

# DIFFERENTIATION PERCENTAGE ANALYSIS PIPELINE MANUAL

## *INTRODUCTION – WHAT THE PIPELINE DOES AND WHAT WILL YOU NEED*

This pipeline offers you to measure the percentage of cardiomyocytes in cell culture (by identifying amount of Alpha Sarc Act positive cells using TILE SCAN immunofluorescence images from ZEISS confocal microscope). Possibly it could also be used to identify percentage of cells positive for some other protein expression – in dependence on learning modifications and differences in between protein localisation.

The counting of cells is based on principle 1 nucleus equals 1 cell. The nuclei which do colocalise within the area occupied (area covered by Alpha Sarc Act signal which is used as a marker for CMs) are counted as cells, the rest is stated as background.

## **SOFTWARE REQUIREMENTS – What softwares will you need to run this pipeline?**

- 1) **ILASTIK – better to use on ODEON shared desktop.**
- 2) **CELL PROFILER** – version ideally 4.2.1 – if you use newer versions, please do not make any changes in the pipeline which you would like to later use on ODEON shared computer – it does not work properly with pipelines created in newer versions e.g. version 4.2.5 – but vice versa it is okey (moving back from older version to newer version is okey if you succeed to open this pipeline in the older version of CP).
- 3) Git Bash – advantage but not necessarily needed. You can also use Windows Power Shell.

In this document you will find brief description how to work with this pipeline - from image export to final processing. Sometimes it is too detailed, so please feel free to skip parts you don't find useful since you already know them. :)

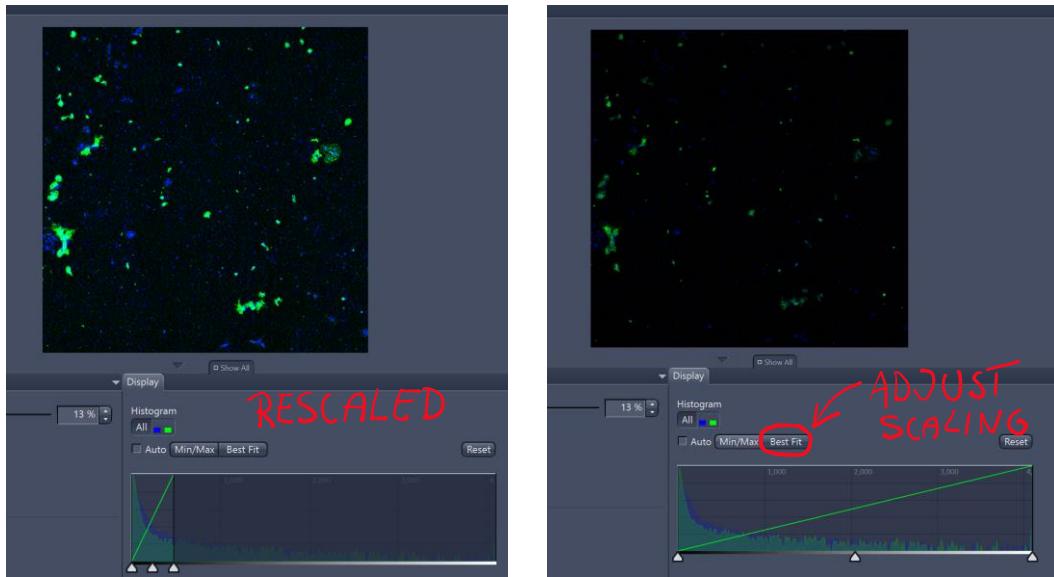
Katerina Jirakova, 15-17/11/2023

Supervisor: Mgr. Bc. Vladimir Vinarsky, Ph.D.

Mechanobiology of Disease

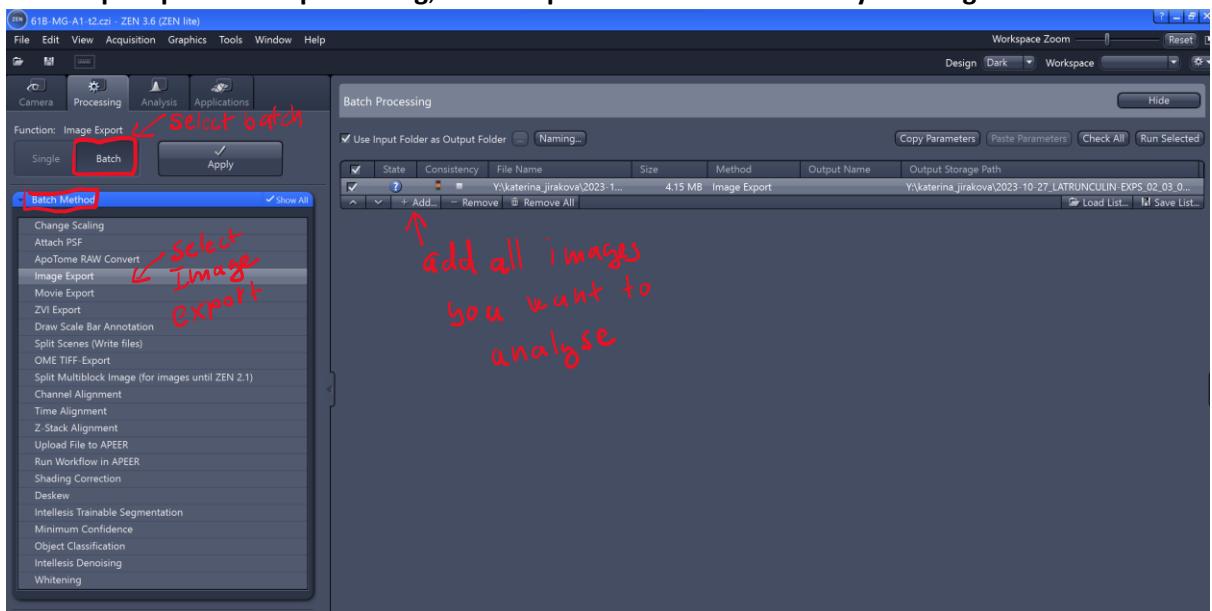
## GETTING READY – PREPARING YOUR IMAGES

- 1) Prepare your **TILE SCAN images** (requirements: **DAPI** staining, **Alpha Sarc Act** staining – maybe staining of other protein which visualize all the cellular area could be also used instead – in case of problems you can try to adjust the learning or send me the images and I will try to add them to the learning). Also creating new pipeline for the protein and adding it to the main pipeline is a possibility.
- 2) Open your images in Zenn Blue and adjust the scaling to **Min/Max** and save the adjusted images.

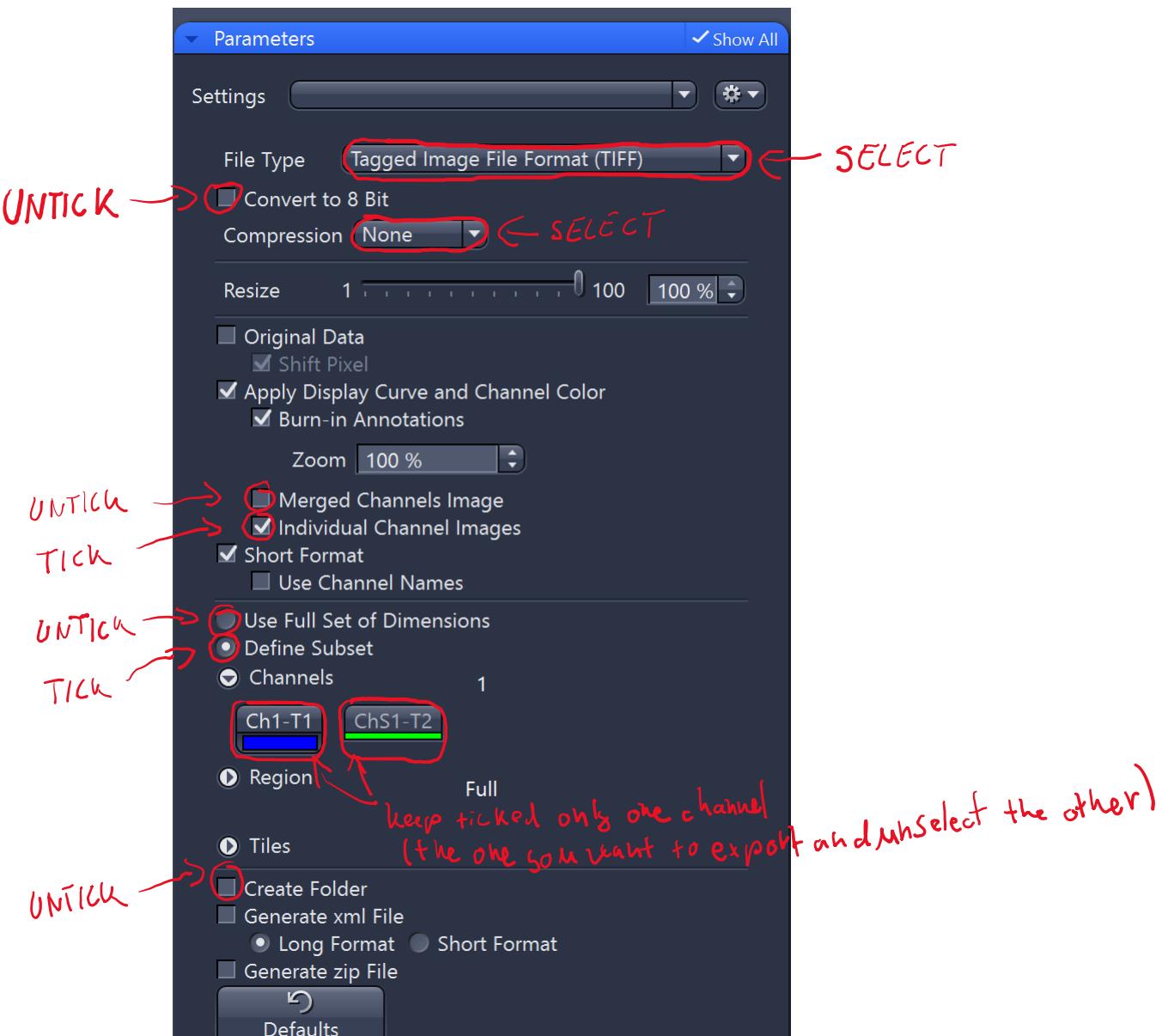


- 3) Export images for **Alpha Sarc Act** and **DAPI** channels from Zenn Blue – images from both channels should be located in separate folders (as a result there will be two **folders – 2-sarc-act-tiffs** and **3-dapi-tiffs** in which the **tiffs for all images for the particular channel** will be located) – brief info of how the folder should look like is below at the end of step 3.

**First step – open BATCH processing, select export method and load all your images.**



Select first image and set export parameters – follow the image attached below.

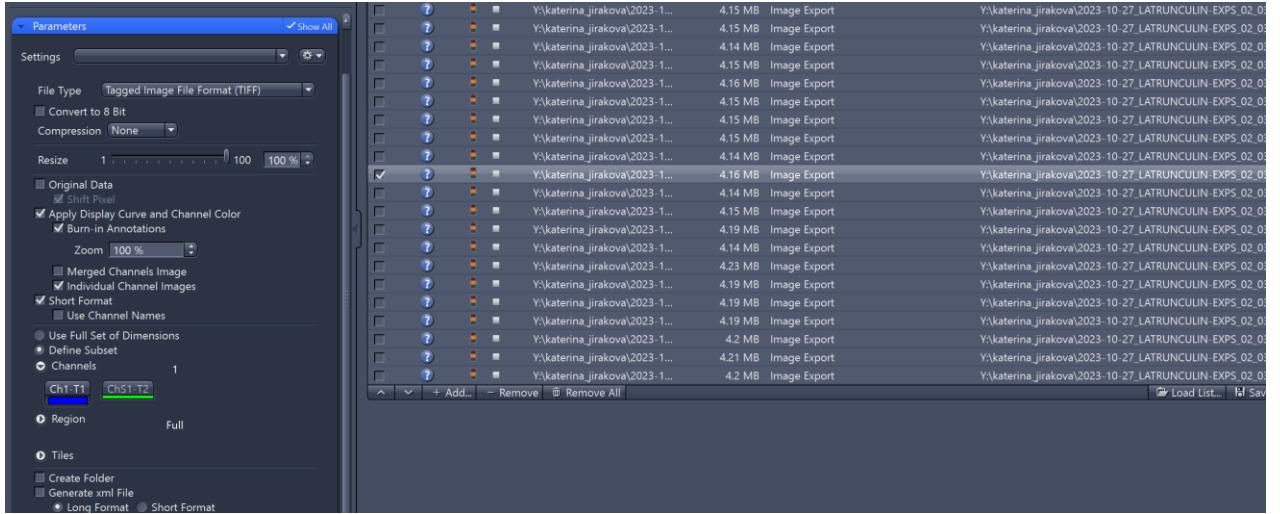


**Apply selected set of parameters to all loaded images.**

**Tick to select all loaded images.**

**Paste the parameters** to change the setting of all images.

**Check if it worked properly – open some image which had different setting before pasting new setting.**



Choose output folder for all images.



