U-Net based ZO-1 image processing for advanced immunofluorescence analysis - end user documentation

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Contents

Chapter 1

Introduction

Based on the idea to compute unbiased measurements for immunofluorescence images a multi-step image processing pipeline was developed. This system replaced the error-prone and time-consuming analysis by hand that couldn't provide numerical values for the image characteristics. The methods can be used to evaluate immunofluorescence images stained with antibodies against ZO-1. This documentation should serve as a step-by-step instruction to process and analyze own image-datasets. For more detailed information of the methods and the theoretical background please have a look publication and the source code.

To better understand the whole system the following text gives brief explanations about the individual processing steps. First, the image capturing takes place. The previously stained microscope preparations are captured and saved as individual files. The second step is the image segmentation using a U-Net deep learning network. This step is executed in ImageJ/Fiji and detects the relevant image components. The third and fourth step are run in Mathematica. It performs the modeling algorithms and the measuring of the image components. Afterwards, the images can be divided into individual groups (e.g. genotype or different treatment) and the gathered data is statistically quantified and visualized.

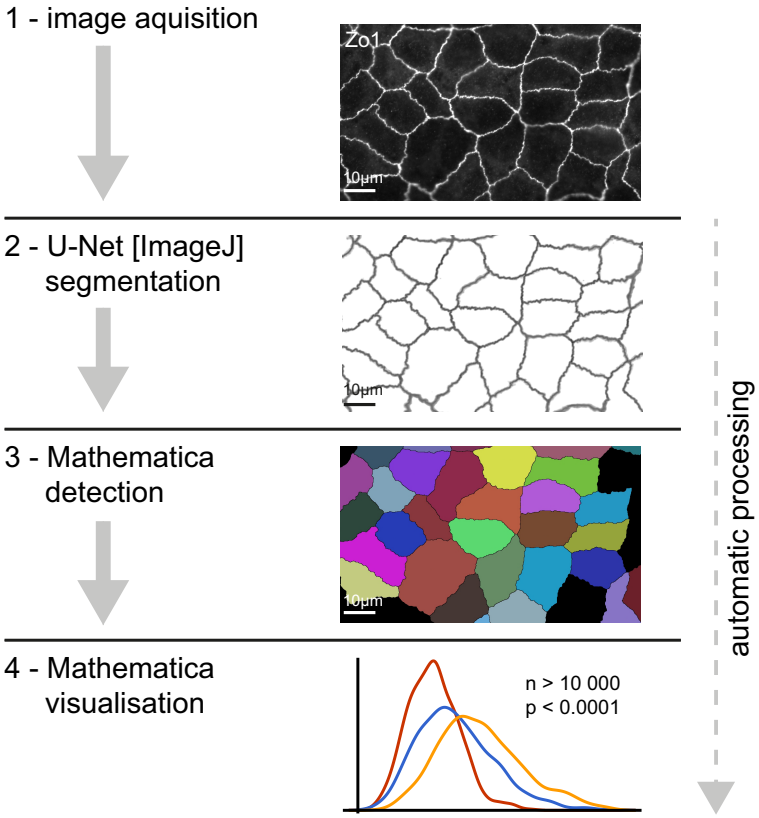


Figure 1.1: Processing steps of the whole workflow

Chapter 2

Preparation

All necessary files, portable software and documentation can be found on github:

They should be copied to the storage of each local machine to avoid problems. Please read the whole documentation carefully.

Chapter 3

Image acquisition and preprocessing

This chapter covers all relevant information for image capturing with immunofluorescence microscopes and the necessary file naming and folder structure for the automatic image pipeline.

3.1 Capturing process

For the published work the images were captured with a Carl Zeiss Axio Observer Z.1 microscope. To avoid problems with later processing steps preferably use similar settings:

- objective: Objektiv i Plan-Apochromat 63x/1.4 Oil DIC M27
- optovar: 1x Tubelens
- exposure time: manually set and consistent for each dataset
- file format: Carl Zeiss Image (*.czi)

This will result in following image dimensions:

- image size (pixel): 1388 x 1040
- image size (scaled): 142.10µm x 106,48µm
- scaling: 0.1024µm/pixel

Preparations with multiple staining's and different wavelengths can be captured in one step and saved to one file; the wavelength channels can later be separated from each other. If necessary, different focus plains can be captured and saved to individual files. For file naming please see section 3.2.

Based on the preparation condition the image quality may vary and affect the processing. Especially some of the ZO-1 antibodies produce weak stainings and image capturing can be quite a challenge. The best possible preparations and image sections should be saved. However, cherry picking should be avoided. The strength of the automatic image processing lies in the large number of images and big datasets. The user introduced bias should be as low as possible. Also, the focus point should be exact on the relevant structures. Out of focus image parts might be ignored in the automatic processing or lead to incorrect results.

If you use other microscope vendors the file format may change. Larger adaptions to the ImageJ script and the U-Net processing may be necessary.

3.1.1 Special Case: stitched images

Stitched images can be captured. They consist of multiple contiguous image tiles. This increases the amount of data in a single image and reduces the selection bias. The number of image tiles can usually be selected in the microscope software and is automatically captured. Stitched images in 3x3 configuration appeared to show the best results regarding capture time, processing speed and information gain. Other configurations up 10x10 are possible without difficulty.

The remaining microscope configurations, as described above, can stay the same. The image size varies depending on the number of tiles. The scaling 0.102µm/pixel should stay the same or will otherwise be rescaled be the U-Net.

3.2 File naming and folder structure

Most of the scripts and software expect an exact folder name and will interrupt, if something variate. Therefore, it is extremely important to use a consistent naming scheme. The following rules should be convenient to organize the files and are essential for the whole processing.

3.2.1 Folder names and structure

This is an example how the folder-tree should look like:

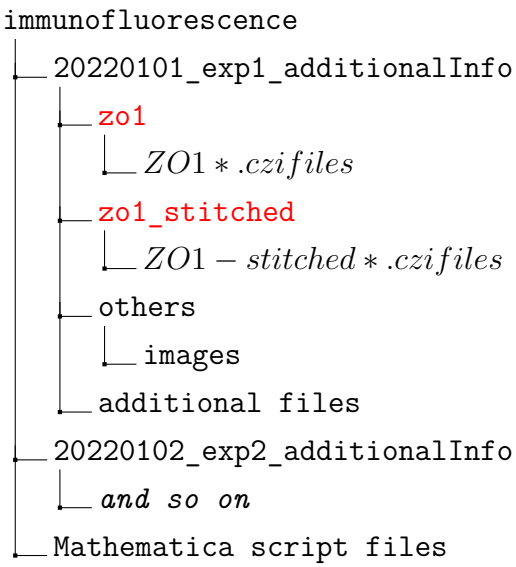


Figure 3.1: Foldertree

All Folders marked in red need to have the exact name, otherwise the scripts won´t work. Other folders can be individually changed by the user. Long folder names can be a problem one some

operating system and should be avoided!

For each experiment, dataset or staining an own folder can be created. These folders can begin with the date in the style YYYYMMDD for easy identification and sorting. Additional information can be added to this folder name. For stitched images the suffix '_stitched' needs to be added to the folder name (e.g. dapi_stitched). The file names can be individually chosen.

Additional files (for example: text- or excel-files) can be stored in the folders. They are ignored during processing.

Chapter 4

U-Net segmentation

This chapter lists all steps necessary to use the U-Net segmentation via ImageJ. To avoid the multiple pitfalls that might interrupt the processing the following chapter should be studied very carefully.

4.1 Initial Preparation

The technical implementation of the U-Net deep learning requires two components: A Server running the necessary deep learning framework and performing the segmentation step of the images. Furthermore, another computer running ImageJ as a client software to communicate with the server and sending the image files. ImageJ can be run on nearly any computer.

4.2 U-Net Server

The details on setting up the U-Net server are slightly complicated and will not be discussed at this point. Please have a look at [2] and [3].

4.3 ImageJ/Fiji

ImageJ is a widely used software for medical and scientific image editing and analysis. Fiji is an adapted version of ImageJ with an extended set of plugins. For the U-Net client implementation Fiji with the U-Net plugin is used in the following version, updated versions of ImageJ should in theory work without problems.

- Fiji ImageJ 1.53b + Java 1.8.0_212
- U-Net Plugin v20190926220201
- HDF5_Vibez Plugin v20150214134118

4.4 First use on new Client machine

On every new client computer/user account a few basic settings need to be configured when using ImageJ for the first time.

4.4.1 Delete ImageJ configuration

To avoid problems with older ImageJ versions and settings the configuration file should be deleted.

1. On windows machines move to following folder: C:/Users/Username/.imagej/
2. Delete the 'IJ_Prefs.txt' file

4.4.2 Bio-Formats configuration

The Bio-Formats plugin handles the loading and pre-processing of microscope image files. For the Zeiss 'czi-files' a few specific adjustments need to be made for automatic processing.

1. Open ImageJ
2. Click on 'File' → 'Open' and open a random czi-file. This should open the import options dialogue window
3. Select the options as shown in fig. 4.1. Especially 'Split channels' is important
4. Click ok and close the opened image
5. Click on 'Plugins' → 'Bio-Formats' → 'Bio-Formats Plugins Configuration'
6. Click on the 'Formats' tab and select 'Zeiss CZI' in the list
7. Check all boxes as shown in fig. 4.2. This will hide the import options dialogue window when opening a file

4.4.3 U-Net plugins settings

The U-Net plugin handles the connection to the remote U-Net server and the transfer of the image files opened for segmentation. Before using the script for automatic processing, the plugin needs to be run by hand for one time to write relevant options in the configuration file. These steps can also be used if you want to segment a single image e.g. for fast analysis or to monitor the quality of the trained model.

1. Open ImageJ
2. Click on 'File' → 'Open' and open a random czi-file.
3. Click on 'Plugins' → 'U-Net' → 'Segment Current Image (Hyperstack)'
4. Enter the fields as shown in fig. 4.3. The model-file (filename ends with *.modeldef.h5) and RSA-keyfile should be stored on your local machine. The file path can be selected with the folder button.

