

# FUNDAMENTALS OF LIGHT MICROSCOPY AND IMAGE PROCESSING

FUNDAMENTALS OF LIGHT MICROSCOPY AND IMAGE PROCESSING  
1<sup>ST</sup> EDITION  
COURSE AT ITQB NOVA 2023

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**BEFORE YOU BEGIN:**

- Download Fiji on your computer (<https://imagej.net/software/fiji/downloads>)
- Install the required plugins in Fiji:
  - Choose **Help → Update** and wait for it to complete;
  - In the ImageJ Updater, press **Manage Update Sites**;
  - Check the following sites and press Close:
    - BIG-EPFL
    - NanoJ-SQUIRREL
    - NanoJ-Core
    - bUnwarpJ
    - Fast4DReg
    - PTBIO (CellPose)
    - TrackMate
    - IJPB-Plugins (MorpholibJ)
  - Press **Apply Changes**.

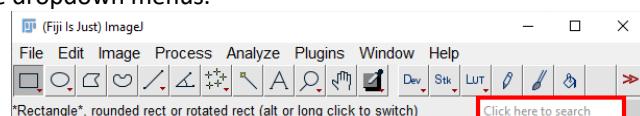
If you have any issues or questions, please don't hesitate to ask us!

**DATA DOWNLOAD**

Please download the data from this link: <https://we.tl/t-1qzfmjvD1p>

**IMAGEJ TIPS**

- Use the **search bar**. Every tool in Fiji/ImageJ can be found quickly through by searching its name rather than looking through the dropdown menus.



- Use keyboard **shortcuts**. These are displayed next to the commands in the dropdown menus and in the search results. Here are some common shortcuts:

- **Ctrl/Cmd + Shift + C**: Brightness and Contrast
- **Ctrl/Cmd + T**: ROI Manager
- **Ctrl/Cmd + Shift + D**: Duplicate image
- **Ctrl/Cmd + Shift + X**: Crop image to selection

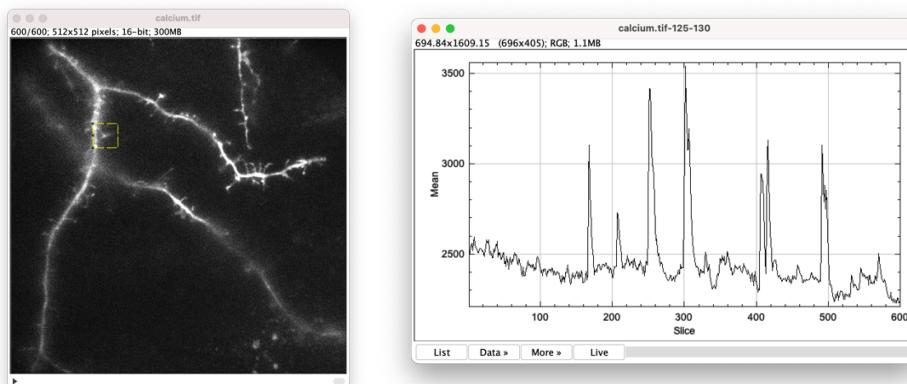
You can find the list of shortcuts in **Plugins → Shortcuts → List Shortcuts**.

- If you find yourself using the same command repeatedly, you can set up **custom shortcuts**. Use **Plugins → Shortcuts → Add Shortcut** to add a hotkey for a given command.

**EXERCISES 1: TECHNIQUES OF IMAGE ANALYSIS. 10:30 - 13:00 (2.5)****1.0 TIME LAPSE FLUORESCENCE DATA ANALYSIS (15 MIN)**

This section explains how to look at intensity profiles across videos, or images with a temporal dimension.

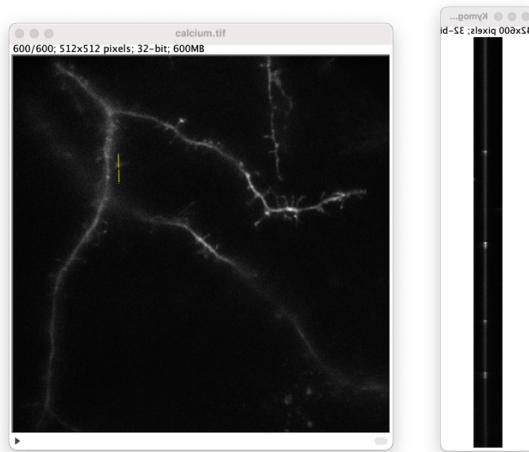
- a) Open the image “**calcium.tif**”. This is a video of neuronal activity using a fluorescent calcium sensor.
- b) Play the video and look for regions that light up repeatedly.
- c) Use the **Rectangle** annotation tool to select an active region.
- d) Choose **Image → Stacks → Plot Z-axis Profile**
  - What do you see?



e) Use the **Straight Line** annotation tool and draw across the region of interest.

f) Choose **Analyze → Multi-Kymograph** and press **Ok**.

- What do you see?

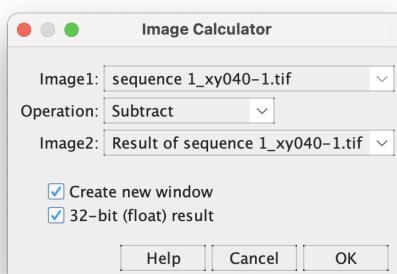


Try these steps on different regions and see the differences in activity.