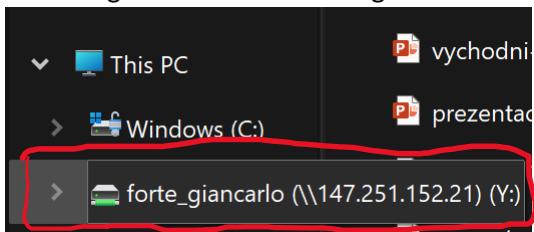
Create folder for your project and put there two folders for the image export – 2-sarc-act-tiffs and 3-dapi-tiffs. In order to prepare for analysis, create folder inner structure following this template:

This PC > RAID6 (E:) > PROJECTS > forte_giancarlo > katerina_jirakova > 2023-11-02_Diff-efficiency-from-latrunculin-tilescans_Exp02-03-04 >				
Name	Date modified	Type	Size	
1-source-images	11/15/2023 10:56 PM	File folder		
2-sarc-act-tiffs	11/7/2023 10:32 PM	File folder		
3-dapi-tiffs	11/7/2023 11:34 PM	File folder		
4-pipeline	11/15/2023 9:29 PM	File folder		
5-sarc-act-outputs	11/15/2023 10:51 PM	File folder		
6-dapi-outputs	11/15/2023 10:57 PM	File folder		
7-CP-FINAL-OUTPUTS	11/15/2023 10:58 PM	File folder		

Once you're done with exporting images from Zenn move the folder with your data to ODEON shared computer – you can connect to the shared storage from Windows File Explorer.

NOTE: Sometimes there are issues with connection to the shared computer (especially when running training in ILASTIK later). When the connection freeze closing the Remote Desktop application simply by the cross in upper corner and then opening it again functions on my computer perfectly and is faster than waiting for the application to load again – since sometimes it does not load at all).

Attending ODEON shared storage from Windows File Explorer.



Example of folder path on ODEON.

```
📁 This PC > forte_giancarlo (\\\147.251.152.21) (Y:) > katerina_jirakova > 2023-11-02_Diff-efficency-from-latrunculin-tilescans_Exp02-03-04
```

Now **download the most actual pipelines for analysis** – you'll find the most updated version on Google Drive in this folder:

Sdíleno se mnou > ICRC-academy-2022 > IMAGE-ANALYSIS\_PIPEL...

Název	Vlastník	Naposledy u...
CMS_area	já	29. 8. 2023
cms_area_CP-VV-pipeline-modified	já	21. 9. 2023
ILASTIK_PRECLASSIFICATION-FOR-VV-YAP1-LOCALISATION-PIPELINE	já	3. 9. 2023
ILASTIK-OBJECT-IDENTIFICATION-AND-MEASUREMENTS	já	7. 9. 2023
nuclei_counting	já	11. 8. 2023
percentage-of-cms-all-pipelines	já	22:45
YAP1_localisation_VV_pipeline	já	31. 8. 2023

in this folder find  
the most actual pipeline  
and DOWNLOAD  
with learning images  
(if you don't want  
to run headless)

Note: In older versions you need to find learning images following the path you find in README file located in the folder with pipelines (after downloading you will need to show ILASTIK where the learning images are located (where you saved them after downloading)). In new versions you'll download ZIP file which contain both pipelines and learning images – it should be enough to unzip it. You should upload the downloaded pipeline with all its relevant attachments to the folder 4-pipeline.

Name	Date modified	Type	Size
1-source-images	11/15/2023 10:56 PM	File folder	
2-sarc-act-tiffs	11/7/2023 10:32 PM	File folder	
3-dapi-tiffs	11/7/2023 11:34 PM	File folder	
4-pipeline	11/15/2023 9:29 PM	File folder	
5-sarc-act-outputs	11/15/2023 10:51 PM	File folder	
6-dapi-outputs	11/15/2023 10:57 PM	File folder	
peli	11/15/2023 10:58 PM	File folder	
2-lr			

## PROCESSING ALPHA SARC ACT IMAGES – GETTING STARTED WITH ILASTIK

Now you decide whether you would like to use ILASTIK in headless mode – no learning images needed but also no possibility to edit learning– or whether you would like to teach ILASTIK from the app-learning-mode – learning images needed, slower, you can edit learning.  
It is also possibility to edit the learning, then close app and run the pipeline headless.

### Running headless

For headless processing I have prepared bash file (can be run in **Git Bash**).

Unfortunately, in Windows the thing with opening files without opening ILASTIK repetitively with every single image is quite sophisticated ([some discussion regarding the topic](#)). Finally, I ended up with also having script in **Windows Power Shell**, yet you need to write names of all files you would like to process.

The disadvantage of bash is that Git Bash is not installed on the shared ODEON desktop, otherwise I do prefer the BASH one.

**Both scripts are available on Google drive.** Path to scripts on Google Drive:

Sdíleno se ... > ICRC-academ... > IMAGE-ANALYSIS... > .bat-scripts-adju... ▾

Typ ▾ Lidé ▾ Změněno ▾

Název	Vlastník	Naposledy u...	Velikost	
final-bash-ilastik-headless-script.sh	GIT BASH	já	18:12	393 b
final-shell-ilastik-headless-script.bat	Windows Power Shell	já	18:37	150 b

Command .bat file for **Windows Power Shell**:

```
set PATH_TO_ILASTIK="C:\Program Files\ilastik-1.4.0-gpu\ilastik.exe"
call %PATH_TO_ILASTIK% --headless --project=MyProject.ilp s01c2.tif s02c2.tif
```

Briefly what it does:

In the **first step** you set **the location of ILASTIK software on your computer** – to allow Shell to open ILASTIK.

**Second** line: Telling Shell to **open ILASTIK** from the designed location, then telling Shell to run **ILASTIK headless**, then parameter **--project** - write the **name of your pipeline** you want to run. Behind the parameters at the end of the command line just write the **names of your image files** – it is not needed to add anything else.

In this case the files names are “s01c2.tif” and “s02c2.tif”.

Command .sh file for **Git Bash**:

```
# Ilastik command line path
ilastik="C:/Program Files/ila.../ilastik.exe"

# Run Ilastik on the current image
"$ilastik" \
    --headless \
    --project="C:/lab/pokus-headless/MyProject.ilp" \
    --output_format="tif sequence" \
    --output_filename_format="outputs/{nickname}_{result_type}_{slice_index}.tif" \
    *.tif \
    
echo "Batch processing complete!"
```

Briefly what it does:

**First section:** Tell Bash **where is ILASTIK application on your computer**.

**Second section:** Opens ILASTIK application, then tells ILASTIK to run **headless**, then **opens your project** (the certain pipeline located in some folder), states how the **outputs should look like** and **to which subfolder they should be exported**, say **how the outputs should be named**, say to do the processing for **all .tif files in the folder**.

Ending: Lets you know when the processing is completed.

### Running in app-learning-mode

Open folder 4-pipeline and open the proper ILASTIK (has the .ilp suffix) pipeline for Alpha Sarc Act signal detection.

X	DAPI_ILASTIK.ilp	11/15/2023 9:26 PM	ilastik project	10,863 KB
X	PERCENTAGE_OF_CMs_CP_improved02.cpproj	11/15/2023 9:26 PM	CellProfiler Project	1,517 KB
X	README_AND_NOTES.docx	11/15/2023 9:26 PM	Office Open XML Do...	192 KB
X	SarcAct_ILASTIK.ilp	11/15/2023 10:55 PM	ilastik project	10,957 KB
X	universal_batch_to_be	Type: ilastik project Size: 10.6 MB	2023 9:26 PM	Windows Batch File 1 KB

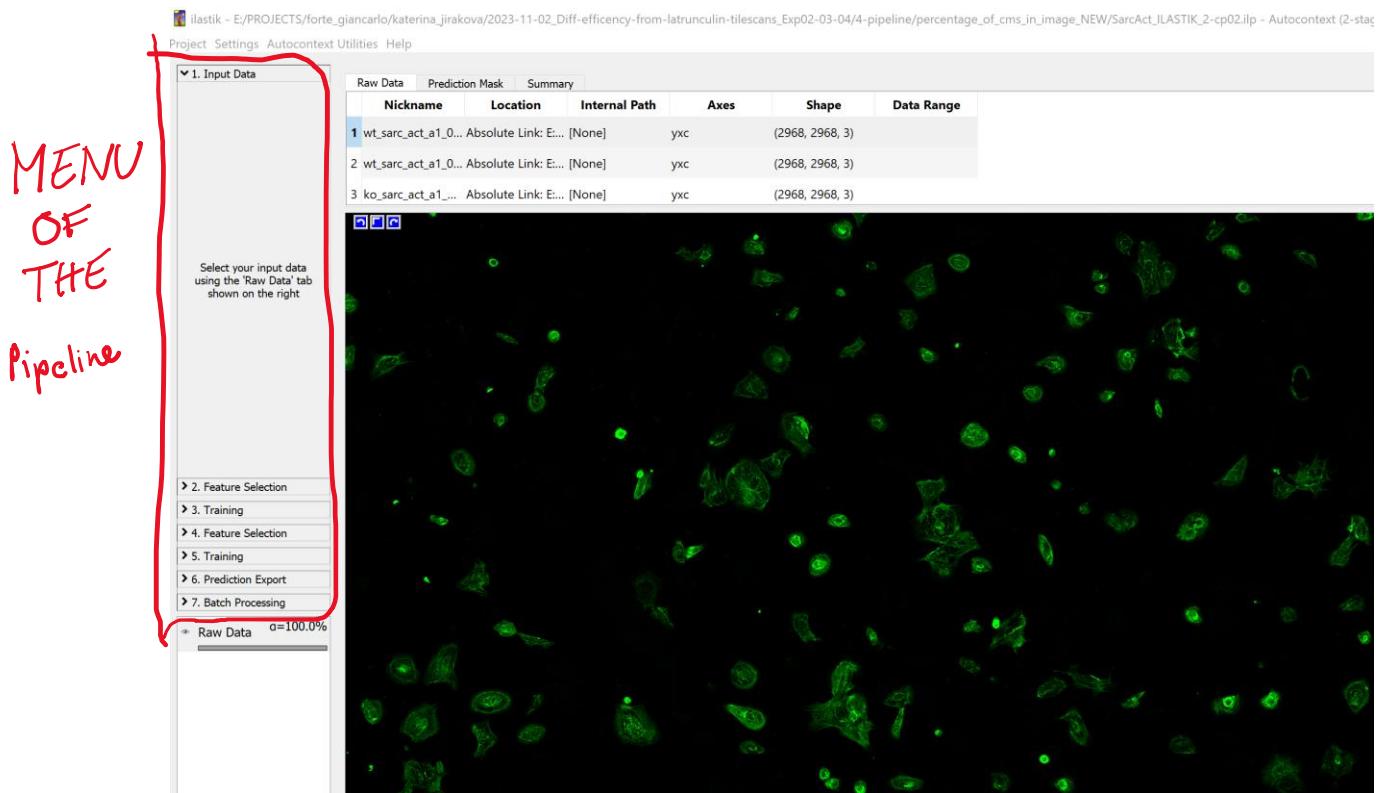
If you decide to change something in the pipeline using learning, keep in mind to:

- Do the learning in both learning phases (you will get better results than if you will do only the first phase)
- Switch off the life update mode before making changes (the computer will get stuck in case of bigger data extension).

