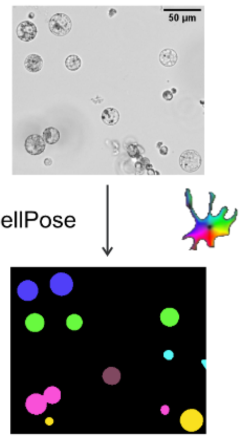
User-guide for the analysis of cell wall regeneration
post-protoplasting to obtain single plant cells

Q-Warg

1-Prepare raw data (TIFF)

```
Experiment_x
   ├── raw_microscopy_file
   ├── Replicate__Condition1.tif
   ├── C1-Replicate__Condition1.tif
   ├── Replicate__Condition2.tif
   ├── C1-Replicate__Condition2.tif
   └── ...
```

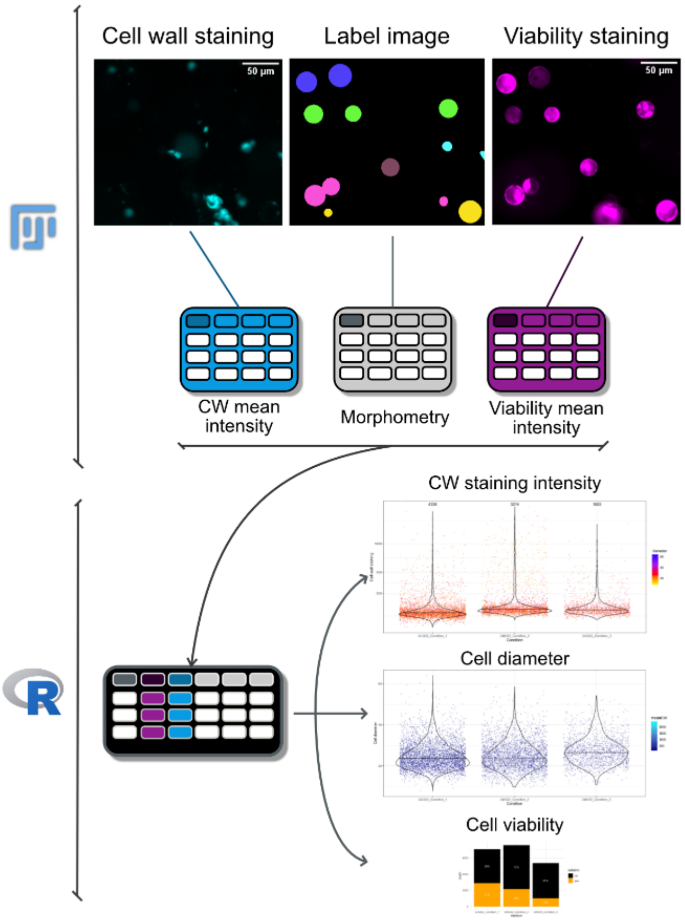
2-Segmentation



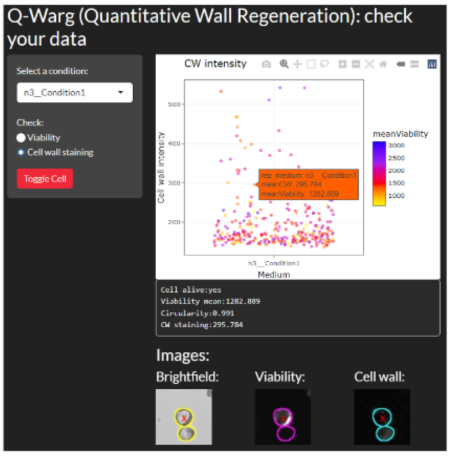
3-Organize files

```
Experiment_x
 ├── RAW_DATA
 │    ├── raw_microscopy_file
 │    ├── Replicate__Condition1.tif
 │    ├── Replicate__Condition1_cp_masks.png
 │    ├── C1-Replicate__Condition1.tif
 │    └── ...
 └── ANALYSIS
      ├── Replicate__Condition1
      │    ├── Replicate__Condition1.tif
      │    └── Replicate__Condition1-labels.png
      ├── Replicate__Condition2
      │    ├── Replicate__Condition2.tif
      │    └── Replicate__Condition2-labels.png
      └── ...
```

4-Analysis



5-Interactive data checking



Q-Warg (Quantitative Wall Regeneration): check your data

Select a condition:
n3__Condition1

Check:
○ Viability
● Cell wall staining

Toggle Cell

```
Cell alive:yes
Viability mean:1282.889
Circularity:0.991
CW staining:295.784
```

Images:
Brightfield: Viability: Cell wall:

Procedure overview and quick guide

Here we describe step-by-step how to operate the image analysis workflow to quantify cell wall (CW) regeneration after protoplast extraction to help the user to screen quickly and efficiently CW regeneration media. This workflow uses different components under the form of ImageJ (Fiji) macro and R scripts.

1- **Raw data preparation**: save_as_tiff.ijm
   a. Save raw images as TIFF with the 3 channels: brightfield, viability and cell wall staining
   b. Save a TIFF image with only the brightfield channel to be used for the segmentation

2- **Segmentation with CellPose**:
   a. Load the brightfield image into CellPose
   b. Run the segmentation
   c. Save the label image as PNG

3- **Organizing files for Q-Warg**: R_orga_folder.R
   a. Prepare a raw data image that will stay untouched by the pipeline
   b. Prepare the folders to be used with the pipeline
      i. Each folder contains the 3-channels image and the corresponding label image
      ii. Rename files to fit Q-Warg requirements

4- **Analysis**:
   a. Run the ImageJ macro CWRegenerationQuantification.ijm to extract quantification data: viability and cell wall staining intensity, morphometry information (such as size and circularity)
   b. Run the R script QWARG-notebook.Rmd:
      i. Tidy data
      ii. Set viability threshold
      iii. Plot data
      iv. Save data table and plots of cell wall mean intensity and cell size

5- **Interactive data checking**:
      v. Reorganize the folders
      vi. Check data: observe the cells corresponding to the quantitative data

See below for the detailed guide.

Pre-requisites:

Software & plugins required:

- Anaconda
- CellPose
- Fiji (https://fiji.sc/)
  - MorphoLibJ (“IJBP-Plugins” update site)
- RStudio (https://posit.co/download/rstudio-desktop/)
  - Libraries:
    - data.table
    - rlist
    - hrbrthemes
    - viridis
    - tidyverse
    - tcltk
    - shiny
    - plotly
    - magick
    - autothreshold
    - patchwork

Workflow scripts:

- save_as_tiff.ijm
- R_orga_folder.R
- CWRegenerationQuantification.ijm
- QWARG-notebook.Rmd

Data:

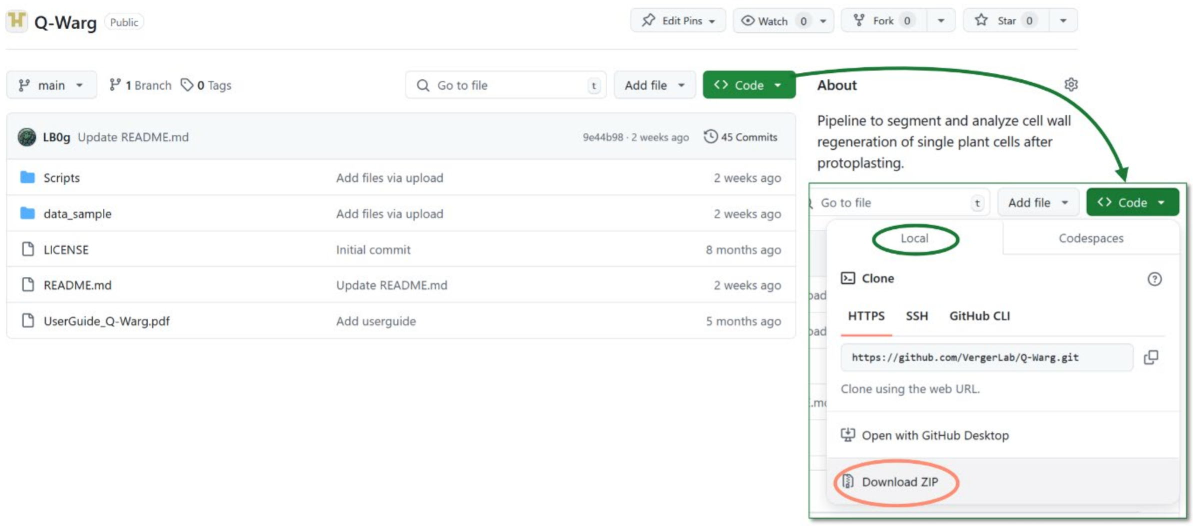
- The original image in TIFF with the 3 channels:
  - Brightfield (should always be channel 1)
  - Viability staining
  - Cell wall staining

Installation

1- GitHub

Both CellPose and Q-Warg are available on GitHub.

- Go on the repository page with the provided link.
- Click on Code
- Choose Local
- Download ZIP: the repository will be downloaded in the download folder of your computer.
- Extract all files from the compressed folder to your folder of choice



LB0g Update README.md	9e44b98 · 2 weeks ago	45 Commits
Scripts	Add files via upload	2 weeks ago
data_sample	Add files via upload	2 weeks ago
LICENSE	Initial commit	8 months ago
README.md	Update README.md	2 weeks ago
UserGuide_Q-Warg.pdf	Add userguide	5 months ago

About

Pipeline to segment and analyze cell wall regeneration of single plant cells after protoplasting.

https://github.com/VergerLab/Q-Warg.git

Clone using the web URL.

2- CellPose

We used CellPose for segmentation, but alternative segmentation tools could be used as well as long as the output is (or is made) compatible with the rest of the workflow.

Anaconda

CellPose requires a python environment that can be provided by Anaconda.

Download and install anaconda here: https://www.anaconda.com/download/success.

CellPose

Detailed guidelines to install CellPose can be found on the readme file of the CellPose repository in GitHub: https://github.com/mouseland/cellpose

3- Download Q-WARG from GitHub

All scripts can be found in the Scripts folder of the GitHub Q-Warg repository found here: https://github.com/VergerLab/Q-Warg.git

4- To use ijm files

Fiji and MorpholibJ

To install Fiji/ImageJ, see https://fiji.sc/

To install the plugin MorphoLibJ, turn on the IJBP-Plugins update site:

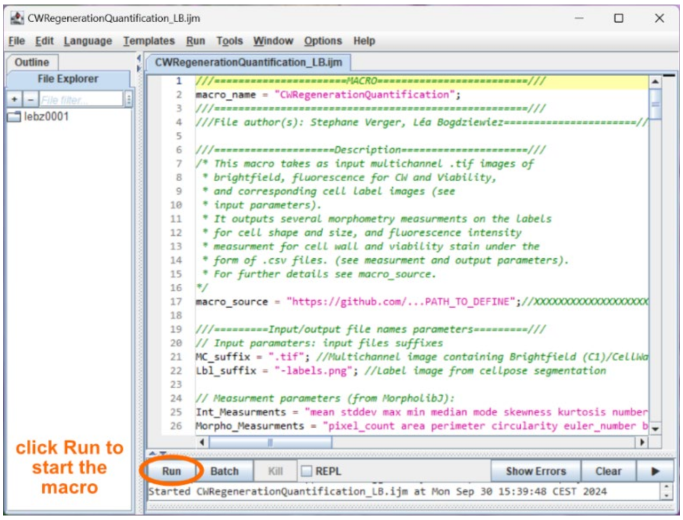
1. Go in the "Help" menu of Fiji and click on "Update…". This will open the "ImageJ updater".
2. In "ImageJ updater", click on "Manage update sites".
3. In the list, find e.g. "IJPB-Plugins" and click in the square next to it to add it.
4. Then close the window and apply changes on "ImageJ updater".
5. Once this is done, Fiji needs to be restarted.