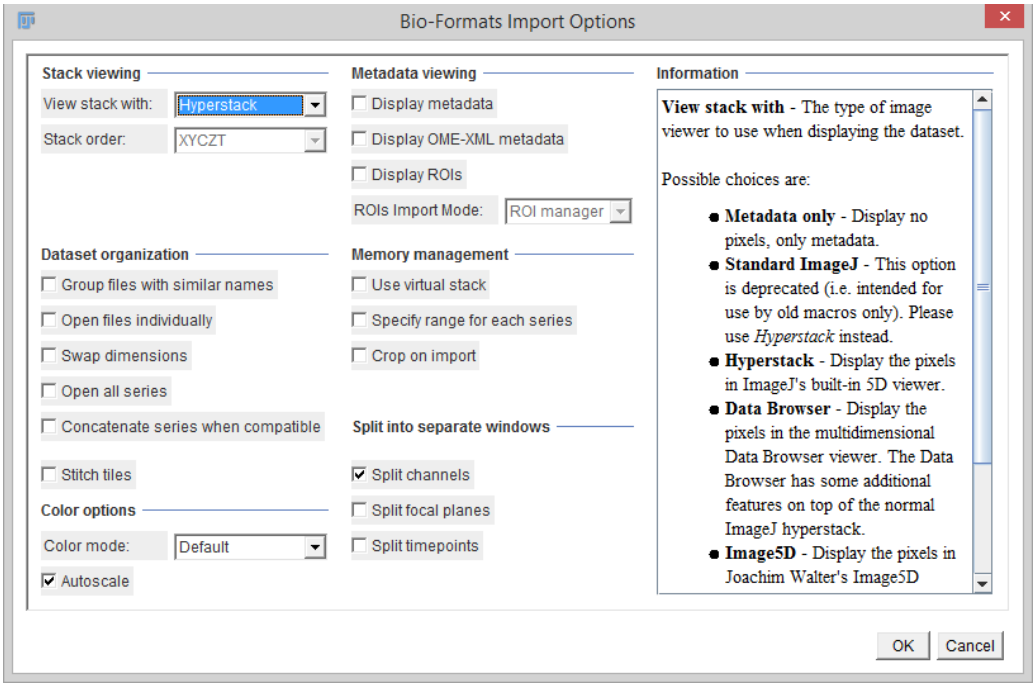


Figure 4.1: Bio-Formats import options

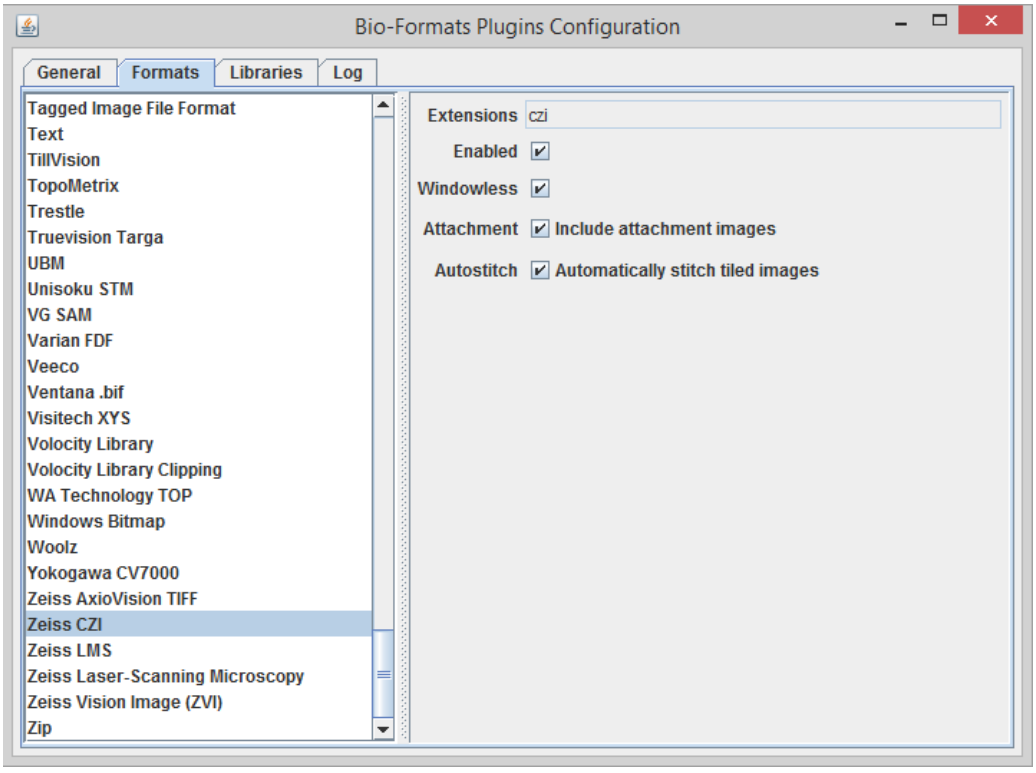


Figure 4.2: Bio-Formats Plugins configuration

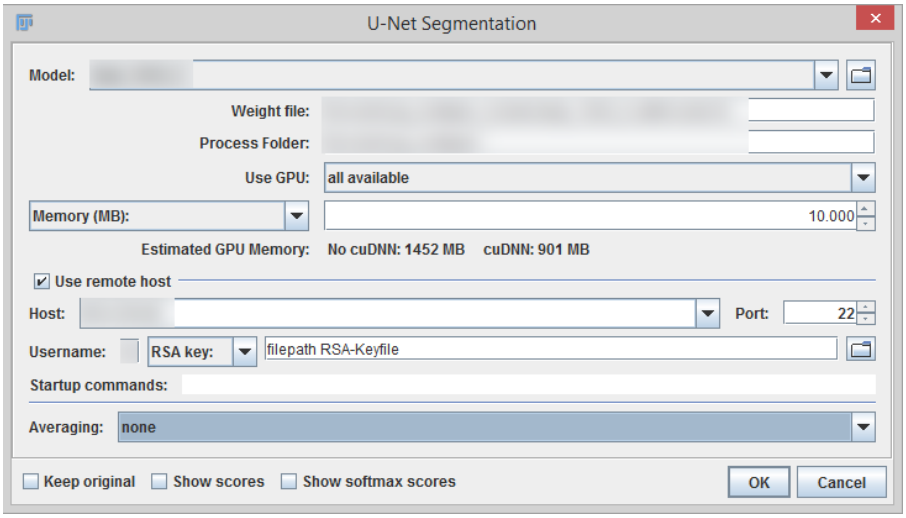


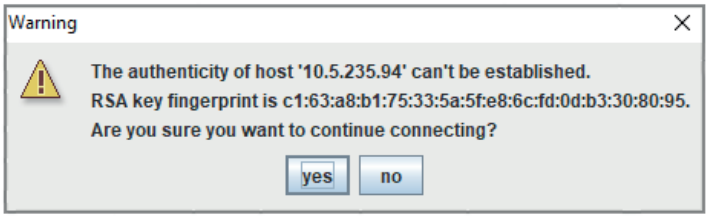
Figure 4.3: U-Net segmentation plugin with filled in settings

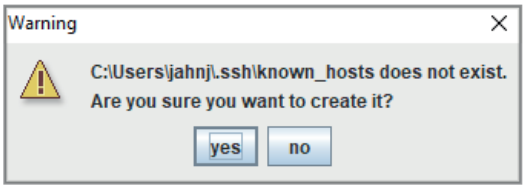
5. Click and wait until segmentation is finished (will open a new windows with the segmented image)
6. When executing the plugin for the first time a few dialogs need to be accepted by clicking on 'yes'. These dialogs are shown in fig. 4.4.
7. Close all images

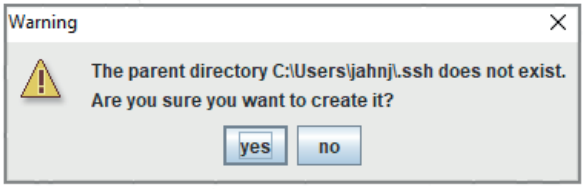
4.4.4 Adapt Script to new machine

The provided script for ImageJ combines all functions from image loading, U-Net segmentation and file saving. It is the essential part in the automated workflow. It contains a few basic options, such as file paths, that need to be manually set for each machine.

1. Open ImageJ
2. Click on 'File' → 'Open' and open the provided file 'U-Net-Processing-Library_v2.ijm' (or newer version number). Alternatively, you can drag and drop the file into ImageJ
3. This opens a new window showing the source code of the script (see fig. 4.5)
4. Change the server settings and adapt the file paths of the RSA- and model-files to the paths of your local machine (line 19-30, see fig. 4.5 - line number might change in newer versions of the script)
5. The processing step size (line 37-38) can be reduced when the computer has a small amount of memory
6. Click on 'File' → 'Save as…' and save the changed file to your machine

1.


2.


3.


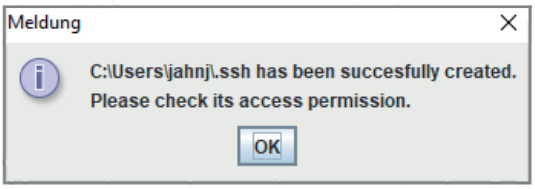
4.


Figure 4.4: Dialogs to confirm when starting the U-Net segmentation for the first time