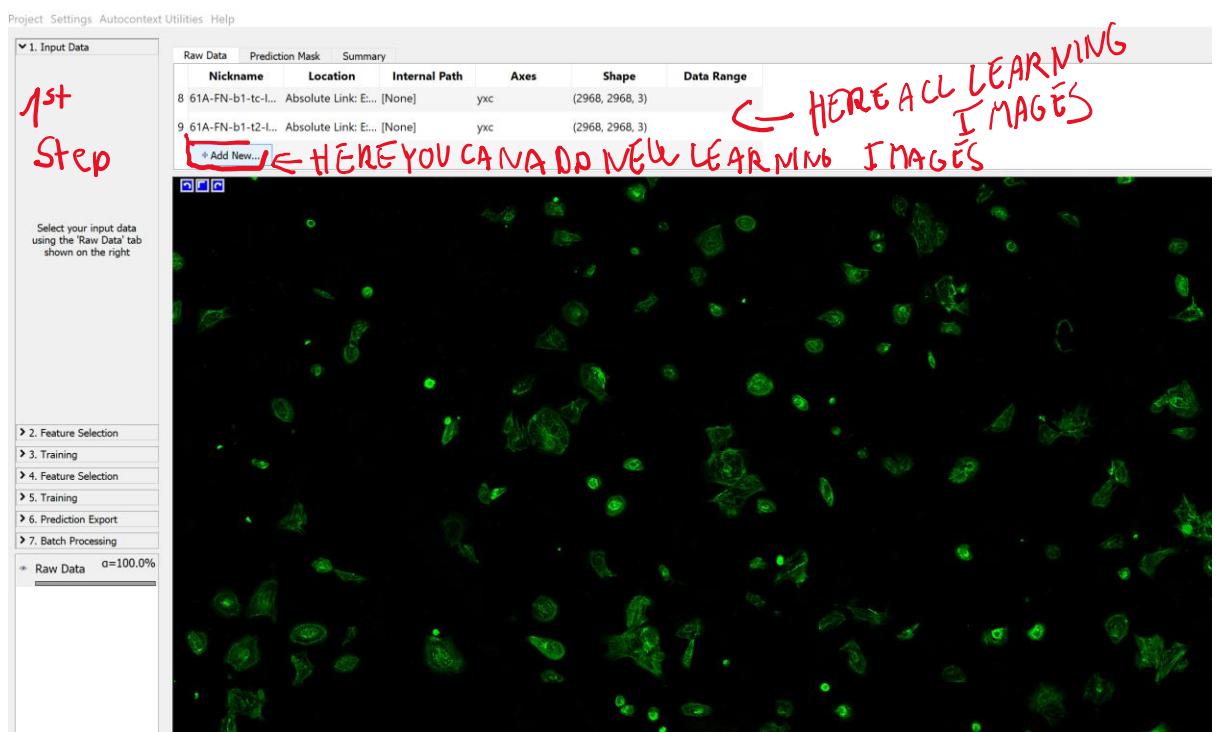
## Brief introduction to machine learning in ILASTIK

You can open ILASTIK pipeline directly by double clicking (there are no problems on ODEON shared computer with it as with CP – noted later).

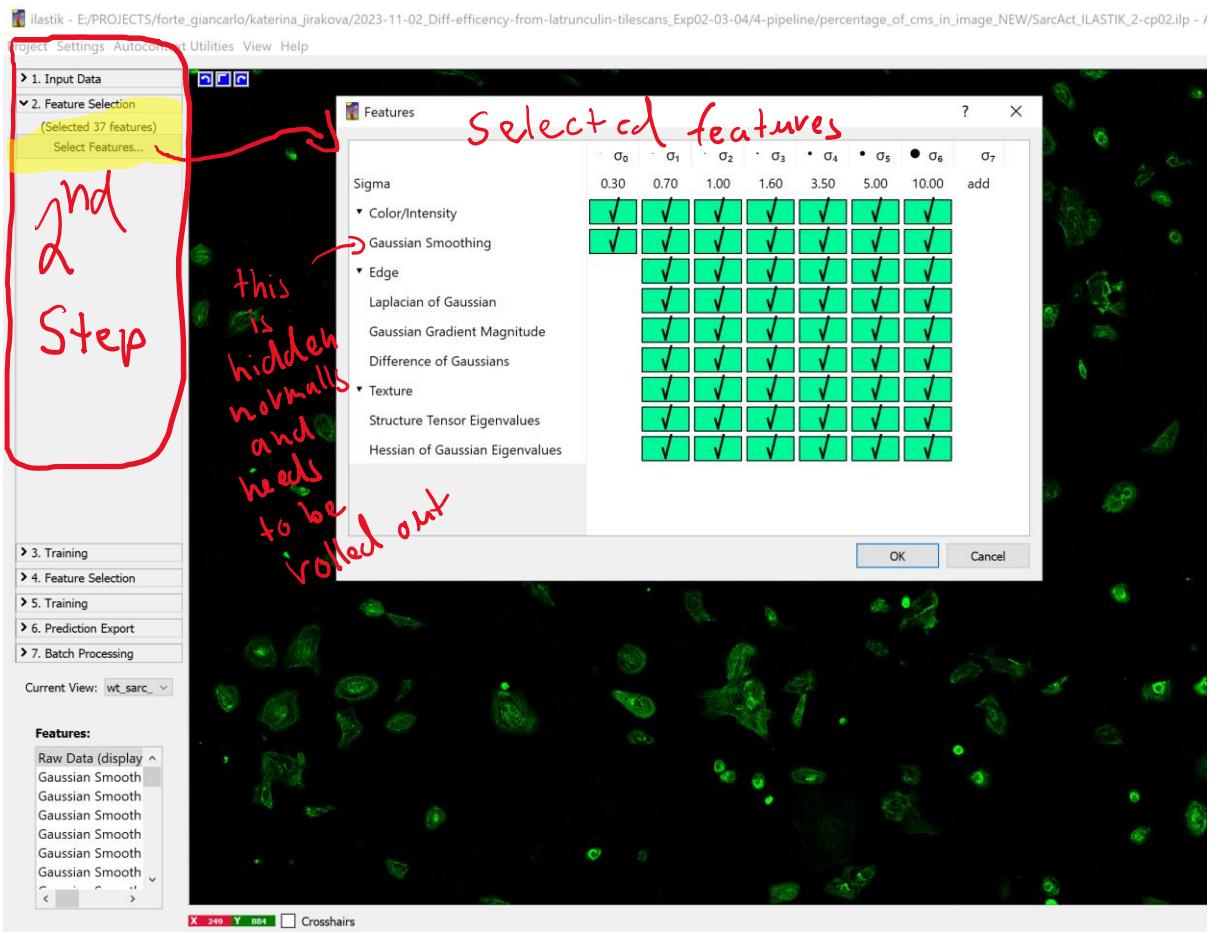
On the left side of your screen, you have the pipeline itself. You can move in between the steps simply by clicking.



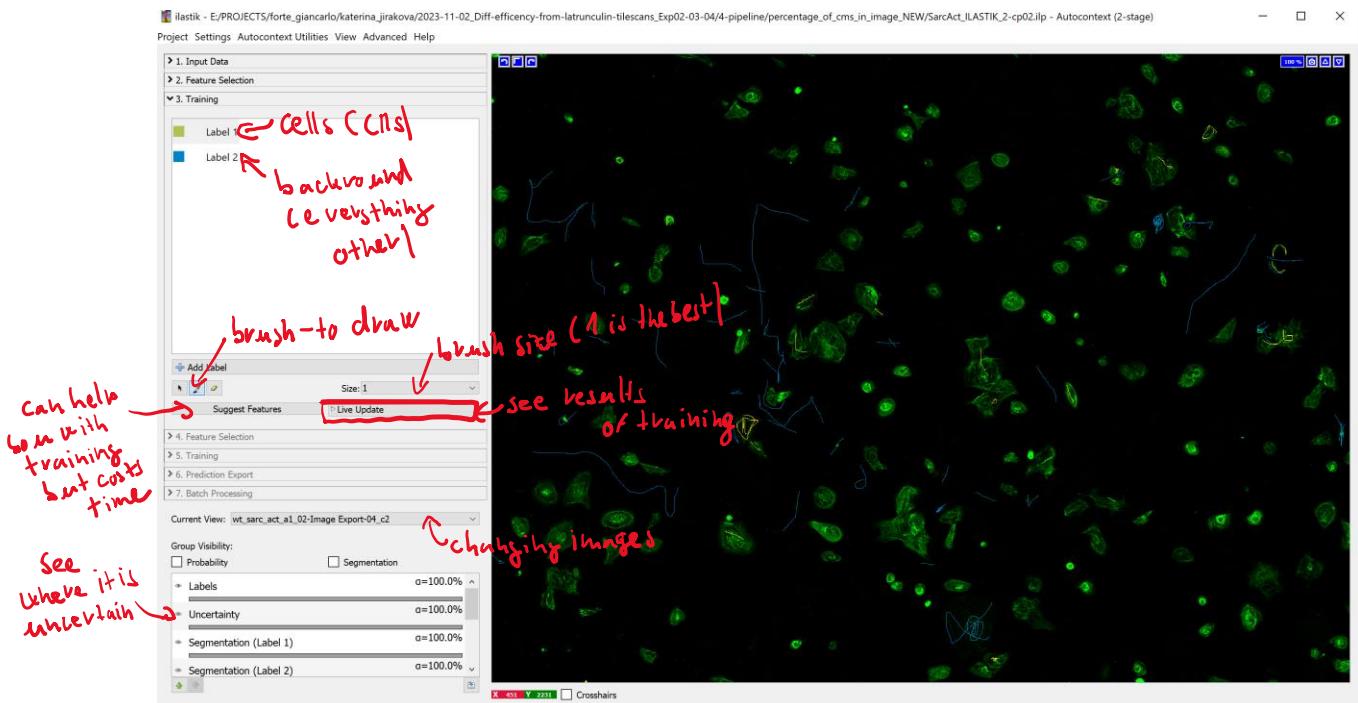
In the first step you see all uploaded learning images and you can also add new ones.