If more explanation needed, see https://imagej.net/update-sites/following

Macro

Both imageJ macro CWRegenerationQuantification.ijm and save_as_tiff.ijm are in the downloaded folder **Scripts**.

- Drag & drop the file on Fiji user interface to open a macro
- Start the macro by clicking Run in the macro window



```
///=====================MACRO========================///
macro_name = "CWRegenerationQuantification";
///================================================///
///File author(s): Stephane Verger, Léa Bogdziewiez====================//

///===================Description====================///
/* This macro takes as input multichannel .tif images of
 * brightfield, fluorescence for CW and Viability,
 * and corresponding cell label images (see
 * input parameters).
 * It outputs several morphometry measurments on the labels
 * for cell shape and size, and fluorescence intensity
 * measurment for cell wall and viability stain under the
 * form of .csv files. (see measurment and output parameters).
 * For further details see macro_source.
*/
macro_source = "https://github.com/...PATH_TO_DEFINE";//XXXXXXXXXXXXXXXXXXX

///========Input/output file names parameters========///
// Input paramaters: input files suffixes
MC_suffix = ".tif"; //Multichannel image containing Brightfield (C1)/CellWa
Lbl_suffix = "-labels.png"; //Label image from cellpose segmentation

// Measurment parameters (from MorpholibJ):
Int_Measurments = "mean stddev max min median mode skewness kurtosis number
Morpho_Measurments = "pixel_count area perimeter circularity euler_number b
```

```
Started CWRegenerationQuantification_LB.ijm at Mon Sep 30 15:39:48 CEST 2024
```

click Run to start the macro

5- RStudio

- Install RStudio see: https://posit.co/download/rstudio-desktop/
- Install the packages needed to run the different scripts. Copy/paste the following code in the console of RStudio:

```
# Define the packages you want to install
packages <- c("data.table", "rlist", "hrbrthemes", "viridis", "tidyverse", "shiny",
"tcltk",  "plotly", "magick", "autothreshold", "patchwork")
# Find out which packages are already installed
installed_packages <- rownames(installed.packages())
# Determine which packages are not installed
packages_to_install <- setdiff(packages, installed_packages)
# Install the packages that are not yet installed
if (length(packages_to_install) > 0) {
  install.packages(packages_to_install)
} else {
  cat("All packages are already installed.\n")
}
```

The code is also available in the downloaded Q-Warg-Scripts folder as Install_R_packages.R.

> *The code checks if the required packages are installed and adds the ones that are not. For more information on how to install packages, see* https://cran.r-project.org/doc/manuals/r-release/R-admin.html#Default-packages .

Once RStudio and the scripts are downloaded, double-click on the script QWARG-notebook.Rmd to open it with RStudio. It is then ready to use.

Q-Warg: Using the pipeline

The Q-Warg pipeline can be fully tested using a sample dataset available in the GitHub repository. This dataset, located in the data_sample folder, includes the original TIFF images for 3 replicates under 2 experimental conditions and their corresponding CellPose labels (in data_sample_labels.zip).

Using these files and the provided scripts, you can run the complete Q-Warg workflow to verify its functionality before applying it to your own data. The user guide includes screenshots that illustrate the expected output at each step of the process.

1- Prepare raw data

Files naming:

- All files' names should have the following format: `Replicate__conditionX.tif`
- Replicate can be the date or nx. It is needed for the plots later.
- Use "__" to separate Replicate from condition. It should only be used to separate replicate from the used condition.
- Do not use "--" (simple – is fine) to not interfere with the scripts.
- Do not use spaces or dots (except for file extension) in the path of files names

If you plan on making only one replicate for screening, put the date as first part of the file name (e.g.: `240213__medium2`).

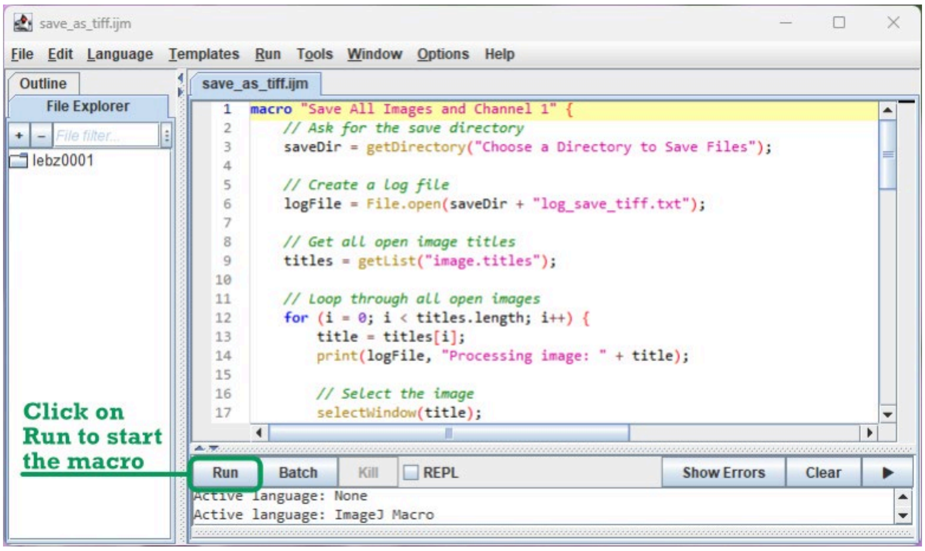
Do not change the names of the files after starting using the pipeline. It is a crucial parameter onto which several operations are based.

Save 3-channels images as TIFF:

The aim of this step is to convert proprietary format files issued from microscopes (like .lif for Leica or .czi for Zeiss) into tif files. TIFF files will then be used in to run the pipeline.

Option 1: (automated)

- Open images in Fiji by dragging and dropping the file onto Fiji user interface
- Open the macro save_as_tiff.ijm by dragging and dropping the file onto Fiji user interface



```
macro "Save All Images and Channel 1" {
    // Ask for the save directory
    saveDir = getDirectory("Choose a Directory to Save Files");

    // Create a log file
    logFile = File.open(saveDir + "log_save_tiff.txt");

    // Get all open image titles
    titles = getList("image.titles");

    // Loop through all open images
    for (i = 0; i < titles.length; i++) {
        title = titles[i];
        print(logFile, "Processing image: " + title);

        // Select the image
        selectWindow(title);
```

```
Active language: None
Active language: ImageJ Macro
```

Click on Run to start the macro

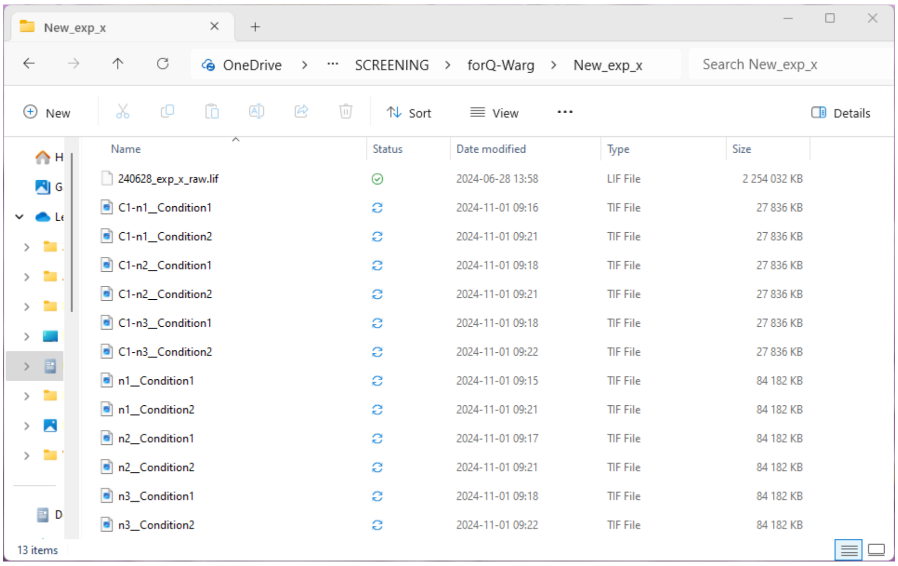
    - Run the macro by clicking Run:
        - Choose a folder where all images will be saved as tiff: 3-channels images and the C1 brightfield channel alone to be used in CellPose for segmentation.
- A log file is saved to check if every image has been saved as expected.

Option 2: (manual)

- Open images:
    - Open images in Fiji by dragging and dropping the file onto Fiji user interface
    - To save: File > Save As > Tiff...
- Save the brightfield (BF) image independently to be used for the segmentation step:
    - Image > Color > Split Channels
    - Save as TIFF the Brightfield channel
- Close images

We advise keeping a raw data folder containing all raw images and the saved TIFF.

The folder should have an organization like:



Name	Status	Date modified	Type	Size
240628_exp_x_raw.lif	✓	2024-06-28 13:58	LIF File	2 254 032 KB
C1-n1__Condition1	↻	2024-11-01 09:16	TIF File	27 836 KB
C1-n1__Condition2	↻	2024-11-01 09:21	TIF File	27 836 KB
C1-n2__Condition1	↻	2024-11-01 09:18	TIF File	27 836 KB
C1-n2__Condition2	↻	2024-11-01 09:21	TIF File	27 836 KB
C1-n3__Condition1	↻	2024-11-01 09:18	TIF File	27 836 KB
C1-n3__Condition2	↻	2024-11-01 09:22	TIF File	27 836 KB
n1__Condition1	↻	2024-11-01 09:15	TIF File	84 182 KB
n1__Condition2	↻	2024-11-01 09:21	TIF File	84 182 KB
n2__Condition1	↻	2024-11-01 09:17	TIF File	84 182 KB
n2__Condition2	↻	2024-11-01 09:21	TIF File	84 182 KB
n3__Condition1	↻	2024-11-01 09:18	TIF File	84 182 KB
n3__Condition2	↻	2024-11-01 09:22	TIF File	84 182 KB

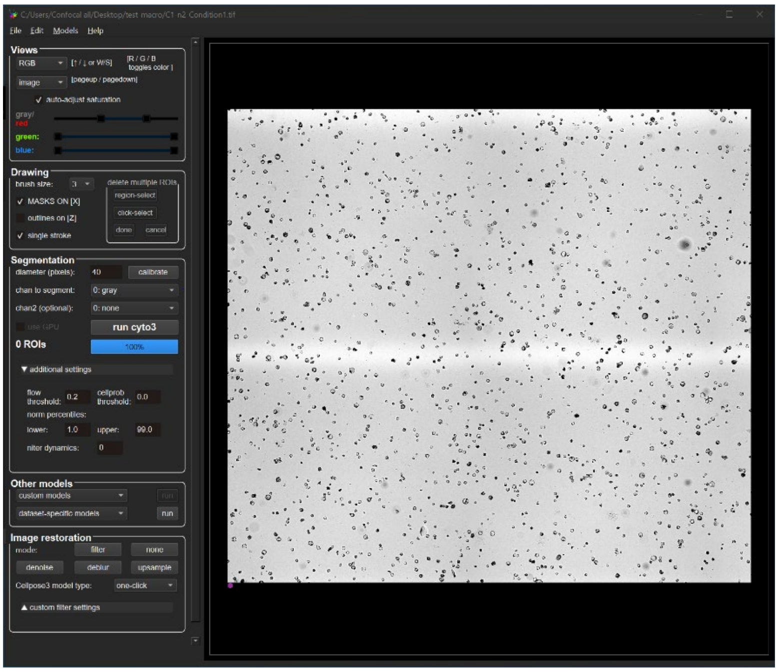
13 items

2- Cell segmentation: CellPose

Cell segmentation is required for extracting data from the images. CellPose, more specifically cyto3 generalist model, is working very well for our samples. This model should work for most of the isolated cells issued from protoplasting. However, any segmentation program can be used as long as it gives a PNG label image to be used in the next steps of the pipeline.

