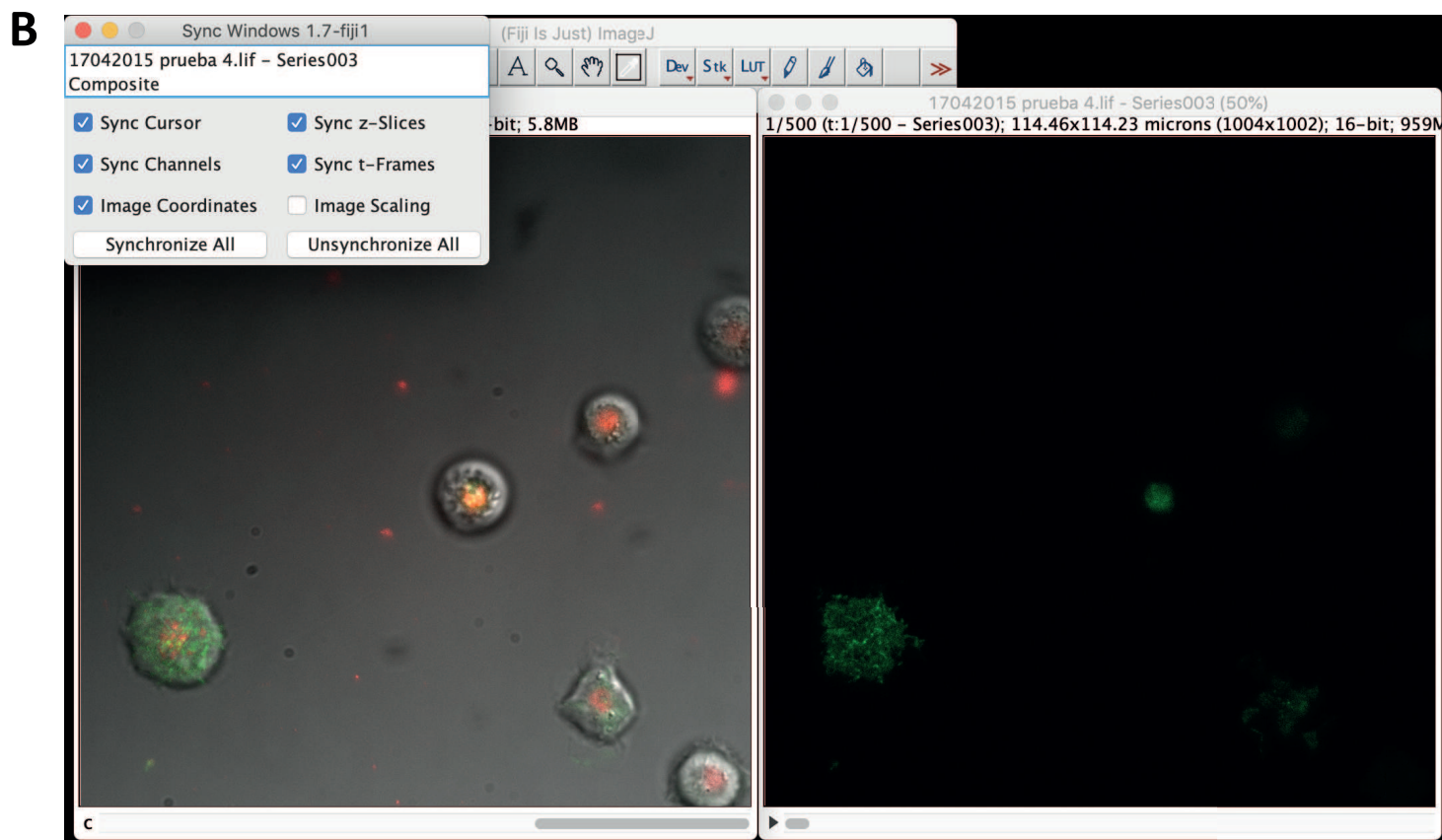
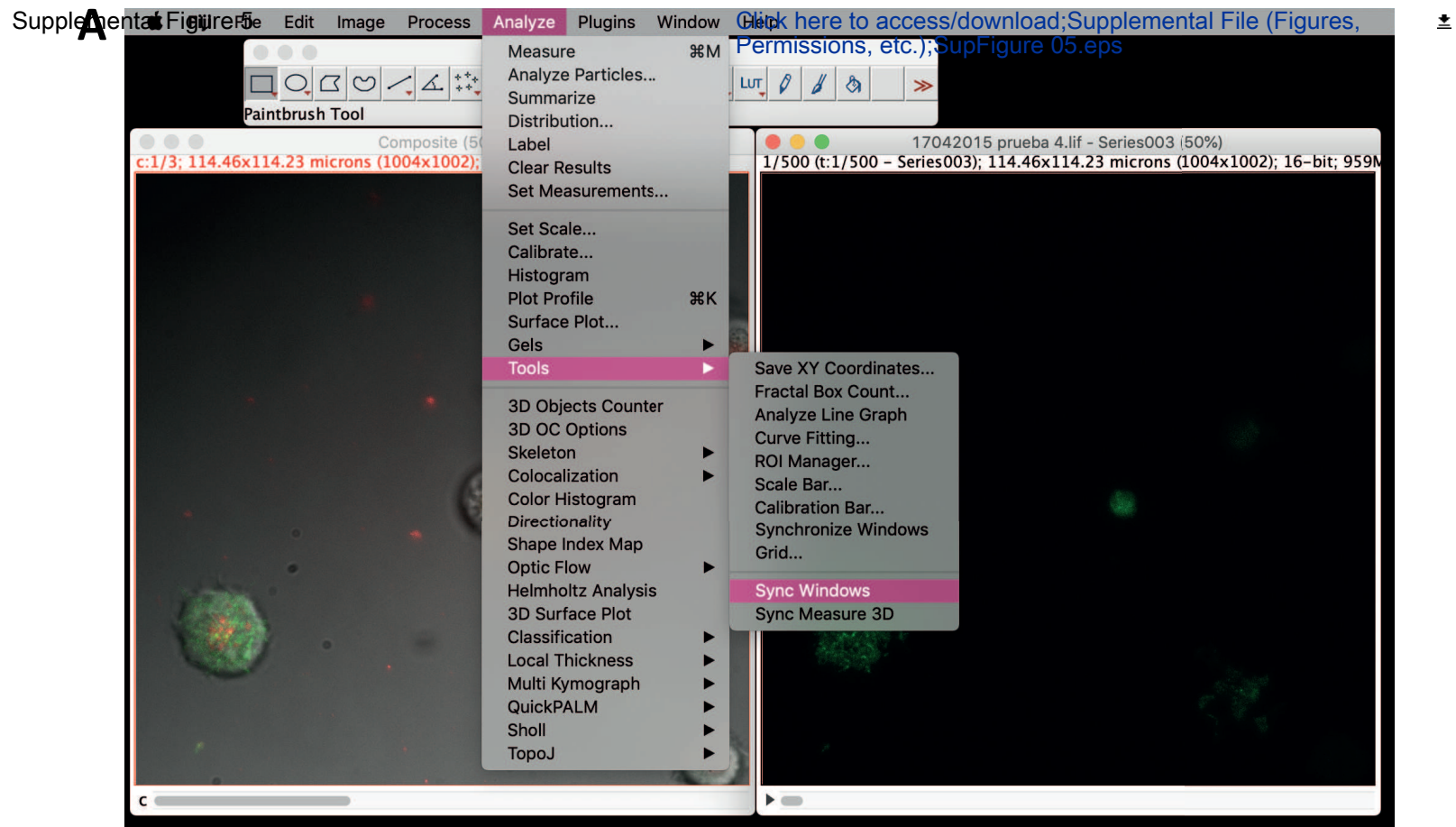
Steps 5.1.1-5.1.8 have been rewritten according to the editorial comments.



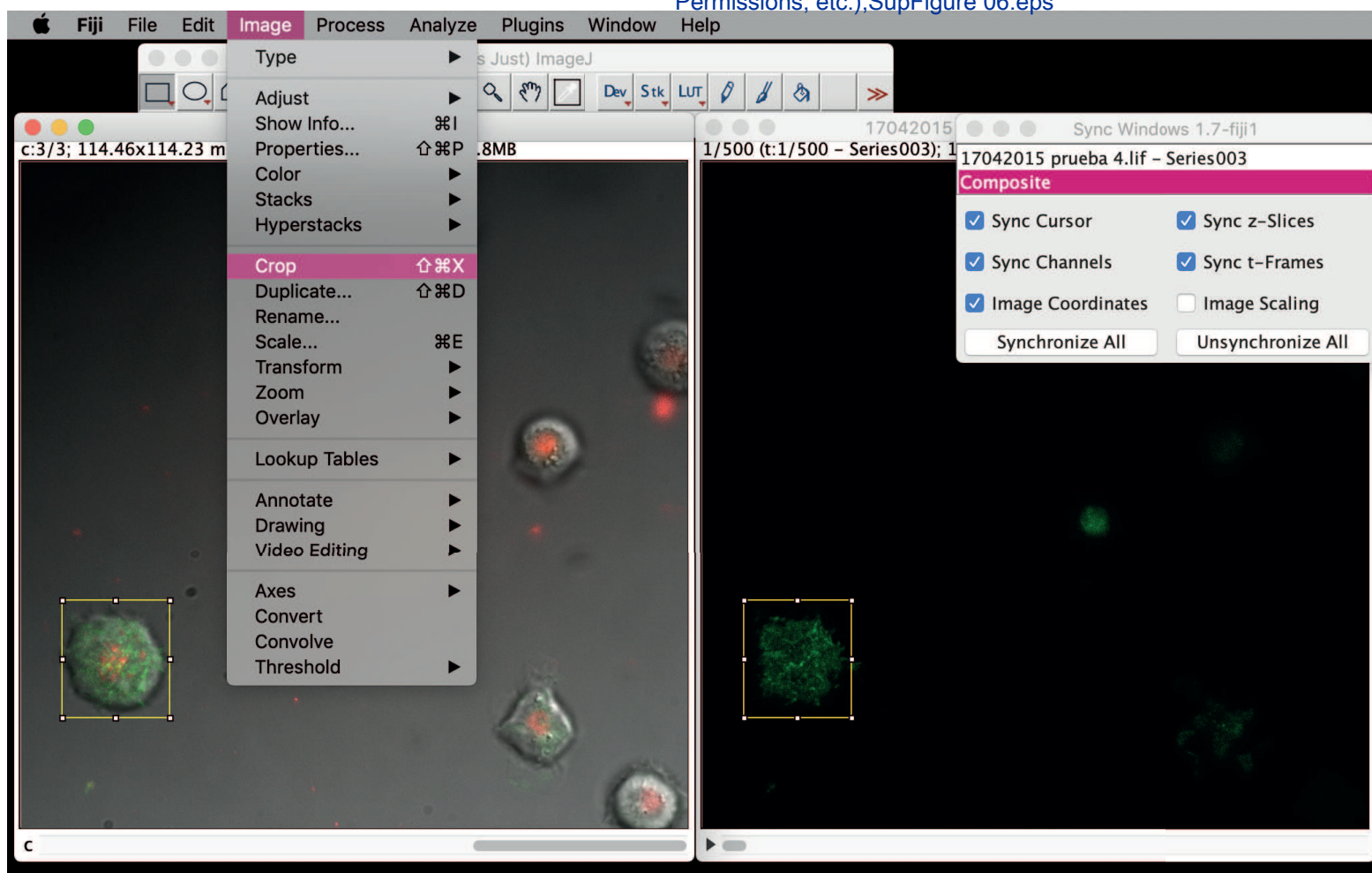
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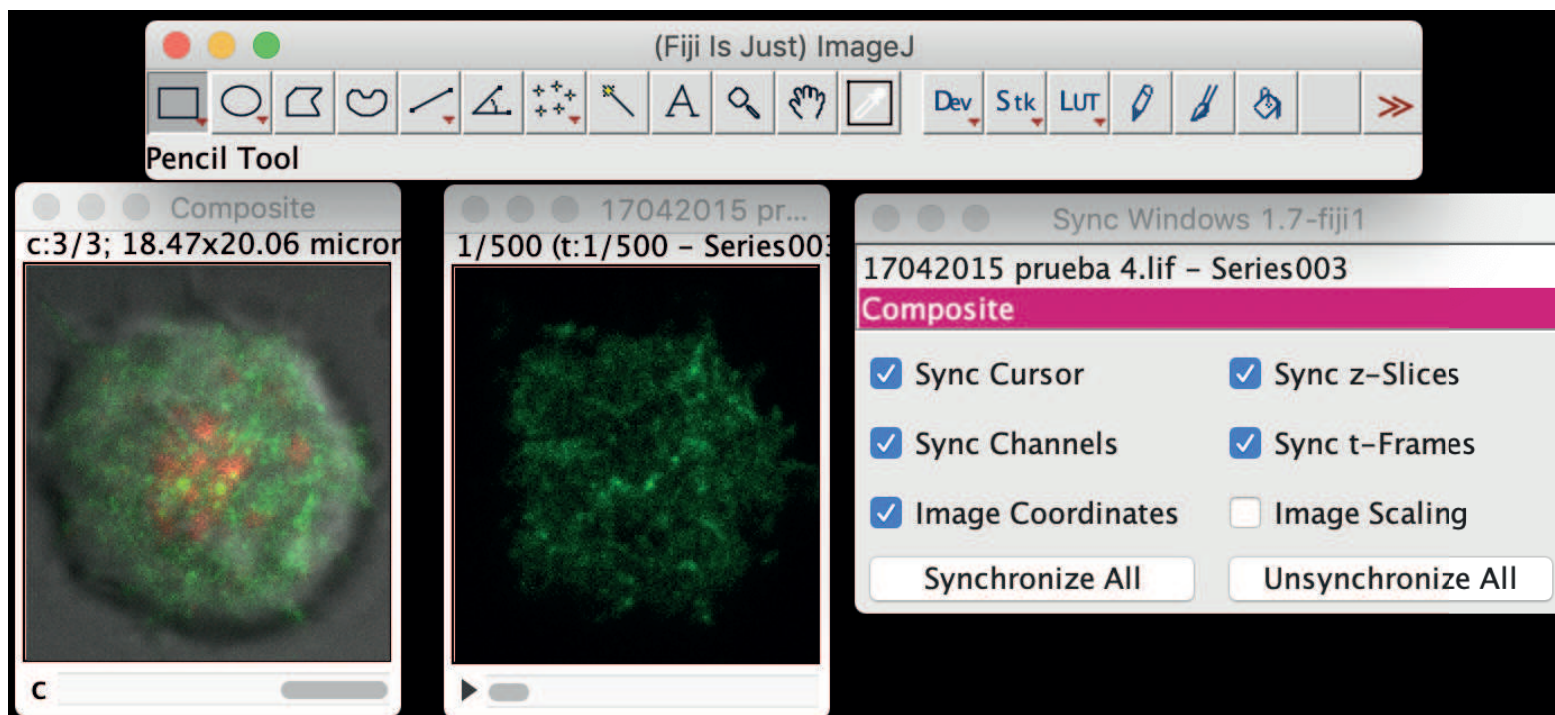