### 1.1 ILLUMINATION ARTIFACT CORRECTION (15 MIN)

In this exercise you will see how to remove the illumination artifacts appearing in transmitted light microscopy.

- Open the image “sequence 1\_xy040-1.tif”.
- Duplicate the stack: Choose **Image → Duplicate...**
- Apply a Gaussian filter with a very high sigma to filter out the details and calculate the illumination artifact: Choose **Process → Filters → Gaussian Blur...** Choose **sigma = 100**
- Subtract the illumination artifact to the original image: **Process → Image calculation → Subtract the filtered image to the original image and choose a result with 32-bit float precision.**



d) Save the results as a tif image: Choose **File → Save as**



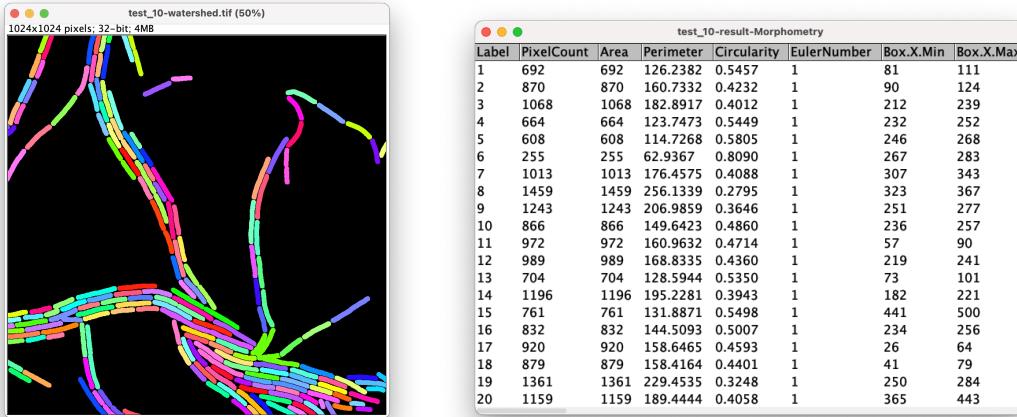
## 1.2 INSTANCE SEGMENTATION OF CELLS USING CELL CONTOURS. (20 MIN)

In this exercise you will post-process the **semantic mask** (each label corresponds to a specific part of the bacteria cell) of bacteria *Bacillus Subtilis* to obtain an **instance segmentation** mask (each label corresponds to an independent bacteria cell in the image).

- Open the image **test\_10.tif**. This image has pixel values 0 (background), 1 (interior of bacteria) and 2 (each bacteria cell boundary). It may open as a black image, so open **Image → Ajust → Brightness/Contrast** to adjust the visualization (DO NOT CHANGE PIXEL VALUES).



- Duplicate the image: **Image → Duplicate...** (call it **markers**)
- Threshold the markers image manually by choosing only the inner side of the cells (label == 1): **Image → Threshold → Set (lower = 1, upper = 1) → Apply**
- Threshold the original image by generating a mask that contains all the pixels belonging to the cell (labels == 1 and 2): **Image → Threshold → Set (lower = 1 and upper=2) → Apply**
- Run the marker controlled watershed segmentation from MorpholibJ: **Plugins → MorpholibJ → Segmentation → Marker-controlled Watershed.**
  - Input: the binarized input image
  - Markers: the binarized markers
  - Mask: the binarised input image
- Analyse the segmentations by running **Plugins → MorpholibJ → Analyse → Analyse regions**



### OPTIONAL HOMEWORK FOR FURTHER DIFFICULTY: IMAGEJ MACRO RECORDING

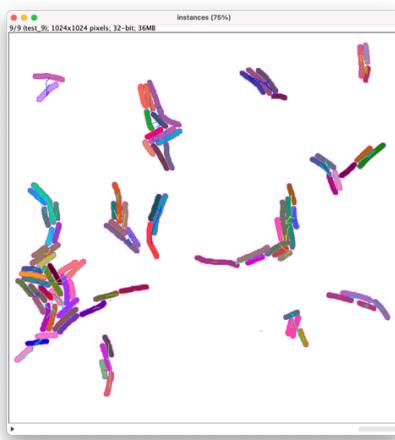
ImageJ can record your actions while using the software and translate it into code. For this, we use the macro recorder.

- Go to Plugins → Macro → Record
- Repeat all the steps of the previous exercise.
- Go to the macro recorder and click on **Create**.
- Clean the macro and create a **Macro 1** that repeats the same workflow as you did. For this you can close all the images, open **test\_10.tif** again and run the macro to see if it works.
- Using this, write a macro that processes the images in **target\_boundaries** and **saves the resulting image**. Tips:

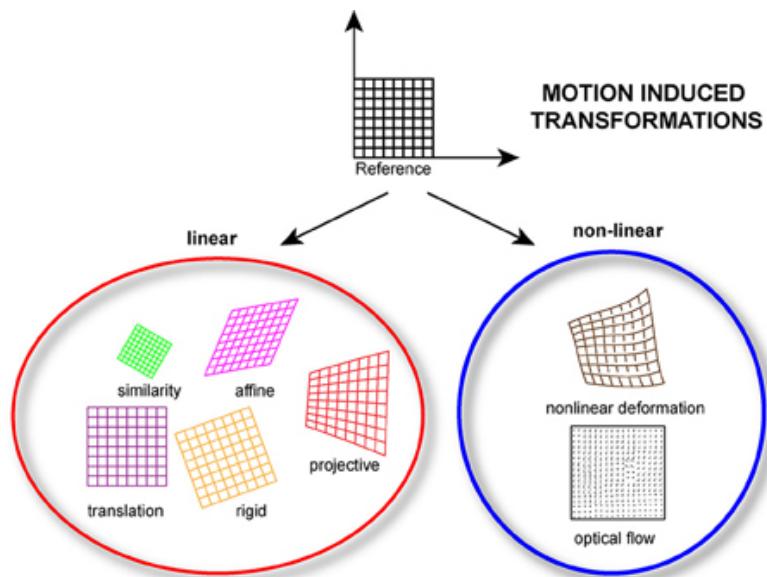
```
input = getDirectory("Choose the folder containing the images");
list_files = getFileList(input);
output = getDirectory("Choose the folder in which the images will be saved");
for (i = 0; i < list_files.length; i++) {
    current_file = list_files[i];
    path_current = input + File.separator + current_file;
    if (endsWith(current_file, ".tif")) {
        open(path_current);
        ...
        saveAs("Tiff", output + File.separator + current_file);
        run("Close All");
    }
}
```

### 1.3 TRACK CELLS USING INSTANCE SEGMENTATIONS WITH TRACKMATE (15 MIN)

- Drag and drop the folder “instances” containing the instance masks that can be obtained with the previous optional homework exercise.
- Run TrackMate: Plugins → Tracking → TrackMate
  - Choose **Label Image Detector** and click next
  - Choose **Preview**
  - Choose **Simple LAP Tracer (this one does not allow for mitoses/splits)**
  - Click next and visualize the results
  - Now try using **LAP Tracer** and try finding a good configuration for the splits so cell divisions are well identified.
    - What issues do you identify with bacteria? What could we do it to overcome this issue?



## 1.4 REGISTRATION



### 1.4.0 LINEAR REGISTRATION FOR DRIFT CORRECTION (30 MIN)

#### 1.4.0.0 DRIFT CORRECTION IN TIME LAPSE ACQUISITIONS WITH STACKREG (10 MIN)

In this exercise you will see how to remove the drift from the microscope in 2D time lapse movies. We can use StackReg or Fast4Dreg, depending on the data we need to analyse. We will see both.

- Open the image sequence **1\_xy040-1.tif**
- Run the registration: **Choose Plugins → Registration → StackReg → Rigid body.**
- Repeat the process with the images **confocal\_ROI1.tif**

#### 1.4.0.1 MULTICHANNEL VOLUME REGISTRATION (FAST4DREG) (20 MIN)

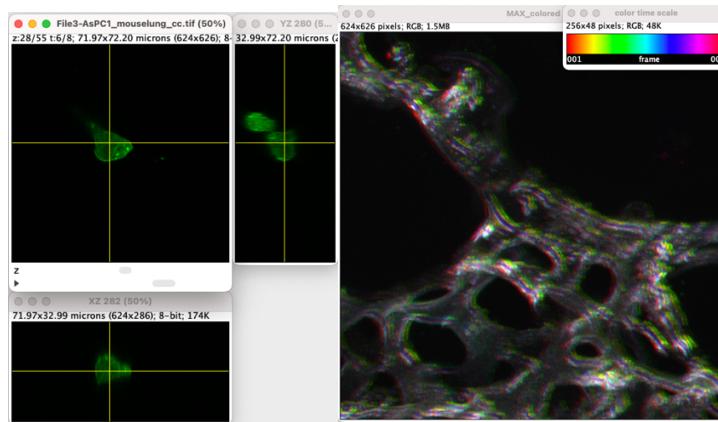
For this exercise we will use the example data of cancer cells (AsPC1 expressing Lifeact-mNeonGreen) migrating inside the lung vasculature (lung endothelial cells were labelled using Alexa Fluor 488) provided by the Fast4Dreg original

paper<sup>1</sup>. The images were acquired using an Airyscan confocal LSM880 microscope equipped with a 63x water objective.

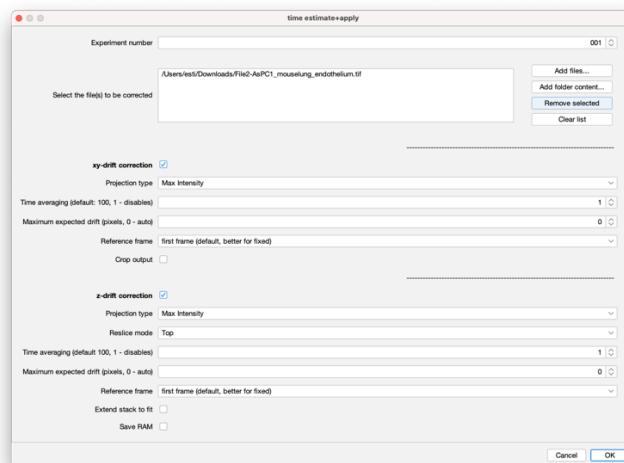
In this example, the time lapse acquisition is as follows: For each time point, the volume is calculated, and for each z-slice of the volume, both channels are acquired. Thus, the drift in Z and in (X,Y) is the same for both channels.