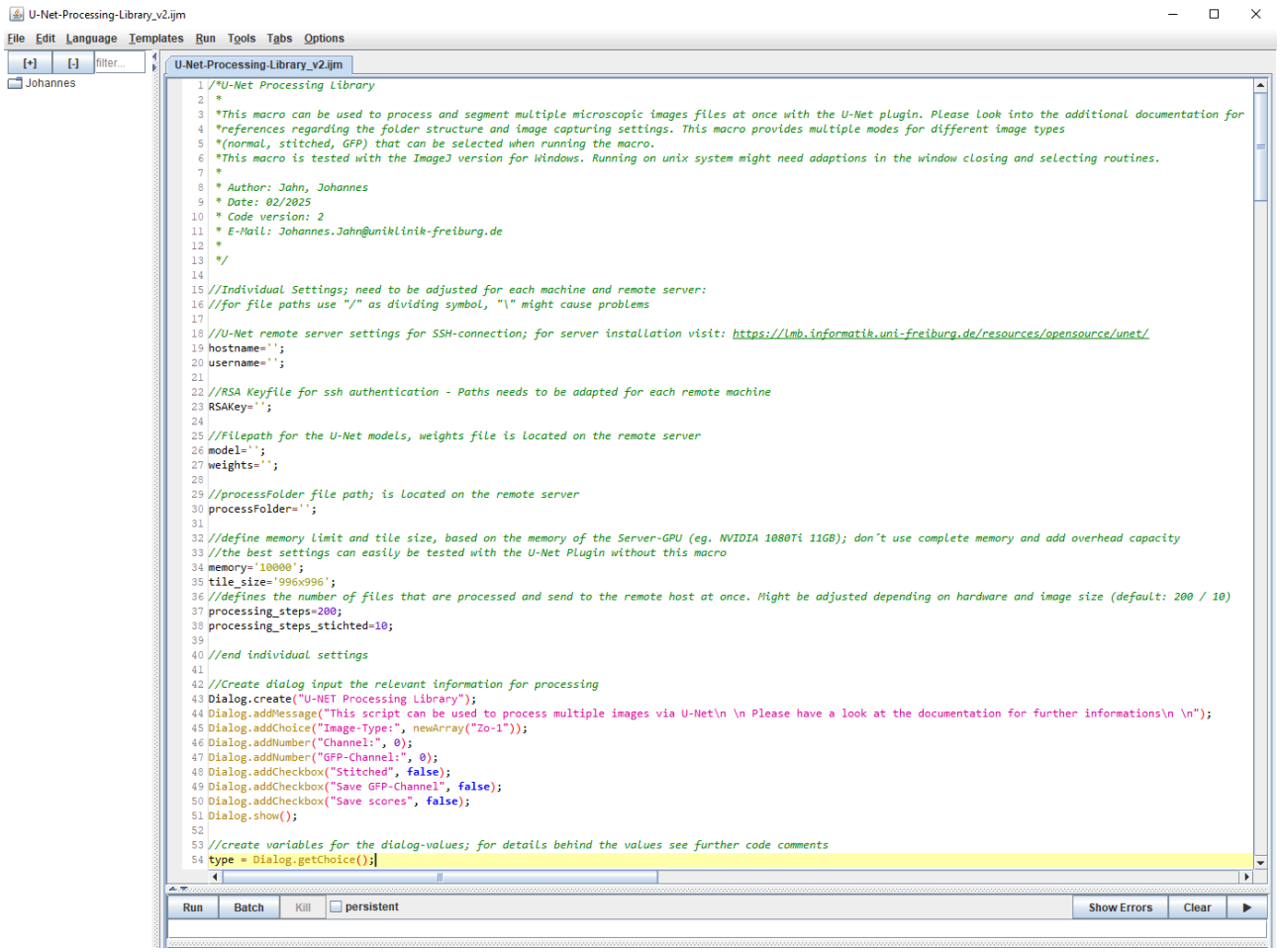


Figure 4.5: U-Net processing script opened in ImageJ showing parts of the source code

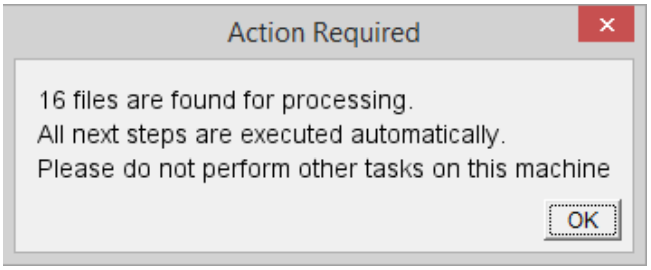


Figure 4.6: Dialog window showing the number of detected files before starting the U-Net processing

4.5 Automatic image segmentation via U-Net

To minimize the necessary user interaction and to provide an easy application a script for ImageJ was developed. As described above it combines and automates all essential steps in the U-Net segmentation workflow. The execution of the script is fairly easy and only needs a few input settings by the user.

4.5.1 Start the script

Now the actual U-Net processing can start. After setting up the parameters the script will process the input folder with all subfolders completely automatic. Depending on image size, number of files and network speed this might take a while (usually 15min for 200 single images, stitched images take longer). When finished a dialog box is displayed.

1. Open ImageJ
2. Click on 'File' → 'Open' and open the provided file 'U-Net-Processing-Library_v2.ijm'. Alternatively, you can drag and drop the file into ImageJ
3. This opens a new window showing the source code of the script (fig. 4.5). Click on 'Run' in the bottom left part of the window
4. Select the settings you want to use to process the folder and press 'OK' (see fig. 4.6) (Description of the settings is written in section 4.5.2)
5. Select the folder you want to process in the opened dialogue window (Please refer to the folder guidelines section in 3.2.1)
6. The next dialog window reports the number of files found in the selected folder. Press on 'OK' to start the processing (fig. 4.6)
7. Wait till the whole process is finished and it will show the final dialog box. You can see the current processing steps in the bottom part of the ImageJ main window. Depending on the server-connection and the amount of images it may take a while

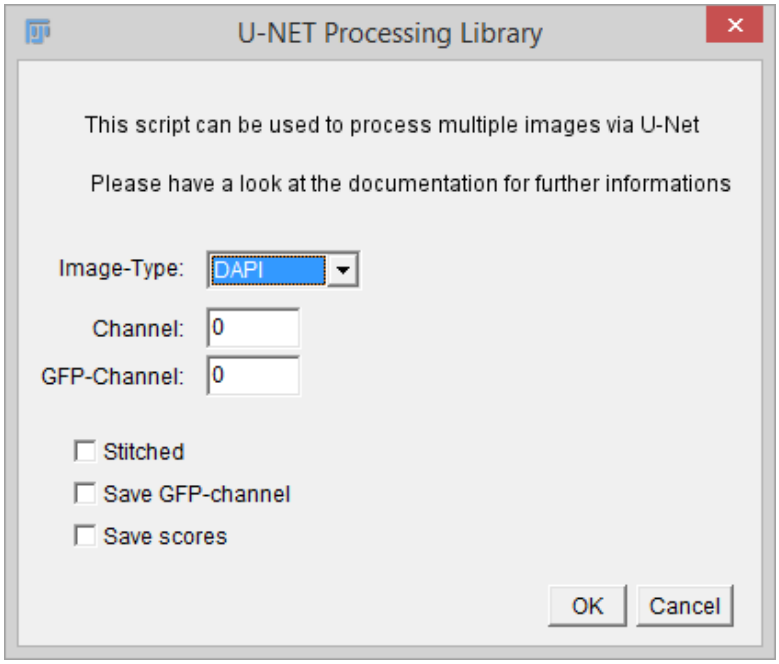


Figure 4.7: Script dialog window to select the settings for automatic processing

4.5.2 Script settings

The script can handle various settings for different experiments and image formats. At the beginning of the script-run a dialog window let the user select the desired settings for the specific dataset (see fig. 4.7). Below the details for each selectable option are shown:

- Image-Type: The dropdown list lets the user select the antibody staining for the images. For multiple fluorescence channels in one image the script needs to be run multiple times.
- Channel: For images with multiple fluorescence channels the number of the channel matching the selected antibody staining/image type is specified. The channel numbering starts with the '0'. For example: image file with two channels: DAPI: channel 0; ZO-1: channel 1
- GFP-Channel: For images with GFP fluorescence the number of the GFP-channel is specified. It only needs to be inputted if the 'Save GFP-Channel'-option below is checked.
- Stitched: If checked the script will search for stitched images and apply necessary preprocessing options.
- Save GFP-Channel: If checked the script will process the specified GPF-channel and save the channel in a separate file. For some experiments this can be used to get additional information about different genotypes in one image.
- Save scores: If checked the script saves the score image generated by the U-Net plugin. It can be used to validate the segmentation output. For usual analysis this option is not needed.

4.5.3 Processing folders and validation overlays

Inside each image folder the script will create a set of subfolders to store the processed data. These will also be used for further processing steps in Mathematica. The filename of the newly created files mostly consists of the original image-filename with an added suffix. Hence, the original filename should be carefully composed. Following files are stored inside the created folders:

- gfp: this folder stores the processed GFP image.
- processing: Used by Mathematica (see chapter 5 and 6) to save the processed image
- processing_overlay: Used by Mathematica to save additional images for validation purposes
- processing_xlsx: Used by Mathematica to save the computed values in an excel file (*.xlsx)
- segmented_overlay: Stores an overlay image, that can be used validate the U-Net output. It overlays the original image (s/w) with the U-Net segmentation (green). Example shown in fig. 4.8
- zo1_seg: This folder contains the segmentation images generated by the U-Net. These are 16-bit tiff-files. Hence, most image viewers (Windows/Photoshop) have problems viewing these files. ImageJ can open these images without trouble. Example shown in fig. 4.8
- zo1_seg_score: This folder stores the additional image file, that shows the segmentation scores. Please use ImageJ to open these files. Example shown in fig. 4.8

4.6 Common Errors

Unfortunately, the whole process is not completely error-free. Most problems occur with the server connection to the U-Net server or missing files. The most common errors and their solution are described below. For other errors restarting ImageJ or the operation system might help. Otherwise, a fresh installation following all steps of this documentation can help.

Error Message ImageJ: In rar cases opening ImageJ or the U-Net processing script might cause an error. This is shown in an additional console window (example shown in fig. 4.9). Restarting ImageJ and/or the operating system should fix this problem.

No files are found or the number doesn't match the input: The script will display an error message if no files for processing are found. In this case check if the right input folder was selected, if the subfolders match the required name scheme (see 3.2.1) and if all microscopic image files are correctly placed. Rerun the script after resolving these problems. If the number of files doesn't match the expected quantity, likely a typo or other problems with the folder name might cause image files not to be found.

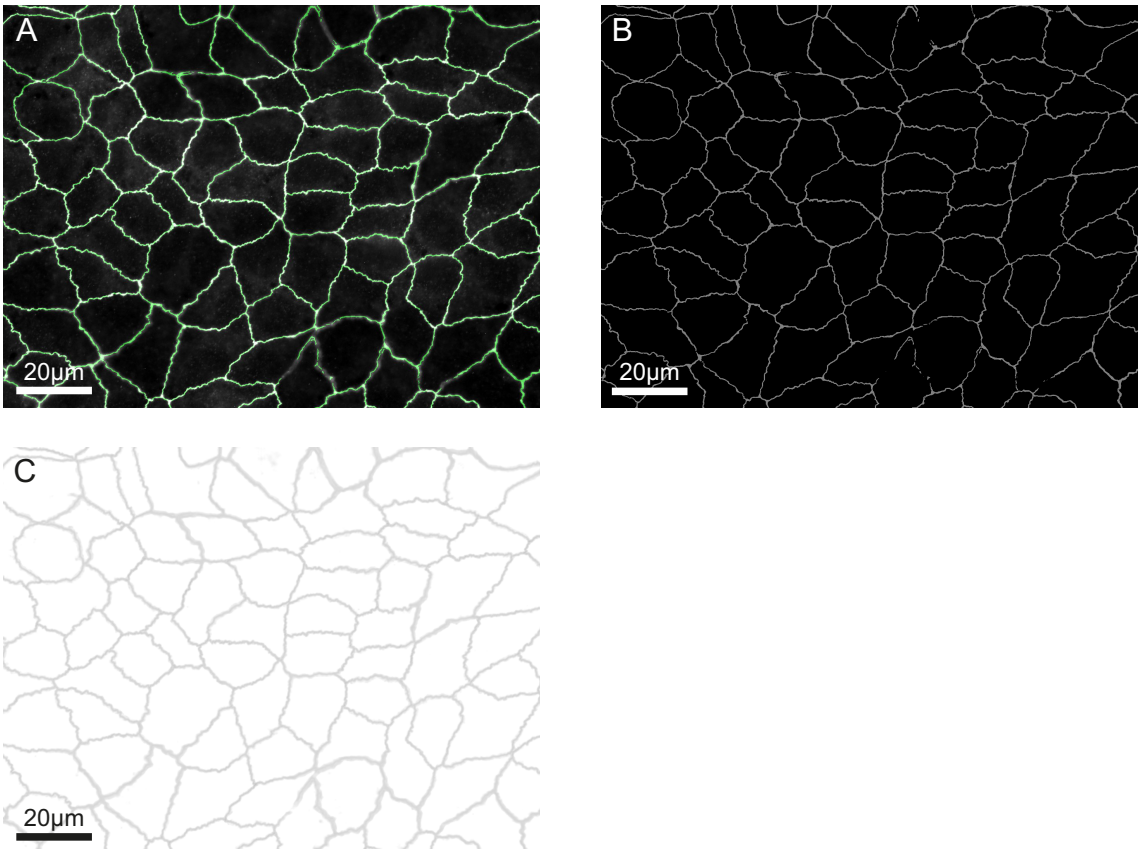


Figure 4.8: A: validation overlay showing the raw image and the U-Net segmentation in green; B: U-Net segmentation, in this case showing the detected cell borders; C: This image shows the scores computed by the U-Net network - based on these values the segmentation is generated.

