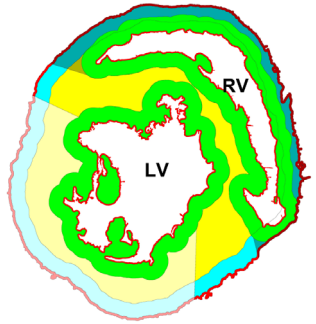
QuantSeg Macro

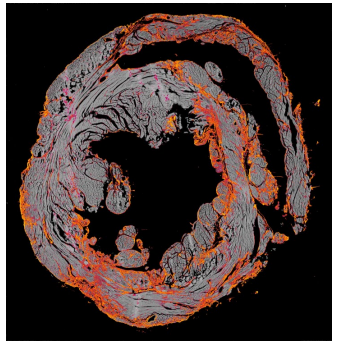
Manual

Overview



- <u>Heart Segmentation</u>
  - Segmentation here means the macro-based, fully automated subdivision of a transversely or longitudinally sectioned heart (PSR-stained) into different microanatomical regions
  - A heart can be subdivided according to its **wall layers** (epicardium, subepicardium, myocardium, subendocardium, endocardium), according to **laterolateral composition** (right half of the heart, septum, left half of the heart) as well as a **combination** of both
  - The data generated from this can then be used for collagen quantification, whereby a separate analysis can be performed for **each region**; this allows anatomically differentiated collagen evaluation for each heart
  - The macro was developed for **transversally sectioned murine hearts**, in which exactly **two** lumina separated by a septum can be seen (right & left ventricle)
  - In principle, however, it should also work **for other species** (as long as the heart is anatomically comparable) and to a **limited extent** also for **longitudinally sectioned hearts** (here too, the left & right ventricles can lie next to each other separated by a septum; the atria, which are often still cut, can be ignored by the macro if the user sets this; the problem here is often the shape of the ventricular lumina, which differs from the transversal plane; this can lead to problems with laterolateral segmentation).

Overview



- <u>Collagen Quantification</u>
  - Distinguishes between parenchyma and collagen based on the clear color contrast in brightfield images (yellow vs. red)
  - Outputs the values *Collagen*, *Area Fraction*, *Collagen Area*, *Mean Collagen Intensity*, *Max Collagen Intensity*, *Mean Spot Area*, *Spot Count*, and *Parenchymal Density* which can be used for statistical analysis as required

Initialization File

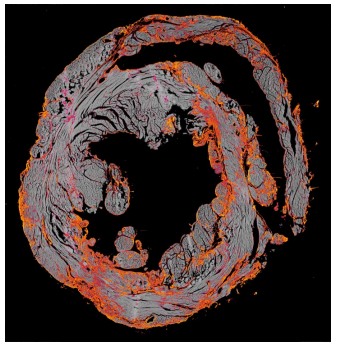
- The QuantSeg Macro is extremely complex, comprising over 5700 lines of code
- It was developed to offer the greatest possible range of functions, flexibility and user-friendliness
- Not all features are always needed, so the user must be able to switch them on and off as required (also to save runtime per image)
- The macro is based on mathematics and geometry behind the scenes; certain preset numerical parameters are required (e.g. thresholds for collagen quantification or area values for segmentation)
- These parameters could have been fixed in the code - but this would have either massively limited the flexibility (if they cannot be edited) or the user-friendliness (if you have to change the source code every time to configure the macro)
- Therefore, there is the **initialization file** where all these parameters are listed and can be adjusted
- This is re-read each time the macro is executed
- To find and set up the file, see the installation guide
- After each change, the file must be saved so that the macro can read in the new parameters. The file can remain open while the macro is running

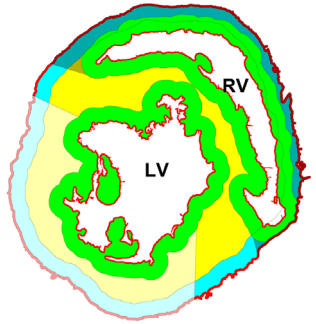
Folder structure

- The .ini file can be saved separately from the images; the path must always be specified directly in the source code
- Each image to be analyzed must be stored in a separate folder with the same title as the image (without a file extension such as .tif)
- If the function is used to perform the segmentation with a low-resolution, compressed image, but the collagen quantification is then performed with a high-resolution version, the compressed image must be named with "_Scale" appended to it
- Example: The image **#89-1234_knockout.tif**, which is to be analyzed, is located in the folder **#89-1234_knockout**. The segmentation is to be performed with a compressed version, which is also located in the same folder and is named **#89-1234_knockout_Scale.jpg**
- This folder structure must be strictly adhered to, as must punctuation and capitalization. It is strongly recommended to avoid spaces in the file name
- Any deviation from this leads to error messages and unexpected behavior

Initialization File

[Mode Selectors]

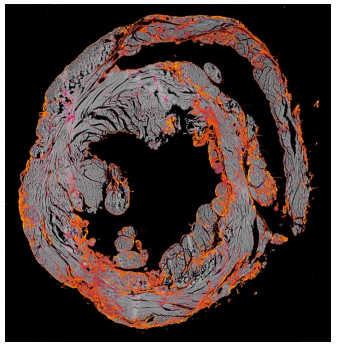


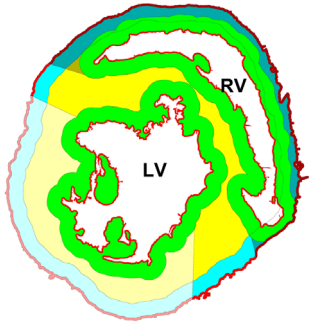


- To tell the macro what it should do, one can use the mode selector parameters to set which actions should be performed
- Parameters like these can be thought of as a switch; `1` means "on", `0` means "off“
- The following modes are available:
  - `PERFORM_SEGMENTATION`
  - `PERFORM_ANALYSIS`
  - `PERFORM_ANALYSIS_WITHOUT_SEGMENTATION`
- For the possible combinations, see the next slide (parameters there in the same order as above)

Initialization File

Combinations of [Mode Selectors]



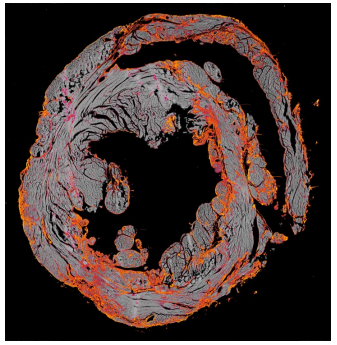


1 0 0	0 1 0	0 0 1	1 1 0	1 0 1
Only a segmentation is performed. The macro then ends.	A differentiated collagen quantification is performed on the basis of existing segmentation data. For this, a .zip file with the ROIs must be in the same folder (see below)	A collagen quantification of the entire section is performed without segmentation.	First a segmentation is carried out, then a differentiated collagen quantification is performed on the basis of it.	First a segmentation is carried out, then a collagen quantification without regard to segmentation data.

`PERFORM_ANALYSIS` (i.e. with segmentation) always additionally analyzes the section as a whole. The combinations 0 1 1 and 1 1 1 are therefore superfluous and produce an error message.