**a)** Open **File2-AsPC1\_mouselung\_endothelium.tif** and make a projection with the maximum intensity (**Image → Stacks → Z-project**). Visualise the drift on each of them by running **Image → Hyperstacks → Temporal-Color Code**

**b)** Open **File3-AsPC1\_mouselung\_cc.tif** and visualise the drift in (x,y,z) with the orthogonal views: **Image → Stacks → Orthogonal views**



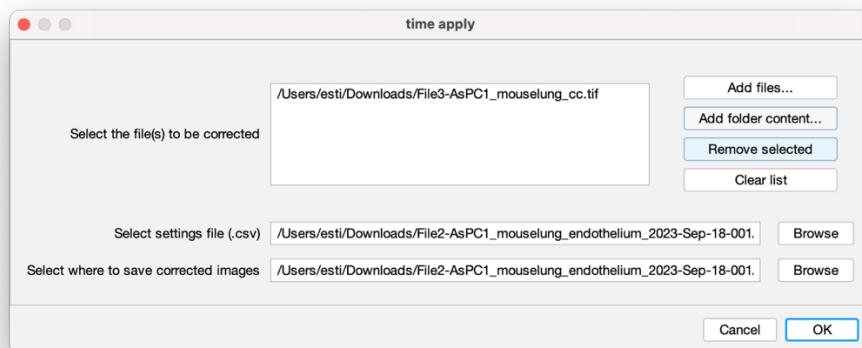
**c)** Calculate the transformation needed to correct the drift in (x,y) and z of the acquisition using the more stable channel that lacks movement (the vasculature). Run **Plugins → Fast4DReg → time estimate + apply**. Choose the vasculature to estimate the drift correction (**File2-AsPC1\_mouselung\_endothelium.tif**). This will create a new folder with the corrected time-lapse volume and the transformation parameters stored as a .csv file.



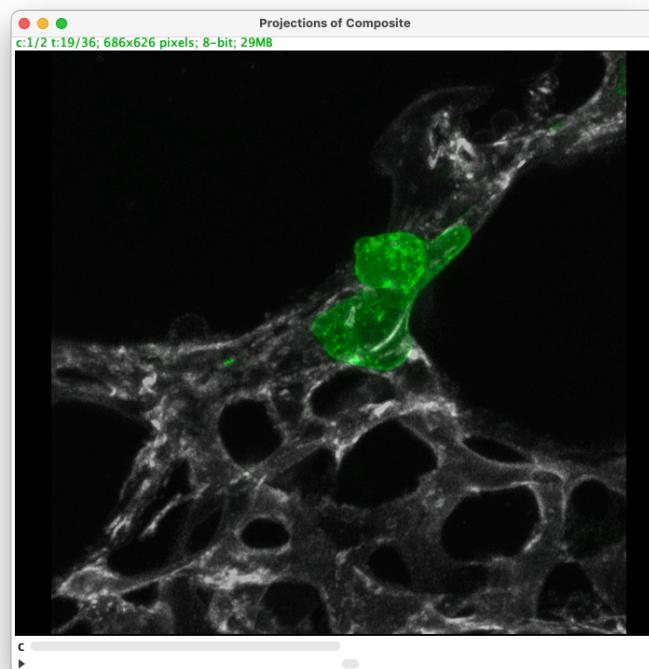
<sup>1</sup> Pylvänäinen et al., Fast4DReg – fast registration of 4D microscopy datasets, Journal of Cell Science, 2023

<https://doi.org/10.1242/jcs.260728>

d) Apply the estimated drift correction to **File3-AsPC1\_mouselung\_cc.tif** with Plugins → Fast4Dreg → time apply. Visualise the obtained results.

