Biofilm Imaging and Structure Classification Automatic Processor (BISCAP): User Manual

Version 1 (January 7 2022)

Disclaimer

The supplied version of the software has been tested, and no undesired behaviour/interactions with the Windows operating system have been detected. Depending on the size and complexity of images being processed, automatic processing may require a significant utilization of available processing resources. Users are made aware that BISCAP automatically reads and writes a set of images and Excel files to disk, within the reference root folder(s) specified by users. In no event, shall the authors be liable for any damage caused by the use of this software.

Authors

All Python routines and the architecture of BISCAP were devised, implemented and tested by Diogo A. C. Narciso. The architecture of BISCAP was further refined, implemented and tested by Nuno O. Dias as a fully independent Graphical User Interface (GUI). Ana Pereira, F. G. Martins and Luis F. Melo contributed to the review, testing and improvements of Python routines and the GUI.

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Definitions

2D OCT image: 2-dimensional image obtained from an OCT scan along the unique vertical axis (z) and a single horizontal axis (x), and commonly referred to as a B-scan. 2D OCT images include a total of N_z and N_x discretized positions in the vertical and horizontal axes, respectively. They include a total of N_z*N_x pixels, where each pixel is associated with a unique grayscale intensity.

Background: All pixels in 2D OCT images with grayscale intensities below a pre-defined threshold intensity.

Biofilm: All biomass pixels defining a continuous set of pixels extending from the substratum.

Biomass: All pixels in 2D OCT images with grayscale intensities above a pre-defined threshold intensity.

Grayscale intensity: All pixels in 2D OCT images are assigned a grayscale intensity ranging from 0 (black) to 255 (white).

OCT: Optical Coherence Tomography.

Pixel: The smallest element of 2D OCT images, in accordance with the definition of 2D OCT image.

Substratum: The surface where biofilm is attached to. In the context of OCT, for any horizontal position, the substratum is generally associated with the vertical position in 2D OCT images where the highest grayscale intensity along the vertical direction is detected.

Threshold intensity: Pre-defined or calculated grayscale intensity such that all pixels with grayscale intensities above/below this value are classified as biomass/background, respectively.

Nomenclature

A distinct word formatting is used in this document to distinguish between the key elements of BISCAP, as follows:

- Screens: **bold**
- User-defined parameters: *italics*
- Actionable buttons: underline arial
- Files and folders: “under brackets”

1. Introduction

BISCAP was developed to extract information from 2D biofilm images. This includes biofilm structural parameters, and other useful visual outputs for detailed biofilm analysis. This work was done specifically in the context of 2D OCT image acquisition and processing, for which no software (apart from some reported custom-made MATLAB scripts) is currently made available to the biofilm research community.

This work was performed to comply with two fundamental guiding principles. Firstly, all image processing tasks should be made as automatic as possible, thus generally reducing the time required to obtain the information of interest, and with the least possible burden to their users. Secondly, and given the intrinsic subjectivity reported in the field on threshold selection, automatic routines were developed to remove subjectivity as much as possible from image processing.

The development work comprised two distinct stages. Firstly a set of Python routines were developed, tested and improved to deliver the proposed goals. This work consisted mainly of code writing and testing its performance on a large set of 2D OCT images. In the second stage, all code was built into a GUI, of which BISCAP is the main deliverable. This format is particularly attractive to researchers not familiar with Python, since BISCAP does not require any programming knowledge, and instead, all tasks are managed and executed via a set of intuitive screens and buttons. This manual covers specifically the practical aspects of the utilization of BISCAP. Additional details on the routines developed may be found at "10.1093/bioinformatics/btac002", where the original publication reporting BISCAP is available. This includes the main manuscript and an appendix (Appendix 1), which we will refer to in the course of this manual.

No specific installation steps are required for BISCAP. It suffices to download the standalone executable file to any location on disk. Note that while BISCAP is built on Python code, it is not necessary to download or install Python or any of its libraries, since the single standalone file is packaged to include all the necessary features. At this stage only a Windows compatible application is provided. Once downloaded, BISCAP may be immediately opened and utilized.

2. Basics

BISCAP was designed to automatically process 2D OCT biofilm images. More concretely, these must be grayscale images in tiff or png formats, obtained from OCT scans along a vertical plane (B-scans). An example is shown in Figure 1, where a biofilm image is presented in its more conventional form (left), and an equivalent digital representation highlighting its data structure is also shown (right). All processing steps in BISCAP are executed via the automatic manipulation of these 2D matrices.

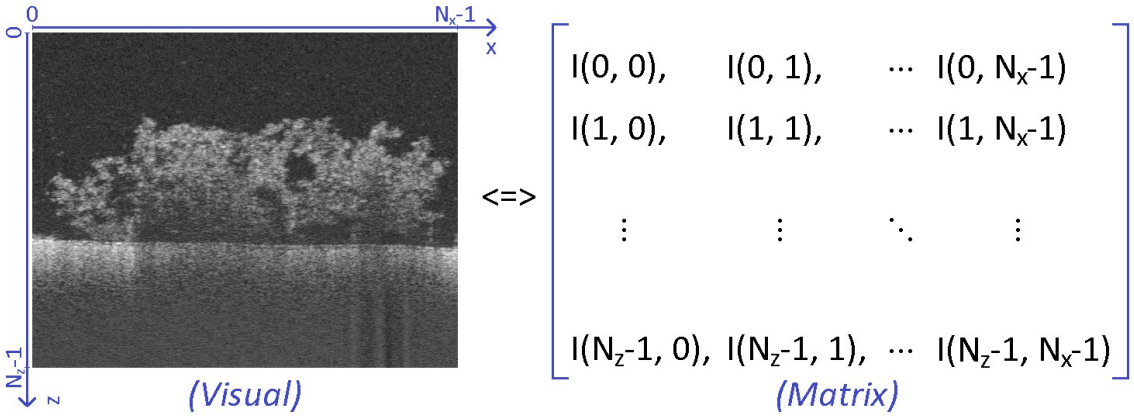


Figure 1: Example of 2D OCT biofilm image (left). These images are imported and processed in BISCAP as equivalent 2D matrices (right), where all matrix entries include the corresponding pixel grayscale intensities.

The total number of pixels in the vertical/horizontal directions in 2D OCT images are denoted as N_z and N_x. respectively. Consistently with the standard convention in the field, the vertical axis (z) increments from the top (z=0) to the bottom (z=N_z-1), and the horizontal axis (x) increments from the left (x=0) to the right (x=N_x-1).

BISCAP includes three image processing functions:

- Pre-processing (Section 3)
- Automatic processing (Section 4)
- Post-processing (Section 5)

Two distinct modes/workflows for full image processing are built into BISCAP, making use of these three basic functions in a slightly different way, as follows:

- Single image processing (Section 6)
- Multi-images processing (Section 7)

On launching BISCAP, the **Home** screen is loaded as illustrated in Figure 2, from where one of the two modes above may be selected. However, since both modes share the three functions above with only slight variations between them, these functions are presented first (Sections 3–5) and then their utilization in the context of the two modes (Sections 6, 7) is discussed. Quick-start tutorials for the two modes are then presented in Sections 8 and 9.

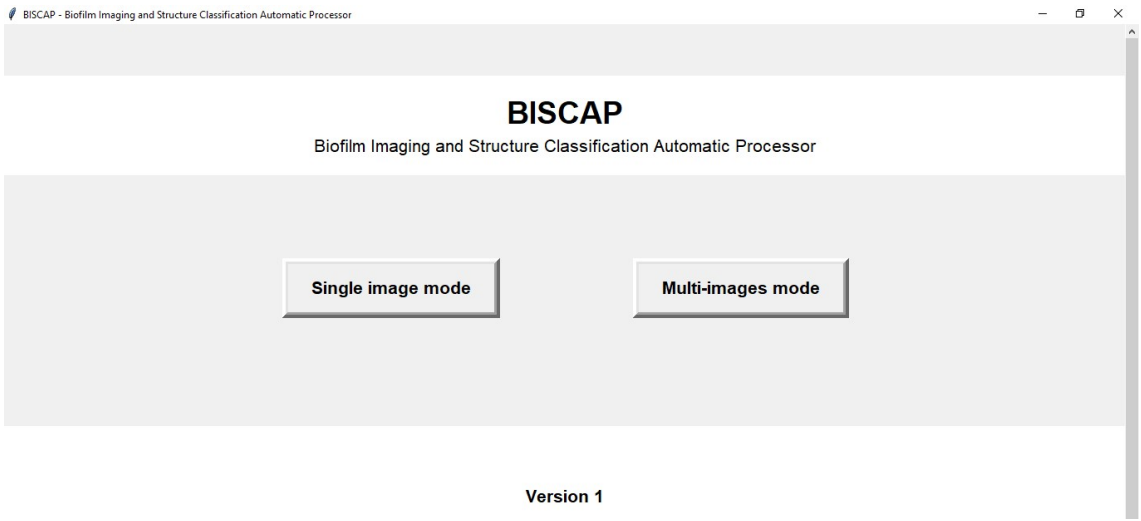


Figure 2: **Home** screen in BISCAP. The single image and multi-images modes are accessible from this screen.

It is assumed that all biofilm image files are saved to folders including exclusively tiff or png files obtained directly from 2D OCT scans. Upon conclusion of image processing, and taking the root folder for any given image as the reference, an additional "inputs" folder is created within the reference root folder to save all auxiliary results obtained during pre-processing. Similarly, an additional "outputs" folder is also created within the reference root folder including all results from the automatic processing and post-processing stages. This folder structure must be kept as described above to ensure BISCAP delivers the desired functionality.

To facilitate image processing in BISCAP, images are resized to fit on screen, which may at times not preserve the expected height/length ratio. Note that all images delivered by BISCAP do preserve this ratio, and are available for additional checking via the standard image display applications.