Initialization File

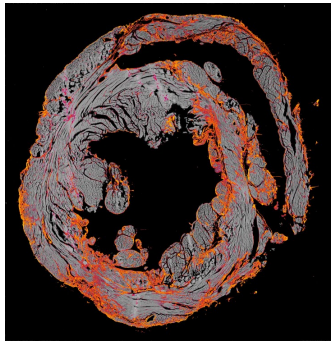
[Segmentation Dimensions]



- Segmentation is based on saving the various regions as so-called *regions of interest*, or *ROIs* for short
- During each segmentation, all possible regions are always segmented, regardless of the settings
- During the subsequent collagen quantification, a decision can then be made as to which dimensions of the section should be evaluated according to the user's needs
- The following settings are available:
  - `DIFFERENTIATE_RSL`
  - `DIFFERENTIATE_STRATA`
  - `DIFFERENTIATE_COMBINATION`
- These also work like switches with `0` & `1`

Initialization File

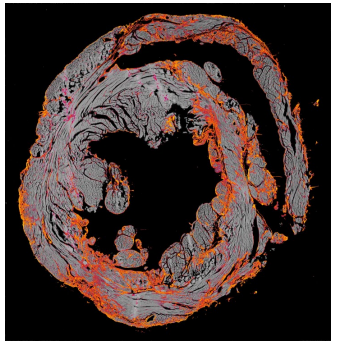
[Segmentation Dimensions]



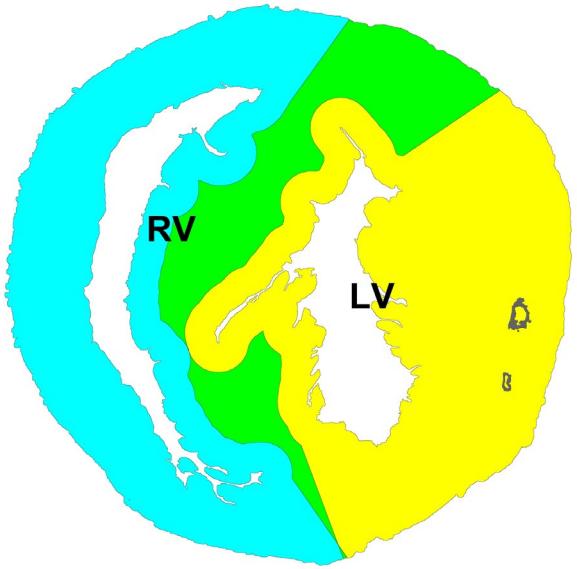
- `DIFFERENTIATE_RSL`
  - RSL stands for **R**ight **S**eptum **L**eft
  - If this option is activated, separate collagen values are output for the right and left heart and for the septum
- `DIFFERENTIATE_STRATA`
  - Strata here means the different wall layers of the heart muscle
  - If this option is activated, separate collagen values are output for the epicardium, subepicardium, myocardium, right and left subendocardium and right and left endocardium
- `DIFFERENTIATE_COMBINATION`
  - RSL and strata are now combined here, so there are individual values for:
    - Right, left & septal epicardium
    - Right, left & septal subepicardium
    - Right, left & septal myocardium
    - Right & left subendocardium
    - Right & left endocardium

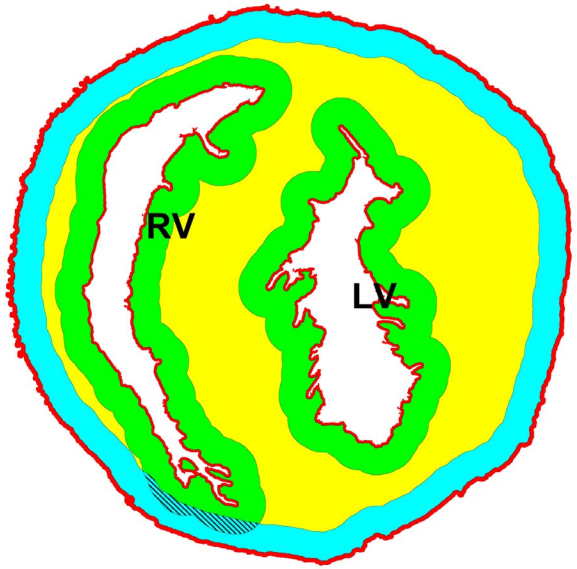
Initialization File

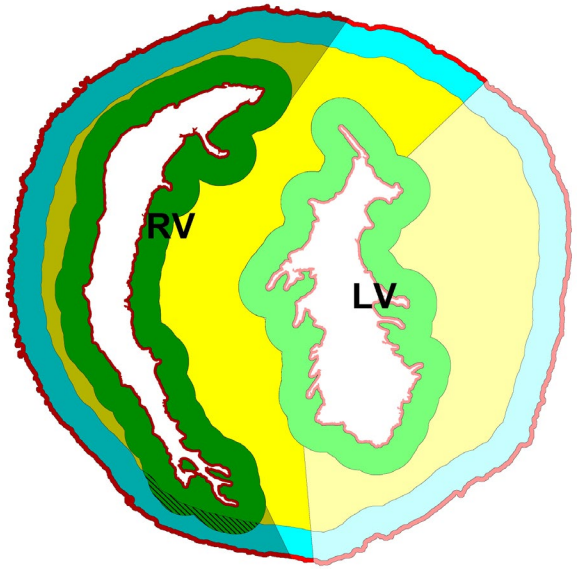
[Segmentation Dimensions]



- The three parameters have an additive effect on each other, so they can be freely combined
- For example, if `DIFFERENTIATE_STRATA` and `DIFFERENTIATE_COMBINATION` were activated at the same time, the epicardium would be output as a whole, as well as separated into right, left and septal epicardium
- Values that are present in two dimensions (e.g. right & left endocardium in `DIFFERENTIATE_STRATA` and `DIFFERENTIATE_COMBINATION`) are only output once in these cases







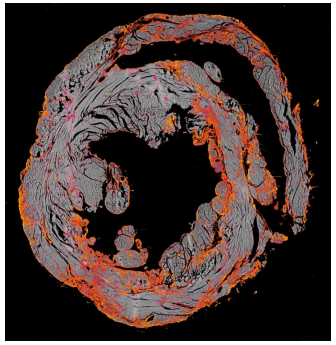
DIFFERENTIATE_RSL

DIFFERENTIATE_STRATA

DIFFERENTIATE_COMBINATION

Initialization File

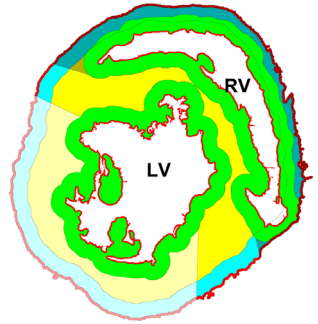
Visualization of [Segmentation Dimensions]



- Legend for the visualizations on the previous slide:
  - `DIFFERENTIATE_RSL`
    - Right heart, septum, left heart, perivascular collagen
  - `DIFFERENTIATE_STRATA`
    - Epi- & endocardium, subepicardium, myocardium, subendocardium, perivascular collagen
  - `DIFFERENTIATE_STRATA`
    - See `DIFFERENTIATE_STRATA`
    - Right heart = dark hue, septum = medium hue, left heart = bright hue

Heart Segmentation

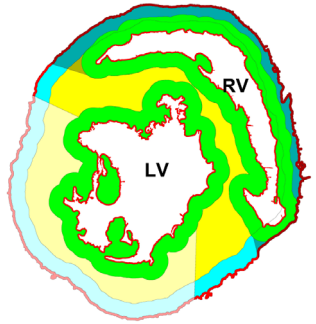
Necessary Images



- In order to segment a heart, an overview image of a PSR two-chamber section is required
- Resolution of stitched micrographs produced by modern digital microscopes can often exceed 200 megapixels which is far too large to create a segmentation from (this level of detail is not required for this task, and the RAM of most PCs is far from sufficient leading to FIJI crashing); images of this size are therefore reduced in size by the macro beforehand
- Compressed, lower-resolution overview images can also be used for segmentation

Heart Segmentation

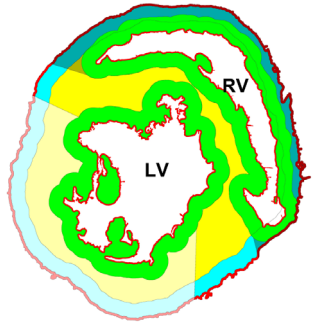
[Structure Recognition]



- The segmentation is based on recognizing a heart silhouette and two "holes" of a certain size within it and constructing the different regions from them
- In order to distinguish the silhouette of the intensely colored tissue from the pale background, the saturation component of the image is extracted and a brightness threshold is applied to it
- The following parameters are relevant for this:
  - `SILHOUETTE_IDENTIFICATION_LOWER_THRESHOLD=38`
  - `SILHOUETTE_IDENTIFICATION_UPPER_THRESHOLD=255`
- For example, all pixels with a saturation of 38 and higher (255 is the brightest value in an 8-bit image, i.e. white) are assigned to the heart, the rest is background
- It is possible to set suitable values manually in `CHOOSE_THRESHOLDS_MANUALLY` mode (see below)