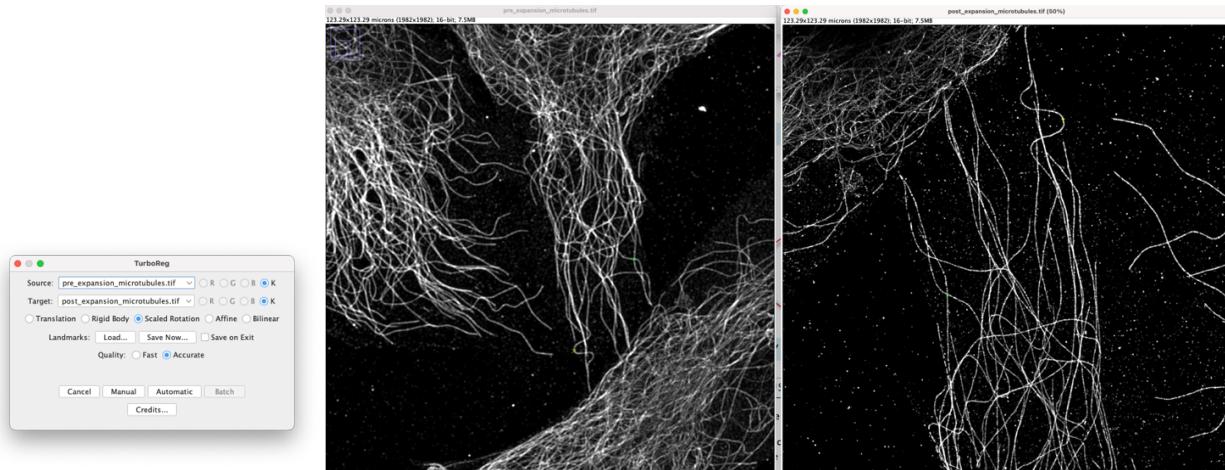


e) Visualise the obtained results: Open the corrected images **File2-AsPC1\_mouselung\_endothelium\_xyzCorrected.tif** and **File3-AsPC1\_mouselung\_cc\_xyzCorrected.tif**. Merge both images using **Image → Color → Merge Channels**. Choose the cell to be in the green channel and the vasculature in the gray channel. Project the 3D volumes with **Image → Stacks → 3D Project**. Choose the brightest point, with respect to the y-axis, to interpolate among frames and to analyse all the time points. It will take some time.



### 1.4.1 ASSESSMENT OF EXPANSION MICROSCOPY USING REGISTRATION (LINEAR AND NON-LINEAR) (20 MIN)

**a)** Identify the structure in the pre-expanded image and colocalize both images (linear registration): Open **pre\_expansion\_microtubules.tif** and **post\_expansion\_microtubules.tif**. Run **Plugins → Registration → TurboReg**. The image we want to transform and register is the pre-expanded one (**source**) and see how much the expansion process altered the structural distribution. A scaled rotation can be defined by two points (we need to manually identify them)

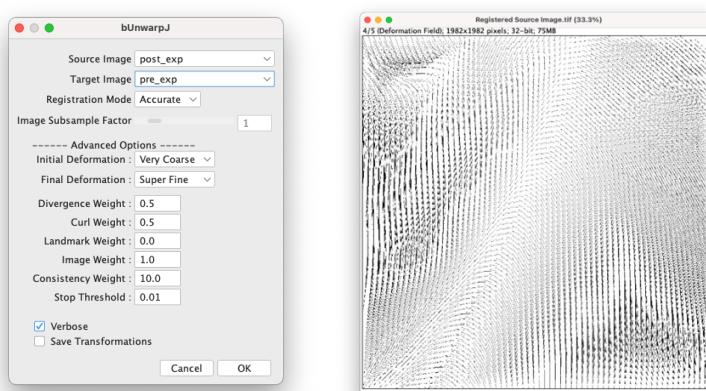


**b)** Save the first frame of the output (i.e., the new registered image in low resolution).

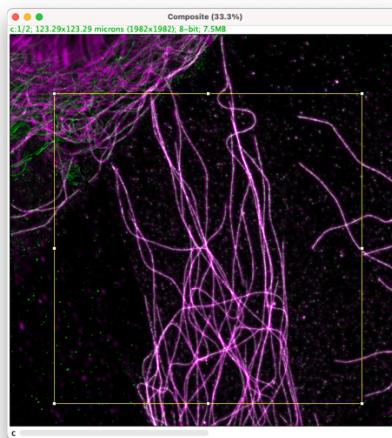
**c)** We will **normalize** and convert to **8 bits** the registered image and the post-expanded one for the following steps: Open the **normalize.ijm** macro file and run it on both images (click run).

**d)** Register both images using elastic transformations: **Plugins → Registration → bUnwarpJ** with the following parameters (note that you can stop the registration whenever you want):

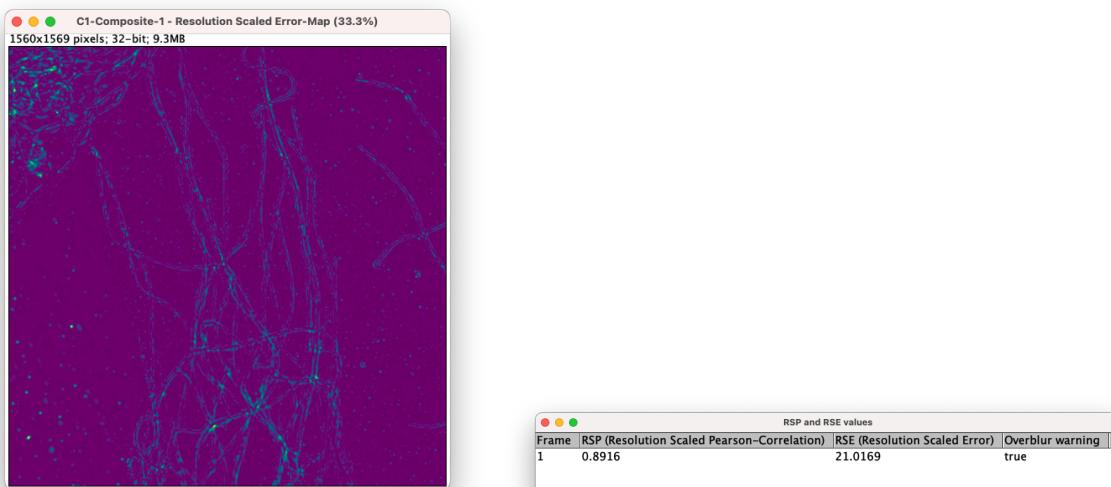
- Register the pre-expanded image (target) with respect to the post-expanded (source).
- Downsample the images once (half the size). This step will speed up the computation and will not significantly compromise the visualization of the transformation.
- Go from a very coarse transformation into a super fine one.