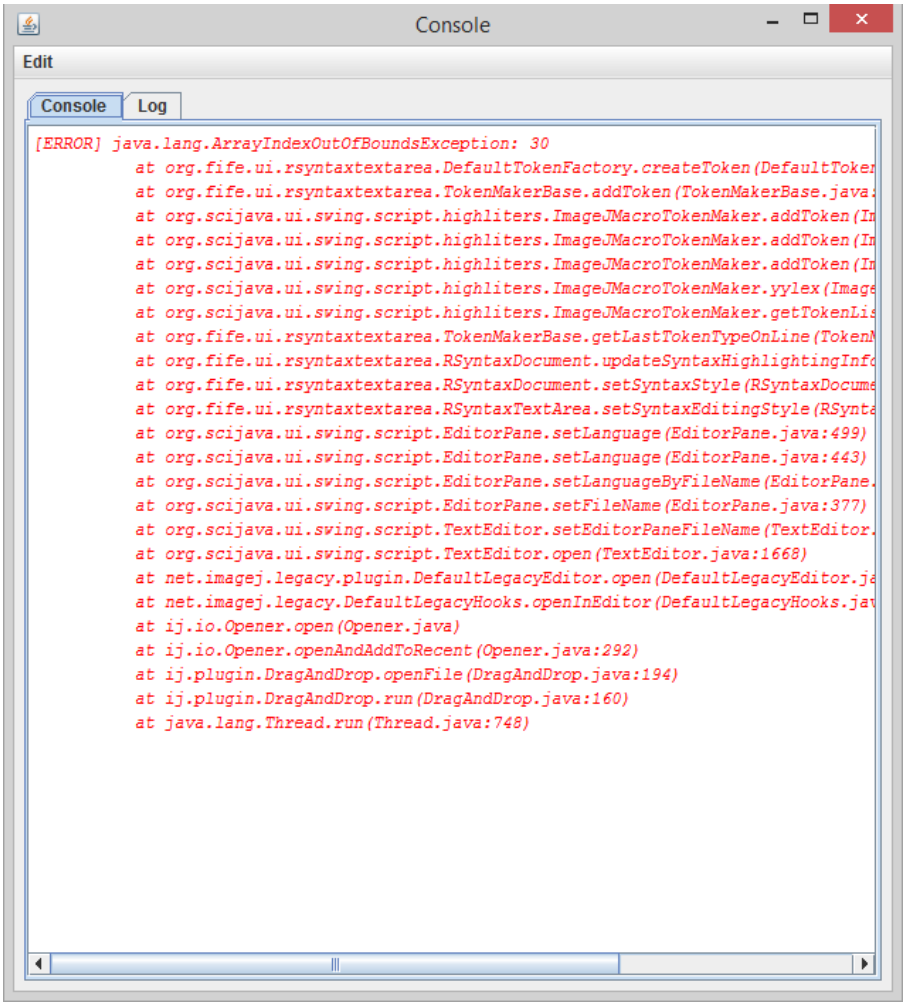


Figure 4.9: ImageJ error message

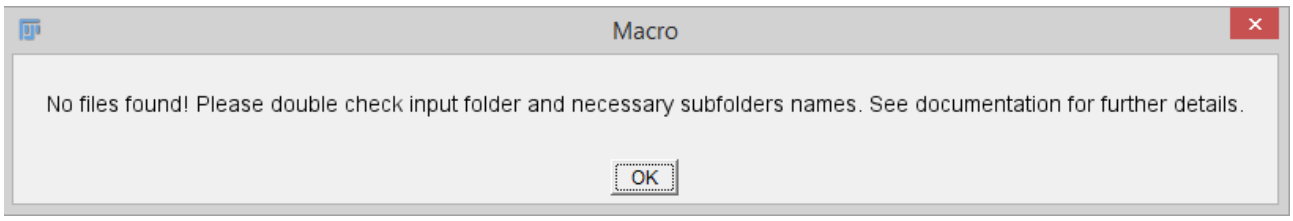


Figure 4.10: Error message when no files are found

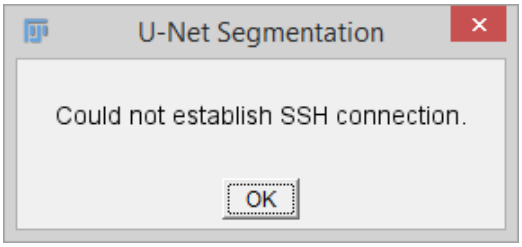


Figure 4.11: Error message when the U-Net server can not be reached

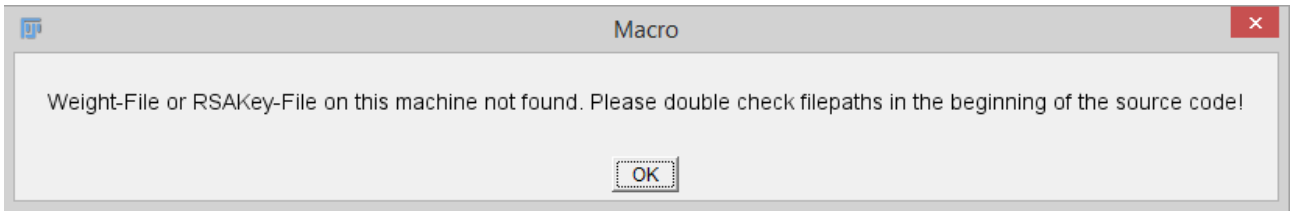


Figure 4.12: Error message when crucial files are not found

SSH connection error: If the U-Net server cannot be reached an error is displayed (see fig. 4.11). After this initial message multiple error messages are displayed which can be ignored. To fix this error double check the network settings. Sometimes ImageJ needs to be restarted, after setting the connection.

Modell/RSA Key not found: The script will display an error message, if essential files for the processing are not found (4.12). The file paths need to be checked (see section 4.4.4) and corrected.

Weight file upload dialog: This window is shown if the weight file (part of the U-Net model) is not found on the server (fig. 4.13). Click on 'Abbrechen' or 'cancel' and check the file paths (see section 4.4.4).

Other errors: Other errors can occur when the depending files on the U-Net server are moved or changed (see fig. 4.14). Make sure the file paths stated in the script are correct and contact the U-Net server administrator for persisting errors . Another rare error is displayed when any step in the script might cause problems (shown in 4.15). In this case restart ImageJ and rerun the script. Check your input files and microscope settings.

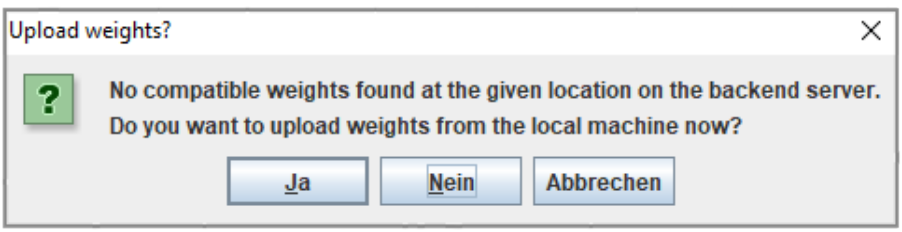


Figure 4.13: Upload dialog when essential parts of the U-Net model are not found on the server or the given file path is wrong

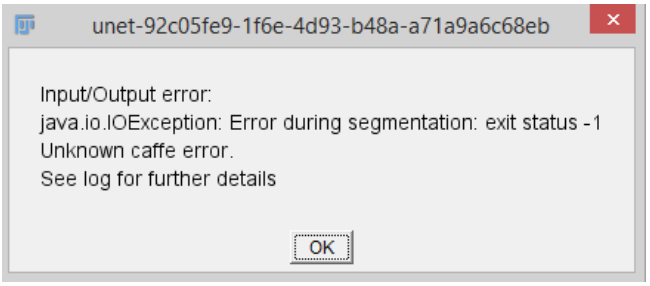


Figure 4.14: U-Net error message

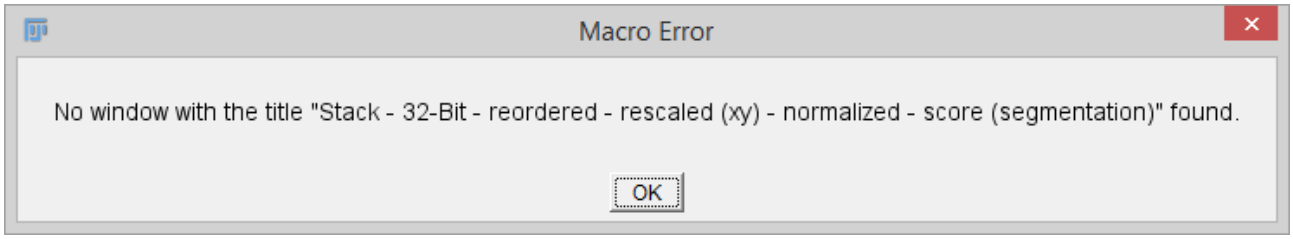


Figure 4.15: Script error showing difficulties in automatic processing

Chapter 5

Mathematica Scripts Part 1 - Detection

This chapter covers the second step in the automatic processing pipeline which applies the modeling functions and algorithms at the previously segmented images.

5.1 Initial Preparation

After finishing the U-Net processing in ImageJ, later steps are executed in Mathematica. Mathematica is a software package developed by Wolfram Research and provides a programming language with lots of advanced image processing and visualization methods. An activated Mathematica installation on the local machine is needed to run the analysis. All scripts containing the processing functions were developed and extensively tested on Mathematica 12. Newer versions should also work. For details on purchasing and installing Mathematica please have a look at the website https://www.wolfram.com/mathematica/.