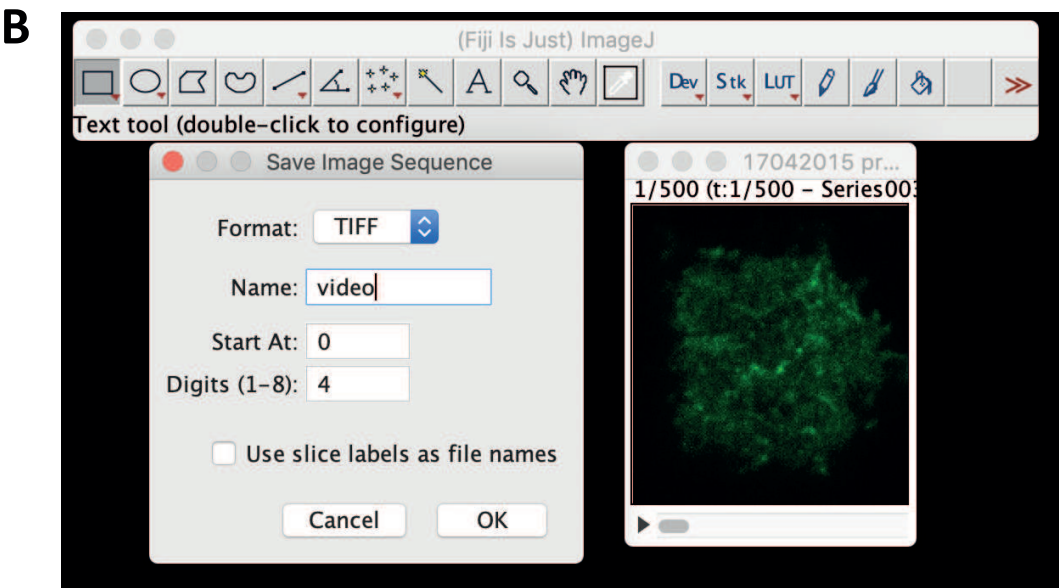
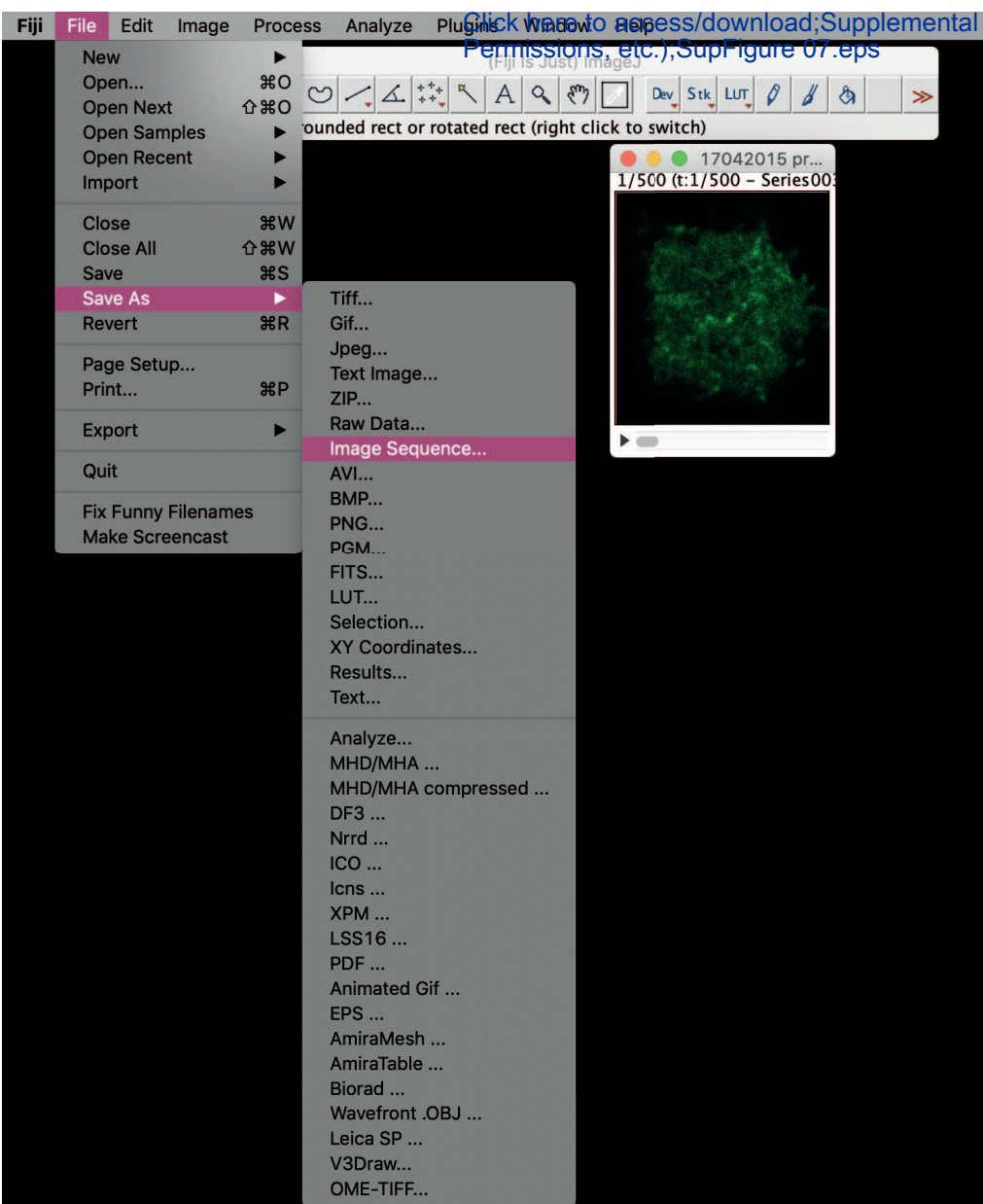


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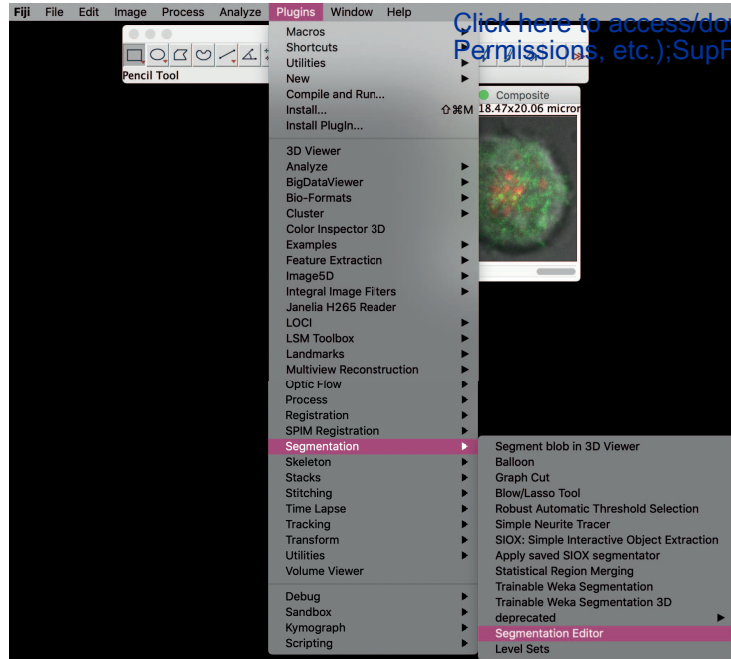


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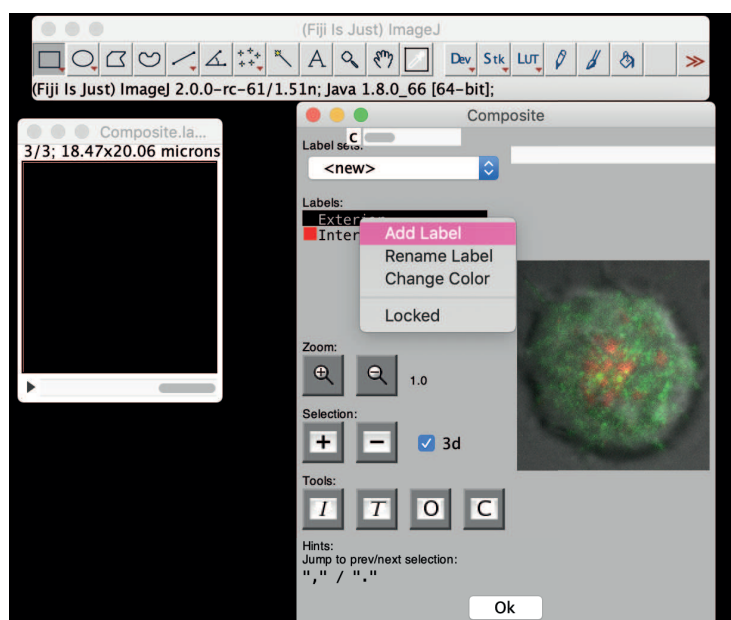


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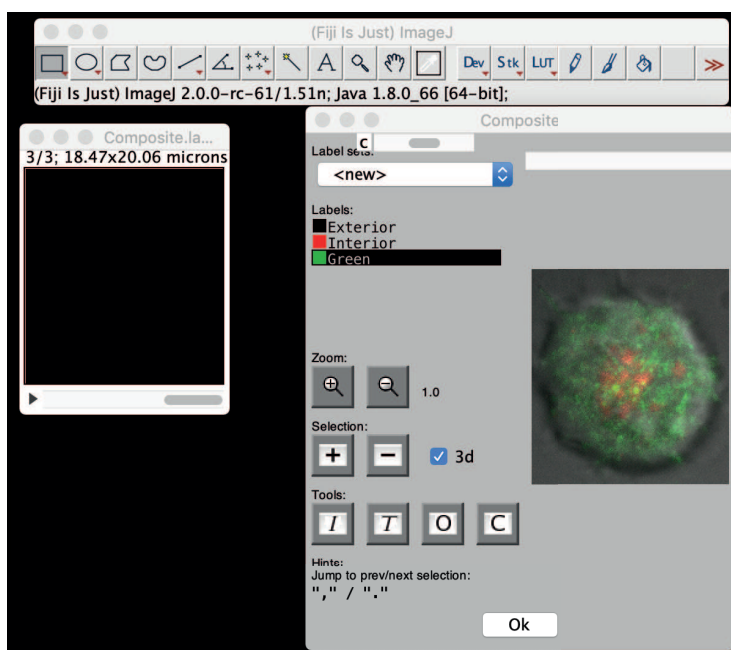