## Second step – Feature Selection – select features based on which the software will be learning.

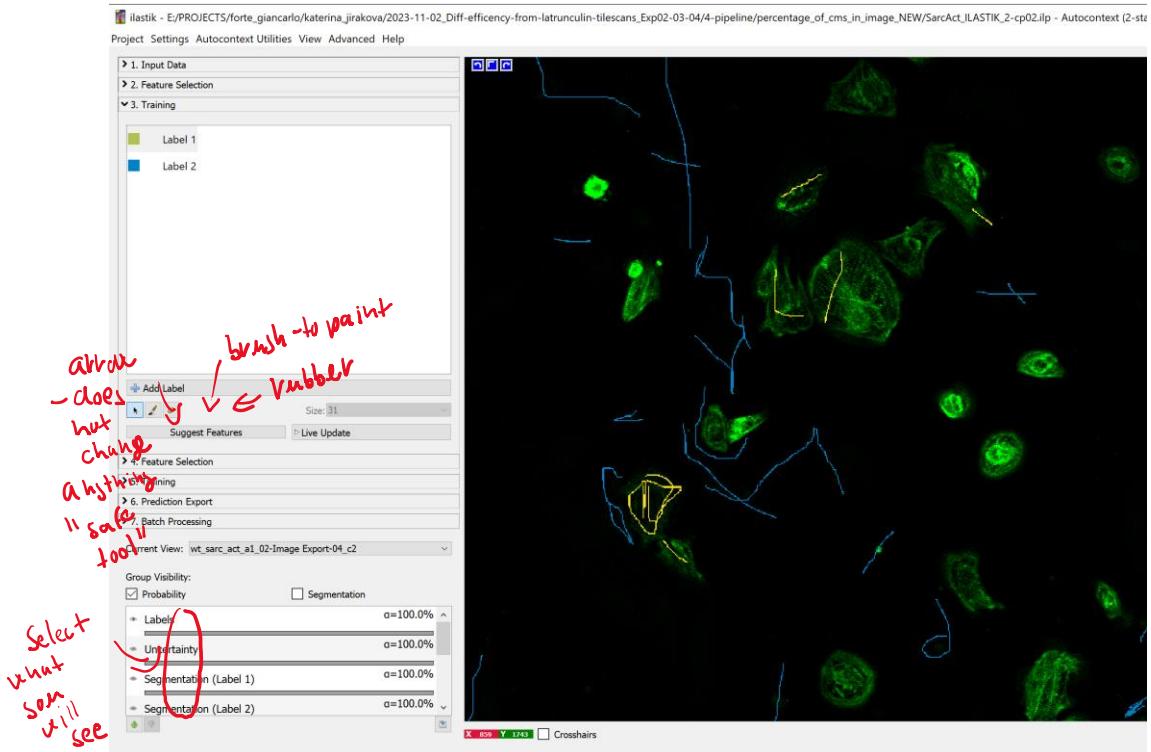


First learning phase. LABEL 1 (yellow = cells = Alpha Sarc Act signal); LABEL 2 = background = non CMs (very low Alpha Sarc Act signal), background)



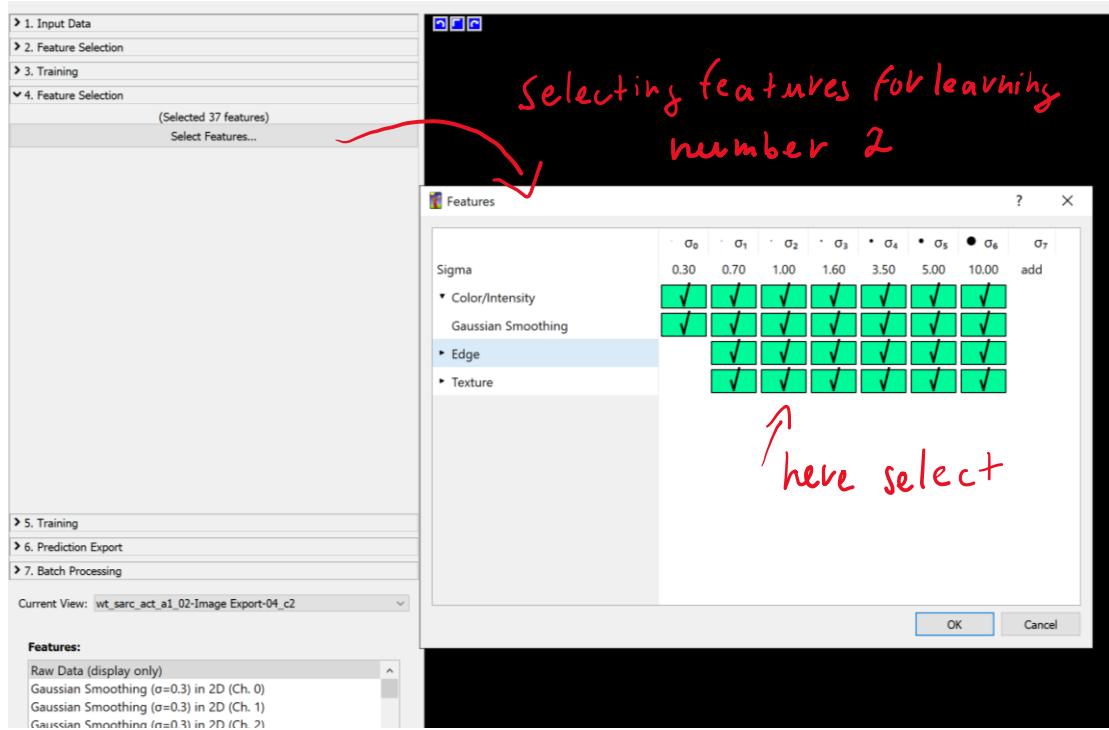
## How to use learning:

- **Brush** – learning simply by drawing what is yellow (foreground) and what is blue (background). Other categories can be easily added by clicking + Add label button if needed. If you click on the certain label button, you can also change the colour in which it displays.
- You switch on updating your changes to learning by clicking button **Live update** – if you train ILASTIK, I highly recommend to switch Live update off and switch it on again after you finish the training step to see the results and decide what you want to train next.
- **Uncertainty** – if you click on the eye icon next to the uncertainty, the software will display the areas of image regarding which it is not sure whether it should count them as foreground or background – so you can hence easily improve learning results.

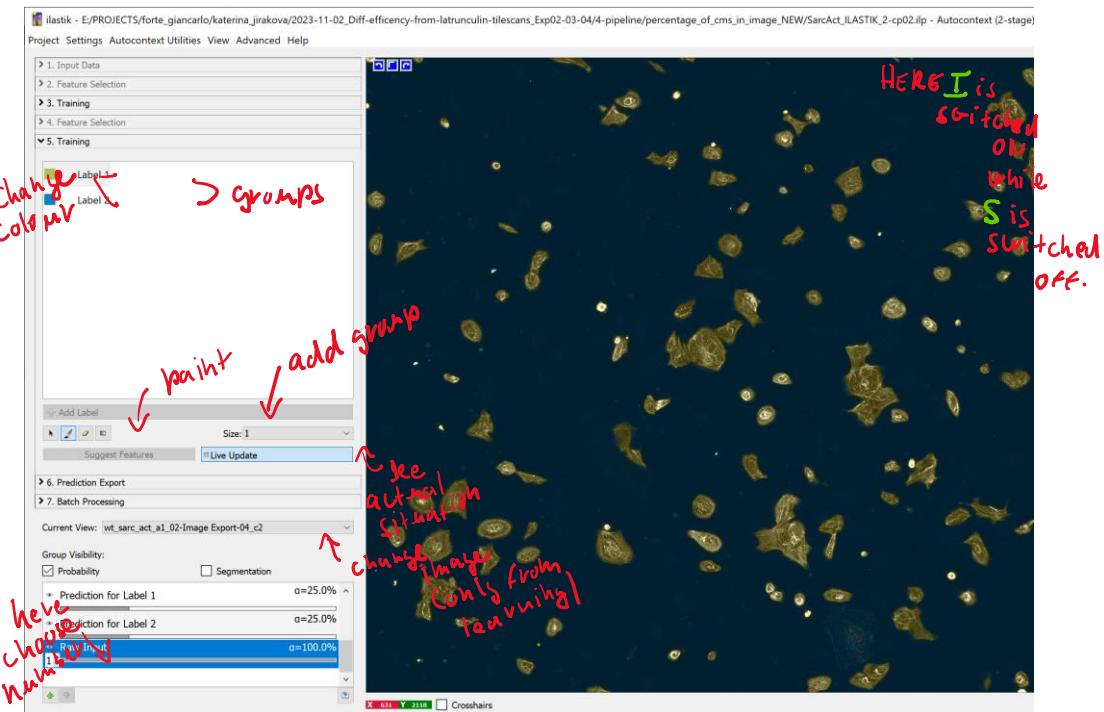


To see what you have drawn press letter i on your keyboard. If you have i switched on, you can also turn on binary mode by pressing letter s – if you want to go back press s again to move to the state where you see the lines and also what it counts as foreground and background but you still see the original image. If you do not want to see the results of learning now, press i again (you can do so even if you have s switched on).

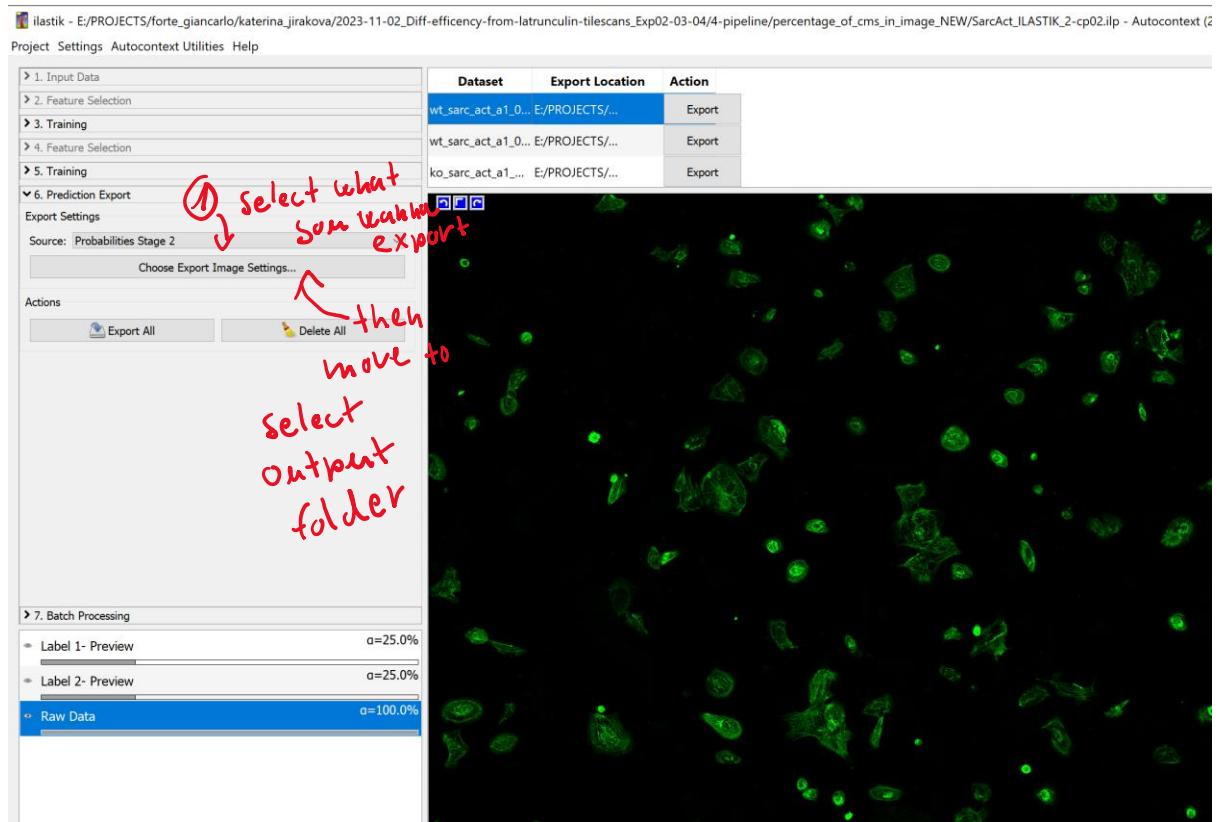
Selecting features no 2 – preparation for the learning step 02.