5.2 The scripts

Source code in Mathematica is organized in so called 'Notebooks'. An own script/notebook was developed, which contains all the functions and methods to process the images. You don't need to know the technical details and using the scripts/notebooks is done in a few easy steps described below (5.4). For technical insights please have a look at the whole commented source code listed under 'Initialising' in the notebook itself.
Following core features are implemented in the scripts: Detect the cells based on the tight junction segmentation. Apply a smooth model and calculate the model parameter R-index showing differences in tight junction structure (riffle vs. smooth). For special experiments the cells can be fragmented into multiple cell border segments and the orientation of these segments based on the neighbor cell genotype is calculated.
The name 'Detection' is referring to the first step in Mathematica, which applies the developed modeling and analyzing algorithms to the segmented images generated by the U-Net.

Every notebook is structured in a similar way. When opening the file it shows the different parts in a plain overview (see fig. 5.1). The header shows the name of the script and basic information like

date of last changes. The 'Change log' and 'Initializing' part should be collapsed. When opening it with the arrow symbol it shows details about the latest applied changed or the whole annotated source code with all necessary functions. The 'Run' part contains the relevant variables, that are definable by the user itself, and the functions to run the script divided in one or multiple code blocks.

5.3 Placing the scripts

Each script will look for the specific file and folder structure (see section 3.2.1). After finishing the U-Net processing the Mathematica script needs to be placed in the parent folder, as shown in the folder tree at 3.2.1. No files or folders need to be changed between between these processing steps. The script will automatically find the right folders containing the segmented images. Multiple subfolders (e.g. for different experiment dates) are automatically detected and processed in one run of the script. Placing the script file at another folder might lead to wrong results picking not all or too many files. Please be sure that only the files/folders you want to process are in the subfolders at the script location.

5.4 Run the scripts

After all preparations the scripts can be run. Depending on image size, number of files and the scripts settings it might take some time and can run hours until finished. A progress bar gives a visual output about the current status. Each code block needs to be run separately. The single code blocks a separated by brackets shown on the right side of the notebook (see fig. 5.1).

1. Open the script placed in the parent folder.
2. Scroll down to the 'Run' section.
3. Right click on the first code block (preferably directly on the text to select the correct block) and click onto 'Evaluate Cell' in the dropdown menu. The first block will close all existing computation kernels which will help avoid side effects with previously run scripts.
4. When asking 'Do you want to automatically evaluate all the initialization cells in the notebook' click on 'Yes'.
5. Wait until execution is finished – the code block brackets on the right side stays bold during the execution.
6. Adapt the settings in the second block if required (see 5.5 for details).
7. Evaluate the second block as described in Step 3 and 5.
8. Evaluate the third block as described in Step 3 and 5. This should display a text with the number of files found for processing. Recheck file structure if numbers don't match the expectations.
9. Evaluate the fourth block as described in Step 3 and 5. This block starts the actual image processing and may take some time until finished.
10. Close the script when all code blocks ran successfully. The script file doesn't need to be saved.

ZO-1 - Detection

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* Date: 11/2021
* Code version: 6.0.0
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Change log

Initializing

Run

1. Quit all Mathematica kernels

```
In[-]:= Quit[]
```

2. Define settings

```
(*define the folder name that contains the segmented ZO-1 images generated by U-Net*)
(*default: "\\zo1_seg\\"*)
segFolderZo1 = "\\zo1_seg\\";

(*set to True if the GFP analysis function should be used,
that can divide the cells into two groups based on the GFP signal. For each ZO-
 1 image a supplementary GFP image showing the same part of the preparation is necessary.
   Cave: the number and alphabetic sorting of the segmented and GFP images need to be identical
     (which should be the case when processing both with the ImageJ script)*)
(*default: False*)
gfp = False;

(*define the folder name that contains the additional GFP images*)
(*default: "\\gfp\\"*)
gfpFolder = "\\gfp\\";

(*set to True if the single branch function should be used. With this function the cell border is divided into subsegments,
that can be individually used for modelling and measurements. When combined with the GFP function it can be used to make a statement about
 the orientation of the cell border in favour of one of the genotypes*)
(*default: False*)
singleBranch = False;

(*set to True if the form analysis function should be used*)
(*default: False*)
formAnalysis = False;

(*The pixel size depends on the U-Net configuration used for training. It defines the size of 1 pixel in µm*)
(*default for ZO-1: 0.1024*)
pixelSize = 0.1024;
```

3. Localisation of image files

```
In[-]:= (*set working directory and search in all subfolders, matching the defined folder name, for image files *)
SetDirectory[NotebookDirectory[]];
files = FileNames["*.tif", "", Infinity];
filesZo1 = Select[files, StringMatchQ[#, __ ~~ segFolderZo1 ~~ __] &];
filesGFP = Select[files, StringMatchQ[#, __ ~~ gfpFolder ~~ __] &];
fileNumberZo1 = Length[filesZo1];
fileNumberGFP = Length[filesGFP];
"Number of files: \nZO-1 segmented: " <> ToString[fileNumberZo1] <> "\nGFP: " <> ToString[fileNumberGFP ]
If [fileNumberZo1 != fileNumberGFP && gfp == True, (gfp = False;
  "GFP-Processing deactivated: file numbers don´t match. Please double check input files")]
```

4. Processing

```
In[-]:= (*side variables for ProgressIndicator*)
counter = -1 * Length[Kernels[]];
SetSharedVariable[counter];

(*call the main function to start the processing. A progress bar shows how many images are already processed. With ParallelTable we utilize
 more CPU cores. For large images or computers with less CPU power switch to the normal Table*)
Monitor[ParallelTable[counter++;
   zoAnalysis[filesZo1 [[i]], If[gfp == True, filesGFP [[i]], {}], gfp, formAnalysis, singleBranch, segFolderZo1], {i, fileNumberZo1 }],
  ProgressIndicator[counter, {0, fileNumberZo1 }]];
```

Figure 5.1: Screen when opening a Mathematica notebook, in this case for the ZO-1 detection script. The brackets on the right side show the separation of the code blocks.

5.5 Script settings

The second code block in the 'Run'-section ('Define settings') of each script lets the user define parameters of the processing. Most of the time the default settings are suitable and don't need to be changed. The following table shows the different variables with a short explanation.

parameter name	default setting	explanation
	ZO-1	
segFolderZo1	'\\zo1_seg \\'	See explanation above: 'segFolderDapi'.
gfp	False	This setting will enable an additional analysis mode. It can only be used if for each image the GFP-immunofluorescence channel is also acquired and saved in an additional file (can be done with the ImageJ script). This can divide the cell population into different groups (e.g. wild type and knock-out). To enable set the parameter to True'.
gfpFolder	'\\gfp \\'	Defines the folder that stores the additional GFP-images. Will only be used if GFP-processing is requested (see above). The folder is usually created by the ImageJ-script and the default setting doesn't need to be changed.
singleBranch	False	This setting will enable an additional analysis mode that divides each detected cell object into subsegments of the cell wall. Measurements are acquired for each subsegment and not the whole cell. In most cases this step is combined with the GFP-analysis and specific cell segments (e.g. between wild type and knock-out cells) can be examined. To enable set the parameter to 'True'.
formAnalysis	False	The form analysis function will create a matrix file overlaying all detected cells. This can be used for advanced evaluation of morphogenesis. In most cases this setting is experimental and can be excluded, otherwise the setting can be changed to 'True'.
pixelSize	0.1024	See explanation above: 'pixelSize'

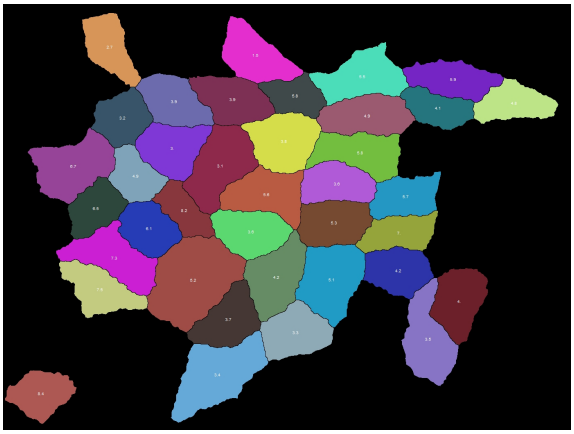
5.6 Interpret output

The detection scripts create multiple files containing the measurements, images showing the modelling effects and images that can be used to validate the output or the processing in general. The following subsection lists all created export files with a short description. The star '*' in the filename is a place holder for the original file name of each immunofluorescence image file.

5.6.1 ZO-1

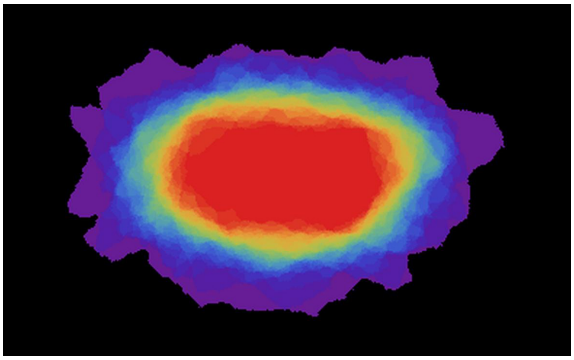
All files created with the ZO-1-Detection script:

zo1/processing/*_original.jpg: The detected, coloured cells are shown with the calculated R-Index displayed in the middle of each cell.

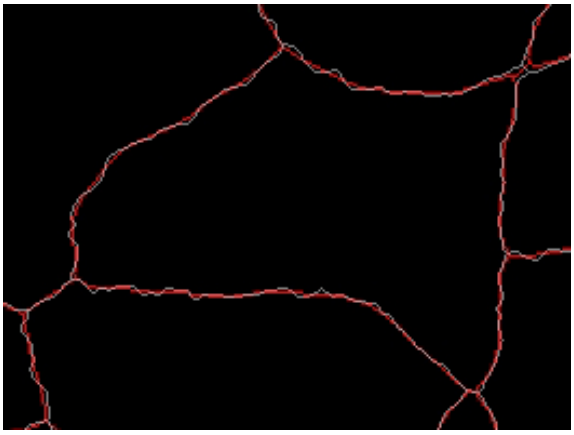


zo1/processing/*_round.jpg: These images show the cell objects after applying the smooth model.

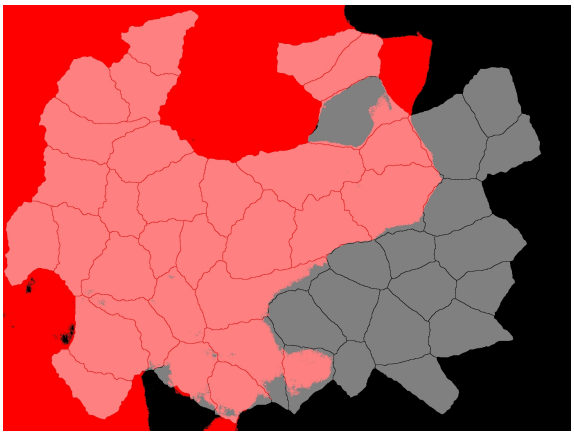
zo1/processing/*_form_analysis _full-cell.jpg: This file is only created if the form analysis function is used. It will overlay all detected cell objects with their centre points and create a head map showing overlapping parts (violet: only a few cells overlap at this pixel, red: most cells overlap at this pixel) It´s a simple way to visualise differences in morphogenesis.