Note: Image files may be saved with any names as required, and generally it is recommended to keep them as short and succinct as possible. The same recommendation holds for paths. In some systems, paths including spaces (e.g. "…\my folder\...") on the folders/paths including the images of interest, may prevent the application from loading images correctly.

3. Pre-processing

Pre-processing is provided as an optional functionality in BISCAP, whereby (raw) 2D OCT images are conveniently trimmed along the vertical axis to include only a central horizontal band including all biofilm pixels. This is achieved via manual user input, and a good understanding of what constitutes biofilm and substratum pixels (in line with definitions presented earlier) is required. While this is an optional step in BISCAP, it is also a highly recommended step, since the minimal effort required in pre-processing generally enables significant improvements on both accuracy and computational efficiency.

Two **Pre-processing** screens were developed: one for the single image mode, and another for the multi-images modes. While there are minor differences between these two screens to accommodate for the distinct workflows in the two modes, their basic functionality is exactly the same and presented in sequence. Additional details on loading images and specific command buttons are discussed separately in Sections 6 and 7.

Once a raw biofilm image is selected, it is displayed accordingly in the **Pre-processing** screen of BISCAP. This screen includes a number of buttons as depicted in Figure 3.

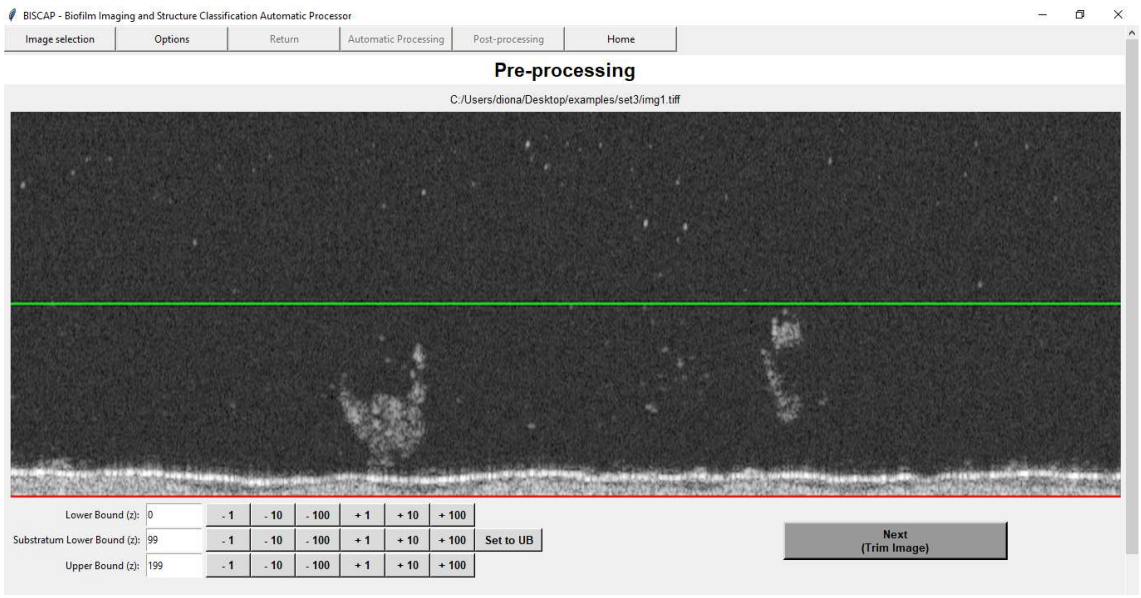


Figure 3: **Pre-processing** screen in BISCAP (single image mode): raw image with default bounds.

Three bounds are defined and visible on the lower left portion of the **Pre-processing** screen, and shown over the displayed raw image. These bounds are defined as follows:

- *Lower Bound (z):* The lower bound of the horizontal band of interest (set in the vertical axis - z); all biofilm pixels must be below this horizontal bound.
- *Substratum Lower Bound (z):* Auxiliary bound for substratum identification (set in the vertical axis - z); all substratum pixels must be below this horizontal bound.
- *Upper Bound (z):* The upper bound of the horizontal band of interest (set in the vertical axis - z); all biofilm pixels must be above this horizontal bound.

By default the *Lower Bound (z)* is set to z=0, and the *Upper Bound (z)* set to $z=N_z-1$. These bounds control the width of the horizontal band of interest and are initialized to include full vertical range of raw images. Following the definitions of bounds presented

above and using the negative and positive increment buttons (-1, -10, -100, +1, +10, +100), all bounds should be set as tightly as possible. Increments of 10 and 100 are usually sufficient for this purpose.

Pre-processing allows additionally to obtain auxiliary information for the automatic processing stage. Specifically, the *Substratum Lower Bound (z)* was defined to minimize the impact of very bright pixels occurring above the substratum, and to enhance the automatic identification of the bottom interface of biofilms. Unlike the lower and upper bounds, this bound does not contribute in any way to trimming raw images, but rather is saved and then used during automatic processing. It is initialized by default at $z=(N_z-1)/2$, and should also be set as tightly as possible for an accurate identification of the bottom interface. Figure 4 illustrates the adequate selection of these bounds.

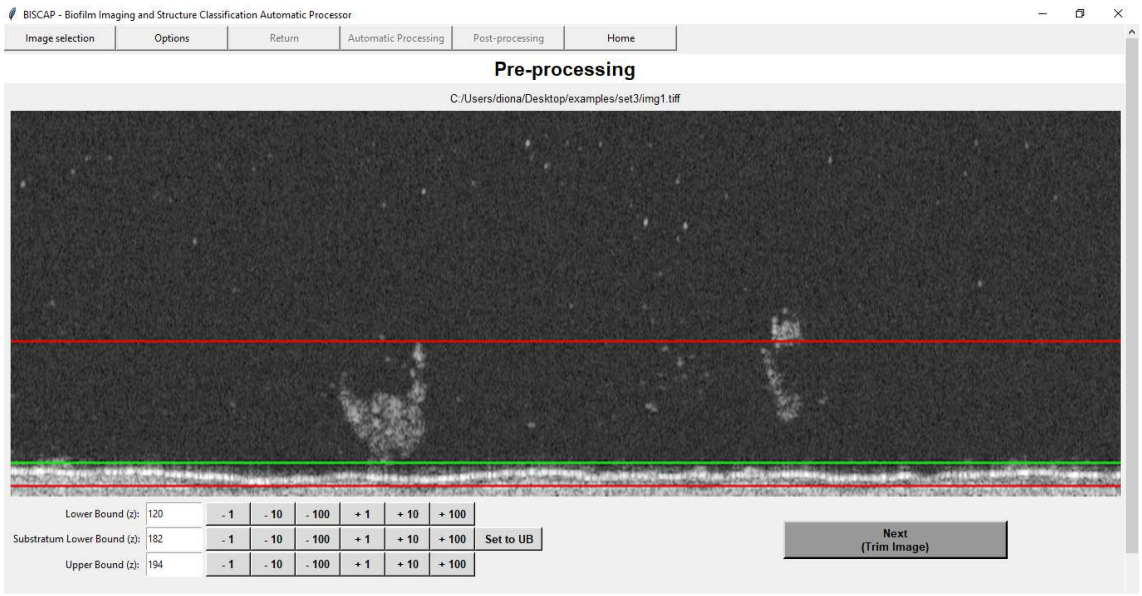


Figure 4: **Pre-processing** screen in BISCAP (single image mode): raw image with user-selected bounds.

Observe that *Lower Bound (z)* ≤ *Substratum Lower Bound (z)* < *Upper Bound (z)*. If a change is made on these bounds via the increment buttons violating this rule, a warning is displayed and the action cancelled. In the extreme case of strongly inclined substratum interfaces, it is possible that *Lower Bound (z)* equals *Substratum Lower Bound (z)*. In this case, the *Lower Bound (z)* should be firstly set, and then using the Set to UB button, the *Substratum Lower Bound (z)* is conveniently made to coincide with the former.

For convenience, bounds' colours and their thicknesses as displayed on screen may be adjusted, via the Options button. To activate any changes made on this window, the Save button must be clicked, which will also close the options window. These options are depicted in Figure 5.

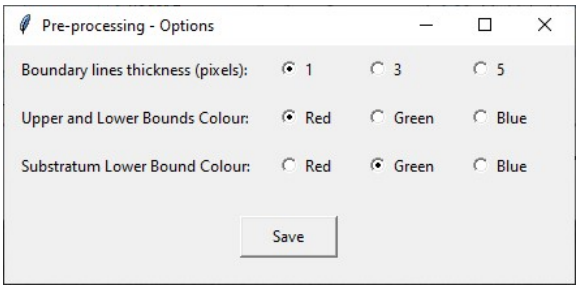


Figure 5: Pre-processing options.

The description presented so far concludes the interactive part of pre-processing. To finalize pre-processing, an additional button (details for the two modes/workflows in Section 6 and 7) takes the specified user-defined bounds and automatically trims raw images accordingly. These images are conveniently displayed in BISCAP for assessment (Figure 6). Pre-processed images are also saved to an "inputs" subfolder within the root folder including the raw image being processed. Pre-processed images are given the same name as the corresponding raw image with appended suffix "_input". All bounds are saved to an Excel file ("inputs.xlsx") and also included in the "inputs" subfolder. Note that if this information is saved for a given image, and the **Pre-processing** screen resumed at a later stage, all bounds will be shown accordingly and not using the defaults presented earlier.

As a final note, the lower bound for image trimming is not defined exclusively via the *Lower Bound (z)*, and in fact an additional gap (in pixels) is used for trimming raw images. This additional gap is necessary for the automatic thresholding module. This is illustrated via Figures 4 and 6: note that the band of pixels defined (Figure 4) does not match the obtained pre-processed image (Figure 6); rather, the pre-processed image extends above the defined bound.