Detailed information about the software and how to use it can be found here: http://www.cellpose.org/ ; https://cellpose.readthedocs.io/en/latest/gui.html

- Drag & drop a brightfield file in the user interface to open the image:



C:/Users/Confocal all/Desktop/test macro/C1-n2-Condition1.tif

diameter (pixels): 40

chan to segment: 0: gray

chan2 (optional): 0: none

0 ROIs

flow threshold: 0.2

cellprob threshold: 0.0

norm percentiles: lower: 1.0 upper: 99.0

niter dynamics: 0

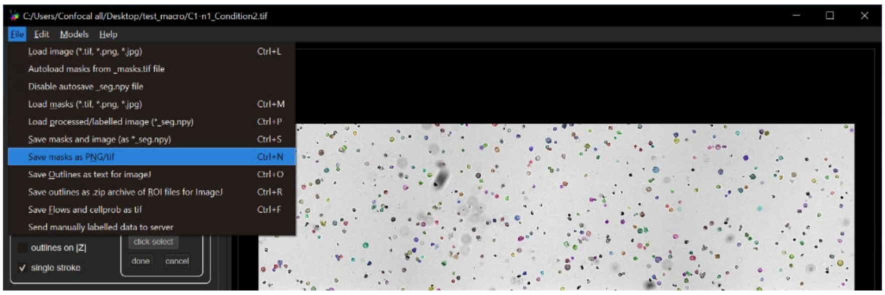
Cellpose3 model type: one-click

- Adjust parameters to fit your cells

Used in the publication: Parameters used for CellPose with cyto3 defaults settings:

  - ○ *Cell diameter (pixels): 40*
  - ○ *flow_threshold: 0.2. "The `flow threshold` parameter is the maximum allowed error of the flows for each mask. The default is `flow_threshold=0.4`. Increase this threshold if CellPose is not returning as many ROIs as you would expect. Similarly, decrease this threshold if CellPose is returning too many ill-shaped ROIs."*

- Run segmentation
- Save labels as PNG in File > Save masks as PNG/tif (the PNG image is automatically saved in the same folder as the original image).



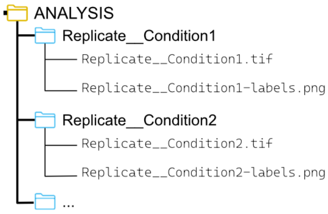
C:/Users/Confocal all/Desktop/test_macro/C1-n1_Condition2.tif

File Edit Models Help
Load image (*.tif, *.png, *.jpg) Ctrl+L
Autoload masks from _masks.tif file
Disable autosave _seg.npy file
Load masks (*.tif, *.png, *.jpg) Ctrl+M
Load processed/labelled image (*_seg.npy) Ctrl+P
Save masks and image (as *_seg.npy) Ctrl+S
Save masks as PNG/tif Ctrl+N
Save Outlines as text for imageJ Ctrl+O
Save outlines as .zip archive of ROI files for ImageJ Ctrl+R
Save Flows and cellprob as tif Ctrl+F
Send manually labelled data to server
outlines on [Z]
✓ single stroke
click select
done
cancel

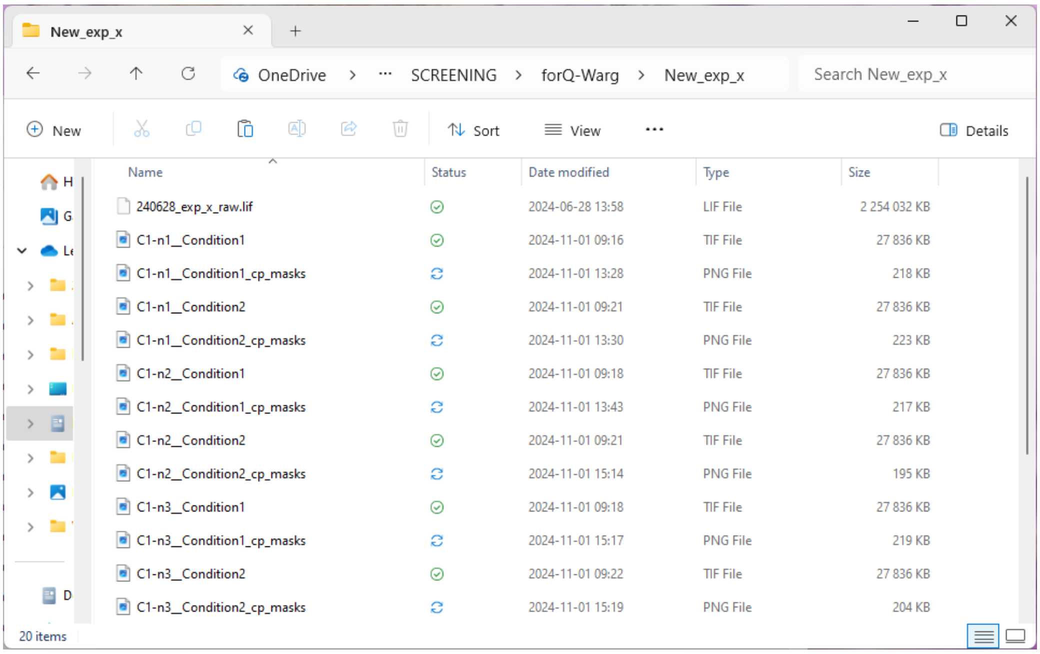
We recommend saving a copy of the label image in the raw data folder.

3- Data organization

The data with which to use Q-Warg should be organized as follows:


ANALYSIS
Replicate__Condition1
Replicate__Condition1.tif
Replicate__Condition1-labels.png
Replicate__Condition2
Replicate__Condition2.tif
Replicate__Condition2-labels.png
...

Each condition has its own folder containing the 3-channels image as TIFF and the label image as PNG. After segmentation with CellPose, the folder resembles:



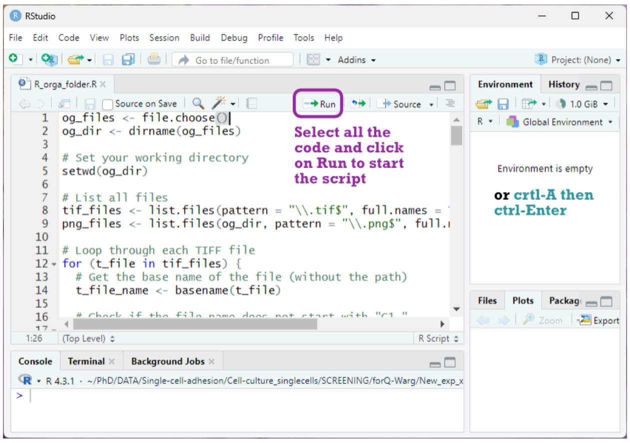
Name	Status	Date modified	Type	Size
240628_exp_x_raw.lif	✓	2024-06-28 13:58	LIF File	2 254 032 KB
C1-n1__Condition1	✓	2024-11-01 09:16	TIF File	27 836 KB
C1-n1__Condition1_cp_masks	⟳	2024-11-01 13:28	PNG File	218 KB
C1-n1__Condition2	✓	2024-11-01 09:21	TIF File	27 836 KB
C1-n1__Condition2_cp_masks	⟳	2024-11-01 13:30	PNG File	223 KB
C1-n2__Condition1	✓	2024-11-01 09:18	TIF File	27 836 KB
C1-n2__Condition1_cp_masks	⟳	2024-11-01 13:43	PNG File	217 KB
C1-n2__Condition2	✓	2024-11-01 09:21	TIF File	27 836 KB
C1-n2__Condition2_cp_masks	⟳	2024-11-01 15:14	PNG File	195 KB
C1-n3__Condition1	✓	2024-11-01 09:18	TIF File	27 836 KB
C1-n3__Condition1_cp_masks	⟳	2024-11-01 15:17	PNG File	219 KB
C1-n3__Condition2	✓	2024-11-01 09:22	TIF File	27 836 KB
C1-n3__Condition2_cp_masks	⟳	2024-11-01 15:19	PNG File	204 KB

20 items

Organize folders:

Option 1: (automated)

- Open the R script R_orga_folder.R by double clicking on it (RStudio will automatically start).
- Run the code:



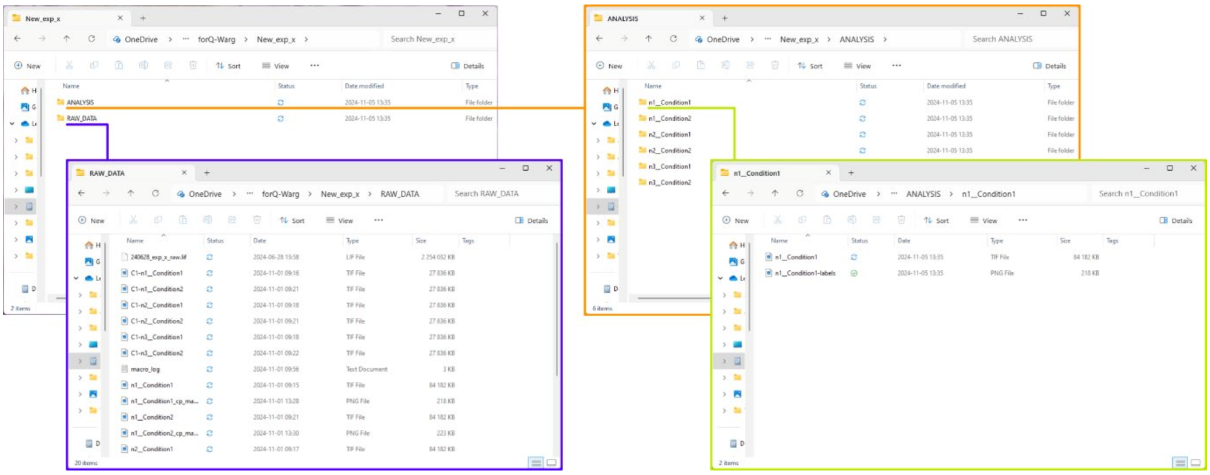
```r
og_files <- file.choose()
og_dir <- dirname(og_files)

# Set your working directory
setwd(og_dir)

# List all files
tif_files <- list.files(pattern = "\\.tif$", full.names = 
png_files <- list.files(og_dir, pattern = "\\.png$", full.

# Loop through each TIFF file
for (t_file in tif_files) {
  # Get the base name of the file (without the path)
  t_file_name <- basename(t_file)

  # Check if the file name does not start with "C1_"
```

```
R 4.3.1 · ~/PhD/DATA/Single-cell-adhesion/Cell-culture_singlecells/SCREENING/forQ-Warg/New_exp_x
>
```

Select all the code and click on Run to start the script

or crtl-A then ctrl-Enter

- A pop-up window will open to choose which folder to organize. Select any file in this folder to get it tidy.