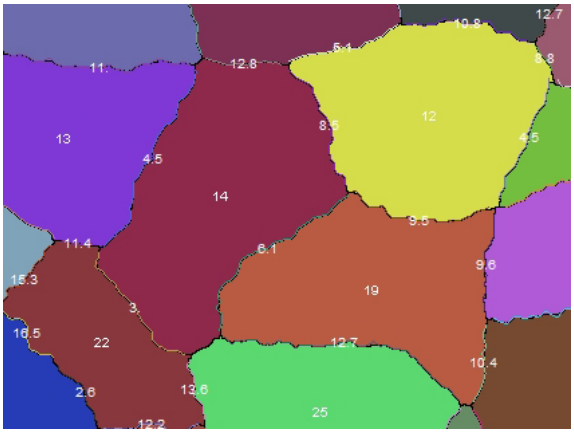
zo1/processing_overlay/*_overlay_riffle_round.jpg: This is a validation image, that overlays the U-Net output with the smoothed cell borders that are created in the modelling process. It can be used to check to quality of the modelling.



zo1/processing_overlay/*_gfp_overlay.jpg: This file is only created if the GFP-processing is requested. This image overlays the GFP-signal (in red) with the detected cells. GFP labelled cells and irregularities in the GFP-signal can be easily spotted.

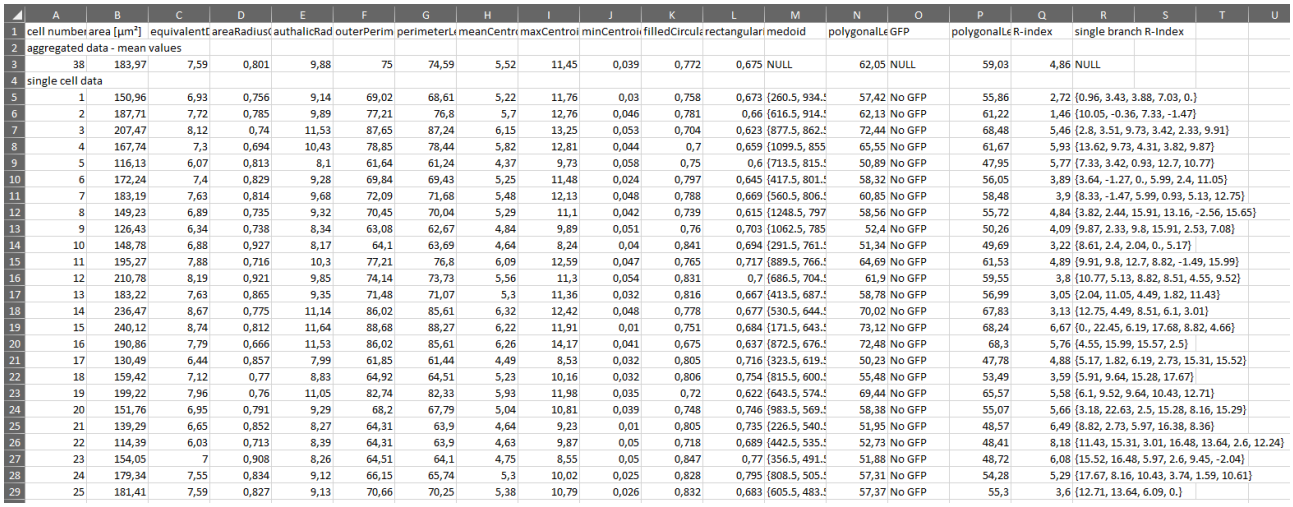


zo1/processing_overlay/*_single_branch.jpg: Only created when the single branch analysis is used. This image shows the detected cells and displays the R-Index for each segment of the cell border. Can be used to evaluate the modelling of the branch analysis. When using additional GFP-information (as shown in the 'gfp_overlay'-file) the differences of the subgroups can be seen.



Excel output file

zo1/processing_xlsx/*_zo1.xlsx: This is the essential file containing all measurements. It's the basis for later visualisation and data processing. See figure 5.2 for an example of the file structure. Row 3 shows the mean values of all single cell objects (that are listed in the second part of the file) and therefore provide key values for the whole image. Following parameter are acquired and shown in the columns (less important parameters are greyed out):



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
1	cell numbe	area [µm²]	equivalentD	areaRadius(	authalicRad	outerPerim	perimeterL	meanCentr	maxCentroi	minCentroi	filledCircula	rectangular	medoid	polygonalLe	GFP	polygonalLe	R-index	single branch R-Index			
2	aggregated data - mean values																				
3	38	183,97	7,59	0,801	9,88	75	74,59	5,52	11,45	0,039	0,772	0,675	NULL	62,05	NULL	59,03	4,86	NULL			
4	single cell data																				
5	1	150,96	6,93	0,756	9,14	69,02	68,61	5,22	11,76	0,03	0,758	0,673	{260.5, 934.5	57,42	No GFP	55,86	2,72	{0.96, 3.43, 3.88, 7.03, 0.}			
6	2	187,71	7,72	0,785	9,89	77,21	76,8	5,7	12,76	0,046	0,781	0,66	{616.5, 914.5	62,13	No GFP	61,22	1,46	{10.05, -0.36, 7.33, -1.47}			
7	3	207,47	8,12	0,74	11,53	87,65	87,24	6,15	13,25	0,053	0,704	0,623	{877.5, 862.5	72,44	No GFP	68,48	5,46	{2.8, 3.51, 9.73, 3.42, 2.33, 9.91}			
8	4	167,74	7,3	0,694	10,43	78,85	78,44	5,82	12,81	0,044	0,7	0,659	{1099.5, 855	65,55	No GFP	61,67	5,93	{13.62, 9.73, 4.31, 3.82, 9.87}			
9	5	116,13	6,07	0,813	8,1	61,64	61,24	4,37	9,73	0,058	0,75	0,6	{713.5, 815.5	50,89	No GFP	47,95	5,77	{7.33, 3.42, 0.93, 12.7, 10.77}			
10	6	172,24	7,4	0,829	9,28	69,84	69,43	5,25	11,48	0,024	0,797	0,645	{417.5, 801.5	58,32	No GFP	56,05	3,89	{3.64, -1.27, 0., 5.99, 2.4, 11.05}			
11	7	183,19	7,63	0,814	9,68	72,09	71,68	5,48	12,13	0,048	0,788	0,669	{560.5, 806.5	60,85	No GFP	58,48	3,9	{8.33, -1.47, 5.99, 0.93, 5.13, 12.75}			
12	8	149,23	6,89	0,735	9,32	70,45	70,04	5,29	11,1	0,042	0,739	0,615	{1248.5, 797	58,56	No GFP	55,72	4,84	{3.82, 2.44, 15.91, 13.16, -2.56, 15.65}			
13	9	126,43	6,34	0,738	8,34	63,08	62,67	4,84	9,89	0,051	0,76	0,703	{1062.5, 785	52,4	No GFP	50,26	4,09	{9.87, 2.33, 9.8, 15.91, 2.53, 7.08}			
14	10	148,78	6,88	0,927	8,17	64,1	63,69	4,64	8,24	0,04	0,841	0,694	{291.5, 761.5	51,34	No GFP	49,69	3,22	{8.61, 2.4, 2.04, 0., 5.17}			
15	11	195,27	7,88	0,716	10,3	77,21	76,8	6,09	12,59	0,047	0,765	0,717	{889.5, 766.5	64,69	No GFP	61,53	4,89	{9.91, 9.8, 12.7, 8.82, -1.49, 15.99}			
16	12	210,78	8,19	0,921	9,85	74,14	73,73	5,56	11,3	0,054	0,831	0,7	{686.5, 704.5	61,9	No GFP	59,55	3,8	{10.77, 5.13, 8.82, 8.51, 4.55, 9.52}			
17	13	183,22	7,63	0,865	9,35	71,48	71,07	5,3	11,36	0,032	0,816	0,667	{413.5, 687.5	58,78	No GFP	56,99	3,05	{2.04, 11.05, 4.49, 1.82, 11.43}			
18	14	236,47	8,67	0,775	11,14	86,02	85,61	6,32	12,42	0,048	0,778	0,677	{530.5, 644.5	70,02	No GFP	67,83	3,13	{12.75, 4.49, 8.51, 6.1, 3.01}			
19	15	240,12	8,74	0,812	11,64	88,68	88,27	6,22	11,91	0,01	0,751	0,684	{171.5, 643.5	73,12	No GFP	68,24	6,67	{0., 22.45, 6.19, 17.68, 8.82, 4.66}			
20	16	190,86	7,79	0,666	11,53	86,02	85,61	6,26	14,17	0,041	0,675	0,637	{872.5, 676.5	72,48	No GFP	68,3	5,76	{4.55, 15.99, 15.57, 2.5}			
21	17	130,49	6,44	0,857	7,99	61,85	61,44	4,49	8,53	0,032	0,805	0,716	{323.5, 619.5	50,23	No GFP	47,78	4,88	{5.17, 1.82, 6.19, 2.73, 15.31, 15.52}			
22	18	159,42	7,12	0,77	8,83	64,92	64,51	5,23	10,16	0,032	0,806	0,754	{815.5, 600.5	55,48	No GFP	53,49	3,59	{5.91, 9.64, 15.28, 17.67}			
23	19	199,22	7,96	0,76	11,05	82,74	82,33	5,93	11,98	0,035	0,72	0,622	{643.5, 574.5	69,44	No GFP	65,57	5,58	{6.1, 9.52, 9.64, 10.43, 12.71}			
24	20	151,76	6,95	0,791	9,29	68,2	67,79	5,04	10,81	0,039	0,748	0,746	{983.5, 569.5	58,38	No GFP	55,07	5,66	{3.18, 22.63, 2.5, 15.28, 8.16, 15.29}			
25	21	139,29	6,65	0,852	8,27	64,31	63,9	4,64	9,23	0,01	0,805	0,735	{226.5, 540.5	51,95	No GFP	48,57	6,49	{8.82, 2.73, 5.97, 16.38, 8.36}			
26	22	114,39	6,03	0,713	8,39	64,31	63,9	4,63	9,87	0,05	0,718	0,689	{442.5, 535.5	52,73	No GFP	48,41	8,18	{11.43, 15.31, 3.01, 16.48, 13.64, 2.6, 12.24}			
27	23	154,05	7	0,908	8,26	64,51	64,1	4,75	8,55	0,05	0,847	0,77	{356.5, 491.5	51,88	No GFP	48,72	6,08	{15.52, 16.48, 5.97, 2.6, 9.45, -2.04}			
28	24	179,34	7,55	0,834	9,12	66,15	65,74	5,3	10,02	0,025	0,828	0,795	{808.5, 505.5	57,31	No GFP	54,28	5,29	{17.67, 8.16, 10.43, 3.74, 1.59, 10.61}			
29	25	181,41	7,59	0,827	9,13	70,66	70,25	5,38	10,79	0,026	0,832	0,683	{605.5, 483.5	57,37	No GFP	55,3	3,6	{12.71, 13.64, 6.09, 0.}			