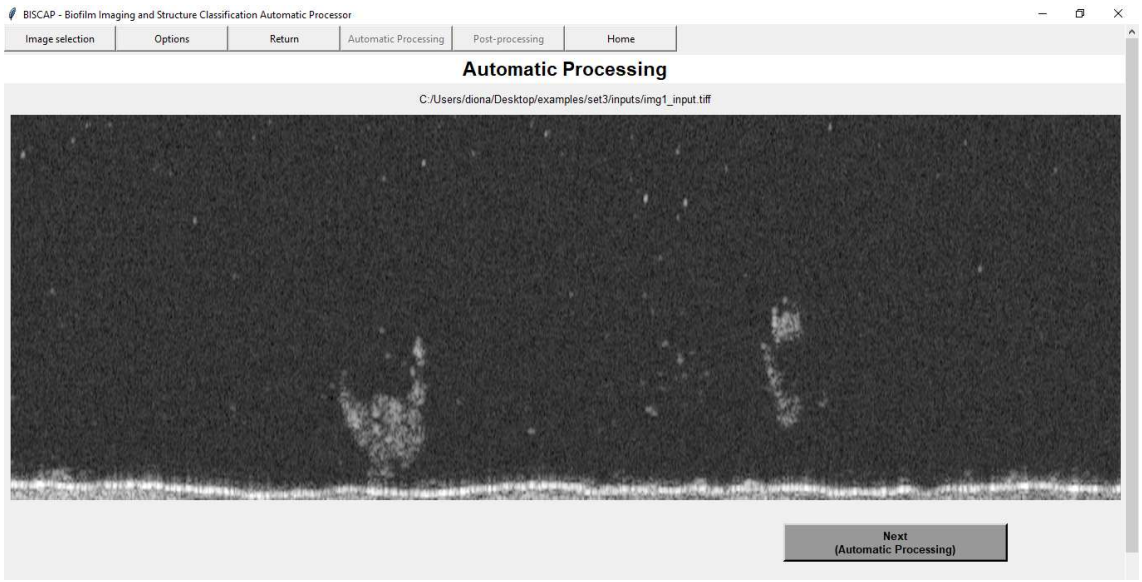


Figure 6: Pre-processed image after bound selection and (raw) image trimming (displays in **Automatic Processing** screen if the single image mode is selected).

4. Automatic Processing

Automatic processing comprises four stages:

- Bottom biofilm interface calculation
- Thresholding
- Biofilm structure calculation
- Top biofilm interface calculation

Detailed descriptions on these four stages are available from the original publication (10.1093/bioinformatics/btac002). In this manual, automatic processing is presented exclusively from a user utilization perspective, and thus not covering any of the specific details of these stages.

Two **Automatic Processing** screens were developed: one for single-image processing and another for multi-image processing. Their core functionality is the same, and specific details for their utilization in the context of the two available modes presented in Sections 6 and 7. It is assumed at this stage that pre-processing was concluded and pre-processed image(s) have been obtained as described in Section 3. Figure 6 depicts the **Automatic Processing** screen.

This task is significantly less interactive than pre-processing, and no user input is strictly required by default. In fact, automatic processing may begin immediately, once pre-processing is concluded. There are however a number of optional settings in this mode, which are presented in detail. This parameter specification window is available from the Options button as shown in Figure 7. All groups of available settings are presented in sequence.

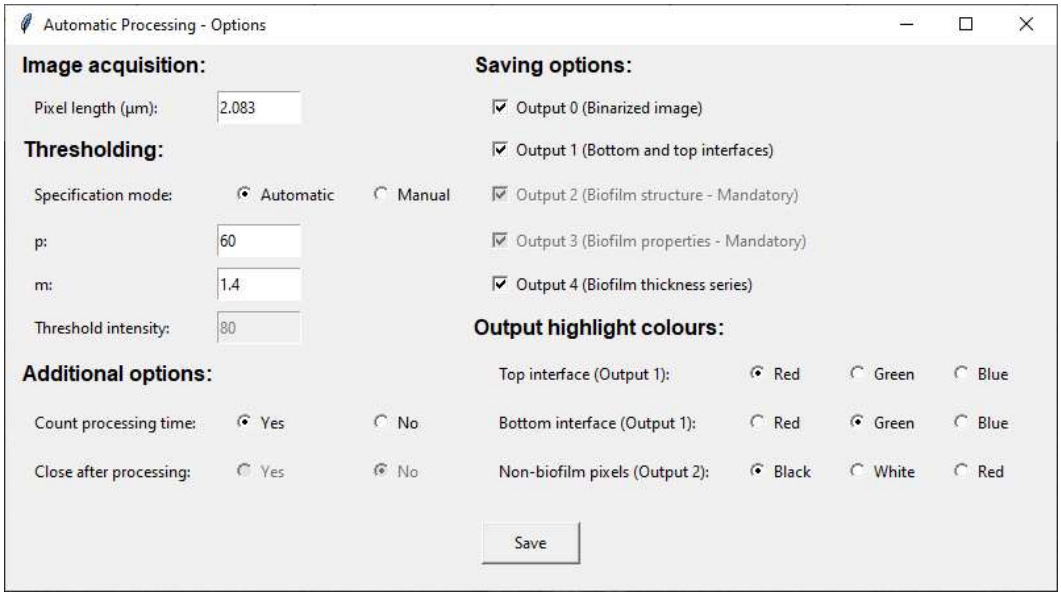


Figure 7: Optional automatic processing settings.

Image acquisition:

A default is given for *Pixel length (μm)*, such that the length of 100 consecutive pixels along the vertical direction equals 48μm. Users must set this parameter to the local image acquisition set-up, and update it whenever necessary.

Thresholding:

This is likely the most important set of processing options, with a noticeable impact on biofilm structure. Automatic and manual threshold *Specification modes* are available. In the first case, parameters p ($0 \leq p \leq 100$) and m ($m > 1$) may be customised. Additional details on these parameters may be obtained from the main manuscript. These have been empirically tuned via a set of 300 biofilm images with highly satisfactory results obtained. Updating these parameters should originate from a rigorous critical analysis of results. In general, the higher are p and m, the higher are the corresponding threshold intensities. Alternatively, users may select the manual specification mode and set a constant *Threshold intensity* in the corresponding box.

Additional options:

If required, users may time the duration of automatic processing (*Count processing time*). An option is also given to close the application on finishing after automatic processing concludes (*Close after processing*). This may be useful in the case when large amounts of images are to be processed and available only in the multi-images mode.

Saving options:

The primary purpose of automatic processing is to deliver a set of useful image and numeric outputs for biofilm analysis. Calculation of outputs 2 and 3 is mandatory, since they include vital information for the final **Post-processing** screen. All other outputs are not strictly necessary, and users may disable their calculation if required.

The full set of available outputs include:

- *Output 0:* Binarized image
- *Output 1:* Bottom and top interfaces
- *Output 2:* Biofilm structure
- *Output 3:* Biofilm structural properties
- *Output 4:* Graphical representation of biofilm thickness

Output highlight colours:

Output 1 includes the *Top interface* and the *Bottom interface*, which are marked with a high contrast colour. The set of *Non-biofilm pixels* in output 2 are marked with a neutral colour to emphasize biofilm structure. These three colours may be customized, where a collection of options is provided for selection.

Details on launching and progress of automatic processing are discussed in Sections 6 and 7 for the two available modes. Once launched and until processing is concluded, a message is displayed accordingly in BISCAP, and buttons remain inaccessible. During this stage BISCAP may appear to freeze, but this is not the case. This is due to the high processing requirements during automatic processing, which may then cause this behaviour. Total processing time depends strongly on the size and complexity of biofilm images and computational power. On a standard desktop machine, a reference of 10-20s per image is presented as a rough reference.

Once automatic processing concludes, all user selected outputs are saved in an "outputs" subfolder within the root folder including the image(s) being processed. All outputs are saved with the same name of the corresponding raw image and with a matching suffix (e.g. "_output1"). Outputs 0, 1, 2 and 4 are saved with the same extension of the raw file (tiff or png) and output 3 is saved as an Excel file.

5. Post-processing

This function allows the convenient visualization of all outputs obtained during automatic processing, and customized biofilm analysis in user-defined regions of interest along the horizontal axis. Two **Post-processing** screens were developed: one for the single image mode, and one for the multi-images mode. Their core functionality is the same. The utilization of this function in the context of these two modes is presented in Sections 6 and 7. The **Post-processing** screen is depicted in Figure 8. It is assumed that at this stage, automatic processing is finalized.

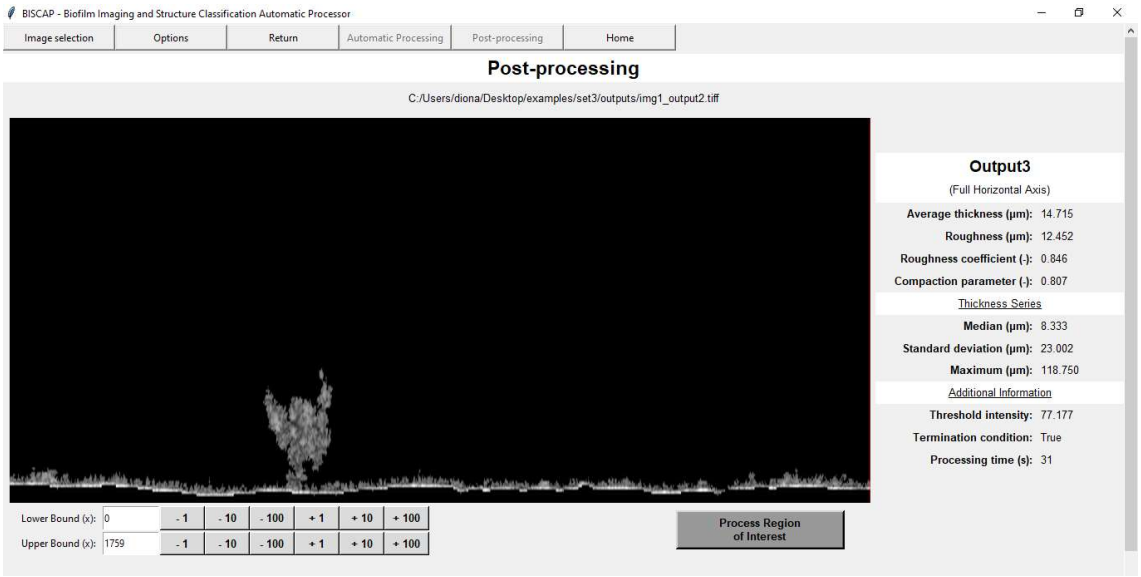


Figure 8: **Post-processing** screen (single image mode).