**e)** Take the registered source image stack and duplicate only the transformed pre-expanded image (**Image → Duplicate**). Now visualize the original post-expanded image and the registered pre-expanded image. You will need to convert to 8bits the transformed image (**Image → Type → 8-bit**). Then run **Image → Color → Merge channels**. In green the post-expanded image and in magenta the transformed pre-expanded image for example.

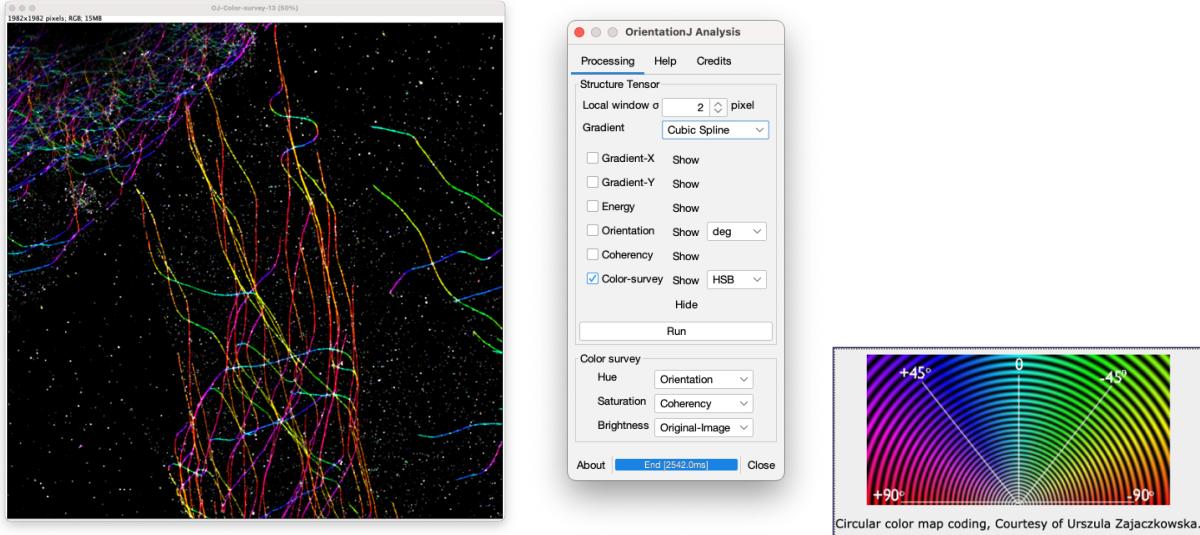


**f)** Compute the difference between the pre-expansion and post expansion. We will crop both images in the center to remove the artifacts in the borders. For this, we take the multi-channel stack we just created. Draw a centered square and duplicate it to create a smaller stack. Split the channels by **Image → Color → Split channels**. Run SQUIRREL analysis with **Plugins → NanoJ SQUIRREL → Calculate Error Map. RSE and RSP – UniQorn! optimizer (beta)**. You can also plot profile plots on top of registered and non-registered data to see the difference.

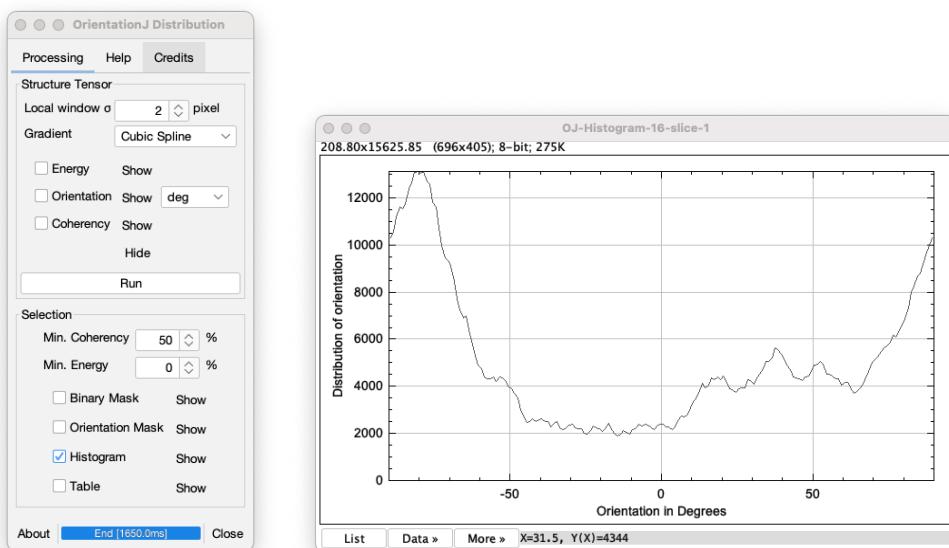


### 1.4.3 MICROTUBULE ORIENTATION DISTRIBUTION. (10 MIN)

a) Open **post\_expansion\_microtubules.tif**. Run **Plugins → OrientationJ → OrientationJ Analysis**. Calculate the orientation of the microtubules in the image and plot them as a color map.