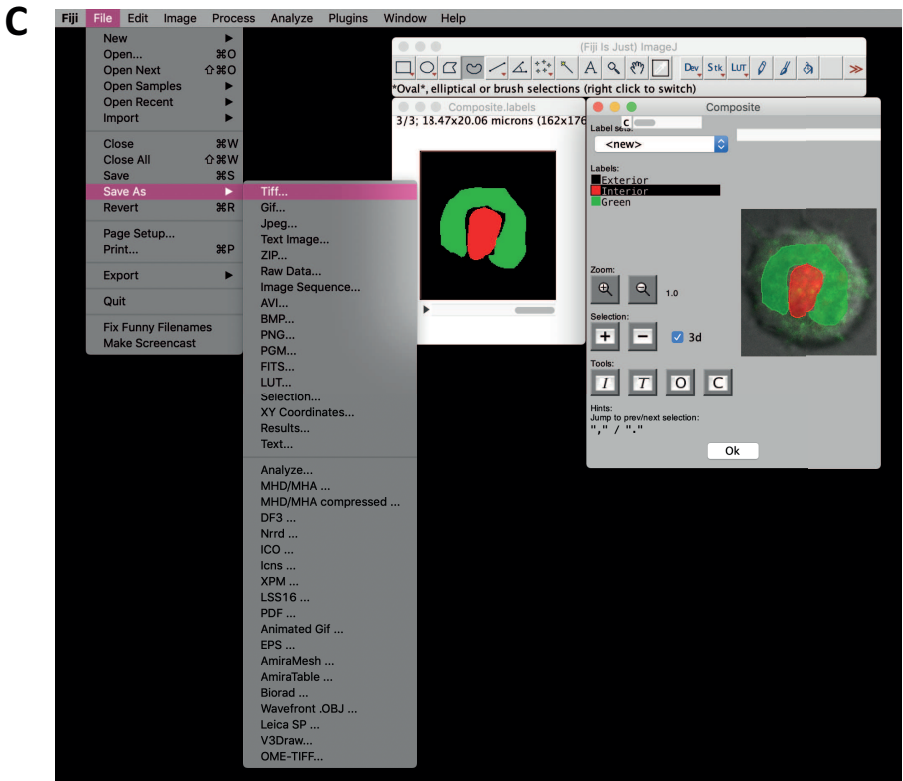
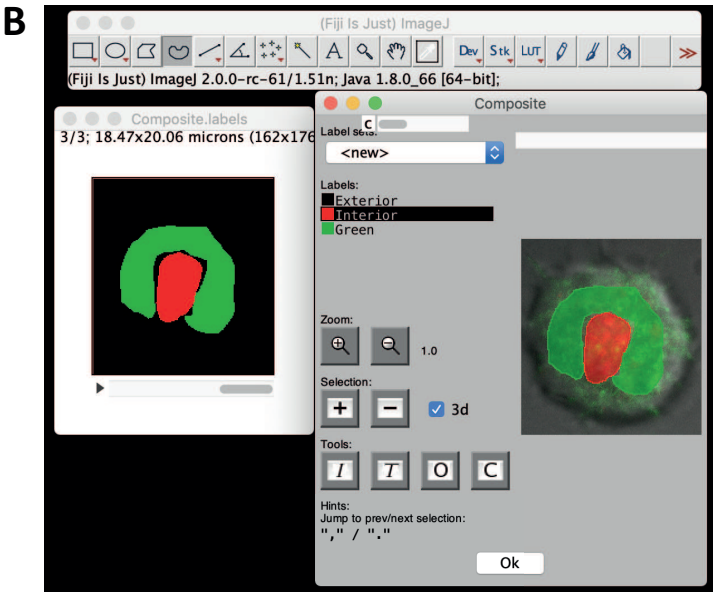
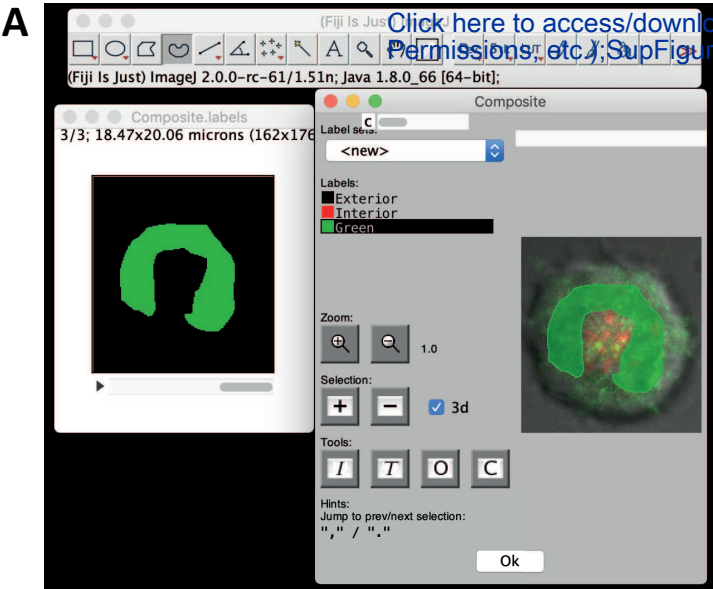
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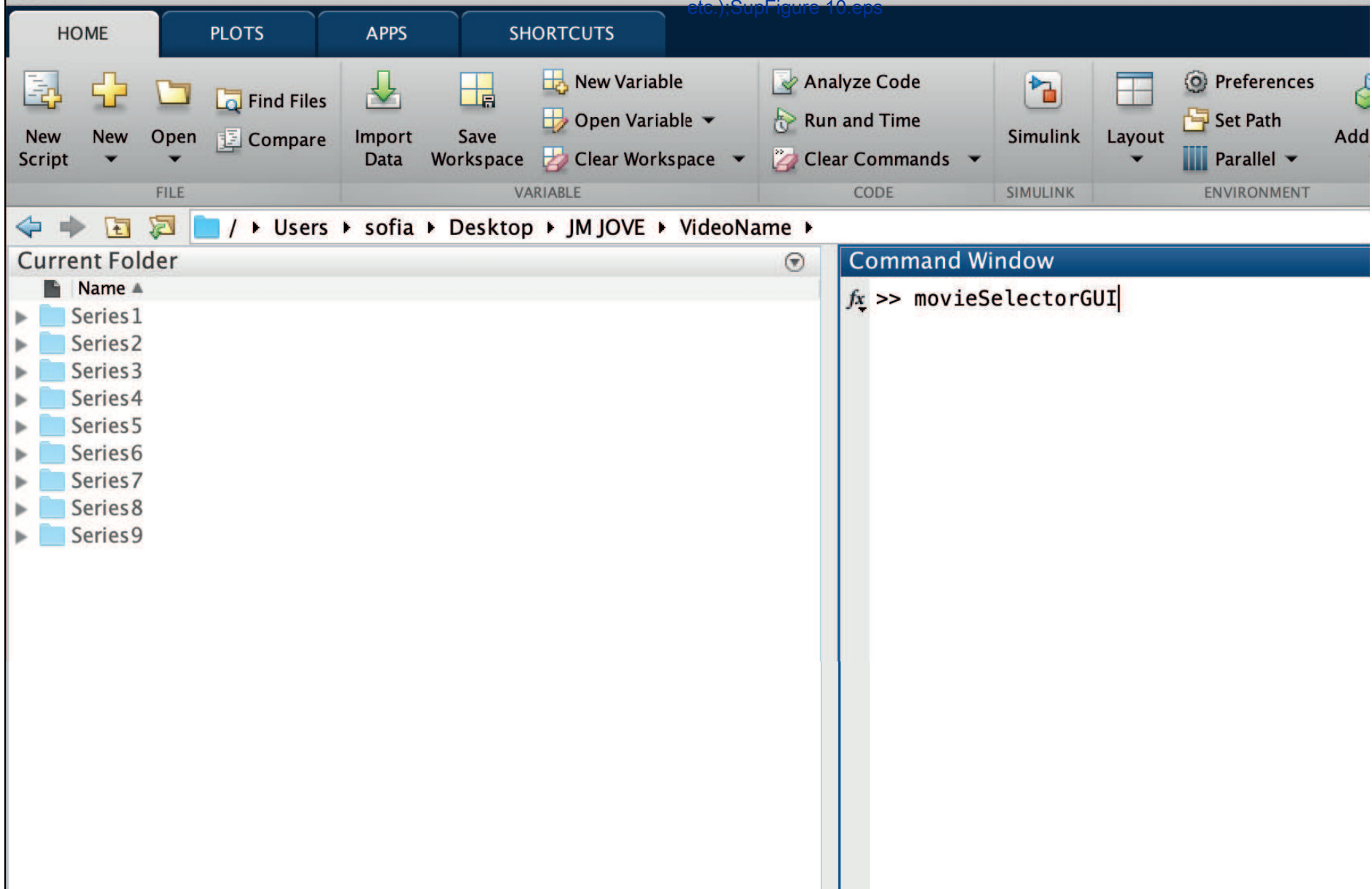
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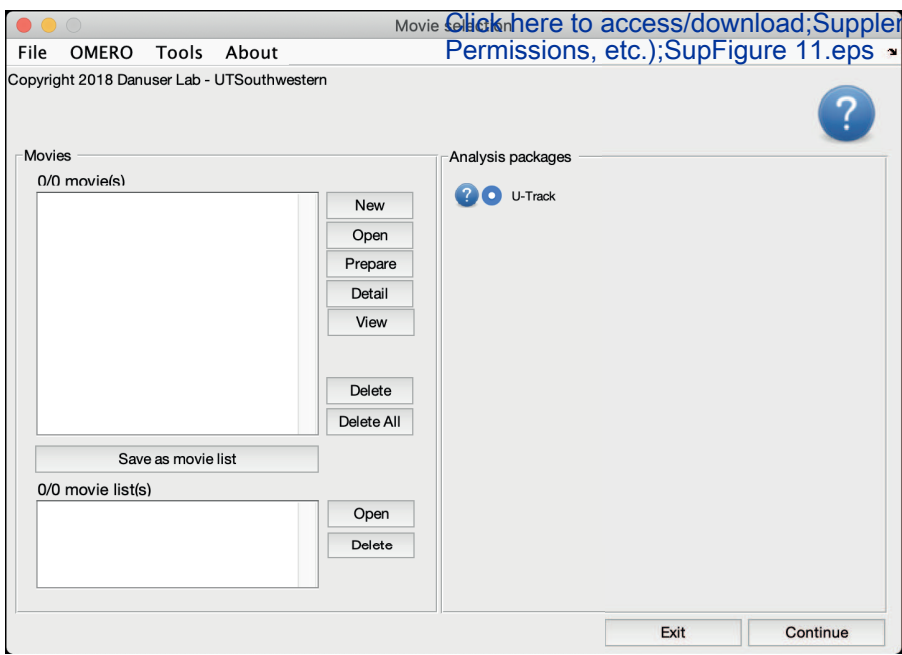


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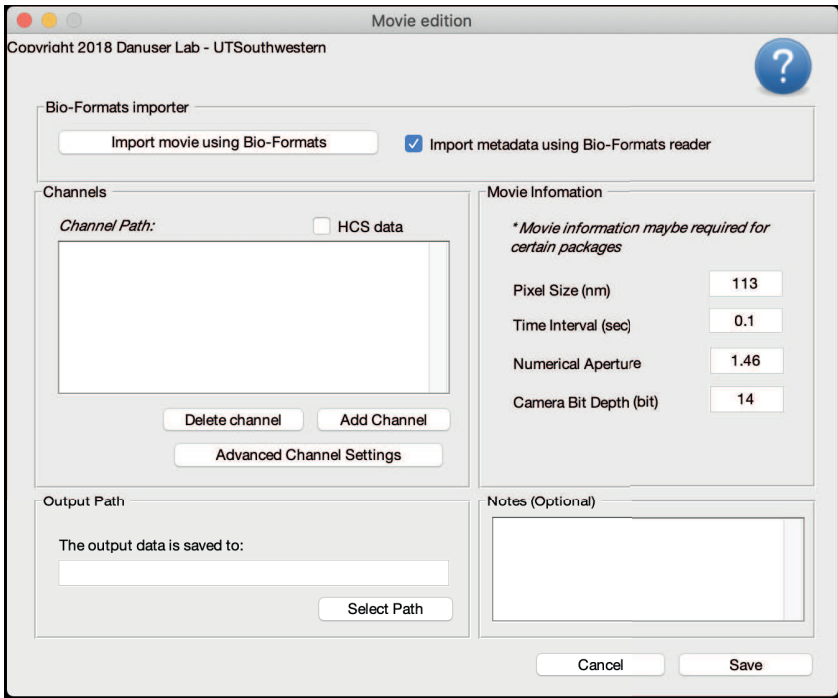




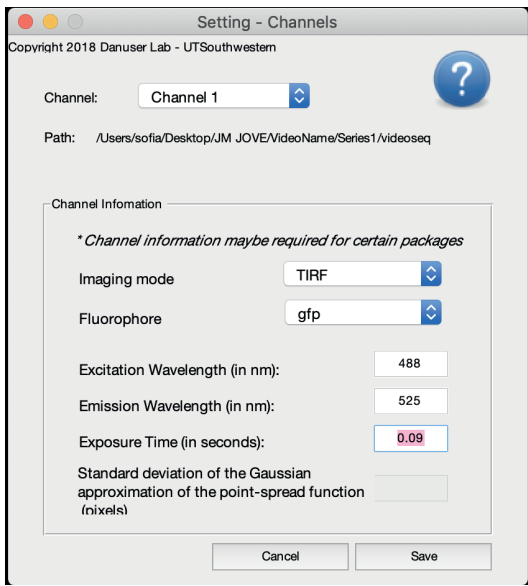




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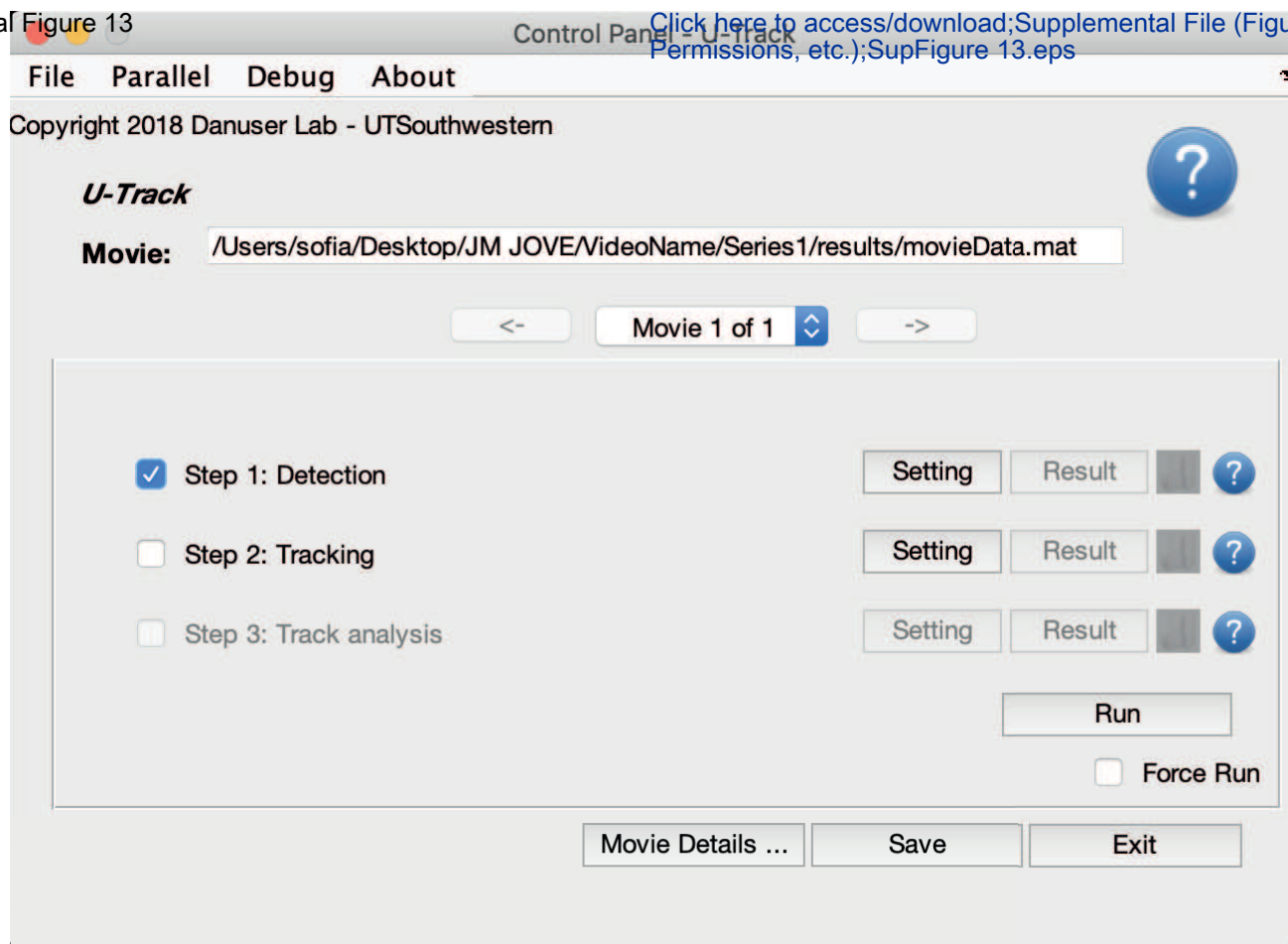