Heart Segmentation

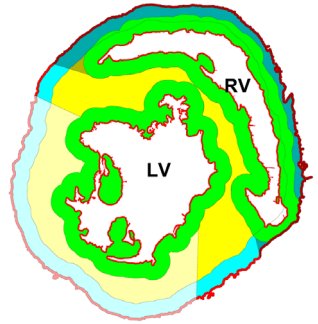
[Structure Recognition]



- The macro needs to know what scale it is dealing with in relation to the size of the section: Is it expecting a heart silhouette of 1,000,000 px^2, of 200,000 px^2 or of 7 mm^2? These numbers differ greatly and depend on the species, the slice itself, the image resolution and the set unit of measurement
- These parameters must therefore be stored in the initialization file; specifically, the following values are preset:
  - `MINIMUM_HEART_AREA=1000000`
  - `MINIMUM_VENTRICLE_AREA=50000`
  - `MAXIMUM_VENTRICLE_AREA=2500000`

Heart Segmentation

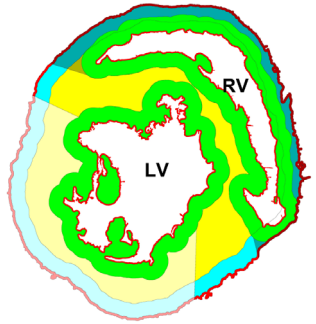
[Structure Recognition]



- It is recommended to determine the proper values for oneselve's purposes empirically, i.e. simply measuring them roughly and then seeing at which values the macro runs smoothly and does not output any errors
- The recognition of the cardiac silhouette and the ventricles is the basis for the construction of all other ROIs; these are ultimately only created by reducing or enlarging and by combining and subtracting existing ROIs
- If the user cannot reliably distinguish which region is which based on the quality of the slice, the macro will not be able to either
- The distinction between which of the two ventricles is the right and which is the left is based solely on the thickness of the adjacent free wall
- The wall thickness is measured at several points for both ventricles and the results are averaged
- The ventricle with the thinner average free wall is declared to be the right ventricle

Heart Segmentation

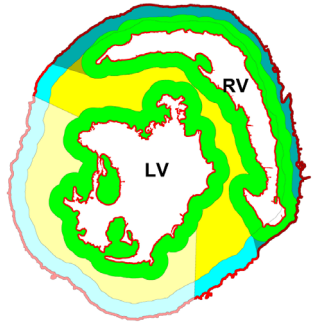
[Structure Recognition]

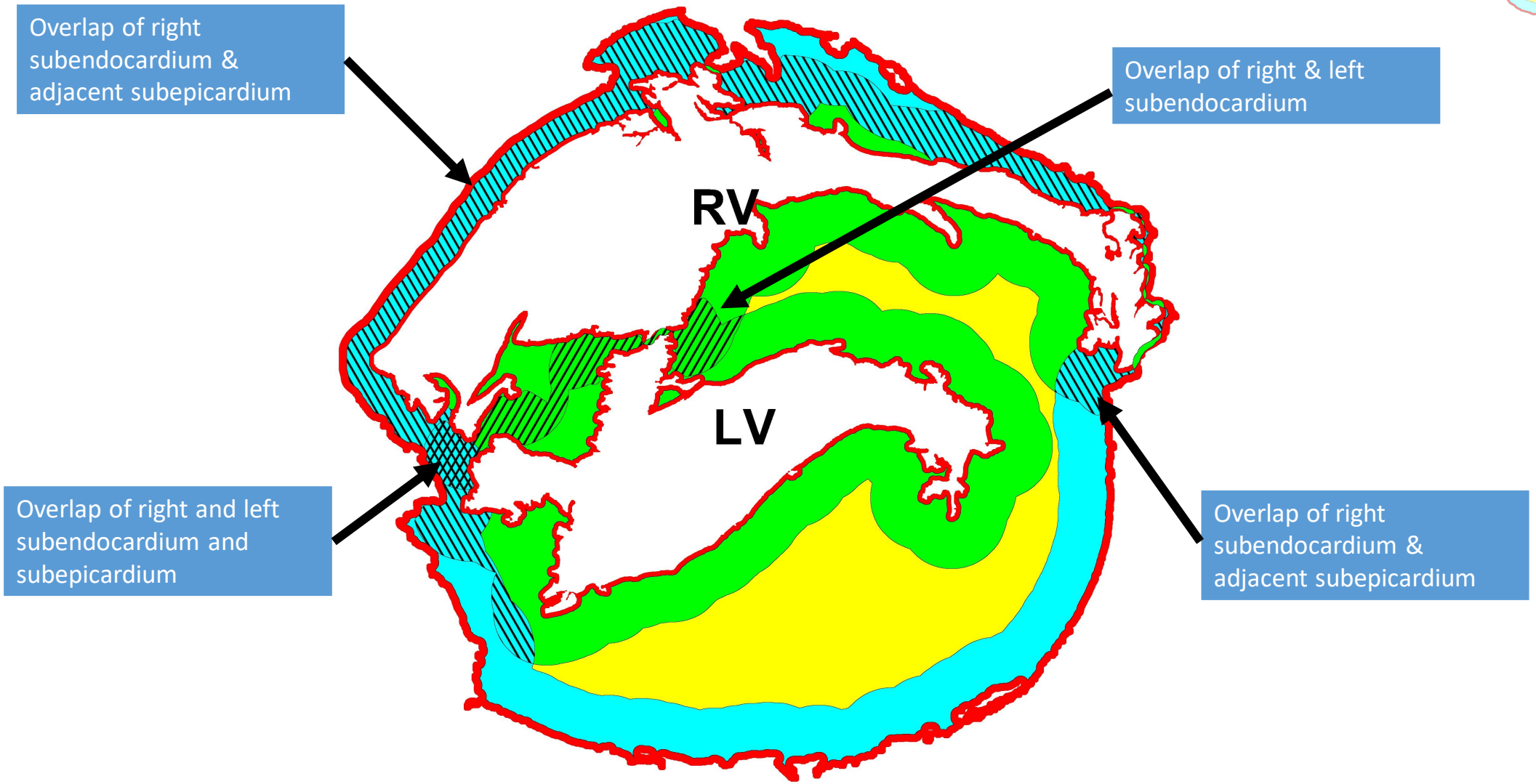


- The following parameters relate to the division into wall layers:
  - `EPICARDIAL_THICKNESS=25`
  - `ENDOCARDIAL_THICKNESS=15`
  - `SUBENDOCARDIAL_THICKNESS=150`
  - `SUBEPICARDIAL_THICKNESS=150`
- The numbers indicate the desired thicknesses of the respective layers, which are strictly adhered to
- The myocardium is constructed from all remaining parts that have not yet been assigned to any other layer
- In thin portions of the wall, it can happen that the same area is counted as both subepicardium and subendocardium; this is an inevitable consequence of the fact that the layer thicknesses are static and are specified by the user
- These areas are marked by hatching (top right -> bottom left: overlap between right & left subendocardium; top left -> bottom right: overlap between subendocardium and adjacent subepicardium; both: overlap between all three regions)
- In hearts with a thin septum, it may be assigned to the right and left heart at the same time; this is marked by horizontal hatching