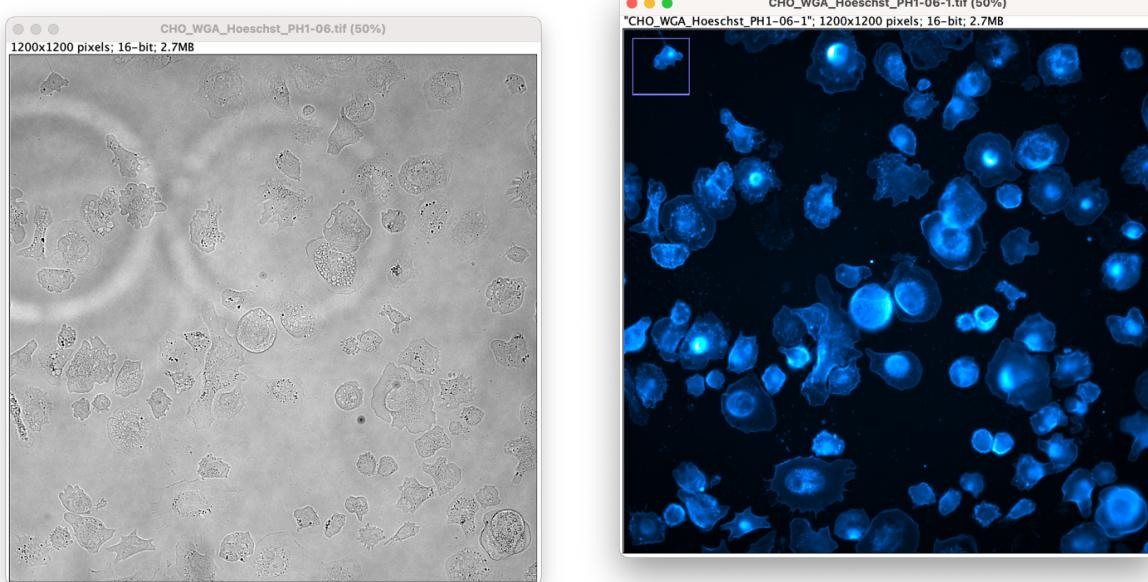


b) Calculate the distribution of the orientations with **Plugins → OrientationJ → OrientationJ Distribution**. You can adjust the threshold for the coherency to ensure that only the orientation of the microtubules is measured. You can display the binary mask (click on **Show**) to make sure that the binary mask is what you expect.



**EXERCISES 2: TECHNIQUES OF IMAGE ANALYSIS – DEEP LEARNING. 15.00 – 17.00 (2 H)****2.0 EASY DEEP LEARNING WITH ZEROCOSTDL4MIC: VIRTUAL STAINING (PIX2PIX) (~1.75 H)**

We will train a conditional generative adversarial neural network (Conditional GAN published as *pix2pix*<sup>2</sup>) to infer the fluorescence labelling corresponding to the cell membrane from phase contrast microscopy images. For this, we will use Chinese hamster ovary cells (CHO) stained with Wheat Germ Agglutinin (WGA) to label glycosilated proteins in the cell surface. The training and test data was obtained by imaging the same field of views with both phase contrast and widefield fluorescence imaging.



For this exercise we will use **Google Drive**, so you need to have an account and some free space to save data (approx. **4 GB**)

- a)** Upload the data of pix2pix to your Google Drive.
- b)** Go to the ZeroCostDL4Mic website: <https://github.com/HenriquesLab/ZeroCostDL4Mic/wiki>
  - b.1)** Look for the pix2pix notebook and open it using Google Colaboratory.
  - b.2)** Once opened, save a copy in your drive: In the top part of the notebook, click on **Files → Save a copy in drive**. **It will save the notebook in your Drive → Colaboratory Notebooks. This new notebook will be opened in a new window.**
  - b.3)** Training of 100 epochs takes approximately 40 minutes. Thus, we will only train: 30 epochs. Lately, you can load the pretrained model and keep training it. From now on, follow the instructions in the notebook.

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<sup>2</sup> Image-to-Image Translation with Conditional Adversarial Networks by Isola et al. on arXiv in 2016 (<https://arxiv.org/abs/1611.07004>)

## 2.1 SEGMENTATION (CELLPOSE) (30 MIN)

[CellPose](#) is a deep learning-based approach to segment cells and cell nuclei in different type of microscopy images. It is possible to run it from code, but also from Fiji directly.