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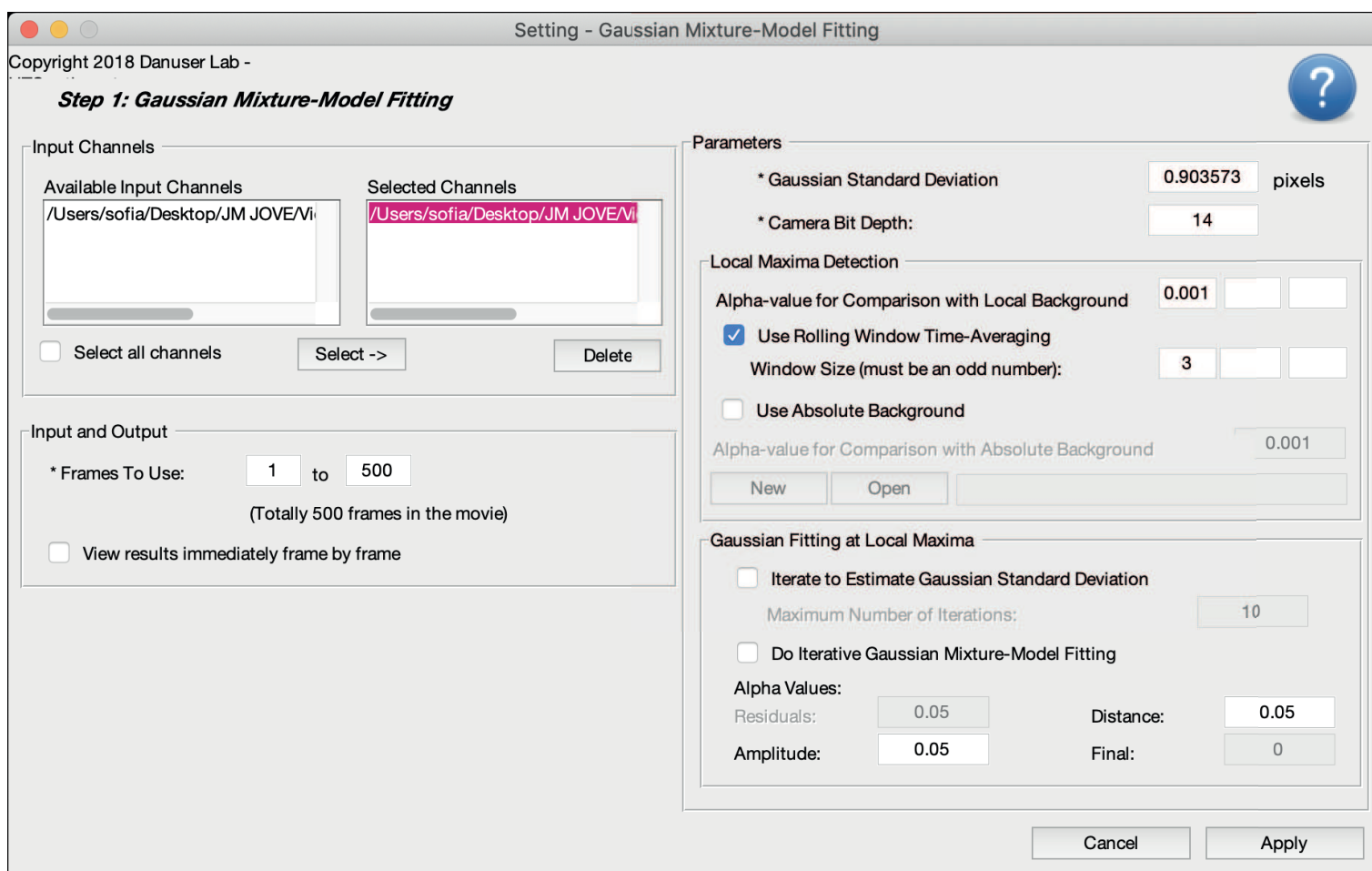


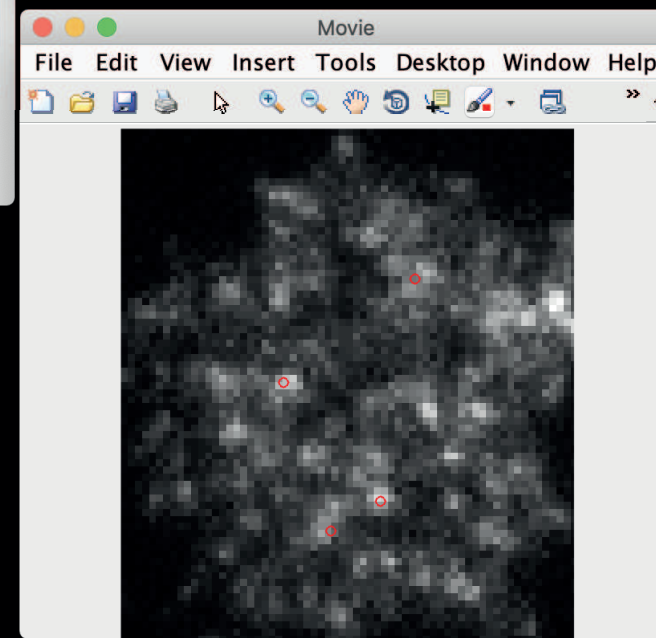
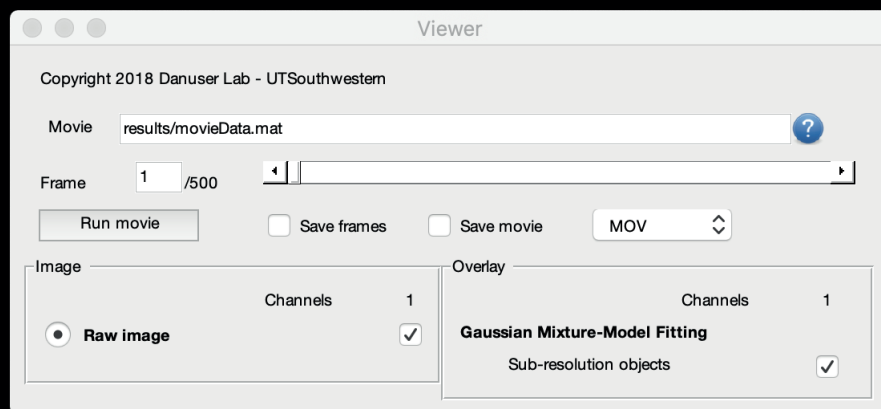
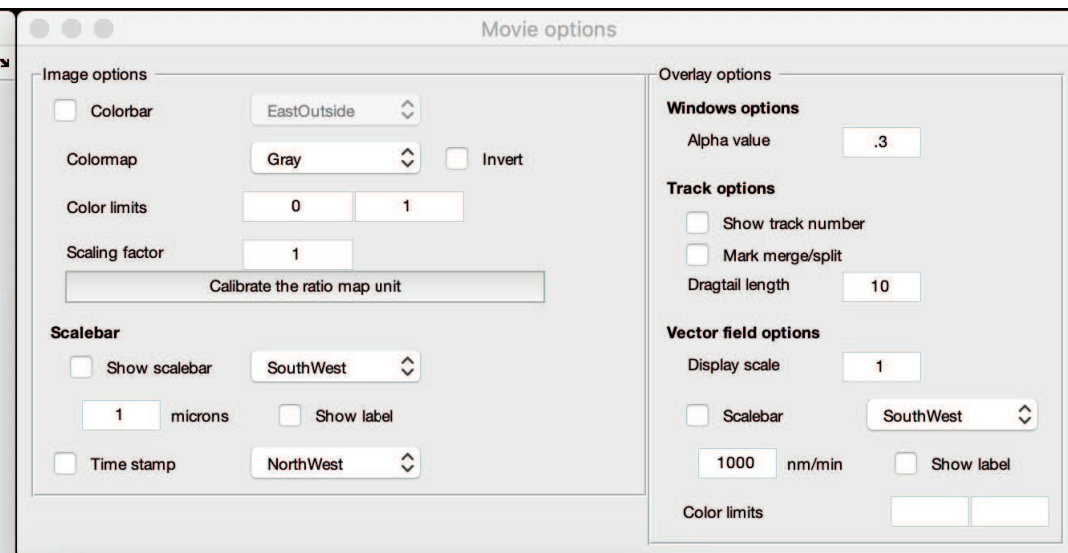
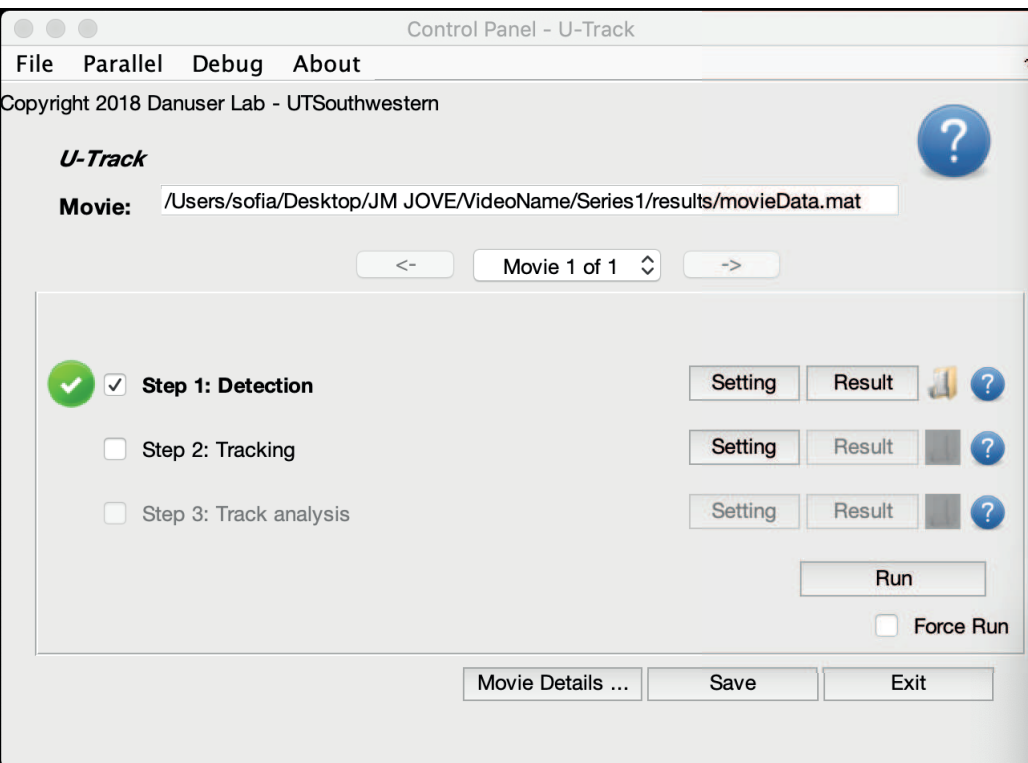