Heart Segmentation

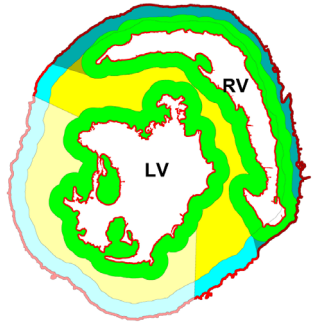
Hatching in case of area overlap





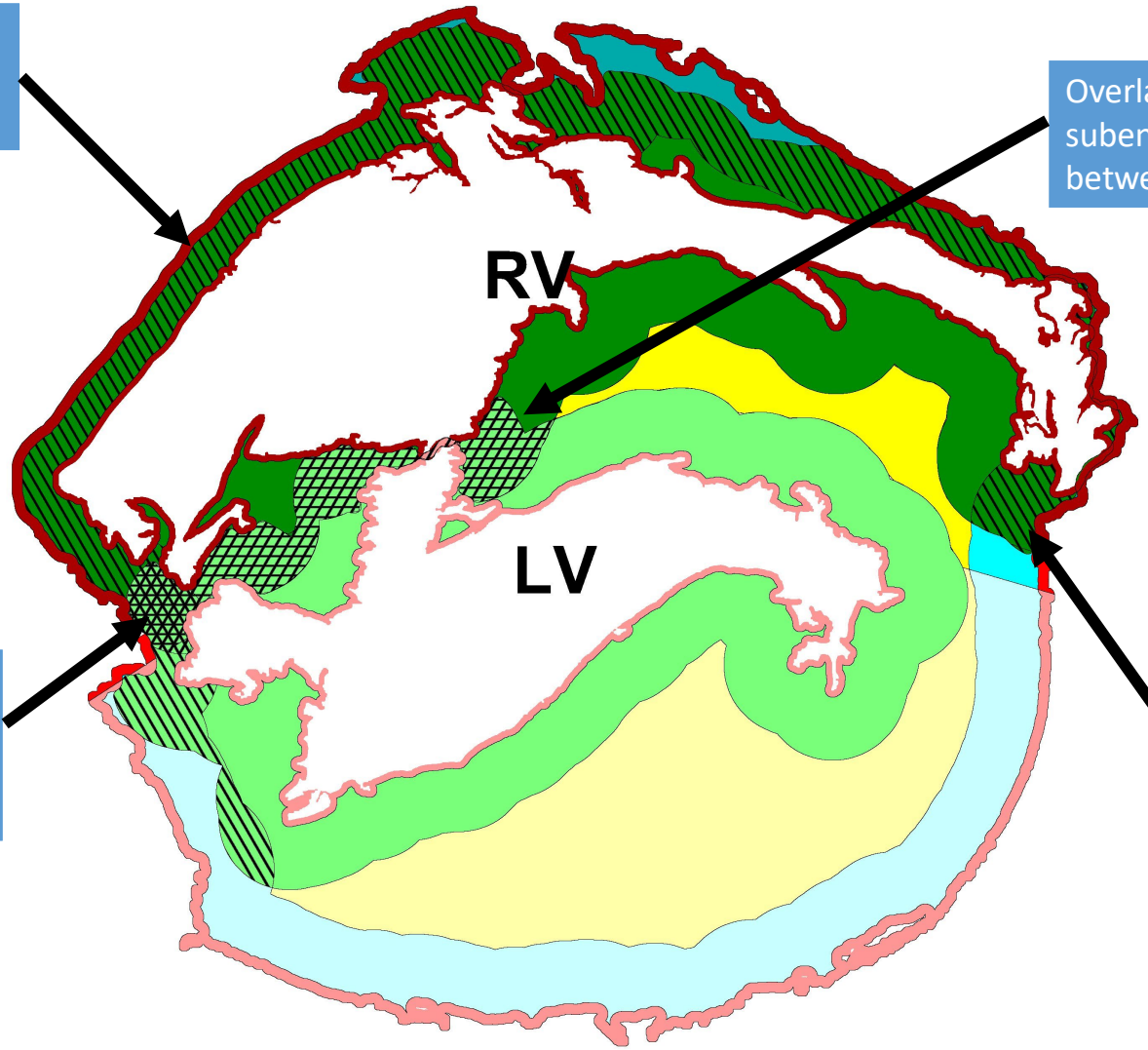
Heart Segmentation

Hatching in case of area overlap



Overlap of right subendocardium & adjacent subepicardium

Overlap of right & left subendocardium and between right & left heart

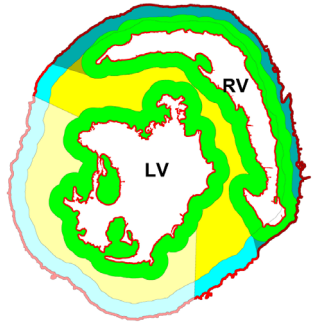


Overlap of right, left subendocardium & subepicardium as well as right & left heart

Overlap of right subendocardium & adjacent subepicardium

Heart Segmentation

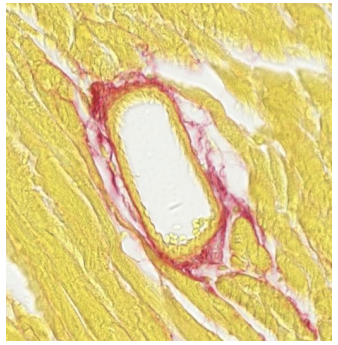
[Structure Recognition]



- Another parameter is available:
  - `SWITCH_VENTRICLES`
- For the demarcation between the left heart, septum and right heart, it is crucial that the ventricle declared as the right ventricle is actually the right ventricle
- The RV usually exhibits a concave-convex shape; this is important for the angle of demarcation between the septum and ventricles
- In the event that the wall of the usually round left ventricle is thinned (e.g. after myocardial infarction), the ventricles can be incorrectly declared and the subsequent subdivision into RSL is also incorrect (boundaries are completely wrong)
- In this case, `SWITCH_VENTRICLES` should be activated; this ensures that the ventricle with the thinner wall is declared as the left ventricle
- In the case of very atypical shapes of the ventricles and even crashes, care should be taken to ensure that the "more curved" ventricle is declared as the right one; if this is anatomically incorrect, the numerical results can also be manually re-declared afterwards

Heart Segmentation

[Perivascular Collagen]

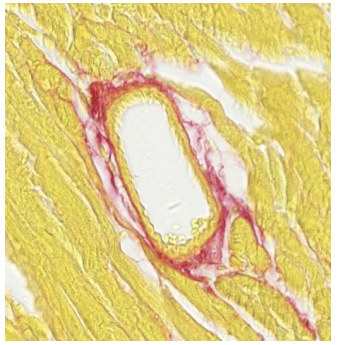


- The perivascular collagen, which usually appears ring-shaped, could potentially distort results
- It is therefore possible to capture this during segmentation and exclude it during subsequent collagen quantification
- The following parameters are available:
  - `EXCLUDE_PERIVASCULAR_COLLAGEN=1`
  - `VASCULAR_RECOGNITION_THRESHOLD=7.5`
  - `MAXIMUM_BLOOD_VESSEL_AREA=40000`
  - `MINIMUM_BLOOD_VESSEL_AREA=700`
  - `MINIMUM_LUMEN_AREA=500`

- `EXCLUDE_PERIVASCULAR_COLLAGEN` switches detection on or off
- The lower `VASCULAR_RECOGNITION_THRESHOLD` is, the sooner a pixel is classified as collagen (value depends on the hue of the coloration, can be between -20 and 60; low positive single-digit values are most useful)

Heart Segmentation

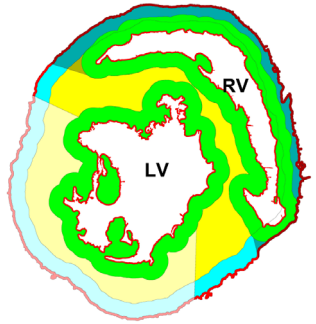
[Perivascular Collagen]



- `MAXIMUM_BLOOD_VESSEL_AREA`, `MINIMUM_BLOOD_VESSEL_AREA` & `MINIMUM_LUMEN_AREA` are the upper and lower limits of the areas of the lumen and vessel cross-section
- The smaller the minimum values and the larger the maximum value, the more structures are classified as perivascular collagen
- This feature is admittedly the least tested and most immature of the macro; it can be used to exclude obvious perivascular collagen from the analysis
- If a real focus is on perivascular collagen, detection and labeling should still be done manually

Heart Segmentation

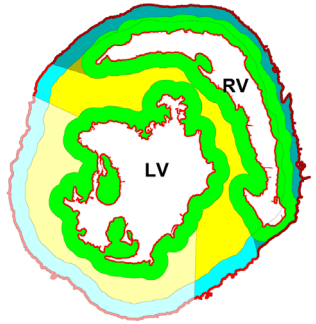
[Left Ventricle Correction]