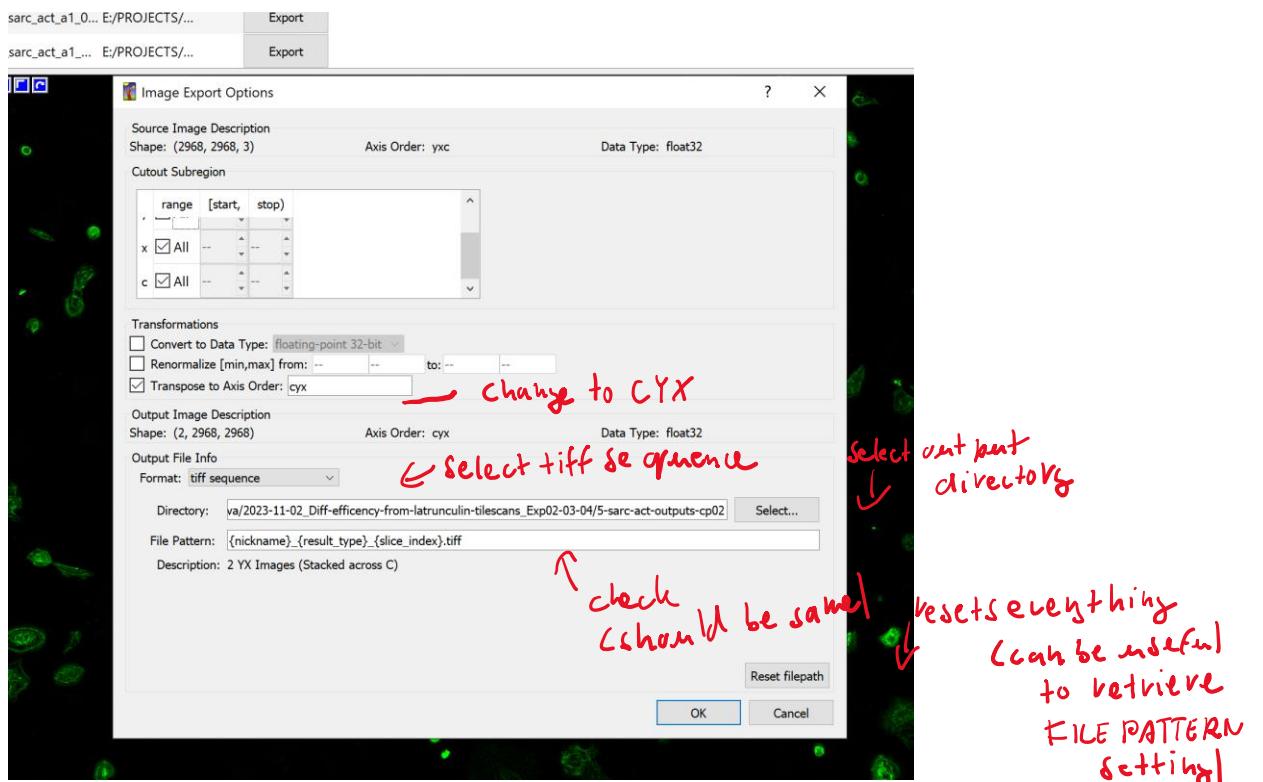
Training no 02 – functions almost the same as the previous training step. In the down left corner you move in between learning visualisations using raw input numbers – there is rolling menu through which you can go through and choose the best one option for you – I always use option **number 1** (selected in this print screen).



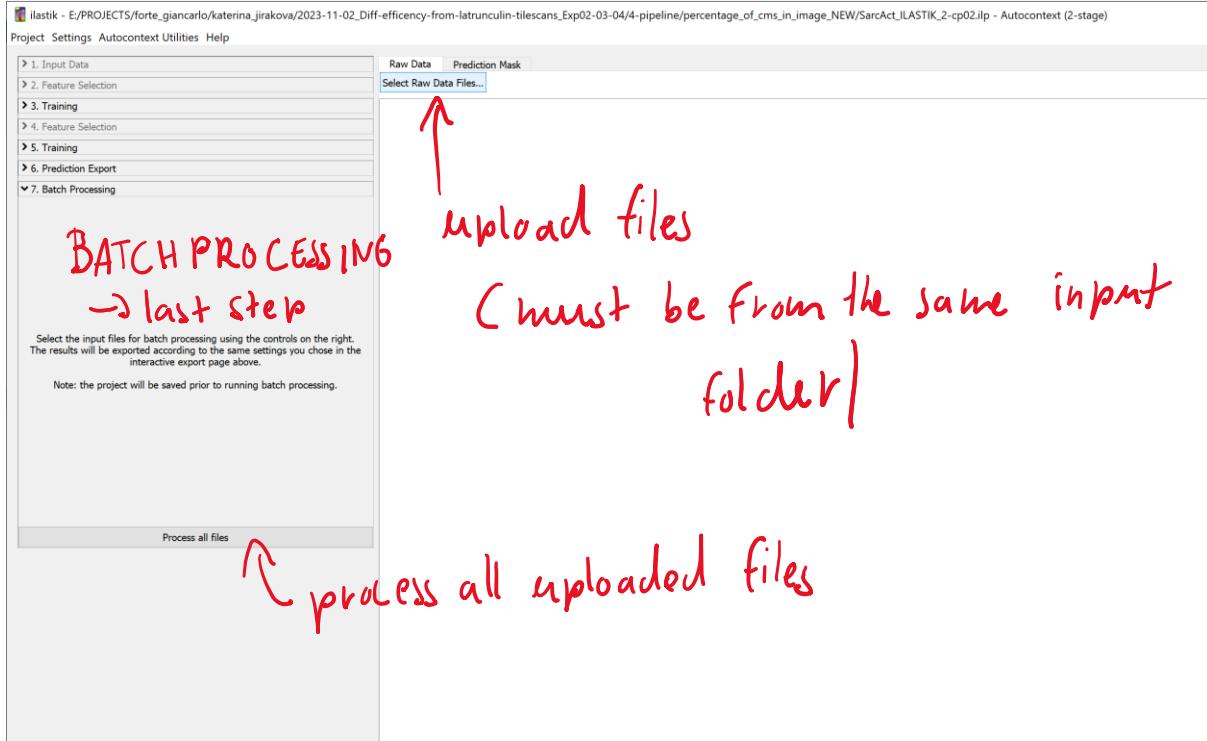
Next it is the time to find locations for your files. Choose **source** – I always export **Probabilities Stage 2** since with other outputs I had problems with analysis in CP software. Then move to “Choose Export Image Settings...” – then see the next image to continue.



Here please pay attention to have **AXIS ORDER changed to CYX**, **FORMAT to TIFF SEQUENCE**, **DIRECTORY – selected the one you want**, **FILE PATTERN the same as in this print screen** if exporting Probabilities Stage 2 (you can retrieve the pattern by clicking button RESET FILEPATH).



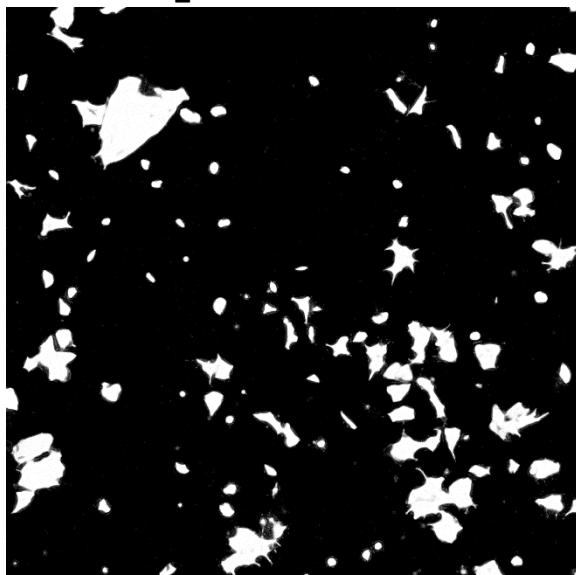
If you do not want to export from headless mode, then click on button **SELECT RAW DATA FILES** and select images you want to analyse using this pipeline (please remember that for one analysis all the images for batch processing **must be located in one input folder**). Once you feel ready press button **PROCESS ALL FILES** – takes a while in this mode.



If encounter any ambiguity, please see [well written supporting documentation on ILASTIK website](#). You can also download ILASTIK from this website.

How should your outputs approximately look like?

Probabilities \*\_0.tif



Probabilities \*\_1.tif



## *SORTING DATA FROM ALPHA SARC ACT PIPELINE – MAKING ORDER IN PROBABILITIES STAGE 2*

Move to your computer and open **Git Bash** (Git Bash is not installed on ODEON shared computer – if you wish you can use Windows Power Shell instead).

**Go to folder 5-sarc-act-outputs** (the folder you designated for Alpha Sarc Act Probabilities Stage 2 export).

**Create new subfolder called prob\_1.**

```
jirak@katerina_hp MINGW64 /y/katerina_j
$ mkdir ./prob_1
```

**Move all probabilities images ending with \_1 to the newly created folder.**

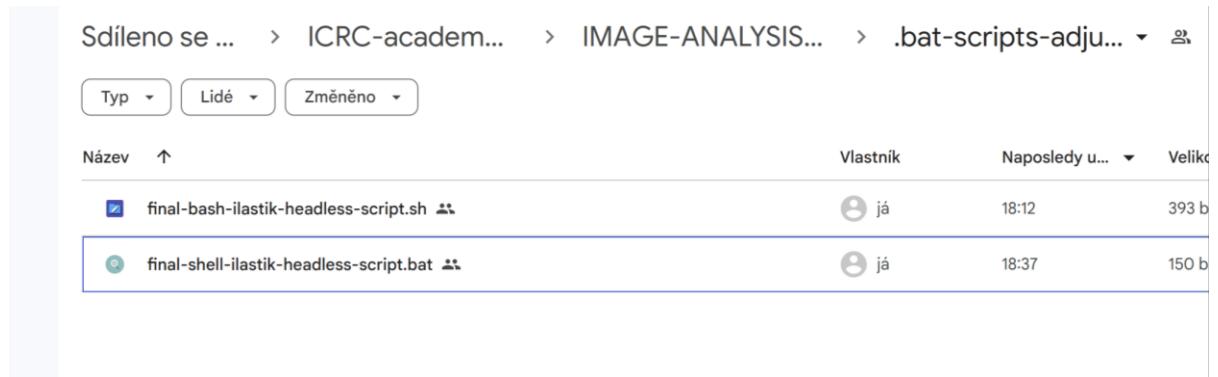
```
jirak@katerina_hp MINGW64 /y/katerina_jirakova/2023-11
$ for i in "*_1.tif"; do mv ${i} ./prob_1; done
```

## *PROCESSING DAPI IMAGES – GOING BACK TO ILASTIK WITH ANOTHER PIPELINE*

Open folder 4-pipeline and open the proper ILASTIK (.ilp) pipeline for DAPI signal detection.

You can run the pipeline headless (using BASH script (primarily) or using the Windows Power Shell script (also a possibility).

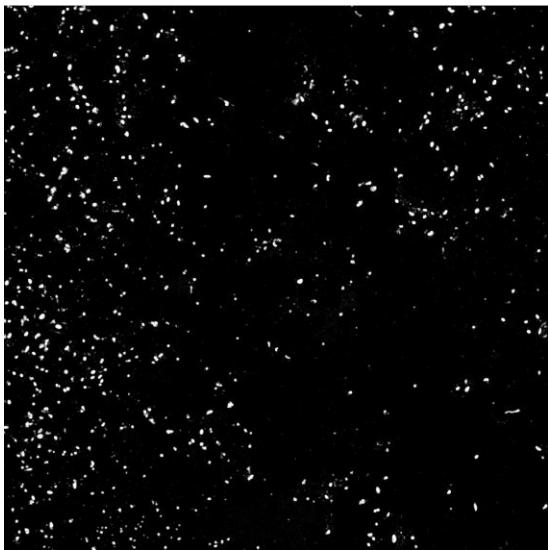
Pipelines are on Google Disk in this folder. For further information please see step “*PROCESSING ALPHA SARC ACT IMAGES – GETTING STARTED WITH ILASTIK*” section above.



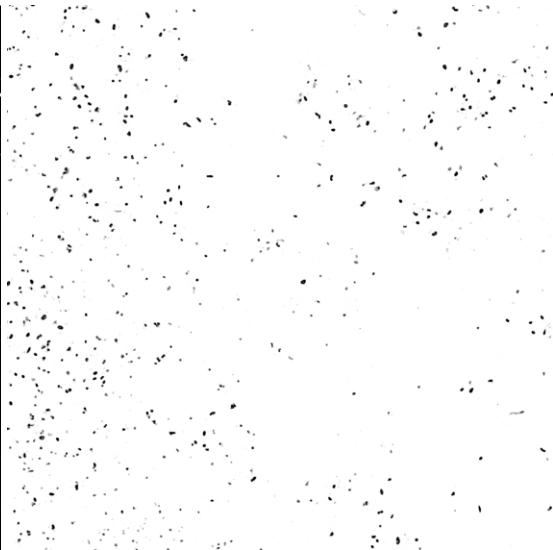
You can also process images in app-learning-mode. Please see above section **Brief introduction to machine learning in ILASTIK**. If you would like to find more about ILASTIK pipelines and processing (also in headless mode) please visit [this website](#). Really good seminar for ILASTIK is [this one](#). For the transition of images from ILASTIK to CP was the material for me [this great video](#).