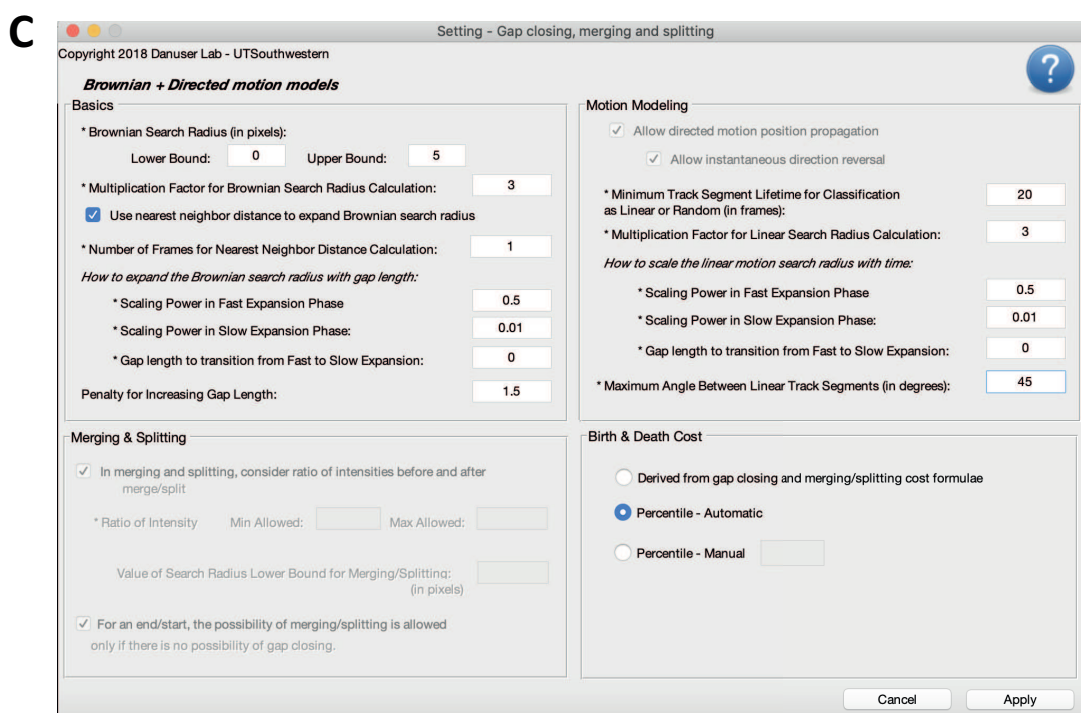
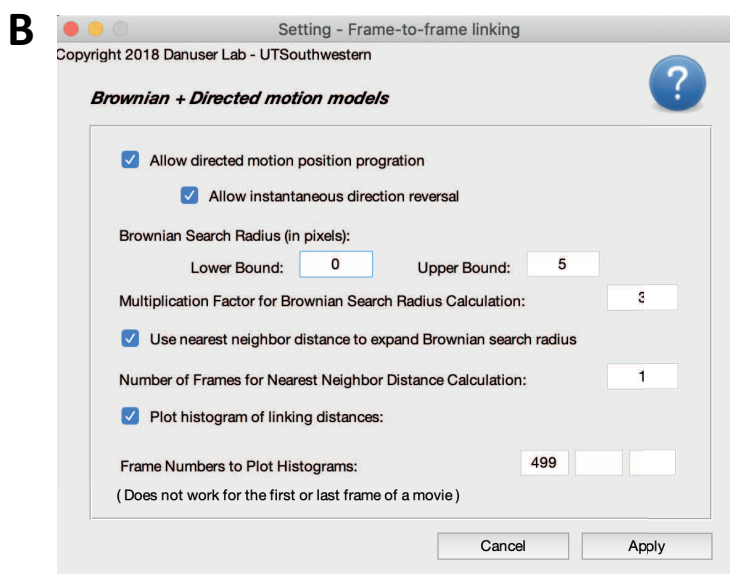
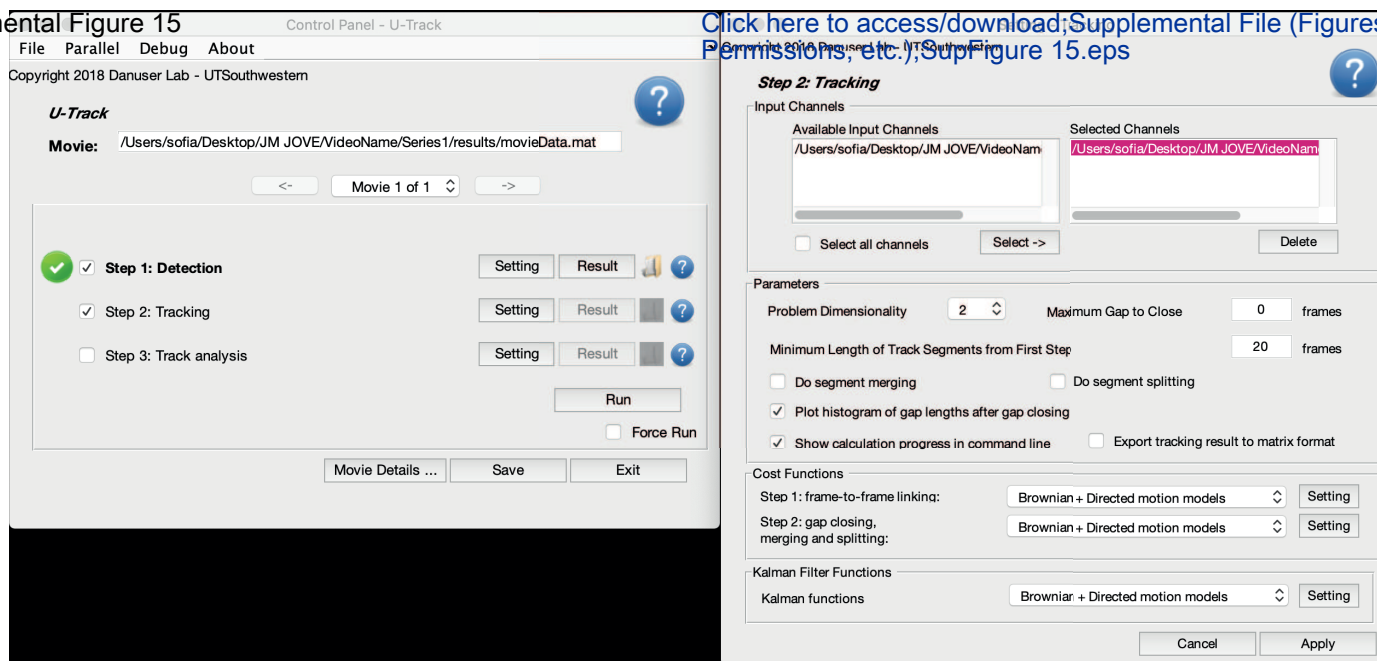
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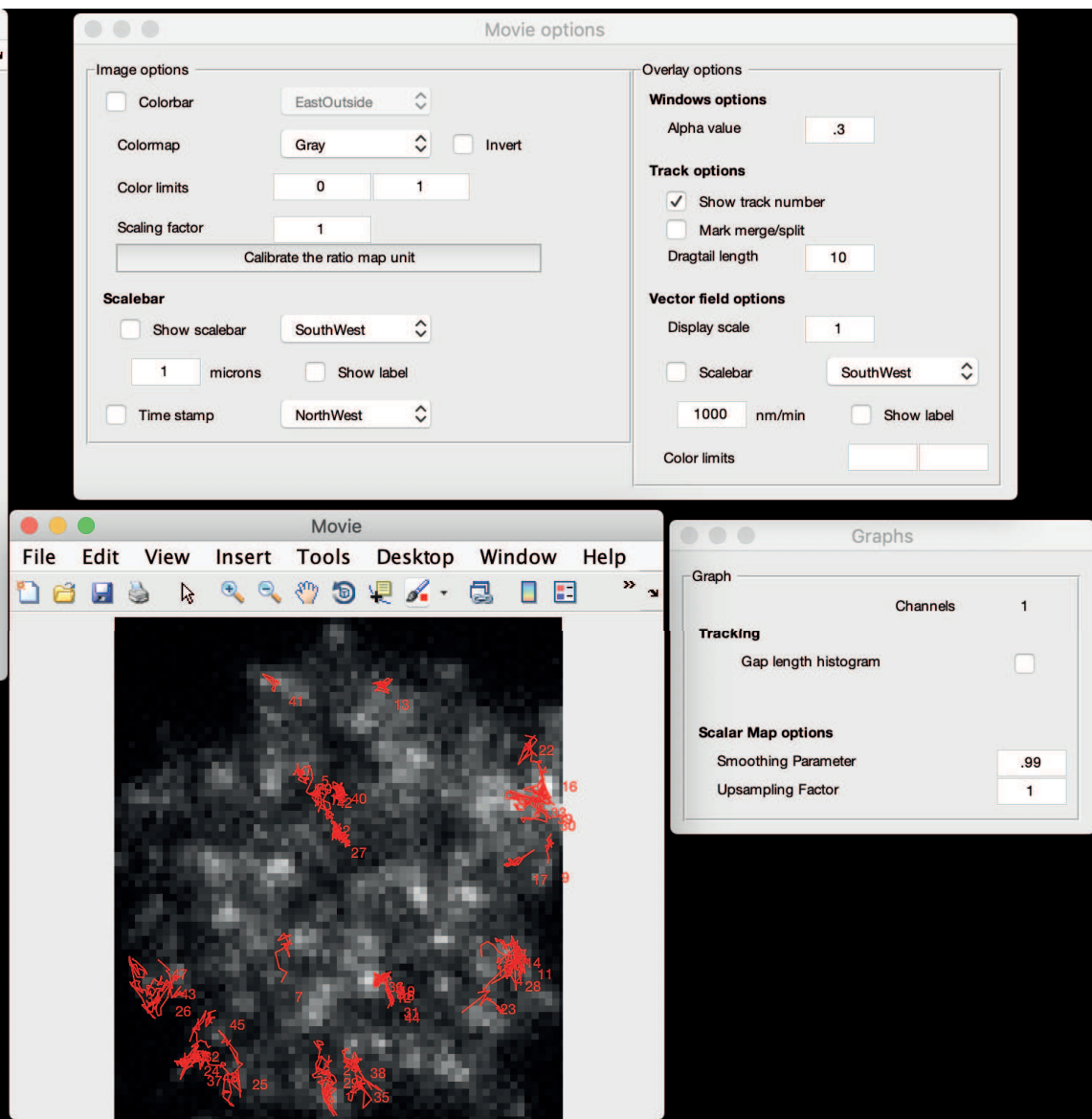
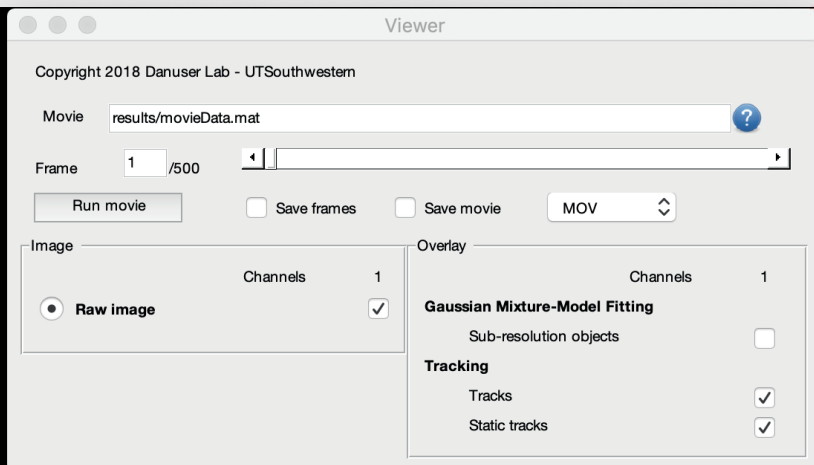
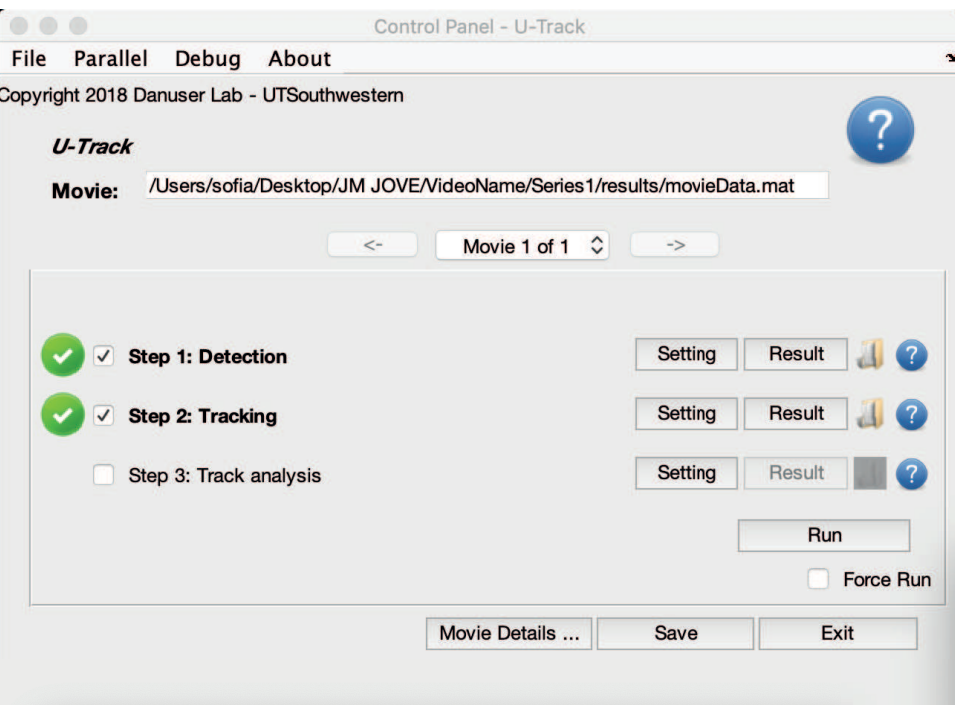