- To differentiate between the right heart, septum and left heart, the macro uses the ROIs of the wall layers, among other things
- Endocardium and subendocardium are automatically assigned to the respective (right and left) side of the heart
- Myocardium, subepicardium and epicardium must be "cut" in order to assign the respective areas
- The lines along which they are cut are generated in a complex process; on the one hand, they must move directly along the border between the subendocardium and myocardium, but further out they must extend straight out of the heart
- The course of this straight section depends on the shape of the ventricles and on which ventricle has been declared as right and left (see `SWITCH_VENTRICLES`)
- Due to the frequently curved appearance of the right ventricle, these straight sections tend to be curved too much around the left ventricle, in a V-shape away from the right ventricle, so to speak
- To mitigate this excessive curvature, these sections are “bent” in the other direction using these parameters

Heart Segmentation

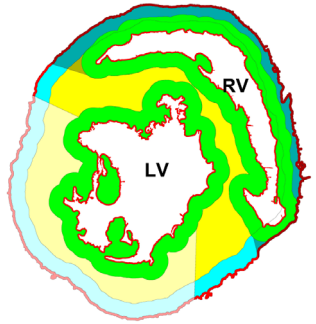
[Left Ventricle Correction]

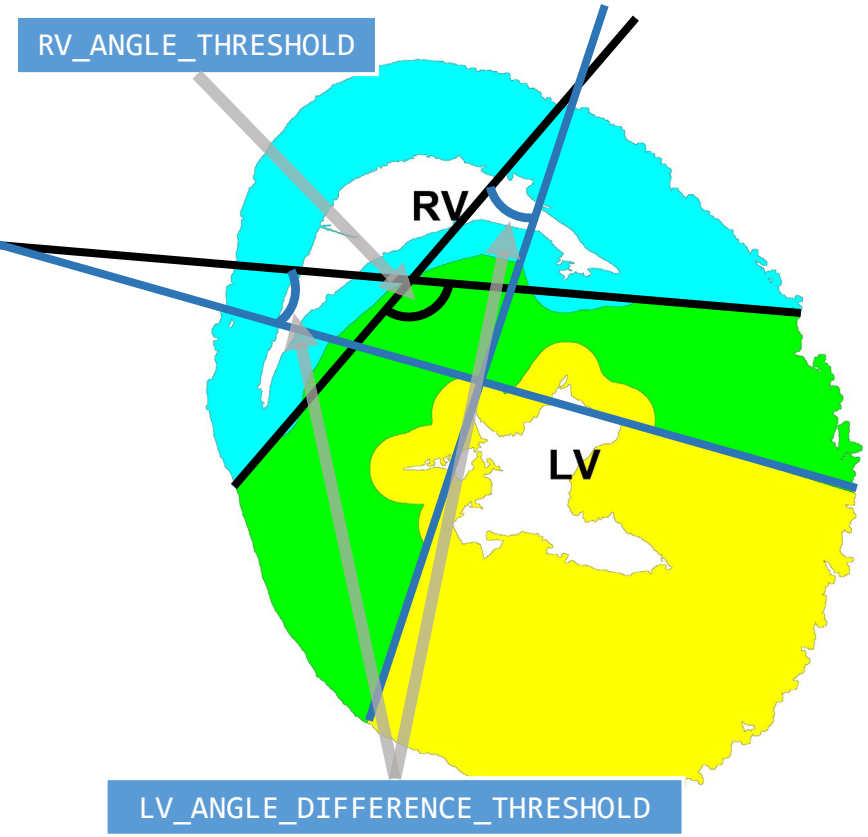


- The following parameters are important for this line correction:
  - `RV_ANGLE_THRESHOLD`
  - `LV_ANGLE_DIFFERENCE_THRESHOLD`
  - `LV_ANGLE_CORRECTION_FACTOR`
- The higher `RV_ANGLE_THRESHOLD` is, the stronger the bend in the right ventricular lines must be for the correction to take effect
- The higher `LV_ANGLE_DIFFERENCE_THRESHOLD` is, the greater the angular difference between neighboring right and left ventricular lines must be for the correction to kick in
- The higher `LV_ANGLE_CORRECTION_FACTOR` is, the more the separation lines are bent in the other direction if they are above the threshold
- One can play around with these values if one is dissatisfied with the result of a segmentation in this respect
- Otherwise, it is not necessary to concern oneself with these parameters

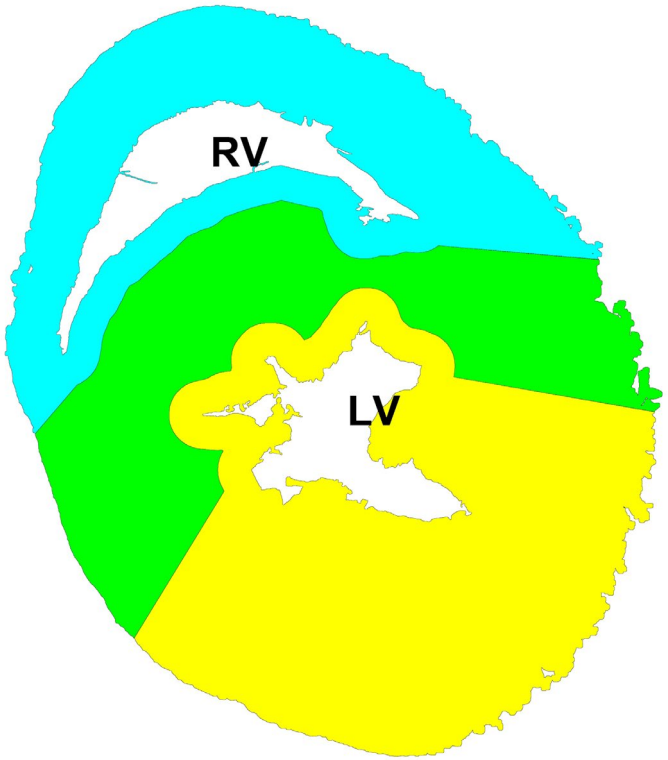
Heart Segmentation

[Left Ventricle Correction]

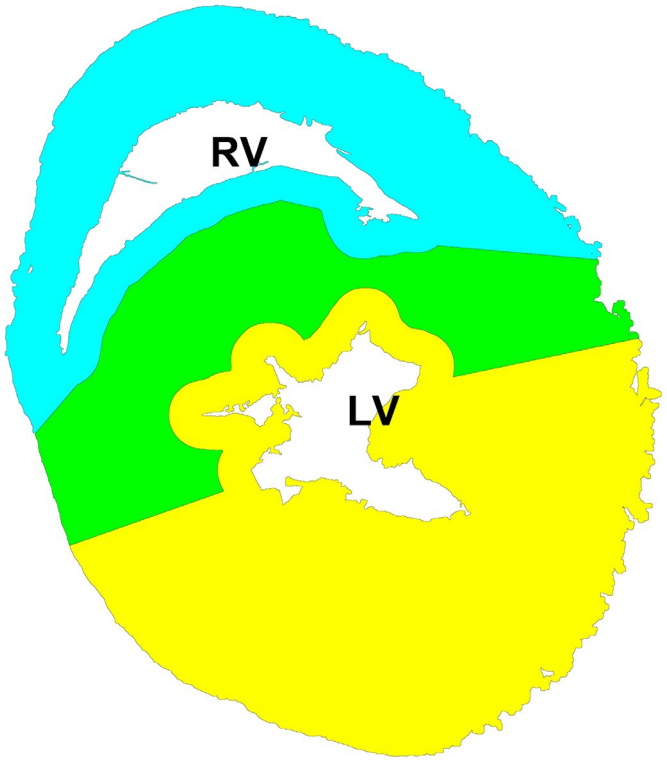




No correction



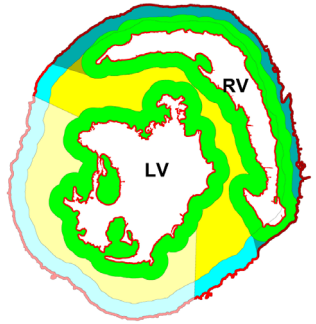
LV_ANGLE_CORRECTION_FACTOR=1.5



LV_ANGLE_CORRECTION_FACTOR=2.0

Heart Segmentation

[Save Options]



- Various files can also be saved as "by-products" of segmentation
- The following parameters are relevant here:
  - SAVE_RSL_SKETCH
  - SAVE_RSL_OVERLAY
  - SAVE_RSL_STACK