New_exp_x — OneDrive > ··· forQ-Warg > New_exp_x >

Name	Date modified	Type
ANALYSIS	2024-11-05 13:35	File folder
RAW_DATA	2024-11-05 13:35	File folder

2 items

RAW_DATA — OneDrive > ··· forQ-Warg > New_exp_x > RAW_DATA

Name	Date	Type	Size
240628_exp_x_raw.lif	2024-06-28 13:58	LIF File	2 254 032 KB
C1-n1__Condition1	2024-11-01 09:16	TIF File	27 836 KB
C1-n1__Condition2	2024-11-01 09:21	TIF File	27 836 KB
C1-n2__Condition1	2024-11-01 09:18	TIF File	27 836 KB
C1-n2__Condition2	2024-11-01 09:21	TIF File	27 836 KB
C1-n3__Condition1	2024-11-01 09:18	TIF File	27 836 KB
C1-n3__Condition2	2024-11-01 09:22	TIF File	27 836 KB
macro_log	2024-11-01 09:56	Text Document	3 KB
n1__Condition1	2024-11-01 09:15	TIF File	84 182 KB
n1__Condition1_cp_ma...	2024-11-01 13:28	PNG File	218 KB
n1__Condition2	2024-11-01 09:21	TIF File	84 182 KB
n1__Condition2_cp_ma...	2024-11-01 13:30	PNG File	223 KB
n2__Condition1	2024-11-01 09:17	TIF File	84 182 KB

20 items

ANALYSIS — OneDrive > ··· New_exp_x > ANALYSIS >

Name	Date modified	Type
n1__Condition1	2024-11-05 13:35	File folder
n1__Condition2	2024-11-05 13:35	File folder
n2__Condition1	2024-11-05 13:35	File folder
n2__Condition2	2024-11-05 13:35	File folder
n3__Condition1		
n3__Condition2		

6 items

n1__Condition1 — OneDrive > ··· ANALYSIS > n1__Condition1

Name	Date	Type	Size
n1__Condition1	2024-11-05 13:35	TIF File	84 182 KB
n1__Condition1-labels	2024-11-05 13:35	PNG File	218 KB

2 items

The script can also be used if the segmentation method is not CellPose if it is a PNG file and it contains the name of the original file. The script removes any extension to the file name and adds -labels.

Option 2: (manual)

- Please organize the folders as described above, creating one folder for each condition. Inside each folder, include only the 3-channel TIFF image and the corresponding label image.
- Naming: (see section 1 for other guidelines)
  - Both images (3-channels TIFF and the PNG label image) must have the same base name e.g.: “Condition_01” (make sure to give meaningful names).
  - The label image must contain “-labels” at the end of the name
  - Do not use spaces

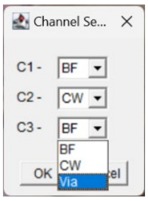
The working folder for the next steps is here called “ANALYSIS”

By using the automated script, the RAW_DATA folder contains already the original images and the label images. Tip: the RAW_DATA folder can be compressed to free up space.

4- CWRegenerationQuantification.ijm (Fiji macro)

The data are now organized and ready to be used for analysis. The next step is to start the quantification for fluorescence intensity and cell morphometry.

- Start the macro (as detailed in the installation part of this guide).
- A pop-up window opens and asks to choose in which order are the channels: for each Cn-, select the corresponding channel (BF: Brightfield; CW: Cell Wall; Via: Viability).



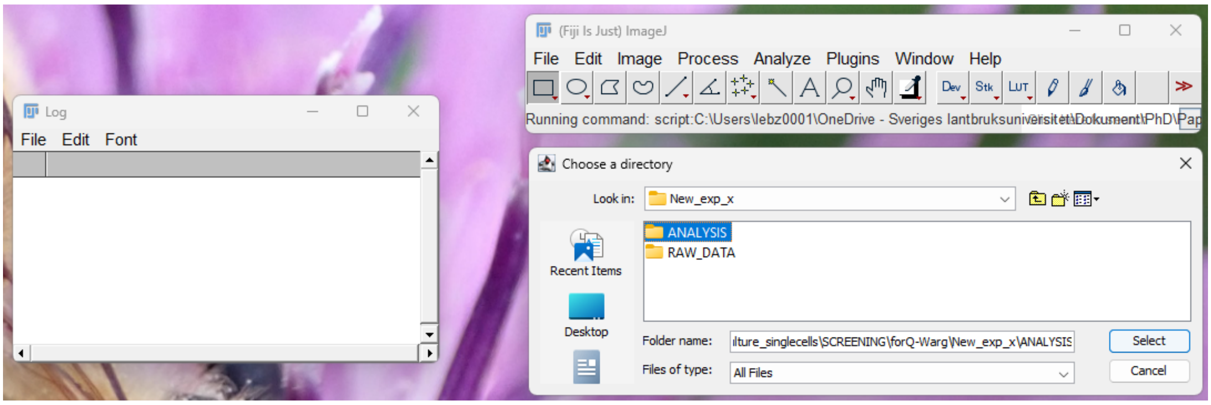
Channel Se...

C1 - BF

C2 - CW

C3 - BF

- Choose the working folder in the pop-up window. *In. the. example. here. »New(exp(x_ANALYSIS¡*



Running command: script:C:\Users\lebz0001\OneDrive - Sveriges lantbruksuniversitet\Dokument\PhD\Pap

Look in: New_exp_x

ANALYSIS
RAW_DATA

Folder name: ilture_singlecells\SCREENING\forQ-Warg\New_exp_x\ANALYSIS

Files of type: All Files

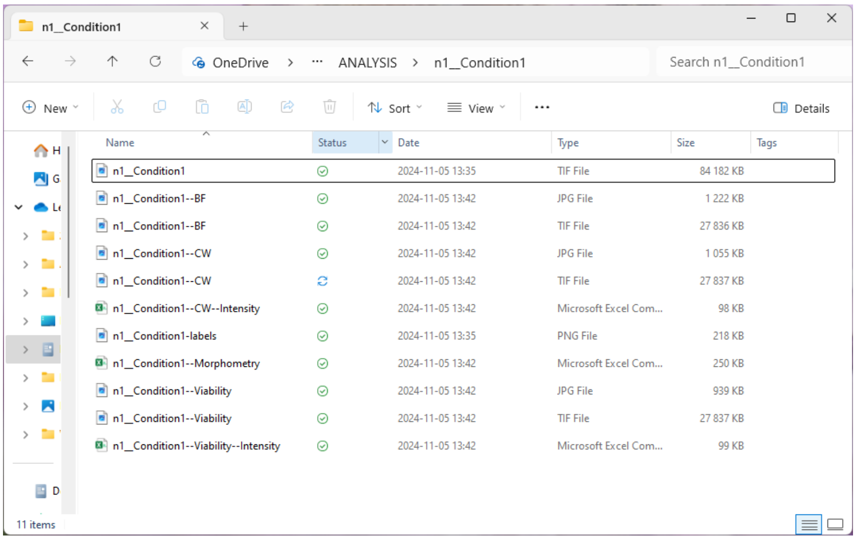
- Let the macro run:
  - Log window opens and allows you to follow the different steps of the process.

If you want to run the macro on only one condition, you need to put your sample folder into a working folder that only contains this folder of interest. The macro will report an error if it is running directly in the sample folder.

When running the macro, try not to use the computer, especially the keyboard, because pop-up windows will come on top of all the other windows open and you might terminate the process by mistake.

  - Check the log window to know when the processing is done

The log is also saved into the working folder. It can be checked to verify all processed conditions.



Name	Status	Date	Type	Size	Tags
n1__Condition1	✓	2024-11-05 13:35	TIF File	84 182 KB	
n1__Condition1--BF	✓	2024-11-05 13:42	JPG File	1 222 KB	
n1__Condition1--BF	✓	2024-11-05 13:42	TIF File	27 836 KB	
n1__Condition1--CW	✓	2024-11-05 13:42	JPG File	1 055 KB	
n1__Condition1--CW	↻	2024-11-05 13:42	TIF File	27 837 KB	
n1__Condition1--CW--Intensity	✓	2024-11-05 13:42	Microsoft Excel Com...	98 KB	
n1__Condition1-labels	✓	2024-11-05 13:35	PNG File	218 KB	
n1__Condition1--Morphometry	✓	2024-11-05 13:42	Microsoft Excel Com...	250 KB	
n1__Condition1--Viability	✓	2024-11-05 13:42	JPG File	939 KB	
n1__Condition1--Viability	✓	2024-11-05 13:42	TIF File	27 837 KB	
n1__Condition1--Viability--Intensity	✓	2024-11-05 13:42	Microsoft Excel Com...	99 KB	

The sample folders now contain many new files:

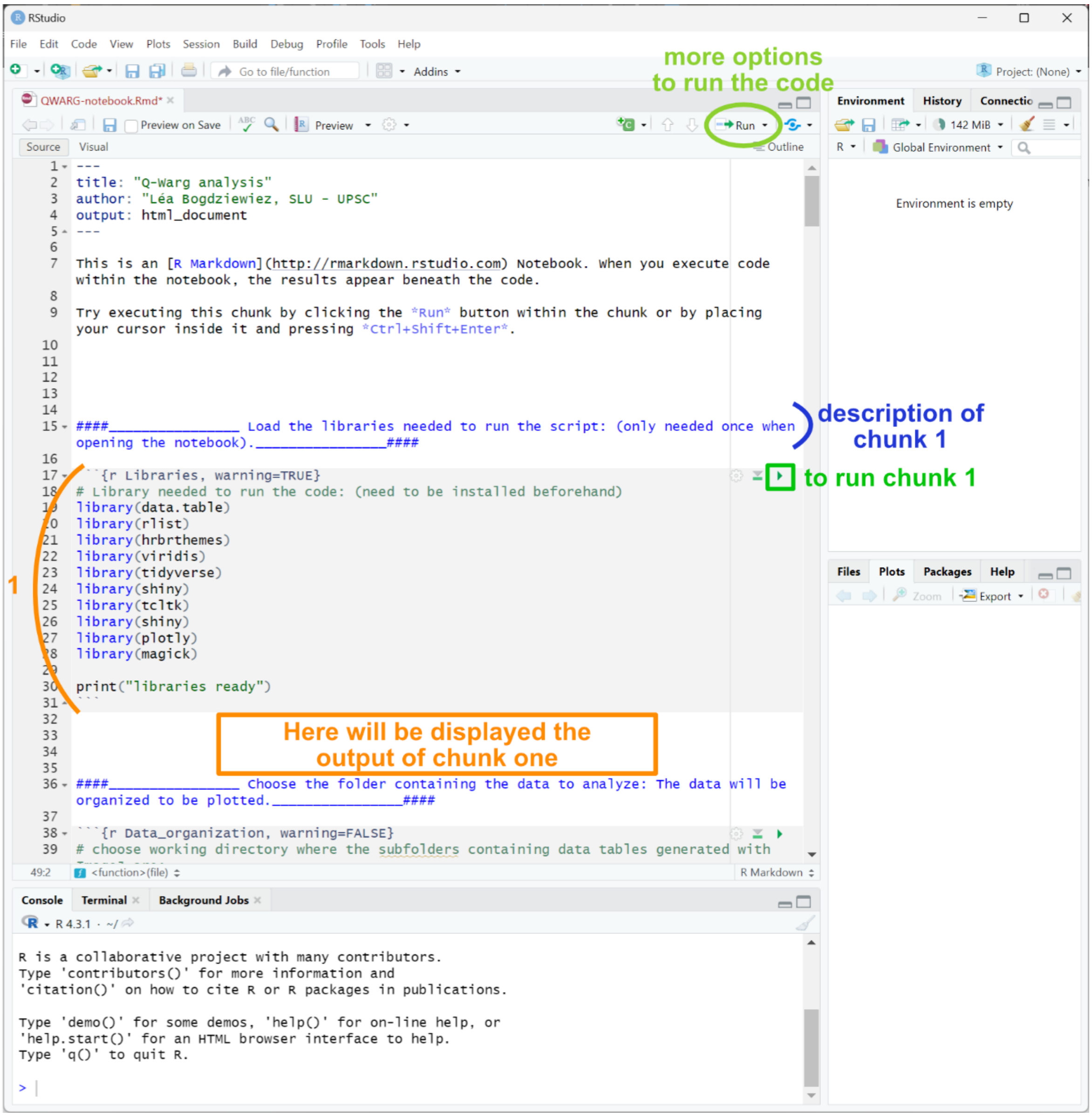
- The macro splits the 3-channel TIFF image and saves each channel independently (for later potential use).
- Each channel in JPG with the segmentation border to be used to check the images in the Q-Warg shiny app.
- CSV files with morphometry (size, circularity, position), fluorescence intensity for viability and for cell wall.