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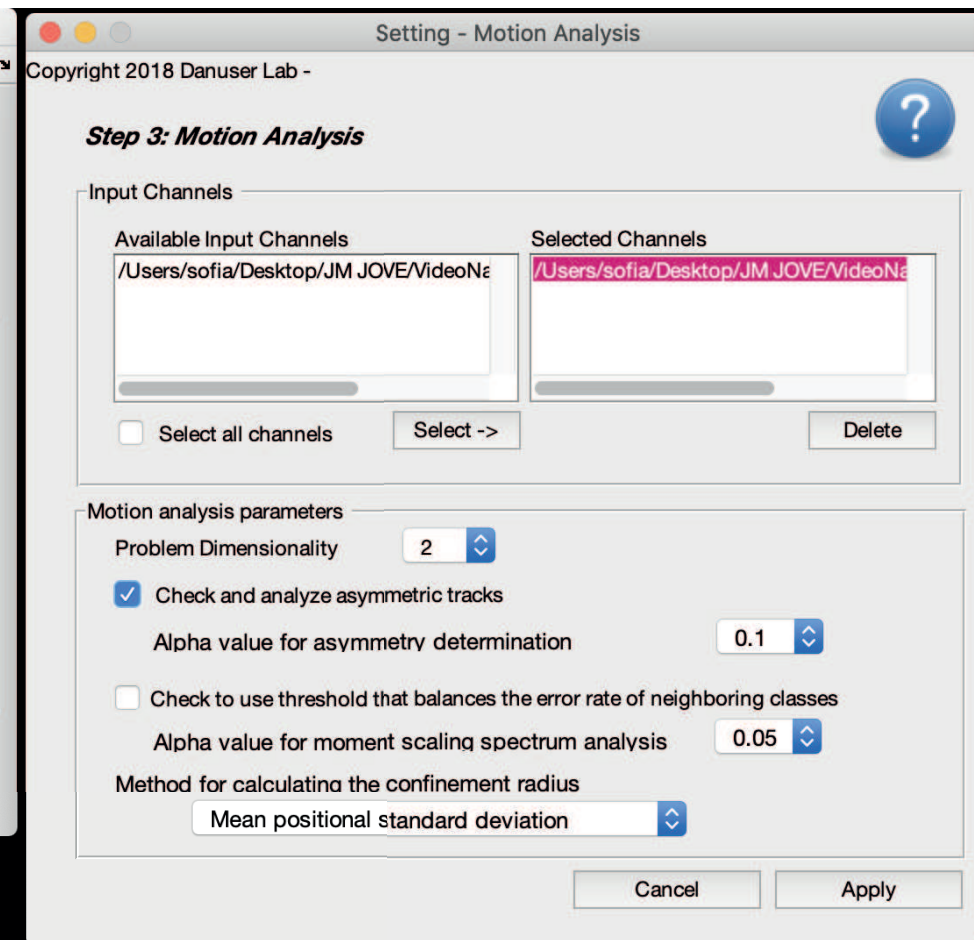
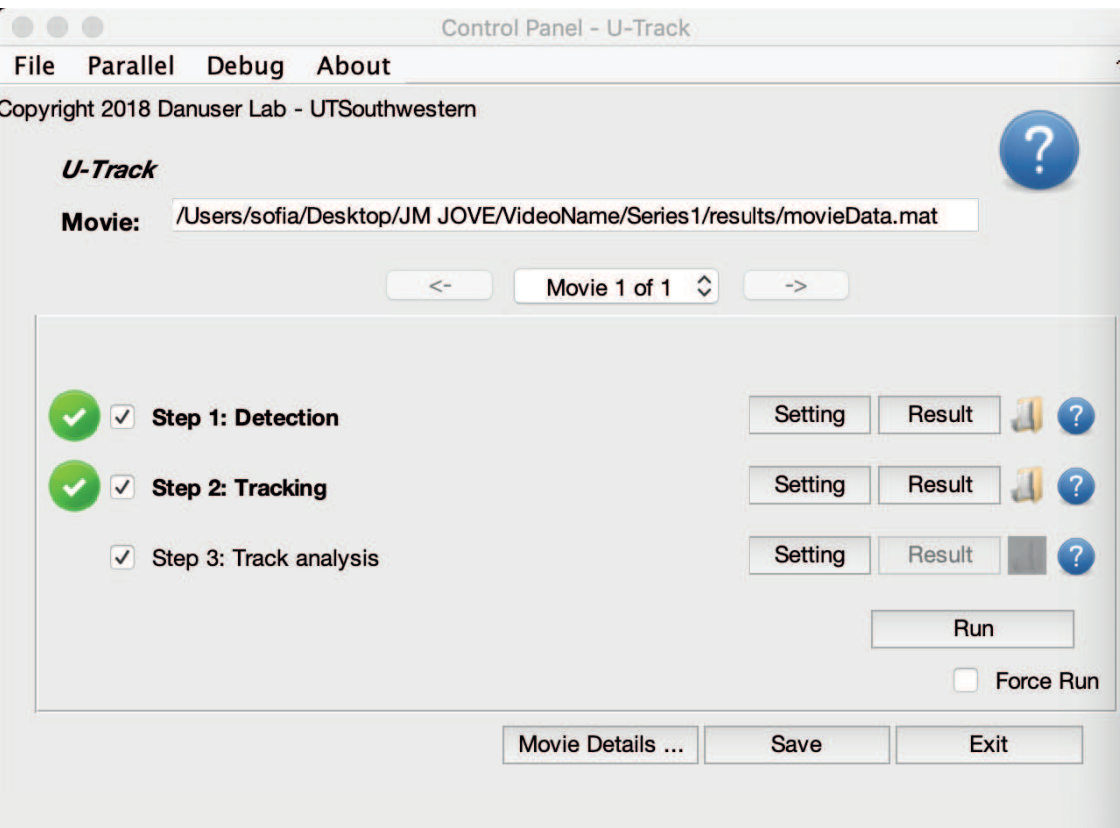




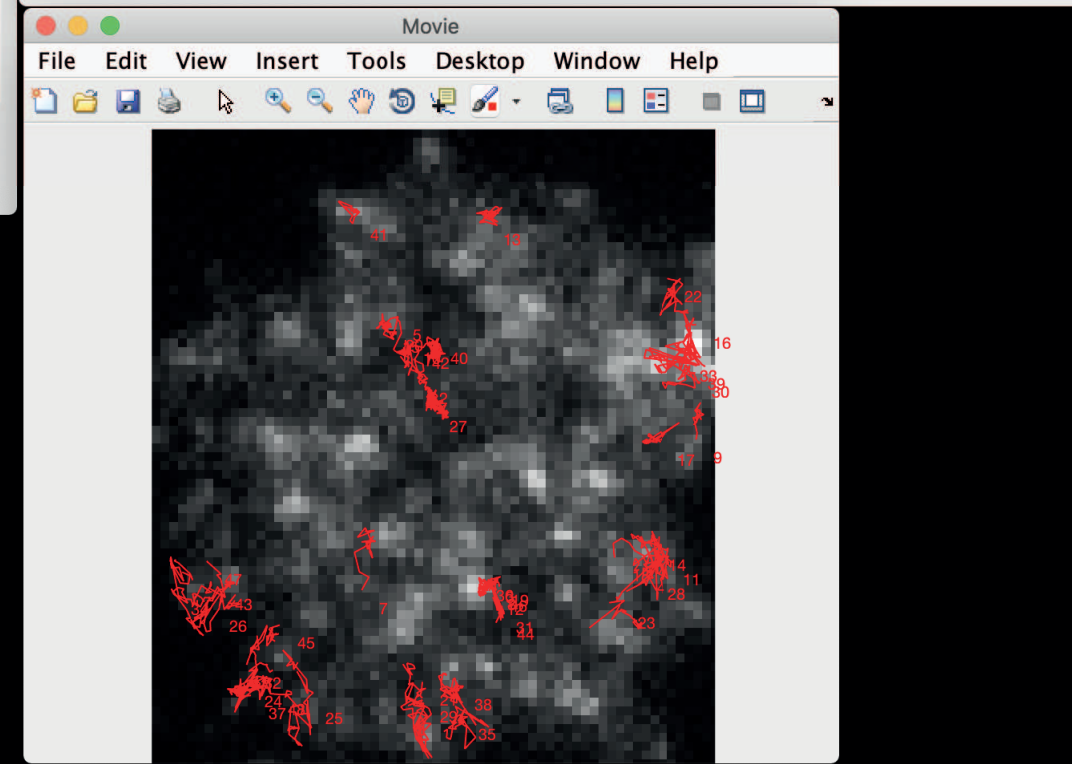
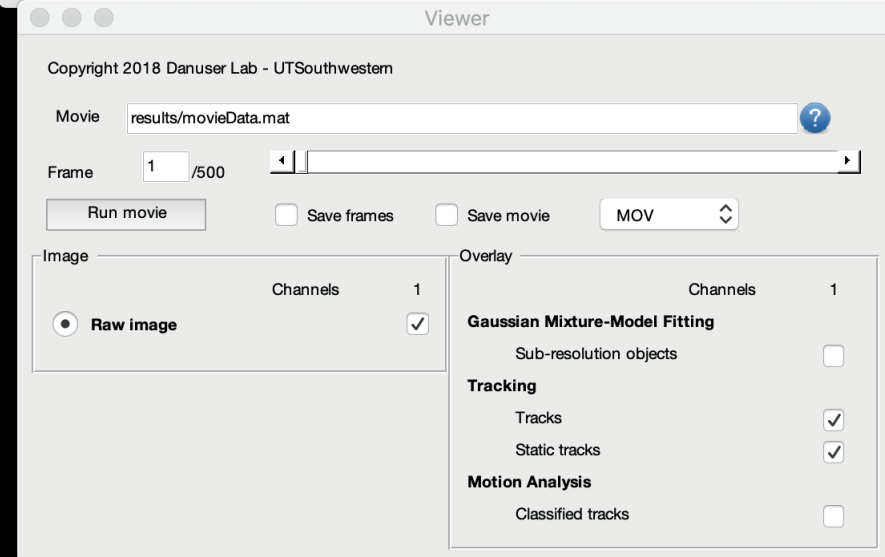
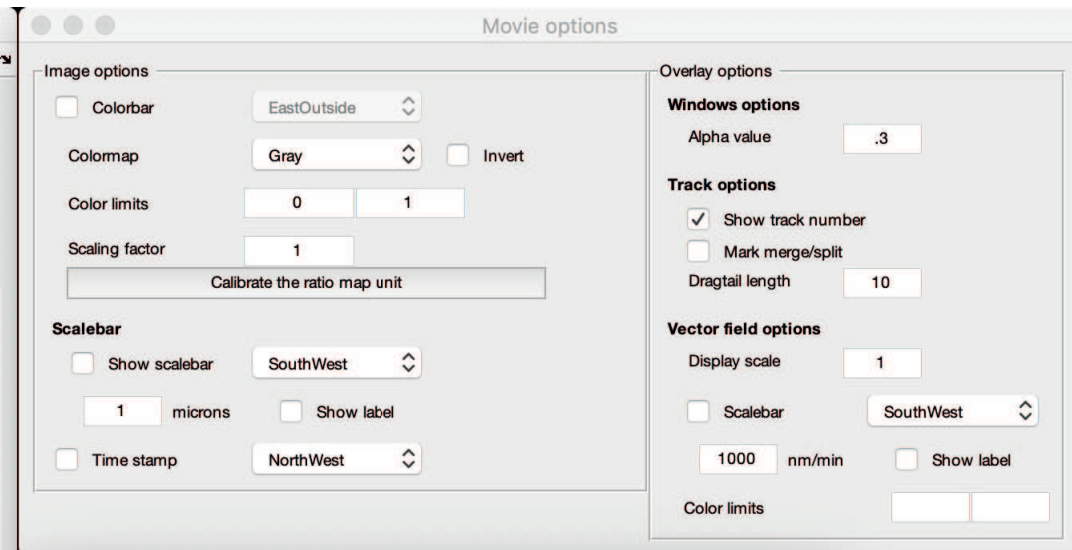
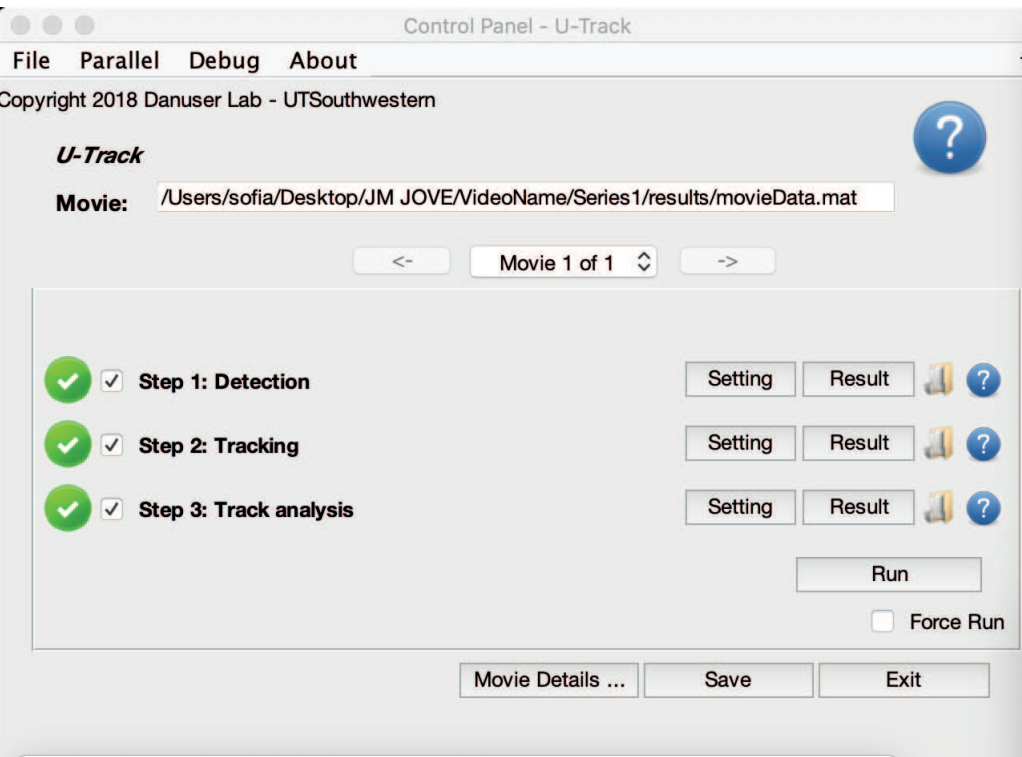