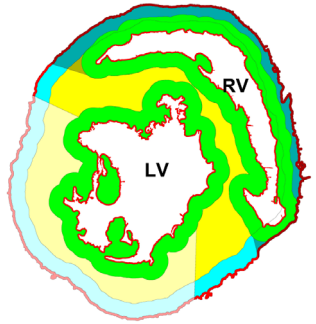
  - SAVE_STRATA_SKETCH
  - SAVE_STRATA_OVERLAY
  - SAVE_STRATA_STACK

  - SAVE_COMBINATION_SKETCH
  - SAVE_COMBINATION_OVERLAY
  - SAVE_COMBINATION_STACK

  - SAVE_ROIS_AS_ZIP

Heart Segmentation

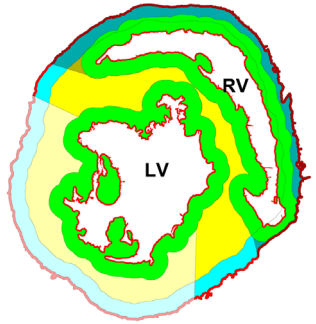
[Save Options]

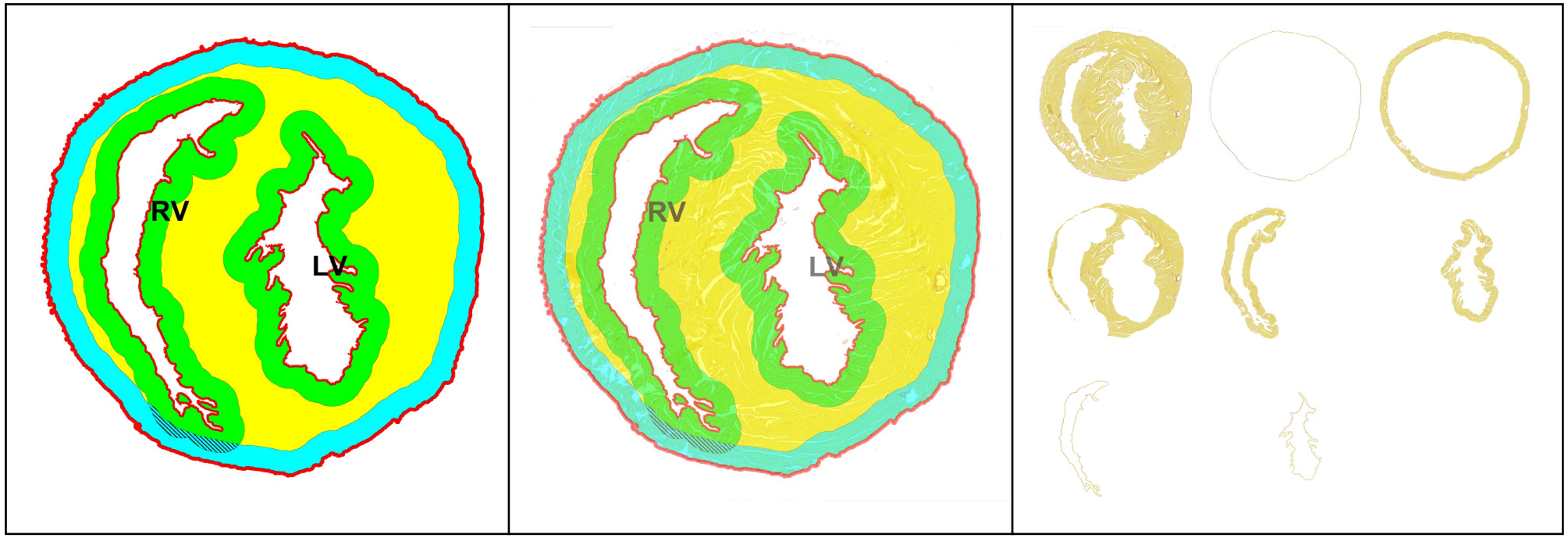


- The parameters labeled "Sketch" refer to the visualizations of the segmentation, as can be seen on slide 10, for example
- The parameters labeled "Overlay" refer to a semi-transparent overlay of "Sketch" over the original image
- The parameters labeled "Stack" refer to the saving of an image stack in which only the respective regions are visible in the brightfield channel
- `SAVE_ROIS_AS_ZIP` ensures that the ROIs of the segmentation are saved in a .zip archive
- These can be reloaded later by FIJI if required
- In this way, a differentiated collagen quantification can also be carried out at a later point in time without having to repeat the segmentation itself
- To use this, the mode selector combination 0 1 0 (see slide 6) must be set, the .zip file must be in the same folder as the source image and have the same name as the folder in question, with "_ROIs.zip" appended.

Heart Segmentation

[Save Options]



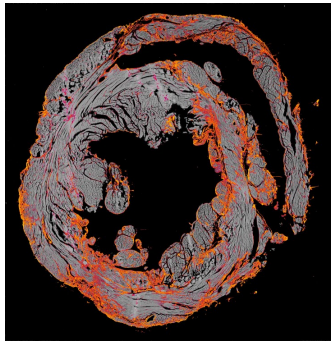


SAVE_STRATA_SKETCH=1

SAVE_STRATA_OVERLAY=1

SAVE_STRATA_STACK=1

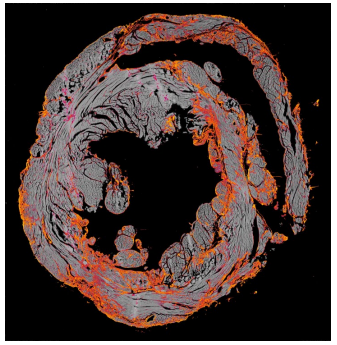
Collagen Quantification
[Analysis]



- General parameters for collagen quantification are stored here:
  - ANALYZE_HIGH_RESOLUTION_IMAGE_AFTER_SEGMENTATION
  - DECREASE_HIGH_RESOLUTION_THRESHOLD
  - DECREASE_WIDTH_OR_HEIGHT_TO
  - SET_SCALE_BEFORE_ANALYSIS
  - SCALE
  - UNIT

- ANALYZE_HIGH_RESOLUTION_IMAGE_AFTER_SEGMENTATION
  - Causes the macro to switch to the high-resolution image after segmenting a compressed copy
  - The main reason for this is that segmentation at very high resolutions either takes a very long time or causes FIJI to crash; however, collagen quantification provides more accurate results at higher resolutions
  - The segmentation data created with the small image is scaled up by the macro so that it fits on the large image exactly

Collagen Quantification
[Analysis]

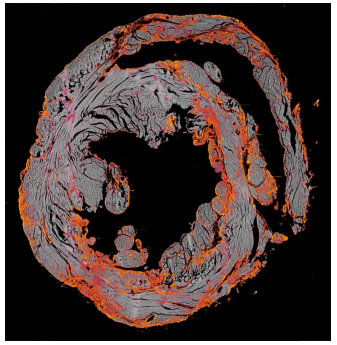


- However, these are not saved; the ROIs for the small image remain in the .zip file, if one was created
- Therefore, if ROIs are to be loaded from this file, the small image must be opened first; the macro then opens the large image automatically and scales the ROIs accordingly

- `DECREASE_HIGH_RESOLUTION_THRESHOLD`
  - Very high image resolutions also cause FIJI to crash during collagen quantification
  - This is why the macro reduces images above a certain size before analysis
  - This parameter specifies the number of megapixels above which the size of the image should be reduced
  - The megapixels are obtained by multiplying the height and width (in pixels) of an image and then dividing by 1,000,000
  - By default, this parameter is set to 36, so all images with more than 36,000,000 pixels are reduced in size
  - Whether FIJI crashes or not depends mainly on the RAM of a PC

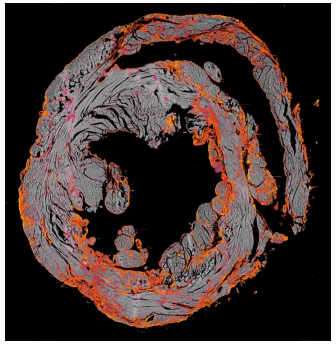
- `DECREASE_WIDTH_OR_HEIGHT_TO`
  - This parameter specifies the edge length (in pixels) to which an image that is too large is reduced in size
  - The longer of the two edges (width or height) is scaled to this value, the smaller one is reduced so that the image ratio remains the same
  - Higher values lead to longer analysis times (depending mainly on the processor), too high values lead to FIJI crashes (depending on the RAM)