Figure 5.2: Example structure of the excel file containing all ZO-1 related measurements

parameter	unit	description
cell number	-	Individual number of each cell
area	µm²	approximate area, where each pixel area is weighted by its neighborhood configuration[1]
equivalentDiskRadius	µm	radius of a disk that has the same area[1]
areaRadiusCoverage	-	fraction of pixels within the equivalent disk radius[1]
authalicRadius	µm	radius of a circle with the same polygonal perimeter length[1]
outerPerimeterCount	µm	number of elements adjacent to the component[1]
perimeterLength	µm	total length of outer pixel sides[1]
meanCentroidDistance	µm	mean distance of all elements from the centroid[1]
maxCentroidDistance	µm	maximum distance of all elements from the centroid[1]
minCentroidDistance	µm	minimum distance of all elements from the centroid[1]
filledCircularity	-	circularity after filling holes[1]. This parameter can be used as a marker for cell morphogenesis. Round cell objects have a circularity close to '1', whereas irregular shaped cells have smaller values.
rectangularity	-	fraction of pixels within the minimal bounding box[1]
medoid	{pixel-position, pixel-position}	coordinate of the closest element to the centroid[1]

parameter	unit	description
polygonalLength	µm	total length of the polygon formed by the centers of the perimeter elements[1]. Most accurate parameter for the cell circumference and one of the key parameters for the R-Index model.
GFP	True/False	Shows the GFP-status of the cells, if an additional GFP-file is available and the processing option is used. Otherwise, the table shows 'no GFP data'.
polygonalLength smooth	µm	Polygonal length for the round cell objects (see above at polygonalLength)
R-Index	-	The key modelling parameter showing the differences in cell border homogeneity and riffle strength.
single branch R-Index	-	List of R-Indices for each subsegment of the cell border if the single branch processing is used. Otherwise, this field is left empty.

Excel output file branches

zo1/processing_xlsx/*_branches.xlsx: This excel file is only created if the single branch processing is used. Like the '*_zo1.xlsx'-file it shows all measurements and is used in later steps. It is based on a similar file structure with row 3 showing the mean values of the whole image. For each single segment the measurements are listed in the second part of the file. See figure 5.3 for an example of the file appearance. Following parameters are listed (less important parameters are greyed out):

parameter	unit	description
branch number	-	Individual number of each cell border segment.
medoid	{pixel-position, pixel-position}	coordinate of the closest element to the centroid[1]
outerPerimeterCount	µm	number of elements adjacent to the component[1]. For the single branch analysis the outerPerimeterCount showed better results when computing the R-Index.
outerPerimeterCount-smooth	µm	Same as above for the smoothed segment.
R-Index	-	Modelling parameter showing the differences in segment riffle.

parameter	unit	description
neighbor cell number	{#} or {#,#}	Shows the number of the neighbour cells. When one of the neighbour cells couldn't be detected only one number is saved.
GFP neighbor cell	{Bool} or {Bool,Bool}	Returns the GFP-status of the neighbor cells, if an additional GFP-file is available and the processing option is selected. Otherwise, the table shows 'no GFP data'.
branch orientation	text	This parameter is only available when GFP-modelling is used. It uses the medoid of the segment to determine if the segment is predominantly pulled to one of the neighbor cells: • non GFP cell pulls • GFP cell pulls. • non distinguishable: Difference too small to make prediction. • Null: only one neighbour cell detected or both with the same GFP-labelling (same genotype).
branch enclosed area	µm²	This parameter is only available when GFP-modelling is used. Both ends of the segment are connected and the enclosed area is computed. Only computed when two neighbor cells of different types are present.
pulled area	µm²	Based on the enclosed area (see one above) the overlapped area with the GFP-cell and non-GFP-cell is calculated. The difference between this two values is the pulled area. When positive the GFP-cells pulls the segment with the stated area. When negative the non-GFP cell pulls the segment.

zo1/processing_xlsx/*_raw-matrix_fullcell.mat: This file is only created if the form analysis function is used. It contains the shape data of each cell object. Might be helpful for advanced morphogenesis studies, currently the file is not commonly used.

zo1/processing_xlsx/*_zo1.txt: If no cell objects are detected in the image (e.g. unsharp microscope image with inadequate U-Net segmentation) a text-file is created instead of the xlsx-files containing the measurements. This way the faulty files can easily be spotted.

	A	B	C	D	E	F	G	H	I	J	K
1	branch number	medoid	outerPerimeter(	outerPerimeter(	R-index	neighbour cell n	GFP neighbour c	branch orientati	branch enclosed	pulled area [µm²]	
2	aggregated data - mean values										
3	128	NULL	26,38	24,43	7,17	NULL	NULL				
4	single cell data										
5	9	{236.5, 1012.5}	21,3	21,09	0,96	{1}	No GFP				
6	12	{299.5, 935.5}	44,75	43,21	3,43	{1}	No GFP				
7	13	{535.5, 936.5}	42,8	38,5	10,05	{2}	No GFP				
8	14	{657.5, 941.5}	57,55	57,75	-0,36	{2}	No GFP				
9	15	{200.5, 956.5}	21,09	20,28	3,88	{1}	No GFP				
10	17	{940.5, 909.5}	21,91	21,3	2,8	{3}	No GFP				
11	18	{888.5, 931.5}	11,67	11,26	3,51	{3}	No GFP				
12	21	{1057.5, 898.5}	48,13	41,57	13,62	{4}	No GFP				
13	23	{985.5, 859.5}	11,57	10,44	9,73	{3, 4}	No GFP				
14	24	{1183.5, 876.5}	23,76	22,73	4,31	{4}	No GFP				
15	25	{360.5, 865.5}	11,26	10,85	3,64	{6}	No GFP				
16	26	{415.5, 866.5}	16,18	16,38	-1,27	{6}	No GFP				
17	27	{260.5, 872.5}	37,89	35,23	7,03	{1}	No GFP				
18	28	{680.5, 854.5}	30,72	28,47	7,33	{5, 2}	No GFP				
19	29	{525.5, 861.5}	19,66	18,02	8,33	{7}	No GFP				
20	30	{770.5, 833.5}	23,96	23,14	3,42	{5, 3}	No GFP				
21	31	{595.5, 856.5}	13,93	14,13	-1,47	{7, 2}	No GFP				
22	33	{1189.5, 829.5}	26,83	25,8	3,82	{4, 8}	No GFP				
23	34	{330.5, 846.5}	5,94	5,94	0	{1, 6}	No GFP				
24	35	{486.5, 797.5}	34,2	32,15	5,99	{6, 7}	No GFP				

Figure 5.3: Example structure of the excel file containing all measurements from the single branch analysis

```
Monitor[ParallelTable[counter++;
    zoAnalysis[filesZo1[[i]], If[gfp == True, filesGFP[[i]], {}], gfp, formAnalysis, singleBranch, segFolderZo1],
    {i, fileNumberZo1}], ProgressIndicator[counter, {0, fileNumberZo1}]];
```

ParallelTable: Iterator {i, fileNumberZo1} does not have appropriate bounds.

ParallelTable:
ParallelTable[counter++; zoAnalysis[filesZo1[[i]], If[gfp == True, filesGFP[[i]], {}], gfp, formAnalysis, singleBranch, segFolderZo1], {i, fileNumberZo1}] cannot be parallelized; proceeding with sequential evaluation.

Table: Iterator {i, fileNumberZo1} does not have appropriate bounds.

Figure 5.4: Mathematica script errors lead to a red error messages below the code block.

5.7 Common Errors

Unfortunately, the execution of the Mathematica scripts is not completely error-free. Compared to the second step in ImageJ errors are significantly rarer. Errors are shown as red text below the error inducing cell block (example in fig. 5.4). In most cases it helps to completely close and restart Mathematica and the script. Get the default version of the script to avoid unknowingly changes in the source code. Check the folder structure and if the script has detected as much image files as expected (number is displayed when executing the third code block in the script). Sometimes only one image file might be faulty and generate the error message with all other images being processed without problems.

Chapter 6

Mathematica Script Part 2 -Visualization

This chapter covers the last step in the automatic processing pipeline which reworks the gathered data and creates basic visualizations.

6.1 Initial Preparation

After successfully running the detection script as described chapter 5, the second step in Mathematica can be executed. The 'Visualisation'-step offers two essential features. It combines the previously generated data to create simple graphs and statistical reports to easily see differences between genotypes or subgroups in the datasets. Additionally, the combined data is saved in an accessible way to use it in further statistical software. The script is build very similar compared to the detection scripts (see section 5.2 and image 5.1).

6.2 Data preparation

Before the scripts can be used the result excel files created with the 'Detection'-script need to be resorted into a new folder structure. The previously used structure, that is automatically created by the ImageJ script, is an easy way to store the image files in a logical order and divided by different experiments. To compare various genotypes the 'Visualisation'-script needs to know which files belong together. The disadvantage: This step needs to be done by hand as it could not be that easily automated as the other steps. The advantage. The user can define exactly what files or genotypes should be compared, which files should be excluded or which different experiments should be mixed. When comparing too many genotypes it can cause trouble with the visualization in the autogenerated graphs. Usually 3-4 genotypes per comparison work without a problem. Multiple comparison folders can created by once, the scripts will automatically process them one by one. The new required folder structure for the visualization step is built as follows:

```
analysis
├── Mathematica ZO-1 visualisation script
├── 20220101/data/Comparsion/1
│   ├── data_input
│   │   ├── genotype/setting 1
│   │   │   └── measurement files (see 5.6.1)
│   │   ├── genotype/setting 2
│   │   │   └── measurement files (see 5.6.1)
│   │   └── and so on
│   └── data_output
└── 20220101/data/Comparsion/2
    └── ...
```

All Folders marked in red need to have the exact name, otherwise the scripts won't work. Other folders can be individually changed by the user, but the folder structure inside the data_input folders needs to stay the same. The 'genotype/setting' name will be used in the graphs to label the data. One exception are the GFP ZO-1 files. These don´t need to be separated by genotype an should be sorted in one folder:

```
analysis ZO-1
├── Mathematica ZO-1 visualisation script
├── 20220101/data/Comparsion/1
│   ├── data_input
│   │   └── all
│   │       └── all measurement files with GFP setting enabled
│   └── data_output
└── 20220101/data/Comparsion/2
    └── ...
```

6.2.1 Different settings

Depending on the settings used in the Mathematica detection script, different result files are created. The possible combinations and the right files for the visualization step are listed in the following part.