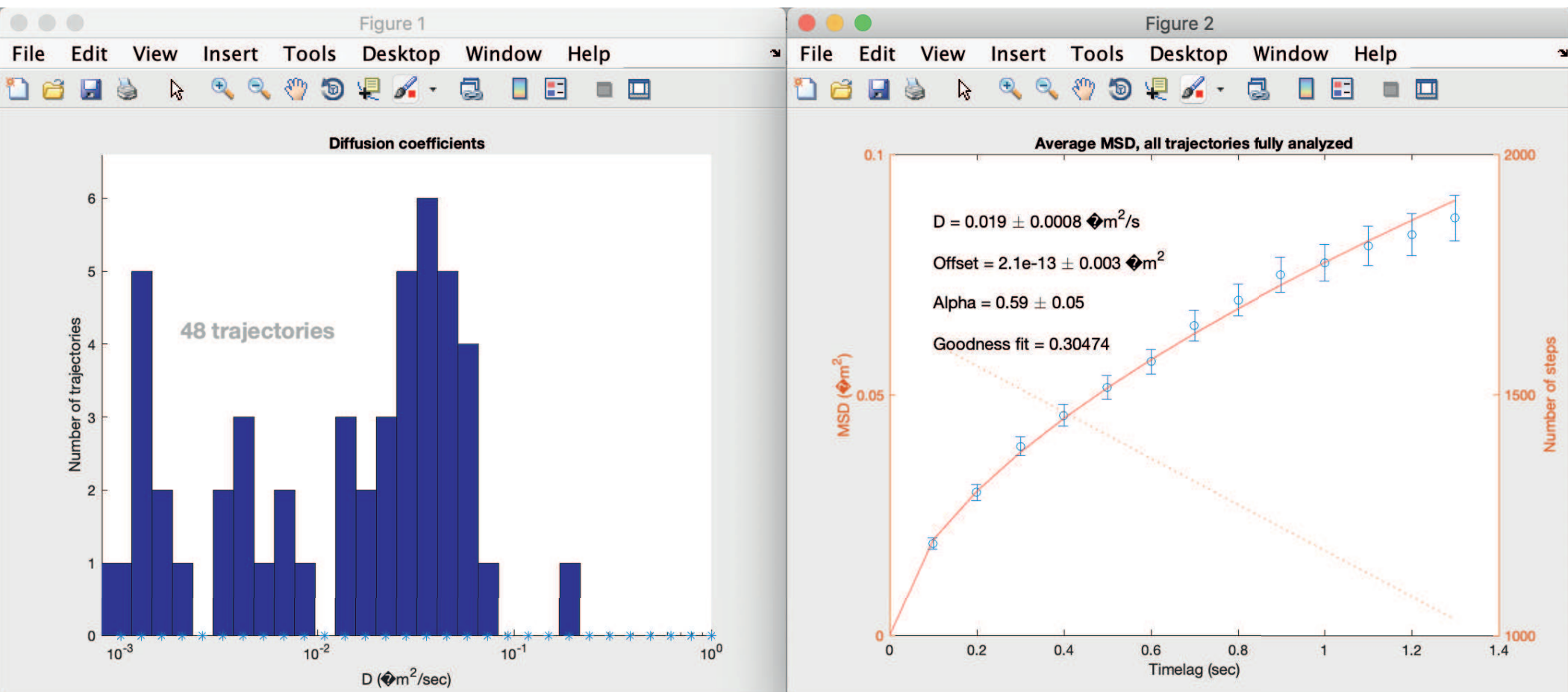


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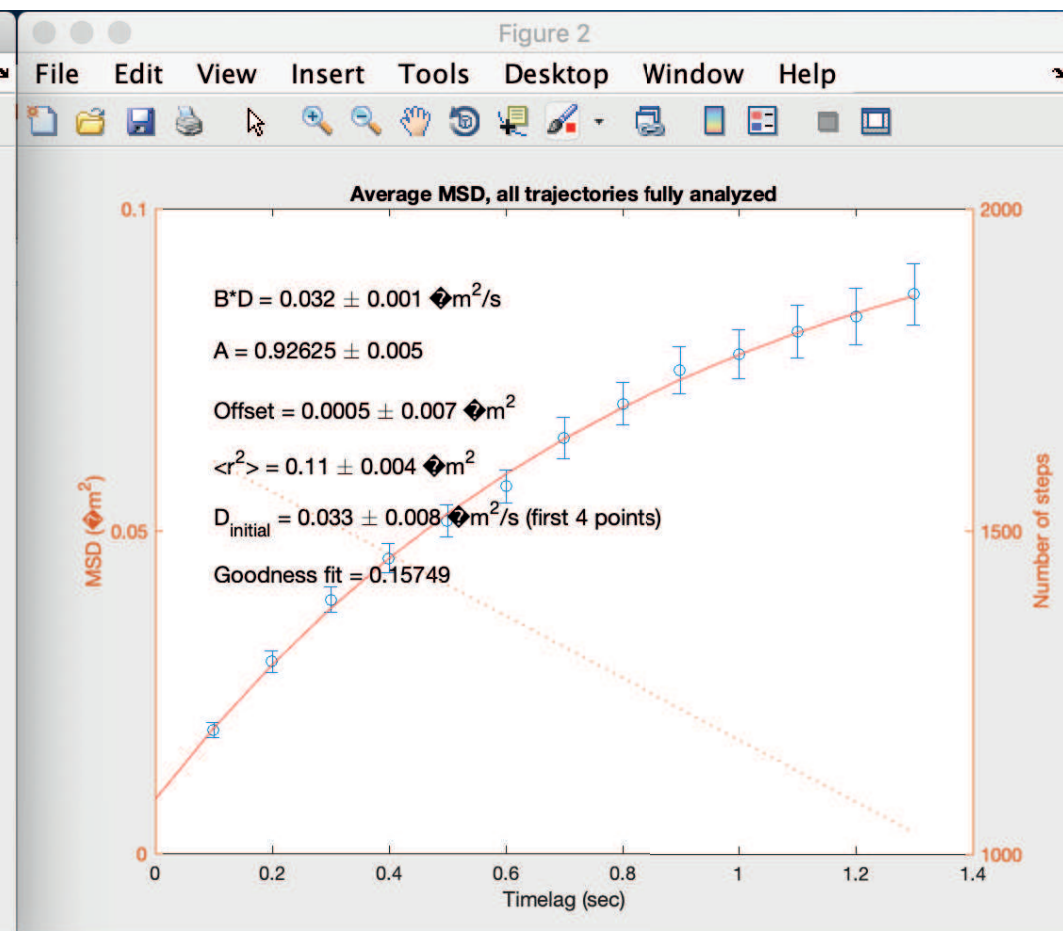
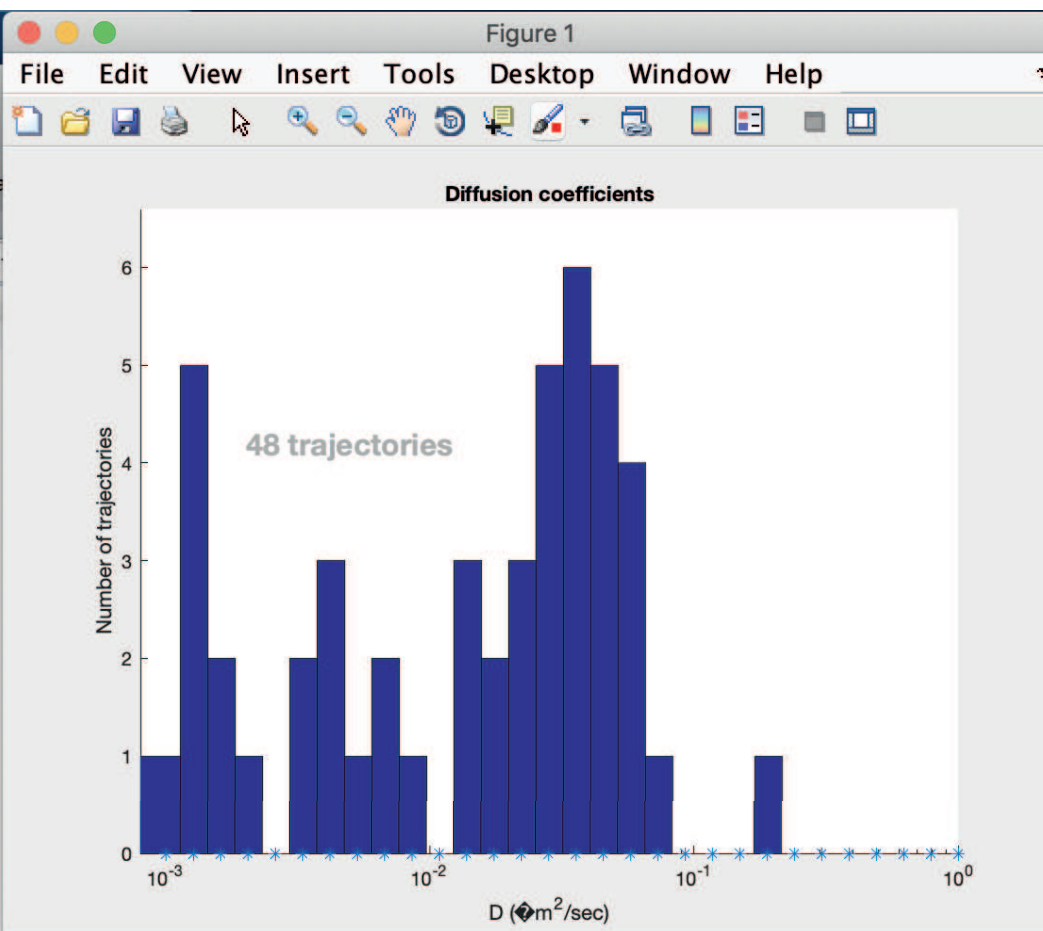


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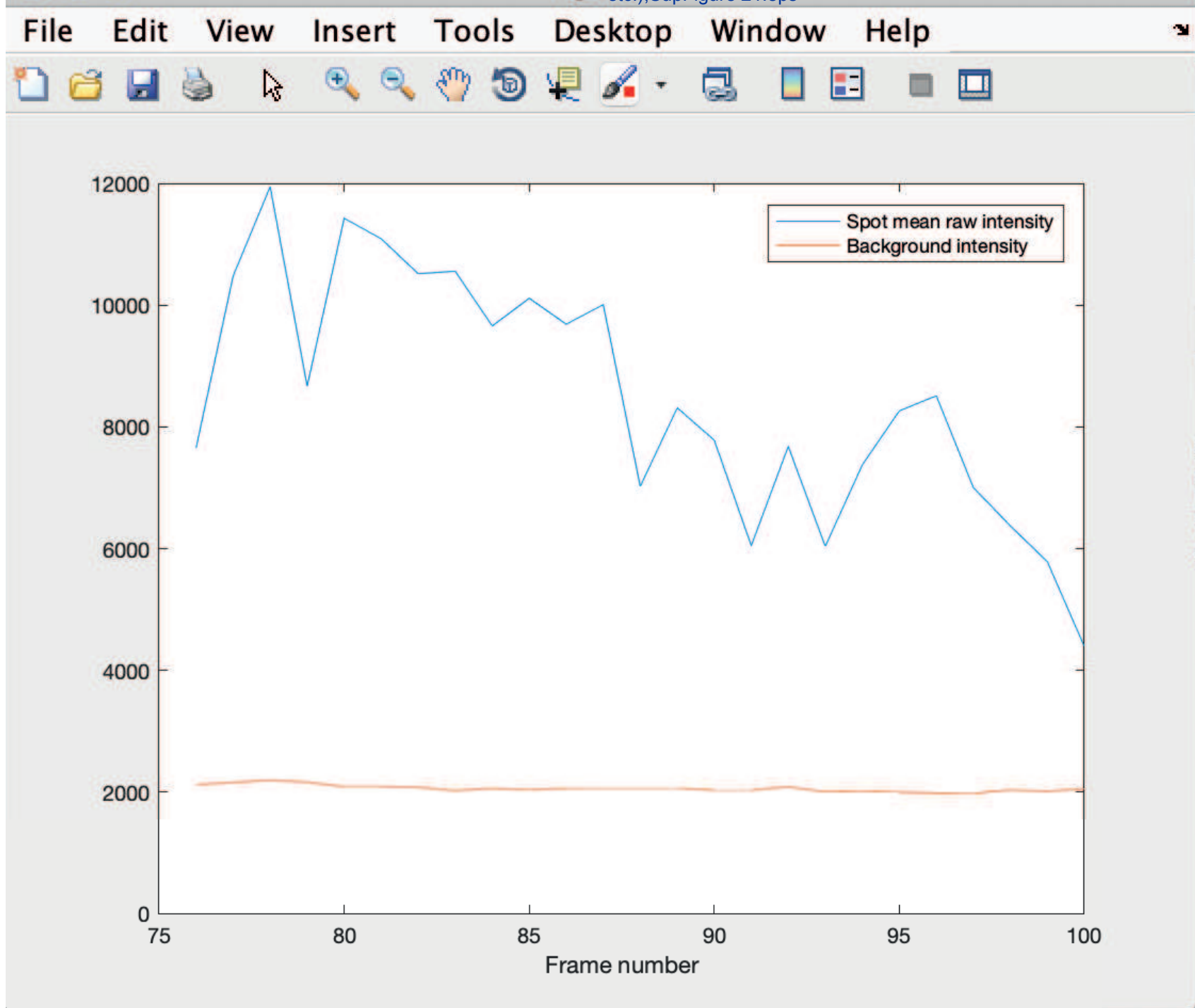