Biofilm structural properties (output 3) are displayed on the right side of the screen. Note that these results are calculated for the full length of the horizontal axis. The key biofilm structural parameters are: (i) average thickness, (ii) roughness, (iii) roughness coefficient, and (iv) compaction parameter. Additional results with respect to the thickness series (maximum, standard deviation and median), the threshold intensity and the total processing time are also displayed. The termination condition for the biofilm's bottom interface module is also reported. Further details on this Boolean are presented in the main manuscript and Appendix 1. Note this is presented as a guideline information only, and a more detailed analysis is usually required to validate the success of this task.

A single image is also displayed to the left of output 3, and occupying the larger portion of the **Post-processing** screen. By default, biofilm structure (output 2) is displayed. From the Options button, all remaining outputs obtained from automatic processing may be selected, by enabling the corresponding option from parameter *Displayed output*. The options window for the **Post-processing** screen is shown in Figure 9.

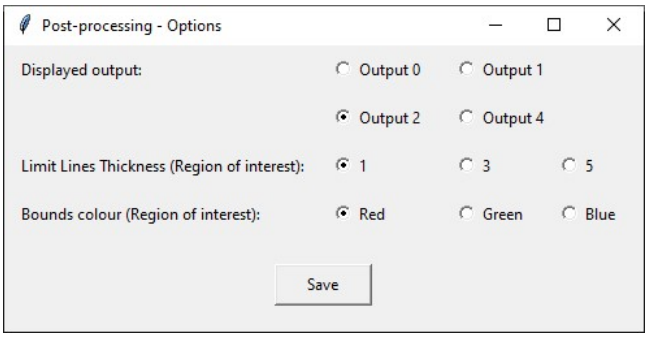


Figure 9: Post-processing options.

Thickness and colour of a set of auxiliary vertical lines are also available from the options window. These vertical lines enable the definition of a region of interest and are shown below the displayed output in the **Post-processing** screen:

- *Lower Bound (x)*: Defines the left vertical edge of the defined region of interest (set in the horizontal axis - x).
- *Upper Bound (x)*: Defines the right vertical edge of the defined region of interest (set in the horizontal axis - x).

By default, *Lower Bound (x)* is set to x=0 and *Upper Bound (x)* is set to $x=N_x-1$. In this case the region of interest coincides with the full horizontal range. Using the increment buttons to the left of these parameters (-1, -10, -100, +1, +10, +100), any region of interest may then be selected, provided that *Lower Bound (x)* < *Upper Bound (x)*.

Once a region of interest is defined, the Process Region of Interest button, calculates all biofilm structural parameters for the present selection. A window is displayed including these results as illustrated in Figure 10. These additional user-defined outputs are also saved in the corresponding "outputs" subfolder. They are saved with the same name as the original outputs for the full horizontal axis and with an appended suffix displaying also the selected bounds (e.g. "_output2_xmin_xmax").

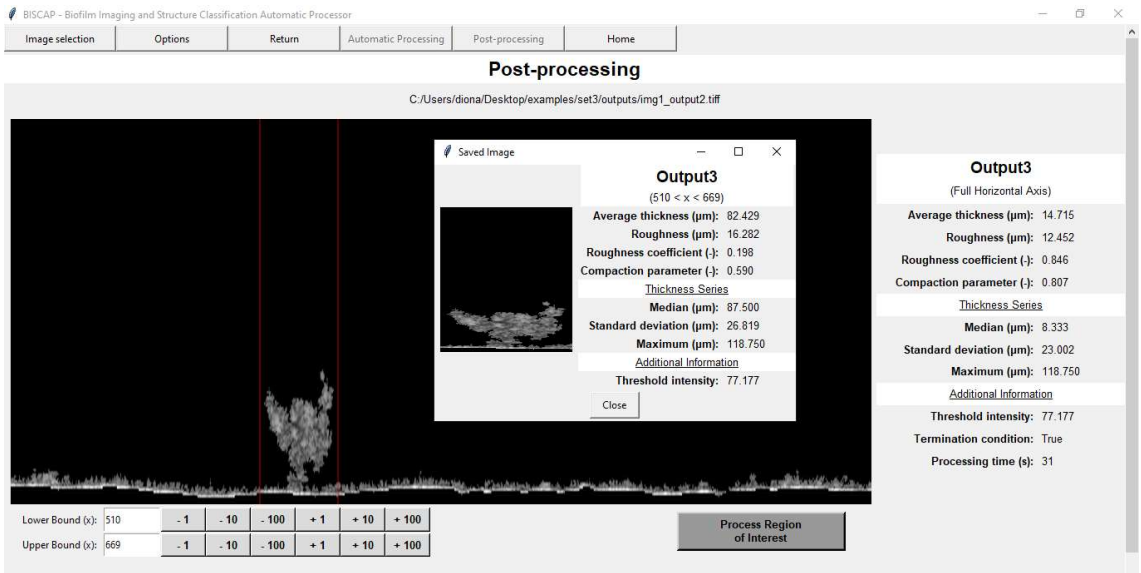


Figure 10: **Post-processing** screen (single image mode): region of interest and detailed biofilm analysis.

6. Single image mode

This mode is accessible via the (initial) **Home** screen. As the name suggests, it is tailored specifically for processing a single biofilm image. The three image processing functions as described in Sections 3-5 are thus displayed sequentially in BISCAP via three screens: **Pre-processing → Automatic Processing → Post-processing**, where each of these screens only becomes available if the necessary actions are completed (e.g. **Automatic Processing** screen available only if pre-processing is concluded).

Pressing the Single image mode button from the **Home** screen displays the **Pre-processing** screen. A set of navigation buttons are permanently displayed while on this mode, enabling users to move through the three available processing functions (subject to the conclusion of all precursor steps). A description of these buttons is presented below:

- Image selection: Clears the selected file from the memory (if any) and opens a window to browse to a 2D OCT image of interest.
- Return: Displays the previous screen according to the natural processing sequence defined above (not actionable in the **Pre-processing** screen – first step).
- Automatic Processing: Displays the **Automatic Processing** screen without re-running pre-processing. Only actionable if corresponding pre-processed image is available.
- Post-processing: Displays **Post-processing** screen without re-running pre-processing and automatic processing. Only actionable if outputs from automatic processing are available.
- Home: Clears the selected file from memory (if any), and displays the **Home** screen.

Image processing begins with the selection of a 2D OCT image of interest. The buttons above enable to some extent navigation between the three screens subject to the availability of relevant results. The buttons effectively executing the main actions as described in Sections 3 and 4 are available in the corresponding screens:

- Next (Trim Image) (**Pre-processing** screen): Takes the defined user-selected bounds and trims the selected raw image. Saves the corresponding pre-processed image and bounds' values, and moves the display to the **Automatic Processing** screen.
- Next (Automatic Processing) (**Automatic Processing** screen): Launches automatic processing of pre-processed image using the select processing options. The **Post-processing** screen is displayed, but no actions available until processing is completed.

The standard workflow for this mode is as follows:

1) Press the Single image mode button from the **Home** screen.
2) Select a 2D OCT image of interest, via the Image selection button.
3) **Pre-processing:** Adjust all bounds as described in Section 4, and once completed, press the Next (Trim Image) button.
4) **Automatic processing:** Adjust any relevant options and press the Next (Automatic Processing) button.
5) **Post-processing:** Select outputs to visualize from the Options button, and define and analyse any region of interest if required.
6) Use the navigation buttons above if necessary to move between screens.

Note: all input and output Excel and image files may be opened and checked independently of tasks executed in BISCAP. However, users are advised to close these Excel files if the main pre-processing or automatic processing tasks are launched. If not, BISCAP is unable to write the Excel files, and the application freezes.

7. Multi-images mode

From a user perspective, the single image mode would be cumbersome and inefficient to process multiple images. This is a consequence mainly of the unavoidable waiting times between processing of any two images, until automatic processing completes. To circumvent this limitation, an alternative workflow was developed for this particular case.

Rather than focusing on a set of images and executing the three processing functions for each of these images as described in Section 6, in the multi-images mode the focus is put on the three processing functions individually, and obtaining the necessary results for all selected images. The workflow is now as follows:

1) Pre-process all selected images.
2) Execute automatic processing for all selected images.
3) Post-process all selected images (if required).

Pre-processing is the only task requiring significant user input. Once completed, during the automatic processing stage all images are processed sequentially with no need for user input and/or supervision, and thus becoming a much more practical approach for image processing. In the end, and if required, users may also post-process results as described earlier.

From the **Home** screen, the **Multi-images mode** screen is accessible from the corresponding button (Multi-images mode). This screen displays four buttons, enabling the navigation to the three available image processing screens (**Pre-processing**, **Automatic Processing** and **Pre-processing**), or back to the **Home** screen. All buttons are actionable at all times. However, the same principles of results availability as described in Section 6 apply in this mode. The utilization of these image processing functions in the context of the multi-images mode is presented in sequence.