How should your outputs approximately look like?

Probabilities \*\_0.tif



Probabilities \*\_1.tif



#### SORTING DATA FROM DAPI PIPELINE – MAKING ORDER IN PROBABILITIES STAGE 2

Apply steps from sorting data from Alpha Sarc Act pipeline on this data.

```
jirak@Katerina_hp MINGW64 /y/katerina_jirakova/2023
$ mkdir ./prob_1

jirak@Katerina_hp MINGW64 /y/katerina_jirakova/2023
$ for i in "*_1.tif"; do mv ${i} ./prob_1; done
```

#### FINAL ANALYSIS – PUTTING EVERYTHING TOGETHER – LET'S MOVE TO CP

Prepare folder 7-CP-FINAL-OUTPUTS for export:

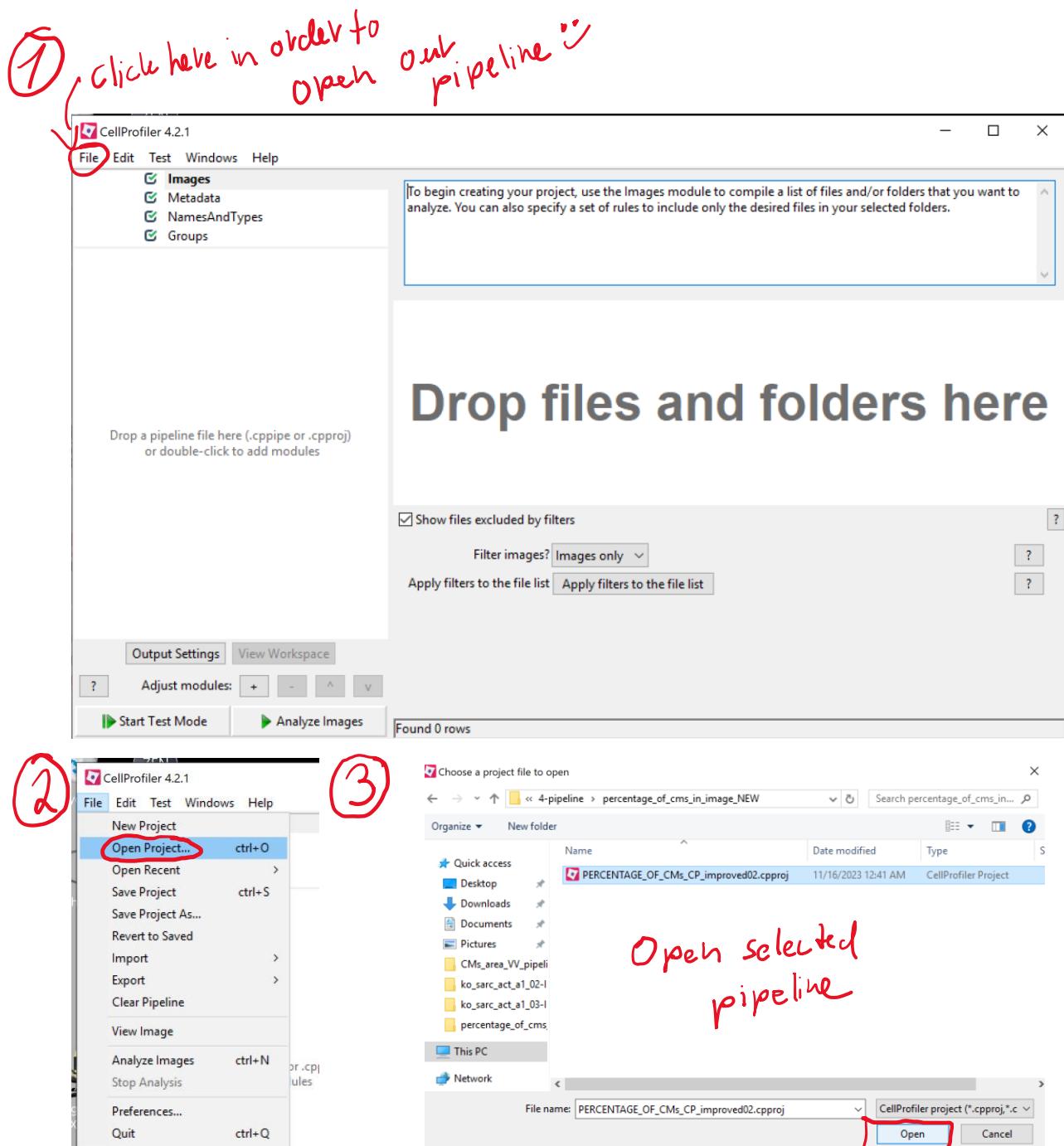
The screenshot shows a file explorer interface. At the top, there's a navigation bar with a path '2023-11-02\_Diff-efficiency-fr...' followed by a red box around the folder name '7-CP-FINAL-OUTPUTS'. Below the navigation bar is a search bar with the placeholder 'Search 7-CP-...'. The main area is a list of files and folders. A red arrow points from the red box down to the list. To the left of the list, there's handwritten text 'SUBFOLDERS TO BE CREATED' with a curly brace underlining '7-CP-FINAL-OUTPUTS' and the three subfolders listed below it. The list has columns: 'Name', 'Date modified', and 'Type'. The contents are:

Name	Date modified	Type
dapi_cm	11/16/2023 1:12 AM	File folder
dapi_overlay	11/16/2023 1:12 AM	File folder
final	11/16/2023 1:13 AM	File folder

Open pipeline in CP and load all your images.

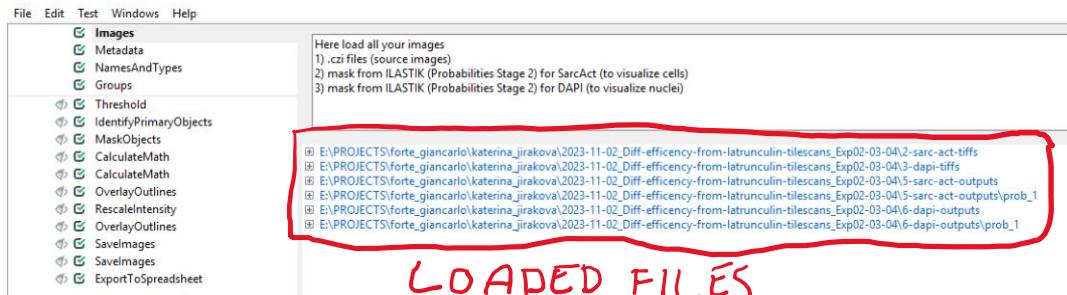
IMPORTANT NOTE: The CP on shared desktop is a bad moody and often makes ugly JAVA errors. To prevent it I highly recommend to first open CP and then from CP open the pipeline – otherwise it makes problems.

How to open project pipeline in CP.

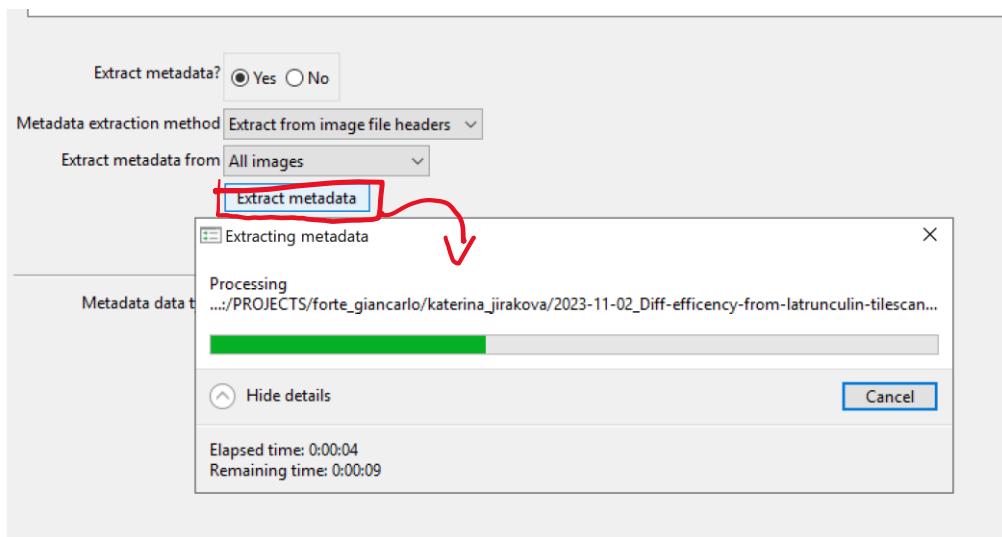


What files will you need to load for each image?

- **SarcAct tiffs**
- **DAPI tiffs**
- **Sarc Act probabilities**
- **DAPI probabilities**



Move to metadata and extract metadata from image file headers.



Next go to Names and types and update grouping.