5- QWARG-notebook.Rmd

Analysis and plots

With the R script, the csv files obtained from the macro are combined and reorganized to obtain information about the living cells that have been segmented. Detailed information about the script can be found as comments directly in the script file QWARG-notebook.Rmd. The notebook is composed of several chunks (part of code) and each one is briefly described.

- Open QWARG-notebook.Rmd by double-clicking on it (RStudio will automatically start).
- Run the code: each chunk (a chunk is a block of code that will be executed as a unit, R name for a code block) can be run by clicking on the play sign at the top of it.



~~~
---
title: "Q-Warg analysis"
author: "Léa Bogdziewiez, SLU - UPSC"
output: html_document
---

This is an [R Markdown](http://rmarkdown.rstudio.com) Notebook. When you execute code within the notebook, the results appear beneath the code.

Try executing this chunk by clicking the *Run* button within the chunk or by placing your cursor inside it and pressing *Ctrl+Shift+Enter*.






####_______________ Load the libraries needed to run the script: (only needed once when opening the notebook)._______________####

```{r Libraries, warning=TRUE}
# Library needed to run the code: (need to be installed beforehand)
library(data.table)
library(rlist)
library(hrbrthemes)
library(viridis)
library(tidyverse)
library(shiny)
library(tcltk)
library(shiny)
library(plotly)
library(magick)

print("libraries ready")
~~~

####_______________ Choose the folder containing the data to analyze: The data will be organized to be plotted._______________####

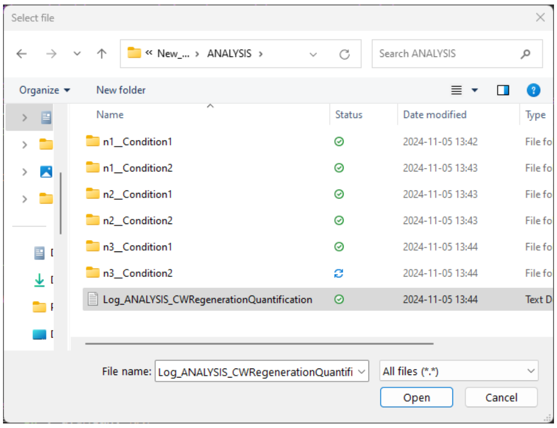
```{r Data_organization, warning=FALSE}
# choose working directory where the subfolders containing data tables generated with
```

```
R is a collaborative project with many contributors.
Type 'contributors()' for more information and
'citation()' on how to cite R or R packages in publications.

Type 'demo()' for some demos, 'help()' for on-line help, or
'help.start()' for an HTML browser interface to help.
Type 'q()' to quit R.