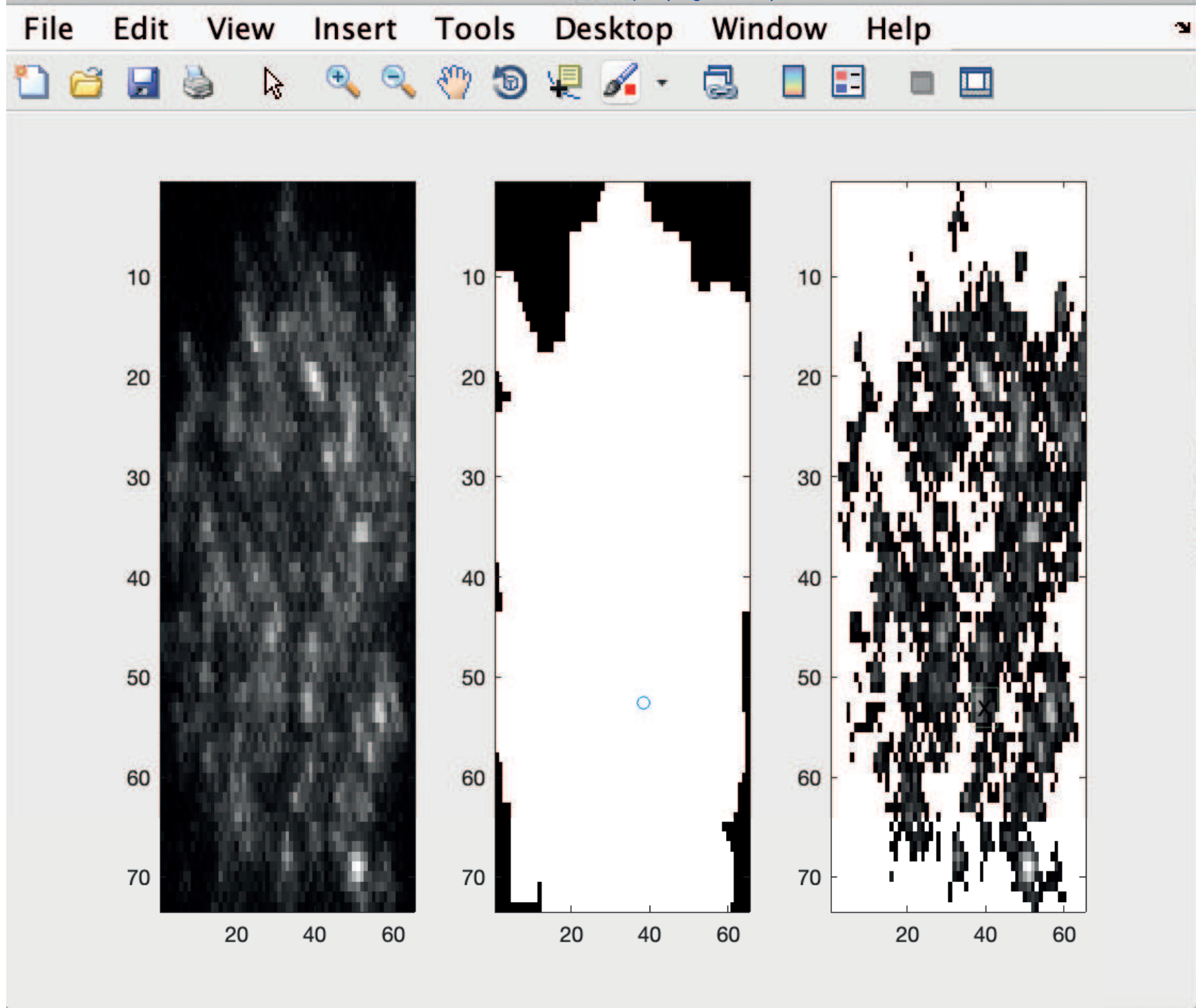
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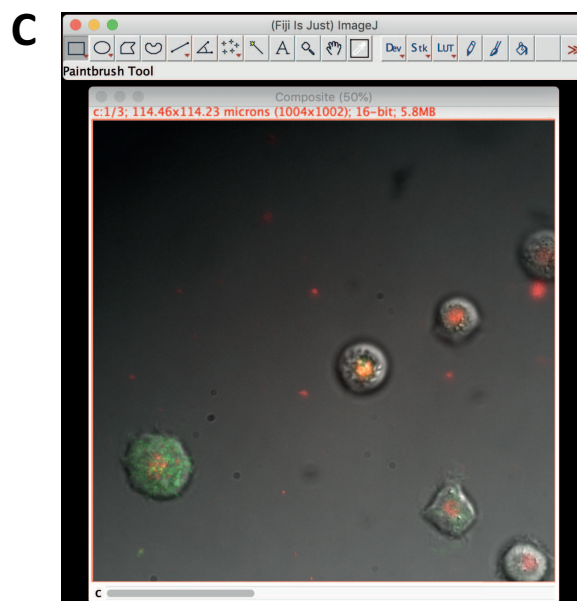
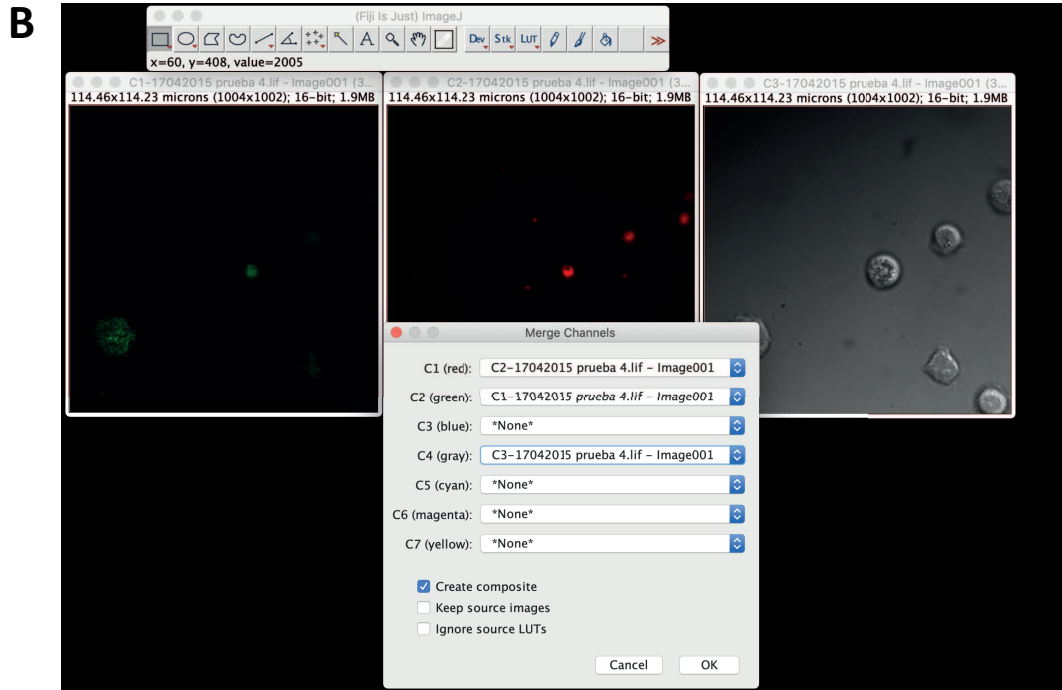
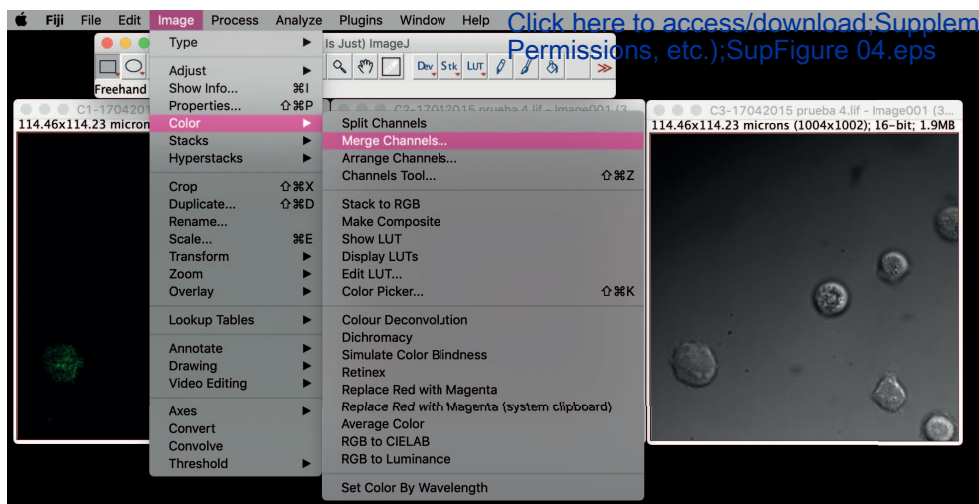
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Supplementary Figure 21



Supplementary Figure 22





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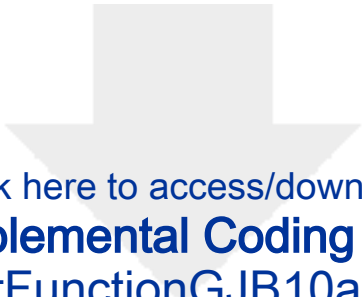
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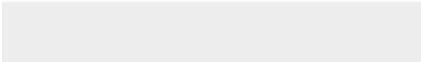



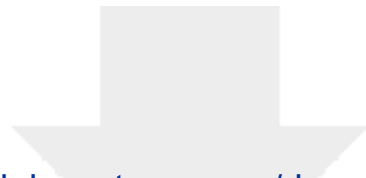
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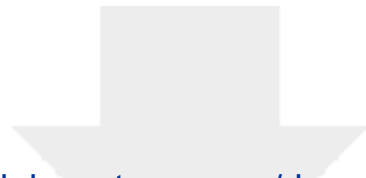


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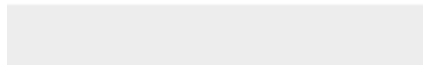


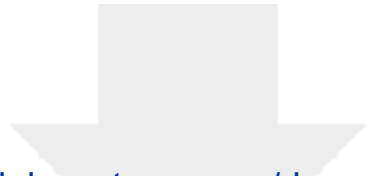


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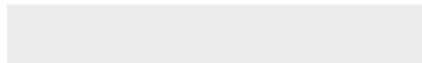
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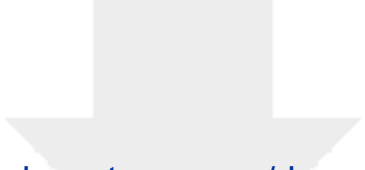
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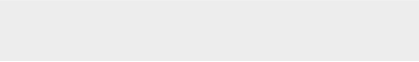
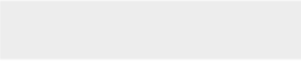


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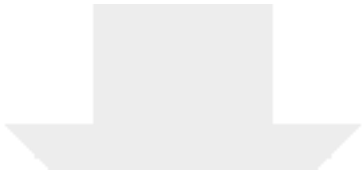
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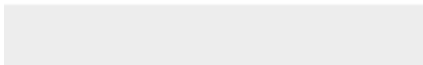
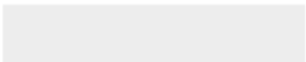


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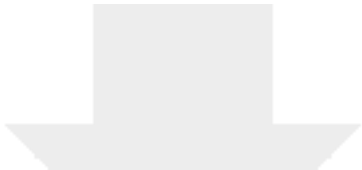


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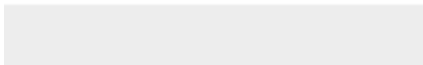
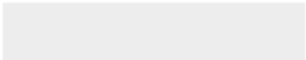


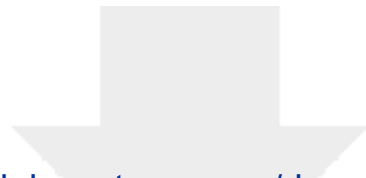


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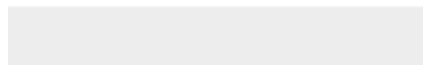
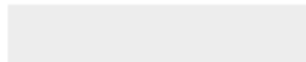
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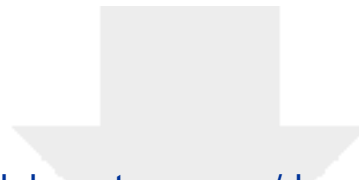




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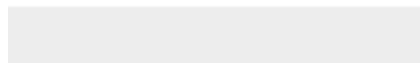
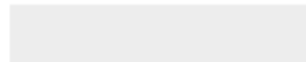


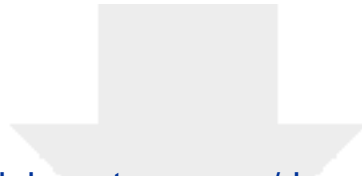


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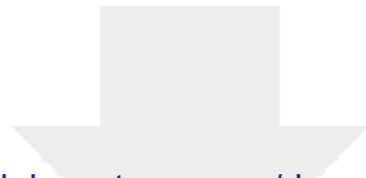
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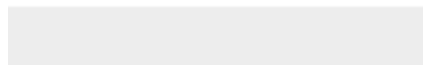
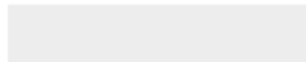


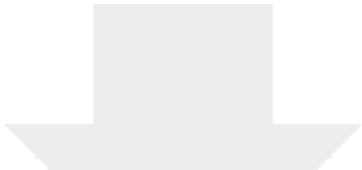


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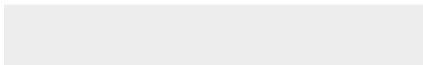
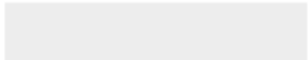


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