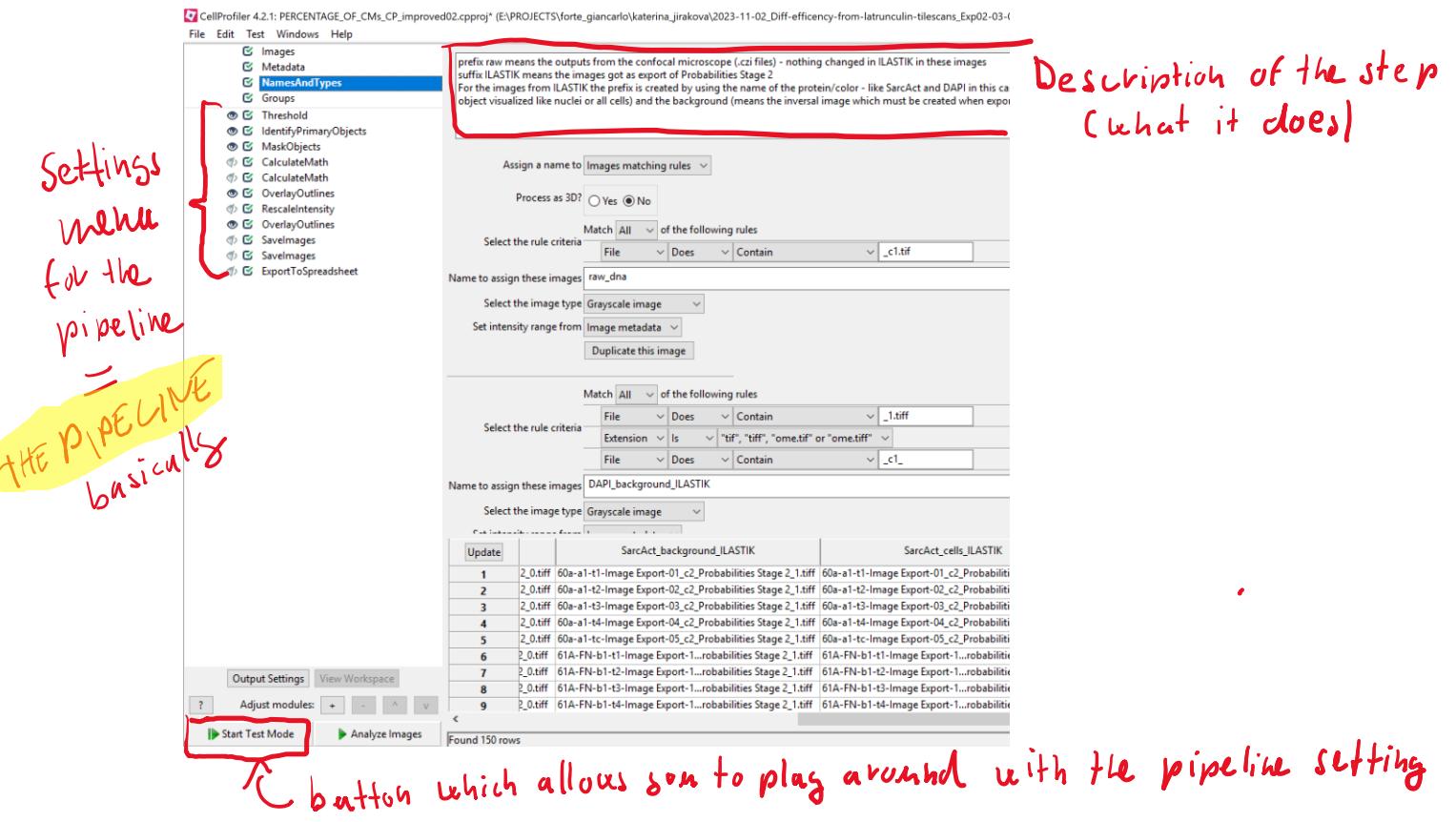
	<input checked="" type="button"/> Update	I_background_ILASTIK	DAPI_nuclei_ILASTIK	SarcAct_background_ILASTIK	SarcAct_cells_ILASTIK
1		port-01_c1_Probabilities Stage 2_1.tif	60a-a1-t1-Image Export-01_c1_Probabilities Stage 2_0.tif	60a-a1-t1-Image Export-01_c2_Probabilities Stage 2_1.tif	60a-a1-t1-Image Export-01_c2_Probabiliti
2		port-02_c1_Probabilities Stage 2_1.tif	60a-a1-t2-Image Export-02_c1_Probabilities Stage 2_0.tif	60a-a1-t2-Image Export-02_c2_Probabilities Stage 2_1.tif	60a-a1-t2-Image Export-02_c2_Probabiliti
3		port-03_c1_Probabilities Stage 2_1.tif	60a-a1-t3-Image Export-03_c1_Probabilities Stage 2_0.tif	60a-a1-t3-Image Export-03_c2_Probabilities Stage 2_1.tif	60a-a1-t3-Image Export-03_c2_Probabiliti
4		port-04_c1_Probabilities Stage 2_1.tif	60a-a1-t4-Image Export-04_c1_Probabilities Stage 2_0.tif	60a-a1-t4-Image Export-04_c2_Probabilities Stage 2_1.tif	60a-a1-t4-Image Export-04_c2_Probabiliti
5		port-05_c1_Probabilities Stage 2_1.tif	60a-a1-tc-Image Export-05_c1_Probabilities Stage 2_0.tif	60a-a1-tc-Image Export-05_c2_Probabilities Stage 2_1.tif	60a-a1-tc-Image Export-05_c2_Probabiliti
6		je Export-1...robabilities Stage 2_1.tif	61A-FN-b1-t1-Image Export-1...robabilities Stage 2_0.tif	61A-FN-b1-t1-Image Export-1...robabiliti	61A-FN-b1-t1-Image Export-1...robabiliti
7		je Export-1...robabilities Stage 2_1.tif	61A-FN-b1-t2-Image Export-1...robabilities Stage 2_0.tif	61A-FN-b1-t2-Image Export-1...robabiliti	61A-FN-b1-t2-Image Export-1...robabiliti
8		je Export-1...robabilities Stage 2_1.tif	61A-FN-b1-t3-Image Export-1...robabilities Stage 2_0.tif	61A-FN-b1-t3-Image Export-1...robabilities Stage 2_1.tif	61A-FN-b1-t3-Image Export-1...robabiliti
9		je Export-1...robabilities Stage 2_1.tif	61A-FN-b1-t4-Image Export-1...robabilities Stage 2_0.tif	61A-FN-b1-t4-Image Export-1...robabilities Stage 2_1.tif	61A-FN-b1-t4-Image Export-1...robabiliti

We have the following groups (6 in total):

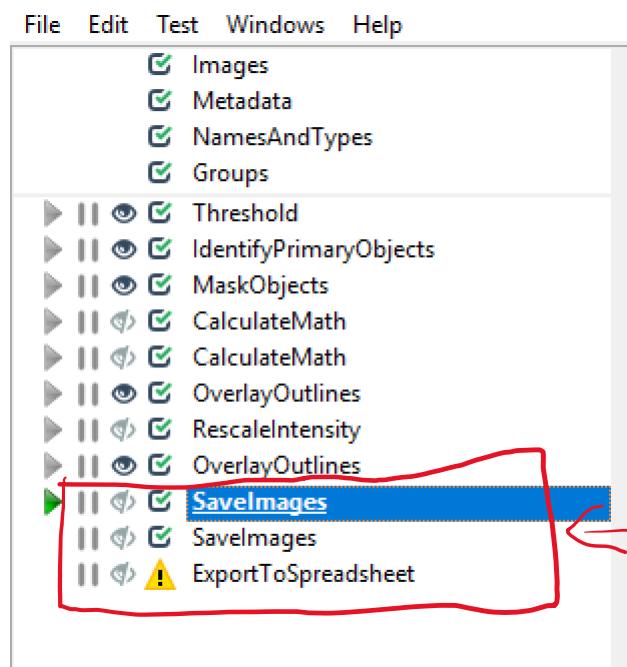
- **DAPI\_background\_ILASTIK** = DAPI probabilities \*\_1.tif
- **DAPI\_nuclei\_ILASTIK** = DAPI probabilities \*\_0.tif
- **SarcAct\_background\_ILASTIK** = SarcAct probabilities \*\_1.tif
- **SarcAct\_cells\_ILASTIK** = SarcAct probabilities \*\_0.tif
- **Raw\_dna** = DAPI tiffs (only Min/Max setting – just exported from Zenn)
- **Raw\_saract** = Alpha Sarc Act tiffs (only Min/Max setting – just exported from Zenn)

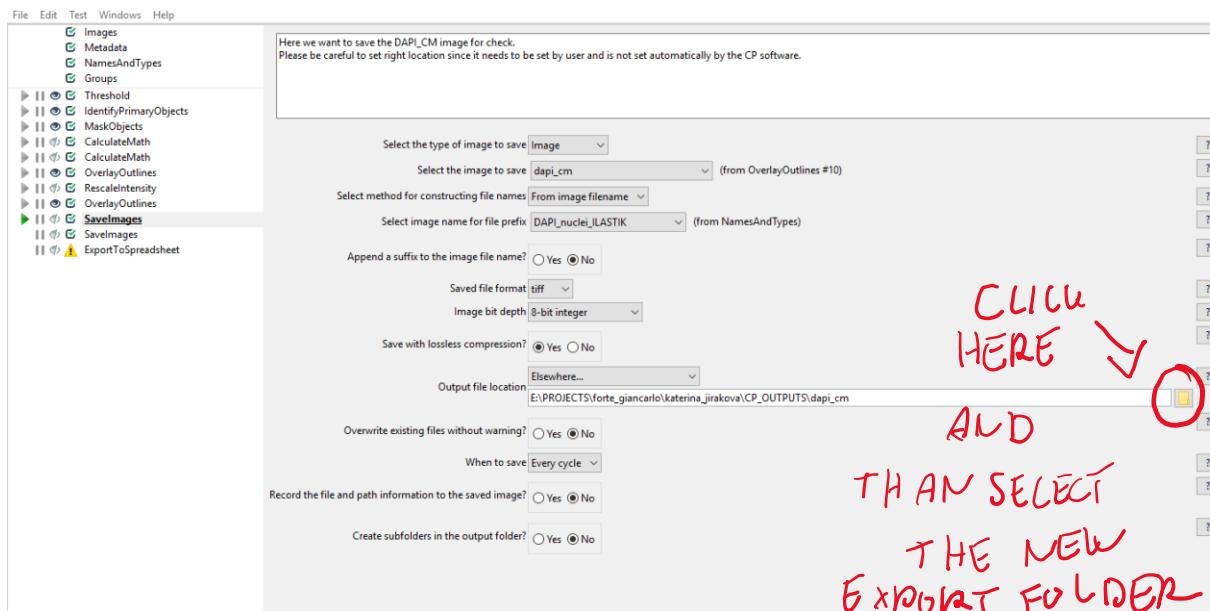
Ignore groups and skip to analysis.

If you want to play around with the setting you can do so in **TEST MODE** – by clicking the eye you will see the results after clicking on the arrow next to the icon of the item in the left menu column. Descriptions for every single step are available in the comments above the detailed setting of the step.

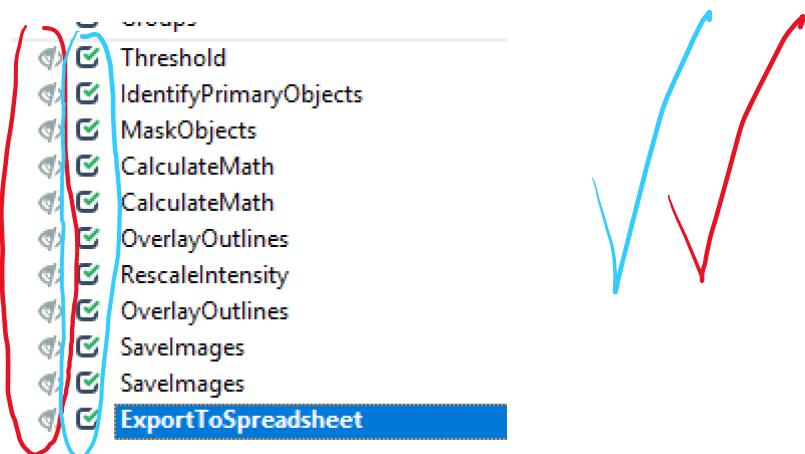


What you need to do is to **change folder addresses for export of images export and tables**.





**Switch off all eye icons** before escaping TEST MODE and starting ANALYSIS MODE – just not to be informed about every single image it analyses. **Check all the boxes are ticked** – means the step in the pipeline will be done.



Press the button **analyse images** and see the results in designated folders.

If you are not happy with the results try to improve threshold values in CP or add additional learning image to ILASTIK and show ILASTIK what is recognised badly.

## RESULTS – EXPLANATION FOR OUTPUT IMAGES

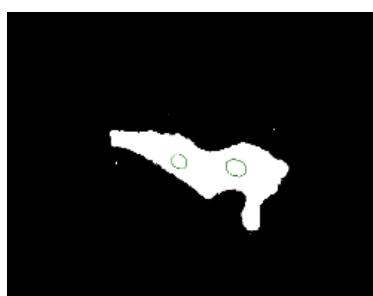
*SUB FOLDERS*

Name	Date modified	Type	Size
dapi_cm	16/11/2023 13:35	File folder	
dapi_overlay	16/11/2023 13:35	File folder	
final	16/11/2023 16:51	File folder	

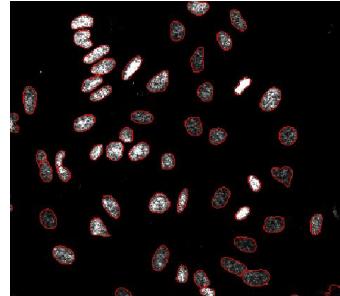
**DAPI\_CM** = nuclei visualised on the Alpha Sarc Act signal areas = visualisation of positive cells (dapi\_cm means dapi cardiomyocytes). Nuclei in green, Alpha Sarc Act signal in white.

**DAPI\_overlay** = visualisation of all recognised nuclei on DAPI rescaled image (we identified nuclei on Probabilities image from ILASTIK so it is a good check that we are not out of focus).

Illustrative crop from DAPI\_CM image.



Illustrative crop from DAPI\_overlay image.



## HOW TO FURTHER PROCESS DATA IN TABS – HOW EASILY FIND DATA FROM YOUR MEASUREMENTS

In folder 7-CP-FINAL-OUTPUTS open subfolder final.

dapi_cm	16/11/2023 13:35	File folder
dapi_overlay	16/11/2023 13:35	File folder
final	16/11/2023 13:35	File folder

In ./7-CP-FINAL-OUTPUTS/final you will find following tables (see the image below). You want to open chart **My\_Expt\_image.csv**.

MyExpt_dapi_cm	16/11/2023 13:35	Microsoft Excel Com...	128 KB
MyExpt_Experiment	16/11/2023 13:35	Microsoft Excel Com...	37 KB
MyExpt_IDENTIFIED_NUCLEI	16/11/2023 13:35	Microsoft Excel Com...	886 KB
MyExpt_Image	16/11/2023 13:35	Microsoft Excel Com...	78 KB

Here you are interested in **columns G and H**.