Collagen Quantification
[Analysis]



- SET_SCALE_BEFORE_ANALYSIS
  - This switch parameter determines whether a scale should be applied to the image before analysis
  - The results of collagen quantification are more meaningful if they refer to µm instead of pixels, for example
  - To avoid having to set the scale manually beforehand, the macro can do this if desired
- SCALE
  - This parameter corresponds to *Distance in pixels* if the scale is set manually via *Analyze -> Set Scale…*
  - Known distance and Pixel aspect ratio are set to 1 by default

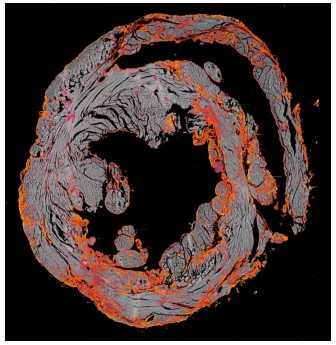
Collagen Quantification
[Analysis]



- `UNIT`
  - This parameter corresponds to *Unit of length* within *Set Scale…*
  - The unit can be entered here as text
  - The field can theoretically also be left empty

Collagen Quantification

[Analysis Thresholds]

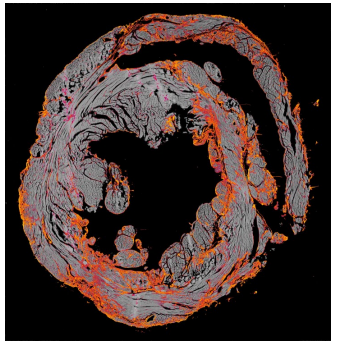


- The thresholds relevant for collagen quantification are stored here; they are primarily dependent on the color hues of the sections
  - `CHOOSE_THRESHOLDS_MANUALLY`
  - `BACKGROUND_IDENTIFICATION_LOWER_THRESHOLD=220`
  - `BACKGROUND_IDENTIFICATION_UPPER_THRESHOLD=255`
  - `PARENCHYMA_IDENTIFICATION_LOWER_THRESHOLD=3`
  - `PARENCHYMA_IDENTIFICATION_UPPER_THRESHOLD=216`
  - `COLLAGEN_IDENTIFICATION_LOWER_THRESHOLD=11`
  - `COLLAGEN_IDENTIFICATION_UPPER_THRESHOLD=100`

- `CHOOSE_THRESHOLDS_MANUALLY`
  - If this mode is activated, the macro stops at every point where a threshold is applied to the image (includes `SILHOUETTE_IDENTIFICATION_LOWER_` & `UPPER_THRESHOLD` from [Structure Recognition])
  - In each case, it is specified which structures should be marked red; the user must try to find the best possible and most generally valid setting

Collagen Quantification

[Analysis Thresholds]



- The selected parameters are saved as a text file in the folder after confirmation and can be entered in the .ini file from there

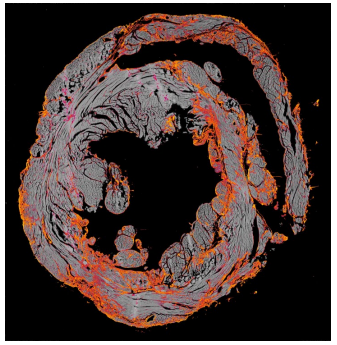
- BACKGROUND_IDENTIFICATION_LOWER_ & UPPER_THRESHOLD
  - Specifies the saturation values (between 0 & 255) between which pixels are assigned to the background
  - The inverted image is used, so high values represent low saturation
  - Default values: 220 & 255

- PARENCHYMA_IDENTIFICATION_LOWER_ & UPPER_THRESHOLD
  - Specifies the hue values (between 0 & 255) between which pixels are evaluated as cardiomyocytes or parenchyma

- COLLAGEN_IDENTIFICATION_LOWER_ & UPPER_THRESHOLD
  - Specifies the intensity values in the a* channel (between -170 & 100) between which pixels are evaluated as collagen

Collagen Quantification

[Save Options]



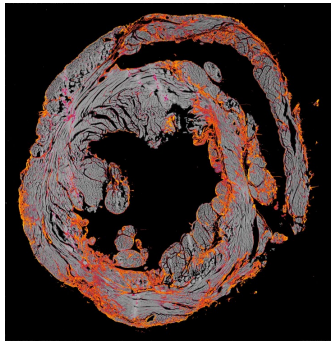
- Under [Save Options] you will find further parameters relating to collagen quantification; various files can also be output here
  - SAVE_FALSECOLOR_AS_TIFF
  - SAVE_IMAGE_WITH_WHITENED_BACKGROUND

  - SAVE_RAW_VALUES_AS_CSV
  - SAVE_RAW_VALUES_AS_XLSX
  - SAVE_PROCESSED_RESULTS_AS_CSV
  - SAVE_PROCESSED_RESULTS_AS_XLSX

- SAVE_FALSECOLOR_AS_TIFF
  - This option causes a false color image of the collagen quantification (see top right) to be saved
  - If different regions were evaluated separately, an image stack is output instead, in which each of the selected regions can be seen individually in the false color representation

Collagen Quantification

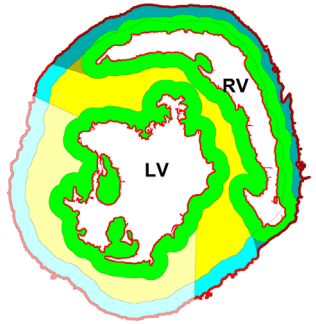
[Save Options]



- SAVE_IMAGE_WITH_WHITENED_BACKGROUND
  - Saves an image that has its original background replaced with pure white
  - Helps with publication of the image as the often off-white or grayish background does not look good on white pages
- SAVE_RAW_VALUES_AS_CSV & _AS_XLSX
  - This option causes the raw values that are subsequently used to calculate the results to be saved
  - This is possible both as a comma-separated values file (.csv) and as an Excel file (.xlsx)
  - FIJI cannot do the latter natively, the Read and Write Excel plugin is required for this; for installation see the installation guide
- SAVE_PROCESSED_RESULTS_AS_CSV & _AS_XLSX
  - This option causes the final quantification results displayed in the FIJI Results window to be written to a file immediately
  - This is possible both as a comma-separated values file (.csv) and as an Excel file (.xlsx)
  - FIJI cannot do the latter natively, the Read and Write Excel plugin is required for this; for installation see the installation guide

Error Messages

General



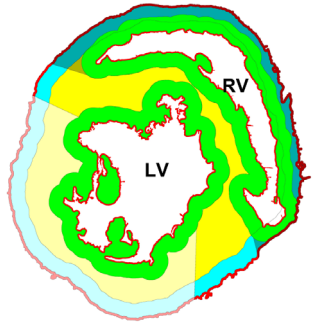
- **„No path to the initiation file has been given. Please check.”**
  - Cause: No path was set in the source code that points the macro to the .ini file
    - **Solution:** Enter path to the .ini file (see installation guide)

- **„[File name] could not be found at the path given. Please check.”**
  - Cause: The path was recognized, but no .ini file was found there under the specified name
    - **Solution:** Check whether the file name corresponds to the one given in the source code

- **„Error: [Path] (Access denied) in line 38. ini_str = File.openAsString(ini_path<)>;“**
  - Possible cause: The path was only entered up to the surrounding folder without giving the name of the file itself
    - **Solution:** Complete the path in the source code, so that the .ini file stands at the end
  - Possible cause: FIJI is not allowed to access or read files at the specified location.
    - **Solution:** Move the .ini file to another folder that FIJI is able to access

Error Messages

General

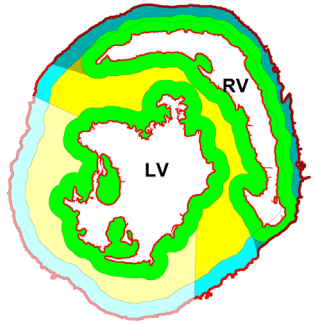


- **„PSR_Segmentation.ini seems to be corrupted. Please check the line in which “…” is set."**
  - Possible cause: The line with the specified parameter was found, but the value there does not make sense or is in the wrong format
    - **Solution:** Check the specified line: Are you sure there is only one number (except "UNIT")? Are there certainly no spaces in the line? Is a dot used as a decimal point? Are only 0 or 1 used for a "switch parameter", no decimal number or similar?
    - If the problem cannot be identified, note down the parameters used and then replace the .ini file by a fresh one from these supplement material