>
```

- Chunk 1: load the libraries, always needed to run other parts of the code.

  If you only need to run the shiny app, or other chunks of the app, you first need to run the first chunk to load the libraries.

- Chunk 2: data frame
  - Define the working folder (here ANALYSIS) in the pop-up window: choose the Log file created when using the CWR macro.



Select file

« New_... › ANALYSIS ›

Search ANALYSIS

Organize ▾ New folder

Name	Status	Date modified	Type
n1__Condition1	✓	2024-11-05 13:42	File fo
n1__Condition2	✓	2024-11-05 13:43	File fo
n2__Condition1	✓	2024-11-05 13:43	File fo
n2__Condition2	✓	2024-11-05 13:43	File fo
n3__Condition1	✓	2024-11-05 13:44	File fo
n3__Condition2	⟳	2024-11-05 13:44	File fo
Log_ANALYSIS_CWRegenerationQuantification	✓	2024-11-05 13:44	Text D

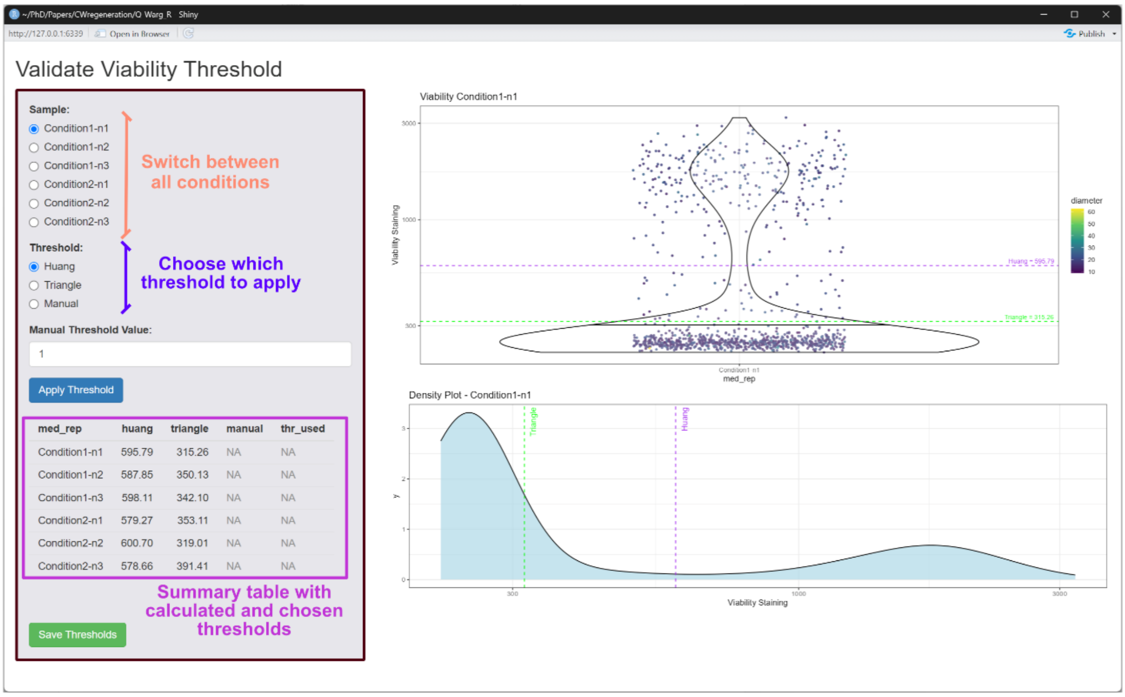
File name: Log_ANALYSIS_CWRegenerationQuantifi

All files (*.*)

Possible issue at this step: the window might open in the background, check the taskbar.

  - Viability threshold is calculated for each condition with both the Huang and the triangle automatic threshold methods.
  - An organized data table is generated (see additional information section for more information).

- Chunk 3: viability threshold
  - An application opens to set the viability threshold for each condition:
  - The viability staining intensity is represented in 2 ways to help estimate which threshold is the most adapted to the data.



~/PhD/Papers/CWregeneration/Q Warg R Shiny

http://127.0.0.1:6339 Open in Browser

Validate Viability Threshold

Sample:

- ● Condition1-n1
- ○ Condition1-n2
- ○ Condition1-n3
- ○ Condition2-n1
- ○ Condition2-n2
- ○ Condition2-n3

Switch between all conditions

Threshold:

- ● Huang
- ○ Triangle
- ○ Manual

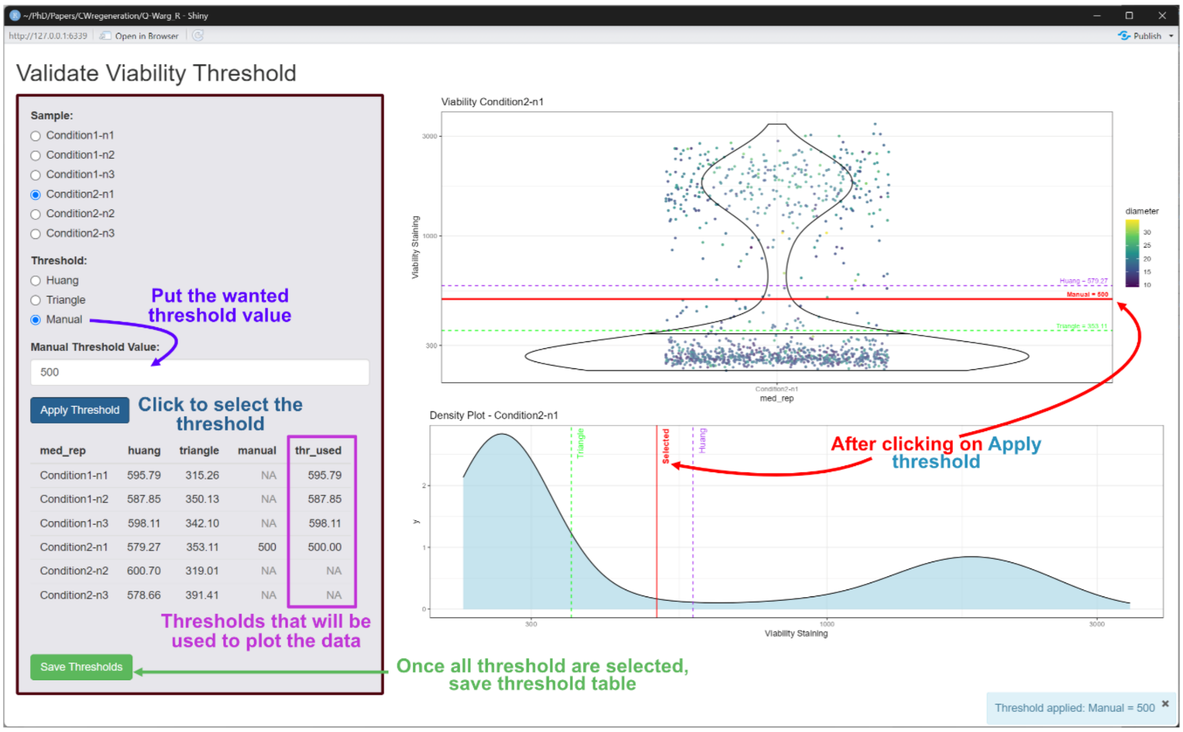
Choose which threshold to apply

Manual Threshold Value: 1

med_rep	huang	triangle	manual	thr_used
Condition1-n1	595.79	315.26	NA	NA
Condition1-n2	587.85	350.13	NA	NA
Condition1-n3	598.11	342.10	NA	NA
Condition2-n1	579.27	353.11	NA	NA
Condition2-n2	600.70	319.01	NA	NA
Condition2-n3	578.66	391.41	NA	NA

Summary table with calculated and chosen thresholds

- ○ Choose which threshold to apply for each condition. Once it has been selected (under Threshold) and if relevant, the manual value written, click on <u>Apply Threshold</u> to save the value in the table. A red line will be added on the plots to show the selected value.
- ○ Click on <u>Save Thresholds</u> to save the table once all values have been added to the last column.



~/PhD/Papers/CWregeneration/Q-Warg_R - Shiny

http://127.0.0.1:6339 Open in Browser

Validate Viability Threshold

Sample:

○ Condition1-n1
○ Condition1-n2
○ Condition1-n3
● Condition2-n1
○ Condition2-n2
○ Condition2-n3

Threshold:

○ Huang
○ Triangle
● Manual

Put the wanted threshold value

Manual Threshold Value: 500

Apply Threshold

Click to select the threshold

med_rep	huang	triangle	manual	thr_used
Condition1-n1	595.79	315.26	NA	595.79
Condition1-n2	587.85	350.13	NA	587.85
Condition1-n3	598.11	342.10	NA	598.11
Condition2-n1	579.27	353.11	500	500.00
Condition2-n2	600.70	319.01	NA	NA
Condition2-n3	578.66	391.41	NA	NA

Thresholds that will be used to plot the data

Save Thresholds

Once all threshold are selected, save threshold table

After clicking on Apply threshold

Threshold applied: Manual = 500

The threshold table will be saved later in the workflow, with a mention of which type of threshold has been used for each condition.

To determine if the segmented objects are living cells, the script uses viability staining and the circularity of the cells. A threshold is applied for cell viability staining. Below this value, the cells are considered dead. This threshold is dependent on the viability staining and the imaging setup so it should be adjusted if needed.

2 automatic thresholding methods are available: Huang or Triangle. Only one method should be used for one data set to obtain consistent results.

As automatic thresholding methods do not consider the values but only the shape of the data, it might lead to thresholding even if there is only a dead population. The app counteracts this with the possibility of adding a manual threshold. The manual threshold should be chosen carefully. For instance, it could be the minimal value of the chosen automated threshold on other conditions.

In addition, to ensure that the object is a cell, a high threshold is used for circularity (0,9). Indeed, after protoplasting, the cells lose their original shape and become spherical. In absence of directional force on the cell, they stay spherical in the early phase of regenerating their cell wall. If experiments are conducted with different cell shapes, it might be needed to adjust this parameter. All the code corresponding to the thresholding can be found in the last part of the chunk 2 (indicated as comment in the code).

- Chunk 4: plot cell wall intensity, cell size and cell viability generation
  - The CW staining mean intensity is represented in 2 plots:
    - With dots color corresponding to the replicate
    - with dots color corresponding to cell diameter
  - The CW staining median intensity
  - The viability staining intensity is represented the same way as the CW staining intensity
  - Live and dead objects count and % of each in the population of segmented objects.

The black percentage cannot be interpreted as a number of dead cells but non-living objects. While dying, a cell might have burst into several parts that have been segmented.

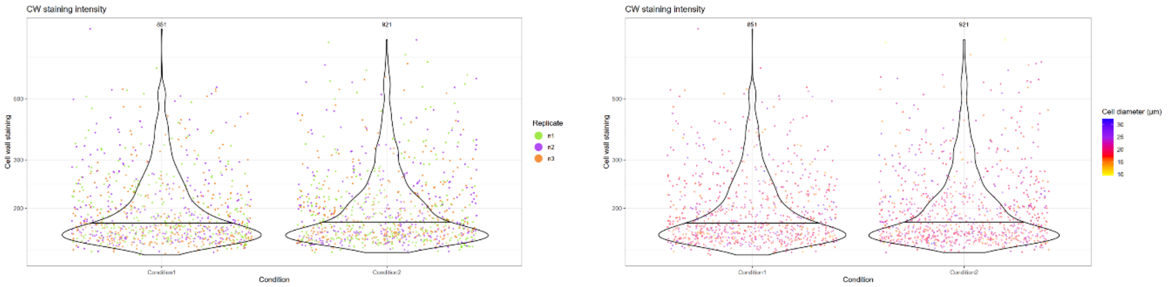
  - Plots with automatic viability thresholds: one dot plot and one density curve per condition.

All plots corresponding to the data sample can be found on GitHub in the data_sample folder.

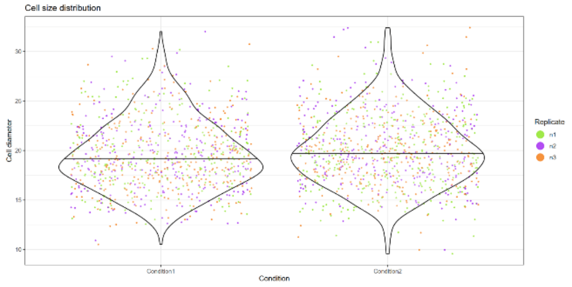
Cell Wall staining intensity

dot color = replicate

dot color = cell diameter

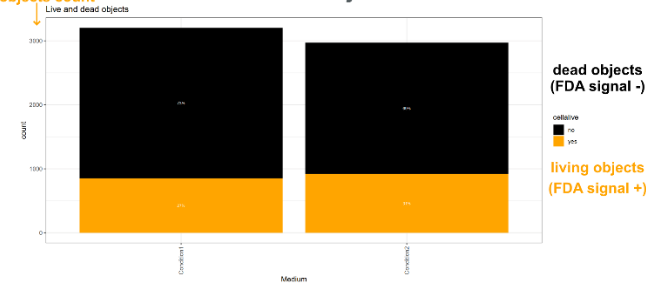


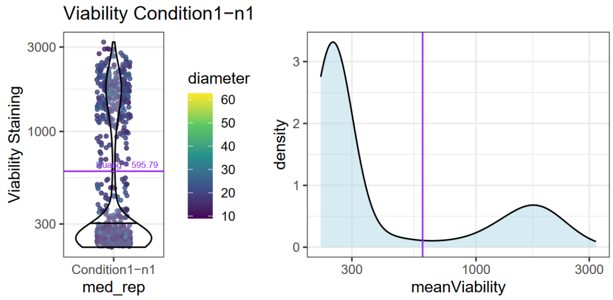
Cell size distribution



Live and dead objects

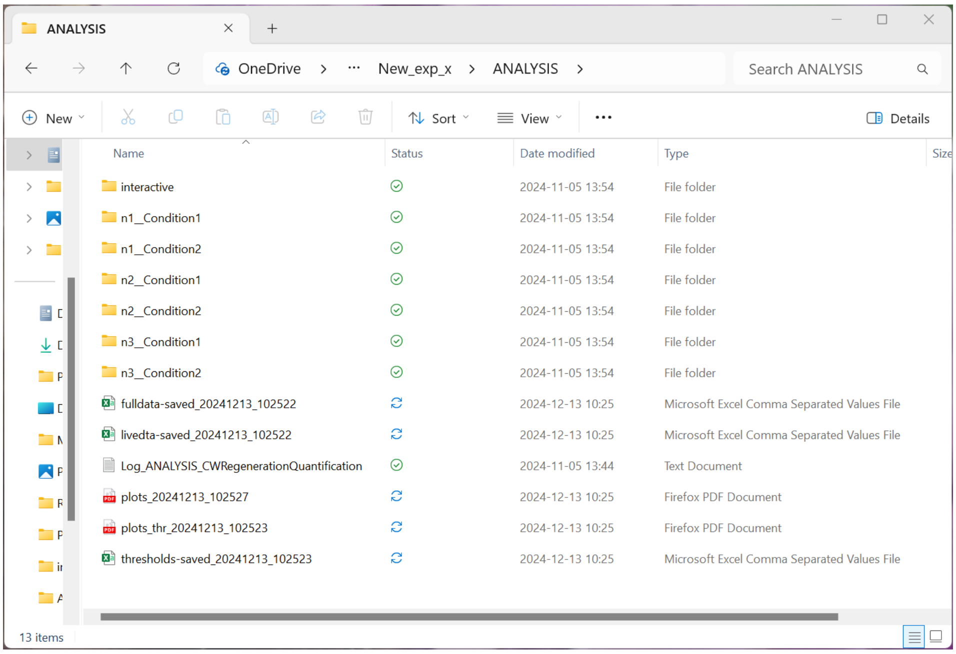
total segmented objects count





- Chunk 5: save plots and data frame
  - Choose a file in the folder where you want to save the plots and data frame, *e¡g¡*: Log_Analysis to save in the Analysis folder (same way as for chunk 2)

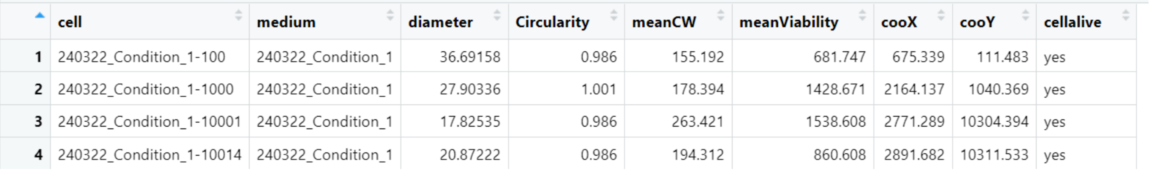
5 files are saved:



Name	Status	Date modified	Type	Size
interactive	✓	2024-11-05 13:54	File folder	
n1__Condition1	✓	2024-11-05 13:54	File folder	
n1__Condition2	✓	2024-11-05 13:54	File folder	
n2__Condition1	✓	2024-11-05 13:54	File folder	
n2__Condition2	✓	2024-11-05 13:54	File folder	
n3__Condition1	✓	2024-11-05 13:54	File folder	
n3__Condition2	✓	2024-11-05 13:54	File folder	
fulldata-saved_20241213_102522	↻	2024-12-13 10:25	Microsoft Excel Comma Separated Values File	
livedta-saved_20241213_102522	↻	2024-12-13 10:25	Microsoft Excel Comma Separated Values File	
Log_ANALYSIS_CWRegenerationQuantification	✓	2024-11-05 13:44	Text Document	
plots_20241213_102527	↻	2024-12-13 10:25	Firefox PDF Document	
plots_thr_20241213_102523	↻	2024-12-13 10:25	Firefox PDF Document	
thresholds-saved_20241213_102523	↻	2024-12-13 10:25	Microsoft Excel Comma Separated Values File	

13 items

- Plots described in the chunk 3 section:
  - Data plots
  - Viability threshold plots
- threshold_saved.csv: contains the selected threshold values.
- full_data_saved.csv: contains all data, for live and dead objects.
- livedta_saved.csv: data for living cells only. It should look like this:



	cell	medium	diameter	Circularity	meanCW	meanViability	cooX	cooY	cellalive
1	240322_Condition_1-100	240322_Condition_1	36.69158	0.986	155.192	681.747	675.339	111.483	yes
2	240322_Condition_1-1000	240322_Condition_1	27.90336	1.001	178.394	1428.671	2164.137	1040.369	yes
3	240322_Condition_1-10001	240322_Condition_1	17.82535	0.986	263.421	1538.608	2771.289	10304.394	yes
4	240322_Condition_1-10014	240322_Condition_1	20.87222	0.986	194.312	860.608	2891.682	10311.533	yes

The names of the files contain date and time. The plots and data table can be made and saved several times without replacing the older ones.

A more detailed explanation of the data tables can be found in the additional information section at the end of this user guide.

Q-Warg shiny app

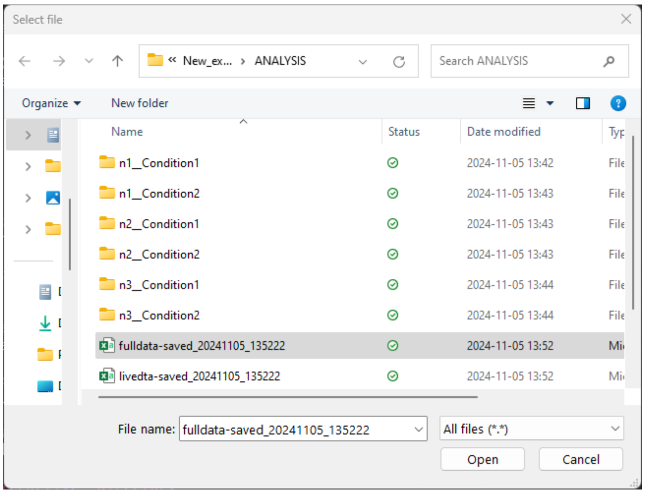
The aim of this app is to check that the analysis pipeline worked as expected: each point of the plot corresponds to a living cell and the viability threshold is correct. It allows you to load the full data file obtained with the previous script and click on each point of an interactive graph to see the corresponding cell in the 3 channels of the original picture.

To access both the images and the data table, the data must be reorganized. All the JPG images and the livedta-saved should be in one folder here called "interactive".

Reorganize data in the working folder:

Option 1: (automated)

- Run Chunk 6 in the QWARG-notebook.Rmd
- Choose the same working directory as before: choose any file in the Analysis folder. E.g. fulldata_saved.csv

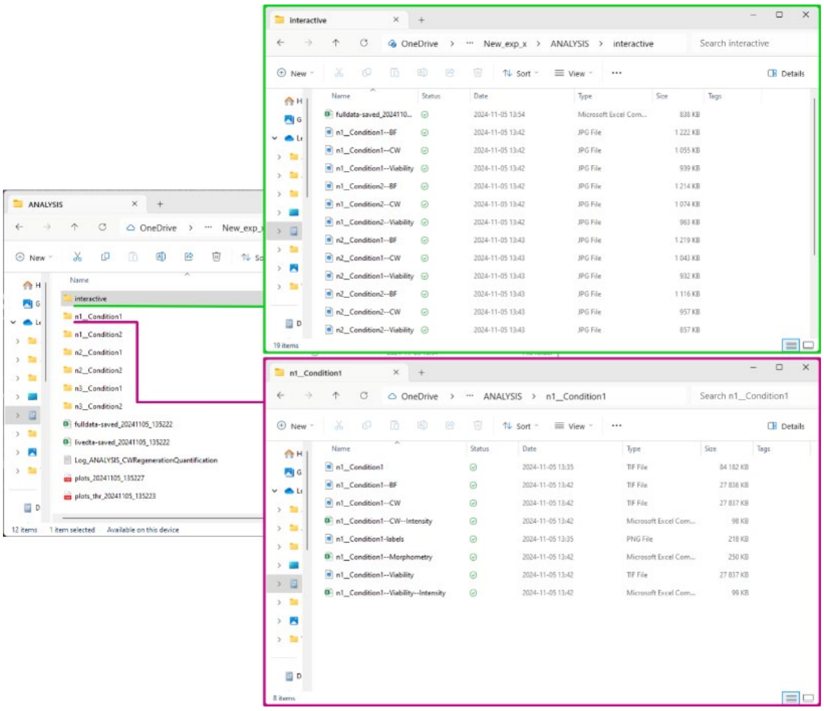


Name	Status	Date modified	Typ
n1__Condition1	✓	2024-11-05 13:42	File
n1__Condition2	✓	2024-11-05 13:43	File
n2__Condition1	✓	2024-11-05 13:43	File
n2__Condition2	✓	2024-11-05 13:43	File
n3__Condition1	✓	2024-11-05 13:44	File
n3__Condition2	✓	2024-11-05 13:44	File
fulldata-saved_20241105_135222	✓	2024-11-05 13:52	Mi
livedta-saved_20241105_135222	✓	2024-11-05 13:52	Mi

File name: fulldata-saved_20241105_135222

All files (*.*)

- The interactive folder is created, JPG files are moved inside, and the fulldata_saved csv file is copied.



Name
interactive
n1__Condition1
n1__Condition2
n2__Condition1
n2__Condition2
n3__Condition1
n3__Condition2
fulldata-saved_20241105_135222
livedta-saved_20241105_135222
Log_ANALYSIS_CWRegenerationQuantification
plots_20241105_135227
plots_thr_20241105_135223

12 items   1 item selected   Available on this device

Name	Status	Date	Type	Size	Tags
fulldata-saved_2024110...	✓	2024-11-05 13:54	Microsoft Excel Com...	838 KB	
n1__Condition1--BF	✓	2024-11-05 13:42	JPG File	1 222 KB	
n1__Condition1--CW	✓	2024-11-05 13:42	JPG File	1 055 KB	
n1__Condition1--Viability	✓	2024-11-05 13:42	JPG File	939 KB	
n1__Condition2--BF	✓	2024-11-05 13:42	JPG File	1 214 KB	
n1__Condition2--CW	✓	2024-11-05 13:42	JPG File	1 074 KB	
n1__Condition2--Viability	✓	2024-11-05 13:42	JPG File	963 KB	
n2__Condition1--BF	✓	2024-11-05 13:43	JPG File	1 219 KB	
n2__Condition1--CW	✓	2024-11-05 13:43	JPG File	1 043 KB	
n2__Condition1--Viability	✓	2024-11-05 13:43	JPG File	932 KB	
n2__Condition2--BF	✓	2024-11-05 13:43	JPG File	1 116 KB	
n2__Condition2--CW	✓	2024-11-05 13:43	JPG File	957 KB	
n2__Condition2--Viability	✓	2024-11-05 13:43	JPG File	857 KB	

19 items

Name	Status	Date	Type	Size	Tags
n1__Condition1	✓	2024-11-05 13:35	TIF File	84 182 KB	
n1__Condition1--BF	✓	2024-11-05 13:42	TIF File	27 836 KB	
n1__Condition1--CW	✓	2024-11-05 13:42	TIF File	27 837 KB	
n1__Condition1--CW--Intensity	✓	2024-11-05 13:42	Microsoft Excel Com...	98 KB	
n1__Condition1-labels	✓	2024-11-05 13:35	PNG File	218 KB	
n1__Condition1--Morphometry	✓	2024-11-05 13:42	Microsoft Excel Com...	250 KB	
n1__Condition1--Viability	✓	2024-11-05 13:42	TIF File	27 837 KB	
n1__Condition1--Viability--Intensity	✓	2024-11-05 13:42	Microsoft Excel Com...	99 KB	

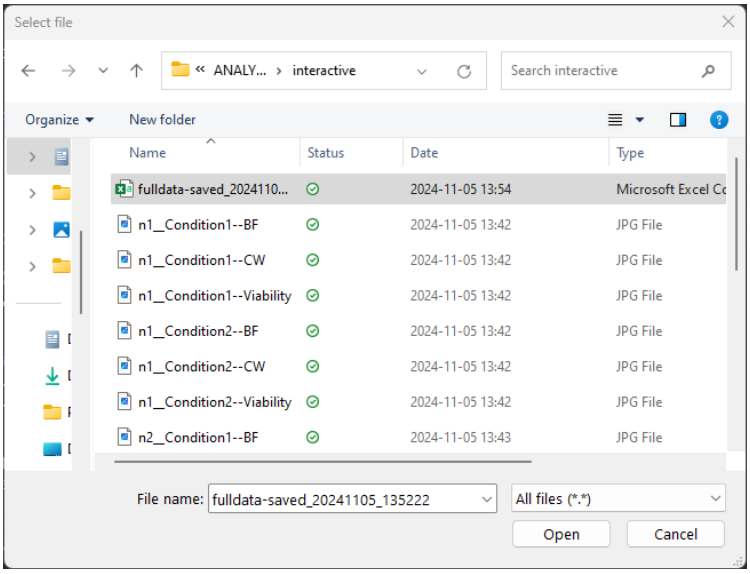
8 items

Option 2: (manual)

- Create a new folder called interactive.
- Move by hand all JPG images from the different sample folders and the livedta-saved csv file into this folder.

  *The name of the folder is not important, if using Option 2 it will be named "**interactive**" by the script. The crucial point is to keep the naming of all files, otherwise the app will not be able to connect the images with the quantifications.*

- Open the application by running Chunk 7
  - Choose any file in the interactive directory in the pop-up window



Select file

« ANALY... › interactive

Search interactive

Organize ▾ New folder

Name	Status	Date	Type
fulldata-saved_2024110...	✓	2024-11-05 13:54	Microsoft Excel Co
n1__Condition1--BF	✓	2024-11-05 13:42	JPG File
n1__Condition1--CW	✓	2024-11-05 13:42	JPG File
n1__Condition1--Viability	✓	2024-11-05 13:42	JPG File
n1__Condition2--BF	✓	2024-11-05 13:42	JPG File
n1__Condition2--CW	✓	2024-11-05 13:42	JPG File
n1__Condition2--Viability	✓	2024-11-05 13:42	JPG File
n2__Condition1--BF	✓	2024-11-05 13:43	JPG File

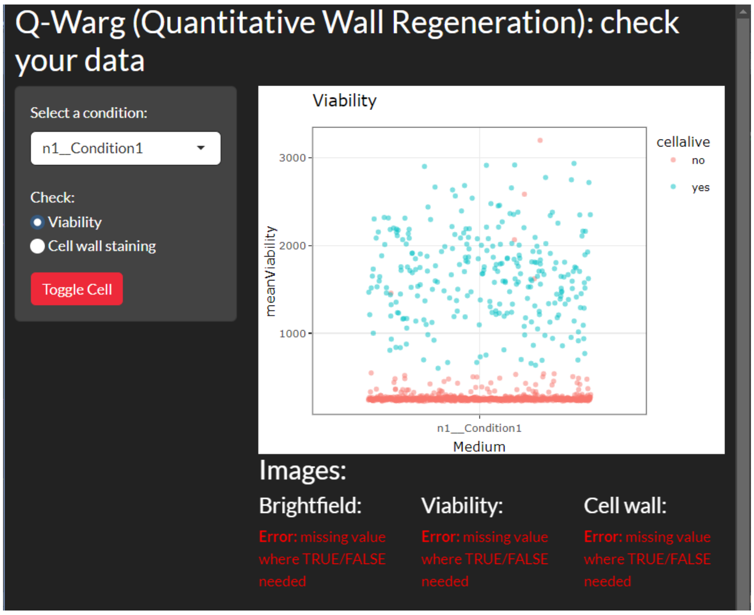
File name: fulldata-saved_20241105_135222

All files (*.*)

Open Cancel

An issue that can arise at this step: the window might open in background, check the taskbar.

- The app window is opening.



Q-Warg (Quantitative Wall Regeneration): check your data

Select a condition: n1__Condition1 ▾

Check:

● Viability

○ Cell wall staining

Toggle Cell

Images:

Brightfield: Error: missing value where TRUE/FALSE needed

Viability: Error: missing value where TRUE/FALSE needed

Cell wall: Error: missing value where TRUE/FALSE needed

On the left panel, a rolling menu allows you to choose which condition to check. Only one condition at a time can be visible.

Two different plots can be displayed:

- Viability plot with the viability staining intensity in Y axis. All segmented objects are plotted, the color of the dot shows if the point corresponds to a dead or a living object.
- Cell wall staining intensity (Y axis), the color of the dot corresponds to the viability mean intensity. Only living objects are displayed.

The plots are interactive, hovering the mouse over a data point will display information about it:

- The condition it comes from
- CW mean intensity
- Viability mean intensity

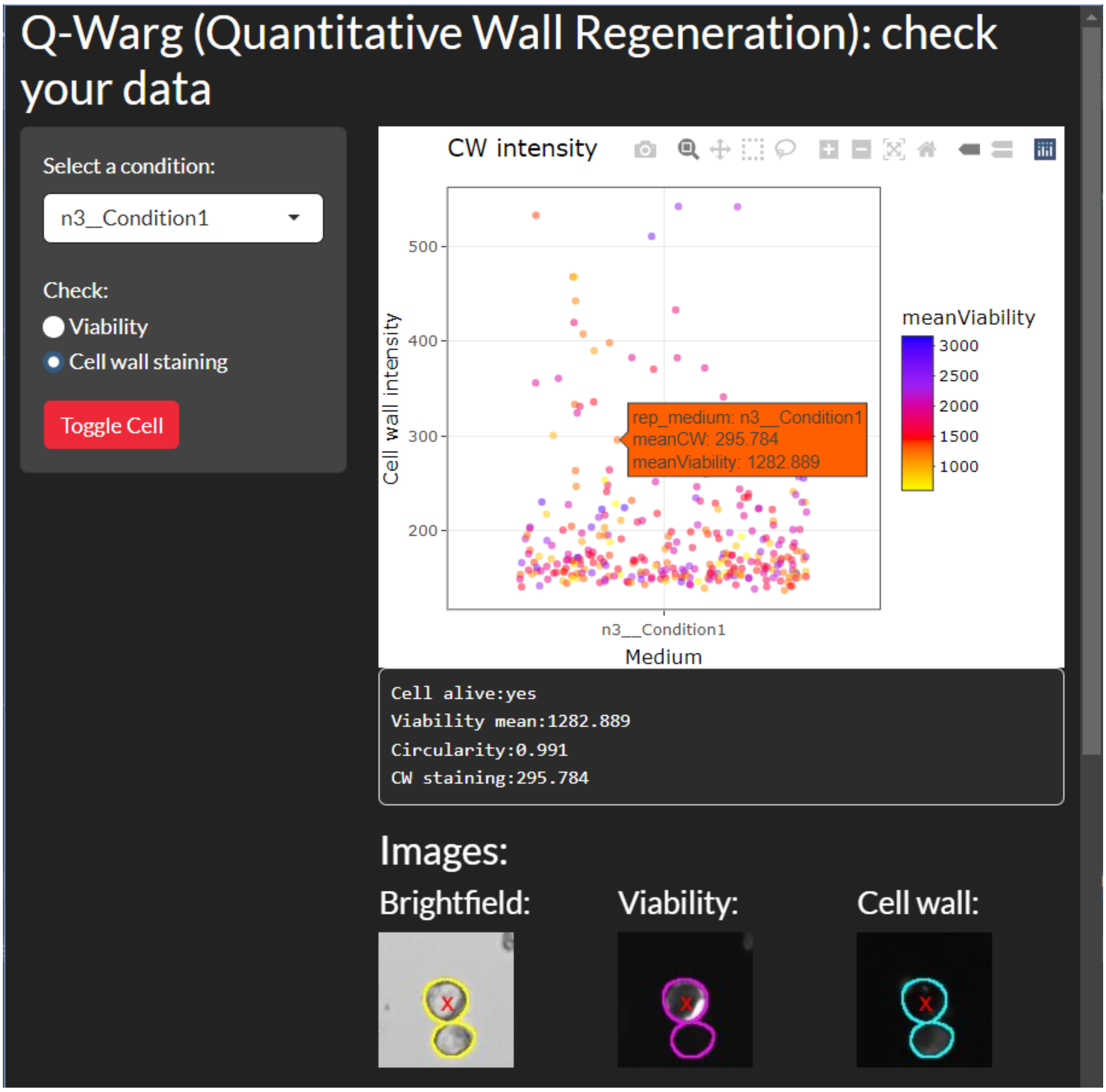
By clicking on one data point, other information will be displayed below the plot:

- If the cell is alive yes/no
- Viability mean intensity
- Circularity: to check if the object is considered dead because of its viability staining or the circularity threshold (default=0,9).
- CW staining mean intensity

Below, the 3 channels of the segmented image are displayed with the object corresponding to the clicked point in the middle of the image. We can then check if the segmentation is well made thanks to the contour line that is represented (yellow for BF image, magenta for viability, cyan for CW).

Before clicking on a data point, the error message (Error: missing value where TRUE or FALSE needed) should be written (see previous image). It just means that a point must be selected to show an image. If another error message is displayed, it probably means there is an issue in the naming of the JPG images in the interactive folder.

A red button "Toggle cell" makes a cross appear on the images to check which cell is the one designated by the point in the plot.



Q-Warg (Quantitative Wall Regeneration): check your data

Select a condition: n3__Condition1

Check:

○ Viability

● Cell wall staining