ZO-1 without single branch or GFP functionality: Sort the '*_zo1.xlsx' files from the 'zo1/processing_xlsx/'-folder in the different genotype folders.

ZO-1 with single branch analysis: Sort the '*_branches.xlsx' files from the 'zo1/processing_xlsx/-folder' in the different genotype folders.

ZO-1 with GFP analysis: In this case the files don't need the separation into different genotypes since the GPF information will provide this categorization (GFP positive cells = genotype 1, GFP negative cells = genotype 2). Sort the '*_zo1.xlsx' files from the 'zo1/processing_xlsx/'-folder in one folder inside the 'data_input'-folder e.g. named 'all', as shown above in the folder tree.

ZO-1 with both single branch and GFP processing: In this case the files don't need the separation into different genotypes since the GPF information will provide this categorization (GFP positive cells = genotype 1, GFP negative cells = genotype 2). Sort the '*_branches.xlsx' files from the 'zo1/processing_xlsx/'-folder in one folder inside the 'data_input'-folder e.g. named 'all', as shown above in the folder tree.

6.3 Placing the scripts

When all comparison folders are created and the required files are sorted in the new file structure the Mathematica scripts need to be placed in the parent folder, as shown in the folder tree in section 6.2. The script will automatically detect all 'data_input' folders.

6.4 Run the scripts

The execution of the Mathematica notebook is similar to the previous part (5.4). Depending on the number of comparison folders it may take a few minutes until the script is finished. Overall, it should be much faster compared to the detection step, were postprocessing and modeling generates a more heavy workload. Again, each code block needs to be run separately and in order given by the script structure.

1. Open the script placed in the parent folder beside all the analysis folders.
2. Scroll down to the 'Run' section.
3. Right click on the first code block (preferably directly on the text to select the correct block) and click onto 'Evaluate Cell' in the dropdown menu. The first block will close all existing computation kernels which will help avoid side effects with previously run scripts.
4. When asking 'Do you want to automatically evaluate all the initialization cells in the notebook' click on 'Yes'.
5. Wait until execution is finished – the block markers on the right side stays bold during the execution.
6. Adapt the settings in the second block if required and available (see below for details).
7. Evaluate the second block as described in Step 3 and 5.
8. Evaluate the third block as described in Step 3 and 5. This will load all files that were selected for analysis.

9. Evaluate the fourth block as described in Step 3 and 5. This block starts the processing and will generate the new output files.
10. A fifth block starts the optional 'cutoff'-analysis, which means it will exclude values larger than the predefined cut off. It´s especially useful for the R-index to exclude outlier that alter the visualisation.
11. Close the script when all code blocks ran successfully. The script file doesn't need to be saved.

6.5 Script settings

The second code block in the 'Run'-section scripts lets the user define parameters of the analysis step. Most of the time the default settings are suitable and don´t need to be changed:

parameter name	default setting	explanation
	ZO-1	
gfp	False	Set value to True if the GFP setting was used.
singleBranch	False	Set value to True if the single branch setting was used.
fileMeanCutoff	0	When using the fileMean analysis (see section 6.6.1) the scripts lets the user define a cut off value. Background: It could be problematic if an image with only 1 detected cell is weighted the same as a file with 20 detected cells. Images with less detected cells than the defined value are excluded.
outliersCutoff	20	Value for cut off analysis. Defines to maximum value that is included, larger values are dismissed. For R-index values larger 20 are most likely detection errors and can be excluded. Is only applied to the specific outputted pdf.
outliersParameter	17 (for non single branch), 5 (for single branch)	Parameter that will be used for cut off analysis. Mostly used for R-index (see default), but every other parameter should work. The number is equivalent to the column number in the excel files.

6.6 Interpret output

All new files are saved inside the 'data_output' folder. Depending on the used script and input files many different excel- and pdf-files are created. For the first time this might look overwhelming, but behind that is a clear pattern based on a couple different analysis modes. In favour of a simple and fast way of reviewing the datasets all possible parameters are created and can be inspected with a few clicks. Therefore, the user doesn't need to specify the parameters when running the scripts and can decide later which parameters are more relevant for the exact question. For the daily work only a few parameters might be relevant and other files can be ignored.

6.6.1 ZO-1 - Output

Depending on the different settings we can apply for the Zo-1 detection our visualization script will generate slightly different output. The parameters are described in subsection 5.6.1 and 5.6.1.

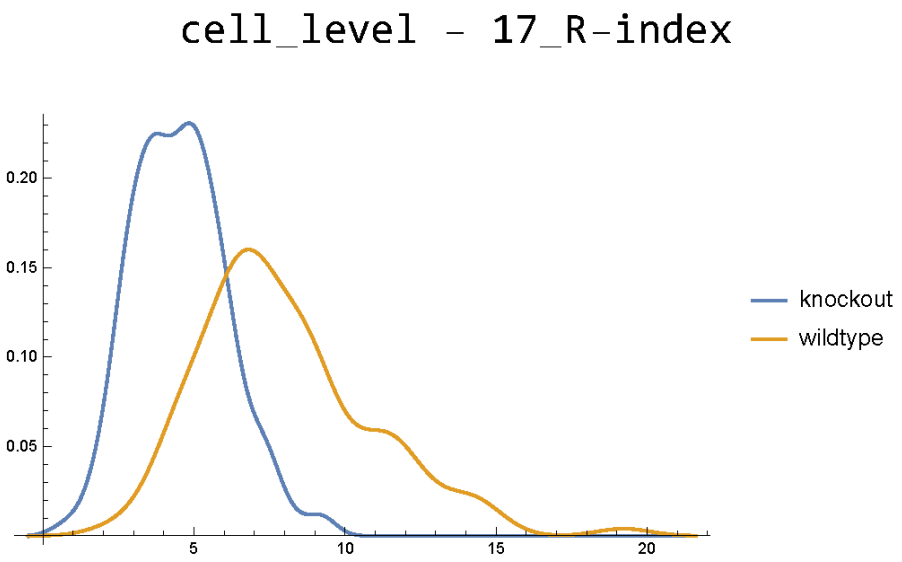
ZO-1 without single branch or GFP functionality

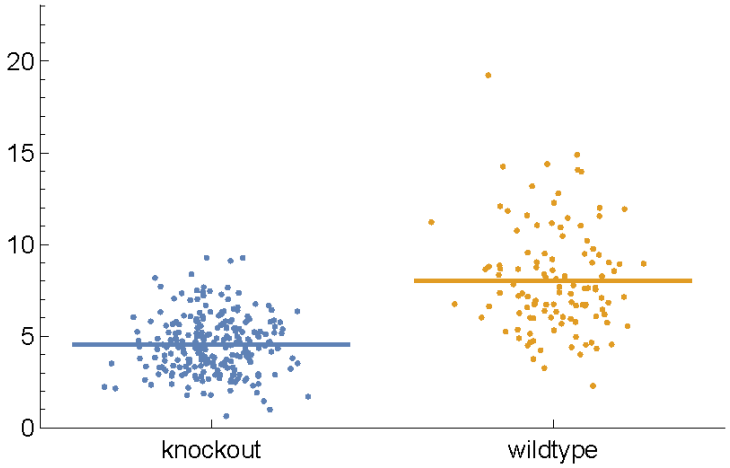
The standard and most used setting will generate 3 different types of files:

Excel-files: These files simple reformat the inputted data, that is divided in single files from each image, into one file. The number and parameter name are shown as a first part of the file name. Every column inside the file corresponds to one image file with all values listed in this row. These newly created files can be easily used in other software.

Cell level PDF-files: These files give a simple visualization and statistical analysis of the data and the particular parameter. Each object is included in the visualization with its own data point. This leads to a wider spreading but represents the whole biological appearance. In addition to the cell_level'-prefix the filename shows the name and number of the parameter. Inside this PDF-file (example shown in fig.6.1) a smooth histogram (y-axis values are arbitrary) and a point cloud plot gave an easy to view visualization of the dataset. Each point in the cloud plot represents one cell object. The mean value of the whole dataset is shown as a bold bar in the cloud plot. Below that, the first table shows the key parameters of the analyzed dataset and genotypes. A second table shows an automatically generated statistic analysis between the genotypes. Additionally, the cut off analysis can be run that excludes measurements larger than the predefined value and negative values. The suffix 'cutoff' followed by the cutoff-value is included in the file name.

File level PDF-files: Like the cell level files but only the mean values from each image are used – every image creates one data point. When a specific 'fileMeanCutoff'-value is defined, images with less cell objects are excluded. The cut off value is also stated in the file name.





genotyp	# files	# cells	17_R–index mean
knockout	10	236	4.54373
wildtype	10	113	8.02416
total	20	349	6.28394

compared groups	statistic		
knockout / wildtype		Statistic	P-Value
	Mann-Whitney	3370.	1.33511×10^{-29}

Figure 6.1: Example of the output PDF that shows the measurements of the cell-level ZO-1 analysis

ZO-1 with single branch analysis

The single branch analysis outputs an object-level PDF-file showing the visualized measurements and an excel file containing the raw data of the R-index values. Other parameters are not collected. The cut off analysis (see above) can also be applied to exclude values larger than the predefined threshold, which might lead to a nicer visualization. Negative values (are considered as modeling errors) are excluded for all visualizations and in the raw data excel file.

ZO-1 with GFP processing

This setting generates an output very similar to the ZO-1 without single branch and GFP functionality. The genotypes are not defined by the folder structure since the files provide the GFP true or false classification. File level analysis is not possible in this setting.

ZO-1 with both single branch and GFP processing

This analysis mode outputs multiple object-level PDF-files showing the visualized measurements and excel files containing the raw data of the R-index values, the branch orientation (only raw data excel file) and the 'pulled area' parameters. The cut off analysis (see above) can also be applied to exclude values larger than the predefined threshold. Negative values (are considered as modeling errors) are excluded for all visualizations and in the raw data excel file. File level analysis is not possible in this setting.

6.7 Common errors

As described in section 5.7 the execution of the Mathematica scripts is not error-free. In most cases it helps to completely close and restart Mathematica and the script. Get the default version of the script to avoid unknown changes in the source code in the previously used one. Check the folder structure of the input data and if the script has detected as many processing folders as expected (is displayed when executing the third code block in the script). Sometime only one excel file might be faulty and leads to an error message. If doable check all input files to detect abnormalities in the table structure.

Appendix A

Acknowledgments

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