- **"No value for “…” could be found. Please check PSR_Segmentation.ini.“**
  - Possible cause : The line containing the specified parameter could not be found
    - **Solution:** Search for the line in question: Was it deleted by accident? Is there an error in the line‘s name? (e.g. `MINMUM_HEART_AREA` instead of `MINIMUM_HEART_AREA`)
    - If the problem cannot be identified, note down the parameters used and then replace the .ini file by a fresh one from these supplement material

Error Messages

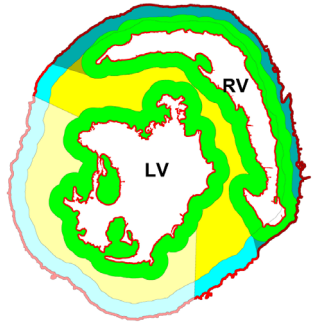
General



- **„No image is open. Please open one and click OK.”**
  - Cause: No image is opened
    - **Solution:** Before running the QuantSeg Macro, an image has always to be opened beforehand, e.g. by drag & drop into the FIJI main window

Error Messages

Heart Segmentation



- **“”PERFORM_ANALYSIS” and “PERFORM_ANALYSIS_WITHOUT_SEGMENTATION” are mutually exclusive. Please consult the manual and check PSR_Segmentation.ini.“**
  - Cause: 0 1 1 oder 1 1 1 were specified as [Mode Selectors]. These combinations are not allowed (see slide 6)
    - **Solution:** Check .ini file and choose a legal combination of [Mode Selectors]

- **„No heart silhouette could be detected.“**
  - Possible cause: `MINIMUM_HEART_AREA` has been chosen too big
    - **Solution:** Estimate or measure a common heart‘s area (measured in the image‘s unit) and change value accordingly
  - Possible cause: `SILHOUETTE_IDENTIFICATION_LOWER_THRESHOLD` & `_UPPER_THRESHOLD` are chosen incorrectly
    - **Solution:** Use `CHOOSE_THRESHOLDS_MANUALLY` and find a suitable pair of values
  - Possible cause: The section touches one or multiple margins of the image without space inbetween.
    - **Solution:** Increase the canvas size so that space is created or paint it in manually.

Error Messages

Heart Segmentation

- **"More than one putative heart silhouette could be detected. How do you wish to continue?"**
  - Possible cause: `MINIMUM_HEART_AREA` has been chosen too small
    - **Solution:** Estimate or measure a common heart's area (measured in the image's unit) and change value accordingly
  - Possible cause: The heart is torn to multiple, clearly separated pieces
    - **Solution:** Paint over the gaps using a blue pencil; the width can be adjusted by right-clicking on the pencil icon in FIJI
  - Possible cause: Besides the heart, other, strongly colored structures are present in the image
    - **Solution:** Paint over the additional structures using a white pencil; the width can be adjusted by right-clicking on the pencil icon in FIJI

Error Messages

Heart Segmentation

- **"Either only one or no ventricle candidate could be identified. How do you wish to continue?"**
  - Possible cause: The wall of a ventricle is not continuous and the ventricular lumen is connected to the surrounding background
    - **Solution:** Paint over the gaps using a blue pencil(„Draw to complete non-continuous ventricle walls; the width can be adjusted by right-clicking on the pencil icon in FIJI
    - **Solution:** Temporary adjustment of the internal parameters for the detection algorithm. Can help if the gap is only narrow („Try again with changed processing “)
  - Possible cause: The ventricle is not contained in the section or torn off, but the user knows where it would lie normally
    - **Solution:** The boundary of the missing ventricle can be drawn manually („Draw freehand selection representing the missing ventricle(s)“)
  - Possible cause: `MINIMUM_VENTRICLE_AREA` is chosen too big
    - **Solution: :** Temporary reduction of this value for the following run („Try again with temporarily reduced MINIMUM_VENTRICLE_AREA value”)
    - **Solution:** Estimate or measure a common ventricle‘s area (measured in the image‘s unit) and change value accordingly („Recheck parameters in PSR_Segmentation.ini”)

Error Messages

Heart Segmentation

- **"More than two ventricle candidates could be identified. How do you wish to continue?"**
  - Possible cause: `MINIMUM_VENTRICLE_AREA` is chosen too small
    - **Solution:** Temporary increase of this value for the following run („Try again with temporarily increased MINIMUM_VENTRICLE_AREA value")
    - **Solution:** Estimate or measure a common ventricle's area (measured in the image's unit) and change value accordingly („Recheck parameters in PSR_Segmentation.ini")
  - Possible cause: The ventricular lumen is divided into two parts by a intensely colored structure
    - **Solution:** Paint over the structure using a white pencil („Draw to connect bisected ventricle sections"); the width can be adjusted by right-clicking on the pencil icon in FIJI
    - **Solution:** Temporary adjustment of the internal parameters for the detection algorithm. Can help if the gap is only narrow („Try again with changed processing ")
  - Possible cause: Another lumen is visible, e.g. an atrium, a cavity in the tissue or a large vessel that should not be counted as a ventricle
    - **Solution:** Paint over the structure using a white pencil or connect to a ventricle lumen („Draw to connect bisected ventricle sections"); the width can be adjusted by right-clicking on the pencil icon in FIJI
    - **Solution:** Evaluate the superfluous structure manually and delete it within the ROI Manager („Exclude wrongly detected candidate in ROI manager manually")

Error Messages

Heart Segmentation

- **“Converter – Supported Conversions: 8-bit -> 16-bit* (…)"**
  - Possible cause: Something was painted on the initial image before the macro was started; in some cases the macro continues to run without problems, in others the macro terminates very quickly and outputs an error; this is probably a bug in FIJI
    - **Solution:** If something is to be painted in right at the beginning, the image should be edited and then saved. In this way, the drawing is written directly into the image data and is then no longer treated as an "overlay" by FIJI. In this case, a copy of the image to be analyzed should be processed in order not to lose the original.

Error Messages

Heart Segmentation

- **“The macro cannot determine automatically which of the ROIs named “temp-...” represent the Right and Left Heart. The reason for this is that, most likely, both ventricles are located extremely close to each other. Please review all ROIs in question, delete all wrong ones and rename the correct ones “29-LeftHeart” and “30-RightHeart”, respectively.”**
  - Cause: When creating the ROIs for the left and right heart, additional, surplus ROIs are often created by splitting the predecessor ROIs. The macro must decide which of these can be discarded and which correspond to the regions being searched for. For geometric reasons, this cannot be done automatically if the ventricles are very close to each other at one point. In this case, the user must decide manually which ROIs are the correct ones. Experience has shown that this error only occurs very rarely.
    - **Solution:** Click all ROIs within the ROI Manager that are labeled „temp-…“ and check which one corresponds to the right and left ventricle, respectibely. Then rename both (*Rename…* -> „29-LeftHeart“ or „30-RightHeart“) and delete the other ones.

Error Messages

Segmentation/Collagen Quantification Interface

- **“A ROI archive could not be found in the image directory. Please make sure that the file exists and is named correctly (folder name + "_ROIs.zip")."**
  - Cause: [Mode Selectors] 0 1 0 was set, meaning a differentiated collagen quantification without prior segmentation. In this case, the QuantSeg Macro needs segmentation data from a previous run that is saved within the same folder as the image as a .zip file.
    - **Solution:** Check whether [Mode Selectors] are set correctly. In case collagen quantification is supposed to be done without regards for segmentation data, `PERFORM_ANALYSIS_WITHOUT_SEGMENTATION` must be used
    - **Solution:** Check whether the surrounding folder contains the necessary .zip file containing the segmentation ROIs and whether it is named correctly (see slide 26). For example, a folder **#89-1234_knockout** must contain **#89-1234_knockout_ROIs.zip** so that it can be found