```
Cell alive:yes
Viability mean:1282.889
Circularity:0.991
CW staining:295.784
```

Below the images, text is displayed: “Clicked Point Coordinates” corresponds to the coordinates of the point in the plot. It is based on the Y value that the script will find back the right coordinates in the jpg image corresponding to the chosen condition. “Picture coordinates” correspond to the cooX and cooY columns of the data table.

Troubleshooting:

Issues with the code:

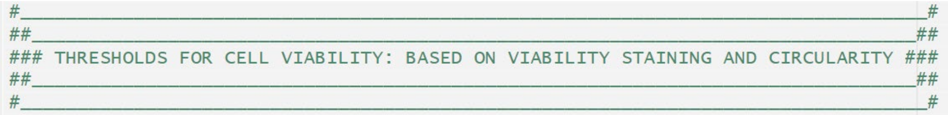
Most errors that could arise are linked to the naming of the files. To reduce this risk, use all automated options of this pipeline and the guidelines for original image naming:

`Replicate__condition.tif`

No use of **other** double _ nor double -, do not use dots and spaces in the names. It goes also for folder names: if there is a space in the path of the experiment data folder, it might create trouble with the code.

R:

Several parameters can be modified in the code. The parts of code that can be easily changed are highlighted with comments in the code with the following structure:

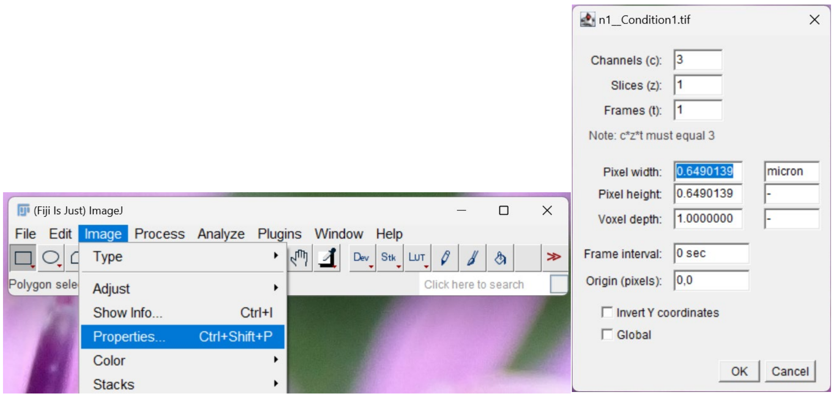