7.1 Pre-processing

The **Pre-processing** screen is depicted in Figure 11:

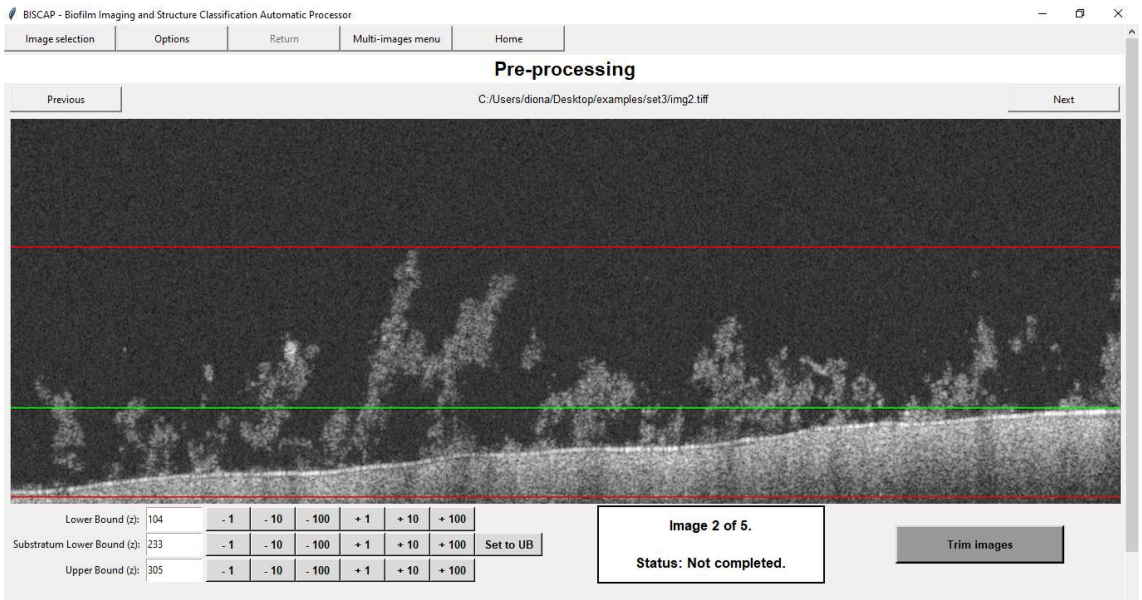


Figure 11: **Pre-processing** screen (multi-images mode).

Additionally to the main functionality presented in Section 3, the **Pre-processing** screen includes also four navigation buttons, as follows:

- Image selection: Clears the selected files from the memory (if any) and opens a window to browse to a folder of interest.
- Return: After executing pre-processing, trimmed images are displayed and increment buttons are not actionable. At this stage, the Return button becomes actionable, and if pressed, BISCAP resumes the selection of horizontal bounds.
- Multi-images menu: Clears the selected files from memory (if any), and displays the main **Multi-images mode** screen.
- Home: Clears the selected files from memory (if any), and displays the **Home** screen.

Rather than selecting a single file, a folder must be selected in this mode. All images within the selected folder are loaded to BISCAP and available for pre-processing. A single image is displayed at the time, and using the Next and Previous buttons, users may visualise all images in the selected folder. Then, using the increment buttons as discussed in Section 3, all bounds must be adequately adjusted. Once completed, the Trim images button, delivers and saves the pre-processed images from raw images as discussed in Section 3.

7.2 Automatic Processing

The **Automatic Processing** screen is depicted in Figure 12:

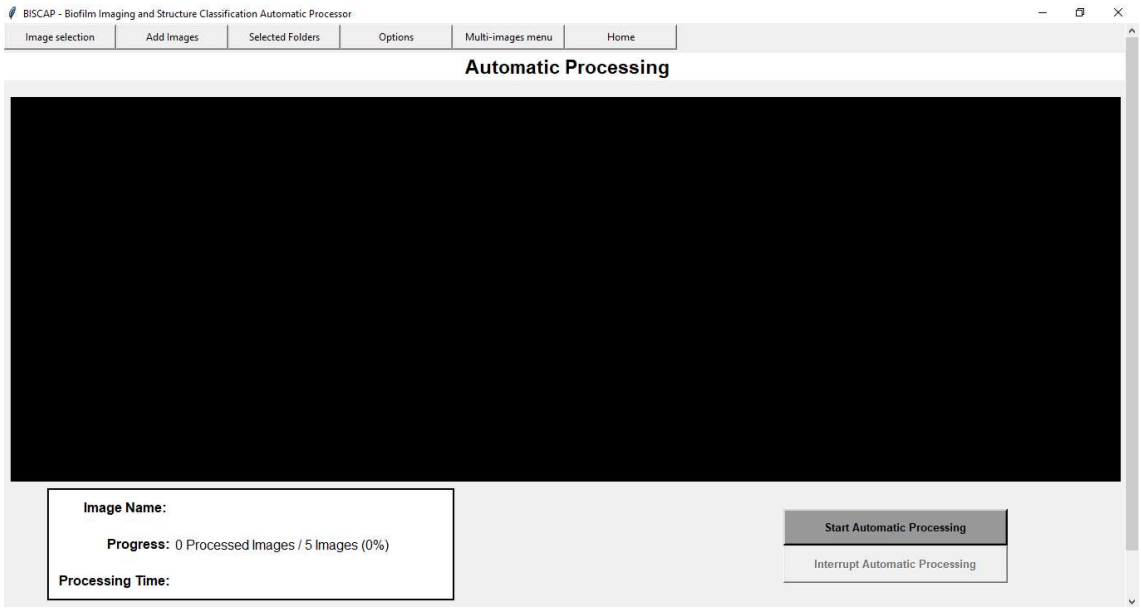


Figure 12: **Automatic Processing** screen (multi-images mode).

Additionally to the navigation buttons already discussed in Section 7.1, two extra buttons are available in the **Automatic Processing** screen, as follows:

- Add Images: Any previously selected file(s) are kept, and a window is displayed to select an additional folder and loading biofilm images therein.
- Selected Folders: Displays all currently selected folders for the purpose of automatic processing.

Any number of folders is allowed. The only requirement is that pre-processing was completed for all images in the selected folders. If this is not the case, a warning is displayed accordingly. Next, BISCAP sequentially processes all images and saves all results to disk. To achieve this, it suffices to adequately set the processing options as discussed in Section 4, and then launch automatic processing, via the Start Automatic Processing button. To cancel this action, the Interrupt Automatic Processing button may be used during processing. In this case, only a fraction of all results requested initially are saved to disk.

During automatic processing, the current pre-processed image being processed is displayed on screen and its name shown below. Progress towards completion is also shown on the lower left corner, and if enabled, the total processing time is also displayed when the activity completes. On completion, a summary Excel file ("outputs.xlsx") is saved for all selected directories in the corresponding "outputs" subfolders, additionally to the Excel files (output3) saved individually for all images processed. This provides a convenient access to all results and facilitates their comparison.

7.3 Post-processing

All navigation buttons as presented in Section 7.1 are available in the **Post-processing** screen. Users must select a folder for which results from automatic processing have been obtained at an earlier stage using the Image selection button. If a folder is selected not satisfying this requirement a warning message is displayed. All results for the selected folder are loaded to BISCAP as a gallery of images. Using the Previous, Next and Options buttons all results may be visualised as described earlier. Additionally, regions of interest may be defined for all outputs as described in Section 5.

7.4 Workflow

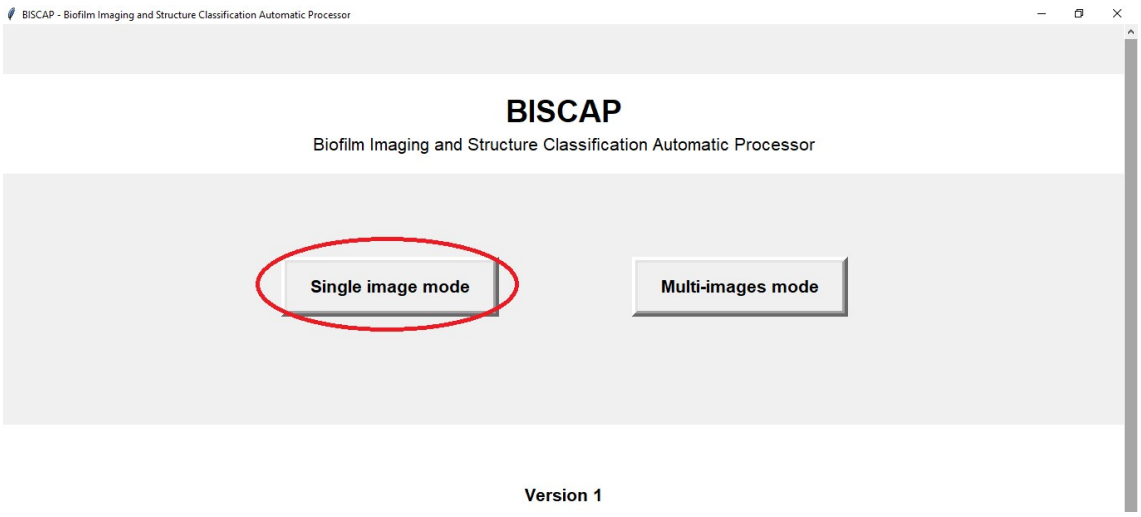
To conclude, the standard workflow for this mode is as follows:

1) Press the Multi-images mode button from the **Home** screen.
2) **Pre-processing:** Press the Pre-processing button from the **Multi-images mode** screen. Select a folder, and adjust all bounds of biofilm images therein as described in Section 7.1. Once completed, press the Multi-images menu button.
3) **Automatic processing:** Press the Automatic Processing button from the **Multi-images mode** screen. Select the same folder as in step 2, and launch automatic processing as described in Section 7.2. Once completed, press the Multi-images menu button.
4) **Post-processing:** Press the Post-processing button from the **Multi-images mode** screen. Select the same folder as in step 2, and browse through the gallery of images to check results and define any regions of interest as described in Section 7.3. Once completed, press the Home button.