**Column G** contains values about number of **identified nuclei** which equals to the total number of nuclei in the image, whereas **column H** contains values called **dapi\_cm** – to simplify these are nuclei localised within the areas of Alpha Sarc Act signal (exactly nuclei which have at least 90 % of their area within the area of Alpha Sarc Act signal).

A	B	C	D	E	F	G	H	I	J	K
Channel_D	Channel_D	Channel_S	Channel_S	Channel_ra	Channel_ra	Count_IDENTIFIED_NUCLEI	Count_dapi_cm	ExecutionT	ExecutionT	ExecutionT
-1	-1	-1	-1	-1	-1	246	93	0	0	7.046875
-1	-1	-1	-1	-1	-1	181	55	0	0	16.04688
-1	-1	-1	-1	-1	-1	472	167	0	0	16.60938
-1	-1	-1	-1	-1	-1	451	133	0	0	16.14063
-1	-1	-1	-1	-1	-1	487	123	0	0	17.85938
-1	-1	-1	-1	-1	-1	943	52	0	0	18.92188
-1	-1	-1	-1	-1	-1	1094	38	0	0	17.4375
-1	-1	-1	-1	-1	-1	951	40	0	0	18.1875
0	-1	-1	-1	-1	-1	977	32	0	0	17.125
1	-1	-1	-1	-1	-1	2769	142	0	0	16.34375
2	-1	-1	-1	-1	-1	279	18	0	0	19.42188
3	-1	-1	-1	-1	-1	884	56	0	0	16.46875
4	-1	-1	-1	-1	-1	244	23	0.015625	0	15.98438
5	-1	-1	-1	-1	-1	462	25	0	0	17.17188
6	-1	-1	-1	-1	-1	1888	111	0	0	17.375
7	-1	-1	-1	-1	-1	381	106	0	0	17.64063
8	-1	-1	-1	-1	-1	316	73	0	0	15.46875
9	-1	-1	-1	-1	-1	394	141	0	0	17.4375
0	-1	-1	-1	-1	-1	369	123	0	0	17.5
1	-1	-1	-1	-1	-1	704	266	0	0	16.23438
2	-1	-1	-1	-1	-1	128	47	0	0	16.98438
3	-1	-1	-1	-1	-1	234	79	0	0	18.25
4	-1	-1	-1	-1	-1	192	88	0	0	17.4375
5	-1	-1	-1	-1	-1	130	52	0	0	17.59375
6	-1	-1	-1	-1	-1	512	274	0	0	16.42188

In order to get the percentage of differentiation efficiency in your culture **create** new chart called **differentiation-efficiency.csv** in the subfolder final. Then **select columns G and H and copy them** to the new chart.

A	B	C	D	E	F	G	H	I
Channel_D	Channel_D	Channel_S	Channel_S	Channel_ra	Channel_ra	Count_IDENTIFIED_NUCLEI	Count_dapi_cm	ExecutionT
1	-1	-1	-1	-1	-1	246	93	0
2	-1	-1	-1	-1	-1	181	55	0
3	-1	-1	-1	-1	-1	472	167	0
4	-1	-1	-1	-1	-1	451	133	0
5	-1	-1	-1	-1	-1	487	123	0
6	-1	-1	-1	-1	-1	943	52	0
7	-1	-1	-1	-1	-1	1094	38	0
8	-1	-1	-1	-1	-1	951	40	0
9	-1	-1	-1	-1	-1	977	32	0
0	-1	-1	-1	-1	-1	2769	142	0
1	-1	-1	-1	-1	-1	279	18	0
2	-1	-1	-1	-1	-1	884	56	0
3	-1	-1	-1	-1	-1	244	23	0.015625
4	-1	-1	-1	-1	-1	462	25	0
5	-1	-1	-1	-1	-1	1888	111	0
6	-1	-1	-1	-1	-1	381	106	0
7	-1	-1	-1	-1	-1	316	73	0
8	-1	-1	-1	-1	-1	394	141	0
9	-1	-1	-1	-1	-1	369	123	0
0	-1	-1	-1	-1	-1	704	266	0
1	-1	-1	-1	-1	-1	128	47	0
2	-1	-1	-1	-1	-1	234	79	0
3	-1	-1	-1	-1	-1	192	88	0
4	-1	-1	-1	-1	-1	130	52	0
5	-1	-1	-1	-1	-1	512	274	0

**Copy also one column which contains the names of the images.**

When done with copying count it in EXCEL (as following). Change the **format** of all cells in **column D** to **percentage with 2 digits**.

	A	B	C	D
1	FileName_DAPI_nuclei_ILASTIK	Count_IDENTIFIED_NUCL	Count_dapi_cm	PERCENTAGE OF CMs
2	60a-a1-t1-Image Export-01_c1_Probabilities S	246	93	=\$C2/\$B2
3	60a-a1-t2-Image Export-02_c1_Probabilities S	181	55	
4	60a-a1-t3-Image Export-03_c1_Probabilities S	472	167	
5	60a-a1-t4-Image Export-04_c1_Probabilities S	451	133	
6	60a-a1-tc-Image Export-05_c1_Probabilities S	487	123	
7	61A-FN-b1-t1-Image Export-10_c1_Probabiliti	943	52	
8	61A-FN-b1-t2-Image Export-11_c1_Probabiliti	1094	38	
9	61A-FN-b1-t3-Image Export-12_c1_Probabiliti	951	40	
10	61A-FN-b1-t4-Image Export-13_c1_Probabiliti	977	32	
11	61A-FN-b1-tc-Image Export-14_c1_Probabiliti	2769	142	
12	61A-MG-A1-t1-Image Export-06_c1_Probabilit	279	18	
13	61A-MG-A1-t2-Image Export-07_c1_Probabilit	884	56	
14	61A-MG-A1-t3-Image Export-15_c1_Probabilit	244	23	
15	61A-MG-A1-t4-Image Export-08_c1_Probabilit	462	25	
16	61A-MG-A1-tc-Image Export-09_c1_Probabilit	1888	111	
17	61B-FN-B1-t1-Image Export-16_c1_Probabiliti	381	106	
18	61B-FN-B1-t2-Image Export-17_c1_Probabiliti	316	73	
19	61B-FN-B1-t3-Image Export-18_c1_Probabiliti	394	141	
20	61B-FN-B1-t4-Image Export-19_c1_Probabiliti	369	123	
21	61B-FN-B1-tc-Image Export-20_c1_Probabiliti	704	266	
22	61B-MG-A1-t1-Image Export-21_c1_Probabilit	128	47	
23	61B-MG-A1-t2-Image Export-22_c1_Probabilit	234	79	
24	61B-MG-A1-t3-Image Export-23_c1_Probabilit	192	88	
25	61B-MG-A1-t4-Image Export-24_c1_Probabilit	130	52	
26	61B-MG-A1-tc-Image Export-25_c1_Probabilit	512	274	
27				

Copy equation so as to other cells to calculate the percentage.

	A	B	C	D
1	FileName_DAPI_nuclei_ILASTIK	Count_IDENTIFIED_NUCL	Count_dapi_cm	PERCENTAGE OF CMs
2	60a-a1-t1-Image Export-01_c1_Probabilities S	246	93	37.80%
3	60a-a1-t2-Image Export-02_c1_Probabilities S	181	55	30.39%
4	60a-a1-t3-Image Export-03_c1_Probabilities S	472	167	35.38%
5	60a-a1-t4-Image Export-04_c1_Probabilities S	451	133	29.49%
6	60a-a1-tc-Image Export-05_c1_Probabilities S	487	123	25.26%
7	61A-FN-b1-t1-Image Export-10_c1_Probabiliti	943	52	5.51%
8	61A-FN-b1-t2-Image Export-11_c1_Probabiliti	1094	38	3.47%
9	61A-FN-b1-t3-Image Export-12_c1_Probabiliti	951	40	4.21%
10	61A-FN-b1-t4-Image Export-13_c1_Probabiliti	977	32	3.28%
11	61A-FN-b1-tc-Image Export-14_c1_Probabiliti	2769	142	5.13%
12	61A-MG-A1-t1-Image Export-06_c1_Probabilit	279	18	6.45%
13	61A-MG-A1-t2-Image Export-07_c1_Probabilit	884	56	6.33%
14	61A-MG-A1-t3-Image Export-15_c1_Probabilit	244	23	9.43%
15	61A-MG-A1-t4-Image Export-08_c1_Probabilit	462	25	5.41%
16	61A-MG-A1-tc-Image Export-09_c1_Probabilit	1888	111	5.88%
17	61B-FN-B1-t1-Image Export-16_c1_Probabiliti	381	106	27.82%
18	61B-FN-B1-t2-Image Export-17_c1_Probabiliti	316	73	23.10%
19	61B-FN-B1-t3-Image Export-18_c1_Probabiliti	394	141	35.79%
20	61B-FN-B1-t4-Image Export-19_c1_Probabiliti	369	123	33.33%
21	61B-FN-B1-tc-Image Export-20_c1_Probabiliti	704	266	37.78%
22	61B-MG-A1-t1-Image Export-21_c1_Probabilit	128	47	36.72%
23	61B-MG-A1-t2-Image Export-22_c1_Probabilit	234	79	33.76%
24	61B-MG-A1-t3-Image Export-23_c1_Probabilit	192	88	45.83%
25	61B-MG-A1-t4-Image Export-24_c1_Probabilit	130	52	40.00%
26	61B-MG-A1-tc-Image Export-25_c1_Probabilit	512	274	53.52%
27				
28				

Now you have counted the percentage per image – it's time to divide the images in groups following the experiments. Let's create new sheet in our chart.

7	61B-FN-B1-t1-Image Export-16_c1_Probabiliti	381	:
8	61B-FN-B1-t2-Image Export-17_c1_Probabiliti	316	:
9	61B-FN-B1-t3-Image Export-18_c1_Probabiliti	394	:
10	61B-FN-B1-t4-Image Export-19_c1_Probabiliti	369	:
11	61B-FN-B1-tc-Image Export-20_c1_Probabiliti	704	:
12	61B-MG-A1-t1-Image Export-21_c1_Probabilit	128	
13	61B-MG-A1-t2-Image Export-22_c1_Probabilit	234	
14	61B-MG-A1-t3-Image Export-23_c1_Probabilit	192	
15	61B-MG-A1-t4-Image Export-24_c1_Probabilit	130	
16	61B-MG-A1-tc-Image Export-25_c1_Probabilit	512	
17			
18			
19			
20			

Current sheet  
↓  
images

Newly created sheet  
↓  
experiments

On experiments sheet divide values in groups regarding the experiments and get the results.

A	B	C	D	E	F
60a		61A-FN	61A-MG	61B-FN	61B-MG
37.80%		5.51%	6.45%	27.82%	36.72%
30.39%		3.47%	6.33%	23.10%	33.76%
35.38%		4.21%	9.43%	35.79%	45.83%
29.49%		3.28%	5.41%	33.33%	40.00%
25.26%		5.13%	5.88%	37.78%	53.52%
<b>DIF EFFICIENCY</b>	<b>31.66%</b>	<b>4.32%</b>	<b>6.70%</b>	<b>31.56%</b>	<b>41.97%</b>
)					

You can also add statistics for WT and KO differentiation efficiency separately.

Another example how you can process:

A	B	C	D	E	F
Count_IdentifierPrimaryObjects	Count_dapi_cm	PERCENTAGE_OF_CMs	FileName_DNA		
358	200	55,87%	ko_sarc_act_a1_01-Image Export-01_c1.tif		
326	209	64,11%	ko_sarc_act_a1_02-Image Export-02_c1.tif		
448	323	72,10%	ko_sarc_act_a1_03-Image Export-03_c1.tif		
713	356	49,93%	wt_sarc_act_a1_01-Image Export-04_c1.tif		
1230	580	47,15%	wt_sarc_act_a1_02-Image Export-05_c1.tif		
<b>KO PERCENTAGE</b>	<b>WT PERCENTAGE</b>				
64,02%	48,54%				

MANUAL ENDS.