```
#__________________________________________________________________________#
##________________________________________________________________________##
### THRESHOLDS FOR CELL VIABILITY: BASED ON VIABILITY STAINING AND CIRCULARITY ###
##________________________________________________________________________##
#__________________________________________________________________________#
```

● Check the installation of the libraries

● Interactive Q-warg shiny app: the coordinates of the images need to be converted into pixels for the program to find the cell corresponding to the clicked data point. The value might be different depending on the imaging system. This can be modified in the first part of the code corresponding to the Q-Warg app: chunk 6. First, we need the converting factor from micrometers to pixels:

In ImageJ go to Image > Properties…:



n1_Condition1.tif

Channels (c): 3
Slices (z): 1
Frames (t): 1
Note: c*z*t must equal 3

Pixel width: 0.6490139 micron
Pixel height: 0.6490139 -
Voxel depth: 1.0000000 -

Frame interval: 0 sec
Origin (pixels): 0,0

□ Invert Y coordinates
□ Global

Report the pixel width for cooX and pixel height for cooY in the code:

```
donnees$cooX_px <- donnees$cooX/0.6490139

donnees$cooY_px <- donnees$cooY/0.6490139
```

If the cells appear too small or too big in the images displayed in the app, it is possible to adjust it. In chunk 6, server definition: functions `output$zoomed_bf`, `output$zoomed_via` and `output$zoomed_cw`. The functions are highlighted thanks to comments in the code.

ImageJ:

- Check and update the IJPB plugin (see installation section of this user guide)

Issues with the data:

Segmentation:

Improper segmentation can lead to incorrect intensity measurements like measuring adjacent cells signal or a smaller part of the cell.

Parameters can be adjusted depending on the microscopy images and the starting biological material (e.g.: size and flow threshold).

If using CellPose, help can be found here: https://cellpose.readthedocs.io/en/latest/faq.html

A model can also be trained to increase segmentation efficiency:
https://cellpose.readthedocs.io/en/latest/train.html

Other segmentation software or plugins can be used as the trainable Weka segmentation tool from ImageJ. https://imagej.net/plugins/tws/

Viability threshold:

Depending on the viability marker (FDA, other staining or presence of fluorescence in fluorescent cell line…) and the image acquisition, the way of determining the viability might not be optimal in the original code.

R script can be modified to use another automated thresholding method using the same autothreshold library:

https://www.rdocumentation.org/packages/autothresholdr/versions/1.4.2/topics/auto_thresh

The circularity cut off can also be changed to take more or less "round" objects: change 0.9 for another value in the following code line:

```
wdta$cellalive <- ifelse(wdta$meanViability > wdta$huang & wdta$Circularity > 0.9, "yes", "no")
```

The code corresponding to the viability is at the end of chunk2.

Additional information

● The fulldata-saved file contains:

- med_rep: name of the medium/ condition followed by the replicate
- cell: replicate, medium/condition and cell label
- Medium: name of the medium/condition tested
- Replicate: name of the replicate or date of the experiment (what is before __ in the file naming)
- rep_medium: replicate and condition/medium
- diameter: calculated with the perimeter measured by the macro in the morphometry csv file
- Circularity: measured by the macro in the morphometry csv file
- meanCW: mean intensity of the cell wall measured by the macro in the CW-Intensity csv file
- meanViability: mean intensity of the cell wall measured by the macro in the Viability-Intensity csv file
- cooX: coordinate of the cell in the image on the X axis (used for the Q-Warg-shinyapp)
- cooY: coordinate of the cell in the image on the Y axis (used for the Q-Warg-shinyapp)
- huang: values of automated threshold for each medium/condition and replicate
- cellalive: yes or no (if no, the object might be a debris and not a cell)

● In R scripts, it is asked to select a file in the working directory. Other function(s) exist to directly select the folder but for an unknown reason, it might not work on all computers. As the choose.files() function is more robust, it is the one used here.

● In this user guide, the focus is made on the cell wall regeneration and CW staining intensity. However, the pipeline can be used to check other staining or fluorescence markers. For instance, it could be used to check the efficiency of transformation of fluorescent reporters. The CW channel will be replaced by a fluorescent marker. Be aware that all naming will still contain CW, but it will represent the chosen signal.

● All scripts are commented for easier comprehension and modifiability/flexibility.