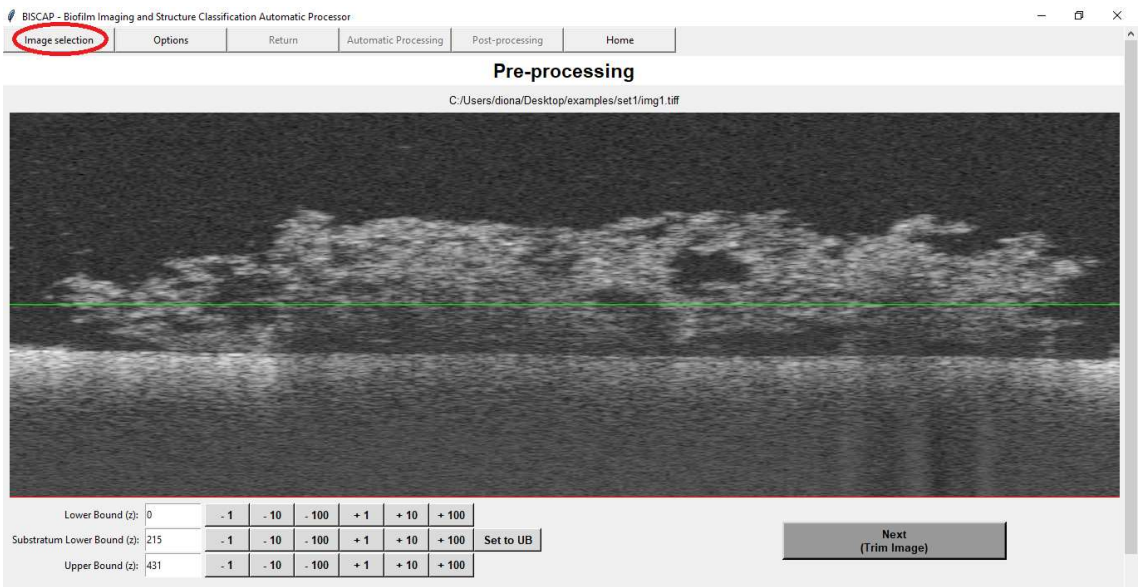
8. Tutorial 1: Single image mode

It is assumed that the complete set of examples was downloaded from "https://github.com/diogonarciso/BISCAP" and unpacked to an adequate root location. In the tutorials presented, folders and files are referred to via relative paths to this root location (e.g. "…\examples\set1"). The full sequence of steps for the first tutorial is presented in sequence.

1) **(Start)** Open BISCAP (note that the standalone version provided for review typically requires a few seconds to display).
2) **(Select mode)** In the **Home** screen click the Single image mode button.



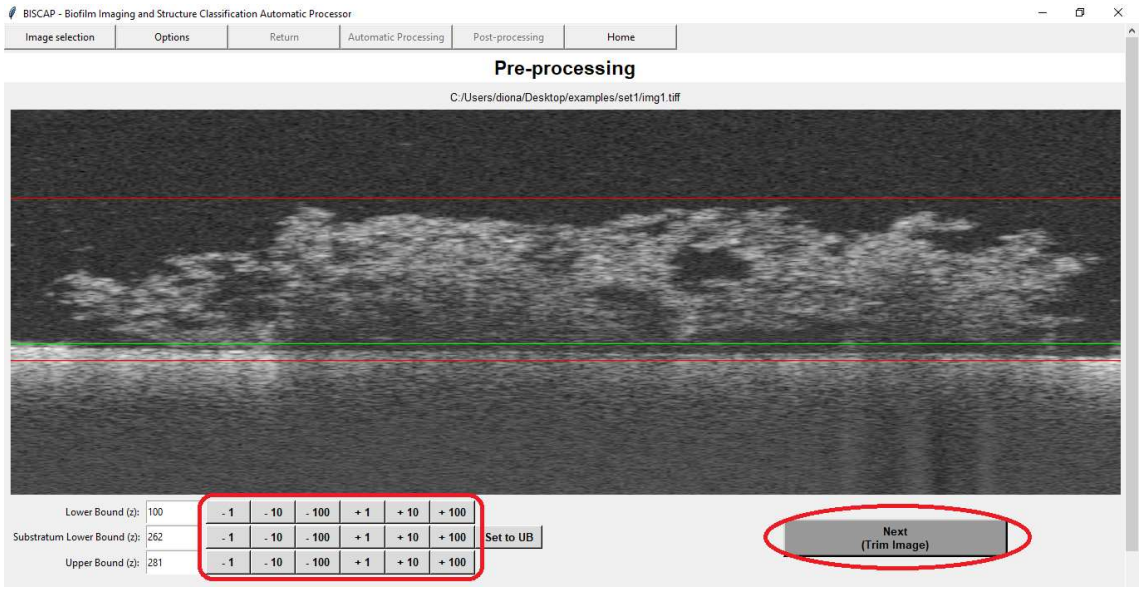
3) **(Image selection)** Click the Image selection button and browse to "…\examples\set1\img1.tiff" (example used in Appendix 1 for illustration purposes). "img1.tiff" is then displayed on the initial **Pre-processing** screen.



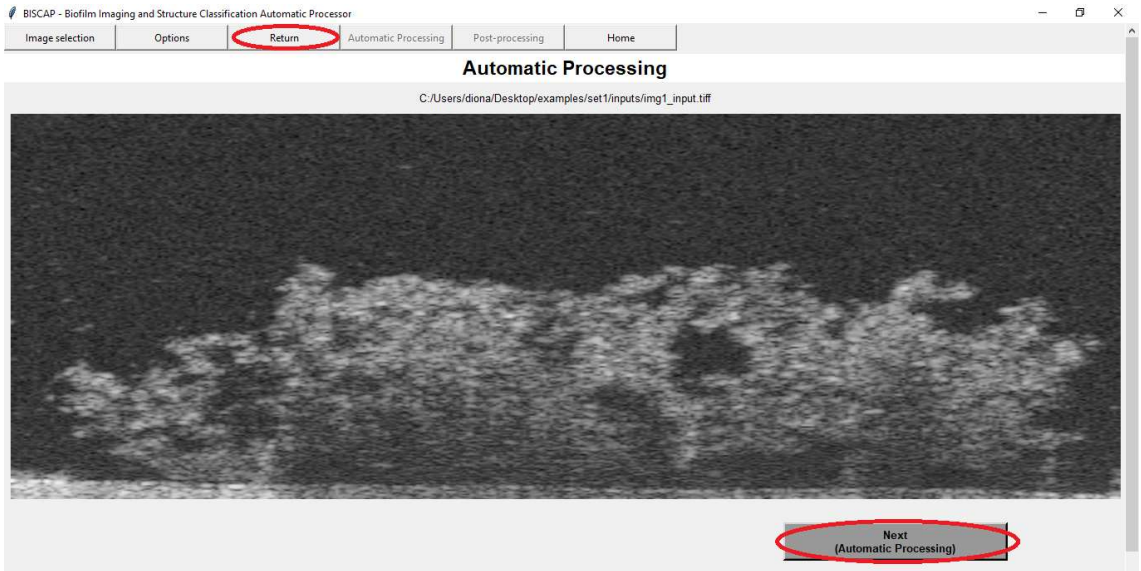
4) **(Pre-processing)** Using the increment buttons (-1, -10, -100, +1, +10, +100), set all bounds such that:
   - All biofilm pixels are below the *Lower Bound (z)*;

- All substratum pixels are below the *Substratum Lower Bound (z)*;
- All biofilm pixels are above the *Upper Bound (z)*.

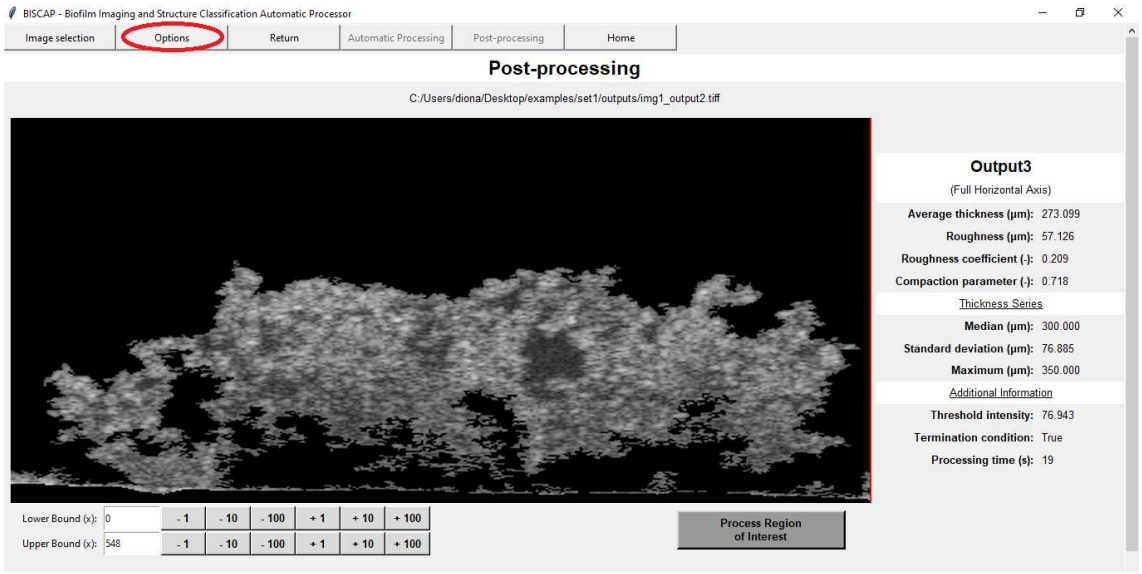
For reference, values used in the original contribution for this example are: *Lower Bound (z)* = 100, *Substratum Lower Bound (z)* = 262 and *Upper Bound (z)* = 281. When the specification is complete click the Next (trim image) button.