- a) We first need to create a Conda (Python) environment that has all the code needed. Open the terminal and run the following commands in order:

```
conda create -n cellpose
conda activate cellpose
python -m pip install cellpose
```

- b) In the terminal, during the installation, you can see the path where this environment is located in the computer. In my case, it is “/Users/esti/mambaforge/envs/cellpose”.

```
Last login: Wed Sep 20 00:31:10 on ttys005
esti@estimacbookair ~ % conda create -n cellpose python=3.10

Collecting package metadata (current_repodata.json): done
Solving environment: done

==> WARNING: A newer version of conda exists. <==
    current version: 22.9.0
    latest version: 23.7.4

Please update conda by running

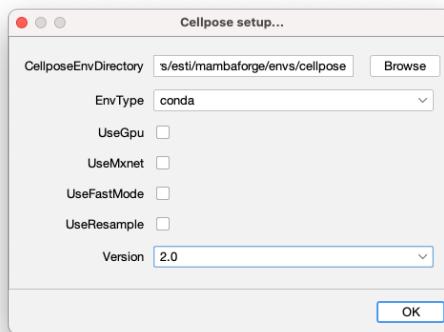
$ conda update -n base -c conda-forge conda

## Package Plan ##

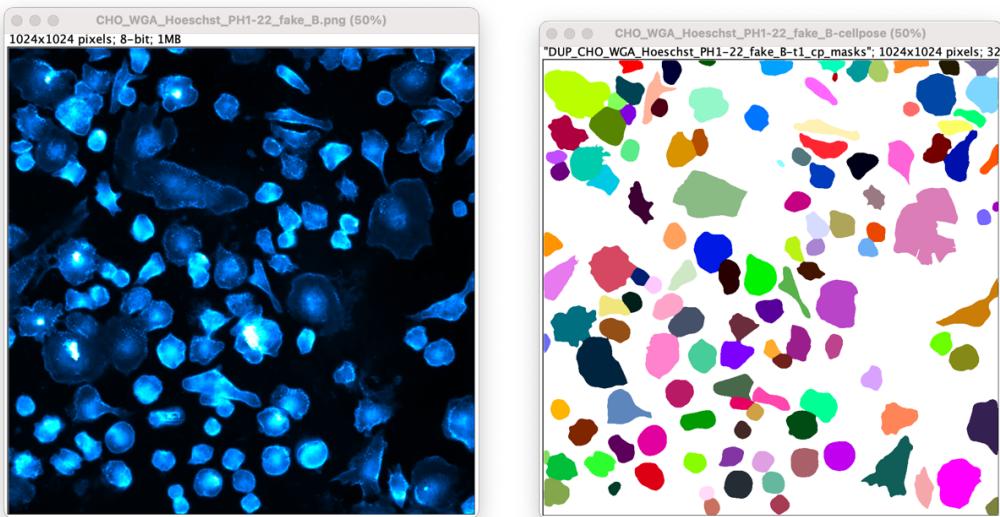
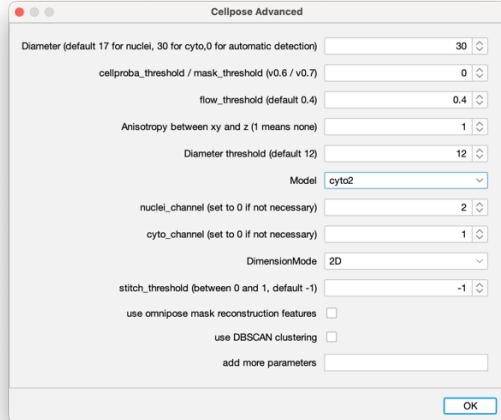
environment location: /Users/esti/mambaforge/envs/cellpose

added / updated specs:
- python=3.10
```

- c) Open Fiji and set up the Cellpose environment: **Plugins → BIOP → Cellpose → Cellpose setup**. Copy the path to your conda environment (step before), uncheck the GPU box and select the version 2.0.



- d) Open one of the artificially labelled images from the previous exercise. Use cell pose to obtain the masks in Fiji: **Plugins → BIOP → Cellpose → Cellpose Advance**. Choose the model for Cyto2 and run it. Wait a bit.



## SOLUTIONS

### SOLUTION FOR THE MACRO 1:

```
// Threshold the inner part of the bacteria cells that will represent the markers.
run("Duplicate...", "title=markers");
setThreshold(1, 1, "raw");
setOption("BlackBackground", true);
run("Convert to Mask");

// Threshold the binary image corresponding to the bacteria cells.
selectWindow("test_10.tif");
setAutoThreshold("Default dark");
setThreshold(1, 2, "raw");
setOption("BlackBackground", true);
run("Convert to Mask");

// Marker controlled watershed.
run("Marker-controlled Watershed", "input=test_10.tif marker=markers mask=test_10.tif compactness=0 binary calculate use");
```

**SOLUTION FOR MACRO 2:**

```
input = getDirectory("Choose the folder containing the images");
list_files = getFileList(input);
output = "/Users/esti/Documents/instance-segmentation/output";
File.makeDirectory(output);

for (i = 0; i < list_files.length; i++) {
    current_file = list_files[i];
    path_current = input + File.separator + current_file;
    if (endsWith(current_file, ".tif")) {
        open(path_current);
        // Threshold the inner part of the bacteria cells that will represent the markers.
        run("Duplicate...", "title=markers");
        setThreshold(1, 1, "raw");
        setOption("BlackBackground", true);
        run("Convert to Mask");

        // Threshold the binary image corresponding to the bacteria cells.
        selectWindow(current_file);
        setThreshold(1, 2, "raw");
        setOption("BlackBackground", true);
        run("Convert to Mask");

        // Marker controlled watershed.
        run("Marker-controlled Watershed", "input=" + current_file + " marker=markers mask=" +
            current_file + " compactness=0 binary calculate use");
        saveAs("Tiff", output + File.separator + current_file);
        run("Close All");
    }
}
```