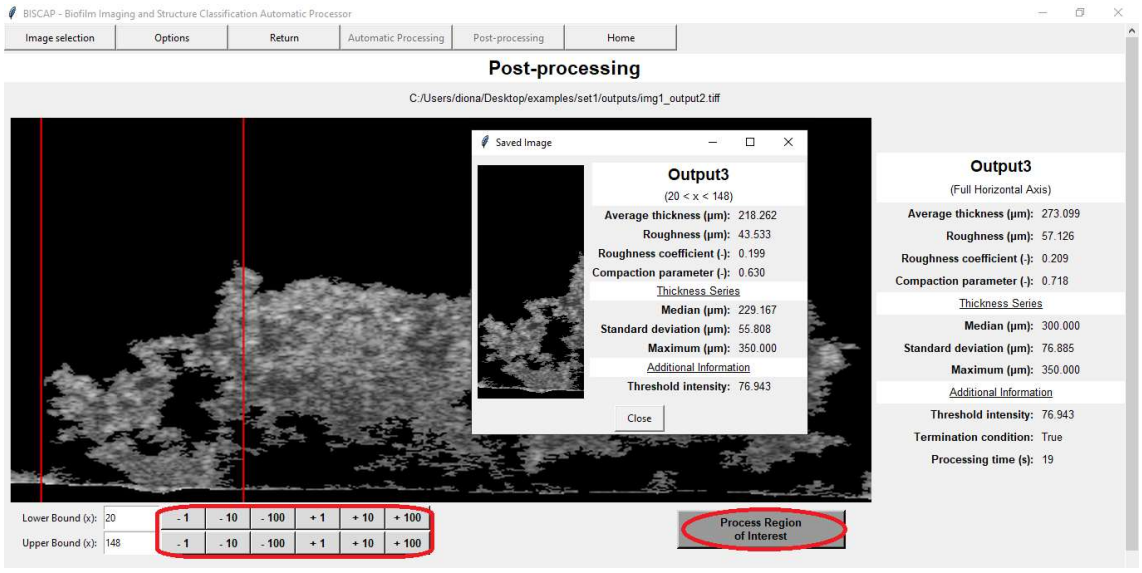
5) **(Automatic Processing)** The pre-processed image is displayed, and if a correction is required on the defined bounds click the Return button to make the necessary adjustments (step 4). When complete, click the Next (Automatic Processing) button to begin automatic processing.



6) **(Results display)** All results from automatic processing are displayed in the **Post-processing** screen, including by default outputs 2 (grayscale image) and 3 (numeric results on the right side of the screen). From the Options button, select and visualise the remaining outputs (0, 1, 3 and 4).

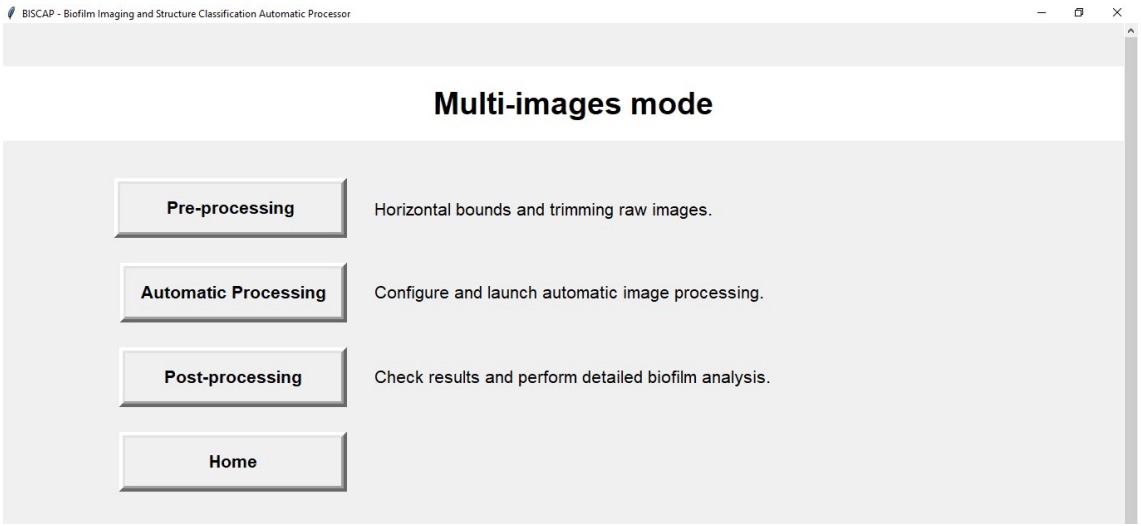


7) **(Post-processing)** Using the increment buttons (-1, -10, -100, +1, +10, +100), set the *Lower Bound (x)* and the *Upper Bound (x)* to define any region of interest. 20 and 148 are suggested, for the two parameters, respectively. Then, click the Process Region of Interest button to display and save the corresponding region of interest and structural parameters.

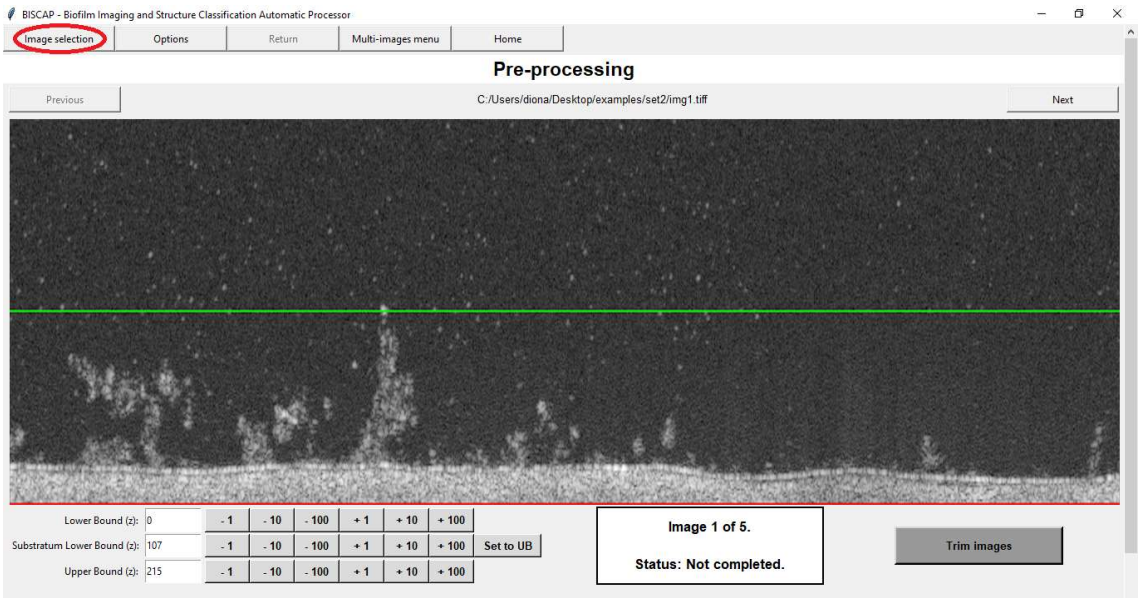


9. Tutorial 2: Multi-images mode

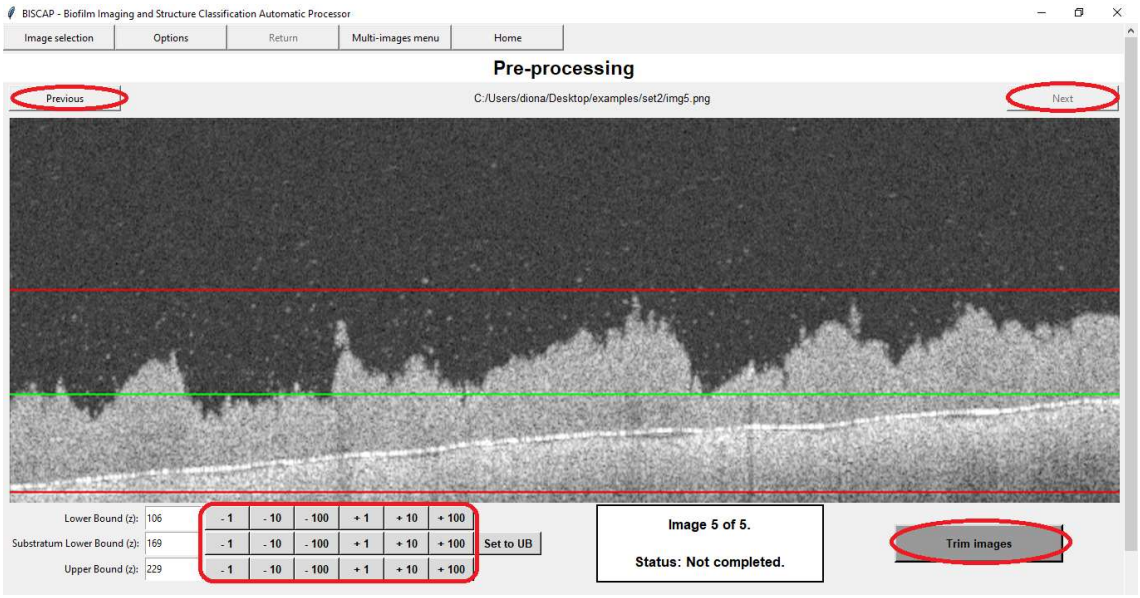
1) **(Start)** Open BISCAP.
2) **(Select mode)** In the **Home** screen click the Multi-Images mode button. The **Multi-images mode** screen is displayed, providing access to the 3 key image processing functions and corresponding screens: (i) **Pre-processing**, (ii) **Automatic Processing**, and (iii) **Post-processing**.



3) **(Pre-processing – part I)** Click the Pre-processing button. In the **Pre-processing** screen, click the Image selection button and browse to "…\examples\set2". This folder includes all examples used for illustration in Section 3 of the main manuscript.



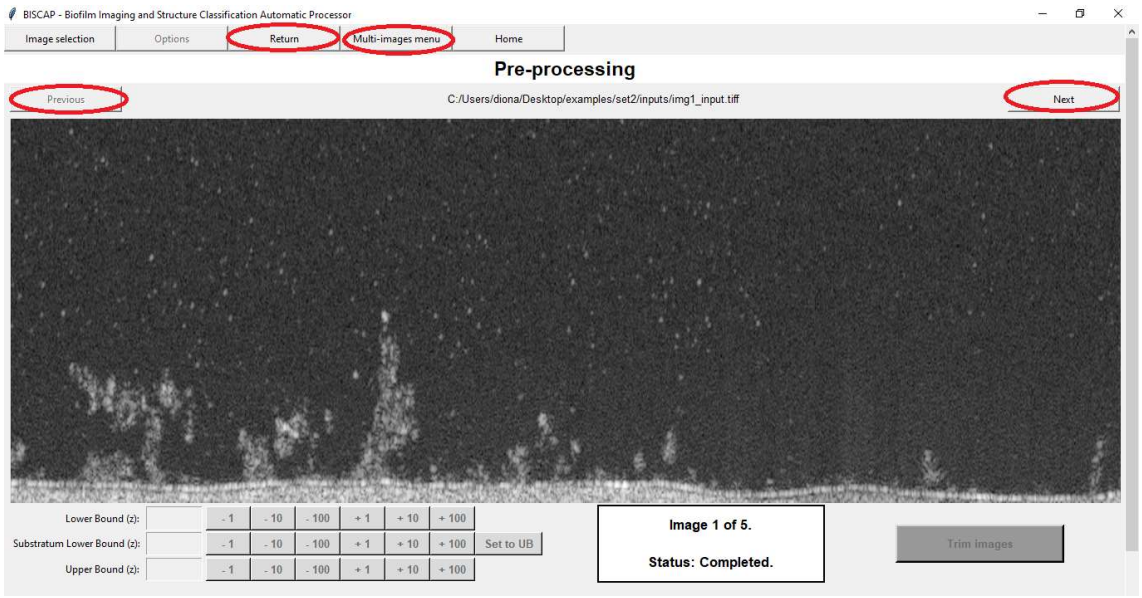
4) **(Pre-processing – part II)** Using the Next and Previous buttons, and all increment buttons available on the bottom of the **Pre-processing** screen, set all bounds in accordance with the guidelines in Sections 3 or 8.



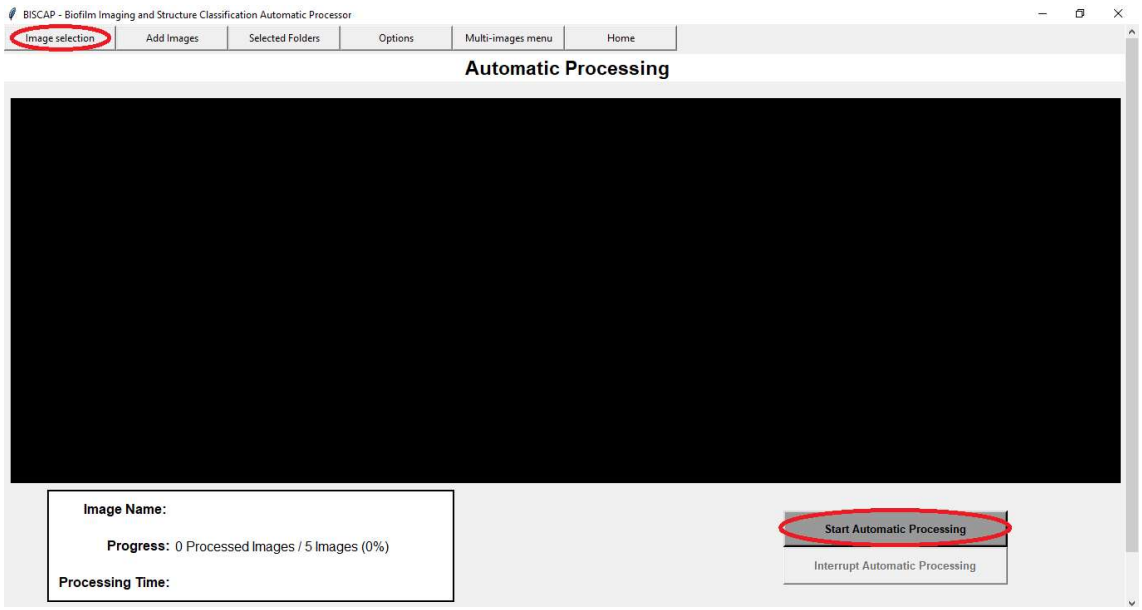
For reference, the bounds used for this set of images as presented in the main manuscript are presented in the Table below:

Image	img1	img2	img3	img4	img5
Lower Bound	103	105	104	106	106
Substratum Lower bound	189	362	254	141	169
Upper Bound	205	399	301	179	229

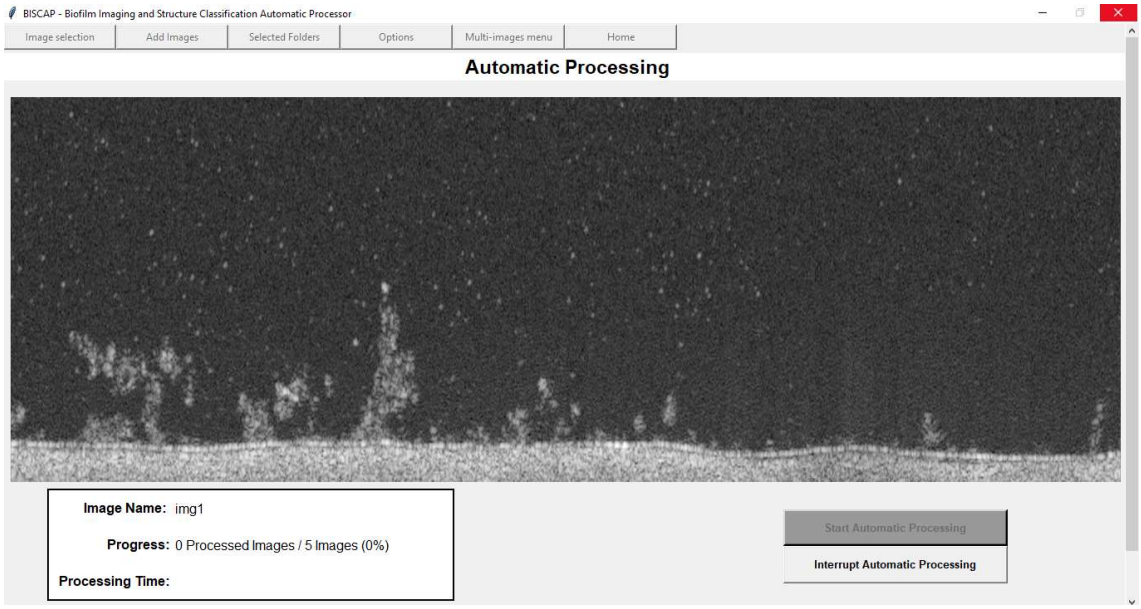
5) **(Pre-processing – part III)** Click the Trim Images button to automatically trim all raw images accordingly to specifications in step 4. All pre-processed images may then by visualized using the Next and Previous buttons. For any additional adjustment, click the Return button. When completed, click the Multi-Images mode button to return to the main **Multi-images mode** screen.



6) **(Automatic Processing – part I)** Click the Automatic Processing button. In the **Automatic Processing** screen, click the Image selection button and browse to folder "…\examples\set2". Click the Start Automatic Processing button to begin automatic processing. To process additional files (optional), click the Add Folder button and select a folder of interest (e.g. "…\examples\set3").



7) **(Automatic Processing – part II)** BISCAP processes in sequence all images selected. The current pre-processed image under automatic processing is displayed, and the progress towards completion is shown below. On completion, click the Multi-Images mode button to return to the **Multi-Images mode** screen.



8) **(Results display)** Browse to the "…\examples\set2\outputs" folder, and open the "outputs.xlsx" file to check and compare the image processing results for the 5 images in the selected root folder.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Ave	rage thickd	ian thickr	lev. thickr	ax. thickne	Roughness	ness coef	action para	Threshold	nation Co	ocessing Time	
2	img1	28.32572	12.5	36.17679	160.4167	25.80559	0.91103	0.73311	77.42008	FALSE	39.25	
3	img2	88.65768	8.33333	126.1986	337.5	113.6516	1.28192	0.56471	92.94994	FALSE	101.3906	
4	img3	91.24681	37.5	108.3924	347.9167	89.84252	0.98461	0.61846	87.41214	TRUE	64.76562	
5	img4	53.2803	52.08333	13.61217	95.83333	10.8141	0.20297	0.98876	96.11722	TRUE	26.14062	
6	img5	106.5594	106.25	23.88725	175	19.37727	0.18184	0.99149	95.6978	TRUE	37.51562	

9) **(Post-processing)** Click the Pre-processing button. Then, click the Image selection button and browse to folder "…\examples\set2". Using the Previous and Next buttons select any image within the selected folder, and then repeat steps 6 and 7 as presented in the first tutorial to visualise all outputs and define any region of interest.